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Neural Stem Cells

Methods and Protocols

Second Edition

Edited by

Leslie P. Weiner

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Neural Stem Cells

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SECOND EDITION

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Preface

Since the publication of the first edition, there has been an explosion of interest and technology in the study of neural stem cells (NSCs). There are still many questions related to stem cell properties and NSC lineage and differentiation. The clinical application of NSCs to neurogenetic and neurodegenerative diseases is not far off. However, the fundamental biological questions related to transplantation have not yet been solved. The need to be certain of which cells are best in a given situation; what directs their differentiation, migration, and survival; and their tumorigenic potential have to be answered before transplantation is attempted in a human disease.

This edition has revised and updated many of the chapters from the first edition. The specific chapters related to isolation and culturing of NSCs, the cellular and molecular biology of NSCs, and particularly those chapters that are relevant to transplantation have been retained.

Cutting edge new chapters showing methods for both human and rodent NSCs have been added. These chapters cover topics such as NSCs from human embryonic stem cells, immortalized human NSCs, nuclear transfer, and single-cell polymerase chain reaction. Chapters on karyotyping, migration, apoptosis, and a thorough protocol for the use of NSC markers will be of invaluable aid to new investigators, fellows, and students.

The combination of NSC isolation, culturing, functional assessment, gene expression studies, and genetic manipulation chapters will allow investigators to produce pure populations that can serve as a means of understanding the biology of NSCs as well as adapting them for transplantation into disease models. The NSCs and lineage cells such as neurons, oligodendrocytes, and astrocytes have to be produced and be shown to reach their targets in the diseased or injured central nervous system to either influence endogenous stem cells in repair or to replace damaged or dead cells.

As outlined in the first edition, we believe that this collection of methods, protocols, and background information will be useful to stem cell scientists and students. Each chapter now has a summary, an expanded introduction for background, and a considerable bibliography to help locate experiments that use similar techniques. The extensive index also will aid investigators in comparing different approaches to the same problems.

We acknowledge the help of Professor John M. Walker, series editor, for guidance in assembling this volume. We are particularly indebted to Drs. Tanja Zigova, Paul R. Sanberg, and Juan R. Sanchez-Ramos for providing such an excellent first edition that served as a model and springboard for the second edition. Finally, this volume could not have been completed without the hard work, intellectual input, and persistence of Ms. Raimonda Apeikis.

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I

Introduction

1

Definitions and Criteria for Stem Cells

Leslie P. Weiner

Summary

The working definition of a stem cell includes self-renewal and the ability to differentiate into several cell types. There are also aspects of clonality and potency. Stem cells can be derived from early embryos after the formation of the blastocyst or from fetal, postnatal, or adult sources. Neural stem cells (NSCs) arise from embryonic ectoderm that forms neuroepithelial cells. The neuroepithelial cells generate radial glia that produce fetal and adult NSCs within the central nervous system (CNS). Adult NSC and restricted progenitors are found in the several regions of the CNS throughout life. Human embryonic stem cells, with their ability for self-renewal, clonal capacity, normal karyotype, and potential to form NSCs, easily may be the best source of NSCs and progenitors for treating disease. However, the complexity of NSCs, neural patterning, and the formation of multiple populations of neurons, astrocytes, and oligodendrocytes warrant the need for intense studies to characterize these cells and to define the microenvironment that will be needed to support them in the diseased CNS. Ways to produce well-defined populations, avoid oncogenicity, and ensure survival need to be clarified before clinical application can begin.

Key Words: Adult stem cell; NSC-derived from human embryonic stem cell; neurogenesis; wnt; sonic hedgehog; basic helix-loop-helix protein; Olig1; Olig2.

1. Introduction

Stem cells have the capacity to self-renew and generate differentiated cells. Self-renewal is defined as the ability to generate daughter cells identical to their mother. The daughter can also produce progeny with more restricted potential. Thus, a stem cell can divide to generate one daughter cell that is a stem cell and another daughter cell that can produce differentiated cells. This definition is easily applied to the totipotent embryonic stem cells that are derived from

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the blastocyst's cell mass ***(1,2)***. Such application is less clear when trying to distinguish between adult progenitor cells that have a reduced self-renewal capacity and true adult stem cells. Adult stem cells may have the capacity to only produce one lineage of differentiated cells ***(3–5)***. They are best defined as displaying significant proliferative ability that lasts the lifetime of the animal.

Embryonic stem cells have characteristics that include self-renewal, clonal capacity, multilineage differentiation, normal karyotype, proliferation to produce large numbers, and the ability to be frozen and thawed. Adult stem cells are a single cell that can self-renew and generate differentiated cells of a specific lineage and that have a capacity to reconstitute tissues or organs. There is evidence of plasticity, namely, the cell fate is not permanent but flexible. Related to this concept is that of transdifferentiation or the formation of tissues or lineages that are distinct from that of the founder stem cell ***(6)***.

The other important aspects include elevated levels of telomerase and maintenance of telomeres ***(7)***. Furthermore, stem cells have remodeled chromatin, suggesting significant epigenetic influences ***(8)***. The molecular definitions of stem cells as they relate to signal transduction, transcription, and posttranslational changes are not yet completely defined.

Proof of existence of stem cells postnatally is evidenced by tissue growth, differentiation, and maintenance. Stem cells seem to exist and persist in many somatic tissues. Perhaps the two areas where this is most evident are the hematopoietic system and the central nervous system (CNS). In the hematopoietic system, huge numbers of blood cells are produced throughout life. Reservoirs of progenitors can be found in cord blood, peripheral blood, and bone marrow. A single cell can repopulate the entire spectrum of blood lineages ***(9)***.

2. Neural Stem Cell (NSC)

CNS stem cells have been isolated from the embryonic, fetal, and adult nervous systems. The functions of NSCs are to generate additional stem cells and to give rise to a differentiated progenitor cell. CNS stem cells can generate lineages of neurons as well as glia, such as astrocytes and oligodendrocytes. The CNS lineage has specific functions and cellular interactions, and these cells must migrate to specific locations in the neuraxis.

2.1. Neurogenesis

The ectodermal neural plate forms during embryonic gastrulation, takes the shape of a neural tube, and consists of neuroepithelial cells. When neural precursors develop, they already have a restricted fate. This is due to a patterning

process that positions them along the neuraxis. The patterning genes seem to trigger a cascade of genes that determine neural differentiation at a specific time and place during development.

It is not certain what are all the factors required for converting ectoderm to neural tissues ***(10)***. Inhibition of bone morphogenic protein (BMP) signaling seems to be critical ***(11)***. BMP is a member of the transforming growth factor-β (TGF-β) superfamily of growth factors. In the embryo, noggin, chordin, and follistatin are all inhibitors of BMP ***(11–13)***. The blockage of BMP occurs before and during gastrulation. Other molecules that have thus far been defined include inhibition of wnt, another growth factor ***(14)***, as well as the production of growth factors such as fibroblast growth factor (FGF) that contribute to the regulation of differentiation in vivo ***(15,16)***.

The best data on neural patterning is in spinal cord development. The initial axis is a dorsal-ventral axis, with one being the ventral pole of the neural tube and the other being the dorsal pole ***(17)***. In the ventral plate, sonic hedgehog (shh) induces or suppresses selective genes, usually transcription factors, and results in the formation of motor neurons ***(18)***. This occurs in the ventral domain of the entire spinal cord with different motor neuron types in the anterior-posterior axial level ***(19)***.

There are a series of genes of the basic helix-loop-helix(bHLH) that encode neural lineage genes ***(20)***. Inhibition of such genes results in differentiation to astrocytes and oligodendrocytes rather than to neurons ***(21)***. The best studies of this class of genes relate to Olig1 and Olig2 ***(22)***. There is evidence that glial differentiation genes are suppressed at the time neuronal differentiation is occurring.

2.2. Fetal and Adult NSCs

Adult NSCs were first found in the rodent striatum and subsequently in the subventricular zone and in the embryonic mammalian forebrain during neonatal development ***(1–3)***. These multipotent glial cells (radial glia) are present in the subventricular zone (SVZ) ***(23)***. Radial glia disappear in the SVZ with maturation, and are replaced by multiciliated ependymal cells that line the walls of the lateral ventricle.

Radial glia serve as progenitors in avian and reptilian nervous system*s* and in the developing mammalian brain. There is very little evidence that they are a significant source of neurons in the adult mammalian brain ***(24)***.

There are data that in the adult brain stem cells can be formed in the subgranular zone (SGZ) of the hippocampal dentate gyrus. Astrocytes from this region can produce oligodendrocytes and NSCs that show self-renewal and restricted differentiation. The SVZ, SGZ, and the olfactory bulbs are

the main sources of adult NSCs. However, NSCs can also be found in the neocortex striation, olfactory tubercle, amygdala, substantia nigra, and spinal cord ***(25–30)***.

3. Conclusions

The complexity of using blastocyst-derived human embryonic stem cells to make usable NSCs for study and possible future transplantation requires a better understanding of neural patterning and differentiation factors than is currently known. The presence of adult stem cells, within different regions of the brain, suggests a potential therapeutic mechanism for repair and regeneration. We must know whether specific microenvironmental factors can be manipulated and whether endogenous NSCs can be induced to proliferate, migrate, and differentiate into specific neuronal populations or growth factor-secreting cells, as well as astrocytes and oligodendrocytes.

By studying neurogenesis, fetal, postnatal, and adult neural stem cells, factors that stimulate or inhibit differentiation and ensure cell survival can be determined. This volume outlines methods and protocols that will allow investigators to pursue the important avenues needed to understand NSCs and lineage cells. Eventually, NSCs can be engineered, cloned, and transplanted into diseases such as amyotrophic lateral sclerosis, Alzheimer's disease, Parkinson's disease, multiple sclerosis, stroke, and CNS injury from trauma.

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2

Neural Stem Cells and the Future Treatment of Neurological Diseases: *Raising the Standard*

Jaime Imitola and Samia J. Khoury

Summary

Neural stem and progenitor cells offer great potential for treatment of neurological disorders. The current strategies of isolation, expansion, and characterization of these cells require in vitro manipulations that can change their intrinsic properties, specifically with the acquisition of chromosomal abnormalities. We have analyzed the rationale of using neural stem cells in neurological disorders, the caveats of current isolation and in vitro culture protocols of neural precursors. Addressing these challenges is crucial for translation of neural stem cell therapy to the clinic.

Key Words: Neural stem cells; neural progenitors; neurospheres; self-renewal; multipotency.

1. Introduction

Neural stem cells (NSCs) are defined as multipotent and self-renewing precursors that reside in specialized molecular microenvironments or niches in the mammalian central nervous system (CNS). The fundamental properties of stem/progenitor cells are self-renewal, multipotency, and differentiation and migration, thereby providing a source of differentiated cells during development and in the adult ***(1,2)***. Alteration in neural progenitor cell properties of migration and proliferation is the basis of some CNS human disorders ***(3,4)***. The recent interest in neural stem cells has been driven primarily by the prospect of using them to treat genetic and acquired diseases of the CNS. For this goal to be feasible, NSCs used therapeutically must demonstrate the

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ability to differentiate along specific lineages, to migrate long distances, to survive in the milieu of the injured brain, and to decrease proliferation once repair has been achieved. Additional properties of NSCs have recently been uncovered, where a beneficial paracrine effect was achieved by transplanted NSCs and by embryonic stem (ES) cells ***(5–7)***. NSCs have also been shown to have immumodulatory properties ***(8)***. These additional properties make NSCs an attractive source of material for clinical transplantation, however these properties may be modified in vitro ***(9)***.

In this chapter, we review the technical challenges that confront the NSCs field, and we make a case for standardization and improvement of existing protocols for isolation and culture of NSCs. We address the following questions: (1) What is the rationale for using stem cells in neurodegenerative diseases? (2) What are sources of NSCs? and (3) Does the in vitro propagation of NSCs impact their intrinsic properties and safety?

2. Rationale for Using Exogenous Stem Cells for Neurodegenerative Diseases

Many diseases have been suggested as targets for stem cell therapy ***(10)***, and neurological disorders, especially neurodegenerative disorders, are at the top of the list. Despite the enthusiasm for the use of stem cells in neurological disorders, detailed analysis of the realities and peculiarities of NSCs and the target disease is necessary to increase the likelihood of clinical success ***(11)***. Several types of stem cells have been proposed for neurological disease therapy, including embryonic, neural, skin and mesenchymal stem cells. We focus this discussion on NSCs.

What neurological disorders are realistically treatable with NSCs? For example, in Alzheimer's disease, the pathology is a multifocal neurodegeneration that would require the use of multiple transplants in the same patient, which may be problematic unless we can achieve reasonable engraftment by systemic or intraventricular administration. It could be argued that a paracrine or chaperone effect may be beneficial in Alzheimer's disease to preserve remaining neurons, even if cell replacement is not achieved. However, more research is required to demonstrate whether the paracrine effect observed in some studies of NSCs and ES cells could be of any benefit in Alzheimer's disease models.

Another equally challenging disease is multiple sclerosis, where not only oligodendrocytes and axons are destroyed but also neurons are dysfunctional ***(12,13)***. Therefore, transplanted NSCs will need to differentiate into oligodendrocytes and neurons. The feasibility of using NSCs has been demonstrated in animal models of dysmyelination as well as chemical-and immune-mediated demyelination. Importantly, NSCs injected intravascularly successfully

ameliorated disease ***(14)***, circumventing the need for multifocal injections. The potential clumping of neurosphere-derived cells and the number of cells needed may pose some risks of thrombus formation that have to be examined in vivo with appropriate clinical trials.

Conversely, focal neurological disease may be more amenable to NSC therapy, especially when a single biochemical defect is apparent. Parkinson's disease has been proposed as a good candidate for cell replacement therapy ***(10)***. However, even in Parkinson's disease the need for specific dopaminergic differentiation has been difficult to achieve with NSCs, requiring modification of NSCs with transcription factors such as Nurr ***(15)*** or the use of ES cells ***(16)***, which have the potential to persist as undifferentiated conglomerate of cells in vivo but with the risk of tumorigenic transformation ***(17)***.

3. Mobilization of Endogenous Stem Cells

In some CNS diseases, the endogenous NSCs participate in the process of repair, and one goal of therapy is to enhance this plasticity ***(18,19)***. Thus, we need to understand the molecular programs that need to be activated to initiate and enhance repair ***(20)***. In humans, NSCs have been identified in the hippocampus and the subventricular zone (SVZ), where a ribbon of astrocytes displays attributes of NSCs when isolated in vitro ***(21)***. Some progenitor cells have been successfully isolated from the white matter, and they seem to be multipotential ***(22,23)***. To successfully mobilize endogenous stem cells requires knowledge of the molecular cues that are necessary for directed migration and differentiation, but even if this is achieved, the distance that the cells have to travel from the niches to reach the area of injury may pose another hurdle. Caution needs to be exercised when using growth factors to mobilize NSCs, because Jackson et al. ***(24)*** reported the generation of glioma-like growth in the SVZ by infusion of platelet-derived growth factor.

4. In Vitro Generation of NSCs for Preclinical Studies

In the early 1990s, researchers were able to isolate neural precursors and stem cells from the adult or embryonic brain and to expand them in vitro with cytokines such as fibroblast growth factor (FGF)-2 and epidermal growth factor (EGF) and to generate sufficient numbers for experimental transplantation ***(25,26)***. From the experimental work of the past 14 years and after more than 4,000 primary papers published in the field of NSCs, it seems that neural stem and progenitor cells can survive, differentiate, and integrate seamlessly in the host. More research is required to conclude whether appropriate neural circuitry can be restored with these transplanted NSCs.

Because of the need to expand neural stem cells in vitro, a debate is ongoing among researchers as to whether in vitro manipulations change the properties of stem/progenitor cells. Some cells that are restricted progenitors in vivo, such as *dlx-2* or type C cells, can acquire stem cell properties such as multipotency in vitro ***(27)***. Moreover, in vitro propagation with FGF-2 may change some of the intrinsic properties observed in vivo ***(28)***. It could be argued that if multipotency and other properties are maintained in vitro, the cells should be operationally defined as precursor cells useful for therapy; however, we should keep in mind that in vitro conditions may change NSCs biology in a way that could be detrimental to the patients.

5. Use of Neurospheres

In contrast to hematopoietic stem cells, where a small number of stem cells can regenerate multiple cells types, NSCs do not regenerate with the same efficiency, and it is not possible to use a small number of cells to regenerate the entire repertoire of neurons, oligodendrocytes, and astrocytes. NSCs have been propagated by using FGF-2 and EGF in low binding culture vessels where they form clusters of cells, termed neurospheres. The numbers of neurospheres have been used as a surrogate for NSC activity in vivo, although this is controversial ***(29)***. Investigators are using the propagation of neurospheres obtained from individuals with brain abnormalities as surrogate in vitro models for understanding CNS disease and development ***(3,4)***.

Cells from neurospheres are multipotential and self-renewing, although the location and number of the true stem cells within a neurosphere are a matter of debate. Therefore, neurospheres represent a nonclonal population of neural precursors cells that includes neurosphere-forming cells and progenitors that exhibit physiological capacity to respond to FGF 2-and EGF ***(4)***. Moreover, in vitro expansion may modify the properties of NSCs, sometimes after only a few passages, which can indicate transformation and the potential to induce brain tumors ***(30,31)***. Aneuploidy is a recognized phenomenon of NSC cultures that also happens in vivo with no apparent detriment to the normal differentiation of neurons. In addition, it can be seen in ES cells dissociated with trypsin ***(9)***. Neurospheres can be transformed by culture after 10–15 passages ***(9).*** Furthermore, one or two tumorigenic hits can render neural progenitors cells highly oncogenic, leading to gliomas ***(32)***. Therefore, when expanding NSCs for clinical use, genetic examination has to be done to clarify their tumorigenic potential by karyotyping; but in some cases microdeletions may be present, therefore more expensive techniques such as comparative genomic hybridization may have to be used.

Neurospheres and neurosphere assays are under scrutiny because of the long-held assumption that neurospheres are clonal. Although it is true that stem cells cultured in vitro at a dilution of one cell per well can form neurospheres that are multipotent and self-renewing, the clonal efficiency of these cultures is very low. Thus, most investigators culture neurospheres under the so-called "clonal dilution" of 10–20 cells per microliter. At these dilutions, the neurospheres are not really clonal, and a recent report demonstrated significant fusion of neurospheres at this density ***(33)***. That neurospheres seem to be hetererogenous, perhaps by fusion of different cells, may call into question earlier observations of molecular and cellular heterogeneity of clonal neural stem cells.

An alternative source of NSCs has been the immortalization of neural progenitors. Cells immortalized by oncogenes may pose *a priori* risk for clinical use. However, these immortalized neural progenitors cells may be more homogenous than neurospheres, because they are clonally derived and genetically marked with a single insertion site of genetic marker. Regardless of the source of stem cells, whether exogenous cells, ES cells, ex vivo-propagated NSCs, or in vitro-immortalized neural stem cells, in vitro manipulation is necessary. Taking lessons from the embryonic stem cell field, we would recommend the same rigorous methodology for culture and passaging the NSCs. Several strategies have been suggested to avoid using cells with altered biological properties, such as isolating specific subpopulation of cells by fluorescence-activated cell sorting sorting(FACs) of ES cells. Avoiding contamination with animal products and the use of defined medium are also required.

6. Future Perspectives

New approaches have to be implemented to follow the fate of transplanted NSCs in vivo: Bioluminescence has been used to follow NSC migration ***(34)*** and MRI techniques are being optimized to image NSCs noninvasively ***(35,36)***. More research is needed to examine the effect of rejection on the fate of transplanted NSCs. Another important area for investigation is the paracrine and immunomodulatory function of certain stem cells that can be therapeutically valuable even if integration of the cells in the CNS does not occur.

7. Conclusions

NSCs are an invaluable tool to understand brain development and repair. There is a need to standardize methods and protocols in the entire field. Even if stem cells fail to fulfill their promise as a source for cell replacement, research in this field will lead to a better understanding of the mechanisms of neurodegeneration and the limitations of intrinsic brain repair. The need for

standardized protocols is important not only for the epistemological benefit of the field but also for the correct translation from the bench to the bedside. Finally, we need to address the long-term survival of these cells by creating a more permissive environment in the diseased brain. Thus, NSC therapy may need to be administered in conjunction with other treatments that target the milieu.

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II

Isolation and Culture of NSCs

3

Neural Differentiation of Human Embryonic Stem Cells

Mirella Dottori and Martin F. Pera

Summary

Embryonic stem cells (ESCs) are pluripotent and capable of indefinite self-renewal in vitro. These features make them a highly advantageous source for deriving any cell type of the central and peripheral nervous system. We describe neural induction of human (h)ESCs, by using the bone morphogenic protein inhibitor protein noggin. Neural progenitors derived from noggin-treated hESCs can be propagated as neurospheres and further differentiated in vitro and in vivo to mature neurons and glia. This complete protocol of neural differentiation, from hESCs to mature neuronal cells, can be used as an in vitro model to study human neurogenesis and neurodegeneration.

Key Words: Neural differentiation; embryonic stem cell; human; neurosphere; noggin; neural progenitor.

1. Introduction

The derivation of human ESC (hESCs) has opened up exciting possibilities for their therapeutic use for treatment of neurodegenerative diseases and injury. Induction of neural progenitors from hESCs has been widely reported, and it usually involves conversion of hESCs into embryoid bodies followed by growth factor treatment, or growing hESCs on a stromal cell monolayer that induces neural differentiation ***(1–8)***. In our laboratory, we have established a well-characterized in vitro model of human neural differentiation derived from hESCs. Noggin is a bone morphogenic protein (BMP) antagonist, and its role in neural induction (the default model of neurogenesis) was first described in *Xenopus* (for review, *see* **ref. *9***). Treatment of hESCs with the BMP antagonist

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noggin for 14 days results in the efficient induction of neural stem cells (NSCs) as shown by the expression of neuroectodermal markers Sox2, Pax6, and nestin ***(5)***. After noggin treatment, NSCs can be mechanically isolated from colonies and cultured in suspension in neural basal media (NBM) and growth factors fibroblast growth factor (FGF) and epidermal growth factor (EGF). Under these conditions, the noggin-treated cells convert with high (>80%) efficiency to spherical clusters of cells known as "neurospheres." Neurospheres derived from noggin-treated hESCs can be further differentiated in vitro to give rise to mature neurons and glia. Furthermore, it has been shown that transplantation of neurospheres derived from noggin-treated hESCs into the brains of Parkinsonian rodents resulted in their integration and differentiation into neural and glial cells ***(10)***. Overall, this neural induction protocol is highly efficient and very useful for research studies. Using a range of culture systems, we can identify, characterize, and monitor various stages of neuronal differentiation from hESCs to mature neurons and glia.

The protocol outlined below provides for neural differentiation of hESCs, beginning with hESCs grown on a feeder cell layer, with neural induction by noggin-treatment, followed by culture of NSCs as neurospheres, and finally,

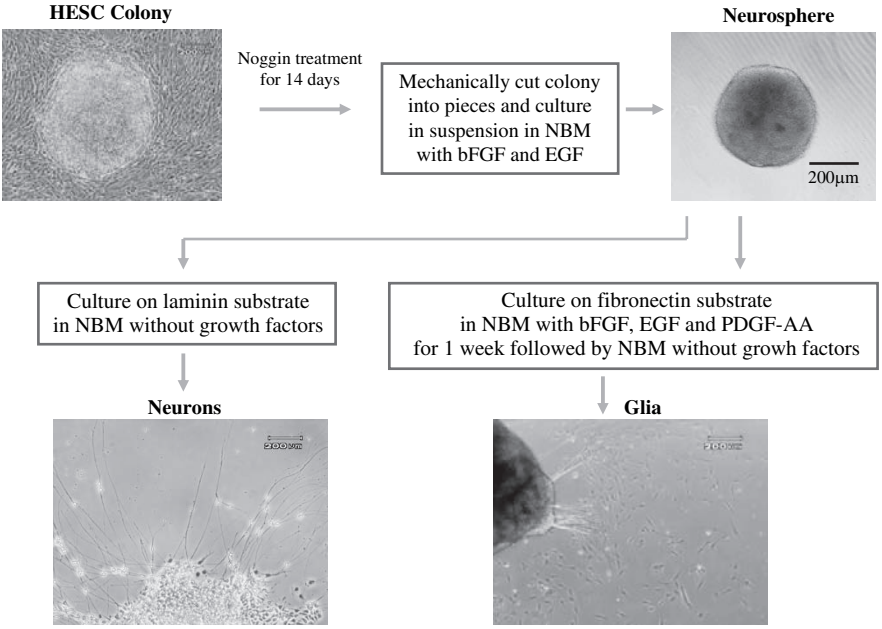

Fig. 1. Schematic diagram showing overall neural differentiation protocol from undifferentiated hESC to mature neurons and glia. Images taken by Kathryn Davidson and Mirella Dottori.

their differentiation to mature neurons and glia (*see* **Fig. 1**). This simple protocol is easily subjected to modifications, such as supplementation of other growth factors at the same or later stages of neural induction by noggin treatment. Because the overall protocol can be dissected to different stages of neural differentiation, it allows flexibility with modifying the technique as well as ease in investigating the readout to reach the desired endpoint.

2. Materials

2.1. Preparation of Culture Media and Solutions

2.1.1. General Regents for Culture Media and Solutions

The culture media for mouse embryonic fibroblasts (MEFs), hESCs, and neurospheres share some common reagents. The abbreviations and source of these reagents are as follows:

1. Dulbecco's modified Eagle's medium (DMEM; Invitrogen, Carlsbad, CA).
2. Fetal calf serum (FCS; Hyclone Laboratories, Logan, UT) (*see* **Note 1**).
3. Insulin-transferrin-selenium solution (ITS-A; Invitrogen). ITS-A supplement consists of 1 g/l insulin, 0.67 mg/l sodium selenite, 0.55 g/l transferrin, and 11 g/l sodium pyruvate.
4. L-Glutamine (L-Glut; Invitrogen).
5. Phosphate-buffered saline solution without calcium and magnesium (PBS^-; Invitrogen). PBS^- consists of 2.67 mM potassium chloride, 1.47 mM potassium phosphate monobasic, 137.93 mM sodium chloride, and 8.06 sodium phosphate dibasic.
6. Phosphate-buffered saline solution with calcium and magnesium (PBS^+; Invitrogen). PBS^+ consists of 0.901 mM calcium chloride, 0.493 mM magnesium chloride, 2.67 mM potassium chloride, 1.47 mM potassium phosphate monobasic, 137.93 mM sodium chloride, and 8.06 sodium phosphate dibasic.
7. Penicillin-streptomycin solution (Pen/Strep; Invitrogen). Pen/Strep solution consists of 5,000 U/ml penicillin and 5,000 µg/ml streptomycin.

2.1.2. Preparation of Mouse Embryonic Feeder Culture Media (F-DMEM)

1. Supplement 1× DMEM with 10% heat-inactivated FCS, 2 mM L-Glutamine, and 0.5% Pen/Strep.
2. Mix well and sterilize using a 0.22-µm filter.

2.1.3. Preparation of Mitomycin C Solution

1. Dissolve 2 mg of mitomycin C (Sigma-Aldrich, St. Louis, MO) in 4 ml of distilled H_2O and filter sterilize using 0.22-µm filter.
2. Keep solution protected from light and store at 4°C.

3. When ready to use, dilute to 10 μg/ml in F-DMEM. Mitomycin C is cytotoxic, mutagenic, and carcinogenic; appropriate safety measures must be adhered to for use and disposal of this agent.

2.1.4. Preparation of Trypsin-EDTA Solution

1. A stock solution of 0.4% EDTA and 2.5% trypsin (Invitrogen) is made in PBS^{-}, followed by filter sterilization.
2. Aliquots can be stored at –20°C. Once an aliquot is thawed, it can remain active at 4°C for up to a week.
3. Working solutions (0.5% trypsin-EDTA) are diluted from stock aliquot with PBS^{-}.

2.1.5. Preparation of hESC Culture Media

1. Supplement 1× DMEM with 20% heat-inactivated FCS, 0.1 mM nonessential amino acids solution (Invitrogen).
2. Nonessential amino acids solution consists of 750 mg/l glycine, 890 mg/l L-alanine, 1,320 mg/l L-asparagine, 1,330 mg/l L-aspartic acid, 1,470 mg/l L-glutamic acid, 1,150 mg/l L-proline, and 1,050 mg/l L-serine, 2 mM L-Glutamine, 0.5% Pen/Strep, 0.1 mM β-mercaptoethanol (Invitrogen), and 1% ITS-A.
3. Mix well and sterilize using a 0.22-μm filter.

2.1.6. Preparation of Dispase Solution

1. A 10 mg/ml solution of Dispase (Roche Applied Science, Indianapolis, IN) is made in prewarmed hESC culture media.
2. The solution is incubated at room temperature for 5 min, gently inverted, followed by 15-min incubation at room temperature.
3. The solution is then sterilized using a 0.22-μm filter, and it can be used or stored at 4°C for up to 2 days.

2.1.7. Preparation of NBM

1. Supplement 1× Neurobasal A (Invitrogen; *see* **Note 2**) with 2% B-27 supplement (Invitrogen; *see* **Note 2**), 1% ITS-A, 1% N2 supplement (Invitrogen; *see* **Note 3**), 2 mM L-Glutamine, and 0.5% Pen/Strep.
2. Mix well and sterilize using a 0.22-μm filter.

2.2. Preparation of Adhesive Substrates

2.2.1. Gelatinized Plates for Culturing MEFs

1. Stocks of 1% gelatin (type A from porcine skin; Sigma-Aldrich) can be made in distilled H_2O and stored at room temperature.

2. For gelatinizing plates, stock solutions are diluted to 0.1% using distilled H_2O and filter sterilized.
3. Plates are coated with 0.1% gelatin and kept at room temperature for at least 1 h.
4. Gelatin solution is aspirated and plates are ready to use for plating cells.

2.2.2. Laminin-Coated Plates for Neural Differentiation

1. Plates are covered with poly-D-lysine (Sigma-Aldrich) solution (10 μg/ml in PBS^+), and they were kept at room temperature for at least 30 min.
2. This solution is aspirated and the plates are washed three times with PBS^+.
3. Plates are then coated with mouse laminin (Invitrogen) solution (5 μg/ml in PBS^+) and incubated overnight at 4°C.
4. The next day, the laminin solution is removed, followed by three washes with PBS^+.
5. Culture media can be added for plating cells/neurospheres.

2.2.3. Fibronectin-Coated Plates for Glial Differentiation

1. Plates are covered with poly-D-lysine solution (10 μg/ml in PBS^+), and they were kept at room temperature for at least 30 min.
2. This solution is aspirated, and the plates are washed three times with PBS^+.
3. Plates are then coated with human fibronectin (BD Biosciences, Franklin Lakes, NJ) solution (10 μg/ml in PBS^+) and incubated overnight at 4°C.
4. The next day, the fibronectin solution is removed, followed by three washes with PBS^+.
5. Culture media can be added for plating cells/neurospheres.

3. Methods

3.1. Culture of hESCs

HESC cells are cultured as colonies on mitotically inactivated MEF feeder layer, in a gelatin-coated organ tissue culture dish (2.89 cm^2; BD Biosciences, San Jose, CA). The MEF feeder layer density is 6.0×10^4 cells/cm^2 (1.7×10^5 cells/organ culture dish). Isolation of MEFs, their inactivation by mitomycin C treatment, and plating onto gelatinized organ culture dishes is described below (*see* **Subheading 3.1.1.**). hESCs are passaged weekly by mechanical slicing of the colonies by using either glass pulled pipettes or a 27-gauge needle (*see* **Subheading 3.1.2.**). hESCs are maintained at 37°C with 5% CO_2 in a humidified incubator. All tissue culture procedures are performed using aseptic techniques in class II biological safety cabinets.

3.1.1. Preparation MEF Feeder Layer

3.1.1.1. Derivation of MEFs

MEFs are derived from embryonic day 12.5–14 embryos. Usually about three embryos are used to obtain a confluent T75 flask (75-cm^2; Nalge Nunc International, Rochester, NY) of fibroblasts.

1. Embryos are extracted from placental tissue.
2. Head and visceral tissue (which includes fetal liver) are removed.
3. Remaining fetal tissue is transferred to a clean dish and washed three times with PBS^- by transferring tissue from dish to dish.
4. Tissue is minced using either scissors or a scalpel blade.
5. 2 ml of 0.5% trypsin is added and mincing is continued.
6. An additional 5 ml of trypsin is added and incubated for 20 min at 37°C.
7. Embryos are pipetted in the trypsin until few chunks remain and then incubated for a further 10 min at 37°C. The aim is to get a single-cell suspension.
8. 10 ml of F-DMEM is added to neutralize the trypsin, and contents are transferred to a 50-ml tube.
9. Contents are mixed well and transferred all to T75 flask (*see* **Note 4**).
10. The next day, the media are changed to remove cell debris and dead cells.
11. When the flask is 70–80% confluent, the cells are frozen down and labeled as passage 0. At this stage, the cells grow very vigorously, so they can be expanded 1:5 (freeze at 1:5). They cannot be used past passage 4, because their growth rate begins to slow down significantly, and they may develop morphological changes.

To use these cells as feeder layers in hESC culture, it is necessary to passage them a few times before use to minimize contamination with residual nonfibroblast cells. Thus, to use these cells as feeder layers, one needs to do the following:

1. Thaw passage 0.
2. Culture for 2 days until confluent and then trypsinize and expand (becomes p1).
3. Culture p1 cells for 2 days and then trypsinize and expand again (p2).
4. Culture p2 cells for 2 days and then trypsinize and freeze at passage 3 (p3).
5. Cells at p3 are used for hESC feeder layer. The cells are mitotically inactivated either by mitomycin C treatment or irradiation.

3.1.1.2. Mitomycin C Treatment of MEFs

1. Aspirate existing media from flask containing MEF cultures.
2. Add mitomycin C (10 μg/ml) made in warm F-DMEM to each flask.
3. Incubate at 37°C for 2.5 h.
4. Aspirate media containing mitomycin C.
5. Wash flask once with F-DMEM and twice with PBS^-.
6. Add 0.5% Trypsin-EDTA solution (*see* **Note 5**) and incubate at 37°C for 3 minutes.

7. Slightly agitate the flask until cells start to detach.
8. Collect the cells with F-DMEM and transfer into a 10–50-ml tube.
9. Spin the cells for 2 min at 2,000 rpm.
10. Aspirate and resuspend the cells with 10–15 ml of F-DMEM.
11. Obtain a 10-µl sample and perform a cell count.
12. Plate cells as required on gelatinized tissue culture plates.

3.1.2. Passage of hESC Colonies

hESCs are cultured as colonies on a feeder layer of mitotically inactivated MEFs, and they are routinely passaged once a week. The method used for hESC passage is by mechanical dissociation of colonies, combined with dispase treatment, to dissect the colony into "pieces." Dissection of colony is performed using a stereomicroscope (Leica MZ6; Leica Microsystems, Wetzlar, Germany) with a 37°C warm microscope stage (Leica Microsystems). Each colony piece is then transferred onto a fresh feeder layer (*see* **Fig. 2**). A detailed description of this method follows:

1. Prepare 2- × 6-cm petri dishes with PBS^+ for two washes.
2. Make dispase solution in hESC media and filter sterilize (*see* **Subheading 2.1.6.**).
3. Change the media of hESC culture to PBS^+.
4. Working with a stereomicroscope, cut hESC colonies into pieces (*see* **Fig. 2**) by using fine glass pulled pipettes or 27-gauge needles. Avoid cutting differentiated portions of partially differentiated colonies (*see* **Fig. 2F**) (*see* **Note 6**).
5. Aspirate the PBS^+.
6. Add dispase solution and leave for 3 min at 37°C on heating stage, or until edges of the cut colony pieces start to detach.
7. Using a p20 pipette, nudge the corner of the colony piece until it complete detaches, collect, and transfer to the PBS^+ wash dish.
8. Once all the colony pieces have been collected and transferred to the wash dish, transfer them to the second PBS^+ wash dish.
9. Finally, transfer the pieces to an organ culture dish containing a fresh feeder layer of mitotically inactivated MEFs and hESC media. Usually, about eight pieces are placed per organ culture dish, evenly spaced and neatly arranged in a circle (*see* **Fig. 2D**).

3.2. Neural Induction of hESCs by Noggin

For neural induction, 500 ng/ml recombinant noggin (R&D Systems, Minneapolis, MN) (*see* **Note 7**) is added to hESC media at the time of hESC colony transfer onto a fresh feeder layer of MEFs (day 0). Cells are cultured for 14 days without passage, and noggin-media are replaced every other day (*see* **Note 8**).

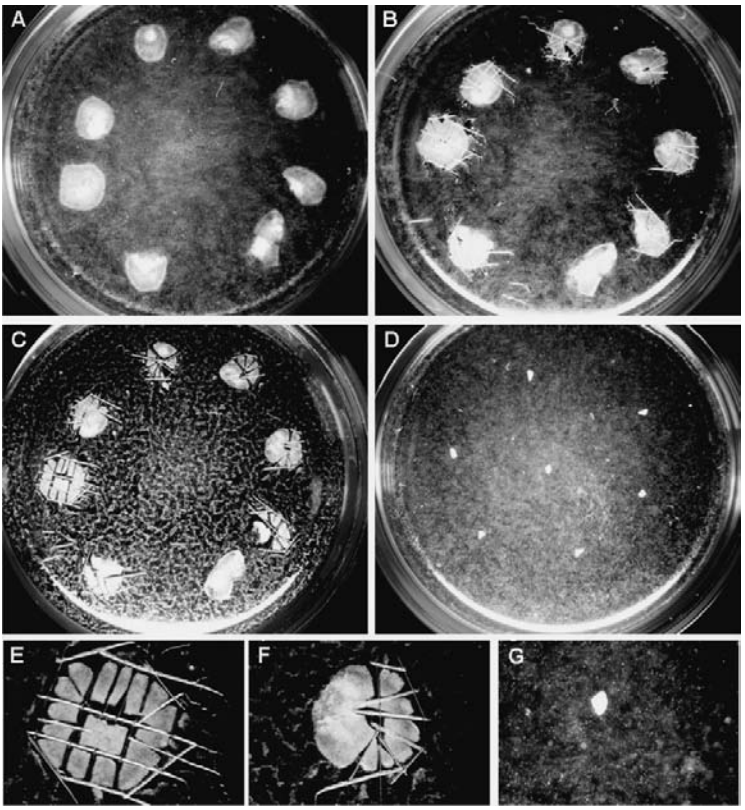

Fig. 2. Propagation of hESC colonies by using mechanical dissection and dispase. After day 7 **(A)**, undifferentiated regions of colonies are cut into small pieces **(B,E,F)**, avoiding areas showing differentiation **(F)**. Dispase is incubated on the colonies until the edges of the pieces begin to lift **(C,E,F)**. Colony pieces are then transferred to an organ culture dish with freshly plated MEFs **(D,G)**. Images taken by Anna Michalska.

3.3. Maintenance of NSCs as Neurosphere Cultures

After 14 days of noggin treatment, colonies are dissected into pieces (as performed for normal hESC passage; *see* **Subheading 3.1.2.**), and then they are transferred to individual wells in a nonadherent 96-well plate to allow neurosphere formation. Neurospheres are cultured in suspension in NBM supplemented with 20ng/ml human recombinant EGF (R&D Systems) and 20 ng/ml human recombinant basic fibroblast growth factor (bFGF; R&D Systems). noggin-treated colonies do still contain some undifferentiated hESCs even after 14 days of treatment (*see* **Note 8**). Contaminating hESC that may be transferred to the neurosphere culture conditions either do not survive or form cystic-like

structures. The latter are easily identified and removed from culture dish. A more detailed protocol of transfer to neurosphere cultures follows:

1. Rehydrate the surface of the 96-well plate with NBM without any growth factors for at least 15 min in the incubator.
2. Aspirate and add NBM with 10 ng/ml bFGF and EGF (100–200 µl/well) and leave in the incubator until ready to plate the pieces.
3. Prepare a petri dish with PBS^+.
4. Wash the day14 noggin-treated hESC cultures with PBS^+.
5. Aspirate and add fresh PBS^+.
6. Using either a pulled glass pipette or 27-gauge needle, cut the center region of the colony (*see* **Notes 8 and 9**).
7. Using a p20 pipette, pick up pieces and transfer them to the PBS dish.
8. Transfer one piece per well.
9. By 3–5 days in culture, the pieces will form a smooth sphere, indicating neurosphere formation. Those that do not form neurospheres will either disintegrate or form cysts.
10. Maintain neurosphere cultures at 37°C with 5% CO_2 in a humidified incubator. Change media every 2–3 days.
11. Neurospheres may be subcultured by dissection by using glass needles in NBM. Dissected neurosphere fragments are returned to 96-well plates and grown as described above.

3.4. Differentiation of Neurospheres to Neurons and Glia

For neuronal differentiation, whole neurospheres are plated onto laminin substrates (*see* **Subheading 2.3.2.**) in NBM without bFGF and EGF supplements. For differentiation biased toward glial lineages, whole neurospheres are plated onto fibronectin substrates (*see* **Subheading 2.3.3.**) in NBM with 20 ng/ml bFGF, 20 ng/ml EGF, and 20 ng/ml human recombinant platelet-derived growth factor (PDGF)-AA; Chemicon International, Temecula, CA) supplements for 1 week, followed by NBM without supplements. Cells are maintained at 37°C with 5% CO_2 in a humidified incubator. Media are changed every other day (*see* **Note 10**).

3.5. Discussion

This simple protocol provides for efficient induction of neural progenitors directly from stock cultures of hESCs. The noggin treatment seems to prime hESCs for neural differentiation; further study is required to define the earliest steps of the induction process. Others have confirmed neural induction of hESCs by noggin ***(11,12)***. Yao and colleagues ***(13)*** demonstrated that noggin could induce neural differentiation of hESCs grown under defined conditions, supporting our previous model of endogenous paracrine BMP signaling in

hESC cultures ***(5)***. Itsykson and colleagues ***(14)*** modified the protocol described herein slightly, and they showed that noggin treatment suppressed expression of markers of endoderm and mesoderm germ layers in a suspension culture differentiation system. When transferred to neurosphere culture, neural progenitors induced by noggin express transcription factors characteristic of anterior central nervous system fates, but they are nonetheless capable of differentiation into a variety of neuronal subtypes that demonstrate appropriate electrophysiological activity ***(14)***. Further study has shown these noggin-induced neural progenitors can also give rise to retinal precursors ***(15)***. Sonntag et al. ***(16)*** combined stromal cell-derived inducing activity with noggin treatment to obtain high yields of midbrain dopaminergic neurons from hESCs.

Recently, several groups have described long-term maintenance of neural stem cells derived from mouse ***(17)*** or human ***(18)*** ESCs in adherent monolayer culture. Such cultures are easier to maintain, manipulate, and study compared with neurospheres, which require manual dissection for subcultivation, cannot be clonally derived in human, and are more difficult to examine microscopically than cells grown in monolayer. It is likely that the combination of noggin treatment with subsequent adherent culture of neural progenitors will greatly facilitate further study of the development and differentiation of the human central nervous system and enhance our ability to produce large numbers of neural progenitors for use in therapy.

4. Notes

1. Fetal calf serum for hESC and MEF cultures need to be batch tested for support of hESC maintenance over at least four passages.
2. The components of Neurobasal A and B-27 supplements are proprietary.
3. N2 Supplement consists of 0.5 mg/ml recombinant insulin, 1 mM human transferrin, 10 mM putrescine, 3 nM selenite, and 2 nM progesterone.
4. In primary cultures of MEF preparations, blood cells, as well as large intact fragments of tissue, may be present along with fibroblasts. Generally, these contaminating cell types disappear after subculture as the fibroblasts overgrow them.
5. The volume of trypsin-EDTA solution used for a T75 (75 cm^2)–T175 (175-cm^2) flask is 2 to 5 ml, respectively.
6. By 7 days after passage, hESC colonies show some regions of cell differentiation, particularly within the center of the colony. These regions are morphologically distinguished by cystic-like structures extending up from the colony.
7. Growth factors (bFGF, EGF, and noggin) are reconstituted according to manufacturer's recommendations to stock concentrations of 50-100 µg/ml. Long-term storage of factors is at –80°C. Stocks can be kept short term (up to 1–3 months) at 4°C.

8. After 14 days of noggin treatment, immunostaining and polymerase chain reaction expression analyses show that hESC colonies consist of a mixed population of hESCs and NSCs, the latter usually found more within the central regions of the colony ***(5)***.
9. After 14 days of culture, the colonies are easily detached by mechanical dissociation from the feeder layer; thus, additional enzymatic treatment is not required.
10. On laminin substrates, neuronal differentiation with axonal outgrowths can be observed usually within 5 days of culturing. Expression of early neuronal markers, such as β-tubulin III, can be observed at this time. On fibronectin substrates, migrating glia expressing glial fibrillary acidic protein can be observed after 2 weeks of culture.

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4

Generating Neurons from Stem Cells

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Summary

Recent work shows that major developmental and clinical processes such as central nervous system regeneration and carcinogenesis involve stem cells (SCs) in the brain. In spite of this importance, the requirements of these SCs and their differentiated offspring (neurons, astrocytes, and oligodendrocytes) for survival and proper function are little understood. In vivo, the SCs themselves interact with their environment. This "SC niche" may be complex because it likely includes cells of the vascular and immune systems. The ability to maintain ***(1)*** and differentiate ***(1–4)*** central nervous system (CNS) SCs in tissue culture where they can be pharmacologically or genetically ***(5)*** manipulated provides a powerful starting point for understanding their behavior. We present detailed information on the methods that permit CNS SCs to differentiate into functional neurons in tissue culture. Important aspects of the culture systems include (1) homogeneity, so that the input and output of a manipulation is known to involve the SC itself; (2) growth in monolayer to visualize and study individual SCs and their offspring; and (3) the use of fully defined culture components to exclude unknown factors from the culture. These conditions support the differentiation of functional, electrically active neurons. These methods allow cell growth and differentiation from normal adult and diseased tissue derived from both animal models and clinical samples. Ultimate validation of such a system comes from accurate prediction of in vivo effects, and the methods we present for CNS SC culture have also successfully predicted regenerative responses in the injured adult nervous system.

Key Words: Stem cells; neurons; proliferation; differentiation.

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1. Introduction

The isolation and the expansion of stem cells (SCs) provide tools to explore critical features of central nervous system (CNS) development and function. To take advantage of these cells, it is necessary to develop precise protocols that allow reproducible growth and standard assays. We present detailed experimental information on the dissection, growth, and differentiation of CNS SCs. The growth conditions do not use serum or other uncharacterized additives, and the cells are always expanded for a limited period. Because the generation of functional neurons requires interactions with astrocytes, we present protocols for growing astrocytes in vitro. These conditions generate functional neurons on a precise schedule in predictable numbers.

2. Materials

2.1. Plate Preparation

1. 10-cm tissue culture dishes (BD Biosciences, San Jose, CA, cat. no. 350003).
2. Poly-L-ornithine (Sigma-Aldrich, St. MO, cat. no. P-3655).
    a. Prepare 5 mg/ml solution in water, make 25-ml aliquots, and store at –20°C for up to several months.
    b. The water used is double distilled and is referred to as "water."
    c. For a working stock, dilute one 25-ml aliquot in 225 ml of water and filter sterilize (*see* **Note 2**) to make a 0.5 mg/ml solution.
    d. Store the working solution at 4°C for up to 1 month.
3. Fibronectin (R&D Systems, Minneapolis, MN, cat. no. 1030-Fn).
4. Filtration apparatus (Corning Life Sciences, Acton, MA, cat. no. 430769).

2.2. Dissection/Plating

1. General dissection instruments, including blade and forceps.
2. Dissection microscope.
3. Pregnant mouse (e.g., CD1 strain) or rat (*see* **Notes 2–5**).
4. CO_2 intoxication chamber.
5. N2 medium for CNS SC culture.
    a. Components: DMEM/F-12 (Mediatech, Herndon, VA, cat. no. 10-090-CV), apo-transferrin (Sigma-Aldrich, cat. no. T-2036), insulin (Sigma-Aldrich, cat. no. I-0516), putrescine (Sigma-Aldrich, cat. no. P5780)—prepare 1 M aliquots in water, freeze at –20°C up to 6 months; sodium selenite (Sigma-Aldrich, cat. no. S5261)—prepare 500 M aliquots in water, freeze at –20°C up to 6 months; progesterone (Sigma-Aldrich, cat. no. P8783)—prepare 100 µM aliquots in 100% ethanol, store at –20°C up to 6 months, penicillin/streptomycin (Invitrogen, Carlsbad, CA, cat. no. 15140-122).

b. Preparation: To one bottle of DMEM/F-12 (500 ml) add 0.05 g of apotransferin, 0.0125 g of insulin (freshly predissolved in 1 ml of 10 mM NaOH), 50 µl of putrescine, 30 µl of sodium selenite, 100 µl of progesterone stocks, and 5 ml of antibiotic solution.
c. Adjust pH to 7.2, if needed.
d. Filter-sterilize and store at 4°C for up to 3 weeks and protect from light.

6. Phosphate-buffered saline (PBS) without calcium, without magnesium (Mediatech, cat. no. 21-040-CV).
7. Basic fibroblast growth factor (bFGF) (R&D Systems, cat. no. 233-FB). Use at a concentration of 30 ng/ml in the N2 medium for mouse cultures (*see* **Note 6**).
8. Bovine serum albumin (BSA) (Sigma-Aldrich, cat. no. A-2058).
9. 0.2% trypan blue (Cambrex Bio Science Walkersville, Inc., Walkersville, MD, cat. no. 17-942E).
10. Laminar flow hood for cell culture.
11. Incubator (humidified, 37°C, 5% CO_2, 5% O_2).
12. 15- and 50-ml conical tubes (Corning Life Sciences, cat. nos. 430053 and 430829).
13. Hemacytometer.

2.3. Passaging

1. Hanks' balanced salt solution (HBSS)/HEPES buffer.

   a. Components: 10× Ca^{2+}- and Mg^{2+}-free HBSS (Mediatech, cat. no. 20-021-CV), $NaHCO_3$, HEPES, and HCl for pH adjustment to pH 7.2.
   b. Preparation: To a sterilized bottle add 800 ml of water, 100 ml of the 10× HBSS, 3.7 g of $NaHCO_3$, 3.9 g of HEPES, allow to dissolve and top up to 1 l with water. Adjust pH to 7.2. Filter, sterilize, and store at 4°C for up to 3 months.

2. Dimethyl sulfoxide (DMSO) (Sigma-Aldrich, cat. no. D-2650).
3. Tabletop centrifuge for 15-ml conical tubes (5 min, 1,000 × *g*, 4°C).
4. Cryo-freezing vials (Corning Life Sciences, cat. no. 430487).
5. Cryogenic liquid nitrogen tank for cell storage.

2.4. Induction of Differentiation

1. Ciliary neurotrophic factor (CNTF) (R&D Systems, cat. no. 557-NT/CF).
2. Neurobasal medium for neuronal survival/maturation.

   a. Components: Neurobasal medium (Invitrogen, cat. no. 12348-017), 200 mM GlutaMAX (Invitrogen, 320-5050AG), sodium selenite (Sigma-Aldrich, cat no. S5261)—prepare 500 µM aliquots in water, freeze at –20°C up to 6 months, insulin (Sigma-Aldrich, cat. no. S5261), penicillin/streptomycin (Invitrogen, cat. no. 15140-122).

b. Preparation: To one bottle Neurobasal (500 ml) add the following: 5 ml of GlutaMAX, 30 µl of sodium selenite, 0.0125 g of insulin (predissolved in 1 ml of 10 mM NaOH), 5 ml of penicillin/streptomycin. Adjust pH to 7.2 if needed and filter-sterilize. Store solution at 4°C for up to 3 weeks. Protect from light.

3. Supplements for Neurobasal medium (to be added immediately before use): B-27 (Invitrogen, cat. no. 17504-044), dilute 50× in 10 ng/µl Neurobasal medium; NT3 (R&D Systems, cat. no. 267-N3), dilute 1,000×; 10 ng/µl brain-derived neurotrophic factor (BDNF) (R&D Systems, cat. no. 248-BD), dilute 1,000×.

2.5. Immunofluorescence Analysis

1. Paraformaldehyde ampules (Electron Microscopy Sciences, Hatfield, PA, cat. no. 15719).
2. Normal goat serum (NGS) (Invitrogen, cat. no. 16210-064).
3. Triton X-100 (Sigma-Aldrich, cat. no. T-8787).
4. Primary antibodies (Abs): anti-Nestin, dilution 1:400 (Chemicon International, Temecula, CA, cat. no. MAB353), mouse monoclonal IgG1 anti-CNPase, dilution 1:200 (Chemicon International, MAB326), mouse monoclonal IgG2a anti-βIII tubulin ("TUJ1"), dilution 1:500 (R&D Systems, MAB1195), rabbit polyclonal anti-glial fibrillary acidic protein (GFAP), dilution 1:500 (Dako North America, Inc., Carpeinteria, CA, cat. no. Z0334).
5. Secondary Abs: Alexa 568 goat anti-mouse IgG1, dilution 1:200 (Invitrogen, cat. no. A-21124), Alexa 488 goat anti-mouse IgG2a, dilution 1:200 (Invitrogen, cat. no. A-21131), Cy5 goat anti-rabbit, dilution 1:200 (Jackson ImmunoResearch Laboratories, West Grove, PA, cat. no. 59883).
6. 4,6-Diamidino-2-phenylindole (DAPI) (Sigma-Aldrich, cat. no. D-8417).

3. Methods

3.1. Culture Dish Preparation

1. Two days before dissection, coat dishes with poly-L-ornithine stock (6 ml/10-cm dish) overnight in the cell culture incubator to reduce contamination risk.
2. The next day, aspirate the poly-L-ornithine and wash the dishes twice for 5 min with 10 ml of PBS. Add fibronectin (1:250, in PBS) to the plate and place the dishes in the incubator overnight (or at least 2 h if urgent).
3. On the day of dissection, aspirate the fibronectin and wash the dishes with PBS twice. Return the plates, containing the final PBS wash, to the incubator until the dissociated tissue is ready for plating. At that time, aspirate off the PBS and add the tissue.
4. Dishes coated with poly-L-ornithine can be stored in the incubator for up to 3 weeks. Dishes coated with fibronectin can be stored for up to 1 week. Fibronectin is susceptible to denaturation; do not let dishes dry.

3.2. Dissection/Plating

1. This protocol applies to a single pregnant CD1 mouse, assuming tissue from 10 fetuses is recovered (*see* **Notes 3–5**).
2. Place a 15-ml Falcon tube that contains 5 ml of N2 medium with added bFGF on ice. Sacrifice the animal by CO_2 intoxication, harvest the embryos, and place them in a 10-cm dish with PBS. The dissection of the fetal brains can be performed with two forceps and a razor blade under a dissection microscope (*see* **Note 7**).
3. Remove the brains from the fetuses and place them in a 10-cm culture dish on ice, with 5 ml of N2 plus bFGF to keep the tissue from drying and to maximize fibroblast growth factor receptor activation. Separate the two hemispheres by using the blade. Using the forceps, remove the cerebral cortices and place them in the Falcon tube containing N2 and bFGF.
4. When all tissue is collected, transport the Falcon tube containing the tissue in a sterile cell culture incubator. Allow the tissue to settle to the bottom of the tube by gravity. Using a hand-held pipette with a 1-ml tip, carefully remove the N2 medium until 1 ml is left. Place the tip of the pipette close to the bottom of the tube and triturate the tissue approximately 12 times, until a homogenous suspension is formed. Allow remaining fragments to sink by gravity for 1 min, and collect the medium leaving the bottom 100 µl.
5. Transfer the mixture to a 50-ml Falcon tube containing 50 ml of cold N2 medium plus bFGF. Mix gently and add 6 ml to each of eight 10-cm dishes that have been coated as described in **step 3.1**, and place in the low oxygen cell culture incubator overnight (*see* **Note 8**).
6. This method should result in approximately one million cells per dish; this can be confirmed by 0.2% trypan blue cytometry. (Expect 70–80% of the cells to exclude trypan blue, indicating that they are alive. Cells should be seen attaching onto the dish within 15 min).

3.3. Daily Care

Twenty-four hours after plating, prewarm 50 ml of N2 with bFGF added and replace the medium in the culture dishes. The next day, add a bolus of bFGF to the medium within the culture dishes. Continue alternating medium/bFGF changes or bFGF additions for a total of 5 days.

3.4. Passaging (see Note 9)

1. After 5 days in culture, the cells should have formed colonies containing a few hundred cells, with cell-free space between colonies. At this stage, the cultures should be approximately 80% confluent, and they are ready for passaging.
2. To passage, place culture dishes in a cell culture hood and wash twice with 5 ml of prewarmed HBSS (do this one dish at a time to avoid cell loss). Aspirate the HBSS and add another 5 ml of pre warmed HBSS containing bFGF. Place the dishes in the cell culture incubator for 5 min.

3. Return the dishes to the cell culture hood to harvest cells. Using a 5-ml pipette, spray the HBSS several times over the entire surface of the dish to lift the cells off. Collect the HBSS containing bFGF plus cells in a 15-ml Falcon tube and pellet by centrifugation (1,000 × *g*, 5 min, 4°C).
4. Resuspend the pellet in the desired volume of N2 containing bFGF and seed new coated plates at the desired cell density. At this point, due to selective expansion of the SC population, the culture contains almost exclusively nestin-positive multipotent precursors.

3.5. Freeze-Storing Cell

1. Alternative to passaging, cells can be frozen in liquid nitrogen for long-term storage. For this, the pellet is reconstituted in 3 ml of cold N2 plus bFGF, and the cells are counted by hemacytometry. The volume of N2 is adjusted to achieve a cell density of 1.1 million cells per 100 µl. Typically, each 10-cm dish may yield 5 million cells before passaging.
2. DMSO is subsequently added to the mixture to a final concentration of 10%. Aliquots (100 µl; 1 million cells) are prepared in cell freezing vials.
3. The vials are immediately placed in a freezing container, precooled to 4°C, and the container is placed in a –80°C freezer overnight. The next day, the cell cryo-freezing vials are placed in a liquid nitrogen tank, where they can be stored indefinitely.

3.6. Thawing Frozen Cells

1. Remove desired number of frozen vials from the liquid nitrogen tank. Place each vial on dry ice, and take the vials to a cell culture hood where they are removed from dry ice and allowed to stand for 30 s at room temperature.
2. Add 1 ml of prechilled (4°C) N2 plus bFGF and let stand for 30 s. Using a 1-ml pipette, gently resuspend and transfer in a Falcon tube with the appropriate volume of prechilled N2 plus bFGF for further plating.
3. Plate the cells in appropriate precoated culture dishes or plates and place in an incubator. The cells will attach within 20 min, at which point, replace the medium with fresh prewarmed (37°C) N2 plus bFGF to wash off the DMSO. Take care when handling cryo-vials because the expanding gas during thawing may cause an explosion.

3.7. Induction of Differentiation

The simplest differentiation paradigm is bFGF withdrawal. When the cells have formed colonies containing several to a few hundred cells each, change the N2 medium but do not add bFGF. Keep changing the medium every second day without adding bFGF. The cells can be fixed at 5 days after withdrawal at which time the differentiation markers are expressed strongly. Alternatively,

differentiation can be induced by the addition of cytokines such as CNTF for 2–5 days, which promotes the glial fate.

3.8. Promotion of Neuronal Survival and Maturation

Differentiation of CNS SCs can also be induced by replacing N2 medium containing bFGF with Neurobasal medium, supplemented with B-27, NT3, and BDNF, which promote the survival and maturation of neurons. Like N2, this medium is also replaced every other day during the differentiation process, and it will generate a greater number of neurons in the culture, which will also be more mature and electrophysiologically active.

3.9. Immunocytochemical Detection

1. Detection of SC and differentiated cell markers can be achieved immunocytochemically, at room temperature. The following protocol is for cells cultured in 24-well plates, and volumes are given per well.
2. Aspirate the medium and fix cell culture plates (self-renewing or differentiated) with 500 µl of 4% paraformaldehyde for 20 min. Wash twice with 1 ml of PBS, 5 min each time. Aspirate PBS and add 0.5 ml of PBS containing 5% normal goat serum and 0.1% Triton X-100, for 20 min to permeabilize and block the cells.
3. Prepare the primary antibody mix in PBS containing 5% NGS, but no Triton X-100. The anti-nestin antibody can be used as a SC marker; the anti-β-tubulin, anti-GFAP, and anti-CNPase antibodies can be combined for a triple staining of differentiation markers.
4. Aspirate the PBS/NGS/Triton X-100 mixture and add the primary antibody mixtures (200 µl). Leave at room temperature for 90 min.
5. Aspirate the primary antibodies and wash with 1 ml of PBS twice, and 1 ml of PBS containing 5% NGS once, each 5 min.
6. Prepare the secondary antibodies in PBS containing 5% NGS, aspirate the last wash and add them to the wells. Incubate in the dark for 40 min.
7. Prepare a fresh solution of DAPI (1:2,000) in PBS, aspirate the secondary antibody, and add the DAPI solution for 3 min. Wash three times with 1 ml of PBS, 5 min each time. The cells are ready for detection with a fluorescence microscope.

4. Notes

1. All solutions and media are filter-sterilized through a low protein binding 0.2-µm filtration apparatus. This process is referred to in this text as "filter-sterilization."
2. This protocol is for mouse CNS SC cultures. Rat cultures can also be established. The equivalent developmental age to mouse embryonic day (E)13.5 is rat E14.5. Rat CNS SCs in culture tend to cluster less than mouse cultures, and they do not always form distinct colonies. However, the times of expansion and differentiation are very similar between the two sources. For the rat cultures, 10 ng/ml bFGF is sufficient.

3. At high cell densities, spontaneous differentiation of the SCs occurs. Therefore, the cultures should not be allowed to expand more than what is stated in this protocol.
4. CNS SC cultures from the adult brain can also be prepared. An established source of adult SCs is the subventricular zone of the lateral ventricles, and these can be dissected from adult (~6-week-old) mouse and rat.
5. This protocol has been adapted to establish cultures from human adult and pediatric brain (tumors and non-neoplastic tissue). Before trituration, we recommend cutting the tissue into ~3-mm cubes; depending on the tissue sample consistency, more trituration may be needed.
6. bFGF and CNTF are purchased "carrier-free," and they are reconstituted in a filter-sterilized solution of PBS containing 0.1% BSA.
7. An advantage of this protocol is the avoidance of enzymatic digestion such as trypsin and collagenase. Enzymatic digestion will cleave critical surface receptors, making the isolated SCs less responsive to essential growth factors and signals such as added bFGF and endogenous or added Notch ligands. Additionally, at the time of passaging, more adherent differentiated cells tend to resist HBSS-induced passaging and they are left behind.
8. CNS SC cultures can also be established on glass coverslips. The protocol remains the same, but due to a lower attachment affinity to glass, some cell loss may be observed.
9. CNS SC cultures retain multipotency for at least two to three passages, allowing for large expansion of the cell population. However, we typically use cells passaged once for consistency in generated data.

Acknowledgments

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5

Isolation of Neural Stem and Precursor Cells from Rodent Tissue

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Summary

Isolation and characterization of neural stem cells and lineage-specific progenitors provide important information for central nervous system development study and regenerative medicine. We describe methods for dissection of rodent embryonic spinal cords by enzymatic separation, and isolation and enrichment (or purification) of neuronal and glial precursors at different developing stages by fluorescence-activated cell sorting.

Key Words: A2B5; E-NCAM; Sox2; neuroepithelial cell (NEP); neuronal restricted precursor (NRP); glial restricted precursor (GRP); astrocyte-restricted precursor (ARP); markers; multipotential stem cells; oligodendrocyte precursor.

1. Introduction

Generation of neurons, astrocytes, and oligodendrocytes in the nervous system involves a sequential process of differentiation. Initially, multipotent stem cells generate more restricted precursor cells, which go through additional stages of differentiation to generate fully differentiated progeny ***(1)***. Precursor cells at each stage of differentiation can be distinguished from each other on the basis of cytokine dependence, functional properties, and antigen expression. Using markers to antigens expressed on the cell surface, live multipotent stem cells, intermediate precursor cells, and differentiated cells can be isolated at various stages of development.

Neural stem cells (NSCs) are most abundant at early developmental stages, with the maximal number being present just after neural tube closure before

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the onset of neurogenesis, and their number declines over subsequent stages of development. However, stem cells persist throughout development, and significant numbers can be isolated even from the adult cortex. Rat embryonic day (E)10.5 (mouse E8.75–9.0) caudal neuroepithelial (NEP) cells represent the earliest multipotent NSCs. At this stage, the majority of cells express nestin but not any markers characteristic of differentiated cells. In addition, fibroblast growth factor receptor 4, Frizzled 9, and SRY box-containing gene 2 (Sox2) also are specifically expressed in NEP cells ***(2)***. In the presence of fibroblast growth factor (FGF) and chick embryo extract (CEE), NEP cells can be maintained in an undifferentiated and homogenous state in culture for >3 months. Cultured NEP cells can readily differentiate into neurons, astrocytes, and oligodendrocytes upon withdrawal of CEE and reduction of FGF concentration ***(3)***.

Neuronal restricted precursor (NRP) cells can be isolated from embryonic spinal cord ***(4)*** and selected regions of the adult brain ***(5)***. In tissue culture or by transplantation, these cells can differentiate into various types of neurons but not oligodendrocytes or astrocytes ***(4,6)***. NRP cells are most abundant at E13.5 in the rat or E11.5 in the mouse, around the onset of neurogenesis. NRP cells can be maintained over multiple passages in culture in the presence of FGF and neurotrophin-3 (NT-3). NRP cells can be identified and isolated by their expression of polysialic acid-neural cell adhesion molecule (E-NCAM), a cell surface antigen ***(4)***.

The tripotential glial restricted precursor (GRP) cells can be identified as early as E12.0 (but they are more abundant at E13.5 in rats and at E11.5 in mice) in the developing rat spinal cord ***(7,8)***. These cells can be identified and isolated by their expression of cell surface antigen A2B5 before the expression of any oligodendrocyte or astrocyte differentiation markers. GRP cells can be maintained in culture for prolonged periods in the presence of FGF, and they can differentiate into oligodendrocytes and two distinct astrocyte populations under specific culture conditions ***(7,8)***. Corresponding to in vivo development, upon a second wave of gene expression of Nkx2.2 ***(9)*** and Olig2 ***(10,11)***, GRP cells differentiate into oligodendrocytes. When Olig2 is downregulated, GRP cells give rise to astrocytes ***(12,13)***. More recently, astrocyte-restricted precursor (ARP) have been identified to serve as an intermediate type of precursors during the development from GRP toward mature astrocytes ***(14)***. ARPs are characterized by transmembrane glycoprotein CD44 expression, they can be generated from GRPs, and they give rise solely to astrocytes in vitro and in vivo after transplantation ***(14)***.

NEP, NRP, GRP, oligodendrocyte precursors, ARP cells, and other identified neural stem and progenitor cells have been studied by transplantation into rodent spinal cords or central nervous system (CNS) trauma models, to provide clues for cell-based therapy for CNS injuries and neural degenerative diseases

(reviewed in **ref. *15***; ***(16–23)***). Results indicate that appropriate cell sources such as immediate precursors of oligodendrocytes and astrocytes might serve as better candidates for therapy in terms of their survival, migration, and possible functional utility of the transplanted cells compared with less mature precursor or stem cells, such as NEP cells.

Human fetal tissue- or human embryonic stem cell-derived NSCs and their derivatives, also have been obtained, characterized, and used in transplantation studies ***(24–29)***. These stem and progenitor cells share many, although not all, characteristics with their rodent counterparts and extensive characterization studies are needed before they can be used for clinical trials.

We describe the isolation of NEP, NRP and GRP cells by dissecting out embryonic spinal cords at specific stages of development and by using cell surface markers to select defined populations of cells. The first strategy, enzymatic separation, is used to isolate rat E10.5 (mouse E9.0) spinal cords. The second strategy, mechanical dissection, is used for older stage embryos (rat E13.5–E14.0, mouse E11.5–E12.0) to obtain spinal cords for NRP and GRP mixed cultures. Finally, fluorescence-activated cell sorting (FACS) is used to enrich NRP and GRP cells with cell surface antibodies such as anti-E-NCAM and anti-A2B5. Rat embryos are used to describe these procedures; however, the isolation of the corresponding cells in mouse and from human tissue is essentially the same ***(30)***.

2. Materials

2.1. Animals

E10.5, E13.5–E14.0 timed pregnant Sprague-Dawley rats (Simonsen Laboratories, Gilroy, CA) (*see* **Note 1**).

2.2. Culture Media and Solutions

1. NEP basal medium: The following are added to Dulbecco's modified Eagle's medium (DMEM)/F-12 medium (Invitrogen, Carlsbad, CA, cat. no. 11320-033) to give the final indicated concentrations:
    a. 1× N2 supplement (100× stock) (Invitrogen, cat. no. 17502-014).
    b. 1× B27 supplement (50× stock, without vitamin A) (Invitrogen, cat. no. 12587-010).
    c. 1 mg/ml bovine serum albumin (BSA) (Sigma-Aldrich, St. Louis, MO, cat. no. A-2153).
    d. 20ng/ml recombinant human FGF-basic (PeproTech, Rocky Hill, NJ, cat. no. 100-18B) (*see* **Note 2**).

e. 1× penicillin-streptomycin (100× stock) (Invitrogen, cat. no. 15070-063).
f. Sterilize the medium by 0.22-μm filter (Corning Life Sciences, Acton, MA, cat. no. 28199-788).

2. Fibronectin-Coated Tissue Culture Dishes or Cover Glasses:

a. 35× 10-mm tissue culture dishes (Corning Life Sciences, cat. no. 25382-348).
b. Cover glasses (Fisher Scientific, Pittsburgh, PA, cat. no. 12-545-82 or 12-545-84).
c. Fibronectin (Sigma-Aldrich, cat. no. F1141) is diluted in distilled, deionized water to a final concentration of 20 μg/ml. Fibronectin solution can be used repeatedly for up to 2 months if stored at 4°C.
d. Coat the dish or cover glass with 1 ml of fibronectin solution at 4°C overnight. Rinse the dish with culture medium once before use (*see* **Note 3**).

3. Poly-L-Lysine/Laminin-Coated Tissue Culture Dishes or Cover Glasses:

a. Poly -L-lysine (Sigma-Aldrich, cat. no. P-1274) is diluted in distilled, deionized water to a final concentration of 15 μg/ml. The solution can be used repeatedly for up to 3 months if stored at 4°C.
b. Laminin (Invitrogen, cat. no. 23017-015) is diluted in distilled, deionized water to final concentration of 15 μg/ml. The solution can be used repeatedly for up to 2 months if stored at 4°C.
c. Coat the dish or cover glass with 1 ml of poly-L-lysine solution at room temperature for at least 30 min. Remove the poly-L-lysine. Rinse the dish with distilled water and incubate the dish with l ml of laminin solution at room temperature for 4 h or at 4°C overnight. Rinse the dish with culture medium once before use.

4. Enzymatic Solution for Dissection: The following are added to 10 ml of 1× Hanks' balanced salt solution, without calcium and magnesium, (Invitrogen, cat. no. 24020-117) to give the following final indicated concentrations:

a. 2 mg/ml dispase II (Roche Diagnostics, Indianapolis, IN, cat. no. 165859).
b. 1 mg/ml collagenase type 1 (Worthington Biochemicals, Freehold, NJ, cat. no. 4196) (*see* **Note 4**).
c. Sterilize the solution using 0.22-μm syringe filter (Millipore Corporation, Billerica, MA, cat. no. SLGS 025 0S).

5. Growth Factor Stock Solutions for Maintaining and Differentiating Cells:

a. FGF-basic (PeproTech, cat. no. 100-18B): make 1 μg/ml stock solution in Leibovitz's L-15 medium (Invitrogen, cat. no. 11415-064) with 1 mg/ml bovine albumin, Path-O-Cyte 4 (BSA) (MP Biomedicals, Irvine, CA, cat. no. 81010).
b. 1 μg/ml recombinant human NT-3 (PeproTech, cat. no. 450-03): make 10 μg/ml stock solution in distilled water.

c. Platelet-derived growth factor-BB, human (Upstate Biotechnology, Lake Placid, NY, cat. no. 01-309): make 5 μg/ml stock solution in 10 mM acetic acid with 1 mg/ml BSA.
d. Store the growth factors at –20°C as working aliquots. The shelf life of the above stock solutions is 1 year at –20°C.

6. Phosphate-Buffered Saline (PBS), pH 7.4, without calcium and magnesium (Invitrogen, cat. no. 10010-023).
7. Trypsin-EDTA, 0.05% (Invitrogen, cat. no. 25300-054)

a. Tissue culture H_2O (Sigma-Aldrich, cat. no. W-3500).
b. Fetal bovine serum (FBS), heat-inactivated at 56°C for 30 min (Invitrogen, cat. no. 26140-079).
c. Dimethyl sulfoxide (DMSO) (Sigma-Aldrich, cat. no. D-2650).

2.3. Antibodies

1. Anti-E-NCAM antibody, obtained from 5A5 hybridoma (Developmental Studies Hybridoma Bank, Iowa City, IA) supernatant, 1:5 in NEP basal medium.
2. Anti-A2B5 antibody, obtained from A2B5 hybridoma (American Type Culture Collection, Manassas, VA, cat. no. CRL-1520) supernatant, 1:20 in NEP basal medium.
3. Anti-nestin antibody, obtained from Rat-401 hybridoma (Developmental Studies Hybridoma Bank) supernatant, 1:1 in NEP basal medium.
4. Anti-Sox2, 1:200 (R&D Systems, Minneapolis, MN, cat. no. MAB2018).
5. Anti-β-III-tubulin, 1:500 (Sigma-Aldrich, cat. no. T8660).
6. Alexa 488 goat anti-mouse IgM, 1:200 in NEP basal medium (Invitrogen, cat. no. A21042).
7. Alexa 568 goat anti-mouse IgG, 1:1000 in NEP basal medium (Invitrogen, cat. no. A11031).

2.4. Instruments and Others

2.4.1. Dissection Tools

1. Micro three-well spot plate, Pyrex brand (Fisher Scientific, cat. no. 21-379).
2. Tungsten needle, bent 90° (Fine Science Tools, Foster City, CA, cat. no. 9718).
3. No. 5 fine forceps (Fine Science Tools, cat. no. 11252-20).
4. Fine scissors (Fine Science Tools, cat. no. 14060-09).
5. Short-stem Pasteur pipette (Fisher Scientific, cat. no. 13-678-20B).

2.4.2. Other Materials

1. 100- × 15-mm petri dish (Fisher Scientific, cat. no. 08757100D).
2. Cryogenic vials (Nalge Nunc International, Rochester, NY, cat. no. 5000-0012).
3. 15-ml sterile conical tubes (ISC Bioexpress, Kaysville, UT, cat. no. C-3317-1).

4. 12- × 75-mm polypropylene tubes (Fisher Scientific, cat. no. 14-959-1A).
5. Cell scraper (Fisher Scientific, cat. no. 3010).
6. Benchtop centrifuge (Fisher Scientific, cat. no. 05-375-46).
7. 70-µm cell strainers (Fisher Scientific, cat. no. 08-771-2).
8. FACSstarPlus cell sorter (BD Biosciences Immunocytometry Systems, San Jose, CA).

2.5. CEE Preparation

2.5.1. Materials

1. Chicken eggs.
2. Minimal essential medium (2×) (Invitrogen, cat. no. 11935).
3. Hyaluronidase, 10 mg/ml stock at –20°C (Sigma-Aldrich, cat. no. H3884).

2.5.2. Procedure of Making CEE

1. Incubate chicken eggs in a humidified incubator at 37°C for 11 days.
2. Wash eggs with 70% ethanol.
3. Macerate approximately 10 embryos at a time by passing them through a 30-ml syringe into a 50-ml Corning tube. This should produce about 25 ml of volume.
4. Add the same volume of Eagle's minimal essential medium and incubate at 4°C for 45 min on a rotary wheel.
5. Add sterile hyaluronidase to the final concentration of 2% and centrifuge at 30,000 × *g* for 6 h at 4°C.
6. Filter the supernatant through a 0.22-µm filter system with a sterile prefilter paper on top of the filter membrane (provided by the manufacturer).
7. Aliquot and store at –80°C. The CEE should last for up to 2 years if stored as working aliquots at –80°C.

3. Methods

3.1. Isolation and Culture of NEP Cells

NEP cells are isolated enzymatically from E10.5 caudal neural tubes and plated on fibronectin-coated dishes as adherent monolayer cultures. The entire procedure, from removal of embryos to collection of intact tubes, should not take more than 40 min for one litter of embryos.

3.1.1. Removal and Dissection of Embryos

1. Euthanize E10.5 pregnant rat with CO_2.
2. Make an incision from the lower abdomen upward to the chest. Hold one horn of the uterus and dissect the uterus out. Remove the uterus and place it in a 100- × 15-mm petri dish containing 20 ml of ice-cold PBS.
3. Transfer the dish to a sterile dissection hood.

4. Under the dissecting hood, cut the muscle wall of the uterus by using a pair of scissors. Make sure to cut through the flat end of the uterus.
5. The embryos usually "pop out" from the uterus (*see* **Note 5**).
6. Remove the attached membranes from the embryos.
7. Remove the head and the tail with a bent (90°) tungsten needle. Angle the cut so that little non-neural tissue remains at the ends.
8. Collect the trunk segment and transfer them with a short-stem Pasteur pipette to a Micro three-well spot plate containing ice-cold PBS.

3.1.2. Removal of Neural Tubes

1. Rinse the segments with medium several times with sufficient force to remove any attached membranes.
2. Add 1 ml of the enzymatic solution to the well and incubate the neural tubes at room temperature for about 5 to 10 min (*see* **Note 6**).
3. As soon as the ends of the tubes seem to separate from connective tissue, replace enzymatic solution with 1 ml of NEP basal medium supplemented with 10% CEE and wash the tubes with the medium several times.
4. Gently triturate the trunks with a Pasteur pipette until the neural tubes come off from the remaining tissues. The neural tube can be easily distinguished from other tissues by its transparency and tubular appearance.
5. Individual tubes should be collected as soon as they separate from the embryos and transferred to a separate dish that contains medium. As little medium as possible should be transferred with the tubes (*see* N**ote 7**).
6. In general, the tubes should be clean and have no adherent somites or membranes. If some somites are still attached, remove them with no. 5 fine forceps.

3.1.3. Dissociation and Plating of NEP Cells

1. Collect the neural tubes with a Pasteur pipette in a 15-ml conical tube.
2. Add 2 ml of trypsin-EDTA and incubate the neural tubes at 37°C for 3–5 min (*see* **Note 8**).
3. Neutralize the trypsin with 5 ml of NEP basal medium containing 10% CEE.
4. Spin the neutral tubes down at 300 × *g* at room temperature for 5 min. Discard the supernatant.
5. Resuspend the cells in 100 µl of NEP basal medium.
6. Dissociate the cells by very gentle trituration three to five times (*see* **Note 9**).
7. Plate the cells at high density on the fibronectin-coated dishes in NEP basal medium containing 10% CEE.

3.1.4. Maintenance and Testing of Undifferentiated NEP Cells

1. Culture the NEP cells at 37°C in a 5% CO_2.
2. The properties of NEP cells can be tested by immunocytochemistry. Cells should express Sox2 (*see* **Fig. 1**) and lack all lineage-specific markers, including E-NCAM, A2B5, and β-III-tubulin.

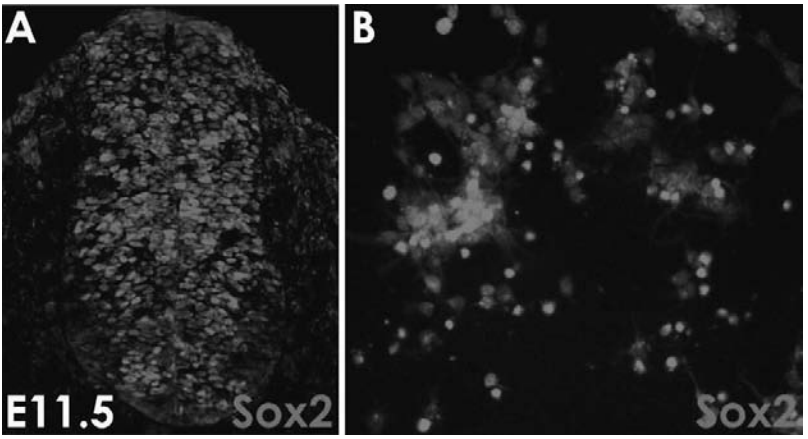

Fig. 1. NEP cells are abundant in E10.5 rat neural tube. Multipotent NEP cells exist in E10.5–11.5 caudal neural tube, and all cells express Sox2 (**A**). E10.5 rat NEP cells were dissociated and plated at high density and grown in NEP basal medium containing 10% CEE. When the cells were fixed and labeled for Sox2, most of the cells are Sox2-immunoreactive (**B**; 4,6-diamidino-2-phenylindole staining for nuclei).

3.1.5. Passaging NEP Cells

1. At 80% confluence, split the culture at a dilution of 1:4 (*see* **Note 10**).
2. Remove the culture medium.
3. Treat the cells with 1 ml of trypsin-EDTA solution for 2–3 min in a 37°C incubator.
4. Add 3 ml of NEP basal medium with 10% CEE to stop trypsinization (*see* **Note 11**).
5. Spin the cells down at 300 × *g*.
6. Resuspend the cells in 100 µl of culture medium.
7. Plate the cells on new fibronectin-coated dishes (*see* **Note 12**).

3.1.6. Freezing NEP Cells

1. When the dish reaches 80% confluence, remove the culture medium. Add 1 ml of trypsin-EDTA solution for 2–3 min at 37°C.
2. Add 3 ml of NEP basal medium with 10% CEE to stop trypsinization.
3. Spin the cells down at 300 × *g*.
4. Remove the medium. Gently resuspend the cells in 10% DMSO/80% NEP basal medium/10% CEE (4°C) at 10^6/ml (*see* **Note 13**).
5. Transfer the cell suspension to prechilled cryogenic vials. Store the vials at –140°C.

3.2. Isolation and Culture of Neuronal-Restricted and Glial-Restricted Precursor Cells

To obtain NRP and GRP cells, the spinal cord is dissected mechanically from an E13.5 or E14.0 embryo. At this stage, the spinal cord contains approximately 10% NEP cells, 70% NRP cells, and 20% GRP cells. The cells can be plated on poly-L-lysine/laminin- or fibronectin/laminin-coated tissue culture dishes or cover glasses. The entire procedure should take about 1 h for one litter of embryos.

3.2.1. Removal of Spinal Cord

1. Remove the E13.5 uterus as described for the E10.5 uterus.
2. Transfer the uterus to a 100-mm petri dish containing fresh ice-cold PBS. Move the dish to a dissecting hood. Cut throughout the length of the uterus with sterile scissors and remove the embryos.
3. Hold the embryo by the head using a no. 5 forceps. Under a dissecting microscope, use a second no. 5 forceps to loosen the spinal cord gently from the surrounding tissue.
4. Begin the dissection at the level of the midbrain where the overlying tissue is minimal.
5. Separate the overlying skin all the way down to the caudal end of the embryo.
6. Separate the connective tissue along the lateral side of the spinal cord until the spinal cord is clearly visible. Proceed along the entire rostrocaudal axis. If necessary, repeat on the other side.
7. Gently insert one forceps under the spinal cord to separate it from underlying connective tissue. Proceed to separate the spinal cord beginning rostrally and extending as far caudally as possible.
8. Cut the spinal cord at the caudal end to free the cord from the remaining trunk.
9. Once the cord is clean, remove the head to free the spinal cord (*see* **Note 14**).

3.2.2. Dissociation of Spinal Cord Cells

1. Collect about three spinal cords in each 15-ml conical tube (*see* **Note 15**).
2. Add 3 ml of trypsin-EDTA and incubate the spinal cords at 37°C for 10 min, shaking the tube occasionally (*see* **Note 16**).
3. Remove the trypsin-EDTA. Add 2 ml of NEP basal medium.
4. Gently triturate the spinal cords with a Pasteur pipette (*see* **Note 17**).
5. Carefully pick undissociated floating tissue (mainly non-neural tissue) by using a P1000 pipette. Try to avoid removing any liquid at this step.
6. Spin the cells at 300 × *g* at room temperature for 5 min. Remove and discard the supernatant.
7. Resuspend the cells in 100 µl of NEP basal medium.
8. Dissociate the cells by gently triturating.
9. Plate the cells at 50% confluence on poly-L-lysine/laminin- or fibronectin/laminin-coated dish or cover glass in NEP basal medium.

3.2.3. Maintenance and Testing of Lineage-Restricted Precursor Cells

1. Incubate the spinal cord cells at 37°C in a 5% CO_2 atmosphere.
2. To keep the cells dividing, add 20 ng/ml FGF every 2 days.
3. To enhance neuronal cell survival, add 10 ng/ml NT-3 every 2 days.
4. Test the immunocytochemical properties of the cells by staining the cells with anti-A2B5, anti-NCAM, and anti-β-III-tubulin antibodies. About 70% of cells are A2B5-positive cells and 20% of cells are NCAM-positive in the initial culture.

3.2.4. Passaging and Freezing the Cells

Passage and freeze the E13.5 cells as described for E10.5 (*see* **Note 18**).

3.3. Enrichment of NRP and GRP Cells

In this procedure, E13.5 spinal cord cells (mouse E12.0) are enriched for NRP (NCAM+) or GRP (A2B5+) cells by FACS. Unwanted cells are removed, and the needed cells are collected during the process. The collected cells are plated on poly-L-lysine/laminin- or fibronectin/laminin-coated dishes or cover glasses. The cells can be enriched to 95–99% purity. This sorting method for enriching NRPs and GRPs has been found to be more efficient than the immunopanning method that we described in the first edition of this chapter.

3.3.1. Sorting Procedure

1. Isolate cells directly from embryonic E13.5 spinal cords (*see* **Subheading 3.2.**) or from cells already in culture by incubating in 0.05% trypsin-EDTA for 1 min.
2. Triturate the cells into single-cell suspension (*see* **Note 19**), add 2 ml of DMEM/F-12 to dilute out the trypsin (*see* **Note 20**).
3. Transfer the cells to a 15-ml conical tube, centrifuge at 300 × *g* by using a benchtop centrifuge for 5 min at room temperature.
4. Resuspend the cells with 1–2 ml NEP basal medium with 20 ng/ml basic fibroblast growth factor (bFGF), label this tube as "experiment." Before adding primary antibodies, aliquot 10–20 µl of cells into another 15-ml tube and add equal volume of NEP basal medium as for experiment tube. This tube will serve as negative control during sorting experiments.
5. Add primary antibodies to stain cells in experiment tube. For sorting of NRP cells, add E-NCAM antibody at 1:5 (*see* **Note 21**); for sorting of GRP cells, add A2B5 antibody at 1:20 (*see* **Note 21**). Antibodies should be diluted in NEP-basal medium supplemented with bFGF. Mix well by gently pipetting up and down.
6. Gently shake both experiment and negative control tubes at 4ºC (or on ice) for 1 h.
7. Centrifuge both tubes at 300 × *g* for 5 min. Remove supernatant.
8. Resuspend cells with 2 ml of NEP basal medium and centrifuge. Repeat this step two to three times for both tubes.

9. Add secondary antibody Alexa 488 goat anti-mouse IgM at 1:200 diluted in NEP basal medium with bFGF to both experiment and negative control tubes.
10. Gently shake both experiment and negative control tubes at 4ºC (or on ice) for 1 h. Centrifuge both tubes at 300 × *g* for 5 min. Remove supernatant.
11. Resuspend cells with 2 ml of 5% FBS in PBS and centrifuge at 300 × *g* for 5 min. Repeat this step two to three times for both tubes.
12. Resuspend cells in 1 ml of 5% FBS in PBS. The cell concentration should be around 0.5–1 × 10^7/ml.

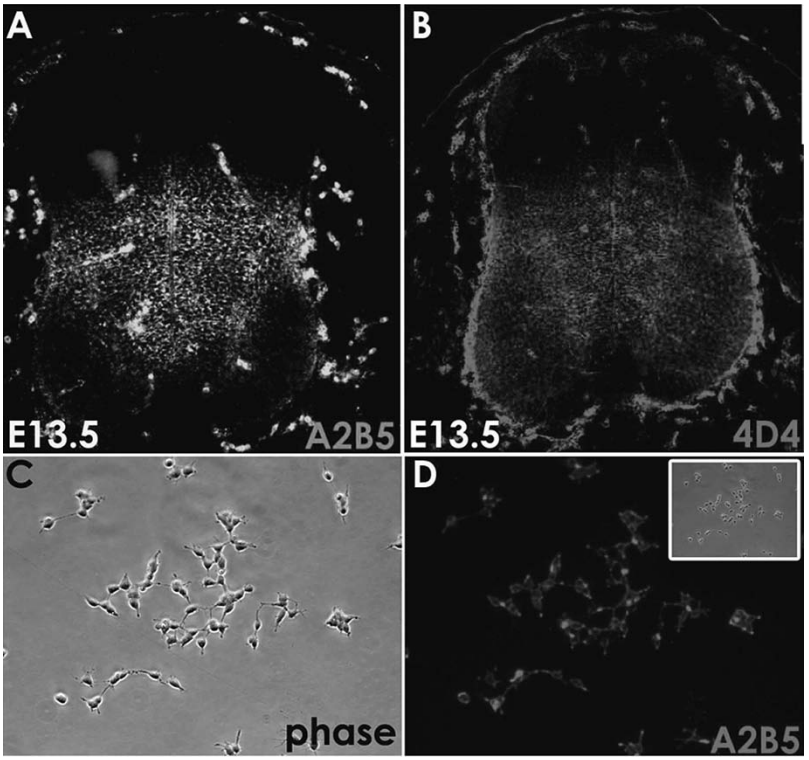

Fig. 2. GRP cells exist in E13.5 neural tube, and they can be purified by FACS with A2B5-specific antibodies. GRP cells were detected in E13.5 rat spinal cord as shown by A2B5 antibody staining (**A**) and by immunostaining of monoclonal antibody 4D4, a newly characterized antibody that recognized the same antigen in tissue culture as A2B5 antibody (**B**). When purified using A2B5 antibody by FACS, A2B5+ cells were obtained from dissociated E13.5 rat spinal cord cells. The cells were plated and grown on poly-L-lysine/laminin-coated dish for 24 h and observed under fluorescence microscopy (**C,D**). Live staining showed that >99.9% cells are A2B5+ (**D**). Inset shows the overlay of phase contrast and the fluorescence staining.

13. Filter the cells through a 70-μm strainer and collect cells by using a 12- × 75-mm polypropylene tube that contains 1 μl propidium iodide.
14. Add 1 ml of NEP basal medium with bFGF to a 12- × 75-mm polypropylene collecting tube that will be used to collect enriched E-NCAM+ NRP cells or A2B5+ GRP cells. Prepare more than one tube if needed.
15. Send the cells to flow cytometry laboratory. Cells from the negative control tube will be used to set up gating parameters. Usually, a FACSstarPlus cell sorter is used to sort cells from experiment tube at 4°C at a rate of 2,500 cells/s.
16. Sorted E-NCAM+ NRP cells or A2B5+ GRP cells will be seeded onto poly-L-lysine/laminin-coated dishes or cover glasses in NEP basal medium with bFGF. Cells should be plated onto several coated dishes/cover glasses for convenience of subsequent experiments.
17. The next day, take one dish or one cover glass to check the purity of sorted cells by live cell staining of E-NCAM or A2B5. About 90–99% of the cells should express E-NCAM or A2B5 (*see* **Fig. 2**).
18. Continue to grow these cells for other experiments or freeze these cells.

4. Notes

1. Equivalent mouse and human embryonic ages to isolate NEP, NRP, and GRP cells are as follows: in mice, E8.75–9.0 for NEP cells and E11.5–12.0 for NRP and GRP cells. In humans, embryonic week (EW)5 for NEP cells, EW8–16 for NRP cells and EW16–24 or later for GRP cells. Dissociation time is longer and the amount of cell death is much higher when adult tissue samples are used.
2. Because some cultures require the reduction of FGF, NEP medium also can be made without FGF and be supplemented with FGF later.
3. Dishes also can be coated at 37°C for 4 h. However, the fibronectin solution cannot be reused.
4. Make fresh enzymatic solution each time before dissection. Higher concentration of papain or dispase alone also has been used successfully by us and others to dissociate neural tube cells, but we prefer the combination of collagenase and dispase for rat neural tubes. Trypsin also can be used to dissect mouse neural tubes.
5. If an embryo does not come out, apply gentle pressure at the other end of the sac and squeeze the embryo out.
6. Incubation time is variable depending on the individual researcher.
7. Neural tubes are very sensitive to the enzymes. Even a trace of the enzymes can kill the cells.
8. The NEP cells plated in serum-free medium are very sensitive. Overexposure to trypsin can result in decreased survival.
9. Do not try to over dissociate the cells. Vigorous mechanical treatment may kill the cells.
10. NEP cells grow rapidly, doubling every 4–6 h in the presence of bFGF and CEE. NEP cells should be passaged every 2 days. Never let cells reach confluence, because it causes the cells to differentiate.

11. The most common problem for passaging NEP cells is that cells seem healthy when plated but die overnight or fail to grow. This is due to the residual trypsin. Care needs to be taken to avoid this. Soybean trypsin inhibitor and higher concentration of CEE can be used to neutralize the remaining trypsin.
12. Keep the cells at relatively high densities to ensure a high rate of cell division and to minimize differentiation.
13. Optimal freezing protocol can be determined by individual laboratories.
14. The meninges exist as a thin layer and are often overlooked. This could be a possible source of contaminating cells. It is difficult to remove the thin meninges sheet once both ends of the spinal cords are free. Therefore, let the tube remain attached to the head and peel off the meninges while holding on to the head.
15. Do not put too many spinal cords in one tube, otherwise the cells will not dissociate very well.
16. Incubating time is variable depending on the individual laboratory.
17. Do not triturate the spinal cords too harshly, because this decreases cell viability.
18. It is hard to replate the frozen NRP cells. Try to use 50% of serum instead of 10%.
19. Make sure the cells are dissociated into single cells. Otherwise, the purity is reduced dramatically.
20. No serum is used to neutralize the trypsin, because serum might inhibit subsequent neural differentiation.
21. The optimal antibody dilution needs to be determined by individual researcher. The concentration of the primary antibodies should be around 1 μg/ml to 5 μg/ml.

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6

Adult Rodent Spinal Cord-Derived Neural Stem Cells: *Isolation and Characterization*

Lamya S. Shihabuddin

Summary

Self-renewing multipotent stem/progenitor cells have been isolated from various areas of the adult central nervous system, both neurogenic and non-neurogenic, in vitro by using a variety of mitogens and culture conditions. Mitogenic growth factors stimulate the proliferation of multipotent stem/progenitor cells from embryonic and adult rodent, primates, and human. Neural stem cells were isolated from the adult rat spinal cord as follows: Adult rat spinal cords were dissected, and tissue was mechanically and enzymatically dissociated. The cell suspension was filtered and further purified from debris and enriched for progenitors by using a Percoll density gradient. The dissociated cells were grown on polyornithine- and laminin-coated plates in N2 medium containing fibroblast growth factor-2. The cells grew as attached layers. Cultures were passaged by trypsinization, and rapidly proliferating cells that could be passaged, frozen, and thawed were isolated. Clonal cultures derived from single founder cells identified by marker genes generate neurons, astrocytes, and oligodendrocytes, confirming the multipotent nature of the parent cell. Subcloning analysis showed that after serial passaging, recloning, and expansion, these cells retained multipotentiality, indicating that they are self-renewing.

Key Words: Stem cells; FGF-2; spinal cord; adult; rat.

1. Introduction

The generation of neuronal and glial cell types found in the mature central nervous system (CNS) involves the massive proliferation of ventricular zone cells of the fetal brain and spinal cord ***(1)*** that stop dividing early in development and terminally differentiate. Most neurons in the adult CNS are terminally differentiated and last through the life span of the animal. However, recent

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studies indicate that cell proliferation in the adult CNS is ubiquitous, but it is primarily confined to the production of glia, with the exception of discrete regions in the hippocampus and subventricular zone *(2–4)*, where neurogenesis persists. The discovery that undifferentiated proliferative cells could be isolated from the adult mammalian CNS under favorable cell culture conditions, and induced to differentiate along both glial and neuronal lineages, revolutionized our thinking about the limited ability of the CNS to replace neural cells lost to injury or disease.

Stem cells are able to differentiate into all the different types of cells in a given tissue, while maintaining a pool of themselves *(5)*. During development of the CNS, in analogy with the hematopoietic system, maturation of neural stem cells involves a continuing loss of potential and restriction of commitment before, finally, a lineage of fully differentiated cells is established. Isolation and long-term culturing of multipotent neural stem/progenitor cells that upon differentiation, generate the major building blocks of the CNS: neurons, astrocytes, and oligodendrocytes have been advanced by the finding that some mitogenic growth factors have a proliferative effect on these cells *(6–8)*.

Multipotent stem cells that respond to epidermal growth factor (EGF) or basic fibroblast growth factor (FGF)-2 have been isolated from both neurogenic *(9,10)* and non-neurogenic regions *(6,11)* of the adult mammalian CNS by using different culture systems (reviewed in **ref.** *12*). Isolated and cultured stem cells provide an important source of cells for in vitro studies to address issues related to development, as well as in transplantation studies to explore their potential as a source of donor cells for therapeutic purposes.

In this regard, neural stem/progenitor cells have aroused a great deal of interest for their therapeutic potential in neurological disorders *(13)*. After transplantation into the CNS, neural stem/progenitor cells migrate throughout the CNS, differentiate exclusively into neural cell types in a region-specific manner, and survive several months *(14–18)*. Adult neural stem/progenitor cells can also be isolated from human postmortem tissue *(19)*, potentially providing an alternative source of cells for therapeutic applications.

Methods for isolation of stem cells from the brain have been described previously *(20)*. Here, we describe a method for isolating and culturing cells from the adult rat spinal cord, the generation of clonal population, and the characterization of these cells.

2. Materials

2.1. Isolation and Culturing of Stem Cells

1. Phosphate-buffered saline (PBS; 1× and 10×; Invitrogen, Carlsbad, CA).
2. Dulbecco's Ca^{2+}-, Mg^{2+}-free PBS (DPBS, Invitrogen).

3. 0.01% papain: 0.1% protease, 0.01% DNase I (PPD) in Dulbecco's modified Eagle's medium (DMEM; Cellgro, Mediatech, Herndon, VA).
4. DMEM/Ham's F-12 (1:1) medium (Irvine Scientific, Santa Ana, CA) containing glucose and L-glutamine with 10% fetal bovine serum (FBS; Sigma-Aldrich, St. Louis, MO).
5. Continuous density Percoll (GE Healthcare, Little Chafont, Buckinghamshire, UK, and Sigma-Aldrich) gradient 9:1 (v/v) with 10× PBS.
6. N2 supplement culture medium (N2 medium), DMEM/Ham'sF-12 with L-glutamine and (1×) N2 supplement (100×; Invitrogen).
7. Recombinant human basic FGF-2, 20 ng/ml final concentration (PeproTech, Rocky Hill, NJ).
8. Falcon tissue culture dishes (100 and 60 mm).
9. 10 mg/ml poly-L-ornitine hydrobromide- (PORN; Sigma-Aldrich) and 5 mg/ml laminin (Invitrogen)-coated culture plates (PORN/laminin).
10. 15- and 50-ml sterile centrifuge tubes.
11. Sterile filters (0.22 μm; Nalgene, Nalge Nunc International, Rochester, NY).
12. Nylon meshes (pore size 15 μm; Nitex, Tetko, Elmsford, NY).

2.2. Isolation of Clonal Cultures

1. Agarose/trypsin: 1 ml of 3% agarose mixed with 2 ml of ATV trypsin (Irvine Scientific).
2. Sterile Pasteur pipette.
3. PORN/laminin-coated 96-well plates.
4. Serum-free N2 medium supplemented with 50% conditioned medium from a high-density stem cell culture grown for at least 24 h.

2.3. Passaging, Freezing, and Reculturing of Cells

1. ATV trypsin (trypsin-EDTA solution, Irvine Scientific).
2. DMEM/Ham's F-12 (1:1).
3. Dimethyl sulfoxide (DMSO; Sigma-Aldrich).
4. 15-ml sterile centrifuge tubes.
5. Cryovials (1.5 ml; Nalgene, Nalge Nunc International).
6. Freezing chambers (Nalgene, Nalge Nunc International) and liquid nitrogen tank.

2.4. Differentiation and Immunostaining of Stem Cells

1. PORN/laminin-coated glass chamber slides.
2. Serum-free N2 medium containing differentiating agents, such as serum (0.5%), retinoic acid (0.5 μM), or forskolin (10 μM).
3. 0.1 M Tris-buffered saline (TBS).
4. Blocking buffer: TBS containing 10% donkey serum and 0.1% Triton X-100.
5. 4% paraformaldehyde solution (pH 7.4).
6. Antibodies against lineage specific proteins.

7. Fluorescence-conjugated secondary antibodies.
8. TBS containing 10 ng/ml 4,6-diamidino-2-phenylindole (DAPI) for nuclear counterstain.
9. Antifade mounting medium: 25% glycerol, 10% polyvinyl alcohol, and 2.5% 1,4-diazobicyclo-[2.2.2]-octane in 100 mM Tris-HCl (PVA-DABCO).

3. Methods

3.1. Establishment of Stem Cell Cultures from Adult Rat Spinal Cord

Dissect out the adult rat spinal cord, cut it into small pieces, dissociate the tissue pieces by enzymatic digestion to release cells from connective tissues, and then plate (*see* **Fig. 1**). To purify stem cells from debris and to enrich for multipotential stem cells based on buoyant density, Percoll density gradient fractionation can be used.

3.1.1. Dissection of Tissue

1. Anesthetize adult female Fischer 344 rats (3–4 months old) by intraperitoneal injection of anesthesia cocktail: ketamine (44 mg/kg), acepromazine (4.0 mg/kg), and rompun (0.75 mg/kg), and sacrifice by decapitation.
2. Expose the spinal column by making an incision of the skin along the back and clear the rostral opening of the vertebral column from any connective tissue.
3. Cut the vertebral column where it meets the pelvic bone.
4. Fill a 10-ml syringe with sterile 1× PBS and insert the short (1–2 cm) beveled 18-guage needle into the caudal opening of the central canal, push the needle against the dorsal side of the central canal to form a tight seal and quickly compress the plunger. The whole spinal cord will be ejected from the rostral opening of the vertebral column.
5. Place in PBS, clean the spinal cord from meninges and dorsal and ventral roots (*see* **Note 1**). Microdissect sacral, thoracic, lumbar, and cervical areas.

3.1.2. Enzymatic Digestion

1. Cut the microdissected tissue into small pieces (1–2 mm^3).
2. Wash dissected tissue with 5 ml of DPBS three times and remove the final wash by aspiration (*see* **Note 2**).
3. Resuspend the tissue pieces in PPD solution (~1 g of tissue cut into small pieces to 20 ml of PPD) and incubate for 30–45 min at 37°C with occasional shaking to keep the tissue pieces suspended, and triturate with a 5-ml pipette (every 10 min) to break up the large chunk of tissues. Triturate with 5-ml pipette until the cell suspension is free of large tissue pieces (*see* **Note 3**).
4. Remove the remaining tissue pieces by filtration through a nylon mesh. Centrifuge the filtered cell suspension at 1000 × *g* for 3 min and remove PPD solution by gentle aspiration so that the cell pellet is not disturbed.

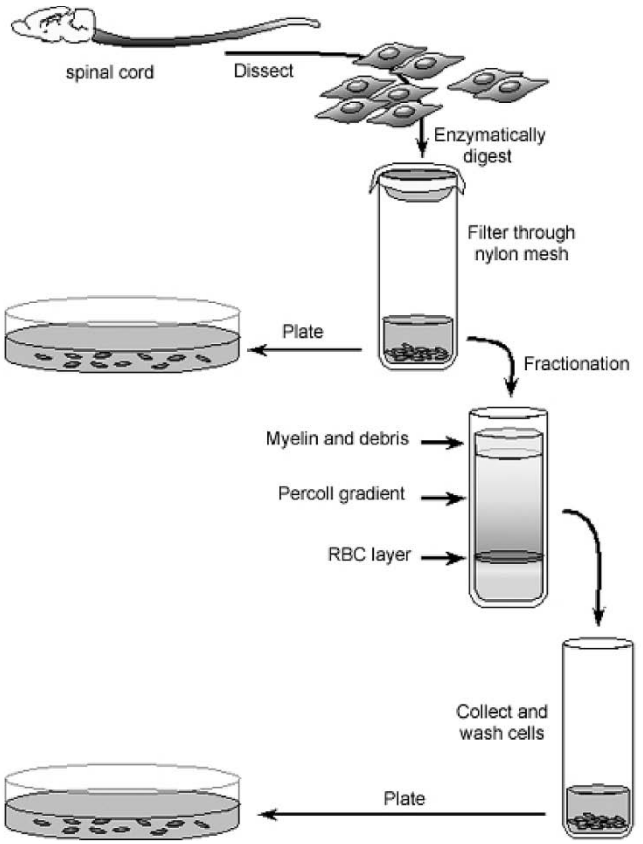

Fig. 1. Schematic diagram of the protocol for isolating stem cells from adult spinal cord. Tissue pieces are subject to enzymatic digestion and mechanical dissociation, filtration to remove large tissue pieces, and then the cell suspension is plated. Alternatively, progenitor population can be enriched and purified from debris by fractionation by using a Percoll density gradient, rinsed well, and then plated.

5. Suspend cells in DMEM/Ham's F-12 containing 10% FBS (10 ml/g starting tissue weight) and wash the pellet three times by centrifugation. Aspirate the medium, and resuspend the cells in N2 medium containing 10% FBS (1–2 ml). The cell suspension contains small pieces of tissue, myelin, and red blood cells. Cells can be plated without further purification, or they can be separated from other cells and debris by Percoll density gradient fractionation.

3.1.3. Percoll Gradient Purification of Stem Cells

A progenitor/stem cell enrichment protocol based on fractionation depending on buoyant density ***(21)***.

1. After washing pellet three times with DMEM/Ham's F-12 containing 10% FBS, resuspend cells in 5.5 ml of DMEM/Ham's F-12 containing 10% FBS.
2. Dilute stock Percoll solution 9:1 (v/v) with 10× PBS.
3. Mix cells with Percoll (1:1).
4. Transfer the cell suspension to an ultracentrifuge tube (14 × 89 mm; Beckman Coulter, Fullerton, CA) and fractionate by centrifugation at 20,000 × *g* for 30 min at 18°C, (12,700 rpm in a SW41Ti Beckman Rotor in an L8-80 M Ultracentrifuge, no brakes).
5. Remove the top myelin and debris layer and take all layers above the bottom red blood cell (RBC) layer (make sure not to take any RBCs) and transfer to a 50-ml centrifuge tube. Immature progenitors/stem cells migrate into the gradient, but they are enriched at the bottom of the gradient, in a band just above the RBC layer.
6. Dilute fivefold in cold PBS and wash cells at least three times by centrifugation at 1,000 × *g* for 3 min. The cell pellet is very small; to avoid losing the cell pellet, leave behind ~1 ml of wash liquid in between aspiration and washes.
7. Resuspend the cell pellet in 1 ml of the appropriate plating medium, count cells, and then plate ~5 × 10^4 cells/cm^2.

3.1.4. Culturing of Rat Cells as Monolayers ***(22,23)***

1. Resuspend cells in N2 medium containing 10% FBS and plate at least 1 × 10^4 cells/cm^2 in 10-cm uncoated tissue culture plates (*see* **Note 4**).
2. The next day, change the medium into serum-free N2 medium containing 20 ng/ml FGF-2.
3. Feed cultures every 3–4 days, and if cell density is low, change half of the medium with fresh medium. Add double amount of FGF-2 to have a final concentration of 20 ng/ml.

3.2. Isolation of Clonal Cultures (10,11)

Clonal cultures are isolated to determine whether single stem cells can generate multipotent progeny that includes both neurons and glia.

1. Plate cells from bulk culture at clonal density (1 cell/2 cm^2 in a 35-mm petri dish) onto PORN/laminin-coated dishes in serum-free N2 medium containing appropriate growth factors.
2. To follow a particular cell, mark its position on the dish by scratching the bottom of the plate. Stem cells migrate while proliferating, so always make sure the cells are well separated.
3. Feed cells every 4–5 days with medium containing 20 ng/ml FGF-2 and supplement with 50% conditioned medium (*see* **Note 5**).
4. When the density of the clones reaches a critical mass (>100 cells/colony), then transfer individual clusters of proliferative cells to 96-well plates (one clone/well)

embedded with agarose/trypsin (see below) by using a glass pipette, or characterize the clonal population by immunocytochemistry.

3.2.1. Genetic Marking of Cells to Establish Clonal Cultures

1. Infect bulk population of stem cells with a replication-defective retroviral vector of choice expressing a marker gene such as green fluorescent protein, *Escherichia coli* Lac Z gene, or alkaline phosphatase ***(24)*** and the selectable marker gene neomycin resistant gene. Incubate the cells in medium containing up to 10% virus stocks for 18–24 h.
2. Plate the cells at ~1% of their initial density in the presence of 100 μg/ml G-418 (Geneticin, Invitrogen) to select for stable transfectant. Usually start with 40 μg/ml G-418 and increase the concentration slowly to 100 μg/ml.
3. Feed cells every 3–4 days until proliferative clusters form. Selection can be stopped when stably transfected cultures are established, but select cells periodically to remove cells that have lost the selectable marker gene.
4. Passage individual clones with agarose/trypsin (see section 3.2.2).
5. Confirm the clonality of cultures by determining the integration site of the retroviral genome within the cellular genome by using Southern blot analysis. Briefly, prepare genomic DNA by lysis of cells and digest with appropriate digestion enzymes. Resolve digested DNA on agarose gels, transfer to nylon membranes, probe with ^{32}P-labled neomycin or transgene-specific probe, and visualize using a phosphorimager.

3.2.2. Agarose/trypsin Method for Transferring Clones

1. Pick clones from plates that have relatively big (>100 cells/clone) and well-separated clones. Mark a clone by marking it on the back of the dish.
2. Melt 3% agarose solution (made in PBS) in a microwave oven, cool down, and mix 1 ml of agarose with 2 ml of ATV trypsin (warmed to 37°C).
3. Remove culture medium. Immediately add agarose/trypsin mixture to dish containing the clones, swirl the plate to gently spread over the cells and allow to solidify 2–3 min.
4. With a sterile Pasteur pipette gently cut around the clones and lift the agarose plugs (with attached cells to them) and transfer them to individual wells of a 24-well-plate containing serum-free N2 medium supplemented with 50% conditioned medium. Gently wash the area of the plug with the medium (~100 μl), and transfer it to the well containing the cells.
5. Change medium every 3–4 days and allow the cells to proliferate until ready to be passaged.

3.2.3. Isolation of Secondary Clonal Cultures

A defining characteristic of stem cells is self-renewal ***(25)***. Subcloning is done to determine whether a clone that originated from a single cell can be

dissociated and replated under the same conditions to yield more than one copy of itself, indicating self-renewing capacity.

For isolation of secondary clones, individual primary clones were dissociated and replated at low clonal density. Single cluster of dividing cells was again transferred to 96-well plates (one clone/well) by using glass pipettes, expanded, and characterized by immunocytochemistry.

3.3. Passaging, Freezing, and Reculturing of Neural Stem Cells

3.3.1. Passaging

1. Add 2.0 ml ATV/trypsin/10-cm plate (add less for small dishes) prewarmed to 37°C. Remove 1 ml and leave 1ml; swirl plate to distribute the liquid evenly.
2. Let sit for 1 min (*see* **Note 6**). Hit the sides of the plate gently to dislodge the cells.
3. Transfer cells to 15-ml sterile centrifuge tube by using DMEM/Ham's F-12. Wash the plate once with DMEM/Ham's F-12 and transfer to the same tube. Centrifuge at 100 × *g* for 3 min.
4. Remove supernatant slowly as not to disturb the cell pellet. Resuspend cells in 2 ml of serum-free N2 medium.
5. Plate portion of cells (split-ratio will depend on initial cell density and the growth rate of cells) on PORN/laminin-coated plates in the same medium containing FGF-2 (*see* **Note 7**).
6. If necessary, freeze cells in liquid nitrogen for long-term storage.

3.3.2. Freezing of Cells

1. Suspend cells in serum-free N2 medium containing 10% DMSO and appropriate growth factors.
2. Aliquot 1 ml in each freezing vial.
3. Put the vials in freezing chambers and place the chamber in –70°C freezer to allow the cells to freeze slowly.
4. On the next day, transfer the vials to a box kept in liquid nitrogen.

3.3.3. Reculturing of Frozen Cells

1. Remove vials from liquid nitrogen and thaw cells quickly in 37°C water bath.
2. Transfer cells to sterile 15-ml centrifuge tube with DMEM/Ham's F-12 medium, centrifuge at 1,000 × *g* for 3 min. Remove supernatant.
3. Wash cells once in the same medium. Resuspend the cells in 1 ml N2 medium.
4. Plate on PORN/laminin-coated plates in serum-free N2 medium containing appropriate growth factors.

3.4. Differentiation and Immunostaining of Stem Cells

3.4.1. Differentiation of Stem Cells

1. Plate cells on PORN/laminin-coated glass chamber slides of 1×10^5 cells/cm^2 (high density) and grow for 24 h in serum-free N2 medium containing FGF-2.
2. Replace the medium with fresh N2 medium without FGF-2 containing differentiating agents, such as serum, retinoic acid (1 mM), or forskolin (10 mM) ***(11)***.
3. Change medium every 2 days and allow the cells to differentiate for 6 days. Fix cells with paraformaldehyde and analyze by immunocytochemistry.

3.4.2. Immunocytochemical Analysis of Stem Cells

Staining procedure is carried on at room temperature unless specified otherwise. Rinses are done for 5 min each.

1. Grow stem cells in PORN/laminin-coated glass chamber slides until 70–80% confluent.
2. Fix cells for 2 min with 2% paraformaldehyde (by removing half of the medium covering the cells and adding an equal amount of 4% paraformaldehyde), then for 10 min with 4% paraformaldehyde and wash three times in 100 mM TBS. Fixed cells can be stored at 4°C for about a week or processed immediately for immunocytochemistry.
3. Preincubate cells for 1 h in TBS containing 5% donkey serum and 0.1% Triton X-100 (blocking buffer).
4. Incubate with pooled primary antibody (polyclonal and monoclonal) diluted in TBS containing 0.1% Triton X-100 (TBS+). If the antibody recognizes cell surface molecules, then exclude Triton from the incubation buffer.
5. After 24–48 h at 4°C, wash cells three times with the blocking buffer and incubate for 2 h in the dark with species-specific secondary antibody conjugated to the desired fluorophores, such fluorescein isothiocyanate, Texas Red, or Cy3 in TBS+. If necessary, the signal for specific antigens can be amplified by using biotin-streptavidin amplification by incubating cells in biotinylated donkey anti-species antibody diluted in TBS+ for 2 h, washing twice in the same buffer, and then incubating in streptavidin conjugated to the desired fluorophore.
6. Wash cells twice in TBS, incubate in TBS containing 10 ng/ml DAPI for 1 min, and then coverslip in PVA-DABCO solution. Analyze cell phenotype by confocal of fluorescence microscopy.

4. Notes

1. Clean dissection of tissues increase the purity of the cultures and decrease contaminating non-neuronal cell populations (i.e. connective tissue) that may eventually overtake the cultures.

2. In the initial isolation and first passage of cultures, all buffers and media used contain 2.5 mg/ml Fungizone (Irvine Scientific), 1× penicillin-streptomycin (100×; Invitrogen) to avoid possible contaminations from dissection.
3. Enzymatic digestion of tissues with PPD for longer than 40 min will lower the yield of stem cells.
4. Survival of rat stem cells grown as monolayer is density dependent, and when plated at <1,000 cells/cm^2, they may not survive even in the presence of FGF-2 (J. Ray, unpublished observation). When rat stem cells are plated at a high density, proliferating cells can be seen within the first 2 weeks, and mixed population of cells is observed. The cultures contain cells that have stem cell morphology with small phase bright cell bodies and short processes and cells with different morphologies. These morphologies include cells with large, flat, phase dark cell bodies and long processes.
5. Factors present in conditioned medium will support the survival/proliferation of cells plated at clonal density. Collect the conditioned medium from high-density stem cell culture after at least 24-h incubation, filter sterilize to prevent accidental contamination with residual cells, and centrifuge at 1000 × *g* for 5 min and keep frozen in aliquots.
6. When passaging, do not trypsinize cultures for longer than 2 min; long exposure to trypsin will result in extensive death of cells. Stem cells detach easily, and short trypsin treatment will enrich passaged cultures with stem cells while leaving behind more differentiated cells.
7. Composition of the substratum is important for the adhesion, survival, proliferation, and differentiation of cells. Factors in serum provide components for cell attachment for the initial plating. After serum withdrawal and the first passage, cells are plated on plates coated with agents such as polymers of basic amino acids, PORN, and cells will attach on basis of charge. Also, laminin, a cell adhesion molecule, can be used in addition to PORN as a substratum.
8. The culturing conditions and requirement of growth factors for the rat, mouse, monkey, and human CNS-derived stem cells are different ***(26)***. The best condition for growing adult CNS rat stem cell, irrespective of region, is to culture as monolayers in serum-free N2 medium containing 20 ng/ml FGF-2 ***(26)***. Growing adult rat spinal cord stem cells on uncoated plates in serum-free medium with FGF-2- and EGF-generated small neurospheres, but they grew poorly and could not be expanded more than four to five passages (L. Shihabuddin, unpublished observation).

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7

Adult Neural Stem Cells

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Summary

Neural stem cells (NSCs) have been identified in the mature central nervous system (CNS), and they reside in specific areas. Cultures of NSCs can be successfully established in vitro by exploiting the NeuroSphere assay. This methodology relies on the continuous exposure of neural cells to mitogens such as epidermal growth factor and fibroblast growth factor-2. Under these conditions, only NSCs and highly undifferentiated progenitors proliferate, whereas committed precursors and terminally differentiated cells are eliminated from the culture. The proper application of this method to the cells allows the establishment of long-term expanding stable NSC lines, starting from different neural tissues as the adult rodent CNS and human brain tumor specimens.

Key Words: Neural stem cell; adult neurogenesis; epidermal growth factor; fibroblast growth factor; cancer stem cell; brain tumor.

1. Introduction

Somatic tissue stem cells (SCs) represent a reservoir of immature cells, endowed with the critical function of replacing the mature cells lost through physiological turnover or by disease. SCs are defined as highly undifferentiated cells, able to proliferate long-term, and, most importantly, to self-renew. At the same time, bona fide SCs are capable of giving rise to the full complement of functional differentiated progeny typical of their tissue of residence (multipotency).

Over the last decade, it has been convincingly demonstrated that the adult brain of both rodents and primates contains areas of active neurogenesis, such as the subventricular zone (SVZ) and the subgranular zone (SGZ) of the dentate

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gyrus (DG) of the hippocampus ***(1–4)***. Besides the SVZ and SGZ, a large number of proliferating cells are found between the hippocampus and the corpus callosum in the adult rodent brain, a region that has recently been named subcallosal zone (SCZ) ***(5)***. From these restricted regions, SCs continuously contribute new neurons and glia to target areas such as the olfactory bulb ***(6,7)***, the corpus callosum ***(5)***, and the hippocampus itself ***(8)***.

Although the existence of putative neural SCs (NSCs) in the adult central nervous system (CNS) has been effectively confirmed in vivo ***(9)***, their propagation, expansion, and characterization in vitro still relies on the development of specific and efficient methodologies by which to assess their proliferation, self-renewal, and differentiation capacity ***(10,11)***. Fortunately, the exploitation of these functional attributes provides a solid basis for the reliable identification of NSCs that has been limited by the relative paucity of identifying antigenic markers ***(12,13)***.

Similar to other renewing tissues, the neurogenic regions of the CNS comprise a hierarchy of different types of cells, i.e., bona fide SCs, transit amplifying progenitors, and mature differentiated cells. Every cell subtype is endowed with specific antigenic and morphological properties that allow identification in vivo ***(14)***. The SC component presenting within this heterogeneous neural population can be isolated by applying an elective in vitro methodology, the neurosphere assay (NSA) ***(15,16)***.

The NSA represents a selective serum-free in vitro assay that has been successfully applied not only to neural tissues of different origin (rodent or human, fetal or adult, normal or pathologic) ***(15)*** but also to non-neural tissues such as the mammary epithelium and the cardiac muscle ***(17,18)***. By taking advantage of the functional features of the different cell populations retrieved within the SC compartment, the NSA selectively enriches for the SC component. In fact, SCs in culture are characterized by the capacity to proliferate and self-renew extensively, thereby giving rise to long-term expanding SC lines. Conversely, transit amplifying progenitors, although displaying limited proliferative capacity, do not self-renew and they are lost throughout extensive subculturing ***(19)***.

Cells from freshly dissociated neural tissues, when plated in the presence of mitogens such as epidermal growth factor (EGF) and fibroblast growth factor (FGF)2, at low cell density (approximately 5×10^4 cells/cm^2) and in the absence of a strong cell adhesion substrate, attach loosely to the substrate and most of them die in 2–3 days. During this time, a very small fraction of cells become hypertrophic, round up, and begin to proliferate while remaining attached to the plate. Then, the progeny of the proliferating cells adhere to each other and form spherical clones. These spherical clusters, due to their increasing mass, eventually detach from the plate, float in suspension, and have

been called "neurospheres." Of note, not all the cells within a neurosphere are true SCs. Only a fraction of this progeny, ranging from 10 to 50% of the total cells generated, retain SC features, whereas the remainder of the cells undergo spontaneous differentiation. Indeed, a neurosphere represents a highly heterogeneous entity made up of SCs, differentiating progenitors, and even terminally differentiated neurons and glia; accordingly, it can be envisioned as the in vitro counterpart of the in vivo neurogenic compartment.

Importantly, neurospheres can be subcultured by mechanical dissociation and by replating under the same in vitro conditions. As in the primary culture, differentiating/differentiated cells rapidly die, whereas the NSCs proliferate, giving rise to secondary spheres that can then be further subcultured. This procedure can be sequentially repeated several times, and because each SC gives rise to many SCs by the time a sphere is formed, it results in the progressive enrichment and stable expansion of NSCs in culture.

When appropriately performed, the NSA allows clear differentiation between SCs and transiently dividing nonstem progenitors. The correct identification of the nature of the sphere-forming cell is a relevant issue. Indeed, committed progenitors, although endowed with limited proliferative capacity, can still produce up to tertiary neurospheres. Thus, to avoid any ambiguity in applying the definition of NSC to a candidate cell, the generation of clonal neurospheres by the NSA needs to be monitored. Both clonogenic and population analysis over an extended period needs to be measured not as absolute time frame, but as the number of subculturing steps that cells undergo in a given timeframe ***(15)***. This value is relative, and it strictly depends on cell-specific characteristics. It has been suggested that the minimum number of passages required to rule out the contribution of committed progenitors to the maintenance of the cell population is the one by which the generation of large quantities of daughter cells is achieved. As a consequence, it is possible to develop serial passage growth curves (i.e., population analysis) on which to base and theoretically extrapolate the SC frequency within the cell culture ***(15)***.

Here, we describe the methods developed and improved by our group for the isolation of different types of adult NSCs, their extensive culturing, and the assessment of their full differentiation potential.

2. Materials

2.1. Reagents for Culture Media

1. Dulbecco's modified Eagle's medium (DMEM)/F-12 (1:1) (Invitrogen; 32500-035).
2. Earle's balanced salts (EBSS) (Sigma-Aldrich, St. Louis, MO, cat. no. E-2888).
3. Glutamine: 200 mM (100×) (Invitrogen, cat. no. 25030-123).

4. Heparin: sodium salt, grade 1A (Sigma-Aldrich, cat. no. H-3393).
5. Glucose (Sigma-Aldrich, cat. no. G-7021).
6. HEPES (Invitrogen, cat. no. 15630-056).
7. $NaHCO_3$ (Invitrogen, cat. no. 25080-060).
8. Selenium (Sigma-Aldrich, cat. no. S-5261).
9. Progesterone (Sigma-Aldrich, cat. no. P-8783).
10. Putrescine (Sigma-Aldrich, cat. no. P-5780).
11. Apo-transferrin (Sigma-Aldrich, cat. no. T-2252).
12. Insulin (Sigma-Aldrich, cat. no. I-1882).

2.2. Growth Factors (GFs)

1. EGF: human recombinant (Peprotech, cat. no. 100-15B).
2. FGF2: human recombinant (Peprotech, cat. no. 100-18B).
3. Leukemia inhibitory factor (LIF; Chemicon International, cat. no. LIF 1010).

2.3. Miscellaneous

1. Phosphate-buffered saline (PBS) (1×) (Invitrogen, cat. no. 14190-169).
2. Penicillin/streptomycin (Invitrogen, cat. no. 15140-130).
3. Papain (Worthington DBA, Freehold, NJ, cat. no. 3120).
4. L-Cysteine (Sigma-Aldrich, cat. no. C-8277).
5. Ovomucoid (Sigma-Aldrich, cat. no. T-9128).
6. DNase (Sigma-Aldrich, cat. no. D-4513).
7. EDTA (Sigma-Aldrich, cat. no. E-6511).
8. Matrigel (GF-reduced) (BD Biosciences, Franklin Lakes, NJ, cat. no. 40230).
9. Laminin (Roche Diagnostics, Indianapolis, IN, cat. no. 1 243 217).
10. Poly-L-ornithine (Sigma-Aldrich, cat. no. P-3655).
11. Fetal bovine serum (Invitrogen, cat. no. 10270-106).

2.4. Instrumentation

1. Rocking platform (SpeciMix, PBI, Milan, Italy, cat. no. 14380).
2. Dissecting microscope (Carl Zeiss, Jena, Germany).
3. Dissecting tools (Martin Instruments, Munich, Germany).
4. Freezing jar (Criostep, Nalgene, PBI International, cat. no. 5100-0001).

2.5. Solutions

These cultures are extremely sensitive to contaminants present in water or glassware. Distilled sterile apirogenic water could be used (but before use, filter sterilize in sterile disposable plastic bottles). Otherwise, you can purchase ultrapure cell culture tissue grade water (Invitrogen, cat. no. 15230-147). A set

of glassware to be used only for tissue cultures should be prepared. Bottles, cylinders, beakers, and other glass should be accurately rinsed several times with distilled water before being sterilized in an autoclave that is used for tissue culture purposes only. We suggest that media and all stock solutions be prepared only in sterile disposable tubes and bottles.

1. 30% glucose: mix 30 g of glucose in 100 ml of water. Filter sterilize. Store at 4°C.
2. 0.2% heparin: mix 100 mg of heparin in 50 ml of water. Filter sterilize. Store at 4°C.
3. 10× stock solution of DMEM/F-12: dissolve the powder in 1 liter of water under gentle continuous stirring. Filter sterilize. Store at 4°C.
4. 3 mM sodium selenite: add 1.93 ml of water to a 1-mg vial of sodium selenite. Mix, aliquot into sterile tubes, and store at –20°C.
5. 2 mM progesterone: add 1.59 ml of 95% ethanol to a 1-mg vial of progesterone. Mix, aliquot into sterile tubes, and store at –20°C.
6. 10× stock solution of hormone mix: combine 40 ml of 10× DMEM/F-12, 8 ml of 30% glucose, 6 of ml 7.5% $NaHCO_3$, 2 ml of 1 M HEPES, and 306 ml of water. Add 400 mg of apo-transferrin. Dissolve 100 mg of insulin in 4 ml of sterile 0.1 N HCl, mix in 18 ml of water, and add all to the hormone mix solution. Dissolve 38.6 mg of putrescine in 20 ml water and add to hormone mix solution. Add 40 µl of 2 mM progesterone and 40 µl of 3 mM sodium selenite. Mix well and filter sterilize. Aliquot in sterile tubes and store at –20°C.
7. GFs stock: reconstitute EGF and FGF2 to have a 500 µg/ml stock. Aliquot into sterile tubes and store at –20°C.
8. Growth medium (for 500 ml): for DMEM/F-12-based medium, mix 375 ml of water, 50 ml of 10× DMEM/F-12, 10 ml of 30% glucose, 7.5 ml of 7.5% $NaHCO_3$, 2.5 ml of 1 M HEPES, 5 ml of 200 mM glutamine, 50 ml of 10× hormone mix, 1 ml of 0.2% heparin, 20 µl of EGF- and/or 10 µl of FGF2-stock (final concentration 20 ng/ml EGF and 10 ng/ml FGF2) and 5 ml PEN-STREP.
9. 10× stock solution of poly-L-ornithine: transfer the content of a 50-mg vial into a bottle containing 333.3 ml of water. Rinse vial twice with the solution, add rinse to solution. Mix well by swirling and sterilize by autoclaving (120°C for 20 min). Aliquot in sterile tubes and store at –20°C.
10. 100× stock solution of Matrigel: thaw a vial of Matrigel overnight at 4°C. Aliquot into sterile tubes (0.5 ml/aliquot) by using refrigerated plastic pipettes and store at –20°C.
11. 100× stock solution of laminin: thaw a vial of laminin solution (1 mg/ml) at room temperature. Aliquot into sterile tubes (20 µl/aliquot) and store at –20°C.
12. 0.1% DNase stock: add 10 ml of water to 10 mg of DNase. Mix well and filter sterilize. Aliquot in sterile tubes (0.5 ml/aliquot) and store at –20°C.

3. Methods

3.1. Isolation of NSCS from the Adult Rodent CNS

Discrete areas of the adult rodent brain, namely, the olfactory bulb and the hippocampus, are endowed with neurogenic activity ***(9)*** (*see* **Fig. 1**). The best-characterized source of NSCs is the SVZ of the forebrain lateral ventricles, which includes the SCs responsible for olfactory bulb neurogenesis. However, NSCs also have been described to reside in the adult mammalian spinal cord, along the ventricular neuraxis ***(20)***, in the SGZ of the DG of the hippocampus ***(4)***, and in the so-called SCZ ***(5)***.

The protocol described here to isolate adult rodent (mouse and rat) NSCs implies the use of enzymatic digestion, followed by mechanical dissociation. For subsequent SC expansion and for the establishment of SC lines, mechanical dissociation is required.

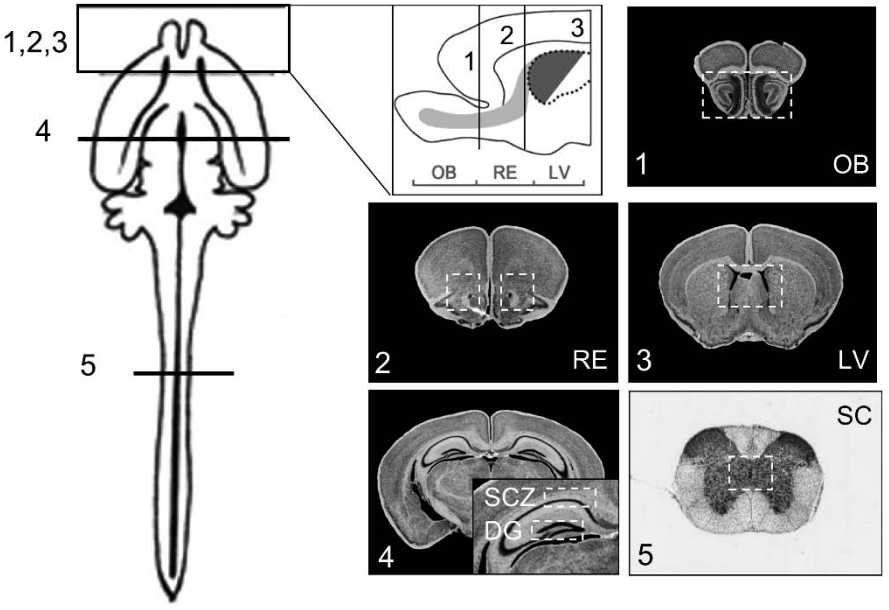

Fig. 1. Localization of the different neurogenic areas of the adult CNS. Distinct regions of the mature CNS are endowed with neurogenic potential. The best characterized is the SVZ lining the two lateral ventricles (*LV*), its rostral extension (*RE*) and its final target area, the olfactory bulbs (*OBs*) ***(1–3)***. Other regions are contained within (DG) or in proximity (SCZ) to the hippocampus ***(4)*** as well as along the spinal cord (*SC*) ependymal canal ***(5)***. The *dashed rectangles* within each section delimit the areas to be dissected for subsequent in vitro culturing.

3.2. Dissection Procedure

3.2.1. Preparation

Sacrificing of animals and removal and dissection of the brain, spinal cord, or both can be performed outside the laminar flow hood. Particular caution should be exercised to avoid contamination.

1. Weigh out papain, cysteine, and EDTA and transfer them into a sterile 50-ml plastic tube. For 50 ml of digestion solution, weigh out 47.2 mg of papain, 9 mg of cysteine, and 9 mg of EDTA. Keep the tube at 4°C until the end of the dissection procedure. The enzyme mix can be stored at 4°C for up to 1 week.
2. Weigh out about 10 mg of ovomucoid (enzyme inhibitor) in a plastic sterile 15-ml tube. Keep the tube at 4°C until the end of the enzymatic incubation period.
3. Add cold PBS to sterile plastic petri dishes: one or two 100-mm dishes to hold tissue; several 60-mm dishes to wash tissues; some 35-mm dishes to hold dissected tissues.
4. Dissection tools can be sterilized in a hot bead sterilizer, in a preheated oven (250°C for 2 h), or by autoclaving (120°C for 20 min).
5. Select tools needed to remove brain and spinal cord (large scissors, small pointed scissors, large forceps, small curved forceps, and a small spatula) or for the tissue dissection (small forceps, curved fine forceps, small scissors, curved fine scissors, and scalpel). Immerse the two sets of tools in 70% ethanol in two beakers with gauze on bottom, to avoid spoiling the tips of the microforceps and scissors.
6. Arrange and check the rocking platform.
7. Warm culture medium to 37°C in a thermostatic water bath.
8. Warm EBSS at room temperature.
9. Begin the dissection.

3.2.2. Removal and Dissection of Brain and Spinal Cord

1. Anesthetize mice by intraperitoneal injection of Avertin (1.2%; 1 ml/100 g), and kill them by cervical dislocation. Using large scissors, cut off the head just above the cervical spinal cord region. Rinse the head with 70% ethanol.
2. Using small pointed scissors, make a medial caudal-rostral cut and remove the skin off the head. Rinse the skull with sterile PBS.
3. Using the small scissors, make a longitudinal cut through the skull along the sagittal suture. Be careful not to damage the brain.
4. Using curved, pointed forceps, grasp and peel the skull of the right hemisphere outward to expose the brain. Repeat for the left hemisphere.
5. Using a small curved spatula, scoop the brain in a petri dish containing PBS.
6. To remove spinal cord: cut the dorsal muscles to isolate the whole backbone; and, by using small pointed scissors, cut the vertebral disks on the lateral sides to expose the cord. Cut the spinal nerves, remove the spinal cord, and transfer it to a petri dish containing PBS.

7. Wash brain and spinal cord twice by subsequently transferring them to new petri dishes containing PBS.
8. To dissect the forebrain SVZ, the SGZ, and the SCZ (*see* **Fig. 1**), place dish containing the brain under the dissecting microscope (×10 magnification). Position the brain flat on its ventral surface and hold it from the caudal side using fine curved forceps. Use scalpel to cut the olfactory bulbs. Make two coronal cuts to dissect an approximately 2-mm-thick slice embodying the lateral ventricles. If you want to dissect tissues of the forebrain rostral extension (RE) and SGZ/SCZ, intermediate cuts have to be made in order to collect coronal slices not including the lateral ventricles (*see* **Fig. 1**). Discard the remaining pieces of the brain and keep the sliced tissue.
9. Shift to a ×25 magnification. Using fine curved microscissors, cut the thin layer of tissue surrounding the ventricles, excluding the striatal parenchyma and the corpus callosum. Make sure not to contaminate the tissue with meninges.
10. Place dissected tissue into labeled 35-mm petri dishes containing sterile PBS.
11. Place the dish containing spinal cord brain under the dissecting microscope (×10 magnification). Carefully remove spinal nerves and meninges.
12. Cut a 1-mm-thick coronal section and dissect out the region surrounding the ependymal canal placing it into labeled 35-mm petri dishes containing PBS.
13. Move into tissue culture laminar flow hood. From this point on, use aseptic technique.

3.3. Dissociation of Digested Neural Tissue and Primary Culture

3.3.1. Dissociation

1. Using fine scissors cut dissected tissue into small pieces.
2. Add 30 ml of EBSS to the tube containing papain/cysteine/EDTA. Vortex until the solution is clear, add 0.5 ml of 0.1% DNAse stock, add a further 20 ml of EBSS, and filter sterilize. Label sterile plastic conic bottom 15-ml tubes (one tube for each CNS region) and add 12 ml of papain/DNAse solution to each.
3. Transfer the pieces of tissues into the 15-ml tubes containing the papain mix solution (*see* **Note 1**).
4. Transfer the tubes to the rocking platform. Incubate at 37°C for 30–60 min, depending on the amount of tissue and on its consistency. Generally, spinal cord requires longer incubation than the subventricular region.
5. At the end of the enzymatic incubation, pellet tissues by centrifugation at 110 g for 10 min.
6. In the meantime, add culture medium to the tube containing ovomucoid to obtain a 0.7 mg/ml solution (10 mg/14 ml). Filter sterilize.
7. Remove almost all the supernatant overlaying the pellets (you should not use the vacuum, because there is a substantial risk of sucking out the tissue as well). Add 3 ml of ovomucoid solution. Dissociate by triturating 20–30 times using a sterile, fire-polished, cotton-plugged glass Pasteur pipette. Let the suspension settle down for 3–4 min. If many undissociated pieces of tissue remain, move cell

suspension to a clean, labeled tube leaving about 1 ml behind. To the latter, add 2 ml of fresh ovomucoid solution by using a new fire-polished, cotton-plugged glass Pasteur pipette, triturate again 20–30 times, until almost no undissociated pieces are left. Let the suspension settle down for 3–4 min. Transfer almost all the volume of this tube to the labeled tube, thereby pooling the cells from both trituration steps (*see* **Notes 2** and **3**).

8. Pellet the cells by centrifugation at 110 g for 10 min.
9. Remove the supernatant leaving behind about 1,000 µl. Using a 1,000-µl pipetteman with the volume set at 800 µl, gently dissociate the pellet 20–25 times.
10. Add 12–14 ml of culture medium and pellet cells by centrifugation at 15 × *g* for 15 min.
11. Discard supernatant and resuspend cells in 200 µl of culture medium. Using a 200-µl pipetteman with the volume set at 180 µl, gently dissociate the pellet 20–25 times. Dilute a 10-ml aliquot from each sample in trypan blue and count in a hemocytometer (initially try a one-half dilution) (*see* **Note 4**).
12. Seed cells at a density of 3,500 viable cells/cm^2in culture medium, in untreated six-well tissue culture dishes (3-ml volume) or 25-cm^2 tissue culture flasks (8-ml volume) (*see* **Note 5**).
13. Incubate at 37°C, 5% CO_2 in a humidified incubator.
14. Cells should proliferate to form spherical clusters, which eventually detach from the plate as they grow larger. These primary spheres should be ready for subculturing 5–10 days after plating, depending on the GF(s) used.

3.4. Isolation of Tumor SCs from CNS Tumors

Recent evidence proposes that one of the cell types frequently hit by oncogenic transformation may be identified with tissue-specific (somatic) SCs ***(21)***. In fact, SCs share important functional properties with tumor cells. These include significant proliferative capacity, extensive self-renewal, wide differentiation potential, and the ability to regenerate tissue of residence/origin. Also, SCs are known to depend upon the same molecular pathways that are often reactivated during tumor initiation and progression ***(22,23)***. In line with these assumptions, the involvement of somatic SCs in the process of tumorigenesis ***(23)*** has been described in different types of brain tumors, including glioblastomas multiforme (GBM), medulloblastomas (MBs), and neuroblastomas ***(24)***. Historically, the existence of putative SCs within highly aggressive CNS tumors as GBMs has been documented by exploiting the NSA ***(25)***. The authors suggested that clonogenic precursors could be identified by means of the NSA within different postsurgery specimens of GBM when exposed to both EGF and FGF2. Subsequently, two other research groups confirmed and extended the original demonstration by proposing that MBs also might contain

a compartment of true SCs ***(25–27)***. All these initial reports, suggestive of the presence of putative TSCs within brain cancers, limited their analysis to the generation of neurospheres up to the third subculturing passage. Thus, these reports did not rule out the chance that the neurosphere-generating cells might be short-term proliferating progenitors rather than bona fide SCs. However, our group and others demonstrated that, indeed, GBMs include neurosphere-forming cells that expand in number exponentially, establishing long-term propagating tumor SC lines. These lines demonstrate extensive self-renewing capability and tumorigenic ability ***(28,29)***.

3.4.1. Collection of Tumor Tissue(s)

Human tumor tissues obtained as postsurgery samples or biopsies should be collected in complete, ice-cold growth medium containing antibiotics. The tumor tissue should be separated from the remainder of the material (hemorrhagic and necrotic areas) and processed as soon as possible (*see* **Note 6**).

3.4.2. Dissection Procedure

3.4.2.1. Preparation

1. It is extremely important to have all the materials and instrumentation ready before starting the dissection.
2. Add cold sterile PBS to sterile plastic petri dishes:
    a. two 100-mm dishes to hold tissues,
    b. several 100-mm dishes to wash tissues,
    c. several 35-mm dishes to hold dissected tissues.
3. Dissection tools can be sterilized in a hot bead sterilizer, in a preheated oven (250°C for 2 h), or by autoclaving (120°C for 20 min).
4. Immerse the different sets of tools in 70% ethanol in separate beakers with gauze on their bottom, to avoid spoiling the tips of forceps and scissors.
5. Arrange the dissecting microscope, the dishes containing PBS and the dissection tools into the laminar flow hood. Keep some sterile petri dishes and PBS ready at hand.
6. Prepare the digestion mix as described in **Subheading 3.2.1.**
7. Warm up culture medium to 37°C in a thermostatic water bath.

3.5. Removal and Collection of Tumor Tissue

1. Place dish under dissecting microscope at high magnification (×25) and dissect out the desired tissue fragment(s) to be used for establishing the culture.
2. Place tissue in a 35-mm dish containing ice-cold PBS.

3.5.1. Dissociation of Tumor Tissues and Primary Culture

3.5.1.1. Preparation

1. Set up the laminar flow hood for the dissociation procedure. If using the same hood used for the dissection procedure, remove everything and thoroughly clean and disinfect the working surface with 70% ethanol.
2. Filter sterilize an appropriate volume of prewarmed culture medium in 50-ml plastic tubes.
3. Label sterile plastic conical-bottomed 15-ml tubes (one tube for each brain region) and add 3 ml of culture medium to each tube.

3.5.1.2. Dissociation

1. Proceed as described under **Subheading 3.3.**, **steps 1–8**.
2. Pellet the cells by centrifugation at 110 × g for 10 min.
3. Remove the supernatant leaving behind about 1,000 µl. Using a 1,000-µl pipetteman with the volume set at 800 µl, gently dissociate the pellet 20–25 times.
4. Add 12–14 ml of culture medium and pellet cells by centrifugation at 15 × g for 15 min. Repeat the procedure twice in order to eliminate tissue-derived debris (*see* **Note 7**).
5. Discard supernatant and resuspend cells in 0.2 ml of culture medium. Using a 200-µl pipetteman with the volume set at 180 µl, gently dissociate the pellet 20–25 times. Add 12–14 ml of culture medium, and pellet cells by centrifugation at 15 × *g* for 15–20 min.
6. Resuspend cells in 0.2 ml of culture medium and dilute a 10-ml aliquot from each sample in trypan blue; count in a hemocytometer (initially try a one-half dilution).
7. Seed cells at a density of 3,500 viable cells/cm^2 in culture medium, in untreated six-well tissue culture dishes (3-ml volume) or 25-cm^2 tissue culture flasks (8-ml volume).
8. Incubate at 37°C, 5% CO_2 in a humidified incubator.
9. Single cells should proliferate to form spherical clusters (primary neurospheres). The time in which primary neurospheres develop in culture ranges from 3 to 30 days after plating, and it depends on the GFs used and the type of tumors (generally, MB cultures generate primary neurospheres faster than GBMs) (*see* **Note 8**).

3.5.2. Differences Between Adult Rodent NSC and Human TSC Cultures

Although the basic protocol given above allows the culturing of both human and rodent NSCs, some differences do exist between species.

1. With few exceptions, adult rodent SCs can be expanded in the presence of either EGF or FGF2 alone, whereas the combined use of these GFs results in a faster growth rate ***(30)***. Conversely, human TSCs require the simultaneous exposure to both EGF and FGF2 to be isolated and expanded efficiently in vitro ***(28)***.

2. The growth rate of human cells is significantly slower than their mouse counterpart, at least at the beginning of the culture. Under our best culture conditions, human TSCs cells display a doubling time ranging between 3 and 12 days, compared with 2–5 days for rodent NSCs.

3.5.3. Propagation of NSCs/TSCs in Culture

3.5.3.1. Subculturing Protocols

Subculturing is one of the most critical steps in NSC growth. As opposed to serum-enriched adherent cultures, neurospheres grow as compact clones floating in suspension. To expand the number of SCs in culture, these clones need to be disaggregated by mechanical dissociation. Upon subculturing, most of the SCs survive and generate secondary spheres, with a variable efficiency depending on the different regions, age, or species of origin. Many parameters may influence subculturing efficiency. Amongst these, loss of cells due to poor or excessively harsh mechanical dissociation, plays a prominent role in decreasing the subculturing efficiency. The protocol described below is essentially identical for different NSC cultures, and it also can be applied to generate clonal SC lines.

1. Tap sides of flasks to be sub-cultured to dislodge spheres and transfer content of the flask to 15ml sterile plastic conical tubes using a sterile plastic pipette. Use 5ml fresh medium to rinse flask and add rinse to the tube.
2. Pellet cell suspension by centrifugation at 110g for 10 minutes (in the case of culture containing high amount of debris, perform centrifugation at 15g for 15–20 minutes to get rid of the debris).
3. Remove the supernatant leaving behind approximately 200 µl.
4. Using a sterilized p200 pipetteman set at the volume to 180 µl. Rinse a 200-µl tip with medium first, to avoid cell sticking inside the tip, and gently triturate pellet: 40–50 times for adult rodent cells, and up to 200 times for human TSCs. Rinse down sides of tube periodically to dislodge undissociated spheres. Slightly tilt the pipetteman and press tip against the bottom of the tube to generate a fair amount of resistance. For cultures containing high amount of debris, dead cells, or both, add 15 ml of culture medium and spin once more at $15 \times g$ for 15 min. Remove supernatant and gently dissociate 10–20 times to disaggregate the pellet.
5. Count viable cells by trypan blue exclusion, and seed cells at the appropriate density in culture medium in untreated tissue flasks: 1×10^4 cells/cm^2 for murine cells and 1×10^4 cells/cm^2 for human TSCs. Subculture when the spheres reach an average diameter of 100–300 µm. If spheres are too small when subcultured, the yield will be low; conversely, if spheres are allowed to grow for too long before being dissociated, the number of dead cells inside the spheres will be too high, and the viability of the culture will be very low. Should this situation be protracted for some subculturing steps, the total number of cells in the culture will progressively decrease, and the culture will be lost. The average subculturing

time will require, approximately, 3–7 days for adult murine NSCs and 3–10 days for human TSCs. The total cell number should increase two to sixfold at each passage for adult murine NSCs and three to tenfold for human TSCs.

3.6. Differentiation of SCs (see Note 9)

One of the cardinal properties of SCs is their capacity to generate all the cell lineages typical of their tissue of residence. Specifically, NSCs should give rise to neurons, astrocytes, and oligodendrocytes, thereby demonstrating their capability to be multipotent. Different experimental paradigms are available to induce undifferentiated NSCs to exit the cell cycle and progressively differentiate into fully mature progeny. The process of differentiation entails qualitative changes, which end up with the generation of committed cells displaying specific phenotypic characteristics as a differentiated morphology and the expression of antigens typical of neuronal or glial cells. Through the process of maturation, the NSC differentiated progeny become functionally active.

Whereas NSCs as undifferentiated cells proliferate and self-renew efficiently in suspension, they need to be plated onto a strong adhesion substrate like laminin or Matrigel with simultaneous removal of GFs to promote their spontaneous differentiation into neuronal and glial cells.

The procedure described below can be applied to different NSC types, and it is based on plating of NSCs in the absence of GFs. Withdrawal of mitogens represents a highly restrictive condition that ultimately might affect the overall survival of rodent NSCs. Often, adult NSCs do differentiate, but cultures start to die 3–4 days after mitogen removal. This shortcoming can be limited by plating NSCs in the presence of FGF2 for 2–4 days. However, because FGF2 is known to prevent the differentiation/maturation process of glial and neuronal/glial precursors, removal of FGF2, and addition of low concentration of serum (1%) are both required to elicit neuronal maturation and to allow the detection of glial antigens.

In contrast, the differentiation process of TSCs is peculiar, and it should take into account relevant tumor cell-specific features. As opposed to normal NSCs, undifferentiated proliferating TSCs express antigenic markers of differentiated neural lineages as Tuj1 and glial fibrillary acidic protein (GFAP), thereby precluding the use of these antigens as reliable markers of differentiation. In addition, these tumor cells are highly resistant to cell death, and, even under differentiating conditions, a fraction of TSCs still undergo active proliferation. Therefore, to properly assess the multipotency of TSCs and to induce effective TSC differentiation, the basic protocol described above needs to be implemented. First, it is necessary to eliminate GFs from the beginning of the differentiation culture to minimize cell proliferation. Second, block of

proliferation and enhancement of differentiation must be obtained by the simultaneous addition to the mitogen-free medium of powerful prodifferentiation factors as LIF, bone morphogenetic protein (BMP)-4, or both ***(31,32)***. Last, the overall time frame of differentiation should be extended up to 15–20 days in vitro. It is worthwhile to note that the detection of differentiated tumor SC progeny, simultaneously labeled for neuronal and glial markers, should be expected, because it is an established marker of aberrant tumor cell differentiation (*see* **Fig. 2**) ***(28,29)***.

3.6.1. Preparation

1. Immerse round glass-coverslips in 70% ethanol. Dry with thin adsorbent paper. Place in a glass petri dish and sterilize in preheated oven (250°C for 2 h).
2. Using sterilized fine forceps, add one coverslip to each well of a 48- or 24-multiwell plate. Alternatively, glass or plastic chamber-slides can be used.

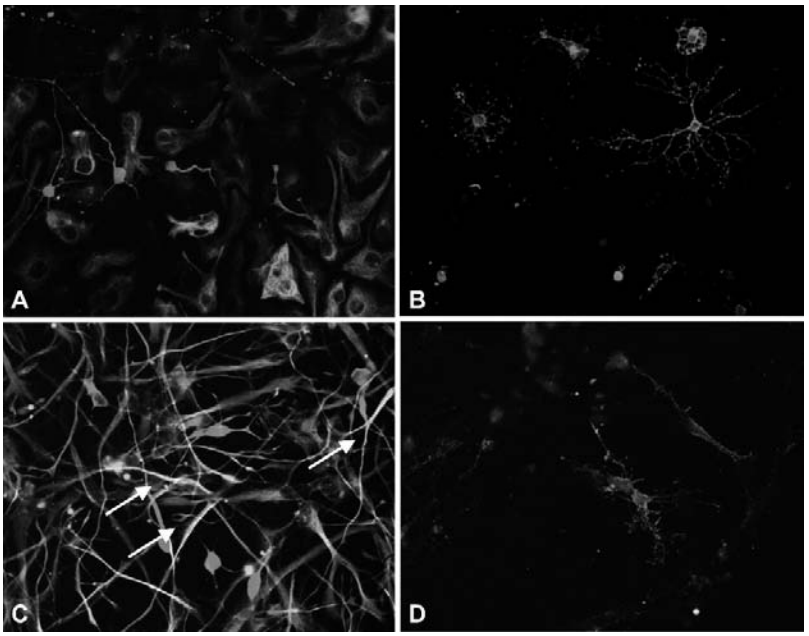

Fig. 2. Differentiation of adult rodent NSCs and human TSCs. Plating of NSCs **(A,B)** and TSCs **(C,D)** in the absence of mitogens and in the presence of serum or specific cytokines prompt their differentiation into Tuj1 immunoreactive neurons (**A,C**), GFAP-positive astrocytes (**A,C**), and galactocerebroside immunoreactive oligodendrocytes (**B**,**D**). Note the presence of cells double labeled for neuronal and astroglial markers in human TSC cultures (**C**; *arrows*). Magnification ×40.

3. Prepare adhesive substrate solutions: dilute laminin or Matrigel (1/100 of the stock solution in control medium (i.e., without GFs).
4. Add 125 µl of the chosen substrate for each square centimeter of well surface. Incubate at 37°C for at least 2 h. Rinse thoroughly with sterile PBS.

3.6.2. Differentiation

1. Tap sides of flasks to dislodge spheres.
2. Remove content of the flask to 15-ml sterile plastic conical tubes by using a sterile plastic pipette.
3. Pellet cell suspension by centrifugation at 110 × *g* for 10 min.
4. To wash cells from GFs, remove supernatant and resuspend cells gently with a plastic pipette in 15 ml of mitogen-free medium. Spin at 110 × *g* for 10 min.
5. Remove the supernatant leaving behind about 200 µl. Using a sterilized p200 pipetteman set at 200 µl, gently triturate the pellet (100–150 times for human TSCs, 50–60 times for adult rodent cells). Count viable cells by trypan blue exclusion. Resuspend cells in the appropriate volume of mitogen-free medium alone (adult rodent NSCs) or (a) containing 1% fetal bovine serum (FBS; adult rodent NSCs); or (b) containing 20 ng/ml LIF or 50 ng/ml BMP-4 (human TSCs). The final number of cells to be plated per well should be contained in 0.5 or 1 ml for 48- and 24-well plates, respectively. Cell density: human TSCs, 30,000–60,000 cells/cm^2; adult rodent NSCs, 50,000–70,000 cells/cm^2. Agitate plate to distribute cells evenly on the coverslips.
6. Incubate at 37°C in a humidified incubator for the appropriate time.
7. The protocol described for dissociated cells can be applied to differentiate single spheres as a whole, which can be plated onto the growth substrate without being previously triturated (*see* **Notes 10–12**).

4. Notes

1. The 12-ml volume of papain solution is sufficient for a good digestion of tissue from two to three mice. Pooling tissues from more than three animals may require a larger volume of papain solution to avoid poor enzymatic digestion.
2. To prevent tissue and/or cell from sticking to the walls of the Pasteur pipette, rinse the pipette several times with medium before every dissociation step, or when a new pipette is used.
3. During trituration, always avoid foaming and bubbles.
4. In primary cultures from adult brain, a large amount of debris is normally present, particularly in spinal cord cultures, together with adherent cells. To reduce debris, you may repeat **steps 9–11**, increasing centrifugation time to 20 min. Generally, debris and adherent cells are eliminated after a few passages.
5. Counting cells is sometimes difficult due to the presence of debris and to the small number of cells that can be isolated. In our experience, this protocol

should yield about 5×10^4cells from the subventricular region of one brain. Thus, if no careful quantification of the primary NSC number has to be carried out, cell suspension derived from two mice may be plated in four dishes of a six-well tissue culture dish, yielding an approximate final cell density of about 3,500 cells/cm^2, or in one 25-cm^2 tissue culture flask, obtaining a final density of about 4,000 cells/cm^2.

6. Dissection should be carried out as quickly as possible (possibly within 2 h) from tissue collection. Over time, tissue becomes soft and sticky and may be difficult to process. If you estimate that more than 2 h is required, keep the tissues at 4°C.
7. In primary cultures from tumor tissues, you should expect the presence of a large amount of debris and of some non-neural adherent cells of the tumor stroma (endothelial cells and fibroblasts). Both debris and adherent cells should disappear after a few subculturing passages.
8. After a few days in culture, cell aggregates may be observed which can be mistaken for primary spheres. This is particularly evident if debris and dead cells are present in excess in the cultures. Cell aggregates are normally large, but the cells that make them up are rather small, phase-dark, and irregularly shaped. Bona fide, primary neurospheres look like small clusters of round, phase-bright cells, which may often be covered and sometimes hidden by debris and dead cells. The early subculturing passages are critical for the selection of proliferating SCs while getting rid of cell aggregates, debris, dead cells, and short-term dividing precursors at the same time (*see* **Subheading 3.5**).
9. Variability between experiments is perhaps the most critical and limiting step for the analysis of NSC differentiation in term of ratio of the lineages generated. The first source of this variability is the heterogeneity of the starting CNS SC population. To increase homogeneity in your adult NSC population, use spheres that have been subcultured at least twice so that in your starting cultures short-term dividing precursors are absent. Generally, an increase in subculturing passages does not affect the proportion of the different cell types produced by SC progeny upon differentiation.
10. To have a good cell yield, do not use spheres that have grown too much (*see* **Subheading 3.5**).
11. By taking into account the growth rate of your cell line(s), harvest spheres at a fixed number of days after the last subculture passage.
12. If dissociation has been efficient, almost the totality of the cells plated after subculturing should be single cells that eventually proliferate in response to GF(s). If you harvest cultures when cells occur mainly as doublets or very small clusters, the culture will result highly enriched in SC and highly undifferentiated precursors.

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8

Olfactory Ensheathing Cells: *Isolation and Culture from the Neonatal Olfactory Bulb*

Susan C. Barnett and A. Jane Roskams

Summary

Olfactory ensheathing cells (OECs), which are glia from the olfactory system, have evolved as attractive candidates for transplant-mediated repair based on long-standing knowledge that the olfactory system is one of the only central nervous system tissues that can support neurogenesis throughout life. After injury and during normal cell turnover, the olfactory receptor neurons (ORNs) die, and new nerves are generated from putative stem cells in the olfactory epithelium. OECs, which reside throughout the olfactory system, guide the ORN axons as they travel through the olfactory mucosa (olfactory epithelium and lamina propria) and the cribriform plate, terminating in synapse formation in the usually nonpermissive environment of the olfactory bulb. It is this ability to support axonal outgrowth throughout life that has made olfactory tissue such a promising focus for repair strategies. Here, we provide a method to purify OECs—from the rat olfactory bulb and in Chapter 9, from the turbinates of the mouse olfactory epithelium.

Key Words: Olfactory system; glia; olfactory bulb; olfactory mucosa; purification.

1. Introduction

The ability to produce highly purified populations of individual cell types is crucial for examining the molecular regulation of cell growth, differentiation, and function. Olfactory ensheathing cells (OECs) are also known as olfactory ensheathing glia and olfactory nerve ensheathing cells. These glia do not fit the classic description of stem cells, but they have several intriguing developmental characteristics that have piqued the interest of the developmental

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neurobiology community as of late. Their ability to support neuronal regeneration, both within the olfactory system and elsewhere in the central nervous system (CNS), has made them an attractive cellular model for transplantation paradigms. Their ability to switch, in vivo, from a nonmyelinating to a myelinating state, has enabled investigators to examine OEC events leading to the onset of myelination.

In trying to purify OECs and examine their characteristics in a controlled in vitro environment, several obstacles have to be overcome. The cellular population of the olfactory bulb consists primarily of oligodendrocytes, astrocytes, OECs, and several types of neurons. The OECs reside in the outer layer of the bulb termed the olfactory nerve layer ***(1–3)*** and also ensheath the olfactory nerve as it travels from the olfactory epithelium to the olfactory bulb. OECs from the olfactory nerve and bulb are antigenically similar, and they share many similar properties in vivo. For example, based on antigenic and morphological criteria, both tissues contain two types of OECs, one type of which has Schwann cell (Sc)-like properties and the other type that has astrocyte-like properties ***(4,5)***. Studies on the OEC from the olfactory bulb have shown that the Sc-OEC has a spindle-like morphology, expresses the low-affinity nerve growth factor receptor (L-NGFr) $p75^{NTR}$ ***(6)***, and contains diffuse staining for the glial-specific intermediate filament glial fibrillary acidic protein (GFAP) ***(7)***. The astrocyte-like OEC has a flattened morphology and expresses the embryonic form of neural cell adhesion molecule (E-NCAM, otherwise known as the polysialic form of NCAM ***(8,9)***), contains fibrous GFAP, and does not express $p75^{NTR}$ ***(5)***. Microarray studies have demonstrated stronger coincident gene expression shared by OECs and Scs than with OECs and astrocytes ***(10)***. It is now generally held that OECs are extremely plastic cells that can rapidly change their antigenic and morphological phenotype and that these changes depend on the in vitro or in vivo environment in which they find themselves ***(11)***. OECs prepared from both sources exhibit different growth rates in equivalent media; different rates of senescence; and, after transplantation, they also demonstrate some differences in their migration patterns and their ability to promote repair ***(12)***.

Here, we outline a procedure to culture a defined subpopulation of OECs—from the nerve fiber layer of the neonatal rat olfactory bulb, although other alternative methods to this (such as $p75^{NTR}$ immunopanning) have been published ***(13)***. It is also worth noting that biologically important aspects of OEC behavior (e.g., their ability to myelinate) also have been shown to vary with the kind of preparation used and the age from which the OECs are prepared ***(14)***. The following chapter outlines a procedure for culturing OECs from the lamina propria of the olfactory epithelium.

2. Materials

2.1. Purification of OECs from the Olfactory Bulb

1. Leibowitz medium (L-15, Invitrogen, Carlsbad, CA) + 25 μg/ml gentamicin (ICN Flow, High Wycombe, UK) (for collecting dissected olfactory bulbs).
2. Hanks' balanced salt solution (HBSS), Ca^{2+}- and Mg^{2+}-free (Invitrogen) (for dissociating tissue through needles).
3. Collagenase, stock of 13 mg/ml in L-15 (~155 U/mg; MP Biomedicals, Irvine, CA).
4. Monoclonal antibodies.
   a. O4 (Roche Diagnostics, Indianapolis, IN; ***(15)***).
   b. Anti-galactocerebroside (anti-galactocerobroside [GalC]; ***(16)***).
   c. Anti-low-affinity nerve growth factor receptor ($p75^{NTR}$) (Roche Diagnostics). A2B5 ***(17)***.
5. Second class-specific antibodies:
   a. Anti-mouse IgM-fluorescein (Southern Biotechnology Associates, Birmingham, AL).
   b. Anti-mouse IgG3-phycoerythrin (Southern Biotechnology Associates).
6. Poly-L-lysine (PLL, <100,000 mol. wt.; Sigma-Aldrich, St. Louis, MO), stock 4 mg/ml, dilute 1:300 in sterile double-distilled water; use at a final concentration of 13.3 μg/ml.

2.2. Generating Astrocyte Conditioned Media (ACM)

1. 0.25% trypsin dissolved in L-15 (Sigma-Aldrich).
2. SD media: soybean trypsin inhibitor (0.52 mg/ml), DNAse (0.04 mg/ml), and bovine serum albumin (3 mg/ml) in Dulbecco's modified Eagle's medium (DMEM) (SD) dissolved in L-15 (all from Sigma-Aldrich). Store as frozen aliquots at –20°C.
3. DMEM + 10% fetal calf serum (FCS).
4. Cytosine arabinoside (Ara-C; Sigma-Aldrich) stock of 1 mM in DMEM.
5. DMEM-BS: DMEM (Invitrogen), 4.5 g/l glucose, and supplemented with 25 μg/ml gentamicin (Invitrogen), 0.0286% bovine serum albumin Pathocyte (MP Biomedicals), 0.5 μg/ml bovine pancreatic insulin (Sigma-Aldrich), 100 μg/ml human transferrin, 0.2 μM progesterone, 0.10 μM putrescine, 0.45 μM L-thyroxine, 0.224 μM selenium, and 0.49 μM 3,3',5-triiodo-L-thyronine (all from Sigma-Aldrich) DMEM-BS ***(18)***.
6. Anti-GFAP antibody (DAKOpatts, Copenhagen, Denmark).
7. Coating coverslips/flasks with PLL: pipette enough PLL to coat the appropriate dish/flask and incubate 0.5 h or overnight in a 37°C incubator. Remove all the PLL and leave the dish/flask to air dry in the hood before use. Dry PLL-coated dishes can be stored for at least a week at room temperature.

3. Methods

3.1. Primary Culture of Astrocytes for Generating ACM

1. Remove the cortex from 1-day-old or newborn rat pups and place in a small petri dish containing 1 ml of L-15 + gentamicin.
2. Dissect off the central tissue (midbrain), followed by the edge tissue and hippocampus, until a flat, white, butterfly-shaped piece of cortical tissue remains. Flip this over and peel off the meninges (*see* **Fig. 1**).
3. Chop up the cortex by using a sterile scalpel and place in a 30-ml plastic sterile universal container (Bibby Sterilin, Stone, Staffordshire, UK). For 15 animals, place the cortices in 2 ml of L-15 and 1 ml of collagenase and incubate at 37°C for 30 min.
4. Add 1 ml of 0.25% trypsin and incubate at 37°C for a further 20 min.
5. Centrifuge the cells at 800 × *g* for 5 min and then remove the supernatant.
6. Add 1 ml of EDTA and 1 ml of 0.25% trypsin to the pellet and incubate for a further 20 min at 37°C.
7. Add 1 ml of SD media from frozen aliquots, pipette up and down to mix, then centrifuge the cells at 1,000 × *g* for 5 min and remove the supernatant.
8. Add 5 ml of DMEM/10% FCS and gently pass through a 5-ml pipette five times (*see* **Note 1**).
9. Count the cells and plate at 1.5–2 × 10^7 cells/75-cm^2 flask in 10 ml of DMEM/10% FCS.
10. Feed and maintain cultures in DMEM/10% FCS the next day to remove debris. Keep feeding twice a week. When cells are completely confluent and no more obvious cell division can occur, proceed with purification.

3.2. Purification of Astrocyte Population

1. To remove top layer of oligodendrocytes, shake the cells on a rocker platform overnight at 37°C (*see* **Note 2**).
2. The next day, feed with DMEM/10% FCS containing 2 × 10^{-5} M Ara-C, to kill off actively dividing oligodendrocytes and progenitor cells.

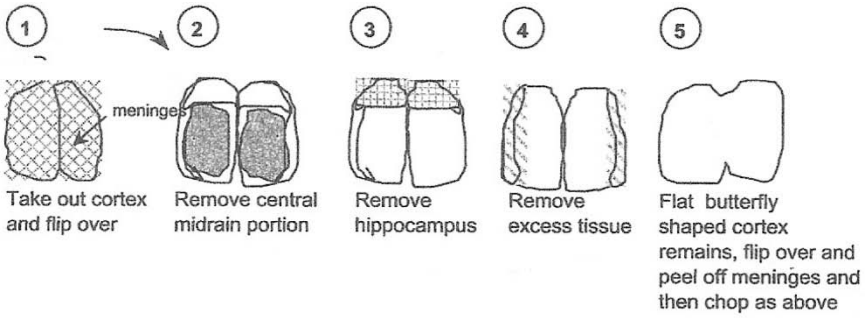

Fig. 1. Dissection of cortex for culturing astrocytes.

3. Feed again the next day with DMEM containing 10% FCS and maintain the cells in this medium until ready to collect conditioned medium DMEM-BS from the confluent astrocytes (*see* **Note 3**).
4. When astrocytes are confluent and pure, add DMEM-BS and leave on for 10 min, then remove and add 10 ml of fresh DMEM-BS for 48 h.
5. Collect the DMEM-BS and replace with DMEM/10%FCS. Spin the ACM at 1,000 × *g* for 5 min.
6. Filter sterilize by passing through a 0.2-µm filter and freeze at –20°C in aliquots of 20 ml. This is your concentrated ACM that is usually added at 1:5 diluted in DMEM-BS to cells. Stocks can be frozen for several months (at –20°C) and once thawed last 1–2 weeks at 4°C.

3.3. Dissection and Enzymatic Digestion of Olfactory Bulb

Purified cultures of OECs are generated from neonatal rats (1–7-day-old pups). It is possible to use fluorescence-activated cell sorting (FACS) as a purification technique for both younger and adult preparations, but cell numbers may be limited from embryonic tissue. However, these cells can be placed in culture and grown to increase cells number in OEC medium and FACS sorted with the anti-$p75^{NTR}$ by using the same method described here. We have used a range of rat strains and routinely use either Sprague-Dawley or Fischer-344 (for in vivo work, inbred strain). For optimal results, we use 15–25 animals per preparation.

1. Decapitate the rats according to humane animal care protocols and rapidly remove the skin and skull to reveal the olfactory bulbs (*see* **Fig. 2**).
2. Remove the olfactory bulbs. The bulbs are readily visible and are separated from the brain using the tip of curved forceps to sever connections at the cribiform

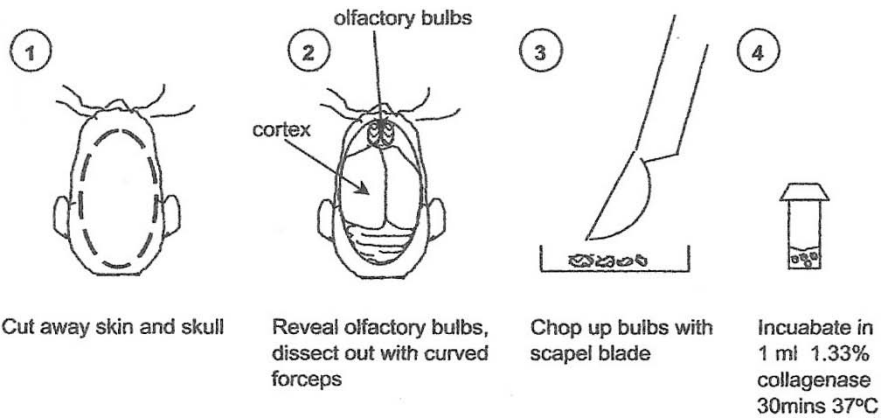

Fig. 2. Dissection of olfactory bulb for culturing OECs.

plate, taking care not to remove any cortical tissue. The bulbs are lightly scooped from the head and placed in a 35-mm petri dish in containing 2 ml of L-15 medium + gentamicin (Invitrogen).

3. Chop the tissue using a scalpel blade with rapid chopping motion moving the dish around in a circle so that all the pieces become evenly cut and the tissue pieces that remain are all roughly the same size of around 0.5 mm^2 (*see* **Fig. 2**). Then, transfer tissue using a sterile plastic pipette into a polystyrene 7-ml Bijou (Bibby Sterilin) containing 500 µl of collagenase (1.33%; MP Biomedicals in L-15 medium) and 1 ml of L-15 + gentamicin and incubate for 30 min at 37°C in the incubator) (*see* **Note 4**).
4. After tissue digestion, generate a single-cell suspension by sequentially passing the cells two to four times through a series of decreasing gauge (G) needles, starting with a 19G needle followed by 20G and ending with a 23G needle, keeping the cell suspension in the Bijou. This must be carried out very gently, because the cells are very fragile. If clumps remain, pass the cells through a 20G needle one additional time.
5. Transfer the final cell suspension into a 15-ml centrifugation tube (Falcon; BD Biosciences Discovery Labware, Bedford, MA), add 4 ml of HBSS, Ca^{2+}- and Mg^{2+}-free media, and spin at 1,000 × g for 5 min at room temperature.

3.4. FACS Purification of OECs

1. Incubate the olfactory bulb suspension for 1 h at 4°C with hybridoma supernatant from the O4 and GalC cell lines, both diluted 1:1 in L-15 or DMEM/1% FCS (*see* **Note 5**).
2. Wash cells once by centrifugation at 1000 × *g* at room temperature for 5 min in DMEM containing 1% FCS.
3. Transfer the cells into a 15-ml centrifugation tube (Falcon; BD Biosciences Discovery Labware) and add 500 µl of DMEM containing 1% FCS and a 1:100 dilution of class specific antibodies IgM-fluorescein and IgG3-phycoerythrein (*see* **Note 6**).
4. Perform secondary antibody incubation for 45 min at 4°C.
5. Wash the cells (**step 2**) twice in DMEM containing 1% FCS, place in snap-top 5-ml sterile tubes at 4 × 10^6 cell/ml, and purify using their O4-positive/GalC-negative profile (*see* **Fig. 3**).
6. After FACS purification, cells are plated onto PLL-coated coverslips in 30 µl to test for purity. Volumes must be kept low to ensure high cell density, because OECs require close cell contact. Approximately 30 coverslips can be made from 20 animals and for each milliliter of antibody solution, we use a cell suspension generated from 10 animals.
7. The optimal growth factor combination for OECs is a mitogen mixture of fibroblast growth factor-2 (50 ng/ml; Peprotech, London, UK), heregulin β1, (50 ng/ml; R&D Systems Europe Ltd., Abingdon, Oxfordshire, UK) forskolin (10^{-6} M; Sigma-Aldrich) and ACM ***(19)***, diluted 1:5 in modified serum-free medium

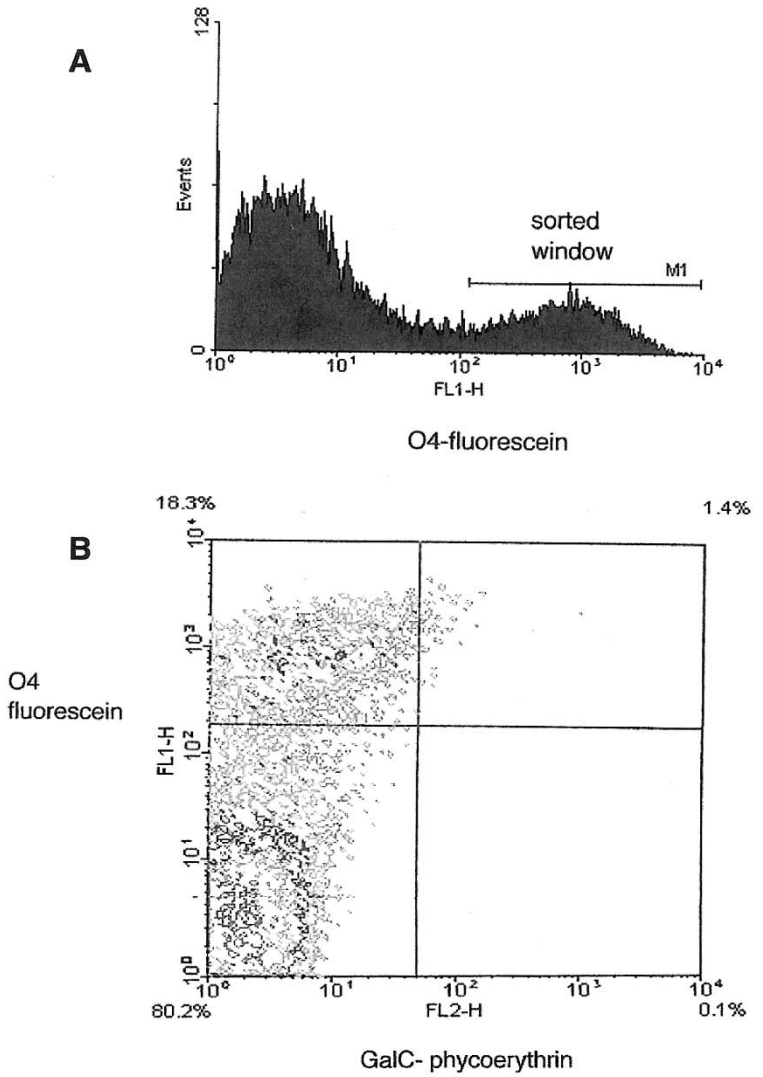

Fig. 3. The one-dimensional profile (**A**) of the O4-labeled cells (using a BD Biosciences FACS Vantage FAC sorter). The *x*-axis represents the fluorescent intensity of the labeled cells, and the *y*-axis represents cell numbers. Using a sort window, the average number of cells that are O4 positive ranges from 6 to 15%. (**B**) Two-dimensional plot of O4-fluorescent intensity against GalC-fluorescent intensity (which is much lower). Using this plot, we draw the sort window around the population of cells that are O4 positive and GalC negative.

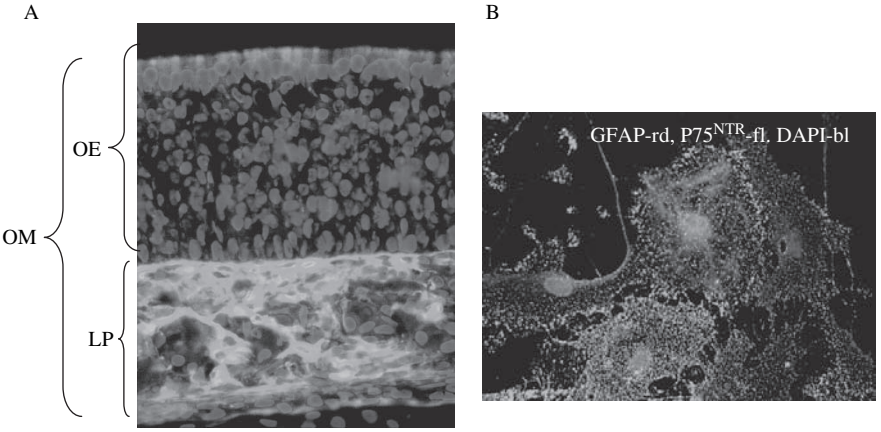

Fig. 4. (**A**) Section of the olfactory mucosa of a postnatal day 7 rat illustrating the location of the OECs within the lamina propria immunolabeled with anti-$p75^{NTR}$ and nuclear 4,6-diamidino-2-phenylindole. **(B)** FACS purified OECs immunolabeled with anti-$p75^{NTR}$, anti-GFAP, and nuclei visualized with DAPI.

(18), which was then diluted 1:1 with DMEM containing 10% FCS (final 5% fetal bovine serum) ***(20)*** (*see* **Notes 7** and **8**). Location of OECs before sort and antigenic and morphological characteristics after a sort are illustrated in **Fig. 4**.

4. Notes

1. This is based on a method of Noble and Murray ***(19)***. Use 2.5 animals per 75 cm^2.
2. When the astrocyte monolayer is confluent, there is often a top layer of contaminating oligodendrocytes and their progenitors. The astrocytes are the flat fibroblast-like cells, with the contaminating oligodendrocytes and O-2A progenitors on top.
3. The monolayers should consist of pure type-1 astrocytes, which can be demonstrated by immunolabeling with the intermediate filament GFAP.
3. One of the antigens that defines the olfactory bulb glial cells is trypsin sensitive (low-affinity nerve growth factor receptor, $p75^{NTR}$), so the cells must be dissociated in collagenase alone.
4. To generate hybridoma supernatant from the O4- or GalC-expressing cell lines, we grow the cells in DMEM containing 10% FCS until 80% confluent. The medium is gently removed (as the cells grow in suspension) and replaced with DMEM-BS or fresh DMEM/10% FCS. Choice of hybridoma supernatant depends on whether the

cells to be immunolabeled are susceptible to differentiation in serum-containing medium. The collection media are left on the cells for 24–48 h and then collected. The supernatant is centrifuged at 1,000 × *g* for 10 min to remove any cells, and the supernatant is collected and filter sterilized through a 0.2-μm filter. The supernatant is usually collected and pooled to give a stock of around 100 ml, which can then be tested on known positive antigen-expressing cells. Once confirmed positive, the supernatant is frozen and stored in 1–2-ml aliquots. Thawed samples remain positive for several weeks when stored at 4°C. The antibodies are also commercially available (Roche Diagnostics); use at recommended concentrations.

5. We have found that OECs express the O4 antibody in vivo ***(5)***, which in turn labels the two types of OECs. We also include GalC in the sort, because O4 also is expressed on oligodendrocyte-type 2 astrocyte lineage cells ***(21)***. To remove the committed oligodendrocytes, we create a sort window around the O4-positive GalC-negative cells. The efficiency of this procedure may be worked out by calculating the number of O4+ cells on the next day.
6. We have found it best to place dissociated embryonic olfactory bulb tissue in culture and allow them to grow for a week in the optimal OEC medium described in the section on FACS purification (*see* **Subheading 3.4., step 7**). From 20 animals, it is reasonable to generate 1.5×10^4 viable cells, and these can be purified using the $p75^{NTR}$ antibody and the FACS.
7. To characterize the resulting OEC population, a range of antibodies can be used. Expression of a panel of antigens on the OEC can define it as Sc cell-like or astrocyte-like ***(5,22,23)***.

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9

Culturing Olfactory Ensheathing Cells from the Mouse Olfactory Epithelium

Miranda Richter, Kathryn Westendorf, and A. Jane Roskams

Summary

Olfactory ensheathing cells (OECs) are not a class of stem cell, but they are a specialized and highly plastic glial cell that can continuously support the neurogenesis and axonal regeneration of olfactory receptor neurons. Because of this, they have been transplanted into sites of spinal cord injury to test their efficacy in promoting repair. They also have been demonstrated to have some ability to support the remyelination of demyelinated axons. Although the majority of these transplantation studies have used OECs prepared from the olfactory bulb (OB-OECs), OECs also can be prepared from the olfactory mucosa, and they are thus a candidate peripherally accessible population of glia that may be effective in promoting repair in a variety of central nervous system lesions. This protocol is designed to produce a highly enriched population of OECs from the lamina propria (LP) of the olfactory mucosa (LP-OECs), which are antigenically similar to OB-OECs and bear some phenotypic similarities to embryonic Schwann cells, but may demonstrate some distinct functional differences.

Key Words: Olfactory mucosa; olfactory ensheathing glia; aldynoglia; regeneration; repair; remyelination.

1. Introduction

The majority of studies testing the properties of olfactory ensheathing cells (OECs) in vitro or after transplantation have used OECs prepared from the nerve fiber layer of the embryonic or neonatal olfactory bulb (OB-OECs). OB-OECs can be prepared by fluorescence-activated cell sorting selection (method 1) ***(1)***, unselected culture of cells from the nerve fiber layer, or immunopanning

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for p75 expression ***(2,3)***. The preparation of OB-OECs is summarized in Chapter 8. Alternatively, a significant fraction of the total OEC population is found within the lamina propria of the olfactory epithelium as they ensheath olfactory receptor axons en route to the olfactory bulb (*see* **Fig. 1**) ***(4)***; potential differences in the biology and regeneration-promoting activities of LP versus OB-derived OECs have recently been reviewed previously ***(5)***. Generating OECs from the lamina propria presents a unique set of obstacles: freeing the lamina propria (LP) tissue from the neuronal olfactory epithelium (OE), the cartilaginous turbinates, and the extracellular matrix (ECM) in which the cells are embedded, and doing this in a way that will minimize fibroblast contamination. Thus, because of the difference in the connective tissue environment of the LP, the method used to culture mucosa-derived OECs is quite different from the method for bulb-derived OECs. Additional steps to minimize cartilage contamination (careful dissection and filtering), digest away ECM (using a defined enzyme treatment), and remove fibroblasts, which certainly will find

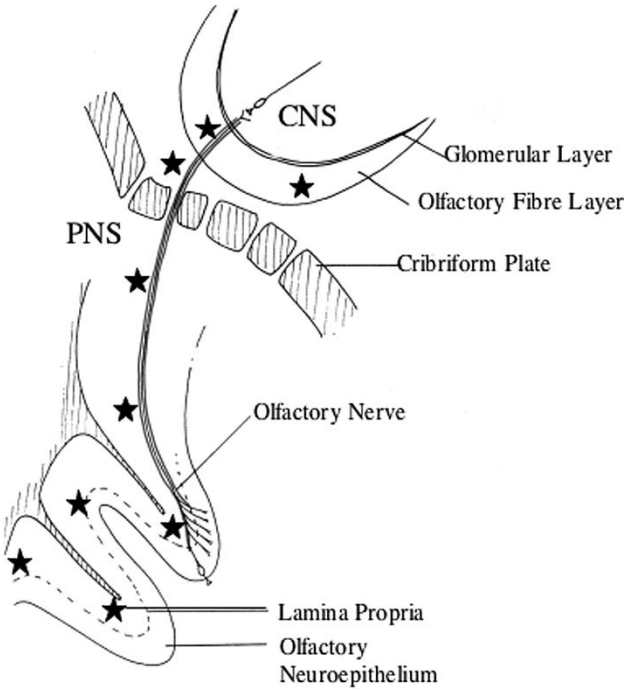

Fig. 1. Distribution of OECs. OECs (*stars*) are distributed throughout the olfactory neuraxis and ensheath the olfactory nerve from the olfactory turbinates (in the peripheral nervous system, *PNS*), through the cribiform plate, and around the olfactory bulb (in the central nervous system, *CNS*).

their way into the culture (cytotoxic lysis of contaminating fibroblasts), are therefore included and suggested. The method outlined below generates a culture of proliferating OECs from either rat or mouse olfactory mucosa that express the same repertoire of antigenic markers as bulb-derived OECs ***(4)***. The procedure outlined below can eliminate the majority of nonglial cells from the OEC culture by passage 3, and it can produce a glial culture that is >90% positive for the coexpression of at least two of the glial markers glial fibrillary acidic protein (GFAP), S100β, and p75, by 4 weeks in vitro. To minimize the time from dissection to cells in vitro, this method uses whole dissection of the OE and LP (combined). If desired, an additional step, whereby OECs also can be cultured from LP digested away from the OE, can be incorporated ***(6)***.

2. Materials

2.1. Dissection of OE

1. Large pair of surgical scissors (15 cm in length).*
2. Fine no. 7 curved forceps (smooth, nonserrated tips, ~11 cm in length tips).*
3. Single edge no. 9 razor blades (one blade for every three pups used + one blade for mincing tissue).*
4. Styrofoam slat: 25 × 15 cm and 3 cm in thickness; can easily be prepared from the lid of a Styrofoam box.
5. 10-mm petri dish (BD Biosciences, San Jose, CA) containing ~15 ml of minimal essential medium (MEM)/D-Val (Invitrogen, Carlsbad, CA).
6. Small pair of surgical scissors.*

*All equipment is sterilized by using a bead sterilizer and spraying with 70% ethanol before use.

2.2. Obtaining Single-Cell Suspension from Epithelium

1. Two sterile 50-ml conical tubes (BD Biosciences).
2. Sterile P1000 tip: cut 4 mm off the tip with a sterile pair of scissors to enlarge the opening.
3. Enzyme Digestion Mix (per pup): 500 µl MEM/D-Val (Invitrogen), 0.5 mg bovine serum albumin (Sigma-Aldrich, St. Louis, MO), 0.6 mg of collagenase D (Roche Diagnostics, Indianapolis, IN), 25 µl (30 U/ml), dispase I (Roche Diagnostics), 25 µl (300 µg/ml) hyaluronidase (Sigma-Aldrich), and 5 µl (10 U/µl) DNAse (Invitrogen) (*see* **Note 1**).
4. Cotton-plugged glass Pasteur pipette, 23 cm (9 in.) in length (Fisher Scientific, Pittsburgh, PA).
5. 40-µm nylon cell strainer (Falcon; BD Biosciences Discovery Labware [Bedford, MA], cat. no. 352340).
6. Initial Plating Medium: MEM/D-Val, 10% fetal calf serum, and 1% penicillin/streptomycin (Penn/Strep) (all from Invitrogen) (*see* **Note 2**).

2.3. Cytotoxic Elimination of Fibroblasts

1. DMEM/Ham's F-12 (Invitrogen).
2. H022-1 hybridoma medium (American Type Culture Collection. Manassas, VA) containing anti-Thy 1.1 (*see* **Note 3**).
3. Rabbit complement (1 mg/ml; Sigma-Aldrich).
4. Growth medium: DMEM/Ham's F-12, 10% fetal calf serum, 1% Pen/Strep (all from Invitrogen).
5. 0.25% trypsin-EDTA (Invitrogen).

2.4. Poly-L-Lysine Coating of Tissue Culture Flasks

1. Poly-L-lysine–coated 75-cm^2 flasks: precoat T75 flask with 4 ml of poly-L-lysine stock solution (50 μg/ml; Sigma-Aldrich) in 15 mM borate buffer solution (pH 8.4).
2. Incubate at room temperature (RT) for 1 h.
3. Aspirate off poly-L-lysine.
4. Rinse twice with phosphate-buffered saline (PBS).
5. Let sit in initial plating or growth medium until ready for use (up to 1 week).

2.5. Antibodies for Verification of OEC Phenotype

1. Rabbit anti-p75 (LNGFR, Chemicon International, Temecula, CA). The widely published anti-rat LNGFR monoclonal (clone Ig192, Roche Diagnostics) will not work specifically in mouse tissue (*see* **Note 4**).
2. Rabbit polyclonal anti-GFAP (Dako North America, Inc., Carpinteria, CA).
3. Anti-S100β (monoclonal, clone SHB1, Sigma-Aldrich), anti-S100 (polyclonal, NeoMarkers, Fremont, CA) (*see* **Note 5**).

3. Methods

3.1. Dissection of OE

1. Place postnatal day (P)5–7 mice in an ice-filled container to preanesthetize. Decapitate P5–P7 mice, two at a time with large surgical scissors, and place the heads on the Styrofoam slat (*see* **Note 6**).
2. Cut the heads in half sagittally. To do so, place a corner of the razor blade into the back of the head and slice down through the head. Slice two heads in half at a time, perform the dissection, and repeat.
3. Dissect out the epithelium. Place the flat edge of the forcep tips against the septum and the outer edge of the olfactory turbinates. Make sure the tips do not go further than the cribiform plate (severing the olfactory nerve at the cribriform plate with a razor blade tip can facilitate removal in one piece). Push down gently, pinch, and peel/tease the epithelium away from the underlying bone and cartilage (*see* **Note 7**).

4. Dissection modification for adult OE: to obtain mucosa from adult OE, the respiratory epithelium at the front of the turbinates should first be cut off and dissected away from the head with a razor blade. The top of each remaining turbinate can be used to anchor forceps, and the gelatinous OE can physically be peeled off into the petri dish.
5. Place the tissue into the 10-mm petri dish containing 15 ml of MEM/D-Val on ice.
6. Pool the dissected tissue from all heads. The remainder of the procedure is performed in a tissue culture hood.
7. Mince epithelium with small surgical scissors followed by razor blade. To do so, tip the dish so that all the tissue collects in one area, and mince with small surgical scissors for approximately 1 min to remove large chunks. Then, lay the dish flat and finely chop the tissue for an additional 2 min with a razor blade to a fine consistency, and no tissue chunks can be seen.

3.2. Preparing a Single-Cell Suspension from OE

1. Carefully pour all of the minced tissue from the dish into a 50-ml conical tube (*see* **Note 8**). Use an additional 10–15 ml of MEM/D-Val to ensure efficient transfer (again, by pouring). Spin at 170 × *g* for 10 min at RT.
2. Aspirate off supernatant. Add 3 ml of fresh MEM/D-Val, and triturate (pipetting up and down) pellet with the precut P1000 tip 25 times. Have the P1000 set at 1,000 µl for trituration.
3. Spin mixture at 460 × *g* for 5 min and aspirate off supernatant. Resuspend pellet by trituration in the enzyme digestion mixture (adjust according to number of pups used).
4. Incubate mixture for 30 min in a 37 °C water bath, with constant agitation at 100 oscillations/min. Also swirl contents manually every 5 to 10 min (*see* **Note 9**).
5. Spin tube at 460 × *g* for 5 min and aspirate off supernatant. Add 2 ml of fresh MEM/D-Val and triturate pellet 35 times with a cotton-plugged Pasteur pipette that has been precoated with fetal bovine serum. Use a pipette-aid (Drummond Scientific, Broomall, PA) at the fast setting, "F" for trituration. Take care not to form bubbles during pipetting.
6. Add 10 more milliliters of MEM/D-Val into tube and swirl gently to bring tissue into suspension.
7. Transfer all digested tissue (by rinsing repeatedly with serum-free MEM/D-Val) and the entire slurry of cells through a 40-µm nylon cell strainer into a new 50-m conical tube.
8. Use another 10 ml of MEM/D-Val to rinse the old conical tube filter as well.
9. Spin collected cells down at 460 × *g* for 5 min.
10. Remove supernatant and resuspend cell pellet in 3 ml of initial plating medium (MEM/D-Val, 10% FCS, and 1% Penn/Strep).
11. Perform a cell count by using trypan blue to determine live cell yield (*see* **Note 10**).

12. Plate cells into T75 flasks (one T75 flask for every 6–10 pups used) precoated with poly-L-lysine and incubate at 37°C with 5% CO_2 for 4 days.

3.3. Cytotoxic Elimination of Fibroblasts

This procedure is usually performed two to three times on successive passages to eliminate all fibroblasts from the culture.

1. Aspirate off media and rinse cells with 10 ml of sterile PBS.
2. Aspirate off PBS and add 10 µl/cm^2 of 0.25% trypsin-EDTA (Invitrogen).
3. Let sit at RT for 2 min, and thentake up the cells with 10 ml of PBS into a 15-ml conical tube (Falcon; BD Biosciences Discovery Labware).
4. Spin cells down at 460 × *g* for 5 min and aspirate off supernatant.
5. Add the following to pellet (*see* **Note 3**):
   a. 1 ml of DMEM/Ham's F-12.
   b. 200 µl of Thy 1.1 hybridoma media (1:5 ratio, determined empirically).
   c. 100 µl (1 mg/ml) of rabbit complement (1:10 ratio; Sigma-Aldrich).
6. Triturate pellet 10 times with a P1000 Pipetman and incubate mixture for 30 min at RT.
7. Spin down at 460 × *g* for 5 min and aspirate off the supernatant. Resuspend the pellet in growth medium (DMEM/Ham's F-12, 10% FCS, 1% Pen/Strep), and plate out cells in poly-L-lysine–coated flasks at a concentration of 6,500 cells/cm^2 (*see* **Note 12**).

3.4. Verification of OEC Phenotype

1. Plate OECs onto poly-L-lysine–coated four-chamber slides at a density of 5,000 viable cells/well.
2. Allow cells to settle and expand for 4 days.
3. Remove media and wash three times with PBS.
4. Fix either with 4% paraformaldehyde (PFA) for 10 min at RT or with ice-cold methanol for 10 min at 4°C.
5. Store at 4 °C for up to 2 weeks in PBS (with 0.1% azide), and test for antigen expression by using standard immunofluorescence protocols. GFAP, S100β, and p75 should be coexpressed in different OEC subsets (degree of staining varies with time in culture, and age from which OECs were prepared) to verify OEC phenotype (*see* **Note 14**).

4. Notes

1. Enzymes and units/pup. Because formulations can change for many commercially available enzymes, international units should be followed and cross-compared across lots. Different quantities of enzymes may be needed for rat versus mouse pups or for neonatal versus adult olfactory mucosa, and quantities

should be determined empirically. Total cell yield is proportional to dispase concentration, but high concentrations of dispase can be detrimental to OEC survival. Hyaluronidase concentration also can specifically enhance OEC yield, but it can be detrimental at high concentrations.

2. Media additions are used to enhance expansion of OECs. OEC expansion can be enhanced by basic fibroblast growth factor, bovine pituitary extract, and forskolin (similar to OB-OECs). The serum-free media supplements B27 and G5 also can be substituted as suitable base media for OEC expansion protocols.
3. Thy1.1 hybridoma. This hybridoma is produced according to standard hybridoma production protocols (roller bottles). After harvesting, hybridoma is significantly more stable at –80°C than at –20°C. Each batch should be tested by dilution series for its efficacy in complement-mediated lysis of primary mouse fibroblasts.
4. Anti-p75 (rabbit polyclonal, Chemicon International). Increased background will be obtained with an overnight incubation (recommended 1 h at RT in 2% serum). If overnight incubations are performed, permeablization is not recommended because a nuclear background signal will be obtained.
5. The antibodies recommended work consistently on mouse LP-OECs in vivo and in vitro. There are several commercially available antibodies from other suppliers that are not as sensitive as the recommended antibodies for both in vivo and in vitro applications. We recommend testing all antibodies on sections of olfactory tissue in vivo , or on Schwann cells in vitro as positive controls.
6. For this preparation, we usually use at least one large litter of mice (10 or more). Mice are generally sacrificed by cervical dislocation before decapitation or simply by decapitation alone after ice pretreatment. Inhalation anesthetic also can be used (this will depend on the animal care protocols of your institution).
7. Be careful not to push the forceps too far down or you will be removing optic tissue along with the epithelium. The general idea is to "scoop" the epithelium out of the head with the tips of the forceps.
8. This methods is used to prevent tissue loss. If you transfer the tissue with a plastic pipette, the tissue will tend to stick to the inside walls.
9. Enzyme incubation. Longer incubations and changes in enzyme concentrations may be required for adult tissue and larger scale preparations. These modifications should be determined empirically within an individual laboratory.
10. Each P5–P7 pup should generate a cell yield of 1.5–3 million cells.
11. Volumes above are for one T75 flask. Multiply the volumes by the number of T75 flasks trypsinized.
12. Penn/Strep can be eliminated from the expansion media after the first passage.
13. It may take as many as three passages with complement-mediated lysis to get rid of the majority of fibroblasts in culture.
14. OEC identity should always be assessed with coexpression of two of these markers. Anti-S100β can cross-react with an antigen found in some fibroblasts, GFAP expression by OECs is highly variable, and p75 expression is context-dependent.

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10

Production and Characterization of Immortal Human Neural Stem Cell Line with Multipotent Differentiation Property

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Summary

We document the protocols and methods for the production of immortalized cell lines of human neural stem cells from the human fetal central nervous system (CNS) cells by using a retroviral vector encoding v-myc oncogene. One of the human neural stem cell lines (HB1.F3) was found to express nestin and other specific markers for human neural stem cells, giving rise to three fundamental cell types of the CNS: neurons, astrocytes, and oligodendrocytes. After transplantation into the brain of mouse model of stroke, implanted human neural stem cells were observed to migrate extensively from the site of implantation into other anatomical sites and to differentiate into neurons and glial cells.

Key Words: Cell line; differentiation; glia; human; immortalization; neural stem cells; neurons; retroviral vector; v-myc.

1. Introduction

Stem cells are multipotent cells that are self-renewing and, under the right conditions or with the correct signals, can give rise to many different cell types that make up the organism. Stem cells hold great therapeutic potential for injury and disease in humans. Two types of mammalian pluripotent stem cells, embryonic stem (ES) cells derived from the inner cell mass of blastocysts and embryonic germ (EG) cells obtained from postimplantation embryos, have been identified and they have been found to give rise to various organs and tissues

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(1,2). In addition to ES cells and EG cells, adult/tissue-specific stem cells could be isolated from various tissues at more advanced developmental stages, such as bone marrow mesenchymal stem cells, hematopoietic stem cells, and neural stem cells (NSCs), among others.

The existence of immature multipotent NSCs has been identified in the embryonic and adult mammalian brain, and the utility of these NSCs for therapies aimed at cell replacement or neuroprotection in disease and injury affecting the brain and spinal cord have been reported *(3–6)*. NSCs the have capacity to grow indefinitely, and they have potential to differentiate into three major cell types of central nervous system (CNS): neurons, astrocytes, and oligodendrocytes. In humans, existence of NSCs with multipotential differentiation capability also has been reported in embryonic and adult human brain *(7–12)*. One of these studies has demonstrated that new neurons are continuously being generated in the adult human CNS *(10)*. In one study, bromodeoxyuridine (BrdU)-labeled proliferating cells that colabeled with neuronal markers were found in granular layer of hippocampal dentate gyrus in cancer patients who had received a BrdU infusion for diagnostic purposes and later died *(10)*. Why, in neurodegenerative diseases or after CNS injury, are the NSCs ineffective in brain repair? Three different factors may underlie the inability of adult NSCs to perform effective repair: (1) Although NSCs are present, their numbers are not enough to perform brain repair; (2) NSCs are unable to reach the pathology site; and (3) the microenvironment in the site of pathology is not favorable for the NSCs to expand and conduct repair. These difficulties in brain repair might be overcome if a sufficient number of NSCs as well as a simultaneous favorable trophic factor environment are delivered to the injury site. Our recent transplantation studies, using conditionally immortalized NSC cell lines in several neurological disease models, indicate that this approach is feasible (*see* below).

In the early days of NSC studies, striatum of adult mice was dissociated into single cells by enzyme treatment and cultured in serum-free medium supplemented with epidermal growth factor (EGF), and a small percentage of these cells formed multicellular aggregates that quickly increased in size and contained a large number of NSCs. These cells were called neurospheres. When neurospheres were dissociated into single cells and subcultured in the presence of the growth factors EGF and basic fibroblast growth factor (FGF), they became new neurospheres owing to active cell division, and they later differentiated into neurons and glial cells *(13)*. A previous study has reported that an established cell line of human NSCs was obtained by repeated subculture of neurospheres derived from a fetal human brain *(14)*, but credibility of the study is in question, because no other laboratories, including ours, could

reproduce the results of the study to generate a stable established cell line of human NSCs by simply growing primary fetal human brain cells continuously.

Recently, stable immortalized cell lines of NSCs have been generated by introduction of oncogenes. These immortalized NSC lines have advantageous characteristics for basic studies on neural development and cell replacement or gene therapy studies ***(8,9,15–18)***: (1) NSC cell line can be expanded to large numbers in culture in short time (24–48-h doubling time); (2) NSC cells are homogeneous since they were generated from a single clone; and (3) stable expression of therapeutic genes can be achieved readily. Immortalized NSCs have emerged as a highly effective source for genetic manipulation and gene transfer into the CNS ex vivo. Immortalized NSCs were genetically manipulated in vitro, survive, integrate into host tissues, and differentiate into both neurons and glial cells after transplantation into the intact or damaged brain ***(8,9,15–18)***.

Primary cultures of fetal human telencephalon cells (at 15 weeks gestation) were infected with a retroviral vector carrying v-myc oncogene, and continuously dividing NSC clones were selected. HB1.F3, one of the newly generated human NSCs, is a clonally isolated, multipotential human neural stem cell line, with the ability to self-renew and differentiate into cells of neuronal and glial lineages in vitro ***(20,21)***. After brain transplantation, HB1.F3 provided clinical improvement in the animal models of stroke ***(22–25)***, epilepsy ***(26)***, Huntington disease ***(27,28)***, Parkinson's disease ***(29)***, lysosomal storage disease mucopolysaccharoidosis VII ***(30)***, and brain tumors ***(31–33)***.

The morphology of the HB1.F3 human NSC line is dependent upon culture medium. In serum-free medium such as UBC1, the cells have a bipolar morphology, but in the presence of serum they become tripolar- or multipolar (8–10 μm; *see* **Fig. 1**). The cloned HB1.F3 and HB1.A4 NSC cells are bipolar or tripolar in morphology (10–12 μm). Cytogenetic analysis of HB1.F3 NSCs showed normal karyotype of human cells with a 46,XX karyotype without any chromosomal abnormality (*see* **Fig. 2B**).

Results of reverse transcription-polymerase chain reaction (RT-PCR) analysis of mRNAs isolated from F3 human NSCs are shown in **Fig. 2C**. Transcripts for nestin (an NSC-specific marker), neurofilament triplet proteins (NF-L, NF-M, and NF-H; cell type-specific markers for neurons), and glial fibrillary acidic protein (GFAP; a specific marker for astrocytes), are all expressed by F3 NSC cell line. However, transcripts for myelin basic protein (MBP), a structural protein and a specific cell type marker for oligodendrocytes, were not demonstrated (*see* **Fig. 2C**). In addition, transcripts for growth factors including nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3), glial-derived neurotrophic factor (GDNF), ciliary neurotrophic factor (CNTF), hepatocyte growth factor (HGF), insulin-like growth factor (IGF)-1, basic (b)FGF, and vascular endothelial growth factor

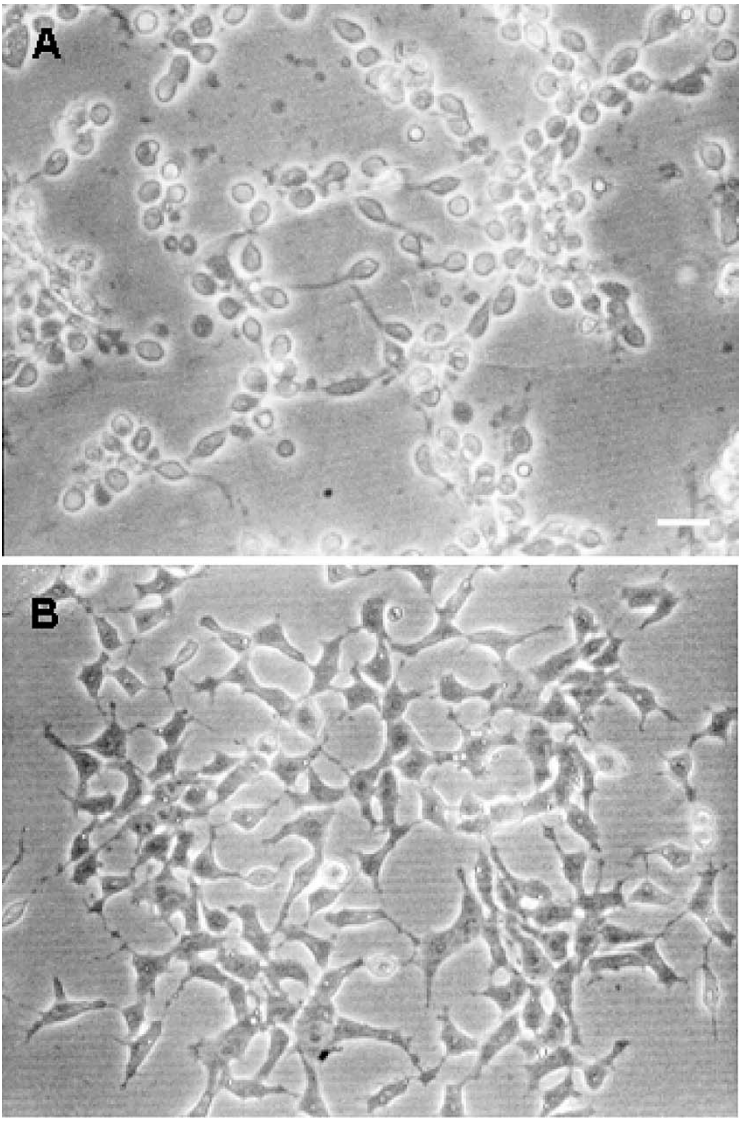

Fig. 1. (**A**) Live cell culture of human cerebrum isolated from 15-week-gestation embryo and grown in vitro for 5 days. The culture contains a large number of neurons, glial cells, and neural stem cells, and represents a starting material for the generation of human NSC lines. Phase contrast microscopy. Bar = 20 μm. **(B)** Immortalized human neural crest stem cells, HB1.F3 (F3), are grown in serum-free medium UBC1 containing bFGF. When F3 cells are grown in serum-containing medium (10% fetal bovine serum), they differentiate into neurons and glial cells.

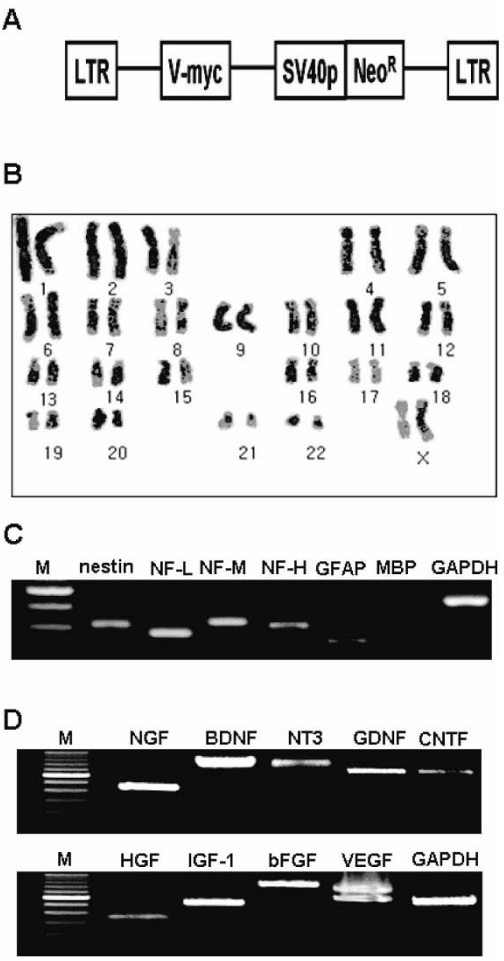

Fig. 2. **(A)** An amphotropic replication-incompetent retroviral vector, pLCN.vmyc, encoding v-myc that was transcribed from the retrovirus LTR plus neo transcribed from an internal SV40 early promoter. **(B)** Karyotype of HB1.F3 human neural stem cell line at passage 7. **(C)** RT-PCR analyses of gene expressed by HB1.F3 human neural stem cell line. **(D)** RNA transcripts for nestin (a cell type-specific marker for NSCs), NF-L, NF-M, NF-H (cell type-specific markers for neurons) and GFAP (a cell type marker for astrocytes) are demonstrated in F3 cells, but transcripts for MBP (a cell type-specific marker for oligodendrocytes) are not. After growth in medium supplemented with sonic hedgehog protein (*shh*), F3 cells express oligodendrocyte-specific antigens such as O4, galactocerebroside, CNPase, and MBP. **(E)** RNA transcripts for growth factors NGF, BDNF, NT-3, GDNF, CNTF, HGF, IGF-1, bFGF, and VEGF are expressed in HB1.F3 human NSC line.

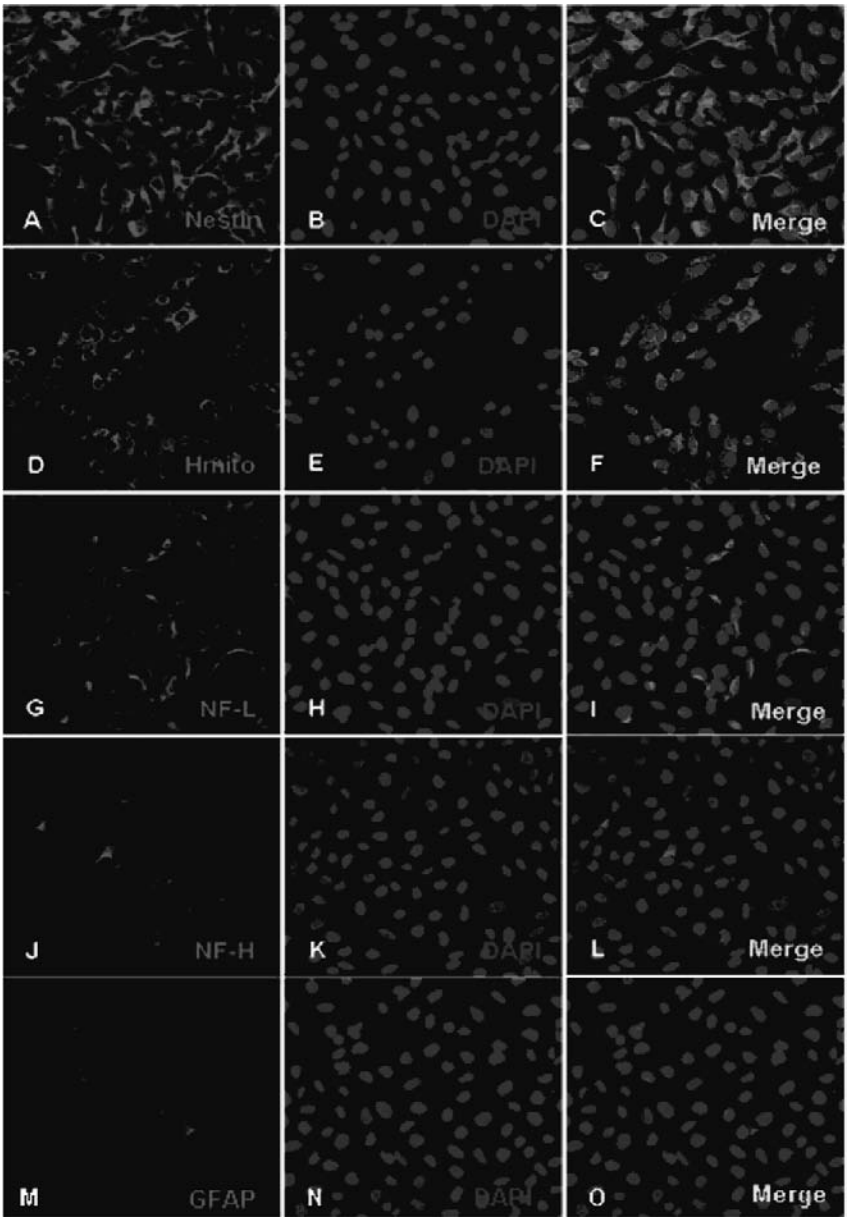

Fig. 3. HB1.F3 human NSCs on coverslips were processed for immunostaining with antibodies specific for nestin **(A–C)**, human mitochondria **(D–F)**, NF-L **(G–I)**, NF-H **(J–L)**, and GFAP **(M–O)**, followed with Alexa Fluor 594 secondary antibody. The nuclei of F3 cells were detected with DAPI. Confocal immunofluorescence images of F3 cells (nestin-positive) indicate that F3 human NSCs have multipotent differentiation capacity and develop into neurons (NF-L- and NF-H-positive) or astrocytes (GFAP-positive) depending upon specific cues provided by local environment in vivo .

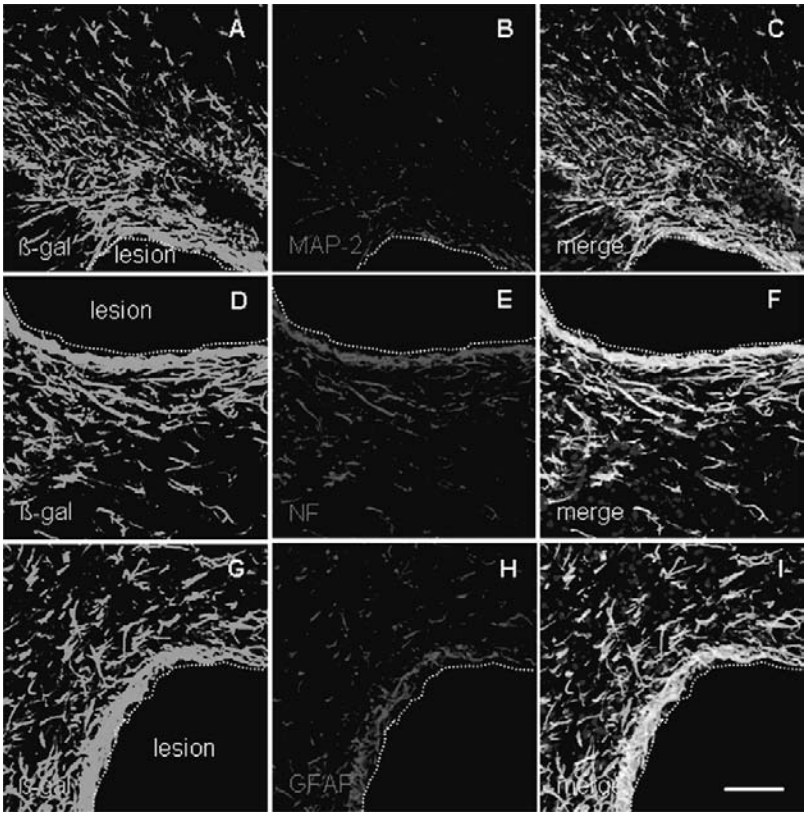

Fig. 4. **(A)** β-Gal–expressing HB1.F3 human NSCs were transplanted into cerebral cortex overlying hemorrhage lesion of adult mouse brain. ICH stroke model was generated by injection of bacterial collagenase solution into the striatum. Four weeks post-transplantation, the β-Gal–positive F3 cells migrated to the border of hemorrhage core and differentiated into neurons (MAP2- and NF-positive) and also GFAP-positive astrocytes. Bar = 20 μm.

(VEGF) are prominently expressed by F3 NSC line, indicating that NSCs are a highly effective source of neurotrophic factors that could provide neuroprotection for neurons assailed by injury or disease (*see* **Fig. 2D**).

All of the HB1.F3 cells grown in DM4 serum-free medium were immunoreaction-positive for nestin (*see* **Fig. 3A–C**) ***(24,25)***, indicating that F3 cells are indeed NSCs as demonstrated ***(19,25)***. F3 cells are reaction-positive by antibody specific for human mitochondria (*see* **Fig. 3D–F**) and for pan-myc antibody (data not shown), indicating that F3 cells are uniquely human origin and contain a copy or copies of v-myc-encoding retrovirus. When F3 cells were grown in serum-containing medium, there were >10–30%

of total cells expressing triplet NF proteins (NF-L, NF-M, and NF-H) (*see* **Fig. 3G–L**), microtubule-associated protein (MAP)2, and β-tubulin III isoform (data not shown). These phenotypes are specifically and exclusively expressed by mammalian nerve cells, including human, indicating that F3 cells could differentiate into nerve cells under suitable culture conditions. F3 cells grown in serum-containing medium also expressed GFAP, a cell type-specific marker for astrocytes (*see* **Fig. 3M–O**), and galactocerebroside (GalC), a surface antigen specific for oligodendrocytes (data not shown), indicating differentiation of glial cells, astrocytes, and oligodendrocytes from F3 cells.

After transplantation into intracerebral hemorrhage (ICH), mouse cerebral cortex Lac*Z*-positive F3 human NSCs migrated selectively to the hemorrhage core and also migrated to the border of the lesion well away from the injection sites. A large population of transplanted F3 NSCs differentiated into MAP2-positive (*see* **Fig. 4A–C**), or NF-positive (*see* **Fig. 4D–F**) neurons in the perihematomal sites. Many of the β-galactosidase (β-Gal)/GFAP double-positive cells also were found along the border of hemorrhagic core (*see* **Fig. 4G–I**). These results indicate that the majority of grafted F3 human NSCs differentiate into neurons and also astrocytes in response to developmental signals provided by the locus of implantation.

2. Materials

2.1. Primary Fetal Human CNS Cell Culture

1. Phosphate-buffered saline (PBS, Sigma-Aldrich, St. Louis, MO).
2. Solution of trypsin (0.25%) and EDTA (0.02%) (Invitrogen, Carlsbad, CA).
3. DNase I (Sigma-Aldrich).
4. Dulbecco's modified Eagle's medium (DMEM) with high glucose (Sigma-Aldrich).
5. Fetal bovine serum (Hyclone Laboratories, Salt Lake City, UT).
6. Gentamicin (10 mg/ml; Sigma-Aldrich).
7. Amphotericine B (2.5 mg/ml; Sigma-Aldrich).
8. bFGF (PeproTech, Rocky Hill, NJ) (*see* **Note 1**).
9. Poly-L-lysine (PLL) (Sigma-Aldrich).
10. Six-well culture plate (BD Biosciences, Franklin Lakes, NJ).
11. 60- and 100-mm-diameter plastic culture dish (Falcon; BD Biosciences Discovery Labware, Bedford, MA).

2.2. Retroviral Vector

1. An amphotropic replication-incompetent retroviral vector, pLCN.vmyc, encoding v-myc that was transcribed from the retrovirus long terminal repeat (LTR) plus neo transcribed from an internal simian virus 40 (SV40) early promoter (*see* **Fig. 2A** and **Note 2**).

2. PASK1.2 cell line was generated by infecting PA317 amphotropic packaging cell line with pLCN.vmyc vector, and successful infectants were selected and expanded (*see* **Note 3**).

2.3. Cytogenetic Analysis

1. Colcemid (Sigma-Aldrich).
2. Ethidium bromide (Sigma-Aldrich).
3. Giemsa solution (Sigma-Aldrich).

2.4. Immunocytochemistry

1. Anti-myc antibody (mouse monoclonal antibody [mAb], Calbiochem, San Diego, CA).
2. Anti-β-Gal antibody (rabbit IgG, Chemicon International, Temecula, CA).
3. For identification of NSCs: anti-nestin (rabbit serum, provided by Dr. K. Ikeda, Tokyo Metropolitan Psychiatric Institute, Tokyo, Japan).
4. For neurons: anti-NF low-molecular-weight protein (NF-L, mouse mAb, Chemicon International), anti-NF medium-molecular-weight protein (NF-M, mouse mAb, Chemicon International), anti-NF high-molecular-weight protein (NF-H, rat mAb, provided by Dr. V. Lee, University of Pennsylvania, Philadelphia, PA), MAP2 antibody (Ab) (mouse mAb, Sigma-Aldrich), and anti-β-III tubulin isoform (mouse mAb, Chemicon International).
5. For astrocytes: GFAP (rabbit Ab, Dako North America, Inc., Carpinteria, CA; rat mAb, provided by Dr. V. Lee; rabbit).
6. For oligodendrocytes: anti-GalC (mouse mAb), O4 (mouse mAb), and anti-cyclic nucleotide phosphodiesterase (CNP, mouse mAb, Chemicon International).
7. Secondary antibodies: biotinylated anti-mouse/rabitt IgG (Vector Laboratories, Burlingame, CA), and Alexa Fluor 487/594-conjugated anti-mouse/rabbit IgG (Invitrogen)
8. 4,6-diamidino-2-phenylindole (DAPI, Sigma-Aldrich).
9. Blocking buffer: PBS containing 0.2% (v/w) Triton X-100 (Sigma-Aldrich) and 5% normal goat serum.
10. Gelvatol, mounting medium (a mixture of polyvinyl alcohol and glycerol, both from Sigma-Aldrich) (*see* **Note 4**).

2.5. Immunoblot Analyses

1. Setup buffer: 25 mM Tris, 190 mM glycine, and 20% (v/v) methanol.
2. Transfer buffer: setup buffer plus 0.05% (w/v) sodium dodecyl sulfate (SDS).
3. Blocking buffer: 5% (w/v) nonfat dry milk in Tris buffered saline/Tween 20 (TBS-T).
4. Antibody dilution buffer: TBS-T supplemented with 2% (w/v) bovine serum albumin fraction V (Sigma-Aldrich).

5. Primary antibodies (*see* **Subheading 2.4.**).
6. Secondary antibody: anti-mouse/rabbit IgG conjugated to horseradish peroxidase (Sigma-Aldrich).
7. Polyvinylidene fluoride membrane (Millipore Corporation, Danvers, MA).
8. Enhanced chemiluminescence (ECL) reagents (Kirkegaard and Perry Laboratories (Gaithersburg, MD).
9. Bio-Max ML film (Eastman Kodak, Rochester, NY).

Table 1
Primers for RT-PCR analyses

Nestin	Sense: 5'-CTCTGACCTG TCAGAAGAAT-3'
	Antisense: 5'-GACGCTGACACTTACAGAAT-3'
NF-L	Sense: 5'-TCCTACTACACCAGCCATGT-3'
	Antisense: 5'-TCCCCAGCACCTTCAACTTT-3'
NF-M	Sense: 5'-TGGGAAATGGCTCGTCATTT-3'
	Antisense: 5'-CTTCATGGAAGCGGCCAATT-3'
NF-H	Sense: 5'-CTGGACGCTGAGCTGAGGAA-3'
	Antisense: 5'-CAGTCACTTCTTCAGTCACT-3'
GFAP	Sense: 5'-CAGAGATGATGGAGCTCAATGACC-3'
	Antisense:5',GTTTCATCCTGGAGCTTCTGCCTCA-3'
MBP	Sense: 5'-ACACGGGCATCCTTGACTCCATCGG-3'
	Antisense: 5'-TCCGGAACCAGGTGGTTTTCAGCG-3'
NGF	Sense: 5'-TCATCATCCCATCCCATCTTCCAC-3'
	Antisense: 5'-CACAGCCTTCCTGCTGAGCACAC-3'
BDNF	Sense: 5"-ATGACCATCCTTTTCCTTACT-3'
	Antisense: 5'-CTATCTTCCCCTTTTAATGGT-3'
NT-3	Sense: 5'-ATGTCCATCTTGTTTTATGTGA-3'
	Antisense: 5'-TCATGTTCTTCCGATTTTTC-3'
GDNF	Sense: 5'-ATGAAGTTATGGGATGTCGT-3'
	Antisense: 5'-TTAGCGGAATGCTTTCTTAG-3'
CNTF	Sense: 5'-ATGGCTTTCACAGAGCATT-3'
	Antisense: 5'-AACTGCTACATTTTCTTGTTGTT-3'
HGF	Sense: V5'-AGGAGAAGGCTACAGGGGCAC-3'
	Antisense: 5"-TTTTTGCCATTCCCACGATAA-3'
IGF-1	Sense: 5'-AAATCAGCAGTCTTCCAACCCA-3'
	Antisense: 5'-CTTCTGGGTCTTGGGCATGT-3'
bFGF	Sense: 5'-GGGTGGAGATGTAGAAGATG-3'
	Antisense: 5'-TTTATACTGCCCAGTTCGTT-3'
VEGF	Sense: 5'-GAAGTGGTGAAGTTCATGGATGTC-3'
	Antisense: 5'-CGATCGTTCTGTATCAGTCTTTCC-3'
GAPDH	Sense: 5'-CATGACCACAGTCCATGCCATCACT-3'
	Antisense: 5'-TGAGGTCCACCACCCTGTTGCTGTA-3'

2.6. RT-PCR Analysis

1. TRIzol (Invitrogen).
2. SuperScript first strand synthesis system (Invitrogen).
3. Primers (*see* **Table 1**).
4. PCR buffer (Invitrogen).
5. dNDPs (Invitrogen).
6. Taq polymerase (Invitrogen).
7. Agarose (Invitrogen).
8. Ethidium bromide (Sigma-Aldrich).
9. FLA 300 Imager (Fujifilm, Tokyo, Japan).

3. Methods

3.1. Primary Fetal Human CNS Cell Culture

Primary fetal human CNS cell cultures are prepared as described previously *(31,32)* (*see* **Note 5**).

1. Brain (15-week-old gestational fetus) tissue blocks are washed twice in PBS supplemented with 1,000 U/ml penicillin and 1 mg/ml streptomycin. Final concentration of penicillin and streptomycin in PBS is 10-fold of what routinely is used in culture medium to block bacterial infection carried over in the brain samples.
2. Small fragments of brain are transferred to a 50-ml centrifuge tube containing 30 ml of PBS with 0.25% trypsin-0.02% EDTA and 40 µg/ml DNase I, and contents are incubated in a shaking water bath for 30 min at 37°C.
3. Remove trypsin solution as much as possible, add 20 ml of culture medium (DMEM with high glucose containing 10% fetal bovine serum (FBS), 20 µg/ml gentamicin and 2.5 µg/ml amphotericin B) to the tube, and gently triturate enzyme-digested tissue 20 times by using a 10-ml pipette followed by trituration by Pasteur pipette for 20 times (*see* **Note 6**). After settling of tissue fragments, 15 ml of the supernatant solution containing dissociated single cells are transferred to a new 50-ml tube, taking care not to include any tissue fragment from the tube bottom.
4. Add 10 ml of culture medium, triturate, remove supernatant, add it to the tube containing the first harvest, and spin the tube at 300 × *g* (1,200 rpm) for 8 min.
5. Remove the supernatant and add 45 ml of culture medium (DMEM with high glucose containing 10% FBS, 20 µg/ml gentamicin, 2.5 µg/ml amphotericin B, and 10 ng/ml bFGF) to the cellular pellet, triturate, and plate in three PLL-coated T75 culture flasks 15 ml each at 10^6 cells/ml cell concentration. Culture medium is changed twice a week.
6. On days 10–12, cultures are washed once with PBS, incubated in PBS with 0.1% trypsin-0.02% EDTA for 5 min at 37°C, dissociated into single cells, and plated 2 ml each in six-well plates (10^5 cells/ml).
7. 3 days later, replace the culture medium with serum-free culture medium consisting of DMEM with high glucose containing 20 µg/ml gentamicin, 10 ng/ml

bFGF, and UBC supplements (containing human insulin, transferrin, sodium selenite, progesterone, hydrocortisone, triiodothyronine, and other nutrients and antioxidants) (*see* **Table 2** and **Note 7**).

8. At this time point, CNS cultures consist of neurons, astrocytes, oligodendrocytes, microglia, and actively dividing neural stem/progenitor cells (*see* **Fig. 1A**), and they are ready for gene transfer experiments.

3.2. Retrovirus-Mediated Gene Transfer

Infection of human CNS cells in six-well plates should be performed twice to improve the infection efficiency by the following procedures:

1. Add 2 ml of supernatant (4×10^5 colony-forming units) from the packaging cell line and 8 μg/ml Polybrene to target cells in six-well plates and incubate for 4 h at 37°C (*see* **Note 8**).
2. Replace infection medium containing retroviral vectors with fresh culture medium; repeat infection process 24 h later.
3. Seventy-two hours after the second infection, select infected cells with 200 μg/ml G-418 (Geneticin, Invitrogen) for 14 days. During each culture medium change, supplement the medium with G-418.
4. At 14 days in culture, terminate G-418 treatment and use normal complete culture medium without G-418 for further propagation of cultures.
5. At various times during the 3–4 weeks in culture, large clusters of cell clones can be identified, isolated, and individually grown in PLL-coated six-well plates.

Table 2
UBC1 Serum-free chemically defined medium

N-Acetylcysteine oleic acid[a]
Albumin progesterone
Ascorbic acid putrescine
Biotin retinol acetate[a]
Catalase sodium pyruvate[b]
Cupric sulfate sodium selenite
Ethanolamine superoxide dismutase
Galactose tocopherol acetate
Glutathione/reduced transferrin
HEPES triiodothyronine
Hydrocortisone trolox
Insulin vitamin B12
Linoleic acid[a] zinc sulfate

[a]Water-soluble forms of retinol acetate, linoleic acid, and oleic acid should be used.
[b]Pyruvate should be excluded when DMEM is used as a basal medium.

6. Generate individual clones by limited dilution and propagate them for further studies. In our study, three individual clones were isolated and designated as human NSC lines HB1.F3, HB1.F5, and HB1.F6.
7. The cloned human NSCs are bipolar or tripolar in morphology (8–10 μm) (*see* **Fig. 1B**).

3.3. Cytogenetic Analysis

Cytogenetic analysis should be performed on established cell lines at various passages.

1. Grow the established cell lines (HB1.F3, HB1.F5, and HB1.F6) in 10-cm culture dish, and treat cells with 0.05 μg/ml colcemid and 5 μg/ml ethidium bromide for 2 h before cell harvest. Detach cells from the dish by a brief trypsin treatment.
2. After centrifugation, add a 4:1 mixture of 75 mM potassium chloride and 34 mM sodium citrate to the cell pellet for 12 min at hypotonic treatment.
3. Fix the cells in methyl alcohol: acetic acid (3:1), followed by Giemsa solution for 1 min for chromosome staining. Three to five karyotypes can be constructed from each cell preparation.
4. Cytogenic analysis of HB1.F3 cell line shows normal karyotype of human cells with a 46,XX karyotype without any chromosomal abnormality (s*ee* **Fig. 2B**).

3.4. Immunocytochemistry

Immunocytochemical determination of cell type-specific markers in human NSC cell line should be performed using antibodies specific for NSCs and their progeny, including neurons, astrocytes, and oligodendrocytes (*see* **Table 3**).

1. Human NSC lines (HB1.F3, HB1.F5, and HB1.F6) grown on PLL-coated Aclar plastic coverslips (9 mm in diameter) were incubated in UBC1 serum-free medium with or without bFGF for 24 h and processed for immunocytochemical staining (*see* **Note 9**).
2. Coverslips bearing NSC cells are rinsed in PBS and fixed in cold acid alcohol (5% glacial acetic acid in 95% ethanol) for 10 min at –20°C. Cultures were incubated in blocking solution (PBS containing 3% Triton-X and 5% normal goat serum) at room temperature (RT) for 20 min. For demonstration of surface antigens O4 and GalC (both cell type markers for oligodendrocytes), coverslips are fixed for 3 min at RT in 4% paraformaldehyde and washed in PBS.
3. The coverslips are incubated with primary antibodies specific for v-myc, β-Gal, human mitochondria, nestin, NF-L, NF-M, NF-H, MAP2, β-tubulin-III, GFAP, CNP, O4, and GalC for 48 h at 4°C.
4. The coverslips are incubated in secondary antibodies Alexa Fluor 488/594-conjugated anti-mouse/rabbit IgG/IgM for 1 h at RT, and then they are counter-stained with DAPI (2 μg/ml, Sigma-Aldrich). After wash in PBS, coverslips are

Table 3
Cell type-specific markers of NSCs and their progeny

Marker	Source
Myc mAb Pan-myc	Calbiochem
β-Gal rabbit cell identification	Chemicon International
Human mitochondria mAb cell identification	Chemicon International
Nestin rabbit NSCs	K. Ikeda
NF-L mAb neurons	Chemicon International
NF-M mAb neurons	Chemicon International
NF-H rat mAb neurons	V. Lee
MAP2 mAb neurons	Sigma-Aldrich
β-Tubulin III mAb neurons	Chemicon International
GFAP rabbit astrocytes	Dako North America, Inc.
GFAP rat mAb neurons	Chemicon International
CNP mAb oligodendrocytes	Chemicon International
O4 mAb oligodendrocytes	S. U. Kim laboratory
GalC mAb oligodendrocytes	S. U. Kim laboratory

mounted onto glass slides by using gelvatol and examined under an Olympus (Tokyo, Japan) laser confocal fluorescence microscope.

5. To demonstrate the fate of transplanted NSCs in rat/mouse models of cerebral hemorrhage stroke, paraformaldehyde perfusion-fixed brains are processed for 30-μm sections in a cryostat, and then the sections are immunostained for β-Gal/MAP2, β-Gal/NF, β-Gal/GFAP (*see* **Fig. 4**), and β-Gal/ myc (*see* **Fig. 5**) double staining as described above.

3.5. Immunoblot Analyses of Cell Types

Solubilized proteins from cultured human NSCs are separated by SDS-polyacrylamide gel electrophoresis (10% acrylamide) and transferred electrophoretically to a polyvinylidene difluoride membrane, and the membrane is processed for proteins specifically expressed by NSCs, neurons, and glial cells as follows:

1. The membrane is incubated for 1 h at RT in TBS-T containing 5% nonfat milk.
2. The membrane is incubated overnight at 4^{o}C in TBS-T containing 5% nonfat milk and primary antibodies.
3. Rinse the membrane four times with TBS-T.
4. Incubate the membrane for 1 h at RT in TBS-T containing peroxidase-conjugated goat anti-mouse/rabbit IgG.

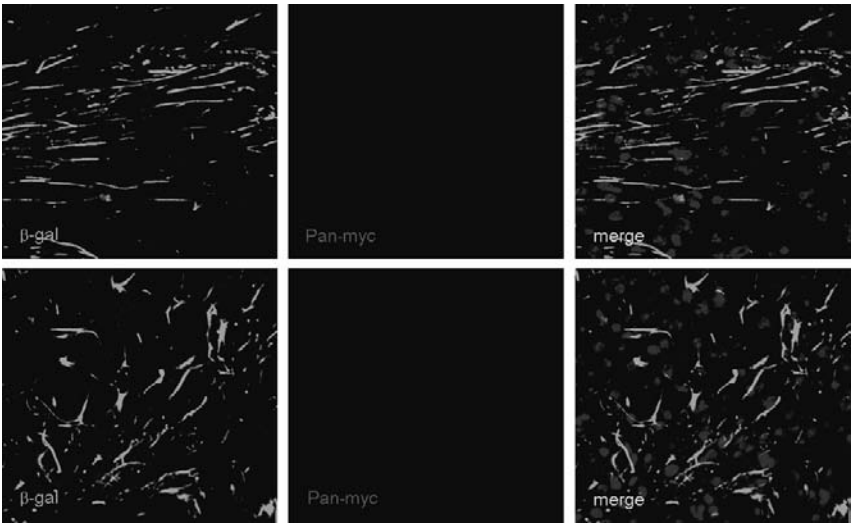

Fig. 5. β-Gal–expressing HB1.F3 human NSCs were transplanted into the brain of adult mouse ICH stroke model, and at 4 weeks post-transplantation, brain sections were processed for double immunostaining of β-Gal/myc. β-Gal–positive F3 cells did not express myc activity any longer, indicating that the v-myc oncogene activity in transplanted F3 human NSC cells has been inactivated and cells no longer undergo proliferation.

5. Rinse the membrane 4 times with TBS-T.
6. Expose the membrane to ECL reagent for 1 min at RT.
7. Expose the membrane to Bio-Max ML film for 2–10 min.

3.6. RT-PCR Analysis of Cell Type-Specific Genes

For analyses of mRNA levels of human NSCs, total RNA is isolated from cultured human NSCs by using TRIzol reagent according to the manufacturer's protocol, and it is the subjected to RT-PCR analysis.

1. First strand of cDNA is synthesized from 1 to 2 μg of total RNA with SuperScript first strand synthesis system for RT-PCR using oligo(dT) primers. Sequences of primers used in the present study are listed in **Table 1**.
2. Reaction mixture (1–4 μl of first strand cDNA, PCR buffer, 200 μM dNTP mix, 2 U of Taq polymerase, and 1.5 mM MgCl2) and 10 pM primers are denatured at 94°C for 2 min.
3. Samples are subjected to 33 PCR cycles (each cycle consists of 30 s at 94°C, 30 s at 50°C, 45 s at 72°C), and then elongated at 72°C for 10 min.
4. PCR products are separated by agarose gel electrophoresis (1.5%) followed by staining with ethidium bromide.

5. Images of the stained RNA gels are acquired using a FLA 3000 Imager. RT-PCR results of mRNA levels for proteins found in human NSCs and their progeny are found in **Fig. 2C,D**.

4. Notes

1. Earlier investigators used culture medium supplemented with EGF (20 ng/ml) or EGF plus bFGF (10 ng/ml) to induce proliferation of NSCs, but many later studies indicated that bFGF (10–20 ng/ml) alone is a competent growth factor for that purpose.
2. An amphotropic replication-incompetent retroviral vector encoding v-myc, pLXN.vmyc, was generated using the ecotropic retroviral vector encoding v-myc, similar to that described previously for generating murine NSC clone C17-2 ***(16)*** and human NSC lines ***(18)***.
3. The supernatants from the PASK1.2 cell line contained replication-incompetent retroviral particles bearing an amphotropic envelope that efficiently infected the human neural cells as indicated by G-418 (neomycin) resistance.
4. Gelvatol could be prepared as follows: measure 10 g of polyvinyl alcohol (Sigma-Aldrich), dissolve it in 0.2 M Tris-HCl buffer (pH 8.5) at 60°C. Next, add 20 ml of glycerol to the solution, mix well, spin at 3,000 rpm for 10 min, and store the solution at 4°C until use as an aqueous mounting medium for immunostained preparations.
5. The permission to use fetal tissue was granted by the Clinical Research Screening Committee involving human subjects of the University of British Columbia, and the fetal tissues were obtained from the Anatomical Pathology Department of Vancouver General Hospital.
6. During the pipette trituration of enzyme-digested brain tissues, tissue fragments often stick to the inside of Pasteur pipette. To prevent this, the inside of Pasteur pipette should be coated with silicone. Aspirate Sigmacote solution (Sigma-Aldrich) briefly with Pasteur pipette briefly up to the neck, expel the solution, air dry, and then autoclave. Sigmacote is a special silicone solution in heptane that easily forms a tight, thin film on glass, is water repellent, and blocks tissue attachment.
7. The most simple form of serum-free medium contains three components, insulin (5–10 μg/ml), transferrin (5–10 μg/ml), and sodium selenite (30 nM), and it is termed ITS. Ready-to-use ITS medium supplement is available from Sigma-Aldrich and other suppliers. More advanced serum-free medium we have used for neural cell cultures (DM4) contains human insulin (10 μg/ml), human transferrin (10 μg/ml), sodium selenite (30 nM), hydrocortisone (50 nM), triiodothyronine (100 pM), and albumin (2 mg/ml) ***(35)***. We use DMEM with high glucose (4 g/l) for the base medium for the serum-free medium. Others often use a 50:50 mixture of DMEM and Ham's F-12 medium.

8. Culture supernatants containing viral vector harvested from the packaging cell line could be stored at –70°C and used for transfection experiments, but it is advised to use a fresh supernatant each time to obtain good results.
9. Aclar plastic round coverslips (9, 12, 18 and 22 mm in diameter) used in the present study are made from transparent Aclar fluorocarbon plastic film, the thickness of which is identical with no. 1 thickness of glass coverslips. Glass coverslips sold by many suppliers are known to contain a minute amount of lead, which is detrimental for cell growth in vitro. Aclar plastic is nontoxic, nondestructible, easy to handle, and culture cell friendly. Aclar coverslips can be purchased from SPL (Seoul, Korea).

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11

Preparation of Neural Progenitors from Bone Marrow and Umbilical Cord Blood

Shijie Song and Juan Sanchez-Ramos

Summary

The bone marrow is clearly much more than a reservoir of stem cells that repopulates blood cell lineages throughout life. The marrow also contains nonhematopoietic stem cells, which are much more versatile than previously appreciated. These nonhematopoietic stem/progenitor cells are found in the bone marrow stromal cell (BMSC) population. BMSCs also are known as colony-forming unit fibroblasts and mesenchymal stem cells (MSCs). MSCs also can be generated from umbilical cord blood and other tissues. MSCs have been shown to express properties of neuroectodermal cells in vitro by many researchers and in vivo after transplantation into the brain and spinal cord. Many investigators have developed variations on the original method described 6 years ago for the preparation of neural progenitors from BMSCs. We bring up to date the materials and procedures used to prepare BMSCs from bone marrow and from human umbilical cord blood for the induction of neural progenitor cells and subsequent differentiation into neurons and glia.

Key Words: Bone marrow; umbilical cord blood; stem cell; hematopoietic stem cell; bone marrow stromal cell; mesenchymal stem cell; neural stem cell; neural progenitor cell; neuron; glia; differentiation.

1. Introduction

In ancient Chinese medicine, the brain was termed the "sea of marrow," the repository of vital energies that flowed from the gonads via the marrow to the brain ***(1)***. Although that ancient perspective now seems absurd, the present evolving view of bone marrow as a source of multipotent cells that can give rise to brain cells and other cells of the body seems just as whimsical.

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The bone marrow is clearly much more than a reservoir of stem cells that repopulate blood cell lineages throughout life. The marrow also contains nonhematopoietic stem cells, derived from marrow stromal cells, which are much more versatile than previously appreciated ***(2)***. Bone marrow stromal cells (BMSCs) are known to provide the structural and functional support for the generation of all the blood cell lineages that arise from hematopoietic stem cells. BMSC consist of morphologically and biochemically distinct cell types: bone marrow fibroblast–reticular cells, adipocytes, osteoblasts, macrophages, and endothelial cells. BMSCs can be cultivated in vitro and contains progenitors capable of generating bone, cartilage, fat, and other connective tissues. These nonhematopoietic stem/progenitor cells found in the BMSC population also are known as colony-forming unit fibroblasts and mesenchymal stem cells (MSC) ***(3)***. The BMSCs per se have been reported to have many characteristics of MSCs; hence, the terms are often used synonymously. MSCs, a homogenous population of fibroblast-like cells purified by Percoll gradient and expanded in vitro, can generate progeny that differentiate into multiple cell lineages, including bone, fat, tendon, and cartilage ***(4)***. MSCs also can be generated from umbilical cord blood and other tissues ***(5,6)***.

BMSCs can be prepared from suspensions of fresh (or postmortem) bone marrow by their selective attachment to tissue culture plastic, or by different depletion methods ***(7)***, and they can be expanded efficiently ***(8–10)***. Even though these adult stem cells are thought to be lineage restricted, which means they should only be able to differentiate into cells committed to their embryonal germ layer origin, there is growing evidence that BMSCs can be induced to "trans-differentiate" into neural cells and other cells types derived from all three embryonal germ layers. A rare multipotent adult progenitor cell (MAPC) was isolated from MSC cultures prepared from rodent bone marrow ***(11,12)***. This cell type was shown to differentiate not only into mesenchymal lineages but also into endothelial and endodermal lineages. Mouse MAPCs injected into the blastocyst contributed to most, if not all, somatic cell lineages, including brain cell types ***(12)***. Another research group using a similar method has isolated multipotent cells called marrow-isolated adult multilineage inducible cells from human postmortem bone marrow specimens ***(13)***.

MSCs (or BMSCs) have been shown to express properties of neuroectodermal cells in vitro by many researchers ***(7,12–23)*** and in vivo after transplantation into the brain and spinal cord ***(24–31)***. Since the original report to document the differentiation of BMSCs into neuron-like cells ***(14)***, many investigators have developed variations on the methods for preparing neural progenitors from bone marrow stromal cells. Most recently, a comparison of various protocols for inducing neural progenitors from bone marrow stromal cells was published ***(22)***. We bring up to date the materials and procedures used

to prepare BMSCs from bone marrow and from human umbilical cord blood for the induction of neural progenitor cells and the differentiation of neurons and glia.

2. Materials

2.1. Growth Medium

1. Dulbecco's modified Eagle's medium (DMEM, Invitrogen, Carlsbad, CA).
2. DMEM is supplemented with the following to reach a final concentration of 2 mM glutamine (100× stock, Invitrogen), 0.001% β-mercaptoethanol (BME), nonessential amino acids, 1 vol/99 vol of DMEM (100× stock, Invitrogen), and 10% donor horse serum (Hyclone Laboratories, Logan, UT) or 10% fetal bovine serum (Hyclone Laboratories).

2.2. Medium for Induction of Neural Progenitor Cells (32)

1. DMEM/Ham's F-12 (1:1; Invitrogen).
2. Supplement DMEM with the following to reach a final concentration of 0.6% glucose, 25 μg/ml insulin, 100 μg/ml transferrin,. 20 nM progesterone, 60 μM putrescine, 30 nM selenium chloride, 2 mM glutamine, 3 mM sodium bicarbonate, 5 mM HEPES, 2 μg/ml heparin, 20 ng/ml epidermal growth factor (EGF), and 20 ng/ml basic fibroblast growth factor (FGF2, or bFGF).

*2.3. Medium for Differentiation into Neurons and Glia**

1. Neurobasal medium (N5) ***(33–35)***.
2. Supplement medium with 5% horse serum, 1% fetal bovine serum (FBS), 100 μg/ml transferrin, 25 μg/ml insulin, 60 μM putrescine, 0.02 μM progesterone, 0.03 μM selenium, 0.5 μM all-*trans*-retinoic acid, 10–20 ng/ml brain-derived neurotrophic factor (BDNF), or 100 ng/ml nerve growth factor (NGF).

*To promote differentiation into glial cells, recombinant human platelet-derived growth factor at 10 ng/ml (rh-PDGF; Promega, Madison, WI) is substituted for BDNF or NGF.

2.4. Preparation of Blocking Solution

1. 7.1 g of Na_2HPO_4 (0.10 M), 0.1 g of sodium azide (very toxic) (0.02%), 25 g of sucrose (5.0 M), 25 g of bovine serum albumin (5.0%), and 0.5 ml of Triton 100 (0.1%).
2. Dissolve ingredients in 450 ml of H_2O.
3. Adjust pH to 7.4 by using 1 M HCl.
4. Bring to final volume of 500 ml.

2.5. Animals

1. BMSCs from C57 BL/6J mice provide the most consistent results.
2. Other strains or species of rodents, such as transgenic mice that express green fluorescent protein (GFP) [C57BL/6J-Tg(ACTbeGFP)1Osb/J; JAX Mice, Bar Harbor, ME] or Fisher rats, have been used as a source of bone marrow.
3. The animals are usually between 6 and 12 weeks of age at the time of marrow harvesting.

2.6. Human Bone Marrow Cells

1. Human bone marrow cells are not easy to obtain for basic research, because they are a scarce resource for treatment of certain leukemias.
2. An alternative source for BMSCs may be the material (bony chips with adherent bone marrow cells, fatty tissue, and debris) retained on the nylon filter through which harvested bone marrow is passed in preparation for bone marrow transplantations.
3. The nylon filters are washed with sterile saline solution five times and centrifuged to remove bone chips.
4. The supernatant is then treated as described under **Subheading 3.2.2.**
5. Other researchers have obtained human bone marrow from cadaveric thoracolumbar (T1–L5) vertebral bodies ***(13)*** or harvested it from routine surgical procedures (pelvic osteotomies) ***(22)***.

2.7. Human Umbilical Cord Blood (HUCB)

1. HUCB can be procured from a number of private or university cord blood banks (*see* **Note 1**).
2. After delivery of the newborn, cord blood is collected from the umbilical cord attached to the placenta, yielding approximately 60–90 ml.
3. The cord blood is usually centrifuged to spin out the heavier red blood cells.
4. The remaining cells are suspended in cryopreservative and stored in liquid nitrogen until needed.

3. Methods

3.1. Mouse Bone Marrow Culture (see Fig. 1)

1. Sacrifice donor mice by cervical dislocation and wash the extremities with ethanol.
2. With sterile scissors, incise the anterior surface of the skin of the extremities cutting from abdomen to the paws.
3. Trim the muscles away, then remove the femur and tibia.
4. Transfer the bones to a beaker containing normal saline with 0.5% bovine serum albumin and keep on ice until all the bones are removed.

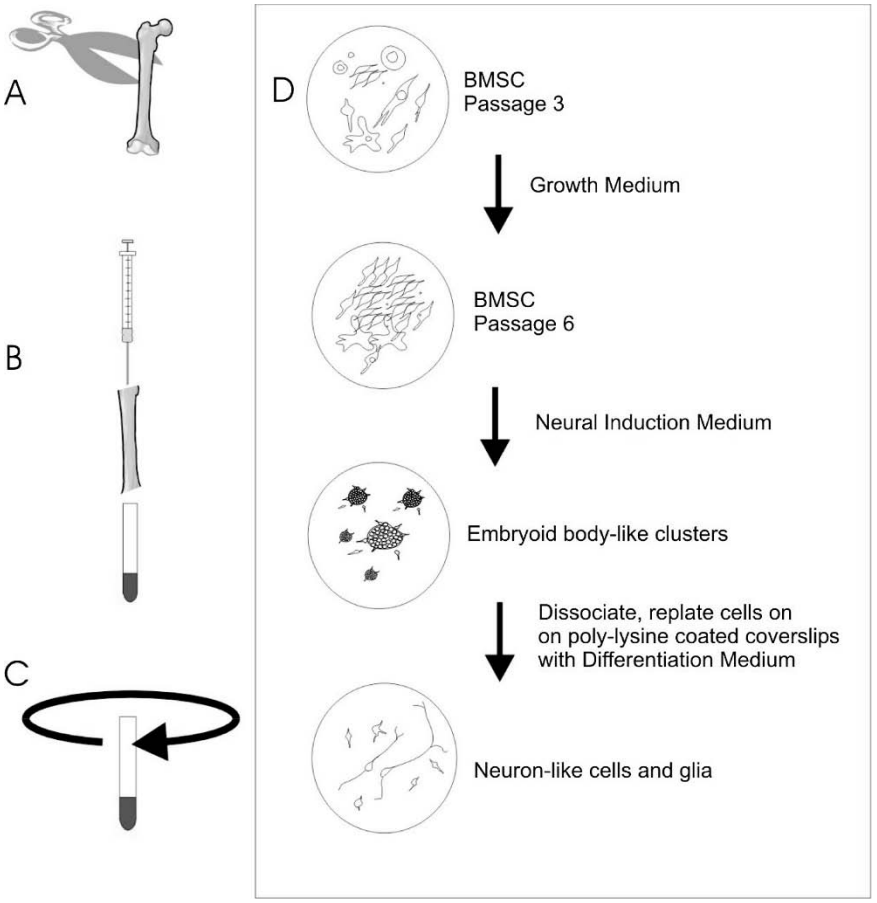

Fig. 1. Preparation of rodent bone marrow stromal cells, induction into neuroectodermal lineages and differentiation. After euthanasia, the limbs of the animals are cleansed with alcohol prior to incision. **(A)** After dissecting away muscle, the long bones of the extremities are visualized and removed. Both ends of the long bones are cut with scissors so that the marrow can be flushed out. **(B)** The tip of a 20–23-gauge needle (attached to a 3-ml syringe) is placed into the proximal end of the femora (and tibia) to flush the marrow through the bone. The flushed material is collected in a 15-ml sterile tube. **(C)** After spinning down the marrow at low speed, the supernatant is discarded, the marrow is resuspended, and plated into culture flasks or dishes. **(D)** The cells are incubated in growth medium. After 4 to 10 passages, cells are harvested, dissociated, and replated in nonadhesive culture flasks in neural induction medium. To trigger differentiation, cells are harvested, dissociated into single-cell suspension, and replated on poly-L-lysine–coated coverslips or culture dishes in differentiation medium.

5. Transfer the femora and tibia to a petri dish under a microbiological safety hood. Carefully cut off the ends of the femora and tibia and avoid splintering the bone. Insert the tip of a 20–23-gauge needle (attached to a 3-ml syringe) into the proximal end of the femora (and tibia) to flush the marrow through the bone. Collect the flushed material in a 15-ml sterile tube.
6. Spin down the cells at 300 × *g* for 5 min. Carefully remove the supernatant flushing medium with a Pasteur pipette and resuspend the marrow in 2–3 ml of "growth medium." (*See* **Subheading 2.1.**) Dissociate cells by mild trituration with a fire-polished Pasteur pipette. Hold the tip of the pipette at a distance of approximately 1 cm from the bottom of the tube.
7. After trituration, spin down cells in a centrifuge at 300 × *g* for 10 min.
8. Suspend the cell pellet in growth medium at 10^6 cells/ml, and plate the cells in culture flasks for 3 days.
9. Replace two thirds of the medium with fresh growth medium two times per week; passage the cells when they reach 80% confluence. This may be repeated up to 10 passages.
10. For induction into neural progenitors, BMSCs are harvested, dissociated in 0.05% trypsin-0.04% EDTA and replated in nonadhesive culture flasks (Nalge Nunc International, Rochester, NY) at a density 10^5 cells/cm^2 in neural induction medium (*see* **Subheading 2.2.**).
11. The formation of clusters of cells that resemble neurospheres becomes evident after 7–14 days. These neurosphere-like structures are expanded for an additional 2–10 weeks (two to four passages or 5 to 30 population doublings). The medium (see **Subheading 2.3.**) is replaced once per week.
12. To trigger differentiation, cells are harvested, dissociated into single cell suspension, and replated on poly-L-lysine–coated coverslips or culture dishes in differentiation medium (*see* **Subheading 2.3.**) for 4–14 days.
13. To promote differentiation into glial cells, plate cells in differentiation medium supplemented with 10 ng/ml rh-PDGF instead of BDNF or NGG for 4–14 days.
14. For visualization of results, fix cells using 4% paraformaldehyde for immunocytochemical staining (*see* **Subheading 3.4.**) or fix with cold methanol or acetone for optimal immunocytofluorescence staining.

3.2. Human Bone Marrow

1. To 3 ml of whole bone marrow collected in heparin or EDTA, add 5 ml of phosphate-buffered saline (PBS) and mix well by inversion.
2. To a 15-ml sterile conical centrifuge tube, add 3 ml of Histopaque-1077 (Sigma-Aldrich, St. Louis, MO).
3. Carefully layer 8 ml of the bone marrow–saline mixture onto the Histopaque-1077 and centrifuge at 400 × *g* for 30 min at room temperature.
4. After centrifugation, use a Pasteur pipette to aspirate the upper layer and discard.

5. With a Pasteur pipette, carefully transfer the opaque interface to a sterile, conical centrifuge tube.
6. To this tube add 10 ml of PBS and mix by inversion.
7. Centrifuge at 300 × *g* for 10 min.
8. Aspirate the supernatant and discard.
9. Resuspend cell pellet with 5 ml of PBS and mix by gentle trituration with a Pasteur pipette.
10. Centrifuge at 300 × *g* for 10 min.
11. Repeat **steps 8–10**. Discard supernatant and resuspend pellet in 3 ml of growth medium.
12. Distribute to three 75-mm flasks with 10 ml of growth medium per each flask for 3 days.
13. Replace two thirds of the medium with fresh growth medium two times per week; passage the cells when they reach 80% confluence. This may be repeated up to 10 passages.
14. From here onward, follow the procedures detailed for mouse BMSCs (*see* **Subheading 3.1.**, **steps 10–14**).

3.3. Human Umbilical Cord Cultures

1. The cryopreserved cells are thawed in a water bath set to 37°C.
2. Cells are spun down (300 × *g* for 10 min) and resuspended in DMEM.
3. An aliquot is taken for counting and the remainder is plated in 75-mm flasks. Details for remainder of the procedure are the same as for bone marrow stromal cells described under **Subheading 3.1.**, steps **8–14**.

3.4. Alternative Method for Neuronal Induction In Vitro

Another method for differentiating neural cells from bone marrow is included ***(14)*** despite criticism of the method for the remarkably rapid change in morphology of cells elicited by dimethyl sulfoxide (DMSO)/butylated hydroxyanisole (BHA) in the neuronal induction media. Rat or human BMSCs were maintained in DMEM/20% FBS.

1. Media are replaced with preinduction media consisting of DMEM/20%FBS/1mM BME for 24 h.
2. Preinduction media are removed, and the cells are washed with PBS.
3. Neuronal induction media-DMEM/2% DMSO/200 μM BHA is used for 30 min to 6 days.
4. To facilitate long-term survival of BMSC-derived neurons, long-term induction media (DMEM/2% DMSO/200 μM BHA/25 mM KCl/2 mM valproic acid/10 μM forskolin/1 μM hydrocortisone/5 μg/ml insulin) is used.
5. Cells are fixed with 4% paraformaldehyde, and immunocytochemical procedures are used for identification of neural cells.

3.5. Coculture of BMSCs with Fetal Midbrain Cultures

To increase the proportion of BMSCs that express neural markers, we have cocultured them with dissociated fetal rat midbrain cultures. Details regarding the preparation of mesencephalic cell cultures from embryonic day 14 rat or mouse fetal brain are beyond the scope of this chapter, but the method we use can be found in a previous publication ***(34)***. BMSCs are first labeled with an appropriate marker before plating with fetal mesencephalic cells (*see* Chapter 28, Cao et al., "Labeling Stem Cells in Vitro for Identification of their Differentiated Phenotypes After Grafting into the CNS"). BMSC also can be distinguished from other cells in cocultures by preparing BMSC from transgenic GFP mice (JAX Mice). When the GFP+ BMSCs differentiate into neurons, they can be distinguished from the neurons derived from fetal mesencephalic cells by virtue of colocalization of the GFP signal with the neuronal marker (e.g., tubulin isotype III [TuJ1] or neuronal nuclei [NeuN]).

3.6. Identification of Neural Cells

Neuron or glial-specific proteins expressed by BMSC-derived neural cells are most readily visualized by standard immunocytochemistry procedures (*see* Chapter 26, Sanjay et al., "Immunocytochemical Analysis of Neuronal Differentiation"). The antibodies and dilutions utilized are listed:

1. Mouse anti-NeuN monoclonal antibodies (Chemicon International, Temecula, CA) 1:100 in blocking solution with normal horse serum, 1:100.
2. Mouse anti-nestin monoclonal antibody (Chemicon International) 1:250 in blocking solution with normal horse serum, 1:100/.
3. Mouse anti-β-TuJ1 monoclonal antibody (Sigma-Aldrich) 1:400 in blocking solution with normal horse serum, 1:100.
4. Mouse anti-microtubule-associated protein-2 monoclonal antibody (Chemicon International) 1:400 in blocking solution with normal horse serum, 1:100.
5. Mouse anti-neurofilament 200 monoclonal antibody (Sigma-Aldrich) 1:40 in blocking solution with normal horse serum, 1:100.
6. Mouse anti-neurofilament 68 monoclonal antibody (Sigma-Aldrich) 1:400 in blocking solution with normal horse serum, 1:100.
7. Mouse anti-human neuronal protein HuC/HuD (anti-Hu) (Invitrogen) 1:100 in blocking solution with normal horse serum, 1:100.
8. Rat anti-Musashi I monoclonal antibody (Chemicon International) 1:500 in blocking solution with normal rabbit serum, 1:100.
9. Rabbit anti-α-internexin polyclonal antibody (Chemicon International) 1:500 in blocking solution with normal goat serum, 1:100.
10. Rabbit anti-glial fibrillary acidic protein polyclonal antibody (Biogenex, San Ramon, CA) 1:100 in blocking solution (without detergent) with normal goat serum, 1:100.

11. Rabbit anti-human fibronectin polyclonal antibody (Sigma-Aldrich) 1:400 in blocking solution (without detergent) with normal goat serum, 1:100.
12. Horse anti-mouse IgG; goat anti-rabbit IgG; rabbit anti-rat IgG (Vector Laboratories, Burlingame, CA). The concentration of biotinylated anti-immunoglobulins is 1:200 in PBS (*see* **Note 2.4**). The concentration of fluorescein- (or other fluorescent molecular) labeled secondary antibodies (Vector Laboratories) is 15 μg/ml.

3.7. Immunocytochemical Staining of Cultures

1. Remove the culture medium and carefully wash cultures two times with PBS.
2. Fix cultures for 30 min in 4% formaldehyde in PBS at room temperature.
3. Wash cultures two times in PBS.
4. Incubate cultures with primary antibodies in blocking solution for 24–48 h at 4°C.
5. Remove medium containing primary antibodies (and store at 4°C for later re-use).
6. Warm up cultures to room temperature and wash three times with PBS.
7. Incubate cultures with second antibody in PBS or blocking solution for 30 min to 2 h (*see* **Note 2**).
8. Wash cultures three times in PBS.
9. Incubate in H2O2 0.3% in PBS for 10 min (*see* **Note 3**).
10. Wash sections in PBS three times and prepare Vectastain reagents at this time (*see* **Note 4**).
11. Incubate cultures in Vectastain reagent preincubated for 30 min at room temperature).
12. Wash sections in PBS three times.
13. Incubate in developing solution (*see* **Note 5**) for 2–5 min with care not to overdevelop the brown stain.
14. Wash sections in PBS two to three times.
15. Pool the waste containing diaminobenzidine and treat with hypochlorite.

4. Notes

1. For more information regarding collection and banking of HUCBs, see www.cordblood.med.ucla.edu.
2. Cultures are routinely incubated with secondary antibody in PBS, but if background staining is high, we incubate the cultures with blocking solution containing serum from the species in which the secondary antibodies were raised.
3. For light microscopic visualization, we use the ABC Vectastain kit (Vector Laboratories) that uses biotinylated secondary antibodies and relies on the high affinity of avidin for biotin. The chromogenic solution contains diaminobenzidine to generate a brown stain.
4. The reagents in the ABC Vectastain kit are prepared (as described in the kit) by adding 9 μl of solution A to 1 ml of PBS, and 9 μl of solution B to 1 ml of

PBS. Both solutions A and B are incubated for 40–60 min at room temperature before use.

5. Prepare the development solution containing diaminobenzidine immediately before use. To 10 ml of PBS, add 10 mg of diaminobenzidine hydrochloride (caution as it is toxic, carcinogenic) and 10 µl of 30% H2O2.

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12

Production of Neurospheres from CNS Tissue

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Summary

The relatively recent discovery of persistent adult neurogenesis has led to the experimental isolation and characterization of central nervous system neural stem cell populations. Protocols for in vitro analysis and expansion of neural stem cells are crucial for understanding their properties and defining characteristics. The methods described here allow for cell and molecular analysis of individual clones of cells—neurospheres—derived from neural stem/progenitor cells. Neurospheres can be cultivated from a variety of normal, genetically altered, or pathological tissue specimens, even with protracted postmortem intervals, for studies of mechanisms underlying neurogenesis, cell fate decisions, and cell differentiation. Neurosphere-forming cells hold great promise for the development of cell and molecular therapeutics for a variety of neurological diseases.

Key Words: Neural stem cells; subependymal zone; transplantation; neurogenesis; culture.

1. Introduction

Until relatively recently, it was widely held, despite isolated reports to the contrary ***(1)***, that de novo generation of neurons in the mammalian central nevous system (CNS) did not persist past perinatal development. This perception drastically changed in the last decade of the twentieth century when it was determined that a persistent germinal zone containing neural stem cells (NSCs) with the capacity to differentiate into neurons and glia existed within the CNS of adult mammals ***(2)***. It is now known that constitutive in vivo genesis of neurons occurs throughout life, and it is restricted primarily to two

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specific regions: the periventricular subependymal zone (SEZ), which generates neurons destined for the olfactory bulb; and the subgranular zone (SGZ) of the hippocampus, which generates neurons destined for the dentate gyrus ***(3,4)***. Although the SEZ and SGZ are the regions of adult neurogenesis, recent studies have revealed that there may be additional, latent, pools of NSCs in other regions of the brain.

In vitro, NSCs can be propagated from a variety of rodent and human tissues, including cerebral cortex, SEZ, hippocampus, and spinal cord ***(5–7)***. Clones of NSCs can be cultivated either as monolayers of substrate-anchored cells ***(6,8)*** or as suspended, spherical structures called "neurospheres" ***(9,10)***. We describe a method that our laboratory has developed for the generation and study of neurospheres ***(5,11)***, which involves cultivating single-cell suspensions in the absence of cell–cell and cell–substrate interactions. This method is based on the theory that our culture conditions will allow for the clonal expansion of cells and that they will maintain cells in a primitive ontogenetic state, because substrate attachment is necessary for differentiation to occur.

Work in our laboratory generally involves postnatal mice (1–8 days), but neurospheres can be generated from differing ages and from a variety of CNS structures in mice. Because neurosphere yield from the SEZ is much higher than other structures, it is technically easier to make a discreet isolation of the SEZ compared with embryonic animals, thereby increasing the signal-to-noise ratio, and the dissociation procedure seems to be far gentler (compared with older animals that have already undergone significant myelination), resulting in better cell survival.

We also have demonstrated that it is possible to generate neurospheres from SEZ after extensive postmortem intervals by using the same methods ***(12)***. However, neurosphere yield declines precipitously if the brain is kept at room temperature. Storing the brain at 4°C dramatically lengthens the neurogenic potential of postmortem tissue such that it is possible to cultivate neurospheres almost 1 week after death.

A major benefit of our protocol is that neurospheres are easily manipulated and lend themselves to many different analyses, both individually and collectively. The plating density that we use is low enough to allow single neurospheres to be quickly removed with a hand-held pipettor. Once isolated, a neurosphere can be used for immunocharacterization or gene analysis, or it can be passaged to generate secondary neurospheres. Populations of numerous neurospheres are also suitable for these purposes and can, in addition, be used for ultrastrucural analysis, long-term cryostorage, or transplantation (*see* **Fig. 4** and **Subheading 3.** for methods). Regarding preparation of neurospheres for transplantation, it is possible to start with a variety of transgenic animals or

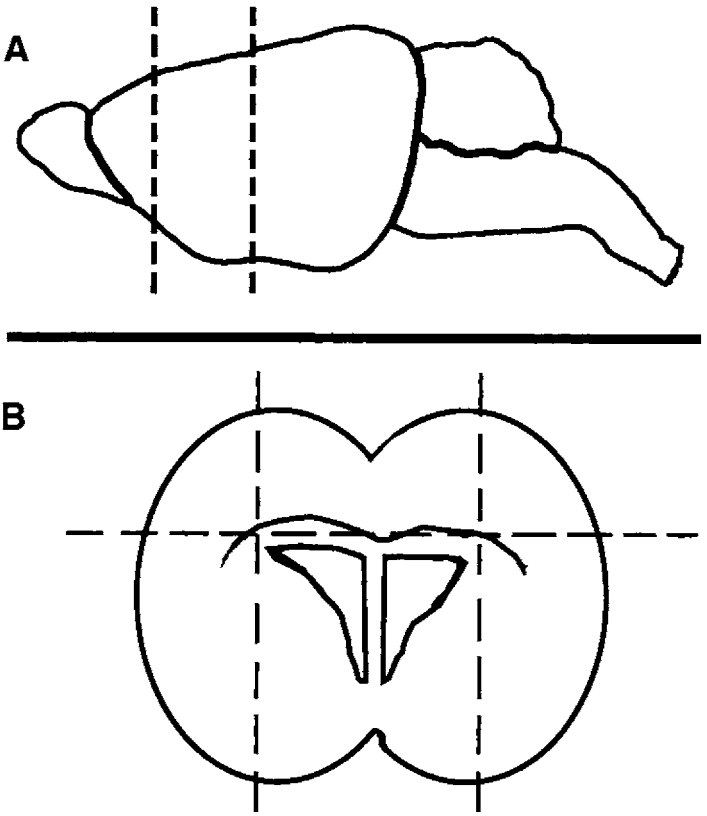

Fig. 1. Schematic of the dissection protocol. Starting with whole brain, make two coronal cuts in the area between the rhinal fissure and the hippocampus (*broken lines*; **A**). Lay the resulting chunk on its posterior surface, and make two parasagittal cuts just lateral to the lateral ventricles, and one horizontal cut at about the level of the corpus callosum (*broken lines*; **B**). Make neurospheres by dissociating the central, rectangular piece of tissue containing the lateral ventricles.

transfected cells that contain marker genes useful for subsequent discernment of donor versus host-derived cells.

Recent evidence has accumulated suggesting that glial cells have stem cell characteristics in vivo and that they may represent the neurosphere-forming cell in vitro. Specifically, certain astrocytes have been shown to undergo mitosis and to give rise to neuroblasts in the adult mouse SEZ ***(13)***. Furthermore, work from our laboratory has demonstrated that subpopulations of cultured mouse astrocytes (derived from a variety of CNS regions) can generate neurospheres in a regionally and temporally restricted manner ***(14)***. Astrocytes cultured from

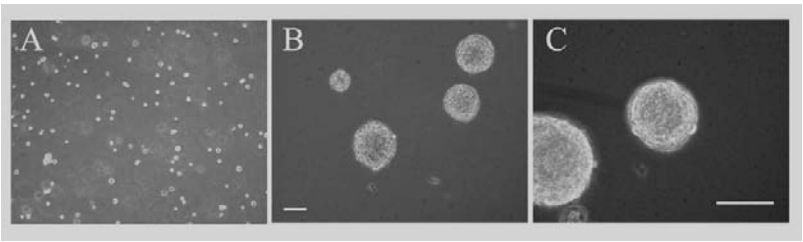

Fig. 2. Phase micrographs of neurospheres in suspension culture. **(A)** Sphere-forming cells immediately after plating (×10). **(B,C)** After 10–14 days, neurospheres are approaching their greatest diameter, and they look like large globes with sharp, phase-contrast bright outer borders. Bar = 40 μm in **B** and 150 μm in **C**.

the cerebral cortex, cerebellum, and spinal cord can generate neurospheres when grown in the presence of growth factors, but only if these cultures are derived from animals younger than postnatal day 11. Astrocytes cultured from the SEZ can generate neurospheres when derived from both perinatal and adult animals. Furthermore, this cell population is amenable to SEZ transplantation and engraftment in a manner remarkably similar to neurosphere cell populations, with migratory, donor-derived progeny present in the rostral migratory stream (RMS) and olfactory bulb weeks after transplantation (*see* **Fig. 3**). The expandability of the adherent monolayer culture system is beneficial in that a significantly larger population of cells can be generated for transplantation in a relatively short period compared with the neurosphere culture system.

2. Materials

2.1. Generation of Neurospheres from Acutely Dissociated SEZ

1. 1× Dulbecco's modified Eagle's medium (DMEM)/Ham's F-12 (Invitrogen, Carlsbad, CA. cat. no. 12500-062).
2. N2 culture supplement (500× stock solution) containing pituitary extract (Sigma-Aldrich, St. Louis, MO, cat. no. P1476; 714 μl/500 ml, 10 mg total), putrescine (Sigma-Aldrich, cat. no. P5780; 8.06 mg/500 ml, 100 μM final), insulin (Sigma-Aldrich, cat. no. I5500), 2.5 mg/500 ml, 67.5 units), progesterone (Sigma-Aldrich, cat. no. P7556; 3.145 mg/ml, 0.22 mg/500 ml), transferrin (Sigma-Aldrich, cat. no. T1428; 12.5 mg/500 ml), and sodium selenite (Sigma-Aldrich, cat. no. S5261; 2.59 μg/500 ml, 30 nM final).
3. 1× DMEM/Ham's F-12 containing 5% fetal bovine serum (FBS; Atlanta Biologicals, Norcross, GA, cat. no. S11150).
4. 0.25% Trypsin-EDTA solution (Invitrogen, cat. no. 15405-012).
5. Ultralow attachment, anti-adhesive six-well plates (Corning Life Sciences, Acton, MA, cat. no. 3471).

6. Fire-polished Pasteur pipettes: prepare medium and narrow bore sets by briefly exposing the tip of the pipette to the flame of a Bunsen burner to narrow the lumen.
7. Add a cotton plug to the proximal end and autoclave before use.
8. 15-ml Falcon tubes (TPP AG, Trasadingen, Switzerland, cat. no. TP91015).
9. Growth factor stock solution:
    a. cultures require supplementation with 20 ng/ml epidermal growth factor (EGF; Invitrogen, cat. no. 13247-010) and 10 ng/ml basic fibroblast growth factor (bFGF) (Sigma-Aldrich, cat. no. F0291) every 2–3 days.
    b. Because each culture well will contain approximately 2 ml of medium, we supplement with 50-µl aliquots of 40× stock (8,000 ng of EGF and 4,000 ng of bFGF in 10 ml of DMEM/Ham's F-12).
    c. Stock can be prepared more concentrated if desired, but we do not recommend a less concentrated stock, because correspondingly larger aliquots will quickly reduce the viscosity of the neurosphere cloning medium.
10. PBS or DMEM/Ham's F-12 containing antibiotic/antimycotic (Sigma-Aldrich, cat. no. A9909).

2.2. Generation of Adherent Monolayers from Acutely Dissociated SEZ

1. PBS or DMEM/Ham's F-12 containing antibiotic/antimycotic (Sigma-Aldrich, cat. no. A9909).
2. N2 culture supplement.
3. 1× DMEM/Ham's F-12 medium containing 5% FBS (Atlanta Biologicals).
4. 0.25% Trypsin-EDTA solution (Invitrogen, cat. no. 15405-012).
5. 15-ml Falcon tubes (TPP AG).
6. T-75 tissue culture flasks (TPP AG, cat. no. TP90076).

2.3. Immunolabeling

1. Standard small-volume pipettor with sterile tips.
2. 12-well culture plates (TPP AG, cat. no. TP92412).
3. 18-mm round coverglass (Fisher Scientific, Pittsburgh, PA, cat. no. 12-546) coated sequentially with poly-L-ornithine and laminin.
    a. Prepare by incubating coverglass overnight at room temperature in H_2O containing 10 mg/ml poly-L-ornithine (Sigma-Aldrich, cat. no. P4957).
    b. Wash three times with H_2O and incubate 8–10 h at 37°C in PBS containing 2.5 mg/ml laminin (Sigma-Aldrich, cat. no. L2020).
    c. Wash three times with PBS.
    d. Plates can be stored long-term in PBS at –20°C or short-term in PBS at 4°C.
    e. Sterilize before use with PBS containing antibiotic/antimycotic and irradiate with an ultraviolet germicidal lamp.

4. 1× DMEM/Ham's F-12 containing 1% FBS.
5. Inverted phase microscope.

2.4. Ultrastructural Analysis

1. Embedding plastic (TAAB, Reading, UK).
2. Electron microscopy (EM) fixative: 0.1 M sodium cacodylate buffer (Electron Microscopy Sciences, Hatfield, PA, cat. no. 12300) containing 2% paraformaldehyde (Sigma-Aldrich, cat. no. P6148), 2% glutaraldehyde (Electron Microscopy Sciences, cat. no. 16350).
3. 2% uranyl acetate (Electron Microscopy Sciences, cat. no. 22400) in 0.9% saline.
4. 1% OsO_4 (Electron Microscopy Sciences, cat. no. 19100) in PBS.
5. Small plastic microcentrifuge tubes.
6. Graded ethanols (50, 70, 80, 90, 100%).

2.5. Gene Analysis

1. Microtip sonicator.
2. 0.6-ml tubes.
3. SuperScript reverse transcriptase (Invitrogen, cat. no. 18053-017), Standard reverse transcription-polymerase chain reaction (PCR) reagents, including: RNase-free water, RNase inhibitor, and RNase-H.
4. Solutions for sterilizing the microtip sonicator: 1 M HCl, 1 M NaOH, 1 M Tris-HCl, and double-distilled H_2O (ddH_2O).

2.6. Transplantation into Neonatal and Adult Mouse Brains

1. 5-µl Hamilton syringe with attached 26-gauge needle (Hamilton, Reno, NV, cat. no. 84851).
2. Avertin (2.5% 2-2-2-tribromoethanol) anesthetic solution (Aldrich Chemical, Milwaukee, WI, T4 840-2) plus 1.5% *tert*-amyl alcohol, in H_2O).
3. Surgical scalpel, forceps, and sutures.
4. Rodent stereotaxic apparatus.

3. Methods

3.1. Generation of Neurospheres from Acutely Dissociated SEZ

The following protocol is the standard method our laboratory has developed to produce neurospheres from mouse and adult human brains ***(5,11)***. Any culture dish configuration can be used, but we prefer six-well plates because: they allow for multiple experimental manipulations of the same sample, they lend themselves to rapid visual screening (without the optical interference common to plates with a smaller well diameter), potential infections are contained within

single wells, and infections can be removed without sacrificing the entire sample.

1. Decapitate mouse pup and briefly dip the head in ethanol (EtOH).
2. Remove the brain and place it on a clean surface suitable for cutting.
3. With a razor blade, make a coronal block, about 2 mm in thickness, in the area between the rhinal fissure and the hippocampus (*see* **Fig. 1A**). Lay the block flat on the cutting surface and use the razor blade to make two parasagittal cuts just lateral to the lateral ventricles, and a horizontal cut to remove the tissue above the corpus callosum (*see* **Fig. 1B**). This procedure leaves a small, rectangular chunk of tissue surrounding the lateral ventricles containing a high density of NSCs.
4. Wash the tissue chunk for several minutes in medium or PBS containing antibiotics/antimycotics. All subsequent work should be performed with sterile materials in a laminar flow hood.
5. Remove antibiotics/antimycotics and incubate tissue in trypsin-EDTA solution at 37°C for 5 min.
6. Gently triturate tissue through a series of descending-diameter, fire-polished Pasteur pipettes to make a single-cell suspension.
7. Add several volumes of DMEM/Ham's F12 containing 10% FBS. Centrifuge to pellet cells and wash several times with fresh medium.
8. Count cells using a hemacytometer.
9. In a 15-ml Falcon tube, combine 6 ml of DMEM/Ham's F-12 medium, 60,000 cells, and 50 µl of 40× growth stock. Add neurosphere cloning medium to bring the final volume to 12 ml.
10. Mix for several minutes by repeatedly inverting the tube.
11. Distribute 2 ml to each well of a six-well plate coated previously with anti-adhesive. The final cell density will be about 1,000 cells/cm^2, although the viscosity of the cloning medium makes precise volumetric measurements diffuse.
12. Add 50-µl aliquots of 40X growth factor stock every 2–3 days. Neurospheres will be visible under phase optics after 7–10 days. True neurospheres are characterized by near perfect spherical shape as well as very sharp, phase-bright outer edges. Importantly, individual cells should not be seen with low-power phase optics (*see* **Note 3** and **Fig. 2**).

3.2. Generation of Adherent Monolayers from Acutely Dissociated SEZ

The following protocol describes our method of generating astrocytic monolayers that can subsequently be used to generate multipotent neurospheres. Once monolayers are established, they can be replated under neurosphere-generating conditions, as described above, where 1–10% of plated cells form neurospheres.

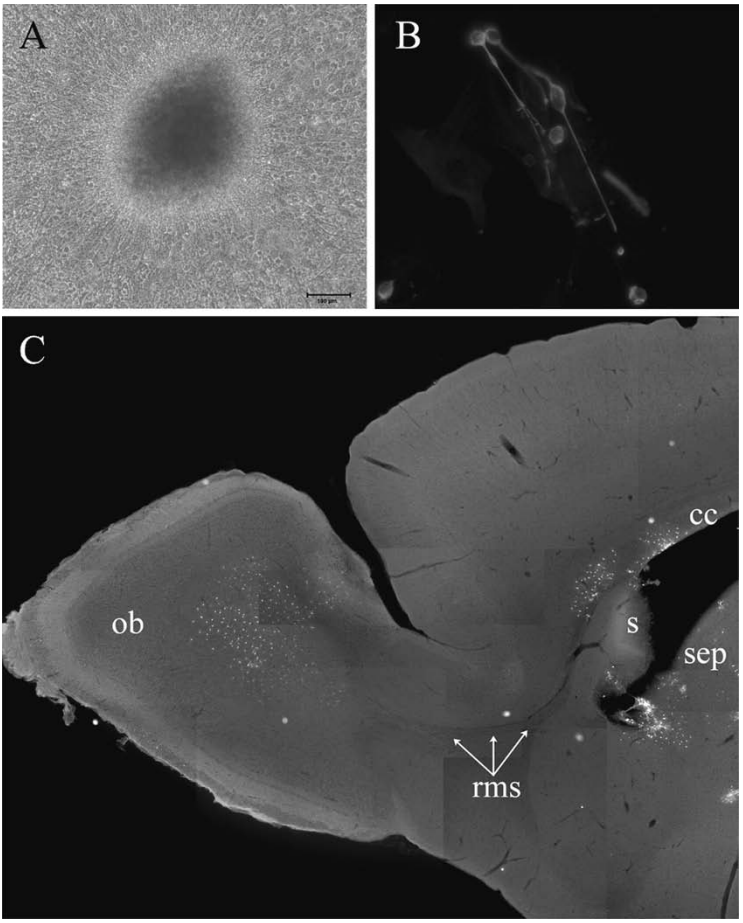

Fig. 3. Characterization and use of neurospheres. **(A)** Phase microscopy of a single neurosphere attached to a coverslip for 5 days. A nearly confluent monolayer of cells has migrated away from the main mass of the neurosphere. **(B)** Immunofluorescence labeling of attached neurosphere cells reveals a population of β-III-tubulin–positive neurons. **(C)** Dissociated neurospheres derived from a GFP transgenic mouse are useful for transplantation because constitutive GFP renders the donor cells easily distinguishable from the host tissue, as seen in this fluorescent photographic montage of ×20 images of a transplant into the lateral ventricle of an adult C57BL/6 mouse 3 weeks after surgery. Bar = 100 μm in A.

1. Decapitate mouse pup and briefly dip the head in EtOH.
2. Remove the brain and place it on a clean surface suitable for cutting.
3. Use a razor blade or microknife to isolate your CNS area or interest (e.g., SEZ; *see* **Subheading 3.1.**).

4. Wash tissue briefly in a 15-ml Falcon tube containing medium or PBS with antibiotics/antimycotics.
5. Remove antibiotics/antimycotics and incubate tissue in 5–10 ml of trypsin-EDTA solution for 37°C for 5 min.
6. Triturate with a 5-ml serological pipette for 2–3 min to break up the tissue into small chunks. It is not necessary to make a single-cell suspension.
7. Add 1–2 ml of FBS to neutralize trypsin and centrifuge cells to form a pellet.
8. Aspirate supernatant and wash by trituration with fresh medium. Pellet and repeat three to four times.
9. Resuspend in DMEM/Ham's F-12 medium containing 10% FBS, plate in T-75 culture flasks (use one flask for each brain), and place in incubator overnight.
10. Remove the culture supernatant and replate into fresh T-75 flasks (discard original flasks which contain primarily microglia).
11. Replace medium every 2–3 days with fresh DMEM/Ham's F-12 containing 10% FBS until astrocytic monolayers become confluent.
12. Remove astrocytes from flasks by aspirating culture supernatant and incubating in trypsin-EDTA for 5–10 min.
13. Collect cells in a Falcon tube, add serum to neutralize trypsin, and proceed with **step 8** (*see* **Subheading 3.1.**).

3.3. Immunolabeling

The following protocol is our standard method for immunolabeling neurospheres (*see* **Note 4**) after they have attached to a favorable substratum and have begun to migrate and differentiate.

1. Place coated coverslips in 12-well plates and put a drop (50–100 µl) of medium near the center of each. Keep in a laminar flow hood.
2. Remove the cover from a six-well plate containing neurospheres and visualize with the inverted microscope (contamination of wells is rare, even though the plate is opened outside the hood, but wash the microscope and pipettor with EtOH first).
3. While looking through the microscope, guide the tip of the pipettor set for 2–5 µl to the neurosphere, and aspirate it into the tip.
4. Eject the neurosphere into the medium on the coverslip. Repeat as often as desired. We typically place 2–10 neurospheres on each coverslip.
5. Place 12-well plates in an incubator. Neurospheres should be attached firmly to the coverslip by the next day, at which time they can be fixed or left to cultivate for a longer period. If the neurospheres are to be cultured for more than 1–2 days, it is important to carefully flood the coverslip with fresh medium after attachment has taken place to prevent evaporation of the media.
6. Wash, fix, and process coverslips for standard immunolabeling, scanning EM, or both (*see* **Fig. 3**).

3.4. Ultrastructural Analysis

We have developed a method for generating electron micrographs of suspended neurospheres. Owing to the need for visually tracking the sample during processing, this method does not allow for the ultrastructural analysis of a single, prospectively identified neurosphere, but rather requires that a large number of neurospheres be processed together before retrospectively choosing individual examples to section and analyze.

1. Liquefy 2% agar by placing in water bath (85°C).
2. Use a transfer pipet to pool several hundred neurospheres in a 15-ml Falcon tube.
3. Centrifuge to pellet neurospheres. Aspirate medium and gently resuspend in EM fixative. Incubate for 30 min at room temperature.
4. Wash two to three times by gently pelleting and resuspending in PBS.
5. After a final pelleting, aspirate as much PBS as possible. Resuspend in a small volume (20–50 µl) of PBS and transfer to a plastic microcentrifuge tube. Quickly add an equal volume of melted agar to the neurospheres and mix gently (work quickly so that the agar does not solidify before being mixed with the neurospheres). Place tube at 4°C for 15 min to harden agar.
6. Cut off the tip of the tube with a razor blade, and use a small spatula to pry the agar plug out. Place the plug into OSO4 for 2 h at room temperature; the osmium will turn the neurospheres brown, rendering them apparent to the naked eye.
7. Rinse for 30 min at room temperature in H_2O and then place into uranyl acetate for 1 h.
8. Dehydrate through graded alcohols, place in embedding plastic, and section for standard transmission EM (*see* **Fig. 4**).

3.5. Gene Analysis

Gene profiling (*see* **Note 6**) of large numbers of pooled neurospheres can be performed using standard techniques for RNA isolation. However, sometimes there may be a desire to examine transcripts present in an individual neurospheres. Because neurospheres consist of at most, several thousand cells, and because these cells are embedded in a dense extracellular matrix, RNA extraction can be tricky, and the normally low RNA yields can be lost if subjected to the additional step of RNA isolation. To address these problems, we have developed a method that combines sonication and RT-PCR without RNA isolation ***(15)***. All procedures must be performed in the same microcentrifuge tube, and the results are much better than those obtained with extraction by either the guanidine cyanide method or freeze-thawing, both of which lead to significant loss of material.

1. Place a single neurospheres in a 0.6-ml tube containing 10 µl of RNase-free water with 5 U of RNase inhibitor. Keep tube on ice.

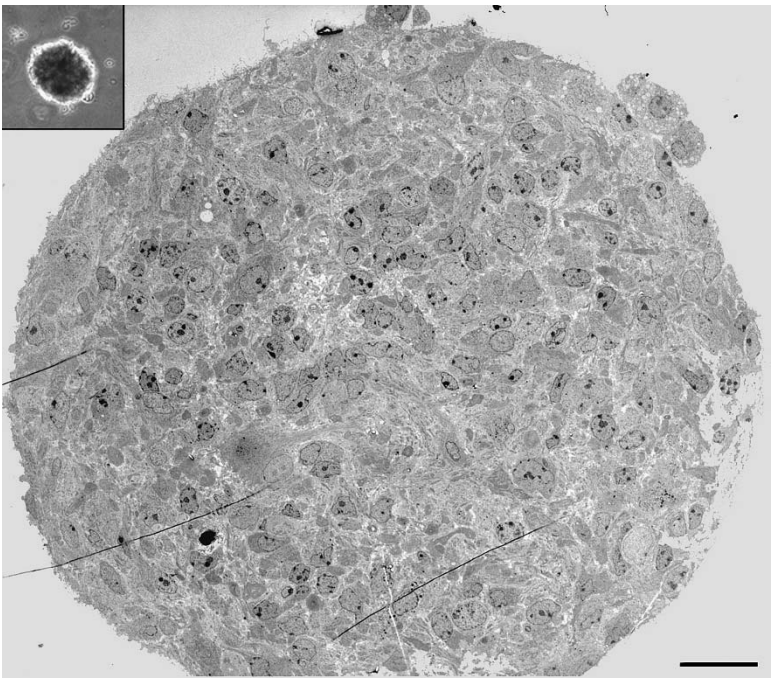

Fig. 4. Ultrastructure of a single neurosphere. Inset contains a lower power phase micrograph of a representative neurosphere derived from surgical biopsy of adult human SEZ. Ultrastructural analysis of these neurospheres (obtained via transmission electron microscopy) reveals a diverse population of cells in different states of differentiation from a presumed stem/progenitor cell to differentiated neurons and glia ***(5)***. Bar = 30 μm.

2. Release RNA by sonicating with microtip sonicator tip (Kontes Glass, Vineland, NJ) by gently touching the surface of the water for 4–10 s. Immediately put the tube back on ice.
3. If working with multiple samples, sequentially wash the sonicator tip between samples in ice-cold 1 M HCl, 1 M NaOH, 1 M Tris-HCl, pH 7.5, and ddH2O.
4. Perform first strand cDNA synthesis by using SuperScript reverse transcriptase (Invitrogen) according to the manufacturer's instructions.
5. Add 4 U of RNaseH to remove the cDNA:RNA hybrid.
6. This solution is now ready to use as template in standard PCR reactions optimized for each primer set.

3.6. Transplantation into Neonatal and Adult Mouse Brains

We routinely transplant dissociated neurospheres derived from transgenic (green fluorescent protein [GFP]) mouse SEZ into the brains of both adult and neonatal C57BL/6 mice in a variety of structures (SEZ, RMS, cortex,

hippocampus, and cerebellum). Although the adult host model is a relevant system for the analysis of adult neurogenesis and regeneration, the neonatal model is an attractive alternative for the analysis of donor cells, because engraftment is significantly more robust in the brains of early postnatal mice (presumably because residual CNS development persists in the days after birth in the mouse).

1. Use a transfer pipette to pool several hundred neurospheres in a 15-ml Falcon tube.
2. Centrifuge to pellet neurospheres. Aspirate medium and resuspend in 2 ml of trypsin-EDTA. Incubate at 37°C for 5 min.
3. Neutralize trypsin by adding approximately 0.5 ml of FBS via a small bore fire-polished pipette. Using the same pipette (the FBS will have coated the inner wall of the pipette and has been found to prevent the trypsinized cells from adhering to the glass), triturate the neurospheres 20 times, or until a majority of the visible clumps are no longer visible.
4. Wash cells in N2 media and then centrifuge to pellet.
5. Determine cell number using a hemocytometer or equivalent technique, then resuspend in a volume of yielding 50,000 cells/µl DMEM/Ham's F-12.
6. Recipient mice should be anesthetized (we prefer to use Avertin; *see* **Subheading 2.6.**), placed into a stereotaxic apparatus, the scalp surgically exposed, and the dura exposed at the required location. Cells (100,000; 2 µl) can be stereotaxically injected into the lateral ventricle via a 5-µl Hamilton syringe attached to a 26-gauge needle at the following coordinates: A-P, –0.2; M-L:, –1.2; and H-D, –2.5. Rostral migratory stream injections can be stereotaxically performed at the following coordinates: A-P, 3.0; M-L, 0.8; and H-D, 3.0.
7. Allow transplanted animals to recover and then return to general housing.
8. For neonatal animals, the transplant technique is somewhat cruder than for adults, and it lacks stereotaxic accuracy. Nevertheless, it is not difficult to direct cells to the large lateral ventricles. As before, load 100,000 dissociated neurosphere-derived cells into a 5-µl Hamilton syringe attached to a 28-gauge needle. Host pups can be cryoanesthetized by placing them at –20°C for 5 min, and cryoanesthesia is maintained during transplantation by placing the animal on a prechilled block. Using the bregma cranial suture as a landmark, the cells can be injected at the desired locale simply by inserting the needle into the skull (we find that the neonatal skull is soft enough for this procedure until around postnatal day 3).
9. Once the desired time for engraftment has been reached, sacrifice transplanted animals via trans-cardiac perfusion using 4% paraformaldehyde. After perfusion, decapitate the animal and remove the brain in its entirety, taking care to leave no pertinent CNS structures behind. Postfix brain(s) overnight in 4% paraformaldehyde then section the tissue in the desired plane and thickness by using a Vibratome or equivalent tissue sectioning equipment.

10. Sections can be processed for immunohistological analysis or directly mounted to glass slides for fluorescent and/or confocal microscopy (*see* **Fig. 3**).

4. Notes

1. We feel it is important to point out that the neurosphere protocol described in this chapter is not for strict clonal analysis. Should the goal of the experiment be to determine the clonal properties of NSCs, the researcher would be best served by plating single cells from primary neural tissue in individual microwells of a 96-well plate or similar cell sequestering protocol.
2. In addition to issues of clonality, the subject of neurospheres as an in vitro manifestation of NSCs and as a model for NSC biology needs to be addressed. As with other tissue-specific stem cells, NSCs are expected to demonstrate three cardinal features: clonal expansion, multilineage differentiation, and extensive self-renewal. The first two features leave little room for interpretive differences. Clonal expansion has a single definition: all cells within a neurosphere are the progeny of a single NSC founder cell. Although it is certainly possible, in high-density cultures, to grow mixed, polyclonal spherical structures that resemble clonal neurospheres, few would disagree that at least the capacity for clonal expansion is a requisite feature of neurosphere if they truly do represent NSCs. Likewise, multilineage differentiation is fairly unambiguous. Neurospheres must be capable of generating progeny belonging to all three neural lineages, and this is generally assayed by phenotypic immunolabeling. Again, although it may be possible to experimentally manipulate the fate of neurospheres progeny by directing differentiation toward one or another lineage, the capacity for multilineage differentiation, whether realized or not, is an essential feature of true neurospheres. It is the third essential NSC feature—extensive self-renewal—that tends to be the most variable and poorly defined as it applies to neurosphere formation ***(16,17)***. What does extensive self-renewal mean? In many studies, neurospheres are dissociated and recultured to generate a number of secondary NSCs. This is clearly evidence of self-renewal, but does it constitute the *extensive* self-renewal that should characterize NSCs? This dilemma has yet to be conclusively resolved, but there are currently studies underway that may allow for the accurate analysis of NSC activity in vitro using the neurosphere assay ***(17)***.
3. The following are suggestions to address some common problems that can arise when trying to grow neurospheres by using our protocol:

    a. False neurospheres: Undissociated tissue pieces can, over time, begin to resemble spheres, which is why it is important to begin with single-cell suspensions. However, these pieces do not have sharply defined outer borders, and they are only rarely spherical enough to be easily confused with true neurospheres. It should be possible to eliminate these pieces from your culture by more thorough trituration with smaller diameter pipettes. Additionally, you can allow the dissociated cell suspension to sediment for several minutes, which permits the larger, undissociated chunks to settle to the bottom of the tube.

The upper portion can then be transferred to a new tube, added to cloning medium, and be plated. We have observed that single, dissociated cells can clump together to form aggregates that resemble neurospheres. These cells, too, lack sharp outer edges, and it should be easy to discern individual cells within the mass using phase optics. If aggregation is a problem, try plating at a lower density.

b. Infection. You may, from time to time, encounter infected wells. Use a repeating pipettor when applying growth factor aliquots, because this will minimize the number of times you need to open the growth factor stock solution. Remember to sterilize your tools and cutting surface with EtOH and to flame before each dissection. Micrococcus infections readily originate from the skin of the donor animal, so take care to thoroughly wash the head in EtOH before removing the brain.

c. Low neurosphere yield. This protocol typically yields dozens of neurospheres in each well, depending on the age of the animal. If you want to increase your yield, try making a cleaner dissection by removing more of the tissue surrounding the SEZ; the less of these other tissues (e.g., striatum, cortex), the greater the percentage of plated cells that will generate neurospheres. It is also possible to plate at a higher cell density, but beware that too high a density will increase the likelihood of forming nonclonal aggregates.

During the dissociation, take care not to triturate too harshly as to lyse the cells. Determine empirically the largest bore pipette that results in a single cell suspension. Also, do not overincubate the tissue in trypsin, as this will eventually lead to cell death.

Finally, it is possible to subclone primary neurospheres by dissociating and recloning them. A single dissociated neurospheres typically will give rise to 5–15 secondary neurospheres. Dissociation can be performed by collecting neurospheres in a tube containing trypsin-EDTA, and triturating with a small-bore pipette. The resulting cell suspension can then be plated in cloning medium as in **Subheading 3.1.7**.

d. Attachment of cells to the culture dish. Occasionally, cells will attach and differentiate on the bottom of culture dishes that have been coated with anti-adhesive, possibly due to cracks or abrasions in the plastic. These attached cells are apparent under phase optics, and they can, in sufficient numbers, form a favorable substrate for the attachment and differentiation of neurospheres. If significant numbers of cells are seen attaching to the dish surface, the remaining suspended cells and neurospheres should be collected and transferred to a new plate.

4. Poor attachment of neurospheres to coverslip. It is not uncommon for a small percentage of neurospheres to not readily attach. Allowing more time for attachment (up to 48 h) often solves this problem. In general, a neurospheres that has not attached after 48 h will never attach. Increasing FBS to 5–10% usually improves attachment; however, higher serum levels alter differentiation. It may be

worthwhile to increase serum concentration to facilitate attachment, and decrease it again after differentiation and migration occurs.

Also, very young neurospheres do not attach readily. Avoid plating neurospheres that have been in culture less than 7 days, and choose those only >50 μm in diameter.

Finally, apply and aspirate solutions (e.g., PBS, fixative) slowly and gently to avoid dislodging lightly attached neurospheres.

5. Contamination of six-well plates. If your plate often becomes infected during the process of removing neurospheres, be sure that you minimize your work directly over the open plate. Maintaining the pipettor at a steep angle as you approach a neurosphere will help. If possible, work in a small room that can be exposed to a germicidal lamp for several minutes before use.
6. Low RNA yield. This method normally yields enough RNA from a single neurosphere to serve as a template for 30–40 PCR runs by using primers for high abundance genes (e.g., housekeeping genes). If you have trouble achieving this level, you may need to adjust the sonication protocol. Sonicating for too long will increase the sample temperature, which increases RNase activity and can lead to reduced yield; too little sonication will not effectively release RNA, again leading to low yield. If you are still unable to sufficiently increase yield, or if your primer set is designed to reveal low-abundance transcripts, it may be necessary to amplify the RNA sample after sonication.

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13

Nuclear Transfer to Study the Nuclear Reprogramming of Human Stem Cells

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Summary

Research of stem cells will enable us to understand the development and function of tissues and organs in mammals. The ability to induce regeneration of new tissues from embryonic stem (ES) cells derived from cloned blastocysts via nuclear transfer can be expected in the not-too-distant future. The fact that there is no way except nuclear cloning for the return of differentiated cells to undifferentiated cells remains an interesting problem to be solved. We describe protocols for the production of cloned calves from bovine ES cells to study nuclear reprogramming ability of stem cells. The frequency of term pregnancies for blastocysts from ES cells is higher than those of early pregnancies and maintained pregnancies after nuclear transfer with bovine somatic cells. We also describe protocols for gene introduction into bovine ES cells in vitro, particularly the human leukocyte antigens (HLA). Bovine ES cells provide a powerful tool for the generation of transgenic clonal offspring. This technique, when perfected for humans, may be critical for neural stem cell transplantation.

Key Words: Nuclear transfer; bovine; ES cell; reprogramming; cloning; stem cells; LIF; HLA; EGF; donor; recipient; transfection; EGFP; pluripotency; differentiation; fusion; microsatellite; PCR; Oct-4; STAT-3.

1. Introduction

Stem cells are undifferentiated cells that can continue self-renewal indefinitely and that also can generate one or more specialized cell types with specific functions in the body. Embryonic stem (ES) cells can be derived from the embryo, and when placed into a developing embryo, they can contribute to all

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three germ layers ***(1,2)***. The ability to induce regeneration of new tissues from human ES cells can be expected in the not-too-distant future ***(3)***. However, isolation of human ES cells has raised ethical questions about the destruction of human embryo. And there is another problem of tissue rejection by an immune system after transfer in patients. These problems could be avoided by using adult-derived pluripotent stem cells that contribute to organs of three germ layers ***(4–6)***. This may be particularly important in producing neural stem cells.

Adult somatic cells are reprogrammable by transferring their nuclear contents into enucleated oocytes ***(7,8)***. The term reprogram refers to methods that convert a differentiated cell into a pluripotent stem cell. However, the full-term birth rates of cloned animals from somatic cells after nuclear transfer are quite low ***(9–11)***. Unsuccessful results from these cloning experiments with somatic cells suggest that the nuclei of differentiated cells cannot support the development of an entire animal. On the contrary, cloned animals have been generated from murine ES cells at higher frequencies than from somatic cells ***(12,13)***. Compared with differentiated somatic cells, stem cells require less reprogramming of their genome, perhaps because many genes critical for development of cloned embryos are already active in the donor cells, and they may not need to be reprogrammed ***(12,13)***.

Nuclear reprogramming research of stem cells will enable us to understand the development and function of tissues and organs in mammals ***(14)***. Enucleated oocytes used for nuclear transfer might contain factors that turn on the switch for reprogramming of somatic cells.

We describe protocols here for the production of cloned calves from bovine ES cells to study nuclear reprogrammable ability of stem cells. The frequency of term pregnancies for blastocysts from ES cells is higher than those of early pregnancies and maintained pregnancies after nuclear transfer with bovine somatic cells. We also describe protocols for gene introduction into bovine ES cells in vitro. Bovine ES cells provide a powerful tool for the generation of transgenic clonal offspring.

2. Materials

2.1. Culture of ES Cells

It is beyond the scope of this chapter to describe the protocols of culture and maintenance of ES cells in detail. Thus, we briefly summarize them. (For detail, *see* **refs. *15–18***).

1. Phosphate-buffered saline (PBS; Sigma-Aldrich, St. Louis, MO, cat. no. D-5773), supplemented with fetal bovine serum (FBS; Invitrogen, Carlsbad, CA, cat. no. 16140-063) and 1% penicillin-streptomycin mix (Pen/Strep, Invitrogen, cat. no. 15140-122) for dissociation solution (*see* **Note 1**).

2. Minimum essential medium (MEM)-α (Invitrogen, cat. no. 12571-048) with 10% FBS (Invitrogen) and 1% Pen/Strep mix (Invitrogen) supplemented with 10 ng/ml epidermal growth factor (EGF; Sigma-Aldrich, cat. no. E-1257) and 10 ng/ml human leukemia inhibitory factor (LIF; Sigma-Aldrich, cat. no. L-5283) for culture medium (*see* **Notes 1** and **2**). The solution of 1,000 ng/ml LIF or EGF is prepared by adding 10 ml pf MEMα to 10 μg of LIF or EGF, respectively.
3. Trypsin-EDTA: 0.25% trypsin, 1 mM EDTA; 0.25% trypsin, 1 mM EDTA·4Na, 1× (Invitrogen, cat. no. 25200-056).
4. Falcon 15-ml conical tubes (BD Biosciences, Franklin Lakes, NJ, cat. no. 2097).
5. Nunc four-well multi dishes (Nalge Nunc International, cat. no. 176740).
6. Fibroblast growth factor (FGF) (Sigma-Aldrich, cat. no. F-3133).
7. Platelet-derived growth factor (PDGF) (Sigma-Aldrich, cat. no. P-3362).
8. Dimethyl sulfoxide (DMSO) (Wako Pure Chemicals, Osaka, Japan; cat. no. 045-07215).
9. 0.22-μm syringe filters (Millipore Corporation, Billerica, MA, cat. no. SLGV 025LS).
10. Humidified incubator.
11. Laminar flow hood.

2.2. Nuclear Transfer

2.2.1. Media

1. PBS (Sigma-Aldrich) with 1% FBS (Invitrogen) and 1% Pen/Strep mix (Invitrogen) for manipulation solution.
2. PBS/collagenase, for removing cumulus cells: add 0.05% (w/v) collagenase (Sigma-Aldrich, cat. no. C-3180) to PBS, filter sterilize, and store at –20°C.
3. Modified (m) TALP medium ***(19)***: prepare as shown in **Table 1**. Filter sterilize and store at 4°C up to 1 week.
4. IVMC-101 medium (Institute for Functional Peptides, Yamagata, Japan) for maturation culture of oocytes, for cutting zona pellucida, enucleation and donor cell injection: add 5 μg/ml cytochalasin B (Sigma-Aldrich, cat. no. C-6762).
5. Fusion medium: 0.3M mannitol, 100 μM $CaCl_2$, 100 μM $MgCl_2$, made in double-distilled water (ddH_2O) and filter sterilized ***(20)***.
6. Hoechst 33342 (Wako Pure Chemicals, cat. no. 539-17191).
7. For nuclear staining, add 1μg/ml Hoechst 33342 to IVMC-101 medium. This solution is light sensitive.
8. Cycloheximide (Sigma-Aldrich, cat. no. C-4859).

2.2.2. Equipment

1. Micropipets: made from hematocrit capillary tubes (capillary tubes for microhematocrits, 75 mm in length, Drummond Scientific, Broomall, PA).
2. Microforge for preparation of micropipets.
3. Stereomicroscope with magnification from ×10 to ×60.

Table 1
Preparation of modified mTALP *(19)*

Stock A component	g/100 ml	Stock B component	g/100 ml	Other component	g/100 ml
NaCl	0.738	$NaHCO_3$	1.30	Pen/Str.	0.1 ml
KCl	0.038	1% phenol red	20 µl	Na-lactate	0.4 ml
NaH_2PO_4	0.006			Na-pyruvate	0.006
$CaCl_2 \cdot 2H_2O$	0.038			Glucose	0.02
$MgCl_2 \cdot 6H_2O$	0.013			MEM-NEAA	1.0 ml
1% Phenol red	20 µl			BME-EAA	2.0 ml
				30 mg/ml glutamine	0.5 ml
				BSA	0.1

[a]Before use, mix stock A, 80 ml; stock B, 16 ml; and dissolve other components. MEM-nonessential amino acids (NEAA; Sigma-Aldrich, cat. no. M7145). basal medium Eagle-essential amino acids (BME-EAA; Sigma-Aldrich, cat. no. B6766).

4. Inverted microscope with a phase-contrast objective equipped with fluorescence and photographic systems (magnification ×40, ×100, ×200, and ×400).
5. Electric pulse generator.
6. Falcon Primaria 60-mm tissue culture dishes (BD Biosciences, cat. no. 3803).

2.3. Transfection of ES Cells

1. Plasmid IRES-Neo-HLA-B-EGFP (RIKEN DNA Bank, Tsukuba, Japan, no. 3441).
2. FuGENE 6 transfection reagent (Roche Diagnostics, Indianapolis, IN, cat. no. 1815091).
3. MEMα medium (*see* **Subheading 2.1., item 2**).
4. Nunc four-well dish.
5. Microtube (Corning Life Sciences, Acton, MA, cat. no. 430909).
6. Geneticin (G-418 sulfate, Invitrogen, cat. no. 11811-023), for selecting a suitable cell line expressing protein of interest. The solution of 20 mg/ml G-418 is prepared by adding 50 ml of ddH_2O to 1 g of G-418, filter sterilize, and store at –20°C. To prepare 200 µg/ml of G-418, combine 9.9 ml MEMα and 0.1 ml 20 mg/ml G-418.

2.4. Immunofluorescence Staining

1. Formaldehyde (37% solution, Sigma-Aldrich, cat. no. F-1635).
2. 4% PBS-buffered formalin: for 100 ml, combine 10.8 ml of 37% formaldehyde and 89.2 ml of PBS and store at 4°C.

3. Mouse rhodopsin-specific antibody (Abcam, Cambridge, UK, cat. no. AB 5417).
4. Mouse pigment epithelium derived factor-specific antibody (Chemicon International, Temecula, CA, cat. no. MAB 1059).
5. Fluorescein isothiocyanate (FITC)-conjugated rabbit secondary antibody against mouse IgG (Sigma-Aldrich, cat. no. F-9887).
6. PBS (*see* **Subheading 2.1.**, **item 1**).

2.5. Reverse Transcription-Polymerase Chain Reaction (RT-PCR)

1. Thermal cycler (model 2400; Applied Biosystems, Foster City, CA; cat. no. 0993-6057).
2. Apparatus for polyacrylamide gel electrophoresis (Nihon Eido Co., Tokyo, Japan; cat. no. NA-1110).
3. Acrylamide (Bio-Rad, Hercules, CA, cat. no. 161-0101).
4. *bis*-Acrylamide (Sigma-Aldrich, cat. no. M-7279).
5. Tris-HCl (Sigma-Aldrich, cat. no. T-1503).
6. Boric acid (Kanto Chemical Co., Tokyo, Japan, cat. no. 04232-00).
7. EDTA (Sigma-Aldrich, cat. no. 09-1320-5).
8. Acetic acid (Sigma-Aldrich, cat. no. 01-0280-5).
9. TRIzol reagents (Invitrogen BV, Groningen, The Netherlands, cat. no. 15596-018).
10. Chloroform (Sigma-Aldrich, cat. no. 06-2320-5).
11. Isopropanol (Sigma-Aldrich, cat. no. 15-2329-5).
12. Ethanol (Sigma-Aldrich, cat. no. 09-0770-5).
13. RNase inhibitor (Invitrogen BV, cat. no. 15518-012).
14. DNaseμ (Invitrogen BV, cat. no. 18162-016).
15. Phenol (Invitrogen BV, cat. no. 15509-237).
16. 50 mM $MgSO_4$ (Invitrogen BV, cat. no. 52044).
17. SuperScript One-Step RT-PCR with Platinum Taq (Invitrogen BV, cat. no. 10928-042).
18. 2× reaction mix (Invitrogen BV, cat. no. 51099).
19. 100 mM dNTP set (Invitrogen BV, cat. no. 10298-018).
20. Reverse transcription (RT)/Platinum Taq mix (Invitrogen, cat. no. 53145).
21. Diethylpyrocarbonate (DEPC)-H_2O. Add 0.2 ml of DEPC (Sigma-Aldrich, cat. no. D5758) to 100 ml of water (or the solution to be treated). Shake vigorously to dissolve the DPEC. Autoclave the solution to inactivate the remaining DEPC.
22. 10× DNase buffer: 200 mM Tris-HCl, pH 8.4, 20 mM $MgCl_2$, 500 mM KCl. For 100 ml, combine 50 ml of 1 M KCl, 1.5 ml of 1 M $MgCl_2$, 10 μl of a solution of 1 mg/ml gelatin, and 10 ml of 2 M Tris-HCl, pH 8.4.
23. A solution of 2.5 mM dNTPs is required for RT: dilute the stock solution of dNTPs 10-fold with DEPC-H_2O.
24. Gateway PCR cloning system (Invitrogen BV, cat. no. 11821-014).
25. QIAquik PCR purification kit (QIAGEN GmbH, Hilden, Germany, cat. no. 28106).

26. Ca^{2+}- and Mg^{2+}-free Hanks' balanced salt solution, 10× (500 ml; Invitrogen, cat. no. 14060-057).
27. Digestion buffer: 20 mM Tris-HCl, pH 8.0, 10 mM NaCl, 10 mM EDTA, 0.5% (w/v) sodium dodecyl sulfate; store indefinitely at room temperature; add 1 mg/ml proteinase K (Merck, Whitehouse, NJ, cat. no. EC3.4.21.14) just before use.
28. 10× amplification buffer (KOD Dash buffer; Toyobo, Kyoto, Japan).
29. 25 mM 4dNTP mix.
30. 0.5 U of KOD Dash polymerase (Toyobo, cat. no. LDP-101).
31. 12% polyacrylamide gel.
32. Automated fluorescent DNA sequencer (Applied Biosystems, Tokyo, Japan, ABI 377).
33. Genescan version 3.1 (Applied Biosystems).
34. Genotyper version 2.1 (Applied Biosystems).
35. Fluorescent dyes; 6-FAM, HEX, and TET (Applied Biosystems).

3. Methods

3.1. Culture and Maintenance of ES Cells

We established bovine ES cell lines (W1, W2, and W3) on a feeder layer of murine STO cells with the addition of LIF and EGF ***(16–18)***. After expansion of bovine ES cells, cells in aliquots were frozen in 10% DMSO/MEMα culture medium as stocks. We describe briefly the protocols of culture and maintenance of ES cells.

1. Thaw cells quickly in a water bath at 36°C.
2. Dilute 2 ml of the suspension of cells with 12 ml of medium in a 15-ml tube.
3. Mix gently and pellet cells by low-speed centrifugation at 700 × *g* for 5 min.
4. Resuspend cells in fresh MEMα for culturing ES cells (*see* **Subheading 2.1.**, **item 2**) and plate on a feeder layer of mitotically inactivated STO cells.
5. Place the dishes in the incubator at 5% CO_2, 38.6°C and replace the medium every second day.
6. After 6–7 days, ES cells that have become confluent are trypsinized and replated on a fresh feeder layer.
7. Cells are to be frozen as described previously ***(18)***.

3.2. Nuclear Transfer

Cloned animals have been generated from murine ES cells at higher frequencies than from somatic cells (*see* **Table 2**). We performed nuclear transfer using bovine ES cells. Cloned blastocysts from ES cells yielded 71% of pregnancy rate 50 days after transfer and developed to term at a frequency of 43% ***(17)***. These values are higher than those for term pregnancies (8–14%)

Table 2
Cloning efficiency of various Cell Types

Source of donor nucleus	Species	Efficiency (%)[a]	References
ES cells	Mouse	10–30	***12,13***
	Bovine	40	***16***
Cumulus cells	Mouse	1–3	***9***
Mammary gland cells	Sheep	3–8	***7***
Fibroblasts	Bovine	8–14	***10***
Lymphocytes	Mouse	0	***11,14***

[a] Transferred embryos/term births.

that have been reported after transfer of nuclei from somatic cells. These observations suggest that reprogramming of a differentiated cell might be extremely inefficient; thus, the development of cloned embryos is likely to fail.

3.2.1. Preparation of In Vitro Matured Bovine Oocytes (21)

1. Aspirate cumulus-oocyte complexes (COCs) from follicles 2–8mm in diameter with an 18-gauge needle into a disposable 10-ml syringe.
2. Culture 10 bovine COCs in small drops of 100-µl aliquots of IVMC-101 medium at 39°C in a humidified atmosphere of 5% CO_2 in air for 22 h.
3. Before nuclear transfer process, the cumulus cells must be removed. Preincubate cultured and matured metaphase II oocytes with 1–2 ml of PBS/collagenase for a few minutes.
4. Remove cells by gently pipetting and transfer mature oocytes into IVMC-101 medium supplemented with 5 µg/ml cytochalasin B.
5. Cut the zona pellucida by the polar-body and the associated metaphase II plate with a small amount of cytoplasm by using with a same microneedle from the cut region of zona pellucida (*see* **Fig. 1A,B** and **Note 3**).
6. Incubate oocytes in a small drop (20–40 µl) of IVMC-101/Hoechst 33342 for 10 min. in the dark and monitor the disappearance of nuclear materials using a fluorescent microscope.
7. Select oocytes that are completely enucleated.

3.2.2. Preparation of Donor Cells

1. Plate ES cells on four-well dish without feeder cells. Culture these cells for 3–4 days in culture medium.
2. Collect ES cells in one well by trypsinization in a centrifuge tube and wash by centrifugation with PBS.
3. Each dissociated donor cells is kept in a microdrop of IVMC-101/FCS (about 40 µl).

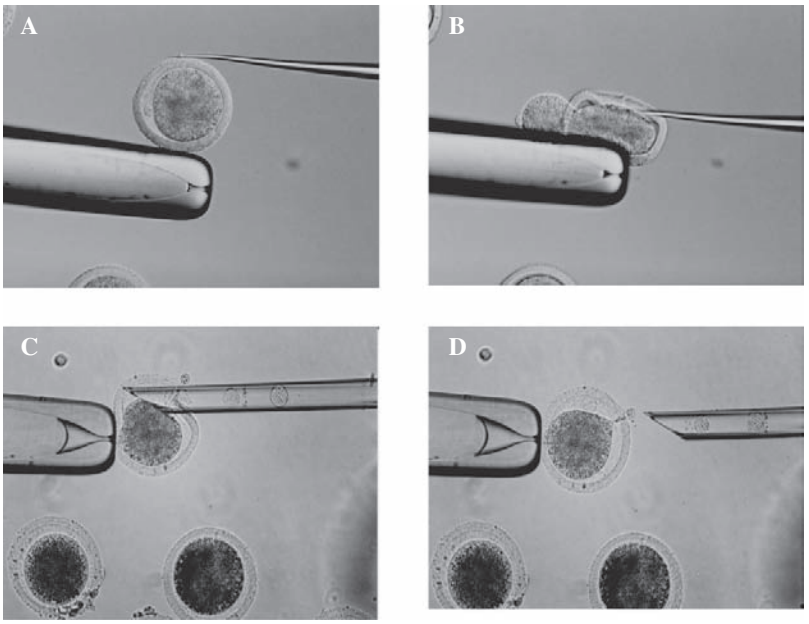

Fig. 1. Procedures for zona cutting, enucleating and donor cell (nucleus) transfer. **(A)** Inserting a microneedle into the perivitelline space and cutting the zona pellucida by rubbing it against the upper edge of the holding pipette. **(B)** Removal of the polar body and the associated metaphase II plate with a small amount of the cytoplasm by using with a microneedle from the cut region of the zona. **(C)** Inserting a donor cell in the injection pipet into the perivitelline space of enucleated oocyte. **(D)** After insertion of a donor cell into the enucleated oocyte. Magnification: all ×200.

3.2.3. Electrofusion ***(20)***

1. Transfer donor cells and enucleated oocytes into manipulation drop of IVMC-101/FCS.
2. Aspirate donor cells into the injection pipette and insert each donor cell into the perivitelline space of enucleated oocyte so as to contact the oocyte membrane (*see* **Fig. 1C,D**).
3. Using microscopic control, transfer the oocyte pairs in a fusion solution (*see* **Note 4**).
4. The membrane of donor cells and cytoplasts are fused by two pulses of 20 V/150 μm of DC pulse, 50-μs duration by using the pair of electrodes connected with electric pulse generator. When all pairs of donor cells and cytoplasts are perpendicularly oriented, switch on DC pulses (*see* **Note 5**).
5. Reconstituted oocytes are activated by incubation in mTALP supplemented with 0.1% bovine serum albumin (BSA) and 10 mg/ml cycloheximide for 5 h.

3.2.4. Culture and Transfer of Reconstituted Embryos

1. Culture in mTALP supplemented with 3% FBS at 38.6°C in a humidified atmosphere of 5% CO_2 in air.
2. After 6 to 7 days of culture, embryos that have developed to normal blastocysts are then nonsurgically transferred into recipient cows.

3.2.5. Parentage Analysis (see **refs. 22–25** and **Subheading 3.5.)**

Fourteen bovine DNA microsatellite markers are used to confirm the genetic identity of cloned calves to the ES cells used for nuclear transfer. Our analysis confirmed that the donor cells were the source of the genetic materials used to produce three cloned male calves (*see* **Table 3**). The basic protocol involves the enzymatic amplification of DNA that corresponds to the microsatellite marker region in genomic DNA by PCR. We describe concisely the protocols of parentage analysis by using with microsatellite markers.

1. Add 300 µl of digestion buffer to the samples of white blood cells from recipient cows, cloned calves and those of donor ES cells at 19th passage, transfer the mixture to a 1.5-ml microtube, and incubate at 55°C.
2. Add 150 µl of a saturated solution of NaCl to the tube and mix vigorously on a vortex mixer. Add 2 vol of 95% ethanol.
3. After centrifugation at 13,000 × *g* for 10 min, wash the precipitates with 70% ethanol and resuspend the pellet DNA in 50 µl of ddH_2O.
4. PCR primers for microsatellite markers are labeled with fluorescent dyes 6-FAM, HEX, and TET.
5. For PCR, mix the following reagents in a 0.5-ml thin-walled PCR tube in a total volume of 15 µl, containing 1.5 µl of 10× PCR buffer, 20 ng of genomic DNA, 0.4 µM each primer, 1.7 mM $MgCl_2$, 10 mM Tris-HCl, 50 mM KCl, 0.2 µM of each dNTP, and 0.75 U of Taq DNA polymerase. Primers were provided by Livestock Technology Association of Japan (Shirakawa, Japan; see **Note 6**).
6. PCR amplification ; perform 30 cycles of denaturation at 94°C for 1 min, with annealing at 55°C for 2 min and extension at 72°C for 20 s.
7. Fractionate the PCR products with a set of internal size markers TS 369 by electrophoresis; the separated fragments are analyzed with an automated fluorescent DNA sequencer. Data are further analyzed with Genescan, version 3.1 and Genetyper, version 2.1 software.

3.3. Transfection of ES Cells

Transgenic ES cells can serve as nearly unlimited sources of donor cells for production of cloned transgenic animals. There are many ways used to transfect cells with DNA constructs. However, we cannot describe the many possible methods for transfection of ES cells. Only those methods considered optimal and routinely used in our laboratory are described. Expression of

Table 3
Analysis of parentage[a]

Marker	Genotypepe of recipient 1	Genotypepe of calf1	Genotypepe of recipient 2	Genotypepe of calf 2	Genotypepe of recipient 3	Genotypepe of calf 3	Genotypepe of cell line
DIK024	231/247	239	231/239	239	239	239	239
BMS1987	111/121	123/125	111	23/125	1111/124	123/125	123/125
DIK069	184/188	182/191	187/189	182/191	178/182	182/191	182/191
BM6026	158/173	167/169	167	167/169	167/173	167/169	167/169
ILSTS093	194/204	198	194	198	186/193	198	198
DIK096	251	251/257	251/257	251/257	244/256	251/257	251/257
BMS60	157/159	148/163	155	148/163	163	148/163	148/163
DIK093	237	230/237	230	230/237	ND[b]	ND	230/237
BM121	124/136	123/125	122	123/125	119/136	123/125	123/125
DIK067	198	199	197	199	ND	ND	199
DIK039	197/204	185/190	190/196	185/190	185/189	185/190	185/190
INRA130	113/115	113	111	113	102/114	113	113
BM4505	239/244	230/244	234/239	230/244	231/233	230/244	230/244
BM7246	143	118	137	138	118	ND	118

[a]For each microsatellite marker, the genotype was determined in terms of the size (bp) of fragments (***22–25***). Almost all satellite markers provided strong support for the genetic identity between the donor ES cells and cloned calves.
[b]ND, not determined.

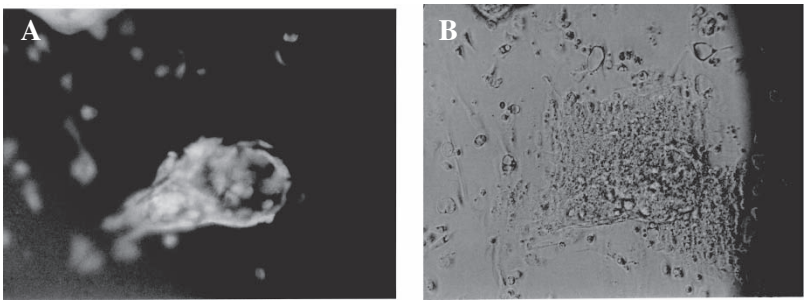

Fig. 2. Expression of HLA-B-EGFP in colonies of transgenic bovine ES cells that were transfected with pIRES-Neo-HLA-EGFP. Distinct expression of EGFP is apparent in ES cells. **(A)** Phase contrast image of the colony. **(B)** Fluorescence image of the same colony. Magnification: both ×200.

human leukocyte antibody (HLA) molecules is expected to inhibit human natural killer (NK) cell-mediated cytotoxicity in xenotransplantation ***(26,27)***. Transgenic cattle carrying a gene for HLA-B could provide a source of donor animals for therapeutic application. Therefore, we have established lines of bovine ES cells that express HLA plus enhanced green fluorescent protein (EGFP) molecules (*see* **Fig. 2A,B**).

1. Plate frozen-thawed cells at 1×10^4 cells/well in a four-well dish and culture 1 day before the transfection process.
2. Add 70 µl of FBS-free MEMα medium to a sterile microtube (*see* **Note 7**).
3. Add 6 µl of FuGENE 6 to this medium. Tap gently to mix.
4. Add 10 µl of Enhancer A to the medium and mix.
5. Add 4 µl of IRES-Neo-HLA-B-EGFP (1 µg/ml) to the reagent and gently tap the tube to mix the contents. Incubate for a minimum of 15 min at room temperature (*see* **Note 8**).
6. Dropwise, add 22 µl of the complex mixture to cells in each well respectively. Swirl the well to ensure even dispersal.
7. Incubate the cell for 1 day and replace the MEMα/FBS culture medium.
8. Expose cells to the MEMα/FBS medium with 200 µg/ml G-418 3–4 days after transfection to select neomycin-resistant transfected cells.
9. The transfection efficiency is confirmed by green fluorescent protein (GFP) expression with fluorescent microscope with GFP fluorescent filter at 488 nm.

3.4. Analysis of Pluripotency of Transfected ES Cells In Vivo

3.4.1. Injection of Transfected ES Cells

1. Around 1×10^5 HLA-B-EGFP cells suspended in serum-free MEMα are injected into anterior eye chamber of 1-month-old SCID mice.

2. Sacrifice mice at 30 days after injection and dissect eye chamber into physiological saline.
3. EGFP-positive colonies of eye chamber are removed by scissors under a dissecting microscope with fluorescent light. Wash them in PBS.
4. Trypsinize pieces of cell colonies with 0.25% trypsin-EDTA and wash by centrifugation.
5. Dispence 2 ml of the suspension of cells into each of four-well dishes covered with MEMα/FBS medium.
6. Culture at 38.6°C in a humidified atmosphere of 5% CO_2 in air.

3.4.2. Immunocytochemical Staining

HLA-EGFP–transfected bovine ES cells that were injected into the eye chamber of SCID mice differentiated to contribute ocular cells instead of tumor cells. Then, we identified their derivatives of engrafted cells by immunocytochemical staining (*see* **Fig. 3A–D**).

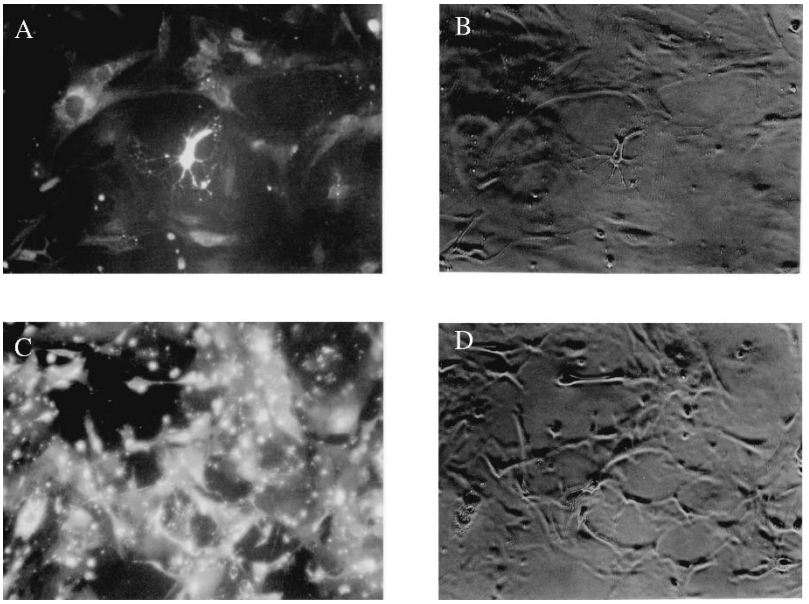

Fig. 3. Expression of markers in ocular precursors derived from HLA-B-EGFP bovine ES cells that were recovered from the eye chamber colonies of SCID mice. **(A)** Fluorescence image of the cells immunostained with antibodies against pigment-epithelium derived factor. **(B)** Phase-contrast image of the same cell colonies. **(C)** Fluorescence image of the cells immunostained with antibodies against retinal rhodopsin. **(D)** Phase contrast image of the same cell colonies. Magnification: all ×200.

1. When cells are nearly confluent, remove medium and wash cells with PBS.
2. Flood wells with 4% formaldehyde and fix at least 1 h.
3. Remove fixative and wash three times with PBS, leaving cells in PBS for 1 h after the third wash.
4. Calculate the volume of solution of primary antibody needed. Each well needs 200 µl. Prepare a solution of 1:50 diluted antibody (*see* **Note 9**). Primary antibodies; mouse anti-rhodopsin (for retinal stromal cells), mouse anti-pigment epithelium-derived factor (for retinal pigment epithelium cells).
5. Remove PBS after final wash and add 200 µl of the solution of antibody to each well. Incubate for 1 h.
6. Flood wells with PBS and leave for 10 min at room temperature.
7. Calculate the volume of FITC-conjugated secondary antibody needed. Prepare a solution of 1:200 diluted secondary antibody (*see* **Note 10**).
8. Secondary antibody: FITC-conjugated rabbit anti-mouse IgG.
9. Remove the solution of primary antibody and wash cells three times with PBS.
10. Add 200 µl of the solution of FITC-conjugated secondary antibody to each well and incubate for 45 min at room temperature (*see* **Note 11**).
11. After incubation, flood each well with PBS and leave for 10 min at room temperature.
12. Remove the PBS and wash cells three times with PBS.
13. Examine the fluorescence caused by FITC, record results, and take pictures (*see* **Note 12**).

3.5. Analysis of Marker Proteins of Pluripotency and Differentiation of ES Cells (18,28)

The expression of octamer (Oct)-4 and signal transducer and activator of transcription (STAT)-3, which play a central role in the maintenance of pluripotency of murine ES cells, was confirmed by undifferentiated bovine ES cells. By contrast, we detected the expression of Nestin, which is a marker protein of neural precursor cells in the differentiated bovine ES cells (*see* **Fig. 4**). We describe briefly the protocols for the analysis of marker proteins of ES cells in an undifferentiated or differentiated state by RT-PCR.

3.5.1. Extraction of RNA with TRIzol

The following procedure was derived from the protocol supplied by manufacturer (*see* **Note 13**).

1. Remove the medium from cell cultures and rinse the dishes three times with PBS. Lyse cells by adding the appropriate amount of TRIzol reagent (i.e., for a 35-mm dish, use 1 ml; for a 100-mm dish, use 6 ml).
2. Collect all the cells and transfer the suspensions to a sterile culture tube. Incubate the sample for 5 min at room temperature.

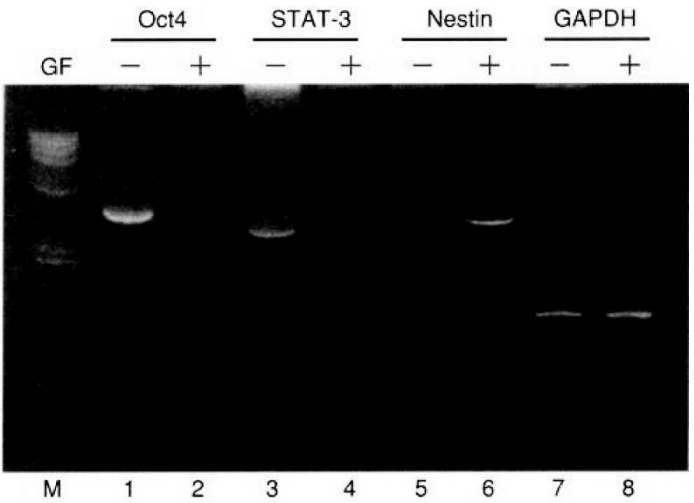

Fig. 4. Analysis of the expression of genes for Oct-4, STAT-3, Nestin ,and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) in colonies of bovine ES cells by RT-PCR. *GF* indicates the inclusion of a mixture of growth factors (FGF, EGF, and PDGF) in the MEMα/FBS medium for 7 days.

3. Add 0.2 ml of chloroform (RNase-free) per 1 ml of TRIzol used (i.e., for a 35-mm dish, use 200 µl; and for a 100-mm dish, use 1.2 ml).
4. Centrifuge at 13,000 × *g* for 15 min at 4°C. The mixture separates into a lower phase (phenol and chloroform), an interface, and a colorless upper aqueous phase. The RNA is in the aqueous phase, and the volume is approximately 60% of the original volume of TRIzol used.
5. Transfer the aqueous phase to a fresh tube. Precipitate the RNA by adding 0.5 ml of RNase-free isopropanol per milliliter of TRIzol originally added (i.e., for a 35-mm dish, use 0.5 ml; for 100-mm dish, use 3 ml). Incubate the mixture for 10 min at room temperature.
6. Centrifuge at 13,000 × *g* for 10 min at 4°C. The RNA should form a pellet.
7. Remove the supernatant and wash the pellet with RNase-free 75% ethanol. Use 1 ml of ethanol for each milliliter of TRIzol used originally (i.e., for a 35-mm dish, use 1 ml; for a 100-mm dish, use 6 ml). Mix the sample on a vortex mixer and centrifuge at 750 × *g* for 5 min at 4°C.
8. Dry the pellet and resuspend it in DEPC-treated water. If it does not dissolve, incubate for 10 min at 55°C.
9. Measure the A_{260} and A_{280}. The ratio of A_{260}:A_{280} should be between 1.6 and 2.0 (*see* **Note 14**).
10. Determine the concentration X of the sample (*see* **Note 15**).

3.5.2. Removing Genomic DNA from RNA

1. Digest 30 µg of RNA so that the volume of RNA plus DEPC-H_2O is 87 µl. Add 10 µl of 10× DNase buffer; 2 µl of RNase inhibitor, and 1 µl of DNase I.
2. Mix and incubate for 15 min at room temperature.
3. Prepare a mixture of chloroform and isoamyl alcohol (24:1, v/v) in a 20-ml Falcon tube: 12 ml of RNase-free chloroform and 0.5 ml of RNase-free isoamyl alcohol.
4. Add an equal volume of phenol plus chloroform to the RNA sample (i.e., for a 100-µl sample, add 50 µl of phenol and 50 µl of chroloform plus isoamyl alcohol; 24:1).
5. Mix vigorously and centrifuge at 13,000 × *g* at room temperature for 5 min.
6. Transfer the aqueous phase (the top phase) to a fresh RNase-free microtube.
7. Add 1/10 volume of RNase-free 3 M sodium acetate (i.e., for a 100-µl sample, add 10 µl of 3 M sodium acetate).
8. Add two volumes of 100% ethanol (RNase-free, –20°C) (i.e., for a 100-µl sample, add 200 µl of 100% ethanol).
9. Mix and incubate at –80°C for 1 h.
10. Centrifuge immediately at 13,000 × *g* for 20 min at 4°C.
11. Wash the pellet with 200 µl of 70% ethanol (RNase-free, –20°C).
12. Centrifuge again for 10 min at 13,000 × *g* at 4°C and air dry the pellet.
13. Resuspend the pellet in DEPC-H_2O and quantify the DNase-treated RNA.
14. Dilute the sample to 1 µg/µl with DEPC-H_2O for RT-PCR.

3.5.3. Denaturation of RNA

Before RT-PCR, the RNA is denatured by heating to facilitate the binding of primers to the template RNA.

1. Mix 1 µl of the solution of RNA with 2 µl of DEPC-H_2O in a 100-µl PCR tube.
2. Place the tube in a thermal cycler and incubate at 65°C for 5 min. Then, lower the temperature to 4°C for at least 5 min before proceeding to the SuperScript One-Step RT-PCR reaction.

3.5.4. Reverse Transcription and PCR with Platinum Taq

The SuperScript One-Step RT-PCR with Platinum Taq system is used for optimizing the synthesis of cDNA and PCR amplification.

1. Mix 25 µl of 2× reaction mix (containing 0.4 mM each dNTP and 2.4 mM $MgSO_4$; *see* **Note 16**), 1.0 µl of RNase inhibitor, 0 µL of solution of template RNA (0.1 µg), 1.0 µl of sense primer (10 µM), 1.0 µL of antisense primer (10 µM), 1.0 µL of RT/PlatinumTaq Mix; and ddH_2O to 50 µL.
2. Program the thermal cycle so that cDNA synthesis is followed automatically and immediately by PCR.

Table 4
Sequences of primers

Gene	Primer (top, sense; bottom, antisense)	Position[a]
Oct-4	5'-TCCCAGGACATCAAAGCTCTGCAGA-3'	(nt 848-873)
	5'-TCTGGGCTCTCCCATGCATTCAAACTGA-3'	(nt 1495-1524)
STAT-3	5'-TCTGGCTAGACAATATCATCGACCTTG-3'	(nt 1908-1935)
	5'-CCCTCTGCCTGTTCTADTCCATTCTCCAT-3'	(nt 2413-2439)
Nestin	5'-TTATTTCCAAACTGCATCAATGAATCT-3'	(nt 4985-5011)
	5'-TCTGATGGGTTTGCTGATGAGGAAGA-3'	(nt 5420-5448)
GAPDH	5'-GGGCTTGGCTTCGGTGACAACACCAAGGCGG-3'	(nt 646-678)
	5'-CGAGCAAAGGCCTCTGCCACCTTGCGGTT-3'	(nt 808-837)

[a]nt, nucleotides.

   a. cDNA synthesis and predenaturation: perform one cycle of incubation at 45°C for 30 min and at 94°C for 2 min (*see* **Note 17**).
   b. PCR amplification: perform 40 cycles of denaturation at 94°C for 15 s, annealing at 55°C for 30 s, and extension at 72°C for 2 min (*see* **Note 18**).
   c. Final extension: one cycle at 72°C for 10 min (*see* **Note 19**).

3. Gently mix and make sure that all the components are at the bottom of each amplification tube. Centrifuge briefly, if needed. Depending on the thermal cycler used, overlay the mixture with silicone oil, if necessary.
4. Analyze the products of amplification by 10% polyacrylamide gel electrophoresis with appropriate molecular markers (*see* **Fig.** 4).
5. Purify the amplified DNA with a QIAquik PCR spin column.
6. Subclone the product of PCR into the Gateway vector and sequence (**Table 4**).

4. Notes

1. It is important to use deionized, ddH_2O in all solutions and procedures. Prepare all solutions for cell culture from tissue culture-grade reagents. Sterilize all final solutions by filtration or prepare from sterile stocks. Use disposable sterile plasticware to prevent microbial and detergent contaminations.
2. This medium contains L-glutamine, ribonucleosides, and deoxyribonucleosides.
3. Carry out micromanipulation in droplets of medium not covered with paraffin oil, because oil is apt to stick to the pipet wall and to destroy the cells.
4. Better skill in the preparation of glass microtools is an important factor to increase the efficiency of nuclear transfer.
5. The important parameters for the efficient electrofusion are the strength of the DC pulse and the pulse duration for the membrane breakdown. A fusion rate that is lower than 70% suggests the need to increase DC voltage, length of duration of pulses, or number of pulses. The extracellular Ca^{2+} in fusion medium increases

the fusion efficiency as well as viability. At least 0.05–1 mM Ca^{2+}should be included in the fusion medium.

6. A genome screening was conducted with a series of microsatellite markers such as DIK 024, 039, 067, 069, 093, 096, BMS 607, 1987, BM 121, 4505, 6026, 724 6, LISTS 093, and INRA 130 ***(22–25)***.
7. The same type of cell culture medium routinely used for culturing cells can be used during transfection. However, serum-free conditions are preferable for obtaining satisfactory transfection efficiency in bovine ES cells.
8. GFP gene system is best suited for *in situ* detection of gene expression, such as localization of fusion proteins within the cells. However, quantitative analysis is limited.
9. For example, for 1,000 µl, dilute 20 µl of the preparation of antibody in 980 µl of PBS.
10. For example, for 1,000 µl, dilute 5 µl of the solution of FITC-conjugated second antibody in 995 µl of PBS.
11. It is essential to protect fluorescent conjugates and labeled dishes from light. Incubate samples in the dark and cover whenever possible.
12. The question whether engrafted HLA-B-EGFP bovine ES cells can protect immune system against cytotoxicity of NK cells remains to be solved.
13. During the extraction of RNA, all equipment and reagents must be free of RNase. Furthermore, gloves are to be changed frequently to minimize the contamination of RNase.
14. Higher quality intact RNA is required for successful synthesis of full-length cDNA.
15. Dilute 1 µl in 100 µl of DEPC-H2O. (X)µg/µl = (*A*260 × 40 × dilution factor)/1,000.
16. Keep all reaction mixtures and samples on ice and also prepare reaction mixture on ice. The 2× reaction buffer provided with the system is optimized to provide higher specific amplification by RT-PCR. The final concentration of Mg^{2+} ion is 1.2 mM.
17. SuperScript II RT is inactivated, Taq DNA polymerase is reactivated, and the RNA/DNA hybrid is denatured during the 2-min incubation at 94 °C.
18. The extension time varies with the size of the amplicon (approximately 1min per 1 kb amplicon).
19. The reaction mixture must be on ice before starting the reaction. A reaction cocktail can be made when multiple reactions are assembled.

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III

In Vitro Studies: A. Cellular Techniques

14

Clonal Analyses and Cryopreservation of Neural Stem Cell Cultures

Angela Gritti, Rossella Galli, and Angelo L. Vescovi

Summary

The discovery of stem cell populations in the adult central nervous system (CNS) that continually produce neurons and glial cells, and the hypothesis that they could contribute to neural plasticity/repair, has opened new and exciting areas of research in basic cell biology and regenerative medicine. The success of these studies relies on understanding the functional features and the normal fate of neural stem cells (NSCs) in vivo as well on the development of in vitro culture conditions enabling isolation, extensive propagation, and rigorous characterization of the "putative" NSCs. The neurosphere assay (NSA) has emerged as a valuable tool for isolating embryonic and adult CNS stem cells and for studying their biology. However, because this assay may select and expand a heterogeneous stem/progenitor cell population, rigorous clonal and serial subcloning analyses are required to detect and document stem cell activity and to unequivocally identify bona fide stem cells. We illustrate and discuss methods for the isolation, propagation, cryopreservation, and functional characterization of NSCs, focusing on the essential issue of their clonogenic capacity.

Key Words: Neural stem cells; neurosphere assay; clonal analysis; cryopreservation; central nervous system.

1. Introduction

For most of the past century, the prospect of replacing lost or damaged cells in the central nervous system (CNS) was hampered by the belief that the mature mammalian CNS was incapable of significant cell turnover. This view

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was supported by a lack of experimental evidence to the contrary. It began to change in the past few decades, due to a series of reports documenting the persistence of neural progenitor cells in restricted germinal layers in the adult vertebrate brain that continually produce neurons and glial cells ***(1–5)***. The presence of de novo neurogenesis in the adult brain suggested the presence of neural stem cells (NSCs) that could sustain this process throughout life. Indeed, a subset of glial cells have been recently described as the primary precursors in vivo, both in rodent ***(6–8)*** and human brain ***(9,10).*** Although the function of endogenous brain cell replacement is not completely understood, it is thought that adult NSCs could contribute to neural plasticity and repair ***(11–13)***. They are often relatively quiescent or slowly proliferating cells, but they retain a significant ability to increase their activity to replace dead and/or injured cells. They seem to accomplish this through the generation of an intermediate, fast-proliferating transit-amplifying cell population ***(14)***. Therefore, although there is deep interest in understanding the organization of proliferative areas in the adult brain and in studying the normal fate of NSCs in vivo, there is at the same time an urgent need of developing in vitro culture conditions enabling the isolation, the extensive propagation, and the rigorous characterization of "putative" NSCs.

Stem cells are endowed with functional characteristics that also provide a basis for their identification, particularly in the CNS, where unequivocal molecular or antigenic markers are not available yet. A widely accepted definition ***(15,16)*** identifies stem cells as (1) capable of proliferation, (2) possessing self-renew capacity over an extended period; and (3) able to generate a large number of progeny that can differentiate into the main cell types of the tissue of origin.

Although a variety of techniques were initially described, the neurosphere assay (NSA) rapidly emerged as a valuable tool for isolating embryonic and adult CNS stem cells and for studying their biology in vitro ***(17–20)***. The NSA relies on a peculiar and selective culture system that does not support the survival and long-term existence of the majority of the cells plated after dissociation of brain tissue but that does support proliferation of a subset of cells (which include both stem and progenitor cells) that respond to epidermal growth factor (EGF), basic fibroblast growth factor-2 (FGF-2), or both, forming clonal aggregates called primary neurospheres ***(21)***. Upon subculturing, these primary clones can generate secondary neurospheres, representing the renewal of the previous population. Under these stable culture conditions, growth factor-responsive cells can be long-term passaged, maintaining stable proliferation and multipotency over time. However, because both stem and progenitor nonstem cells can proliferate after each dissociation step, caution is necessary in performing and interpreting this assay. The definition of a neural cell as a stem cell (or of a population of cells as containing a stem cell) should be

applied only to a founder cell (or population of cells) that (1) self-renew extensively, (2) can be propagated in long-term cultures (more than five passages in vitro), and (3) display steady multipotentiality through the generation of progeny (neurons, astrocytes, and oligodendrocytes). This needs to be several orders of magnitude more numerous than the starting population ***(21)***. In this view, rigorous clonal analysis and serial subcloning assays are essential to document stem cell activity and to identify *bona fide* stem cells. In fact, only if the progeny of an individual clone-founder cell contain cells that give rise to neurons and glia, and, more importantly, contain one or more cells identical to itself (i.e., able to reproduce multipotent progeny), can we confirm the stem cell features of the founder cell.

We illustrate and discuss some methods for the isolation, propagation, and functional characterization of NSCs, focusing on the essential issue of their clonogenic capacity. Once established, stem cell lines can be effectively expanded to obtain a large number of cells that can then be cryopreserved. This allows setting up a homogeneous reservoir of cells that can be further expanded for future experiments. Repeated cycles of freezing and thawing do not affect the CNS stem cell functional properties. Most importantly, tissue from which the cell lines have been established also can be cryopreserved and stored if the need to reestablish a specific cell line were to arise. This is not particularly relevant for murine cell lines, but it is of fundamental importance for human neural stem cell lines, given the ethical and technical difficulties of obtaining large amounts of donor tissue.

2. Materials

2.1. Clonal Analysis

2.1.1. Limiting Dilution

1. Glassware: bottles, cylinders, beakers used only for cell culture (*see* **Note 1**).
2. Culture medium: in 375 ml of water, mix 50 ml of 10× Dulbecco's modified Eagle's medium (DMEM)/Ham's F-12, 10 ml of 30% glucose, 7.5 ml of 7.5% $NaHCO_3$, 2.5 ml of HEPES, 5 ml of 200 mM glutamine (Invitrogen, Carlsbad, CA) 50 ml of 10× hormone mix, 1 ml of 0.2% heparin, 20 µl of EGF- and/or 10 µl of FGF2-stock (final concentration: 20 ng/ml of EGF and 10 ng/ml of FGF2; human recombinant, PeproTech, Rocky Hill, NJ).
3. 10× stock solution of hormone mix: combine 40 ml of 10× DMEM/Ham's F-12, 8 ml of 30% glucose, 6 ml of 7.5% $NaHCO_3$, 2 ml of HEPES, and 300 ml of water. Add 400 mg of apo-transferrin (Sigma-Aldrich, St. Louis, MO). Dissolve 100 mg of insulin (Sigma-Aldrich) in 4 ml of sterile 0.1 N HCl, mix in 36 ml of water, and add all to the hormone mix solution. Dissolve 38.6 mg of putrescine (Sigma-Aldrich) in 40 ml of water and add to hormone mix solution. Add 40 µl

of 2 mM progesterone and 40 µl of 3 mM sodium selenite. Mix well and filter sterilize. Aliquot in sterile tubes and store at –20°C.
4. Humidified chamber consisting of glass or plastic chamber with wet gauze on the bottom to hold a 96-well plate.
5. 96-well plates.
6. Disposable plasticware: flasks, 15-ml conical tubes, pipettes.
7. P200 Pipetman (Gilson, Villier Le Bel, France).
8. Trypan blue.
9. Hemocytometer.
10. Laminin- or Matrigel-coated glass coverslips.

2.1.2. Manipulation of Individual Cells

1. Heat-polished glass microelectrode pipette (40–70 µm inside diameter).
2. Silicon tubing.
3. Screw-driven 500-µL syringe.

2.1.3. Methylcellulose Assay

1. Methylcellulose gel matrix (powder, Dow methocel A4M, premium grade; 4% final concentration) in DMEM/Ham's F-12 or culture medium.
2. EGF and FGF2 stock solutions.
3. 5-ml syringe.
4. 60-mm petri dish.
5. Digital camera or time-lapse cinematography.
6. 5-ml microfuge tubes.

2.1.4. Subcloning Procedure

1. P200 Pipetman.
2. Multiwell plates.
3. Humidified chamber.
4. 5-ml microfuge tubes.

2.2. Cryopreservation

1. Freezing jar.
2. Isopropanol.
3. Freezing medium: 10% dimethyl sulfoxide (DMSO) in culture medium (*see* **Subheading 2.1.1.**, **item 2**).

3. Methods

The following protocols can be performed starting either from primary or serially passaged neurospheres. Differences in the efficiency of the cloning and subcloning procedures might occur depending on the species, the age,

and the CNS region from which NSCs have been derived (i.e., mouse vs human, embryonic vs. early postnatal vs. adult; subventricular zone (SVZ) vs. hippocampus vs. olfactory bulb).

3.1. Clonal Analysis

3.1.1. Limiting Dilution

1. Warm culture medium to 37°C.
2. Prepare the humidified chamber.
3. Tap sides of flask to dislodge spheres and remove content of the flask to 15-ml sterile plastic conical tubes by using a sterile plastic pipette.
4. Pellet cell suspension by centrifugation (110 × *g* for 10 min).
5. Remove the supernatant leaving behind about 300 µl. Using a sterilized P200 Pipetman set at 200 µl, vigorously dissociate pellet to a single-cell suspension.
6. Add 10 ml of fresh culture medium and spin cells (15 × *g* for 15 min).
7. Remove supernatant and resuspend pellet in 0.5 ml of culture medium.
8. Dilute a 10-µl aliquot in trypan blue and count in a hemocytometer.
9. Resuspend cells in culture medium at a cell density of 5 to 10 cells/ml. Use a dispenser to add 100 µl of this cell suspension to each well, frequently resuspending the starting cell solution. To prepare three 96-well plates, you need 300 cells/30 ml of culture medium.
10. Incubate cells at 37°C in the humidified chamber (*see* **Note 2**). Feed cells with fresh, prewarmed medium every 4–5 days. For the first feeding, add 100 µl; if further feeding is needed, carefully replace 100 µl of medium in the well with 100 µl of fresh medium.
11. Carefully inspect plates under the inverted microscope to unequivocally identify and mark wells containing single cells. Make sure to use high magnification to assess that a cell is indeed "single." Wells containing two cells or more should not be further considered for the clonal analysis.
12. Inspect the plate once a week; make sure the pH of the medium does not change excessively (*see* **Note 3**). Many of the cells will die and some will differentiate. Only a small percentage will proliferate to form a clonal sphere that could undergo further subcloning or differentiation. This will require 10–20 days, depending both on the type of cells and culture conditions.
13. For differentiation, transfer the intact clonal sphere onto a laminin- or Matrigel-coated coverslip in the absence of EGF and FGF2 (or in the presence of serum, cytokines, or other molecules whose effect you want to test) and culture for the appropriate time. For serial subcloning, see **Subheading 3.1.4**.

3.1.2. Cloning by Manipulation of Individual Cells

1. Follow **steps 3–8** under **Subheading 3.1.1.**
2. Resuspend cells in culture medium and plate in 33-mm petri dishes in 2 ml of culture medium at a density of 50 cells/cm^2.

3. After 2–6 h, choose viable cells on the basis of round shape, phase brightness, hypertrophic appearance, and lack of processes (*see* **Fig. 1A**).
4. Transfer single cells to a 96-well plate (one cell/well) by using a heat-polished glass microelectrode pipette connected by silicon tubing to a screw driven 500-µl syringe (*see* **Note 4**).
5. Follow the fate of single cells over time (*see* **Fig. 1B–D**). Clonal-derived spheres can be differentiated or can undergo further subcloning, either by repeating this process or by methylcellulose assay.

3.1.3. Methylcellulose Assay

1. Warm the culture medium to 37°C.
2. Prepare a humidified chamber.
3. Prepare methylcellulose gel matrix in DMEM/Ham's F-12.

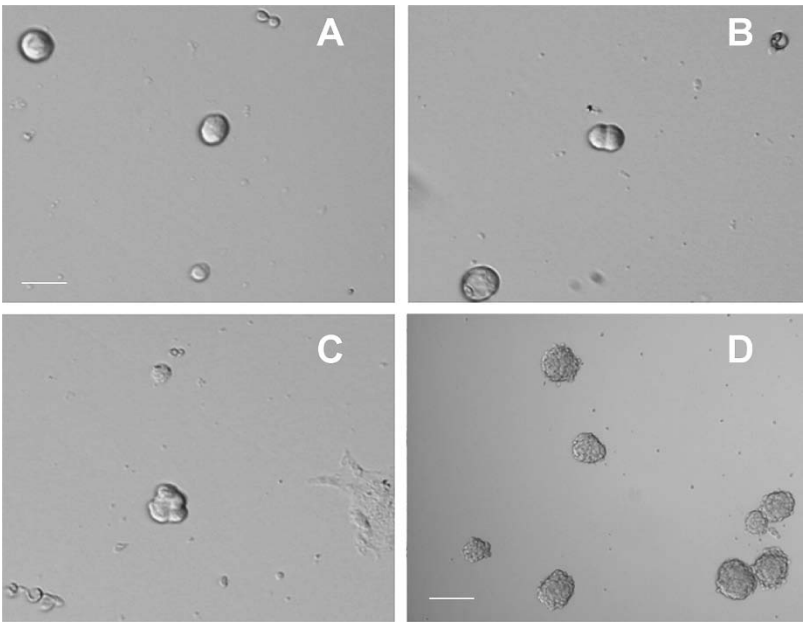

Fig. 1. NSCs proliferate in response to mitogens, giving rise to clonal neurospheres. Undifferentiated precursor cells can be isolated and cultured from the adult mouse brain and the human embryonic CNS. **(A–D)** Representative examples of human-derived precursor cells. When plated as single cells in the presence of EGF and/or FGF2 **(A)**, a subset of these cells divide **(B)**, proliferate **(C)**, and give rise to spheres of undifferentiated cells **(D)**. At this point, spheres can be further subcloned or collected for cryopreservation. Bars = 25 µm **(A–C)** and 200 µm **(D)**.

4. Follow **steps 3–8** under **Subheading 3.1.1.**
5. Make sure that the majority of the cells are single cells by withdrawing an aliquot and checking it under the microscope.
6. Resuspend single cells in growth medium containing 40 and 20 ng/ml EGF and FGF2, respectively. Final cell concentration should be <200 cells/ml.
7. Aspirate 2.5 ml of the cell suspension into a 5-ml syringe.
8. Aspirate 2.5 ml of the methylcellulose gel matrix into the same syringe (*see* **Note 5**).
9. Gently inject the mixture of cells and methylcellulose gel matrix into a 60-mm petri dish, avoiding bubbling and foaming.
10. Using the same syringe, resuspend the mixture multiple times until a semisolid homogeneous gel has formed and the single cells are thoroughly dispersed.
11. The day after plating, inspect the plate to identify single hypertrophic cells. Mark their position on the plate with a fine-tipped marker and take microphotographs over time.
12. When clonal spheres have been generated, perform subcloning of individual spheres by this same procedure or by subcloning (*see* **Note 4**).

3.1.4. Subcloning Procedure

1. Transfer individual clonal spheres (*see* **Fig. 2A**) to 5-ml microfuge tubes containing 300 µl of appropriate medium (one sphere/tube) by using a sterilized P20 Pipetman. Rinse tip with medium first, to avoid cells sticking to the tip walls.
2. Using a sterilized P200 Pipetman set at 180 µl, dissociate spheres to a single-cell suspension (100–150 times for human cells, 50–60 times for adult mouse cells, and 30–40 times for embryonic mouse cells). Rinse tip with medium first, to avoid cell sticking to the tip walls. Press the tip to the bottom or the edges of the well to generate a fair amount of resistance. Avoid foaming and bubbles.
3. Plate all the cell suspension in a clean well of a 96- or 48-well plate (depending on the size of the sphere) and incubate cells at 37°C in the humidified chamber (see **Subheading 2.1.**).
4. Within 1 h of plating, count the number of the single cells obtained by dissociation of each clone under the microscope (*see* **Fig. 2B**). A subset of these cells will proliferate, giving rise to secondary clones (*see* **Fig. 2C**). The cloning efficiency can be calculated by normalizing the number of secondary clones by the total number of cells in the same well, as assessed by direct observation 1 h after dissociation (*see* **Note 6**).
5. Individual secondary clones can either be differentiated to assess their multipotentiality or they can undergo further subcloning.
6. If a clonal cell line has to be generated, secondary spheres derived from a single primary sphere can be pooled, mechanically dissociated to a single-cell suspension, and plated at a cell density of 10,000 cells/cm^2 in the appropriate medium. Subculture until a bulk culture is established.

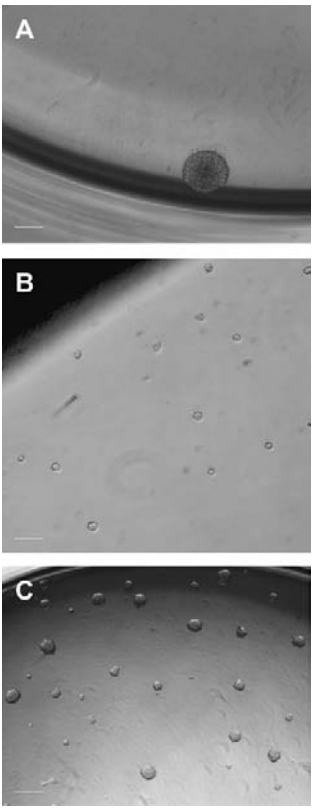

Fig. 2. Individual clonal neurospheres can be subcloned. **(A–C)** Representative examples of SVZ-derived NSCs. Individual clonal spheres generated by limiting dilution from serially passaged adult NSCs **(A)** can be dissociated and single cells replated in the presence of mitogens **(B)**, giving rise after 7–10 days to secondary neurospheres **(C)**. From 50 up to > 200 secondary spheres can be obtained from each individual primary sphere, depending on the number of viable cells plated. The cloning efficiency for adult SVZ-derived NSCs under these culture conditions is in the range of 2–8%. Bars = 100 μm **(A)**, 25 μm **(B)**, and 250 μm **(C)**.

3.2. Cryopreservation of Neurospheres

3.2.1. Preparation

1. Ensure that freezing jar is a room temperature and filled with isopropanol.
2. Prepare freezing medium: 10% DMSO in culture medium (*see* **Subheading 2.2.2.**) (*see* **Note 7**).
3. Label cryovials with date, cell type, and passage number.

3.2.2. Cryopreservation

1. Collect spheres by gentle pipetting and pellet them by centrifugation at 110 × *g* for 10 min (*see* **Note 8**).
2. Remove supernatant and resuspend in 1.5 ml of freezing medium. Swirl gently to resuspend spheres.
3. Transfer cells into labeled 2-ml cryogenic vial(s) and let them equilibrate at room temperature for 10 min.
4. Transfer vial(s) into the freezing jar. Leave the jar at –80°C for a minimum of 4 h to allow a slow and reproducible decrease in temperature (–1°C/min).
5. Transfer vial(s) into a liquid nitrogen tank for long-term storage.

3.2.3. Thawing of Cryopreserved Neurospheres (see Note 9)

1. Warm culture medium in a water bath to 37°C.
2. Quickly transfer cryovial(s) from liquid nitrogen to 37°C water bath and leave until thawed. Swirl the vial(s) to speed thawing.
3. Wipe entire cryovial with 70% ethanol.
4. Slowly transfer cell suspension from cryovial to 15-ml plastic tube containing 5 ml of warm culture medium.
5. Spin cell suspension for 8 min at 110 × *g* and remove most of supernatant.
6. Gently resuspend pellet in fresh medium and plate in flask(s) of appropriate size.

4. Notes

1. A set of glassware to be used only for tissue cultures should be prepared by rinsing accurately several times with distilled water before being sterilized in an autoclave that is used for tissue culture purposes only. We suggest that media and all stock solutions be prepared only in sterile disposable tubes and/or bottles. These cultures are extremely sensitive to contaminants present in water or glassware. Distilled, sterile, apirogenic water should be used. Alternatively, ultrapure cell culture tissue grade water can be purchased from appropriate suppliers.
2. Because small volumes of medium are used in 96-multiwell plates, evaporation of medium is very critical; always hold plates in humidified chambers.
3. NSC cultures are very sensitive to pH changes. The pH of the culture medium should be around 7.4, which is indicated by a dark orange color. If the color is close to violet, leave the medium to equilibrate in the incubator. If the color is light orange/yellow, check the composition and prepare fresh medium if necessary.
4. If you want to generate a clonal cell line, use the limiting dilution protocol and then dissociate single clonal spheres directly in the 96-well plate. Then, transfer the cell suspension in a clean well with fresh medium.
5. Embedding cells in methylcellulose before plating is recommended to avoid cell aggregation.
6. Because both stem cells and progenitors can generate neurospheres, the premise of a one-to-one relationship between neurospheres and stem cells is incorrect, and

estimating stem cell frequency on the basis of neurosphere frequency might give an invalid readout ***(21)***. Thus, the cloning efficiency calculated by normalizing the number of secondary spheres obtained in a well by the total number of cells plated (as assessed by cell count before plating or by direct counting 1 h after dissociation of individual spheres), actually returns the frequency of sphere-forming cells, which include both stem and nonstem cells (progenitors). However, the size of the secondary spheres formed might give further indication about the nature of the sphere-forming cells: progenitor cells should display only a

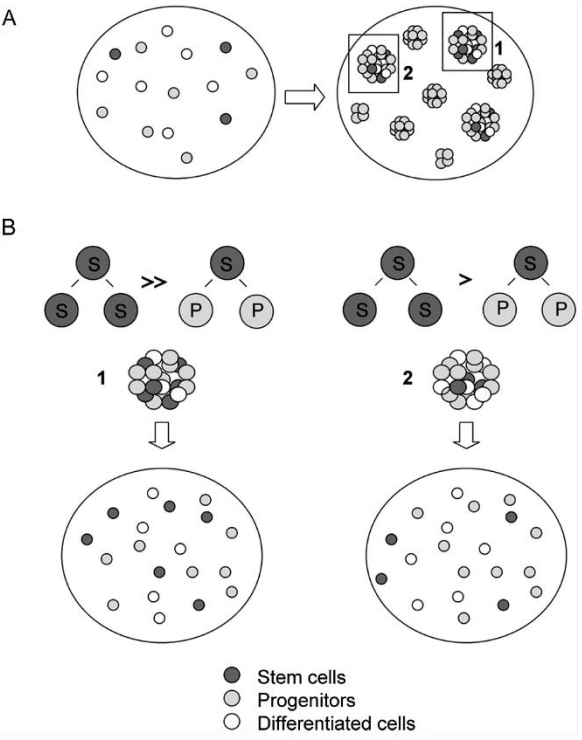

Fig. 3. Cloning efficiency relates to the proliferative mode of stem cells. **(A)** Cloning efficiency returns the number of sphere-forming cells (stem and progenitor cells). The size of the secondary spheres formed might give an indication about the nature of sphere-forming cells, with smaller size suggesting a neurospheres generated by transient amplifying progenitors endowed with limited proliferation ability. **(B)** Increased frequencies of proliferative versus differentiative symmetric divisions occurring within stem cell-derived neurospheres affect the total number of stem cells yielded upon dissociation and results in increased numbers of stem cell-derived neurospheres at the subsequent subcloning steps. *S*, stem cells; *P*, progenitors. Spheres 1 and 2 in **A** correspond to spheres 1 and 2 represented in **B**.

limited proliferative capacity; thus, they should give rise to small-sized spheres with respect to larger spheres resulting from the proliferation of a stem cell. In fact, in our culture conditions, stem cells undergo self-renewal preferentially by symmetric proliferative divisions in which two stem cells are generated at each cell cycle ***(22)*** and thus display enhanced proliferation potential (*see* **Fig. 3A**). Increased frequencies of proliferative versus differentiative symmetric divisions occurring within stem cell-derived neurospheres affect the total number of stem cells yielded upon dissociation (*see* **Fig. 3B**) and result in an increased number of stem cell-derived neurospheres at the subsequent subcloning step. If these conditions are maintained, you will obtain a progressive enrichment in the stem cell population upon serial subcloning steps, which could be assessed either by an increase in the cloning efficiency, or, if working with a bulk cell population, by an upward trend in the growth profile.

7. In our experience, glycerol yields very poor results when freezing NSCs.
8. Do not let spheres grow too large before harvesting for cryopreservation and do not mechanically dissociate spheres before freezing. These procedures increase the number of dead cells and decreases cell viability upon thawing.
9. This same freezing-thawing protocol also can be applied to finely chopped embryonic human neural tissue. Freezing medium composed of 10% DMSO in fetal calf serum guarantees better survival of brain tissue. To start a NSC culture from frozen human brain tissue, follow previously described protocols ***(23)***.

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15

Telomere Neurobiology

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Summary

The ends of chromosomes consist of a hexanucleotide DNA repeat sequence and specialized DNA-binding and telomere-associated proteins. An enzyme activity called telomerase maintains telomere length by using an RNA template (TR) and a reverse transcriptase (TERT) to add the hexanucleotide sequence to the free chromosome end. The structure of telomeres is maintained and modified by telomere repeat-binding factors (TRF1 and TRF2) and proteins known for their role in DNA damage responses, including poly(ADP-ribose) polymerase-1, Werner, and ATM. Telomerase activity can be quantified using a telomere repeat amplification protocol (TRAP) assay, and levels of TERT and telomere-associated proteins are evaluated by immunoblot and immunocytochemical methods. Levels of TERT and telomere-associated proteins can be overexpressed or knocked down using viral vector-based methods. Using the kinds of approaches described here, evidence has been obtained suggesting that telomeres play important roles in regulating neural stem cell proliferation, neuronal differentiation, senescence of glial cells, and apoptosis and DNA damage responses of neural cells.

Key Words: Apoptosis; astrocytes; ATM; neurogenesis; telomerase reverse transcriptase; TRF1; TRF2.

1. Introduction

Highly proliferative cells, including stem cells and cancer cells, express high levels of telomerase, an enzyme activity that adds a six-base DNA repeat sequence (TTAGGG) to chromosome ends and thereby prevents their shortening during successive rounds of mitosis ***(1,2)***. Telomerase activity decreases in association with cell differentiation, and it is generally absent

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from most somatic cells in the adult; shortening of telomeres in such somatic cells may trigger cell cycle arrest in the G1 phase (cellular senescence). In this way, telomere shortening effectively limits the proliferative potential of cells, functions as a tumor suppressor mechanism, and may contribute to the aging process ***(3–6)***. Telomerase consists of an RNA template (TR) and a protein called TERT that possesses reverse transcriptase activity. Several telomere-associated proteins have been identified, including telomere repeat-binding factor (TRF)1, which may inhibit telomerase activity and promote telomere shortening, and TRF2, which may promote maintenance of telomeres and activation of telomere damage response pathways ***(7,8)***. Data obtained during the past several years have provided evidence that telomerase can play important roles in the regulation of cell proliferation, differentiation, and survival. Examples include overexpression of hTERT can immortalize cultured fibroblasts and epithelial cells ***(4)***; telomerase is downregulated during muscle cell differentiation ***(9)***; TERT promotes cell survival (prevents apoptosis) of developing mouse and rat brain neurons ***(10–12)***; and TRF2-related telomere changes regulate the differentiation of postmitotic neurons ***(13)***.

TERT and telomerase activity levels are high in several types of stem cells, including embryonic stem cells ***(14,15)***, hematopoietic stem cells ***(16,17)***, and neural progenitor cells ***(12,18)***. A progressive decrease in telomerase levels seems to occur in association with progressive lineage restriction and cellular differentiation, suggesting a role for telomerase in controlling cell fate. Interestingly, studies of cloned cattle in which donor nuclei from adult fibroblasts are injected into oocytes suggest that telomerase activity and telomere length regulation can be "reprogrammed" ***(19)***. It is therefore important from both basic science and clinical perspectives that we better understand the mechanisms whereby telomerase regulates cell fate. We detail methods for quantifying telomerase activity, and TERT expression in tissue samples and cultured cells. Protocols for inhibiting TERT expression and telomerase activity also are presented.

2. Materials

2.1. Telomerase Activity Assay

1. CHAPS buffer: 0.5% CHAPS, 10 mM Tris-HCl (pH 7.5), 1 mM $MgCl_2$, 1 mM EGTA, 5 mM β-mercaptoethanol, 1 mM phenylmethylsulfonyl fluoride, 1 U/μl RNase Out (Invitrogen, Carlsbad, CA), and 10% glycerol.
2. Eppendorf microfuge.
3. Bicinchoninic acid (BCA) Assay (Pierce Chemical, Rockford, IL).
4. TRAP reaction mixture: 20 mM Tris-HCl (pH 8.0), 1 mM EGTA, 0.005% Tween 20, 1.5 mM $MgCl_2$, 63 mM KCl, 200 μM dNTP mix, 2 U of Taq

polymerase, 10 pmol of TS primer (5´-AAT CCG TCG AGC AFA GTT-3´), 10 pmol of CX-ext primer (5´-GGT CCC TTA CCC TTA CCC TTA CCC TTA-3´), and an internal amplification standard that produces a 135-base pair product ***(20)***.
5. PTC-20P (DNA Engine, MJ Research, Watertown, MA).
6. ROX-500 (GeneScan-500Rox size standard, Applied Biosystems, Foster City, CA).
7. Formamide.
8. ABI Prism 310 capillary electrophoresis unit (Applied Biosystems).

2.2. Reverse Transcription-Polymerase Chain Reaction (RT-PCR) Analyses of TERT and Telomere-Associated Protein mRNA Levels

1. TRIzol (Invitrogen).
2. SuperScript First Strand Synthesis System (Invitrogen).
3. Reaction mixtures: 1–5 liters of the first strand cDNA, PCR buffer (Invitrogen), 200 M dNTPs mix, 2 U of Taq polymerase, 1.5 mM $MgCl_2$, and 10 pM primers.
4. β-Actin.
5. Agarose gel.
6. Ethidium bromide.
7. FLA 3000 Imager (Fuji, Tokyo, Japan).

2.3. Immunoblot Analysis of TERT Protein Levels

1. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (10% acrylamide).
2. Polyvinylidene difluoride (PVDF) membrane.
3. 5% nonfat milk.
4. Tris-Tween-buffered saline (TTBS): dissolve the following in 1 liter of water: 80 g/l NaCl, 29.2 g of Tris base, and 0.5 ml of Tween 20 (pH 7.5).
5. Peroxidase labeled anti-rabbit or anti-mouse secondary antibody.
6. Enhanced chemiluminescence (ECL) reagent (GE Healthcare, Little Chalfont, Buckinghamshire, UK).
7. Hyperfilm (GE Healthcare).

2.4. Immunostaining Methods for Cellular Localization of TERT Protein

1. 4% paraformaldehyde.
2. Phosphate-buffered saline (PBS).
3. 2% Triton X-100.
4. 2% normal goat serum.
5. Biotinylated goat anti-rabbit secondary (Vector Laboratories, Burlingame, CA).
6. Fluorescein isothiocyanate (FITC)/avidin conjugate (Vector Laboratories).
7. Anti-fade solution: 10 μM propylgallate in water.
8. ABC reagent (Vector Laboratories).

9. Nickel-enhanced diaminobenzidine solution (Vector Laboratories).
10. TERT Antibodies: a rabbit polyclonal antibody against human TERT is commercially available from Calbiochem. Results of studies using other TERT polyclonal antibodies have been reported previously ***(10,11)***.

2.5. Overexpression of TERT

1. pBabest 12 retroviral plasmid.
2. Simian virus 40 (SV40) early promoter.
3. Lipofectamine (Invitrogen).
4. Geneticin (G-418, Invitrogen) antibiotic.

2.6. Suppression of TERT Expression and Activity

1. TERT antisense and control oligonucleotides: an antisense oligonucleotide against mouse TERT (5′-GAGGAGCGCGGG TCATTGT-3′) and the scrambled control oligonucleotide (5′-GGAGGACGCTGCGAGTGTT-3′) are purchased from IDT (Coralville, IA) and prepared as 1 mM stocks in sterile deionized water.

2.7. Induction of Selective Telomere Damage

1. Telomestatin.
2. Dimethyl sulfoxide.

2.8. Inhibition of TRF2 Function

1. DN-TRF2 primers: forward primer: 5′-caccagatctaccatggaggcacggctggaagaggc agtcaat-3′ and reverse primer: 5′-gaattcttacttctgctt ttttgttatattggttgt-3′.
2. TOPO cloning vector (Invitrogen).
3. Adenoviral Gateway Destiny vectors (pAD/CMV-V5-DEST or pAD/PL-DEST).
4. Human embryonic kidney 293A cells.
5. ViraPower Adenoviral expression system (Invitrogen).
6. Cesium chloride gradient (1.50, 1.35, and 1.25 mg/ml).
7. Vivaspin 20-ml column (Vivascience, Stonehouse, UK).

2.9. Detection of Telomere Damage

1. 4% paraformaldehyde in PBS.
2. Blocking buffer: 2% nonfat powdered milk, 2% normal serum, and 0.2% Triton X-100 in PBS.
3. Primary antibodies against TRF2 (Imgenex, San Diego, CA) and anti-γH2AX (Upstate Biotechnology, Lake Placid, NY).
4. Alexa 568- or Alexa 633-conjugated secondary antibodies appropriate for the specific primary antibodies.

5. 4,6-Diamidino-2-phenylindole (DAPI) and propidium iodide in PBS containing 1% RNase and 0.2% Triton X-100).
6. Yellow fluorescent protein (YFP)-TRF1 expression plasmid.
7. PermaFluor aqueous mounting medium (Immunon, Pittsburgh, PA).
8. Zeiss LSM510 confocal laser-scanning microscope (or equivalent) with 63× oil immersion or 40× water immersion objective.

3. Methods

3.1. Telomerase Activity Assay

A capillary electrophoresis-based telomeric repeat amplification protocol (TRAP) assay is used to quantify levels of telomerase activity ***(20,21)***. This assay is performed on lysates of tissues or cultured cells using the following protocol.

1. Tissues or cultured cells are homogenized in CHAPS buffer and incubated for 30 min on ice.
2. Samples are centrifuged for 30 min at maximum speed in a microfuge (Eppendorf AG, Hamburg, Germany), and the supernatant is removed; a 5-µl aliquot is removed for protein determination (BCA assay, Pierce Chemical), and the remainder of the sample is stored at –80°C.
3. The reaction is initiated by adding 100 ng of sample protein to a TRAP reaction mixture. The reaction is incubated at 30°C for 30 min to allow telomerase to add telomeric repeats to the TS primer.
4. The telomerase products are amplified by PCR (30 cycles). Each PCR cycle consists of 30°C for 30 min for the first extension cycle, followed by 94°C for 30 s, 50°C for 30 s, and 72°C for 30 s.
5. Two microliters of PCR product is missed with 1 µl of ROX-500 and 22 µl of formamide and analyzed using an ABI Prism 310 capillary electrophoresis unit (Applied Biosystems).
6. Integrated values are summed for telomerase products containing five (one repeat beyond primer dimmer size) to 10 telomeric hexamer repeats and calibrated by dividing by the value for the peak area of the internal amplification standard. All assays are performed in triplicate. Experimental values are typically expressed as a percentage of the value obtained using an equivalent amount (usually 100 ng) of HeLa cell extract.

The published results using the method can be found in **ref.** ***12***.

3.2. RT-PCR Analyses of TERT and Telomere-Associated Protein mRNA Levels

For analyses of mRNA levels, total RNA is isolated from samples of tissue or cultured cells by using TRIzol reagent according to the manufacturer's

protocol (Invitrogen), and it is then subjected to RT-PCR analysis as follows (*see* **Note 1**):

1. The first strand of cDNA is synthesized from 1 to 2 μg of total RNA with the SuperScript First Strand Synthesis System for RT-PCR by using oligo(dT) primers and following recommendations provided by the supplier.
2. Reaction mixture consisting of 1–5 μl of the first strand cDNA, PCR buffer, 200 μM dNTPs mix, 2 U of Taq polymerase, 1.5 mM $MgCl_2$, and 10 pM primers are denatured at 94°C for 2 min.
3. Samples are then subjected 33 PCR cycles (each cycle consists of 30 s at 94°C, 30 s at 50°C, and 45 s at 72ºC), then elongated at 72°C for 10 min. The primers for the internal β-actin control are added to the reaction at the 60°C step of cycle 9.
4. PCR products are separated by agarose gel electrophoresis (1.5%) followed by staining with ethidium bromide.
5. Images of the stained DNA gels are acquired using a FLA 3000 Imager (Fuji). Densitometric analyses are performed using the software provide by the manufacturer. Values are normalized to the level of actin mRNA in the same sample.

The published results of an RT-PCR analysis of TERT mRNA level in HeLa cells can be found in **ref. *12***.

3.3. Immunoblot Analysis of TERT Protein Levels

Solubilized proteins from tissues and cultured cells are separated by SDS-PAGE (10% acrylamide) and transferred electrorphoretically to a PVDF membrane. The membrane is then processed for immunodetection of TERT protein as follows:

1. The membrane is incubated for 1 h at room temperature in a solution of 5% nonfat milk in TTBS.
2. The membrane is incubated overnight at 4°C in TTBS containing 5% nonfat milk plus primary antibody against TERT. The antibody dilution that results in an adequate signal and a low level of nonspecific binding should be established in preliminary studies. Controls for specificity should include preadsorption of the primary antibody with excess antigen.
3. Rinse the membrane four times with TTBS (5–10 ml/wash).
4. Incubate the membrane for 1 h in TTBS containing peroxidase-labeled anti-rabbit or anti-mouse secondary antibody.
5. Rinse the membrane four times with TTBS (5–10 ml/wash).
6. Expose the membrane to ECL reagent for 1 min at room temperature.
7. Expose the membrane to Hyperfilm; the time of exposure that results in the best signal-to-noise ratio depends on a variety of factors, and it must be determined empirically (typical exposures are from 1 to 10 min).

Examples of results obtained using this method can be found in **ref. *10***.

3.4. Immunostaining Methods for Cellular Localization of TERT Protein

Tissues in adult mice or rats are fixed by perfusion with a solution of 4% paraformaldehyde in PBS, followed by an overnight incubation at room temperature in the same fixative. Tissue sections are cut at 30 μm on a freezing microtome, and they are collected in wells of microwell plates containing PBS. Cultured cells are fixed by incubating for 30 min in a solution of 4% paraformaldehyde in PBS (cells can be stored in PBS [4°C] for several weeks; *see* **Note 2**). The tissue sections and cultured cells are immunostained using the following protocol:

1. Tissue sections and cells are incubated for 1 h and 5 min, respectively, at room temperature in PBS containing 0.2% Triton X-100 plus 2% normal goat serum.
2. Tissue sections and cells are then incubated overnight at 4°C in PBS containing 2% normal goat serum plus primary TERT antibody at an appropriate dilution (typically between 1:1,000 and 1:10,000 depending upon the antibody titer).
3. Tissue sections and cells are then washed three times in PBS.
4. Tissue sections and cells are then incubated for 1 h in PBS containing biotinylated goat anti-rabbit secondary antibody.
5. Tissue sections and cells are then washed three times in PBS.
6. For fluorescence-based detection, tissue sections and cells are then incubated for 30 min in PBS containing FITC/avidin conjugate (4 μl/ml). The sections or cells are then washed in PBS, mounted in an anti-fade solution consisting of 10 μM propylgallate in water, and imaged by conventional or confocal fluorescence microscopy.
7. For peroxidase-based labeling, tissue sections and cells are incubated for 1 h in the presence of ABC Reagent (Vector Laboratories), and peroxidase is detected using nickel-enhanced diaminobenzidine solution (Vector Laboratories). Immunoreactivity is visualized and photographed using bright-field optics.

Published results obtained using this method can be found in **ref. *10***.

3.5. Overexpression of TERT

Clones of cell lines stably overexpressing hTERT can be generated using conventional transfection and antibiotic selection protocols. We have produced clonal lines of pheochromocytoma (PC12) cells overexpressing hTERT by using methods similar to those described previously ***(22)***.

1. Cells are transfected with the pBabest2 retroviral plasmid in which hTERT cDNA is under the control of SV40 early promoter ***(23)***. Control cells are transfected with empty vector. Lipofectamine is used to facilitate plasmid uptake.
2. Transfected cells are selected by maintenance in medium containing the antibiotic G-418, and single clones are isolated by serial dilution.

3. Clones overexpressing hTERT protein are identified by telomerase activity assay and by immunoblot and immunocytochemistry by using TERT antibody.

Examples of the characterization of cells overexpressing TERT generated using these methods can be found in **ref. *10***.

3.6. Suppression of TERT Expression and Activity

3.6.1. One Experimental Approach

One experimental approach for studying the function of telomerase is to suppress TERT production by treating cells with antisense oligodeoxynucleotides directed against TERT mRNA ***(10)***. The oligodeoxynucleotides enter the cell and bind specifically to TERT mRNA, thereby suppressing translation and reducing levels of TERT protein.

1. Cultured cells (we have used embryonic mouse brain neurons in primary culture and human tumor cell lines, including SH-SY5Y and HeLa cells, in our studies) are switched to a culture medium lacking serum.
2. Antisense oligonucleotides in a 1 mM stock solution are diluted into the culture medium to a final concentration of 10–25 μM. Control cultures should receive an equivalent concentration of scrambled control oligodeoxynucleotide, and additional cultures should be treated with vehicle (water) only.
3. Preliminary studies should establish the time course of change in TERT protein levels after exposure of the cells to the antisense oligodeoxynucleotides, which can be accomplished by performing immunoblot and immunocytochemical analyses. A time period of exposure that results in a maximum decrease in TERT protein levels should be chosen for subsequent experiments.
4. After exposure to oligodeoxynucleotides, endpoints of interest are measured to determine whether suppression of TERT expression changes those endpoints. For example, we have used this approach to provide evidence that TERT plays a role in promoting the survival of immature neurons in the developing mouse brain ***(10)***.

3.6.2. A Second Experimental Approach

A second experimental approach involves the use of chemical inhibitors of telomerase enzyme activity. Because of their potential use in cancer therapy, there has been considerable effort placed on identifying chemicals that selectively inhibit telomerase ***(24)***. Inhibitors that we have used to study the roles of telomerase in neural development include the reverse transcriptase inhibitor 3'-azido-3'-dideoxythymidine),3,3'-diethyloxadicarbocyanine (AZT, an agent that binds to dimeric hairpin quadruplexes), and the oligodeoxynucleotide TTAGGG, which may bind to the telomerase RNA component and thereby inhibit telomerase activity ***(10,11,25)***. The following is an example of an

approach for using chemical inhibitors to study the role of telomerase in regulating the differentiation of cultured neural cells.

1. A toxicity profile of the chemical is established by incubating the cultured cells in the presence of increasing concentrations of the inhibitor for 48–72 h.
2. Cells are incubated in the presence of subtoxic concentrations of the inhibitor for increasing periods, and telomerase activity is measured and compared with control cultures not exposed to the inhibitor of exposed to an inactive analog of the inhibitor. It is important to establish that the inhibitor reduces telomerase activity before a change in cell proliferation rate or survival.
3. The effects of inhibitors at concentrations and times that decrease telomerase activity on endpoints of interest are then determined.

3.7. Induction of Selective Telomere Damage

Damage to telomeres can be induced by treating cultured cells with telomestatin, a natural product isolated from *streptomyces anulatus 3533-SV4*, which selectively interacts with the G-quadruplex DNA in telomeres ***(26)***. Telomestatin increases DNA cleavage by S1 nuclease at the loop regions of intramolecular G-quadruplex structures formed within telomeric sequences.

1. Telomestatin is prepared as a 500× stock in dimethyl sulfoxide.
2. Telomestatin is administered by direct dilution into the culture medium, and an equivalent volume of dimethyl sulfoxide is added to control cultures.

3.8. Inhibition of TRF2 Function

The normal functions of TRF2 in telomere maintenance and damage response mediation can be inhibited by overexpressing a truncated dominant negative TRF2 (DN-TRF2) form of TRF2 ***(27)***.

1. DN-TRF2 is generated by PCR with forward primer 5'-cac cagatctaccatggaggcac ggctggaagaggcagtcaat-3' and reverse primer 5'-gaattcttacttctgctt ttttgttatattggttgt-3', and it is then ligated with pENTER directional TOPO cloning vector (Invitrogen).
2. The DN-TRF2 cDNA is then subcloned into adenoviral Gateway Destiny vectors (pAD/CMV-V5-DEST or pAD/PL-DEST) by using the manufacturer's protocols modified for coexpression of eGFP reporter genes under cytomegalovirus (CMV) promoter.
3. For cell transfection of adenoviral DN-TRF2 (with or without conjunction of IRES-eGFP reporter gene), vectors are packaged into 293A cells by using the ViraPower Adenoviral expression system according to the manufacturer's protocol (Invitrogen). Adenoviral particles are purified by ultracentrifugation in a gradient of cesium chloride (1.50, 1.35, and 1.25 mg/ml) for 1 h at 15,000 × *g*

first, followed by centrifugation for 18 h at 15,000 × *g* in a solution of 1.35 mg/ml cesium chloride. The band containing mature viral particles is removed and desalted against PBS in a Vivaspin 20-ml column by using the manufacturer's protocol (Vivascience). Infection units used for transfections are 10–200 plaque-forming units/cell.

3.9. Detection of Telomere Damage

Telomere damage can be detected by double-label confocal immunofluorescence analysis in cultured cells stained with an antibody against the DNA damage marker γH2AX in combination with an antibody against TRF2 or expression of YFP-TRF1 ***(13,28)***.

1. Cells are washed once with PBS and fixed in 4% paraformaldehyde in PBS for 30 min at room temperature.
2. After preincubation with blocking buffer (2% nonfat powdered milk, 2% normal serum, and 0.2% Triton X-100 in PBS) for 1 h, cells are incubated with a primary antibody in blocking buffer overnight at 4°C. The primary antibodies include anti-TRF2 monoclonal antibody (Imgenex) and anti-H2AX polyclonal antibody (Upstate Biotechnology).
3. After thorough washing, cells are incubated with Alexa 568- or Alexa 633-conjugated secondary antibodies appropriate for the specific primary antibodies. Cells are further incubated with DAPI in PBS or propidium iodide in PBS containing 1% RNase and 0.2% Triton X-100 for 10 min, and then they are mounted in PermaFluor aqueous mounting medium (Immunon).
4. The cells are examined under a Zeiss LSM510 confocal laser-scanning microscope with 63× oil immersion or 40× water immersion objective.
5. Telomeres also can be labeled by transfecting cells with a YFP-TRF1 expression plasmid ***(28)***.

4. Notes

1. In the RT-PCR analyses, it is important to establish that the RT-PCR products of the correct size do, in fact, correspond to the mRNAs of interest by excising the bands from the gels and sequencing them. Preliminary analyses also are required to determine the optimum PCR conditions that result in a level of amplification that falls within the linear range.
2. For immunoblots and immunostaining, it is important that antibodies not be subjected to multiple freeze-thaw cycles; this can be avoided by diluting the stock with an equal volume of glycerol and storing it at –20°C. To reduce the level of nonspecific binding of the primary antibody to proteins in immunoblots, the concentration of primary antibody can be decreased and the membranes can be washed more extensively with buffer after the primary antibody step. Cross-reactivity of antibodies raised against hTERT peptides with tissues and cultured

cells from mice and rats should be established. It is important to include a positive control (e.g., HeLa cells) and a negative control (e.g., adult brain tissue) in the analyses.

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16

Chromosomal Mosaicism in Neural Stem Cells

Suzanne E. Peterson, Jurjen W. Westra, Christine M. Paczkowski, and Jerold Chun

Summary

Neural stem and progenitor cells (referred to here as NSCs), located in the proliferative zones of embryonic brains, can be seen undergoing mitosis at the ventricular surface. Mitotic NSCs can be arrested in metaphase and chromosome "spreads" produced to reveal their chromosomal complement. Studies in mice and humans have revealed a prominent developmental presence of aneuploid NSCs, whereas other chromosomal defects, such as interchromosomal translocations and partial chromosomal deletions/insertions, are extremely rare ***(1,2)***. Aneuploidy is defined as the loss or gain of whole chromosomes, resulting in cells that deviate from the normal diploid number of chromosomes (46 in humans, 40 in mice). In NSCs, aneuploidy can occur as a result of missegregation during mitosis, through events such as lagging chromosomes, supernumerary centrosomes, and nondisjunction events ***(3)***. The percentage of aneuploid NSCs can be altered by in vivo and in vitro growth conditions as well as through genetic deletion of genes involved in DNA surveillance and repair ***(1,4)***. Aneuploidy can be detected by classical cytogenetic methods such as counting the number of chromosomes visualized by DNA dyes (e.g., 4,6-diamidino-2-phenylindole) by using standard light or fluorescence microscopy. Precise chromosome identification is much more difficult: classical methods using banding patterns or size to assign identity are very time consuming even under ideal conditions, and they are notoriously difficult in mice, which often have ambiguous banding patterns and acrocentric chromosomes. A comparatively new technique that allows the unambiguous identification of chromosomes in mice and humans is "spectral karyotyping" or SKY, developed by Ried et al. at the National Institutes of Health for the study of cancer cells ***(5)***. This technique uses chromosomal "paints" that are hybridized to chromosome spreads to produce a distinct spectral output for each chromosome. SKY offers superior speed and sensitivity in its ability to detect many types of chromosomal defects, including deletions, insertions, translocations, and aneuploidy.

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Key Words: Spectral karyotyping; chromosome; aneuploidy; neural stem cell; mosaicism.

1. Introduction

There are four major procedures involved in this protocol. First, neural stem cells (NSCs) must be isolated, cultured, and harvested. Second, metaphase chromosome spreads are made. Third, the slides are pretreated. And finally, "spectral karyotyping" (SKY) is performed. The underlying procedure behind metaphase cell analysis using SKY is straightforward. SkyPaint, which is a combination of fluorescently labeled nucleic acid probes that recognize specific DNA sequences on every chromosome, is hybridized to chromosome spreads to produce a distinct spectral output for each chromosome. To prepare single chromosome paints, individual chromosomes are first separated by flow cytometry. Degenerate-oligonucleotide polymerase chain reaction is then performed using a specific combination of fluorescent nucleotides for each chromosome. SkyPaint is the resulting mixture of these individual chromosome paints.

Individual chromosomes hybridized with SkyPaint emit a unique "spectral signature" upon fluorescence excitation. The spectral identity is detected using an interferometer attached to a standard fluorescent microscope and pseudo-colors assigned via SkyView (Applied Spectral Imaging, Vista, CA). SkyView is a software program that both counts and identifies chromosomes within the examined spread. SkyView analyzes the spectral image in two dimensions, and it displays each chromosome with a distinct classification color from which it creates a karyotype table (*see* **Fig. 2**).

2. Materials

2.1. Isolation, Culture, and Harvest of NSCs

1. Timed pregnant female mice or NSC line (*see* **Note 1**).
2. Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum (FBS).
3. Basic fibroblast growth factor (bFGF).
4. Colcemid.
5. 40-µm filters.
6. 0.075 M KCl.
7. Fresh fixative (3:1 methanol:glacial acetic acid).

2.2. Slide Pretreatment

1. Pepsin (*see* **Note 8**).
2. 0.01 M HCl.

3. Phosphate-buffered saline (PBS).
4. PBS with 50 mM $MgCl_2$.
5. Formaldehyde.
6. 100% ethanol (EtOH).
7. 10× standard saline citrate (SSC) (87.7 g of sodium chloride, 44.1 g of sodium citrate in 1 l of water, pH 7.0).

2.3. Spectral Karyotyping

1. SkyPaint (Applied Spectral Imaging).
2. Denaturation solution: (70% formamide, 2× SSC, pH 7.0).
3. 37°C slide warmer or heating block.
4. Formamide solution: (50% formamide, 2× SSC, pH 7.0)
5. 4× SSC, 0.1% Tween 20.
6. Concentrated antibody detection (CAD) kit (Applied Spectral Imaging).
7. DAPI.
8. VECTASHIELD antifade (Vector Laboratories, Burlingame, CA).
9. Microscope equipped with an interferometer and SkyView software (Applied Spectral Imaging).

3. Methods

3.1. Isolation, Culture, and Harvest of Cortical NSCs

1. Dissect the cerebral cortex from embryonic day (E)12–14 embryos, and triturate cells with a Pasteur pipette to get a single-cell suspension (*see* **Note 1**). Keep cells on ice.
2. Spin down cell suspension at 300 × *g* for 5 min at 4°C.
3. Resuspend cells in 3 ml of DMEM with 10% FBS, 40 ng/ml bFGF, and 0.1 μg/ml colcemid, and transfer the cells to one well of a six-well plate (*see* **Note 2**).
4. Incubate the cells at 37°C for 3–5 h while slowly rotating (70–80 rpm).
5. Triturate cells again briefly to get a single cell suspension. Wash cells with 10 ml of PBS, and filter the suspension through a 40-μm filter.
6. Spin cells down at 300 × *g* for 5 min, aspirate the supernatant, and flick the pellet to resuspend. Add 10 ml of 0.075 M KCl to the cells.
7. Incubate cells in a water bath at 37°C for 15 min (*see* **Note 3**).
8. Add 3 drops of fixative with a transfer pipette to the cells while flicking the tube between drops.
9. Spin cells at 300 × *g* for 5 min at room temperature. Aspirate most of the supernatant off, and then flick the tube to resuspend the pellet.
10. Add 5 ml of fixative dropwise while slowly vortexing the tube (*see* **Note 4**).
11. Incubate at 4°C until use (*see* **Note 5**).

3.2. Metaphase Chromosome Spreads

1. Warm cells to room temperature. Wash twice with fixative and resuspend cells in 1 ml of fixative (*see* **Note 6**).
2. Set up an 80°C water bath with a flat, thin piece of metal positioned horizontally, about 1.3 cm (0.5 in.) above the water level.
3. Flick cell suspension to resuspend. Take 20 µl of cell suspension and pipette it onto the slide. Hold the slide level for about 15–20 s. As the fixative evaporates, the center of the slide will become granular.
4. Quickly flip over the slide (cell-side down) and very briefly expose it to the steam from the water bath (about 5 cm [2 in.] above the water level in the bath) (*see* **Note 7**).
5. Immediately place the slide on the metal heating plate in the water bath. As soon as the liquid on the slide beads up and is almost gone, remove the slide.
6. Use a microscope to check the spreads for (1) spread density and (2) chromosome morphology (*see* **Fig. 1**).

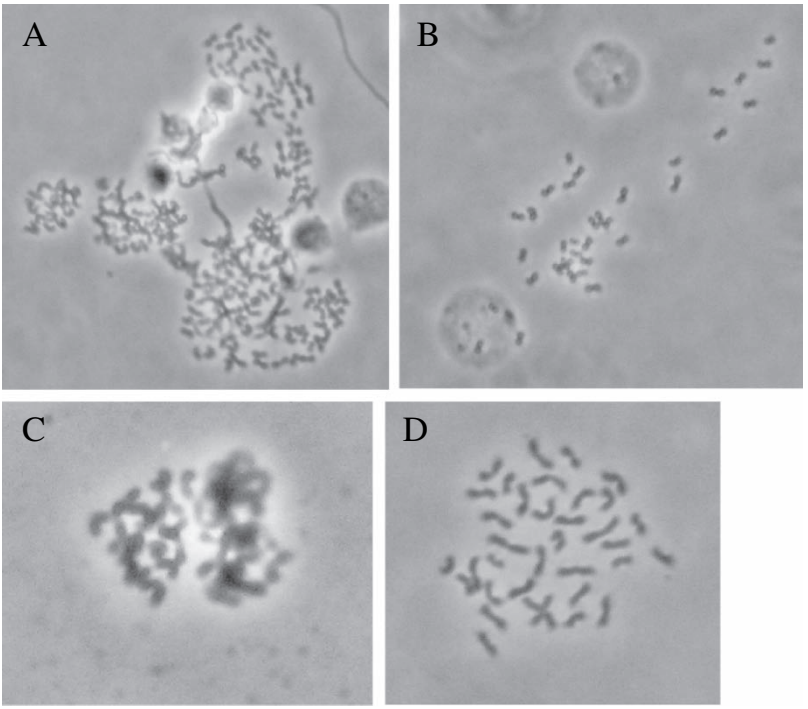

Fig. 1. Examples of metaphase chromosome spreads. **(A)** Overlapping chromosome spreads. The cell density is too high. **(B)** Potentially incomplete metaphase spread. **(C)** Spread containing overlapping chromosomes. **(D)** Great spread. The chromosomes are well separated, but they are contained in a relatively tight circle.

7. Make 5–10 good slides/sample and age them at room temperature for 1 to 7 days.
8. Add 5 ml of fixative to the remaining cell suspension and store at 4°C for up to 1 year.

3.3. Slide Pretreatment

1. Wash aged slides in 2× SSC for 5 min at room temperature.
2. Add 25 µl of 100 mg/ml pepsin to 50 ml of prewarmed (to 37°C) 0.01 M HCl. This results in a final pepsin concentration of 50 µg/ml. Incubate slides in the pepsin solution for 5 min at 37°C (*see* **Note 8**).
3. Wash slides two times with PBS at room temperature for 5 min.
4. Incubate slides in PBS with 50 mM $MgCl_2$ for 5 min at room temperature.
5. Incubate slides in PBS containing 1% formaldehyde plus 50 mM $MgCl_2$ for 10 min at room temperature.
6. Wash slides in PBS for 5 min at room temperature.
7. Dehydrate slides in increasing EtOH concentrations of 70, 80, and 100% for 1 min each.
8. Air dry slides (*see* **Note 9**).

3.4. Spectral Karyotyping

1. Place 10 µl of SkyPaint in a small microfuge tube and incubate at 37°C for 10 min (*see* **Note 10**).
2. Denature SkyPaint for 10 min at 80°C (using water bath or thermocycler) and then incubate for 60 min at 37°C.
3. Dehydrate slides in increasing EtOH concentrations of 70, 80, and 100% for 1 min each. Air dry slides.
4. Denature slides for 1.5 min in denaturation solution at 73°C.
5. Immediately repeat dehydration in 70, 80, and 100% EtOH sequence for 1 min each. Air dry slides.
6. Warm slides on a 37°C slide warmer for 5 min before adding the SkyPaint.
7. Apply 10 µl of SkyPaint to a 24- × 24-mm coverslip. Immediately put coverslips on slides and use rubber cement to seal the edges.
8. Place slides in a humidified box prewarmed to 37°C and allow hybridization to proceed overnight in the dark (*see* **Note 11**).
9. Prepare the wash solutions for the next day and leave at 45°C until use.
10. Remove the rubber cement seal carefully, using forceps, and place slides in 2× SSC until the coverslip comes off.
11. Wash slides three times for 5 min in formamide solution (at 45°C).
12. Wash slides three times for 5 min in 1× SSC (at 45°C).
13. Wash slides once in 4× SSC + 0.1% Tween 20 for 5 min.
14. Prepare staining solution: add 10 µl of reagent 3 and 5 µl of reagent 4 (from the CAD kit, Applied Spectral Imaging) to 1 ml of 4× SSC. Vortex mixture for 10 s, then spin for 2 min in a microfuge to pellet any unwanted fluorescent aggregates.

15. Remove as much moisture as possible from the slides without allowing them to dry out. Add 100 µl of staining solution to the slides and cover with a coverslip.
16. Incubate slides in a dark 37°C humidified chamber for 30 min.
17. Carefully remove coverslips in 4× SSC and wash slides at 45°C in 4× SSC + 0.1% Tween 20, three times for 5 min.
18. Incubate slides at room temperature in 4× SSC with 0.5 µg/ml DAPI for 5 min.
19. Immediately dehydrate slides in increasing EtOH concentrations of 70, 80, and 100% for 1 min each.
20. Air dry slides in the dark.
21. Add coverslip (24 × 50 mm) and VECTASHIELD (antifade) to slides.
22. Use a microscope equipped with an interferometer and SKY software to view and analyze slides (*see* **Fig. 2**).
23. Analyze at least 20, preferably 40, metaphase spreads for each sample.

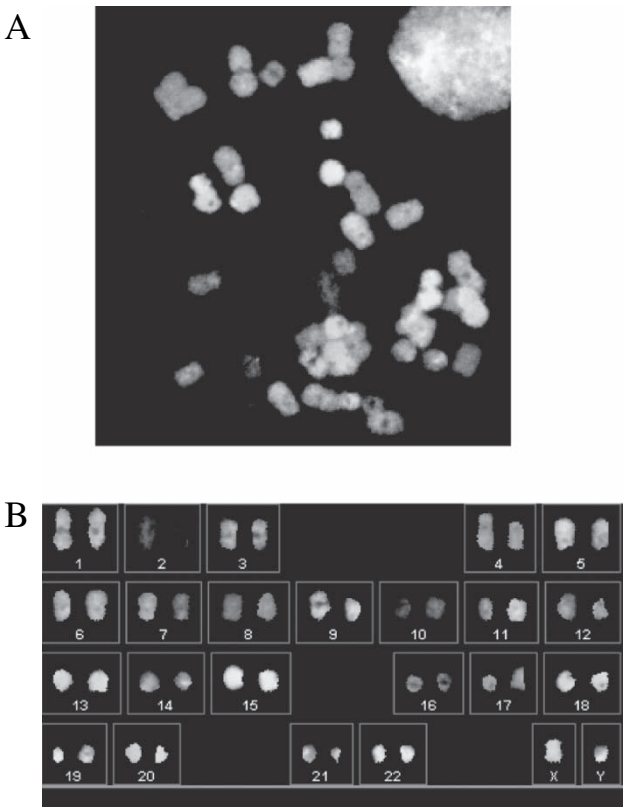

Fig. 2. SKY analysis of a human NSC line. **(A)** Chromosome spread hybridized with SkyPaint. **(B)** Corresponding karyotype table for spread shown in **A**. The karyotype of the cell is 46,XY.

4. Notes

1. Cells must be dividing to be karyotyped via SKY or other methods such as Giemsa banding (G banding). Cortices must be taken from embryos at E12–14 to get a sufficient number of dividing cells. To karyotype cultured NSC lines, make sure a large percentage of cells are dividing at the time of harvest. Treat cells with 0.1 μg/ml colcemid for 3–5 h, and then trypsinize cells to get a single-cell suspension. Proceed to **step 6**.
2. Colcemid arrests dividing cells in metaphase. If few cells are dividing or the colcemid step is omitted, there will likely be an insufficient number of metaphase chromosome spreads for karyotyping. Longer colcemid treatments can be toxic to the cells, and they can cause the chromosomes to become short, making analysis more difficult.
3. This hypotonic KCl solution causes cells to swell, so they will break open when dropped on a slide.
4. It is very important to have a single-cell suspension when the fixative is added. In addition, the cells should be in motion as the fixative is added. If not, the cells will form clumps, making analysis impossible.
5. It is often possible to use cells that have been stored in fixative at 4°C for more than a year.
6. Addition of 1.0 ml of fixative to resuspend the cells is an estimate. Depending on the number of cells in solution, it may be necessary to resuspend them in a larger or smaller volume of fixative. Test with 1.0 ml, and if the spreads on the slide are too sparse or dense, adjust the volume appropriately and then repeat the procedure (*see* **Fig. 1**).
7. Making chromosome spreads is an art, and it is dependent on many uncontrollable variables, such as humidity. If the cells are not spreading well, try some of the following variations in the procedure:

    a. Do not place the slides on the heated metal plate, just allow them to air dry slowly.
    b. Do not expose the slides to steam.
    c. Expose the slides to steam before adding the cell suspension.
    d. After the drying fixative becomes granular, add a few drops of glacial acetic acid to the slide and continue with the procedure.
    e. Try a different fixative, such as 1:1 methanol:glacial acetic acid. If all else fails, try making chromosome spreads on a different day when the atmospheric conditions have changed. For more advice, see http://info.med.yale.edu/genetics/ward/tavi/FISH.html.

8. Pepsin treatment of the slides is crucial, and it may vary with cell type. Some cells contain more cytoplasm than others, and they will need longer pepsin treatments. Thus, pepsin concentration and incubation time should be determined empirically. It is important not to expose the cells to pepsin for too long, because this will make the chromosomes difficult to hybridize. Insufficient

pepsin treatment may cause fluorescent green background haze on the slide after it is hybridized with SkyPaint.

9. At this point, slides can either be hybridized immediately or stored in a desiccator at –20°C for several months.
10. The first time the SkyPaint is thawed it should be incubated at 37°C and vortexed every 3–5 min for 30 min to mix it thoroughly. The paint should then be aliquoted and stored at –20°C. In addition, the paint is light-sensitive, so keep it protected from light as much as possible.
11. The SkyPaint hybridization can be performed overnight or for as long as 2 days.

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17

In Vitro Assays for Neural Stem Cell Differentiation: *Induction of Dopaminergic Phenotype*

Marcel M. Daadi

Summary

Parkinson's disease (PD) is a neurodegenerative disorder characterized by the loss of dopaminergic neurons in the substantia nigra. Symptoms include tremors, rigidity, bradykinesia, and instability. Neural transplantation is a promising strategy for improving dopaminergic dysfunction in PD provided that the dopaminergic neurons are consistently generated from a renewable source of cells. Neural stem cells (NSCs) have the ability to self-renew, generate a large number of progeny, and differentiate into the principal nervous system cell types. As such, stem cells provide an exciting opportunity to understand the basic mechanisms involved in cell differentiation and histogenesis. These mechanisms have translational applications in tissue engineering and biomedicine in general. In vitro differentiation assays are important in cell characterization, in assaying for novel instructive molecules, and in generating specific cell types. We describe differentiation techniques to test NSCs for multipotency and to induce the dopaminergic phenotype in neural stem cell progeny by coculturing them with astrocytes and treating them with conditioned media and basic fibroblastic growth factor.

Key Words: Neural stem cells; stem cell lines development; characterization of cell lines; cellular differentiation; dopamine induction assay; coculture with astrocytes; tyrosine hydroxylase expression; glial-derived conditioned media; cell therapy; Parkinson's disease.

1. Introduction

Neural stem cells (NSCs) are an undifferentiated population of cells residing in the tissue lining the ventricular system of both the embryonic and adult mammalian central nervous system (CNS) ***(1–4)***. When isolated under the

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appropriate genetic or epigenetic conditions, NSCs have the ability to self-renew and produce a large amount of cells that may be well-characterized in vitro and in vivo, cryopreserved, and ready for therapeutic use. Upon transplantation into the adult CNS, NSC progeny integrate within the host tissue and differentiate according to the local microenvironment into any one of the principal CNS cell types such as neurons, astrocytes, and oligodendrocytes. These attributes make NSCs a promising source of cells for cell replacement therapy for neurological disorders. However, this source of neural tissue will not reach the clinic unless it is well characterized both in vitro and in animal models.

As we continuously perpetuate NSCs in vitro, one or more of their properties may to change. Therefore, to ensure product consistency, it is necessary to cryopreserve and characterize a reference stock of cells. Many different assays and techniques may be used to define and characterize NSCs. There is an extensive list of commercially available markers that may be used to characterize NSCs by means of simple immunocytochemistry or fluorescence-activated cell sorting. Other techniques include microarray technology to establish gene or protein expression profiles that also may be used to identify the NSC line. Together, these assays will ensure that the cells are stable and that they maintain the desired characteristics. Functional assays may be used to further define the NSCs and to determine suitability for their intended purpose. Ultimately, these assays will correlate with the animal model data to support the intended therapeutic use.

We have been using different bioassays to test whether a specific NSC line has the potential to express different neurotransmitter phenotypes. Particularly, we investigated the expression of tyrosine hydroxylase (TH), the rate-limiting enzyme in the synthesis of the neurotransmitter dopamine, as a functional correlative marker. Based on the outcome of this assay and the in vivo transplantation studies, the NSC line may be selected for potential use in Parkinson's disease cell replacement therapy.

2. Materials

1. Medium for Culture of NSCs: The culture medium is a serum-free medium composed of 1:1 mixture of Dulbecco's modified Eagle's medium (DMEM) and Ham's F-12 nutrient supplemented with hormone mixture. The media components are DMEM (Invitrogen, cat. no. 430-2100), Ham's F-12 nutrient (Invitrogen, cat. no. 430-1700), glutamine (Invitrogen, cat. no. 320-5030), insulin (Sigma-Aldrich, cat. no. I-5500), putrescine (Sigma-Aldrich, P-7505), progesterone (Sigma-Aldrich, P-6149), sodium bicarbonate (Sigma-Aldrich, S-5761), glucose (Sigma-Aldrich, G-8270), transferrin (Sigma-Aldrich, T-2252), HEPES buffer (Sigma-Aldrich, H-3375), selenium (Sigma-Aldrich, SQ-133), epidermal growth

factor (EGF, Upstate Cell Signalling, Lake Placid, NY); human recombinant), basic fibroblastic growth factor (bFGF) (R & D Systems, Minneapolis, Minnesota, USA). Cell-culture plasticware was purchased from VWR (Brisbane, CA).

2. Medium for Culture of Astroglial Cell Layer: DMEM/Ham's F-12 culture medium supplemented with 10% (v/v) fetal bovine serum was used to establish astrocyte culture. For the astroglial culture, the dissection and preparation protocol we used is similar to the protocol reported by Cole and de Vellis ***(4)***.
3. Antibodies for Characterization of Stem Cell Progeny:
   a. Polyclonal antimicrotubule-associated protein-2 (1:100; Roche Diagnostics, Indianapolis, IN).
   b. Monoclonal anti-β-tubulin (type III, 1:1000; Sigma-Aldrich).
   c. Polyclonal antibody against glial fibrillary acidic protein (GFAP) (1:100; Sigma-Aldrich).
   d. Polyclonal anti-galactocerebroside (GC) (1:1000; Chemicon International. Temecula, CA).
   e. Monoclonal anti-bromodeoxyuridine (BrdU, 1:5; GE Healthcare, Little Chalfont, Buckinghamshire, UK).
   f. Polyclonal anti-GABA (1:5,000; Sigma-Aldrich).
   g. Polyclonal anti-tyrosine hydroxylase (1:1,000; Pel-Freeze Biologicals, Rogers, AR).
   h. Polyclonal antisera to neuropeptide Y (1:500), somatostatin (1:500), substance P (anti-Sub P; 1:100), and methionine-enkephalin (1:100), and glutamate (anti-Glu; 1:500) were from Chemicon International (Temacula, CA).
   i. Secondary antibodies raised in goat against mouse and rabbit immunoglobulins, conjugated to the fluorophore rhodamine isothiocyanate (1:200) or fluorescein isothiocyanate (1:100) (Jackson ImmunoResearch Laboratories In., West Grove, PA).
4. 4,6-Diamidine-2-phenylindole (DAPI) (Roche Molecular Biochemicals).
5. FluorSave reagent (Calbiochem, San Diego, CA).

3. Methods

3.1. Isolation of NSCs

The isolation of stem cells from the nervous system is described in detail elsewhere in this book, and it is not elaborated on here. However, it is worthy to mention that according to the media components and growth factors used, specific cell populations will be selected with well-defined characteristics acquired throughout serial passaging (*see* **Note 1**). We use chemically defined medium (*see* **Table 1**) containing a mitogenic growth factors to isolate a specific population of neural stem cells.

Table 1
Serum-free growth media components

Component	Stock solution	Final conc	Solvent
Glucose	30%	0.6%	Water
Sodium bicarbonate	7.5%	3 mM	Water
Glutamine	200 mM	2 mM	Water
HEPES	1 M	5 mM	Water
Insulin	250 µg/ml	25 µg/ml	0.1 N HCl
Putrescine	60 mM	60 µM	Water
Progesterone	0.2 µM	20 nM	95% Ethanol
Transferrin	1 mg/ml	100 µg/ml	DMEM/Ham's F-12
Selenium	0.3 µM	30 nM	Water
bFGF	10 µg/ml	10 ng/ml	PBS + 0.1 bovine serum albumin
EGF	20 µg/ml	20 ng/ml	Hormone mixture

In the stem cell suspension cultures, the cells are seeded at 100,000 cells/ml in DMEM/Ham's F-12 supplemented with hormone mixture (*see* **Table 1**), EGF at 20 ng/ml, bFGF at 10 ng/ml, or both. For monolayer cultures, cells are plated 25,000 cells/cm^2 on poly-L-ornitine substrate (PLO, 15 µg/ml; Sigma-Aldrich, cat no. P3635). At this early stage of stem cell line establishment, to ensure the continuity of cell supply, it is highly recommended to cryopreserve and characterize a reference stock of cells. A minimum of testing for viability, identity, and freedom from mycoplasma, bacteria, and fungi should be carried out, particularly on the master cell bank (*see* **Note 2**).

3.2. Differentiation of NSC Progeny

The differentiation of NSCs is induced by first removing the EGF/bFGF-containing medium and resuspending the cells in fresh control media. This step is repeated one more time to ensure the complete removal of the mitogenic factors from the media. The single-cell suspension is plated under control (media/hormone mix) or treated conditions at a density of 2.5×10^5 cells/cm^2 on PLO-coated glass coverslips in 24-well Nunclon culture dishes (Sigma-Aldrich). For consistent results, care should be taken when coating coverslips with adhesive substrate (*see* **Note 3**). The treatment used to induce differentiation and test the functionality of the cells varies depending on the desired outcome. For example, brain-derived neurotrophic factor is used to induce process outgrowth and ciliary neurotrophic factor is used to inhibit proliferation and induce astrocyte differentiation. We also cocultured NSCs with astroglia (*see* **Fig. 1**) or treated them with glial-derived conditioned medium (CM) to test

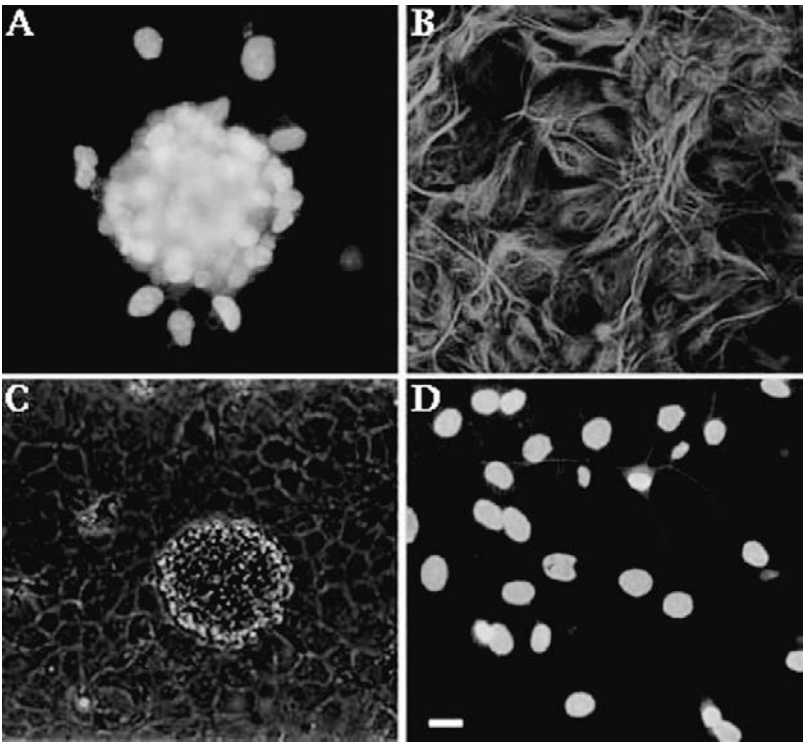

Fig. 1. Coculture of NSCs with astroglia. **(A)** The majority of NSC progeny incorporated BrdU after 24-h exposure. **(B)** An example of an astroglial monolayer immunostained with GFAP and ready for coculture. **(C)** Photo shows coculture of NSC progeny with the astroglial monolayer. **(D)** Photo shows stem cell progeny that are BrdU-immunoreactive, dispersed on the top of the astroglia, and expressing mature neurotransmitter phenotype (Sub P) after 7 DIV. Bar = 20 μm (**A**,**B**,**D**) and 30 μm (**C**).

for the induction of new neurotransmitter phenotype in the neuronal population. To ensure consistency and stability of the stem cell line, this procedure is systematically carried out at each passage, and the number of neurons, astrocytes, oligodendrocytes as well as the neuronal subpopulations expressing specific neurotransmitter phenotypes is measured.

3.2.1. Coculture of NSCs with Astrocytes

We have been using the direct interaction with astroglial microenvironment to test for the potential of NSCs to express new neurotransmitter phenotypes. The isolation of pure astrocytic cultures is carried out ***(5)***. Striatal tissue

derived from postnatal day 1 mice is used to generate astrocyte cultures. After mechanical dissociation, the cells are suspended in DMEM/Ham's F-12 media supplemented with 10% and plated at a concentration of 1.25×10^4 cells/cm^2 culture flask. Once a confluent monolayer is established, cells are enzymatically dissociated (trypsin-EDTA) and plated at 200,000 cells/cm^2 onto PLO-coated glass coverslips in 24-well culture dishes. After 3 to 4 days, cell confluence is established. NSCs are then resuspended in fresh medium and plated on the glial cell layer. To be able to distinguish NSC progeny from the astroglial cells, we labeled them with the DNA replication marker BrdU in the S phase (*see* **Note 4**). BrdU is added at a concentration of 1 μM to 6-day-old NSC culture for 24 h. Clonally derived NSCs (in the shape of sphere of cells) are then washed free from mitogens and BrdU before coculture with astrocytes.

After 7 days in vitro, cultures are processed for single or double immunocytochemistry (for BrdU and each of the neurotransmitter antibody listed in **Subheading 3.**) as follows:

1. All the coverslips are fixed with 4% paraformaldehyde (with 0.1% glutaraldehyde for anti-GABA or Glu) for 30 min.
2. Wash for 10 min, three times with phosphate-buffered saline (PBS).
3. Incubate in the primary antibodies in PBS/10% normal goat serum/0.3% Triton X-100 for 2 h at 37°C, except for anti-BrdU (*see* **Note 5**).
4. Wash for 10 min, three times with PBS.
5. Apply rhodamine- and/or fluorescein-conjugated secondary antibodies prepared in PBS for 30 min at room temperature.
6. Wash coverslips three times (10 min each) in PBS, and then rinse them with water and place them on glass slides.
7. Cover the slides with large coverslip by using FluoroSave as the mounting medium.
8. The number of neurons, astrocytes, and oligodendrocytes is then determined for each NSC clone under a Nikon optiphot photomicroscope (Nikon, Tokyo, Japan), and their NSC origin is confirmed by the nuclear immonostaining of BrdU. New neurotransmitter phenotype expression is systematically evaluated in all cultures and recorded for each cell passage.

3.2.2. Induction of TH Expression in NSCs

This bioassay was used to test the potential of a stem cell line to adopt a dopaminergic fate ***(6)***. Thus, this assay constitutes a first selective criterion for a cell line of potential therapeutic use for Parkinson's disease.

1. Dissociated NSCs are suspended in control medium and plated at a density of 2.5×10^5 cells/cm^2 on PLO-coated glass coverslips in 24-well Nunclon culture dishes with 0.5 ml/well.

2. After 2 h, cultures are treated with the appropriate differentiation inducing agents: bFGF, 20 ng/ml + 75% (v/v) of B49 glial conditioned medium (CM) (*see* **Note 6**).
3. Twenty-four hours later, cultures are fixed and investigated for induction of TH gene expression by using the immunocytochemical technique, described above.
4. Cultures are also left for 7 days in vitro (DIV), and one half of the culture medium is replaced after 3 and 5 DIV before processing for TH immunocytochemistry.
5. The total number of live cell nuclei stained with DAPI and the percentage of TH-immunoreactive neurons is determined for each culture at each passage.

4. Notes

1. The growth medium is critical in the development of a stable NSC line. Variations may exist from lot to lot. This may be due to something as simple as water purity. Thus, it is necessary to set up criteria for testing raw material and to qualify each of the media components (DMEM/Ham's F-12, hormone mixture, and growth factors) for their consistency in growth promotion.
2. Optimal growth medium is a perpetual endeavor. However, changes in cell line characteristics may be due to cross-contamination with other cell lines, to sterility, or, frequently, to mycoplasma contamination. Therefore, cell lines should be routinely karyotyped or subjected to isoenzyme analysis for species identity, and tested for mycoplasma. Kits are available, and several companies offer testing services for reasonable prices. The use of the DNA-specific bisbenzimide fluorochrome Hoechst 33258 (Sigma-Aldrich or MP Biomedicals, Irvine, CA) is the simplest and quickest way to test for mycoplasma contamination.
3. Plating and adherence properties of dissociated NSCs may be inconsistent on PLO-coated glass coverslip. This may be due to the coverslip quality, the coating process, bad batch of coating substrate, bad plates, mycoplasma infection, or many other reasons. Coverslips may float after adding PLO to the well. Pushing them with a pipette to the bottom of the well ensures that the entire surface of the coverslip is covered with PLO. We have experienced consistent results with Nalge Nunc glass coverslips (Nalge Nunc International, Rochester, NY, cat. no. 174950).
4. When using BrdU in the last 24 h of NSC culture, there may be a postmitotic subpopulation of NSC progeny that will not incorporate the DNA marker and thus be undetectable after coculture. To overcome this problem, BrdU may be added earlier to 2–4-day-old NSC cultures. Alternatively, NSCs may be derived from a different species, allowing for the use of species-specific markers or from transgenic mice such as the GFP and ROSA26 that ubiquitously express the markers green fluorescent protein and β-galactosidase, respectively (The Jackson Laboratory, Bar Harbor, ME, stock nos. 003291 and 002292).
5. BrdU immunocytochemistry works best when the antibody is incubated overnight with no acid treatment.
6. The preparation of CM from glial cell lines, such as the B49 glial cell line (***6***) is critical for the successful induction of TH gene expression in stem cell progeny.

The B49 Glial cell culture need to be >95% confluent and looking like a healthy monolayer of cells, with no aggregate before beginning CM preparation. We use CM at 75% (v/v) of the total volume of plating medium.

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In Vitro Migration Assays of Neural Stem Cells

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Summary

We describe three rapid procedures for the in vitro investigation of molecular factors influencing the migration of neural precursors derived from embryonic or postnatal neural stem cells. In the first assay, factors influencing chain migration from the anterior subventricular zone of perinatal mice can be analyzed after explantation and embedding in Matrigel, a three-dimensional substrate mimicking the in vivo extracellular matrix. The second assay enables to assess soluble factors influencing radial migration away from adherent neurospheres in which embryonic stem cells have been expanded. In this example, neurospheres have been derived from the striatum primordium of embryonic mice. Finally, the directed migration of these precursor cells can be analyzed using a chemotaxis chamber assay, in which the directional movement (chemotaxis) of cells across a membrane occurs in controlled conditions. These three assays are useful tools to evaluate the importance of surface molecules and environmental factors, such as the polysialylated form of neural cell adhesion molecule (NCAM) or chemokines such as CXCL12, in the directional migration of neural precursors.

Key Words: Neural stem cells; subventricular zone; chemotaxis; chemokines; CXCL12; PSA-NCAM.

1. Introduction

This chapter focuses on in vitro assays dedicated to the analysis of cell migration from embryonic or postnatal neural stem cells. Here, we describe three assays that reproduce, to some extent, in vivo migration, are rapid and

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easy to perform, and allow the investigator to test the effect of specific factors on the cell migration process. This area of work has considerable interest for a better understanding of neural development and regeneration as well as for the development of repair strategies of the injured or diseased nervous system.

The first model allows investigation of factors influencing cell migration from the anterior subventricular zone (SVZ), where a niche of neural stem cells persists throughout postnatal development until adulthood. Under physiological conditions, these SVZ stem cells essentially develop into neuronal progenitors that migrate along a well-defined pathway called the rostral migratory stream (RMS) to the olfactory bulb (OB) where they differentiate as interneurons ***(1)***. The mode of migration of these neuronal progenitors along the RMS is called "chain migration," because cells migrate closely associated to each other without the guidance of radial glia or axonal processes. Chain migration can be reproduced in vitro by culturing explants from the SVZ in a three-dimensional substrate of extracellular matrix components called Matrigel ***(2)***. Factors can be tested in this assay for their potential influence on migration speed, chain formation, or guidance. Using this assay, we have demonstrated the role of the polysialylated form (PSA) of neural cell adhesion molecule (NCAM), a membrane-bound cell adhesion molecule, in this chain migration process ***(3)***. Indeed, genetic or enzymatic removal of PSA-NCAM has shown the importance of PSA moiety in this process: our analysis of chain migration from NCAM knockout mice and after endoneuraminidase N (Endo-N) treatment demonstrated the function of PSA-NCAM in precursor migration ***(3)***.

The two other assays described here allow investigation of cell migration of precursors derived from embryonic striatal neural stem cells. In contrast to the well-defined mode of migration and migratory route of SVZ-derived precursors, striatal precursor migration is more complex. Some precursors migrate radially, whereas others migrate tangentially and later differentiate into cortical interneurons ***(4,5)***. It is possible to analyze such precursor migration in two in vitro assays. The first assay reproduces a radial mode of cell migration: embryonic striatal precursors are expanded in vitro within neurospheres, and, upon adhesion to a substrate, they extensively migrate radially away from the neurospheres forming a "spokes of a wheel" pattern. The speed of this radial migration can be modified by addition of exogenous factors to the culture medium. This assay allows a combined analysis of neural precursor cell migration and differentiation, because cells start to differentiate while migrating ***(6)***.

In the second assay, the attraction of these precursor cells can be investigated by gradients of soluble factors such as chemokines implicated in the development of the central and peripheral nervous systems ***(6–11)***. One key factor

is the α chemokine CXCL12 (also known as stromal-derived factor 1), which binds to the G protein-coupled receptor CXCR4. A chemotaxis assay based on the classical Boyden chamber principle demonstrated that a CXCL12 gradient chemoattracts cortical and striatal precursors in a concentration-dependent manner ***(6–8)***. By placing a soluble factor in the lower well below the filter and cells to be analyzed for migration in the upper well, a chemical gradient forms that may attract cells to the lower well. Thus, chemotaxis chamber assays are important tools to quantitate the dose-dependent attraction to chemotactic factors and their ability to "retain" cells at high concentration.

Other in vitro assays are used to analyze neural precursor migration, which are not detailed under **Subheading 3.** For example, it is possible to monitor the migratory behavior of neural precursors tagged with a retroviral vector encoding green fluorescent protein within brain slices maintained in culture by time-lapse or sequential fluorescence microscopy analysis ***(12,13)***. Also, the migration of precursors with stem cell-like properties was observed from postnatal SVZ fragments embedded in a collagen matrix toward a gradient of secreted molecules ***(14)***. By using several embryonic slice culture manipulations, Borrel and Marin ***(15)*** demonstrated that the CXCL12-containing meninges are an essential anchorage substrate for the tangential migration of cortical hem-derived Cajal-Retzius cells via /CXCR4 signaling. The chemoattractive effect of meningeal-derived CXCL12 on the migration of these precursors in vitro seems motogenic and results in their proper positioning along the marginal zone in vivo. In the absence of CXCL12/CXCR4 signaling, many hem-derived Cajal-Retzius cells migrate into deep layers of the developing cortex, suggesting that other factors stimulate the initial movement of these cells.

Thus, it is important to keep in mind the complexity of the in vivo environment dictating migration of neural precursors from neural stem cell niches compared with the reductionist approach of in vitro assays, which address the role of one or two factors. In vivo, cell migration is likely to be dictated by a combination of short- as well as long-range guidance molecules, and cells may behave differently whether positioned in their normal developmental context, under pathological conditions, or after transplantation. For example, we observed that overexpression of PSA by genetic engineering in neurosphere-expanded striatal precursors led to a very specific directional migration pattern of these cells after in vivo transplantation in a heterotypic heterochronic environment, whereas no specific effect could be measured in the neurosphere radial migration assay ***(16)***. This highlights the necessity to combine several approaches to dissect out the mechanism of action and hierarchy of molecular factors driving neural precursor cell migration.

2. Materials

2.1. Cell Migration Assay from Postnatal SVZ Stem Cells

2.1.1. Animals and Dissection

1. 3- to 10-day-old CD-1 mice (Janvier, Le genest St Isle, France).
2. Small scissors, small forceps (Moria, Antony, France) and ophthalmic blade for SVZ dissection.
3. 1× phosphate-buffered saline (PBS) sterile (Invitrogen, Cergy-Pontoise, France) and Hanks' balanced salt solution (HBSS) (Invitrogen).
4. Dulbecco's modified Eagle's medium (DMEM; Invitrogen) with 10% fetal calf serum (FCS; Invitrogen)
5. Prepare a solution of 2% (w/v) agarose in water, dissolve by heating it 2–3 min in a microwave and pour it in small petri dishes. Keep it at 4°C until use.

2.1.2. Media, Reagents, and Devices

1. Serum-free medium is prepared as follows: mix DMEM and Ham's F-12 (Invitrogen) in a 3:1 ratio. DMEM/Ham's F-12 is supplemented with 50 µg/ml human transferrin (Sigma, Paris, France), 5 µg/ml insulin (Sigma), 100 µM putrescin (Sigma), 20 nM progesterone (Sigma), 30 nM selenium (Sigma), and antibiotics. Antibiotics used are penicillin-streptomycin (10,000 U/ml; Invitrogen) kept at 4°C; this medium is stable 15 days in the refrigerator. Before use, the medium is complemented with B-27 (Invitrogen) 1:50 as recommended by the manufacturer.
2. Four-well plates are used in this experiment. They are easier to handle.
3. Matrigel (Clontech, Mountain View, CA).

2.2. Cell Migration Assays from Embryonic Striatal Stem Cells

2.2.1. Animals and Dissection

1. Pregnant C57/BL6J mice (Janvier).
2. Small iridectomy scissors and small forceps for dissection (Bioseb, Chaville, France).

2.2.2. Radial Migration Assay from Neurospheres

1. Epidermal growth factor (EGF; Invitrogen) is dissolved at 100 µg/ml in DMEM/Ham's F-12 (use the GlutaMAX-supplemented version of ready-made DMEM/Ham's F-12; Invitrogen) and stored in single use aliquots at –80°C.
2. Fibroblast growth factor (FGF)2 (Valbiotech, AbCys, Paris, France) is dissolved at 100 µg/ml in DMEM/Ham's F-12 and stored in single use aliquots at –80°C. Working solutions are prepared by 2-fold dilutions in DMEM/Ham's F-12 before use.
3. Insulin (Sigma) is dissolved at 2.5 mg/ml in DMEM/Ham's F-12 and stored in single use aliquots at –80°C.
4. B27 medium consists of 48 ml of DMEM/Ham's F-12 (Invitrogen) supplemented with 50 µl of gentamicin (Invitrogen), 500 µl of B27 supplement (Invitrogen), 400 µl of insulin stock solution, and 715 µl of glucose (200 g/l; Invitrogen).

5. Neurosphere medium: 6 ml of B27 medium, 1.2 µl of EGF stock solution (20 ng/ml final), and 1.2 µl of FGF2 working solution (10 ng/ml final).
6. Human CXCL12 α and its truncated form CXCL12 (4-67), which lacks three amino acids critical for binding to CXCR4 on lymphocytes ***(17)***, were synthesized by Dr. F. Baleux (Pasteur Institute, Paris, France), and they are dissolved at 1 mg/ml in DMEM/Ham's F-12 and stored in single-use aliquots at –80°C. These products are also available from Almacgroup (Craigavon, UK).
7. Four- or 12-well plates are coated with poly-L-ornithine, by adding 100 µg/ml in 400 µl of distilled water/well and incubating the whole at least 1 h at room temperature under a hood. The wells are then twofold rinsed with sterile distilled water before use.

2.2.3. Chemotaxis Assay

1. Poly-L-lysine (mol. wt. 300,000; Sigma) is dissolved at 20 mg/ml in sterile water and then stored in single-use aliquots at –20°C.
2. *Bordetella Pertussis* toxin V (PTX; Sigma) is dissolved at 100 µg/ml in sterile water and stored in single-use aliquots at –80°C.
3. PD-98059 (Cayman Chemical Company, Tallin, Estonia) is dissolved at 1 mM in dimethyl sulfoxide and then stored in single-use aliquots at –20°C.
4. The neutralizing anti-CXCL12 K15C antibody was generated by Dr. A. Amara (Pasteur Institute) and is available at the Pasteur Institute.
5. Formol is composed of 4% formaldehyde in distilled water.
6. Cresyl violet is dissolved at 20 g in 200 ml of ethanol, 100 ml of formol, and 700 ml of distilled water. Working solution (0.1% cresyl violet final) is made of 1 vol of stock solution, 1 vol of formol, and 8 vol of distilled water.
7. PVP-free polycarbonate filters containing 8-µm pores (NeuroProbe, Gaithersburg, MD) are coated with poly-L-lysine at 50 µl/ml on both sides. Briefly, 125 µl of poly-L-lysine stock solution is diluted in 50 ml of sterile water and filtered at 45 µm. Polycarbonate filters are immersed, using grips, in 10-mm dishes containing this poly-L-lysine working solution during 18 h at room temperature, and then they were twofold rinsed in sterile water before use.
8. The 48-well microchemotaxis chamber (NeuroProbe) is incubated in 100% ethanol for one night and then twofold rinsed in sterile water. The chamber should be dry overturned on absorbing paper and can be re-used.

3. Methods

3.1. Chain Migration Assay from Postnatal SVZ Stem Cells (see Fig. 1)

Cultures of SVZ explants are performed as described in Wichterle et al. ***(2)***.

1. Three- to 10-day-old CD-1 mice are anesthetized by hypothermia and then euthanized by rapid decapitation. Brains are dissected out and placed in ice-cold HBSS medium (Invitrogen) (*see* **Notes 1–3**).

2. Cerebellum is cut and removed (*see* **Fig. 1A**, dotted line), and the brain is orientated and fixed vertically on the vibratome platform. The brain is fixed using glue (cyanolit glue), and a small piece of 2% agarose is placed behind the brain to facilitate sectioning and avoid brain deformation during sectioning. The glue is placed first on the vibratome platform; the piece of agarose is fixed first and the brain at the end. The brain is orientated with the OB up, the cortex facing the vibratome blade, and the base of the brain against the agarose piece. Let the setting dry for 1–2 min at room temperature.
3. Using a vibratome, 400-µm frontal sections are obtained. Sectioning is performed at 4°C in sterile PBS. The three first sections (sections 1, 2, and 3) include usually only the RMS. Then, the three subsequent sections (sections 4, 5, and 6), in which the lateral ventricles are clearly visible, are the sections where the SVZ will be dissected (*see* **Fig. 1A**). Slices are kept on ice in DMEM/10% fetal calf serum (FCS).
4. SVZ from the lateral wall of the anterior horn of lateral ventricle is microsurgically removed from sections (*see* **Fig. 1A**) by using an ophthalmologic blade (Alcon) under a dissecting microscope, in a small petri dish containing DMEM/10% FCS. The SVZ is then cut into pieces 50–300 µm in diameter with a fine scalpel (four

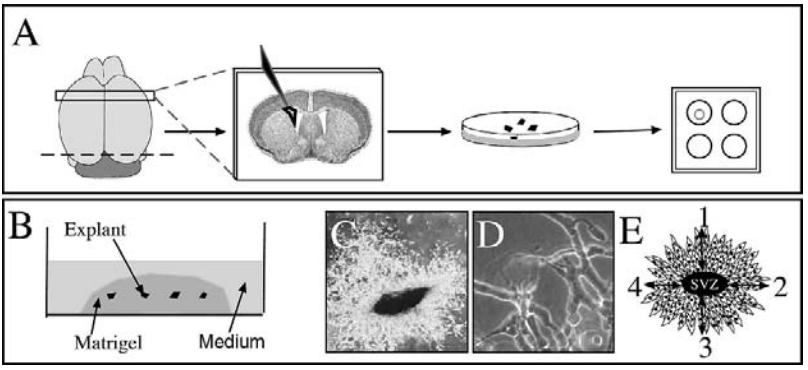

Fig. 1. Chain migration assay. **(A)** Brains are taken out, and vibratome sectioning is performed. Frontal sections containing the SVZ are placed in a petri dish; SVZ fragments are then dissected out, cut into small pieces, and placed in Matrigel **(B)** in a four-well dish. Four explants are arranged in the Matrigel, and medium is added to cover the three-dimensional gel. **(C,D)** Forty-eight hours after culture, the neuronal precursors migrate radially out of the explants **(C)** in a chain-like organization **(D)**, forming a complex three-dimensional network. E. Migration quantification can be obtained by a direct measurement of cell migration around the explant. From the border of the explant to the migration front, four measurements are performed (*1–4*), and the average of those distances allowed to calculate the mean migration distance per explant.

to six pieces from one single SVZ). The explant-containing petri dish is kept on ice before embedding in Matrigel.

5. The explants are mixed with Matrigel in a 1:3 ratio and cultured in four-well dishes as follows: three to four explants are placed in serum-free culture medium (20 µl) on the center of each well of a four-well dish on ice. Matrigel (60 µl) is mixed gently with the medium containing the explants using the tip of the yellow tip. Pipetting is not recommended at this stage to avoid bubble formation (*see* **Notes 4–6**).
6. Use the tip of a needle to align correctly the explants in the drop of Matrigel. The explants should not be in contact. Keep the dish on ice until the four wells are ready.
7. Remove the dish from ice and allow the Matrigel to polymerize at room temperature for 10 min. After polymerization, the gel is overlaid with 0.5 ml of serum-free medium containing B27 supplement (Invitrogen). At this stage, Endo-N (1:1000; AbCyss) can be added in the culture medium (*see* **Fig. 1B**).
8. Cultures are maintained in a humidified, 5% CO_2, 37°C incubator.
9. Cultures are viewed under an inverted microscope at low magnification to observe the overall radial migration around explants (*see* **Fig. 1C**) and at high magnification to appreciate the structure of the chains (*see* **Fig. 1D**). Images from each explant are collected using a video camera and analyzed using appropriate software (Biocom, MetaMorph, Molecular Devices, Sunnyvale, CA). Migration distance measurements are calculated per explant as follows: four measurements (*see* **Fig. 1E,1–4**) are done per explant at 24 and 48 h. The migration distance per explant is obtained by calculating the average of the four values obtained (*see* **Notes 6** and **7**)

3.2. Migration Assays for Embryonic Striatal Stem Cells

3.2.1. Dissection and Preparation of Striatal Stem Cells

Pregnant C57/Bl6 mice are euthanized by cervical dislocation following the Pasteur Institute's animal experimentation regulations. Striata are dissected from embryonic day (E)14 embryos in ice-cold HBSS and mechanically dissociated. This cell suspension is then either processed for neurosphere formation and analysis of radial migration or directly used for the chemotaxis assay.

3.2.2. Assay of Radial Migration Away from Adherent Neurospheres

A. Preparation of Neurospheres.
After mechanical dissociation from E14 striata in DMEM/Ham's F-12, cells are seeded at a density of 85,000 cells/ml in 25-cm^2 flasks containing B27 medium, in the presence of 20 ng/ml EGF and 10 ng/ml FGF2 as mitogens (adapted from Reynolds and Weiss ***(18)***). After 6 days in culture, differentiated neurospheres are generated by adhesion on poly-L-ornithine-coated (100 µg/ml) four-well plates in

the same medium with N2 complement (Invitrogen) in place of B27 and without the two mitogens (N2 medium).

B. Analysis of Radial Migration
   1. Take with a siliconed cone individual neurospheres of approximate same size (80–100 µm in diameter) in 5 µl of culture medium and gently plate them on the center of each well, at the density of one per well. After 15 min, neurospheres adhere to wells, and then complete each well with 500 µl of N2 medium with or without increasing CXCL12 concentrations for the dose response and at 200 nM for other assays. Incubate the plates at 37°C in a 10% CO_2-humidified incubator (*see* **Notes 8–11**).
   2. Capture images at 0, 21, 48, and 96 h with a Zeiss AxioVision camera (Carl Zeiss, Jena, Germany) on the 135 Zeiss inverted microscope (no. 135).
   3. Measure by the MetaMorph software (Molecular Devices) the mean maximal distance covered by migrating cells, expressed as mean "equivalent outer radius" of the neurosphere outgrowth. Three independent experiments are performed where each data point is a pool of 12 to 14 neurospheres and expressed as mean ± SEM with statistical significance measured by the Student's *t* test, using Prism software (GraphPad Software Inc., San Diego, CA).

3.2.3. Chemotaxis Assay (see Fig. 2)

1. Put 29 µl of medium (DMEM, 4.5 g/l glucose, and 1% gentamicin) with CXCL12 α or the truncated and nonchemotactic form of CXCL12, CXCL12/4-67 (2-800 nM) in the lower compartment of the microchemotaxis chamber (*see* **Notes 12–15** and **Fig. 2A**).
2. Cover the lower compartment with the polycarbonate membrane, by using grips. Gently eliminate the bubbles. Then, assemble the chemotaxis chamber.
3. Add 50 µl of 10^6 cells/ml suspension in the upper compartment of each well (*See* **Fig. 2A**). For inhibition studies, the CXCL12 neutralizing antibody K15C (20 µg/ml); PTX, which uncouples Gi proteins; or an inhibitor of the mitogen-activated protein kinase pathway were used at 20 µg/ml, 0.01 µg/ml and 50 µM, respectively.
4. After 4- to 18-h incubation at 37°C in a 10% CO_2-humidified incubator, disassemble the chamber (*see* **Fig. 2B**). Return the chamber and remove the filter. Wash in PBS and scrape off the cells attached to the top surface of the filter, which have failed to migrate through the pore, after the NeuroProbe protocol. This procedure should be done speedily to not let drying and fixing cells to the upper surface of the filter (*see* **Fig. 2C**).
5. Fix the cells by immersing the filter for 30 min at room temperature in PBS containing 4% paraformaldehyde and 0.1% glutaraldehyde (Sigma), in a chemistry hood (*See* **Fig. 2D**).
6. Stain cells by incubating the filter in 0.1% cresyl violet for 30 min. Then, rinse the filter twofold in PBS for 5 min.
7. Put the filter onto a slide, with the side containing the cells in the top position.

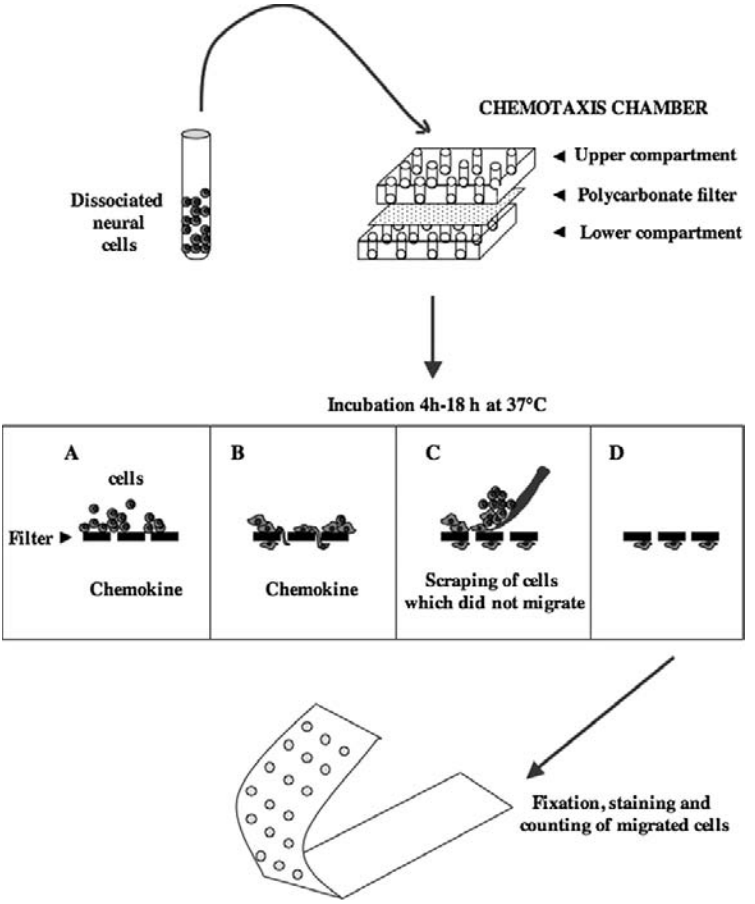

Fig. 2. Chemotaxis assay. Dissociated neural cells are placed in the wells of the upper compartment of the microchemotaxis chamber **(A)**. The lower compartment contains medium without or with chemoattractants, inhibitors, or both. A polycarbonate filter with 8-μm pores separates the upper and lower wells. Four- to 18-h incubation at 37°C in a 10% CO_2-humidified incubator allows directed migration of the cells from the upper wells to the lower wells, toward a chemokine gradient **(B)**. After dismantling the chamber, cells on the top side of the filter, which did not migrate through the pores, are wiped off **(C)**. Cells situated on the lower side of the filter **(D)** are fixed, stained, and counted (see text for details).

8. Count the cells that have migrated through the pore to the lower chamber and adhered to the lower surface of the filter: select five microscopic fields per quadruplicate well, at ×400 magnification, and acquire TIFF images with a CDD video camera (Kodak KAF-1400, Princeton Scientific Instruments, Monmouth

Junction, NJ) mounted on a photomicroscope (Lecia, Wetzlar, Germany), by using NIH Image software (National Institutes of Health, Bethesda, MD).

9. Express the results as mean number of cells migrating per four wells in five microscopic fields ± SE. Calculate the significance by Student's *t* test, by using Prism software (GraphPad Software Inc.).

4. Notes

1. The window for the correct age of mice to be dissected is between 3 and 10 days postnatal. Chain migration is still occurring in vivo after this stage, but we noticed that survival and migration was really poor when tissues were obtained from older mice.
2. This protocol can be done with other mouse strains or adapted for rats.
3. It is better to dissect the animals one by one. Time is important, and the whole procedure should not exceed 2–3 h from the time the animals are sacrificed to the moment explants are placed in the incubator.
4. Under control conditions, the cells start to form chains and to migrate out of the explant 24 h after the beginning of the culture. Migration distance is maximum at the end of the second day. After 48 h, the cells start to differentiate and chains start to collapse.
5. All tips used for the explant manipulation are coated with silicone (Sigmacote, Sigma, cat. no. Sl-2). The dissection tools also can be coated with silicone if necessary. To coat tips or forceps, dip or fill articles to be coated into Sigmacoat, drain off excess liquid and air dry. Dry surfaces are essentially neutral.
6. Fixation with paraformaldehyde and immunolabeling of the cultures are possible but be aware that chains are nicer before fixation, and they should be photographed before fixation. Instead of plating the Matrigel-embedded explants directly onto the plastic surface of a four-well dish, these explants can be plated directly onto coverslips, immunolabeled, fixed, mounted onto glass slides, and observed under a straight microscope. Be aware that Matrigel can give high background and prevent antibodies penetration and fixation.
7. Factors can be tested in this assay for their potential implication on cell migration, detachment, or guidance. To do so, appropriate reagents can be added in the culture medium at the time of explantation. For example, the effect of PSA-NCAM can be tested by addition in the medium of Endo-N enzyme ***(3)***. Reelin detachment/dispersal effect has been shown in vitro by the addition of medium conditioned by control COS cells or COS cells expressing secreted Reelin. In both conditions radial migration is observed and the total migration distance is not affected, but the cells are not forming the typical chain migration structure observed in control ***(19)***. Another possibility is to use a source of producing cells to test the attractive or repulsive activity of a given factor on chain migration. Wong and colleagues ***(20)*** described in such an assay the repulsive activity of Slit protein on neuronal chain migration.

4.1. Concerning Radial Migration Assay Out of Neurospheres

1. Cell migration out of the neurosphere is a very rapid process that can be observed already within the first hour of culture.
2. The use of a plastic surface rather than a glass surface is critical.
3. The size of the neurospheres used in this assay should be kept as constant as possible from one well to another (around 100 μm in diameter). To this aim, the use of a graduated device within the ocular is very helpful.
4. A similar assay has been described for precursors at a later developmental stage: oligodendrocyte progenitors migrating out of oligospheres ***(21)***.

4.2. Concerning the Microchamber Chemotaxis Assay

1. The experiment should include negative controls in the chemotaxis chamber: some of the upper wells containing the cell suspension to be tested should have lower wells containing only cell suspension media without any chemoattractant.
2. The experiment should also include chemokinetic controls: sets of wells in the chemotaxis chamber should contain the chemotactic factor at the same concentration in both the upper and lower wells. In this case, no directional gradient of chemoattractant is present; yet, the cells may show increased motility, a process called chemokinesis, and result in about equal amounts of migrating cells on each side of the filter.
3. To remove any bubbles in the upper wells, suck the well completely dry with a pipette tip, and then refill it.
4. The chemotaxis chamber should be cleaned in distilled water after each use without delay, to prevent proteins from drying on the instrument. The chamber can be reused immediately.

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Measuring Apoptosis in Neural Stem Cells

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Summary

In trauma to, and diseases of, the central nervous system (CNS), apoptotic events are frequently observed in and around areas of damage. Human embryonic stem cells (hESCs) and their progeny have been suggested as possible therapeutic agents in the treatment of CNS diseases. The success of stem cell transplantation not only depends on the capacity of these cells to retain their functionality after transplant into the CNS but also on their ability to resist the *in situ* environmental cues that may lead to apoptosis. Although there are many methods used to detect apoptosis, the assessment of apoptosis in adherent cultures of primary stem cells and their progeny is more limited. We describe a series of protocols we have used to assess apoptosis in these cells.

Key Words: Apoptosis; embryonic stem cells; nuclear morphology; TUNEL staining; caspase Western blot.

1. Introduction

In trauma to, and diseases of, the central nervous system (CNS), there are often marked apoptotic events occurring in and around areas of damage ***(1–6)***. Indeed, in multiple sclerosis (MS), an autoinflammatory, demyelinating disease, active lesions not only show extensive oligodendroglial hyperplasia and hypertrophic astrocytes but also, in the early stages of the disease, significant oligodendrocyte apoptosis ***(7,8)***. Oligodendrocyte precursor cells (OPCs), which have been observed in and around MS lesions, are unable to completely repair

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the accruing damage ***(9–13)***. The lack of repair by OPC has been suggested by many researchers to be due to microenvironmental effects on their viability and functionality ***(14–21)***.

Human embryonic stem cells (hESCs) and their progeny, neural stem cells (NSCs) and OPCs, have been suggested as possible therapeutic agents in the treatment of CNS diseases such as MS, and they have shown promise in models of demyelination ***(22–26)***. The success of stem cell transplantation into the human CNS depends on the capacity of these cells to retain their functionality in this hostile microenvironment; this includes resisting those molecular signals that may lead to apoptosis. Our laboratories, and those of many other investigators, are examining the effects of various CNS-produced factors on apoptosis induction in hESCs, NSCs, and OPCs, and also are developing ways to circumvent susceptibility to these ligands. Thus, high-quality, reproducible methods to assess the induction of apoptosis in hESCs and their derivatives are critical to the future direction of this field.

Cell death can either be the consequence of a passive, degenerative process termed necrosis, or the consequence of an actively driven cell process termed apoptosis ***(27–32)***. These two distinct modes of cell death can be distinguished based on differences in morphological, biochemical, and molecular changes. Cells undergoing apoptosis display typical features, including cell shrinkage, membrane blebbing, changes in membrane lipid configurations, organelle contraction, extensive damage to chromatin, and DNA fragmentation (***30,33–38***). Nuclear fragmentation is associated with DNA-cleavage into oligonucleosomal length DNA fragments after activation of calcium-dependent endogenous endonuclease ***(34,39)***. Although there are many different methods used to detect apoptosis, nuclear fragmentation and chromatin condensation are considered to be the most definitive markers of apoptotic cells ***(28–30,34,39,40)***. Thus, it has become well established that definitive determination of apoptosis should always include analysis of nuclear morphology along with at least one other assay ***(41,42)***.

Assessment of apoptosis in adherent cell cultures, such as hESCs and their derivatives, limits the use of some techniques, such as flow cytometry. We describe a series of techniques that enable you to successfully determine the induction of apoptosis in adherent cultures and to determine whether the treatment of choice induces DNA fragmentation, changes in nuclear morphology, and activation of distinct apoptotic pathways, thereby allowing for the categorical assertion that the factor tested induces apoptosis in the tested cell population. Simultaneous analyses of cell surface phenotype allow for the analysis of differential effects on mixed populations of hESC and progeny, and analyses of lysates of treated cells enable confirmation of a caspase-dependent or caspase-independent apoptotic pathway.

2. Materials

2.1. Cell Culture and Miscellaneous

1. 75-cm^2 flasks or eight-well chamber slides for appropriate growth of cells of interest (BD Biosciences, Franklin Lakes, NJ) (*see* **Note 1**).
2. Appropriate culture medium for starting cell population of interest.
3. Factors to be tested for inducing apoptosis.
4. Staurosporine (*Streptomyces staurospores*) stock solution (Sigma-Aldrich, Saint Louis, MO): 1 mM in 100% dimethyl sulfoxide (Sigma-Aldrich), stored in aliquots at –20°C. Use at a final concentration of 500 nM diluted in cell growth medium (*see* **Note 2**).
5. Wash buffer and diluent: phosphate-buffered saline (PBS), pH 7.4.
6. Fixation solution: freshly prepared 4% paraformaldehyde in PBS, pH 7.4 (*see* **Note 3**).

2.2. DNA Ladder Detection

1. Trypsin (0.05%)-EDTA (0.02%) in PBS, pH 7.4 (Invitrogen, Carlsbad, CA).
2. Neutralizing buffer: 10% fetal calf serum (Omega Scientific, Tarzana, CA) in PBS, pH 7.4.
3. Apoptotic DNA Ladder Extraction kit (BioVision, Mountain View, CA). This kit contains DNA Ladder Extraction buffer, enzyme A solution, enzyme B (reconstituted in 275 µl of distilled, deionized water), ammonium acetate solution, and DNA suspension buffer (*see* **Note 4**).
4. Isopropanol.
5. Ethanol.
6. Tris borate-EDTA (TBE) electrophoresis buffer: 45 mM Tris borate and 1 mM EDTA, pH 8.0.
7. Agarose (Roche Applied Science, Indianapolis, IN).
8. Ethidium bromide (Sigma-Aldrich); use at a final concentration of 0.5 µg/ml.
9. Agarose gel electrophoresis apparatus and power supply (VWR, Scientific Products, West Chester, PA).
10. Ultraviolet light illuminator and photodocumentation system (VWR).

2.3. Terminal Deoxynucleotidyl Transferase dUTP Nick-End Labeling (TUNEL) Staining

1. Permeabilizing solution: freshly prepared 0.1% Triton X-100 in 0.1% sodium citrate.
2. Antibody dilution buffer: PBS, pH 7.4, containing 0.1% Tween 20.
3. Monoclonal or polyclonal antibody specific for antigen of interest.
4. Fluorochrome-conjugated secondary antibody specific for the species and isotype of antibody used in step 3 (*see* **Note 5**).
5. Recombinant DNase I (Sigma-Aldrich): 500 U/ml in 50 mM Tris-HCl, pH 7.5, and 1 mg/ml bovine serum albumin.

6. *In Situ* Cell Death Detection kit, Fluorescein (Roche Applied Science). This kit contains enzyme solution (10× recombinant terminal deoxynucleotide transferase) and label solution (nucleotide mixture) (*see* **Note 6**).
7. VECTASHIELD hard-set mounting medium with 4,6-diamidino-2-phenylindole (DAPI) (Vector Laboratories, Burlingame, CA).

2.4. Hoechst Staining

1. Hoechst 33342 (Sigma-Aldrich) stock solution: 3 mg/ml in H_2O; store at 4°C in the dark. Use at a final concentration of 50 μg/ml.
2. Propidium iodide (Invitrogen) stock solution: 1 mg/ml; store at 4°C in the dark. Use at a final concentration of 20 μg/ml.
3. VECTASHIELD hard-set mounting medium (Vector Laboratories).

2.5. Caspase-Specific Western Blot

1. Ice-cold lysis buffer: 150 mM NaCl, 50 mM Tris, pH 8.0, 2% Triton X-100, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate (SDS), 1 mM EDTA, 1 mM EGTA, 2 mM Na_3VO_4, 1 mg/ml leupeptin (added immediately before use), 20 mg/ml aprotinin (added immediately before use), 0.2 mM phenylmethylsulfonyl fluoride (added immediately before use) (*see* **Note 7**).
2. Total Protein Assay kit (Sigma-Aldrich).
3. 5× loading sample buffer: 50% glycerol, 312.5mM Tris-HCl, pH 6.8, 10% SDS, and 0.05% bromphenol blue.
4. β-mercaptoethanol (Sigma-Aldrich).
5. Heating block (VWR).
6. Precast SDS-polyacrylamide gels (Cambrex Bio Science Rockland, Inc., Rockland, ME).
7. Tris-glycine Laemmli running buffer (Bio-Rad, Hercules, CA).
8. Prestained protein markers of known molecular weights (Invitrogen).
9. SDS-polyacrylamide gel electrophoresis apparatus and power supply (Pharmacia, San Diego, CA).
10. Transfer buffer: 10 mM Tris base, 100 mM glycine, and 20% methanol.
11. Western blotting apparatus (TE 22 Mini Tank Transfer Unit, GE Healthcare Little Chalfont, Buckinghamshire, UK).
12. Nitrocellulose membrane (VWR).
13. Shaker, rocker, or other agitating device (VWR).
14. Western blot washing buffer (Tris-buffered saline/Tween 20 [TBST]): 20 mM Tris-HCl, 150 mM NaCl, and 0.1% Tween 20, pH 7.4.
15. Ponceau Red staining solution (Sigma-Aldrich): 2% Ponceau Red in 30% trichloroacetic acid and 30% sulfosalicylic acid, diluted 1:10 in TBST.
16. Blocking buffer: low-fat milk 5% in TBST, pH 7.4.
17. Monoclonal antibody or polyclonal antiserum specific for the caspase of interest and monoclonal antibody or polyclonal antiserum specific for the loading control (β-actin or glyceraldehyde-3-phosphate dehydrogenase [GAPDH]) (*see* **Note 8**).

18. Alexa Fluor 680-conjugated secondary antibody specific for the species and isotype of the antibodies in **item 17** (Invitrogen) (*see* **Note 8**).
19. Infrared imager (Li-Cor Biosciences, Lincoln, NE).

3. Methods

3.1. DNA Ladder Detection

The presence of a DNA ladder is a widely used measure to confirm the fragmentation of DNA in apoptotic cells ***(39,43,44)***, although it is very important to confirm this with morphological analyses. This very simple procedure involves the isolation of DNA from apoptotic cells, and the subsequent separation by agarose gel electrophoresis, yielding a pattern of oligonucleosomal DNA fragments with steps of approximately 180 base pairs.

1. Grow cells to appropriate confluence in 75-cm^2 flasks, and then reseed flasks with predetermined optimal cell numbers before induction of apoptosis (*see* **Note 1**).
2. Induce apoptosis by the desired method for the required length of time, simultaneously set up positive control (500 nM staurosporine) and negative control (no induction) flasks.
3. Wash each flask three times with wash buffer, and then detach adherent cells by incubating in trypsin-EDTA for 5 min at 37°C (*see* **Note 9**).
4. Neutralize trypsin with neutralizing buffer, pellet cells from each sample by centrifugation for 5 min at 500 × *g*, and then carefully remove all supernatant.
5. Extract each cell pellet with 50 µl of DNA Ladder Extraction buffer (provided with kit) by gentle pipetting for 10 s at room temperature.
6. Centrifuge samples at 1,600 × *g* for 5 min, transfer supernatants to fresh tubes, and then re-extract pellets for a second time with a further 50 µl of DNA Ladder Extraction buffer.
7. Combine both supernatants from each sample, add 5 µl of enzyme A solution (provided with kit), mix thoroughly, and then incubate at 37°C for 10 min.
8. Add 5 µl of enzyme solution B (provided with kit) to each sample and incubate for at least 30 min at 50°C.
9. Add 5 µl of ammonium acetate solution (provided with kit) to each sample and mix well.
10. Add 100 µl of isopropanol, mix sample well, incubate at –20°C for 10 min, and then precipitate the DNA by centrifugation for 10 min at 16,000 × *g*.
11. Remove supernatant, wash the pellet with 70% ethanol, centrifuge again, and air dry the DNA pellet for 10 min at room temperature before resuspending in 20 µl of DNA Suspension buffer (provided with kit).
12. Prepare a 1.2% agarose gel in TBE buffer containing 0.5 µg/ml ethidium bromide and sufficient running buffer also containing 0.5 µg/ml ethidium bromide.
13. Load 20 µl of each DNA sample onto gel and run at 5 V/cm until dye front reaches the edge of the gel.

14. Visualize ethidium bromide-stained DNA with an ultraviolet light illuminator and photograph.

3.2. TUNEL Staining

Although methodologically straightforward and reliable, the detection of a DNA ladder does not provide information on apoptosis at the individual cell level, which is particularly important when assessing apoptotic events in mixed cell cultures such as hESC-derived NSCs and progeny. To this end, *in situ* labeling of apoptosis by using TUNEL is a highly sensitive, very rapid procedure that allows for evaluation of early phases of apoptosis in individual cells ***(40,45–47)***. This technique, using fluorescence light microscopy, allows for the simultaneous enumeration of apoptotic cells and the determination of cell surface phenotype, thereby enabling the analyses of differential effects of apoptotic stimuli on mixed populations of cells.

1. Grow cells to appropriate confluence in eight-well chamber slides, and then induce apoptosis by the desired method (*see* **Note 1**). Retain five noninduced sample wells to be used as negative and TUNEL-positive controls, and a apoptosis positive control well by incubation with 500 nM staurosporine. Label the wells as follows: (a) cells only (no treatment/no stain); (b) cells only plus label without enzyme; (c) negative control, untreated cells with label and enzyme; (d) treatment positive control, staurosporine-treated cells; (e) TUNEL-positive control, DNase I-treated cells; and (f) test treatments.
2. Wash chambers three times with wash buffer (*see* **Note 9**).
3. Fix cells with 200 µl of freshly prepared fixation solution for 1 h at room temperature in a humidified chamber (15–25°C), and then rinse wells three times with wash buffer (*see* **Note 10**).
4. Fixed cells can now be used to assess cell surface phenotype; if not determining cell surface phenotype, go to **step 8**.
5. Block fixed cells in 4% serum from the species in which secondary antiserum was developed. Dilute serum in PBS, pH 7.4, with 0.1% Tween 20, add 200 µl to each chamber, and incubate at room temperature (15–25°C) for 30 min.
6. Rinse each chamber with wash buffer then incubate samples in 200 µl of an optimal concentration of the desired antigen-specific primary antibody for 1 h at room temperature (15–25°C) or overnight at 4°C.
7. Wash chambers thoroughly (six to eight changes), aspirate wash buffer completely, and then incubate samples in 200 µl of an optimal concentration of the desired fluorochrome-conjugated secondary antibody (e.g., phycoerythrin) for 30 min at room temperature (15–25°C) in the dark (*see* **Note 5**).
8. All the remaining procedures should be carried out in the dark.
9. Wash chambers thoroughly (six to eight changes), incubate cells in freshly prepared permeabilizing buffer for 2 min on ice (2–8°C), and then rinse again twice with wash buffer.

10. Incubate cells in TUNEL-positive control wells (step 1, e) with 200 µl of 500 U/ml DNase I for 10 min at room temperature (15–25°C) to induce DNA strand breaks.
11. Rinse wells three times with wash buffer, aspirate wash buffer ensuring removal of all residual liquid, and then remove chamber and silicone gasket from each slide.
12. Rinse slides in wash buffer, dry carefully around each sample, and mark around each sample with a hydrophobic slide marker.
13. Prepare sufficient TUNEL reaction mixture from *In Situ* Cell Death Detection kit (fluorescein) for all chambers to be examined to ensure complete coverage of all cells (30–50 µl/chamber sample). TUNEL reaction mixture should be prepared immediately before use and kept on ice.
14. Per set of reaction mixture tubes (vial 1 and vial 2), remove 100 µl of label solution (vial 2) for no enzyme negative controls (step 1, b), and then add 50 µl of enzyme solution (vial 1) to the remaining 450 µl of label solution to generate 500 µl of TUNEL reaction mixture; mix thoroughly to equilibrate.
15. Add 30–50 µl of PBS to each of the untreated, unstained negative control samples (step 1, a).
16. Add 30–50 µl of label solution to each of the no enzyme negative control samples (step 1, b).
17. Add 30–50 µl of TUNEL reaction mixture to all the remaining negative, test, and positive control samples (step 1, c–f).
18. Incubate all slides in a humidified atmosphere for 1 h at 37°C, and then wash slides three times with wash buffer.
19. Mount slides with VECTASHIELD hard-set mounting medium with DAPI. Add 1 drop (~25 µl) of mounting medium onto slides, and then gently lower coverslip and allow mounting medium to disperse over the entire section. Slides can be viewed immediately, although mounting medium takes 15 min to dry and overnight at 4°C to harden.
20. Analyze slides with a fluorescence microscope. To assess TUNEL staining, use an excitation wavelength of ~488 nm and detection of ~530 nm. To assess cell surface staining, use a secondary conjugate with appropriate detection wavelength that can be separated from that of fluorescein TUNEL staining (e.g., phycoerythrin: excitation, ~488 nm and detection, ~575 nm). To assess DAPI staining, use an excitation wavelength of ~360 nm and detection of ~460 nm.
21. Overlay fluorescent images using commercially available software packages (e.g., Adobe Photoshop, Adobe Systems, Mountain View, CA).
22. Assess the proportion of cells stained with the TUNEL reaction mixture (TUNEL-positive/DAPI-positive) in at least six different fields of view within each chamber slide sample.

3.3. Hoechst Staining

It is very important that nuclear morphology (quantification of chromatin condensation) be analyzed simultaneously or in parallel experiments when

carrying out the TUNEL assay, because this procedure has been known to give false positive signals under some specific conditions ***(48,49)***. To assess nuclear morphology, fluorescence light microscopy with differential uptake of fluorescent DNA binding dyes is a commonly used method for its simplicity, rapidity and accuracy. Using the bisbenzimidazole dye Hoechst 33342 (which penetrates the plasma membrane and stains DNA in cells without permeabilization), the nuclei of apoptotic cells stain with a very bright blue fluorescence, as compared to nonapoptotic cells. These nuclei have highly condensed chromatin, which can show up as crescents around the periphery of the nucleus, or it can be one, or a group of bright apoptotic bodies. Nuclei are scored as either normal (uncondensed chromatin with dull blue fluorescence) or apoptotic (highly condensed chromatin with bright blue fluorescence) ***(50–52)***. Dual staining of cells with propidium iodide (PI), which cannot permeate the plasma membrane of viable cells, allows for discrimination between dead and apoptotic cells.

1. Grow cells to appropriate confluence in eight-well chamber slides, and then induce apoptosis by the desired method; simultaneously set up a positive control well by addition of 500 nM staurosporine. Retain noninduced sample wells to be used as the negative controls (*see* **Note 1**).
2. Aspirate media from wells and rinse three times with wash buffer (*see* **Note 9**).
3. Incubate all samples with 50 µg/ml Hoechst 33342 (~120 µl/chamber) at room temperature (15–25°C), in the dark, for 8 min, and then wash three times with wash buffer.
4. After final rinse, incubate all samples in 20 µg/ml PI (~120 µl/chamber) at room temperature (15–25°C), in the dark, for 3 min, and then wash three times with wash buffer.
5. Remove chamber and silicone gasket from each slide, rinse slides a final time in wash buffer, and then mount slides with VECTASHIELD hard-set mounting medium. Add 1 drop (~25 µl) of mounting medium onto slides, and then gently lower coverslip and allow mounting medium to disperse over the entire section. Slides can be viewed immediately, although mounting medium takes 15 min to dry and overnight at 4°C to harden. Keep slides in the dark at all times.
6. Analyze slides with a fluorescence microscope. To assess Hoechst 33342 staining, use an excitation wavelength of ~360 nm and detection of ~420 nm. To assess PI staining, use an excitation wavelength of ~488 nm and detection of ~575 nm.
7. Cell counts should be taken from at least six random fields of view, and cells should be scored as PI-negative (viable cells) or PI-positive (dead cells). The level of apoptosis is then determined in the PI-negative population only. Nuclei should be scored as either normal (uncondensed chromatin) or apoptotic (highly condensed chromatin, with bright blue fluorescence (crescents or fragmented into discrete apoptotic bodies)) (*see* **Note 11**).

3.4. Caspase-Specific Western Blot

Execution of apoptosis in many mammalian cells requires the coordinated action of a series of aspartate-specific cysteine proteases, termed caspases, which cleave specific substrates, resulting in the systematic disassembly of the dying cell *(53)*. Because both caspase-dependent and caspase-independent pathways have been identified, the characterization of any proteases activated in vivo by your suggested apoptotic stimulus is important *(54–57)*. The presence of activated caspases can be very easily assessed by immunoblotting of whole cell lysates by using antibodies specific for a given caspase. Because caspases are activated by autoproteolysis or heteroproteolysis of the respective proenzyme, the appearance of bands with lower molecular weights, corresponding to the active enzyme, is indicative of their activation.

1. Grow cells to appropriate confluence in 75-cm^2 flasks; use sufficient numbers of flasks to give approximately 2×10^6 cells/treatment (*see* **Note 1**).
2. Induce apoptosis by the desired method for the required length of time; simultaneously set up positive control (500 nM staurosporine) and negative control (no induction) flasks.
3. Wash cells three times in ice-cold wash buffer and aspirate fully after final wash (*see* **Note 9**).
4. Add 300 µl of ice-cold lysis buffer to each flask, remove adherent cells with a cell scraper, and transfer resulting lysate to a precooled microfuge tube. Rinse flask with a further 200 µl of ice-cold lysis buffer, and then combine with the first aliquot of cell lysate.
5. Incubate lysate for 30 min on ice, and then centrifuge cell lysates for 20 min at 18,000 rpm at 4°C. Remove supernatant to a fresh precooled microfuge tube and keep on ice.
6. Determine protein concentration of cell lysates according to the manufacturer's instructions (e.g., Total Protein Assay kit, Sigma-Aldrich). It is important to dilute the cell lysates as necessary to give final protein below the sensitivity range (500 µg of protein) in a final volume of 50 µl (we suggest making a dilution series from 1/10 to 1/100).
7. Mix required volume of each lysate (50 µg of total protein) with one fifth the volume of 5× loading sample buffer, and then add 5% (v/v) β-mercaptoethanol. Freeze remaining lysate at –80°C in aliquots.
8. Heat all samples at 100°C for 5 min, and then load whole sample onto precast poly-acrylamide gel. Load 10 µl of prestained molecular weight markers in a reference lane.
9. Run gel at 100–120 V until dye front reaches the bottom of the gel, use a cooling system to ensure gel does not run too hot.
10. Remove gel from casting plates, and then equilibrate gel and nitrocellulose membrane in ice-cold transfer buffer for 5 min.
11. Transfer proteins onto a nitrocellulose membrane by using a blotting apparatus for 2 h at 50–60 V at constant room temperature (15–25°C).

12. Wash nitrocellulose membrane (3 × 5 min) in TBST with constant agitation before staining for 5 min with Ponceau Red solution.
13. Visualize transfer of protein onto nitrocellulose membrane by washing membrane extensively with water until protein bands appear.
14. Destain membrane completely by repeatedly washing in TBST before incubating in blocking solution for 1 h at room temperature (15–25°C).
15. Wash membrane with TBST for 5 min, and then incubate overnight at 4°C with constant agitation in a cocktail of primary antibodies previously diluted in TBST at predetermined optimal concentrations. The cocktail consists of an antibody specific for the caspase of interest and a second antibody specific for the loading control protein (β-actin or GAPDH) (*see* **Note 8**).
16. Rinse membrane thoroughly in TBST (5 × 5 minutes) before incubating for 1 hour with Alexafluor 680-conjugated secondary antibody diluted in TBST at a predetermined optimal concentration (*see* **Note 8**).
17. Wash membrane thoroughly with in TBST (at least 5 x 5 min), and then visualize specific staining by using an infrared imager (*see* **Note 12**).
18. Collect electronic image of stained membrane and assess presence of bands of appropriate molecular weight by using molecular weight standards.
19. The representative expression of appropriate caspase(s) under different conditions (untreated, various treatment conditions, positive control) can be determined by comparing relative fluorescence intensity of each sample. To accurately ascertain treatment effect, the variation in fluorescence intensity of the loading control for each sample should be taken into account.

4. Notes

1. It is very important to determine the optimal growth conditions of the cells you are testing. Because hESCs and their progeny are adherent cells, preliminary experiments should be performed to determine the appropriate cell density of the starting population so as to avoid the potential influence of cell-to-cell contact on induction, or prevention, of apoptosis. Similarly, the length of time required for reestablishment of cellular characteristics (e.g., morphology, phenotype) after seeding should be determined before induction of apoptosis.
2. Staurosporine is a potent inhibitor of many kinases, including protein kinase C, protein kinase A and protein kinase G, and it is routinely used as a nonspecific method for inducing apoptosis ***(58)***.
3. To prepare 100 ml of paraformaldehyde solution dissolve 4 g of paraformaldehyde in 90 ml of H_2O, add 2 drops of 10 N NaOH, heat for 30 min at 60°C, and then add 10 ml of 10× PBS and adjust pH to 7.4.
4. There are numerous well-published methods for the extraction of DNA for the detection of DNA laddering. The Apoptotic DNA Ladder Extraction kit described in this chapter is a very simple procedure that is suitable for analyses of as few as 5×10^5 cells where there is a high degree of apoptosis, and up to 10^7 cells if there is little apoptosis. Preliminary experiments should be carried

out to ascertain degree of cell death induced by your treatment of choice, so that reaction volumes can be adjusted accordingly. Should you be limited by cell numbers, other techniques have been described in which DNA ladders can be detected in as few as 6×10^4 cells ***(59)***.

5. Use of the fluorescein-dUTP "TUNEL kit" allows for the simultaneous immunofluorescence analyses of expression of other cell specific molecules (e.g., cell surface markers) by using alternate fluorochrome-conjugated antibodies with different emission spectra (e.g., phycoerythrin, phycoerythrin:cyanine-5, and allophycocyanin). The capacity for simultaneous analyses is likely to be limited only by the capacity of the immunofluorescence microscope that you are using. Determine that you have all the necessary filters and lasers to enable these analyses before commencing your experiments.
6. Other *in situ* cell death detection kits are commercially available with different fluorochrome conjugates, allowing for various different fluorochrome combinations in the analysis of TUNEL staining.
7. It is critically important that the lysis buffer, with all inhibitors added, is ice-cold (<4°C) before commencing.
8. To facilitate easy development of the Western blot, it is useful to have both antibodies in the primary cocktail be of the same species and isotype. Multiple commercially available monoclonal antibodies or polyclonal antiserum are available for loading control antigens such as β-actin or GAPDH. Thus, obtaining a specific and isotype matched caspase-specific antibody should be relatively easy. Should this not be possible, the secondary fluorochrome-conjugated antibodies should be chosen carefully, and blocking should be carried out appropriately so as to avoid nonspecific cross-reactivity. Preliminary experiments should always be carried out using positive control samples (e.g., recombinant proteins, positive control cell lysates) to determine the capacity of both primary antibodies to be used simultaneously, and the optimal concentrations of primary and secondary antibodies.
9. Induction of apoptosis in adherent cells may cause these cells to become loose or even detached from the culture vessel. Preliminary experiments should be carried out to determine optimal test conditions (e.g., concentration of test factor, time of incubation) to minimize cell detachment. In any case, following treatment of adherent cells, all assay steps should be carried out with great care, especially when removing and adding liquids so not to dislodge cells.
10. Proper fixation is essential to avoid loss of fragmented DNA, the target of the labeling reaction; thus, it is critically important to use fresh fixation solution. It is also very important to ensure that the fixation solution (and all subsequent solutes) covers the entire chamber and that the entire sample remains submerged for the duration of all the incubations. Check slides frequently during each incubation period to ensure no leakage of solution. To avoid loss from evaporation, chamber slides should be covered and incubated in a humidified chamber.

11. Some of the morphological features of apoptosis can often overlap with those typical of necrosis; therefore, this should always be taken into consideration. Comparison with published, photographic evidence demonstrating apoptotic cells is strongly advised for those new to the evaluation of apoptosis.
12. A Western blot may be stripped and reprobed several times to visualize other caspases or even individual caspase substrates. The key to this process is to use conditions that cause the release of antibody from the antigen without causing a significant amount of antigen to be released from the membrane. There are many published protocols, which are readily available.

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20

Markers of Adult Neural Stem Cells

Eric Wexler

Summary

I provide detailed protocols for conduction and troubleshooting the key steps in our three most used experimental designs: (1) prospectively counting and sorting of human neural stem cells (NSCs)/committed progenitors before placing them in culture; (2) high-throughput methods of quantifying changes in NSC/progenitor proliferation, in vitro; and (3) retrovirally tagging NSCs before differentiation to assess cell fates in individual clones. Detailed troubleshooting of immunohistochemical and fluorescence-activated cell sorting staining is described. Some of these techniques overlap with other chapters in this volume. Ultimately, this provision of complementary technical information should help ensure the reader's experimental success.

Key Words: Human; retrovirus; packaging cell line; neural stem cell; immunochemistry; BrdU; fluorescence-activated cell sorting; proliferation; antibody; differentiation; ELISA.

1. Introduction

One must study neural stem cell (NSC) behavior both in vitro and in vivo because each model system has its own assets and liabilities. For example, in vivo, cytoarchitecture and cellular morphology are important aids in establishing cellular identity; yet, manipulating the microenvironment of individual cells is difficult. The situation reverses in vitro, where it is easier to tag and manipulate NSCs, but harder to be certain of their true phenotype (e.g., radial glia verse astrocyte). We are all skilled and enthusiastic about grappling with the "big picture" of understanding how NSCs function and how they can be

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harnessed for therapeutic benefit. Unfortunately, the more pedestrian experimental difficulties often do us in way before we even get a chance to address the interesting questions. For example, how many of you have thrown away tons of money on panels of antibodies, only to find that they label either everything or nothing at all. Don't worry, we all have a box of these "old soldiers" collecting frost in the freezer. One goal of this chapter is to help you stem the hemorrhaging of time and money by providing our list of tested antibodies (*see* **Subheading 2.3.**). If it keeps you from buying just one more useless antibody, you will have paid for this book more than twice over. Moreover, **Subheading 3.** contains a selection of NSC marker protocols that I think are improvements over some of their more prevalent counterparts. Taken together with other chapters in this volume, I hope that this information helps save you time and money.

2. Materials

2.1. General Reagents and Supplies

1. Fluorescence-activated cell sorting (FACS) filter tubes: 12- × 75- 5-ml tube with 70 μM filter cap, nonsterile (BD Biosciences, San Jose, CA, cat. no. BD352235).
2. DNAse (Sigma-Aldrich, St. Louis, MO, cat. no. D-5025, bovine pancreas).
3. Paraformaldehyde (PFA) (Prill grade) (Electron Microscopy Sciences, Hatfield, PA, or Ted Pella, Redding, CA).
4. Cell proliferation enzyme-linked immunosorbent assay (ELISA), 5-bromo-2'-deoxyuridine BrdU (colorimetric) (Roche Diagnostics, Indianapolis, IN, cat. no. 11 647 229 001; 1,000 tests).
5. Cell proliferation ELISA, BrdU (chemiluminescence) (Roche Diagnostics, cat. no. 11 669 915 001; 1,000 tests).
6. Anti-BrdU-peroxidase, Fab fragment (Roche Diagnostics, cat. no. 11 585 860 001; 15 units).
7. 3,3',5,5'-Tetramethylbenzidine (TMB) Liquid Substrate System for ELISA (Sigma-Aldrich, cat. no. T 0440).
8. 1,4-Diazabicyclo [2.2.2] octane (DABCO) (Sigma-Aldrich, cat. no. D2522).
9. *N*-Propyl gallate (NPG) (Sigma-Aldrich, cat. no. 02370).
10. Preferred antifade agents:
    a. Prolong Antifade kit (Unvitrogen, cat. no. P7481).
    b. Polyvinyl alcohol mounting medium with NPG (Sigma-Aldrich, cat. no. 10979).
    c. Polyvinyl alcohol mounting medium with DABCO (Gelvatol) (Sigma-Aldrich, cat. no. 10981).
11. Alternate antifade agents:
    a. PermaFluor (Shandon Lipshaw, Pittsburgh, PA) (aqueous, sets hard).
    b. FluorSave (aqueous).

c. Fluoroguard (Bio-Rad, Hercules, CA).
d. VECTASHIELD (Vector Laboratories, Burlingame, CA).
e. PolyMount or Aqua-PolyMount (Polysciences, Warrington, PA).
f. MOWIOL (Calbiochem, San Diego, CA).

12. Ethyleneglycol-bis-succinimidyl-succinate (EGS) (Sigma-Aldrich, cat. no. E3257).
13. Ethylene glycolbis(sulfosuccinimidylsuccinate) (Sulfo-EGS) (Pierce Chemical, Rockford, IL, cat. no. 21566).
14. EGTA (Sigma-Aldrich, cat. no. E3889).
15. Sodium borohydride (Sigma-Aldrich, cat. no. S9125).
16. Tetracycline-screened fetal bovine serum (Hyclone Laboratories, Logan, UT, cat. no. SH30070.03T).
17. Polybrene, 4-8 µg/ml (Sigma-Aldrich, cat. no. H-9268).
18. BIT9500 serum substitute (Stemcell Technologies Inc., Vancouver, BC, Canada).
19. Microtiter plates, 96-well, V-bottom (Fisher Scientific, Pittsburgh, PA, cat. no. 07-200-108).
20. Unless otherwise mentioned, all general reagents (e.g., solvents and salts were obtained from Sigma-Aldrich).

2.2. Buffers

1. FACS wash buffer (L15 medium [without phenol red] supplemented with 0.1% sodium azide [to prevent receptor internalization] BIT9500.
2. FACS sort buffer: Hanks' (Ca^{2+}/Mg^{2+}-free): 25 mM HEPES, pH 7.0, 5 mg/ml bovine serum albumin (BSA) fraction V, 1 mM EDTA (0.22-µm sterile filtered), 5 µg/m insulin (human), 100 µg/ml transferrin (human, iron-saturated).
3. DNase working solution: add: 50 Kunitz units/ml DNase buffer (0.15 M NaCl + 4.2 mM MgCl).
4. DNase buffer (50 ml): 46.8 ml of distilled H_2O (dH_2O), 200 µl of $MgCl_2$ (1 M stock), 1,500 µl of NaCl (5 M stock).
5. Nonpermeabilizing staining buffer: Tris-buffered saline (TBS) + 3% BSA or sera from animals where the secondary was generated (usually goat or donkey).
6. PEM "Brinkley buffer" (make 5× stock): 80 mM PIPES, pH 6.8, 1 mM $MgCl_2$, and 1 mM EGTA; store at 4°C.
7. Cytoskeleton buffer (CB) with sucrose (CBS): 10 mM 2-(*N*-morpholino)ethanesulfonic acid, pH 6.1, 138 mM KCl, 3 mM MgCl, and 2 mM EGTA; store at 4°C (*see* **Note 1**).
8. TBS: 0.15 M NaCl, 0.02 M Tris-HCl, Ph 7.4:

TBS (1×)	6 liters	4 liters
Trizma hydrochloride	79.32 g	52.88 g
Trizma base	11.64 g	7.76 g

Sodium chloride	54.00 g	36.00 g
Bring to volume	6 l dH_2O	4 l dH_2O

9. 10× TBS, pH 7.5

	1 liter	5 liters
Trizma HCl	132.2 g	661 g
Trizma base	9.4 g	97 g
NaCl	90.0 g	450 g
dH_2O	855.0 ml	4,275 ml

10. Triton X-100 stock (10%): add a bolus of approximately 2 g of Triton to 50-ml conical tube on balance. Bring up volume to 20 ml with double-distilled H_2O (ddH_2O) and invert repeatedly to dissolve.
11. TBS-Triton (TBST, 0.1–2%) add 1–2 ml of Triton stock to 100 ml of TBS.
12. Saponin stock (10%): dissolve 10 g of saponin (≥25% saponingen content) into 100 ml of ddH_2O at 37°C while gently stirring. Sterile filter (0.22 µl) and store at 4°C.
13. TBS-saponin (TBSS, 0.2%): add 2 ml of Triton stock to 100 ml of TBS.
14. 0.2 M phosphate buffer (1 liter): 5.52 g of sodium phosphate monobasic anhydrous (NaH2PO4) and 21.9 g of sodium phosphate dibasic anhydrous (Na2HPO4); dissolve in 1 liter of ddH_2O.
15. 4% PFA in 0.1 M phosphate-buffered saline (PBS): dissolve 80 g of PFA (Prill grade) in about 500 ml of dH_2O by stirring at 55–70°C, under a fume hood. Add a few drops of 1 N NaOH to depolymerize the PFA. Stir until fully dissolved (there may be a fine white precipitate; this is OK). Once cooled to room temperature, filter through a 0.22–0.4-µm Millipore filter (Millipore Corporation, Billerica, MA). Mix 1:1 with 0.2 M phosphate buffer or 2× PBS. Adjust the pH to about 7.0–7.5 and check with pH paper. Aliquot and store frozen at –20°C. Discard after thawing (*see* **Notes 2–5**).
16. EGS stock solution (100 mM): dissolve 45 mg in 1 ml of bone dry dimethyl sulfoxide (DMSO) (unstable in water due to hydrolysis).
17. Diaminobenzidine (DAB) (reconstitute Sigma-Aldrich ISOPAC with 20 ml of dH_2O): 1 ml of the reconstituted DAB to 9 ml of TBS; add 60 µl of hydrogen peroxide just before incubation). This leads to a 10-min reaction.
18. BrdU: cultured cells: 10 µM for 2–4-h pulse; in vivo: administer 50 mg/kg ip once per day × 6 days.
19. Tissue collecting solution (TCS)/cryoprotectant), 1 liter: 250 ml of glycerin, 300 ml of ethylene glycol, 450 ml of 0.1 M NaPO4, pH 6.7; Store in 4°C refrigerator.
20. Antifade medium (hardening) polyvinyl alcohol and 1,4-diazobicyclo-[2.2.2]-octane (PVA-DABCO)—PVA-NPG: add 2.4 g of PVA (mol. wt. 30,000–70,000) to 6 ml of glycerol. Stir well to mix. Add 6 ml of dH_2O and leave for at least 2 h at room temperature. Add 12 ml of 0.2 M Tris-NCl, pH 8.5. Heat to 50°C for 10 min with occasional mixing. After PVA is dissolved, clarify by centrifugation (5,000×g) for 15 min. Collect supernatant liquid. Add DABCO to 2.5% or NPD to 3% as antifade medium. Aliquot in microtubes and store at –20°C. Stocks of Gelvatol are stable at room temperature for several weeks after thawing. Store aliquots in dark at 4°C for 3 months.

21. Antifade medium (nonhardening): 90% glycerol, 3% *N*-propyl gallate, and 0.1 M sodium borate, pH 9 (*see* **Note**).
22. 4,6-Diamidino-2-phenylindole (DAPI) wash buffer: 1 µl of DAPI stock (10 µg/ml) to 50 ml of TBS.

2.3. Cell Type-Specific Antibody Markers (see Note 7*)*

A systematic analysis of how the following markers were validated is beyond the scope of this practical review. However, you are encouraged to refer to one of the many excellent reviews and primary articles on the topic of human ***(2–4)*** and rodent NSC markers ***(5–11)***.

2.3.1. Proliferation Markers

1. BrdU clone ICR1; rat IgG; 1:250–500 (Accurate Chemical & Scientific, Westbury, NY); recognizes 5-chloro-2'-deoxyuridine (CldU) + BrdU.
2. BrdU clone B441; mouse IgG1; 1:50 in vivo (BD Biosciences PharMingen, San Diego, CA); recognizes 5-iodo-2'-deoxyuridine (IdU) + BrdU.
3. BrdU clone BU-33; mouse IgG1 (does not work well) (Sigma-Aldrich).
4. PCNA clone PC10 (proliferating cell nuclear antigen); mouse IgG2a; 1:100 (Chemicon International, Temecula, CA, formerly Roche cat .no. 1742353) (*see* **Note 8**).
5. Phospho-histone H3; rabbit; 1:500 (Upstate Biotechnology, Lake Placid, NY) (*see* **Note 9**).
6. MCM2 (anti human); goat; 1:200 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) (*see* **Note 10**).
7. Ki-67 (pan specific); rabbit; 1:1000 (NovoCastra, New Castle, UK) (*see* **Note 11**).
8. Ki-67 (MIB3, human specific); Ms IgG1a κ; 1:100 (Dako Denmark A/S, Glostrup, Denmark).
9. Ki-67 (TEC3; mouse specific); rat IgG2a; 1:100 (Dako Denmark AS).
10. MCM2 (anti human); rabbit; 1:300 (BD Biosciences PharMingen).

2.3.2. Multipotent, Mitotic NSCs

1. Nestin (clone rat-401; rodent specific); mouse IgG1; 1:100 (Chemicon International, MAB353 or DHSB Rat 401).
2. Nestin (human specific clone 10C2); rabbit; 1:100 (Chemicon International, AB5922).
3. Nestin (human specific); mouse IgG1; 1:100 (Chemicon International, MAB5326).
4. Sox2; rabbit; 1:1500 (Chemicon Internaional).
5. Sox2; rabbit; 1:100 (Santa Cruz Biotechnology, Inc.).
6. Pax6; mouse IgG: 1:100: (Developmental Studies Hybridoma Bank, University of Iowa, Iowa City, IA).

7. Prominin/CD133+; mouse; 1:200 (BD Biosciences PharMingen).
8. XCR4/CD184; mouse; 1:200 (BD Biosciences PharMingen).
9. Integrin β1/CD29; mouse; 1:200 (BD Biosciences PharMingen).
10. SSEA-1/LEX/CD1; mouse; 1:200 (BD Biosciences PharMingen).
11. TAPA1/CD81; mouse; 1:200; (BD Biosciences PharMingen).
12. CD34; mouse; 1:200 (BD Biosciences PharMingen).
13. CD45; mouse; 1:200 (BD Biosciences PharMingen).
14. CD24; mouse; 1:200 (BD Biosciences PharMingen).
15. Musashi1; rabbit; 1:200 (Chemicon International).
16. Basic lipid binding protein (BLBP); rabbit; 1:3,000; gift from N. Heintz (Rockefeller University, New York, NY).
17. Glial fibrillary acidic protein (GFAP) (*see* **Subheading 3.2.5.**).

2.3.3. Radial Glia

1. GLAST (glutamate transporter); guinea pig; 1:1,000 (Chemicon International).
2. Vimentin; rabbit IgG; 1:500 (Chemicon International).
3. Vimentin; mouse IgG; 1:500 (GE Healthcare, Little Chalfont, Buckinghamshire, UK).
4. RC2; mouse IgM; in vivo: supernatant 1:5; in vivo: ascites fluid 1:150; in vitro: ascites 1:400 (DSHB).
5. BLBP (*see* **Subheading 2.3.2.**, item 16).

2.3.4. Multipotent, Mitotic Glia

1. NG2 chondroitin sulfate proteoglycan; rabbit; 1:200 in vivo; 1:1,000 in vitro (Chemicon International) (NB: sensitive to overfixation; use 2% PFA × 5-min fixation).
2. A2B5; mouse IgM; 1:100 (Chemicon International).
3. Platelet-derived growth factor receptor (PDGFR)α; IgG2b κ; 1:50 (Spring Bioscience, Fremont, CA) (NB: recognizes both human and mouse; requires antigen retrieval if fixed).

2.3.5. Immature Astrocytes

1. S100β; rabbit; 1:1,000 (Swant, Bellizona, Switzerland).
2. S100β; rabbit IgG; 1:500 (Chemicon International).

2.3.6. Mature Glia

1. Glutamine synthetase; mouse IgG; 1:200 (Chemicon International).
2. GFAP; guinea pig IgG; 1:500 (Accurate Chemical & Scientific).
3. GFAP; guinea pig IgG; 1:500 (Advance Immuno).
4. GFAP; guinea pig IgG; 1:2,500 in vitro.
5. 1:1,000 in vivo (Advance Immuno).
6. GFAP; rabbit; 1:500 (Chemicon International).

2.3.7. Immature Oligodendrocytes

1. O4; IgM; 1:1,000 (Chemicon International) (lost with alcohol/acetone fixation).
2. NG2 remains expressed in committed oligo progenitors, but it is turned off in mature oligonucleotides.

2.3.8. Mature Oligodendrocytes

1. RIP; mouse IgG1; 1:5 (Sup) (DSHB).
2. Galactocerebroside (GalC); rabbit; 1:250 (Chemicon International); mature oligodendrocytes.
3. O1; IgM; 1:500 (Chemicon International, MAB344) (best seen in fresh frozen or live tissue; lost with alcohol/acetone fixation).
4. CNPase; mouse IgG1; 1:100 (Chemicon International).

2.3.9. Mitotic, Lineage-Committed Neural Progenitors

1. Type III β-tubulin (Tuj-1): mouse: 1:100 (Chemicon International).
2. Type III β-tubulin (Tuj-1); mouse; 1:500 (Covance, Princeton, NJ).
3. Type III β-tubulin (Tuj-1); rabbit; 1:5,000 (cells) 1:500 (tissue) (Covance).
4. PSA-neural cell adhesion molecule (NCAM); mouse IgM; 1:400 (AbCys, Paris, France, ABC0019): NB: Ms,Hu.
5. PSA-NCAM; mouse; 1:5 (DSHB, AKA ECAM/CD56).
6. Doublecortin (DCX); goat; 1:100–1:500 (Santa Cruz Biotechnology, Inc.).

2.3.10. Postmitotic, Immature Neurons

1. Calretinin; rabbit; 1:300 (Chemicon International).
2. Neuron-specific enolase (NSE); rabbit; 1:100 (Chemicon International) (not neuron-specific).
3. NeuroD1; rabbit; 1:200 (Chemicon International).
4. NeuroD1; goat; 1:200 (Santa Cruz Biotechnology, Inc.).
5. HuC/D; mouse IgG; 1:100 (Invitrogen).
6. NeuN (A60-E4); mouse IgG; 1:10 (gift from Mullen laboratory).
7. NeuN; mouse; 1:100 (Chemicon International) (*see* **Subheading 3.4.**).
8. Tuj-1, DCX, and PSA-NCAM are expressed in early postmitotic neurons (*see* above).

2.3.11. Mature Neurons

1. Map2ab; rabbit; 1:1,000 (Chemicon International, AB5622).
2. Map2abc clone HM2; mouse; 1:500 (Sigma-Aldrich).
3. MAP2ab clone AP20; mouse IgG1; 1:500 (Sigma-Aldrich).
4. Neurofilament 200 kDa; rabbit; 1:250 (Chemicon International).
5. Tau; mouse; 1:100 (Chemicon International).
6. Calbindin; mouse IgG; 1:500 (Roche Diagnostics).

7. Calbindin; rabbit IgG; 1:2,000 (Chemicon International).
8. Calbindin; rabbit; 1:300 (Swant).
9. Tyrosine hydroxylase; sheep; 1:600 (Dynal Biotech, Lake Success, NY).
10. NF145; mouse; 1:500 (Chemicon International).
11. VIP; rabbit; 1:100 (Chemicon International).
12. Choline acetyltransferase; goat; 1:200 (Chemicon International).
13. Tryptophan hydroxylase; sheep; 1:300 (Chemicon International).
14. Parvalbumin; mouse IgG1; 1:1,000 (Sigma-Aldrich).

2.3.12. Vascular Markers

1. Rat endothelial cell antigen (RECA-1); mouse IgG; 1:20 (Harlan, Indianapolis, IN).
2. Smooth muscle α-actin; mouse IgG; 1:3,000 (Sigma-Aldrich).
3. Fibronectin; rabbit IgG; 1:1,000 (Chemicon International).
4. Integrin β3; rabbit IgG; 1:250 (Chemicon International).
5. Von Willabrandt; rabbit IgG; 1:2,000 (Chemicon International).
6. Vascular endothelial growth factor (VEGF)-A; rabbit IgG; 1:1,000 (Chemicon International).
7. rVEGF; mouse IgG; 1:500 (Chemicon International).
8. Flk-1; rabbit IgG; 1:250 (Santa Cruz Biotechnology, Inc.).
9. Flk-1; mouse IgG; 1:500 (Chemicon International).
10. RECA-1; mouse IgG; 1:20 (Serotec, Oxford, UK).
11. Smooth muscle α-actin; mouse IgG2a; 1:500 (tissue), ≤1:1,500 (cells) (Sigma-Aldrich).
12. Von Willebrand; rabbit IgG; 1:2000 (Dako Denmark AS).
13. Endothelin; rabbit IgG; 1:500 (Calbiochem).

2.3.13. Immune Cell Markers

1. Monocyte, microglia (Ox42); mouse IgG; 1:500 (Roche Diagnostics).
2. Activated microglia (ED-1); mouse IgG; 1:1,000 (Sigma-Aldrich).
3. Pan leukocyte antigen; mouse IgG; 1:200 (Sigma-Aldrich).

2.3.14. Other

1. Human nuclei, mouse IgG1; 1:500–1,000 (Chemicon International) (*see* **Note 12**).
2. Anti-green fluorescent protein (GFP); chicken; 1:500 (Chemicon International).
3. Secondary antibodies: species-specific secondary antibodies (donkey when possible) conjugated to Alexa 350, 488, 594, 633 were purchased from Invitrogen and used at 1:1,000–1,200 (cells); 1:500–750 (tissue) (*see* **Note 13**).

3. Methods

Throughout the NSC literature, you come across many scientifically elegant studies, each abounding with aesthetically gorgeous micrographs. However,

you also may have tried to replicate these findings, only to discover that your pictures were, shall we say, "not quite so pretty" or possibly just confusing. Major culprits include bad choice of antibodies, poor immunohistological technique, or sometimes just using the wrong tool for the task. Below, I provide you with the best tool for each experimental task, guaranteeing that you do not make the same mistakes again.

3.1. Prospective NSC Methods

3.1.1. Labeling and Sorting Live NSCs

This section assumes that you have access to a FACS, likely through your university's core facilities, and that their personnel will do the actual cell sorting for you. Therefore, the hard part for you is to label the cells and not kill them in the process. Either the cytoplasm or nucleus is the site of expression for most NSC markers (e.g., filaments, synthetic enzymes, or transcription factors), making them unusable for sorting. The following is a summary of well-described cell surface antigens useful for sorting human NSCs at different stages of development (most, unless noted, are applicable to sorting murine cells, as well):

" Embryonic stem cells: alkaline phosphatase, SSEA3, SSEA4 (*see* **Note 14**)

Endothelia: CD31

NSCs: Prominin/CD133^{+}, CXCR4/CD184^{+}, Integrin β1/CD29^{+}, SSEA-1/LEX/CD15^{+} (*see* **Note 15**), TAPA1/CD81^{+},5E12+ (*see* **Note 16**) (negative selection: CD34^{--}, CD45^{--}, and CD24$^{--/lo}$)

Glial progenitor: A2B5, PDGFRα 9 (*see* **Note 17**)

Mature oligodenrocyte: O1, O4, GalC

Neural restricted progenitor: PSA-NCAM/CD56

1. The first step is to choose a marker from the boxed list based on what cell type you want to select/count (e.g., for multipotent human NSCs, you may choose CD133, Lex, CD34, and CD45).
2. IMPORTANT: Before purchasing antibodies, consult with your FACS technician to determine the capabilities of your machine, specifically which fluorophores it can detect. Whenever possible, you want the antibodies used for FACS to already be conjugated to a fluorophore (*see* **Note 18**). They cost more, so you do not want to buy antibodies that will not work with your machine; however, when properly chosen, they are well worth the expense. (My laboratory finds that BD Biosciences PharMingen has an excellent selection of high-quality prelabeled antibodies against the tetraspan proteins [i.e., any protein starting with the letters CD].)
3. Dissociate cells by using 0.05% trypsin (*see* **Note 19**).

4. Centrifuge at 1,500 rpm in 15-ml conical tubes for 2 min.
5. Decant supernatant and resuspend cell in Neurobasal medium (without phenol red) supplemented with BIT9500 (*see* **Note 20**) to a final concentration of 10^6 cells/ml (*see* **Notes 21** and **22**).
6. Repeat **step 4** then resuspend in 100 µl and transfer to 96-well V-bottomed microtiter plate (*see* **Note 23**).
7. Add 0.1–10 µg/ml of the primary antibody (*see* **Note 24**) diluted in L15 (without phenol red) + 10% BIT9500 (*see* **Note 25**).
8. Incubate on ice for 30 min, away from light (*see* **Note 26**).
9. Spin plate for 2 min to pellet cells, and then resuspend in FACS wash buffer (L15 medium (without phenol red) supplemented with 0.1% sodium azide (to prevent receptor internalization) + 10% BIT9500).
10. Repellet cell and resuspend in FACS wash buffer.
11. Repellet cells and resuspend in FACS sort buffer.
12. Sort within 30 min (*see* **Notes 27** and **28**).

3.1.2. Prospective Marking of NSCs by Using Retrovirus

Retroviruses only infect dividing cells, but once there, irreversibly integrate into the host genome. These properties make them excellent reagents for marking NSCs. However, their greatest drawback is that production can be an extremely labor-intensive undertaking, especially with in vivo experiments that require substantial quantities of virus. I have developed a protocol that does away with many of the preparatory steps such as plasmid maxi preps and large-scale triple transfections with packaging constructs. At the heart of this protocol is a tetracycline-regulated cell line (LZG293) that stably produces a vesicular stomatitis virus G protein (VSV-G) pseudotyped Moloney murine leukemia virus-based retrovirus capable of transducing an exceptionally bright variant of GFP under the control of a powerful promoter (i.e., the fluorescence of single integrant can be seen in NSCs, before and after differentiation ***(13)***). These LZG cells have been selectively expanded based on their ability to produce extremely high-titer virus.

3.1.3. LZG: Retroviral-GFP Packaging Cell Line (see Note 29 for Details on Cell Line)

1. Rapidly thaw LZG cells (available upon request).
2. Transfer cells to 15-ml centrifuge tube and add 10 ml of Dulbecco's modified Eagle's medium (DMEM) + 10% fetal calf serum.
3. Centrifuge in clinical centrifuge at low speed for 2–5 min to gently pellet cells.
4. Repeat **steps 2** and **3**.
5. Completely remove supernatant and resuspend in 5 ml of growth medium without selection [GM(–): DMEM/glutamine/high-glucose/HEPES/heat-inactivated 10%

fetal bovine serum (FBS)/penicillin/streptomycin (Pen/Strep-Fungizone + 2–5 μg/ml tetracycline (*see* **Notes 30** and **31**).
6. Plate into one or two T25 flask(s).
7. At 24 h, remove 95% of medium from flask and transfer to centrifuge tube and pellet cells; resuspend in 5 ml of medium and plate into second T25 flask.
8. Feed initial flask with a fresh 5 ml of GM(–).
9. IMPORTANT: Cells will be stunned for days or even weeks. They are not dead! Be gentle and keep feeding them with some regularity to keep the tetracycline levels high. They will spontaneously start to grow extremely fast. When this happens, they will exhaust their medium, detach, and die.
10. Maintenance: once cells begin to grow rapidly, they should be fed every other day with GM(+) (DMEM/glutamine/high-glucose/HEPES/heat-inactivated 10% FBS/Pen/Strep-Fungizone + 2–5 μg/ml tetracycline, 2 μg/ml puromycin, and 300–500 μg/ml Geneticin [G-418], Invitrogen).
11. Passaging: cells should be split 1:5 when approximately 90% confluent (2–3 days without selection, 3–5 days with selection). After initial thawing and rapid growth, they should be passaged several times (2–3 weeks) in selection because G-418 can take 2 weeks to select. Continue to maintain in GM(+) until ready to make virus (*see* **Note 32**).

3.1.4. Large-Scale Production of Virus for Experiments

1. Expand stably infected producer cells in GM(+/–) medium (e.g., 1–50 10-cm dishes).
2. When sufficient plates are 70% confluent, replace GM with viral medium (DMEM/glutamine/high-glucose/HEPES/heat-inactivated 10% FBS/Pen/Strep-Fungizone).
3. Switch to 32°C incubator to improve titers.
4. Start collecting supernatants at 48 h.
5. Add Polybrene at 4–8 μg/ml (Sigma-Aldrich, cat. no. H-9268).
6. Centrifuge at 1,500 rpm for 5 min to pellet cell debris.
7. Filter through 0.45-μm low-protein binding (polyvinylidene difluoride or cellulose acetate) filter preblocked with GM.
8. Store at 4°C for titering.
9. On the final day, resuspend up all the cells and supernatant together; vortex until cloudy, even consider freeze-thaw cycle, and then transfer to 15-ml tubes; centrifuge and filter.

3.1.5. Titering

1. Seed human embryonic kidney-293 cells or NPCs in a 12-well plate at 50–75% confluence (cells need to be cycling).
2. Put 1 ml of DMEM/10% FBS/4 μg/ml Polybrene into each well 2–12.

3. Add 1 ml of raw viral supernatant to first well. NB: because VSVG env is toxic, the first couple wells of cells will likely die (i.e., always dilute raw virus at least 100-fold for real experiments).
4. Remove 100 µl from well and add to next well, and so on; afterward, move trays gently to avoid detachment.
5. After 1 week, count the infected colonies. The effective titer (infectious particles per milliliter) is the lowest dilution where you get infected colonies. Good titers are 10^6 without concentrating.

3.1.6. Sample Experiment Using Retrovirus to Mark Adults NSCs In Vitro

1. Plate human NSCs onto polyornithine/laminin-coated 10-cm tissue culture dishes at a density of 5×10^4cells/cm^2 into serum-free medium (DMEM/Ham's F-12 + N2 supplement + fibroblast growth factor [FGF]2 [20 ng/ml] + epidermal growth factor [EGF] [20 ng/ml]) (*see* first section of this volume for NSC culture protocols).
2. Allow cells to attach for 24 hours then add 1µl of raw supernatant (≈1000 iu) to the 10ml.
3. Wait 3–4 days to visualize GFP fluorescence. If you want to perform a clonal analysis, your goal is to have roughly 50 infections per plate. If you have significantly greater or fewer infected cells, you may need to adjust the amount of viral supernatant used for infection.
4. Mark each green colony with an alcohol-resistant marker underneath the plate.
5. Withdraw mitogens (FGF2 and EGF) and add proposed differentiation factor (e.g., Wnt11).
6. Allow 1 week for differentiation and then label with cell type-specific markers (*see* below).

*3.1.7. Concentrating Virus by Ultracentrifugation (*In Vivo*):*

1. LZG cells produce titers on the order of 10^6 infectious units (iu)/ml. However, in vivo studies often require retroviral titers of at least 5×10^8 iu/ml. The most efficient means of obtaining this 500- to 1,000-fold concentration is by ultracentrifugation.
2. Pool supernatant that are being stored at 4°C (*see* above).
3. Precool ultracentrifuge rotor to 4°C (1–2 h).
4. If using a swing-bucket rotor (e.g., Beckman SW-41), fill Beckman Ultraclear tubes (or equivalent) to within 2–3 mm from top with supernatant, according to manufacturer's guidelines.
5. Spin at 50,000 × *g* for 90 min at 4°C.
6. The concentrated virus should be visible as a small (1–2 mm) white pellet at the bottom of the tube.
7. Resuspend in DMEM or PBS with 4 µg/ml Polybrene to reduce clumping.
8. Make 40-µl aliquots and freeze at –80°C (*see* **Note 33**).

3.2. High-Throughput Measurements of NSC Proliferation

3.2.1. FACS Counting of BrdU-Labeled Cells

1. Starting material: cultured cells prelabeled for with a 2-h pulse of 10 μM BrdU at some time in the past.
2. Rinse cells in 500 μl of cold PBS and then pellet cells.
3. Incubate in blocking solution (no detergent) for 15 min and then repellet cells.
4. Stain cell surface epitopes, and then pellet cells and transfer to transfer them to FACS tubes on ice.
5. Add 500 μl of PBS and vortex at one-third speed.
6. Add ethanol (EtOH) with Pasteur pipette at 1 drop per second (NB: do not add EtOH too quickly).
7. Incubate on ice for 30 min.
8. Spin at medium speed to pellet cells (at 4°C, if possible), and then decant liquid.
9. Add 1 ml of blocking buffer (no detergent) into each tube, and then pellet cells and decant.
10. Add 1 ml of 1–2% PFA + 0.2% saponin in PBS.
11. Incubate at room temperature for 15–30 min.
12. Add 1 ml of DNAse buffer, and then spin and incubate at room temperature for 15–30 min (use higher speed spin, because postfixed cells are more buoyant than their unfixed counterparts).
13. Incubate in blocking solution (no detergent) for 15 min, and then pellet the cells.
14. Add anti-BrdU-fluorescein isothiocyanate antibody (Roche Diagnostics or BD Biosciences PharMingen).
15. Pipette up and down to resuspend pellet, then and incubate for at least 30 min at 4°C (or may be left overnight).
16. Rinse cells in 500 μl of cold PBS, and then pellet cells and transfer them into FACS filter tubes.
17. NB: this protocol is generally applicable to labeling any intracellular antigen before FACS counting.

3.2.2. BrdU Measurement by ELISA

1. Several excellent ELISA-based BrdU detection kits are commercially available. The easiest (and most expensive) are the one-step colorimetric and luminescent assays from Roche Diagnostics. However, in my work, the two-step kits from Calbiochem seem to yield just as good data. If you are performing many assays, these kits can get very expensive, very quickly. Described below is the more cost-conscious alternative that very closely parallels the Roche colorimetric kit.
2. Seed cells into 96-well flat-bottomed plate at density of between 10^4 and 10^5 cells/cm^2.
3. Pulse adherent cells with 10 μM BrdU for 2–4 h, and then proceed with experimental manipulations. When ready to quantify BrdU incorporation proceed to **step 2**. Leave the diagonal empty (i.e., no cells in A1, B2, C2 ... H12) to be used as controls.

4. Add 50 µl of 4% PFA to each well and incubate for 5 min at room temperature.
5. Invert over sink and tap gently on paper towels to remove liquid. Use inversion for all subsequent steps where liquid must be decanted.
6. Rinse three times with 100–200 µl of 100 mM PBS-glycine.
7. Permeabilize with 0.5% Triton X-100 in PBS for 10 min at room temperature.
8. Rinse three times with 100–200 µl of PBS.
9. Block in 3% BSA + PBS for 30 min at room temperature, and then decant.
10. Dilute anti-BrdU Fab fragment into 100 µl of DNase solution (supplementation with 10 mg/ml BSA is optional).
11. Incubate at 37°C for 1 h.
12. Rinse three times and then add TMB solution.
13. Wait 5–15 min for color to begin developing.
14. Read unquenched reaction at either 370, 620, or 655 nm on an automated microplate reader. Use the diagonal. Blank your reading using the diagonal (i.e., no BrdU control) (*see* **Note 34**).
15. My lab has found that this assay can detect roughly a 10% change in BrdU incorporation.

3.3. Cell Fate Analysis

Two general immunolabeling protocols follow. The first protocol is a simple but reliable means of counting BrdU-labeled neurons and astrocytes by means of flow cytometry. The second protocol can be used.

3.3.1. Immunohistochemistry Protocol for Detecting BrdU and Intracellular NSC-Specific Antigens

1. Perform intracardiac perfusion protocol (*see* **Subheading 3.3.2.**), and then prepare tissue sections by using methods of choice (e.g., cryostat); transfer sections to multiwell plate, and then store them in cryoprotectant at –20°C. With practice, 24-well plates are well suited to storing rat brain sections, and 48- or 96-wells plate can be used for mouse brain sections).
2. Skip to next step if using cultured cells. Otherwise, remove sections from freezer and transfer to new wells with a P10 pipette tip bent at the end and rinse three times (5 min each) with TBS to remove cryoprotectant. Skip to **step 6**.
3. If using cultured cells, they will need to be fixed at this step. To fix cells, add an equal volume of 4% PFA-PBS directly to tissue culture medium and fix for 5 min (*see* **Note 35**). It is important to never make up aldehyde fixative in buffers that contain amine groups (Tris or glycine), because they will inactivate the fixative! Although I recommend aldehyde fixation for most staining, a discussion of fixation methods is included in **Note 36**, and an alternative methanol fixation protocol is outlined in **Note 37**.
4. Wash cells TBS or TBS-glycine (TBS with 100 mM glycine) to bind up free aldehydes.

5. Optional: perform antigen retrieval (*see* protocol below). NB: generally, only necessary when staining tissue sections.
6. Permeabilize cells by incubating in either TBST or TBSS. For a discussion of choosing a permeabilization agent, *see* **Notes 38** and **39**).
7. Incubate with DNase working solution for 30 min at 37°C to uncover BrdU epitope. (This step may omitted if not staining BrdU-labeled cells.) An alternative method for uncovering the BrdU epitope based on HCl denaturation is outlined in **Notes 40** and **41**.
8. Block nonspecific binding sites with 3% normal donkey serum (NDS) in TBS/Triton or saponin for 30 min to 1 h (TBSD-T/S).
9. Transfer sections to primary antibody (anti-BrdU, if using DNase treatment) in TBSD-T or TBSD-S (antibody working dilutions can be found under **Subheading 2.**).
10. Aspirate off and save primary antibody mix for re-use. (Save primary antibody mix for re-use; the mix can be used at least twice).
11. Rinse sections three times with TBS or TBSS to remove residual unbound primary antibody.
12. Incubate the sections in fluorophore-conjugated secondary antibody in TBSD-T or TBSD-S for 1–2 h at room temperature. Incubation chambers should be covered with foil to avoid exposure to light.
13. Aspirate off and save secondary antibody mix for re-use (the mix can be used at least twice).
14. Rinse sections three times with TBS or TBS-S to remove residual unbound secondary antibody.
15. Rinse with TBS two times quickly, 1 × 10 min as above and check for background.
16. Final wash includes either DAPI (for epifluorescent microscopes) or TOPRO-3 (for confocal microscopes) to label nuclei.
17. Mount and coverslip the sections by using Prolong antifade (Invitrogen), PVA-DABCO, or PVA-NPG.

3.3.2. Intracardiac Perfusion

1. Anesthetize/sacrifice animal with ip injection of sodium pentobarbital (Nembutal, 30 mg/kg body weight).
2. Lay the animal flat and make a horizontal incision just below diaphragm (bottom of ribs); then, cut into thorax, through rib cage, vertically, past the heart.
3. Identify the left ventricle (facing you on the right side) of the animal and insert a blunt needle (21–23-gauge for mice and 18–21-gauge for rats) connected to the peristaltic pump into the ventricle and clamp in place. Snip the right atria, which allows the blood and perfusion fluid to exit the circulatory system.
4. Perfuse the animal with ice-cold PBS, pH 7–7.5 (flow rate of 3–5 ml/min), to remove red blood cells that will show up as brightly fluorescent cells throughout your micrographs (50–100 ml/mouse, 150–200 ml/rat).

5. Fix animals by perfusing fresh 4% PFA-PBS, pH 7.3–7.4 (on ice), until the animal becomes stiff, and then continue perfusing for 5–10 min (approximately 100 ml/mouse, 400–500 ml/rat).
6. Block the brain and store in 4% PFA overnight at 4°C.
7. Transfer to 30% sterile-filtered sucrose solution until the brain sinks.
8. Cut 40-µm sections on freezing sliding microtome.
9. Store sections in TCS at –20°C.

3.3.3. Antigen Retrieval of Either Cultured Cells or Tissue Sections

1. Process cells or free floating sections normally and store in 24-well plate.
2. Replace media in each well with 0.5–1 ml of 10 mM sodium citrate, pH 9 (if using slide-mounted sections, place slides into Coplin jar filled with citrate buffer) (*see* **Note 42**).
3. Float plate in 80°C water bath for 5–10 min for cultured cells and for 30 min for floating sections (*see* **Note 43**).
4. Remove from water bath and cool to room temperature on the bench top.
5. Rinse the sections three times for 5 min each in 0.1 M PBS, pH 7.4.
6. Continue with antibody labeling.

3.3.4. Sodium Borohydride Quenching of Autofluorescence

1. Prepare a 1 mg/ml solution of sodium borohydride in ice-cold PBS; inside fume hood (if possible, perform all incubation on ice). The solution will be effervescent, like carbonated water.
2. While the solution is still bubbling, apply to tissue.
3. Close plate and cover with foil.
4. Incubate at room temperature for 15 min on orbital shaker in the fume hood.
5. Discard solution and replace with fresh solution, and then repeat incubation two to four times.
6. Finally rinse three times with PBS, pH 7.4, for 15 min each, at room temperature ***(18)***.

3.3.5. DAB Substrate Staining (in Lieu of Fluorescence)

1. Rinse 2× TBS.
2. 30 min in hydrogen peroxide (1 ml/49 ml of dH_2O; store aliquots in hydrogen peroxide at –20°C; otherwise, use within 1 month if stored at 4°C).
3. Rinse 2× 0.9% saline.
4. 1 h at 37°C in 2–3 M HCl.
5. Quick rinse in TBS, pH 7.4.
6. Block for 30 min in TBS++ (TBS + 3% NDS + 0.3% Triton).
7. Overnight in primary antibody at 4°C.
8. Importantly, if using anti-BrdU, incubate for 48 h (1:500 in TBS++ with NDS reduced to 1%.

9. If Accurate Scientific & Chemical rat anti-BrdU antibody is used, make sure it is the ascites if staining rat tissue TB0030CX (the purified material is fine for mouse tissue OT0030F).
10. Rinse three times in TBS.
11. 2–4 h in biotinylated anti-rat IgG.
12. Rinse three times in TBS.
13. 2 h in AB reagent at room temperature (from Vector Laboratories, PK6100 ABC kit) prepare AB reagent 30 min before use.
14. Rinse three times in TBS.
15. React with DAB (I use the Sigma-Aldrich ISOPAC; reconstitute with 20 ml of ddH_2O, and then add 1 ml of the reconstituted DAB to 9 ml of TBS and add 60 µl of hydrogen peroxide just before incubation). This gives you about a 10-min reaction.
16. Rinse in TBS (or tap water).
17. Mount tissue and let dry overnight.
18. Dehydrate and coverslip under Permount.

3.4. Troubleshooting Immunofluorescent Labeling

3.4.1. Nonspecific Fluorescence

1. Problem: smooth, regular, punctate fluorescence (i.e., bright fluorescent dots everywhere in brain tissue).

   Reason: blood vessels may retain red blood cells when animals are not adequately perfused with saline before fixation. Solution: increase initial PBS perfusion time.
2. Irregularly shaped, bright specs throughout tissue. Reason: accumulation or either primary-secondary antibody complexes or clumps of precipitated secondary antibody.

   Solution: briefly centrifuge the secondary antibody to pull down any antibody clumps, and then add an extra rinse after incubating with primary antibody to remove all unbound primary. This will prevent precipitation of unbound primary–secondary antibody complex.
3. Diffuse tissue autofluorescence: there are two types autofluorescence: natively fluorescent tissue components (e.g., lipofuscin, red blood cells, elastin) and fixation-induced fluorescence, arising from the presence of free aldehyde groups).

   Reason: the presence of unreacted aldehydes can be particularly problematic when using paraffin-embedded tissue or anytime glutaraldehyde fixation is used.

   Solution: this problem can often be addressed using one of the following strategies:

   i. Avoid using glutaraldehyde in the initial fixation, if possible, because this will substantially reduce the number of free aldehydes in the tissue (PFA-fixed cultured cells should never exhibited significant autofluorescence).
   ii. Exogenous amine-containing reagents can react with and neutralize free aldehydes (e.g., 0.1 M glycine in TBS or PBS, pH 7.4, for 1 h at room

temperature). In theory, the proteins included in the blocking steps should subsume the same function, but they may be insufficient because of poor tissue penetration.

iii. Reduce free aldehyde groups to alcohols through treatment with sodium borohydride, a reducing agent (*see* sodium borohydride quenching protocol above).

iv. Should the aforementioned techniques fail, you should consult one of the reviews on troubleshooting autofluorescence for alternative quenching strategies (e.g., use of Sudan Black) ***(19)***. Also, consider the online reference http://www.uhnres.utoronto.ca/facilities/wcif/PDF/Autofluorescence.pdf.

4. Diffuse Labeling of Across Coverslip

Reason: this can be seen when immunolabeling for secreted small molecules that can become fixed to the underlying growth substrate (e.g., amino acid neurotransmitters).

Solution: Wash cells in PBS before fixation.

3.4.2. Poor Staining (*see* **Note 44**)

1. Diffuse absence of NeuN staining, even in hippocampal cell layers.

Reason: NeuN antigen reactivity is reduced after 30 min (or even less) of ischemia ***(20)***.

Solution: improve intracardiac perfusion technique so that as little time as possible elapses between sacrificing the animal and perfusion fixing the brain (NB: ensure that buffers are cold to reduce cellular enzymatic activity).

2. Proliferating cell nuclear antigen (PCNA) staining works well in paraffin-embedded tissue, but not cryostat sections or cultured cells.

Reason: epitope is often not accessible in cryostat section without addition denaturation steps.

Solution: repeat antigen retrieval step twice. Alternatively, for fresh tissue/cells, consider the following two-step fixation procedure (fix in 4% PFA for 5 min at room temperature, and then rinse and refix for 5 min in ethanol-acetic acid) (2:1, v/v) ***(21)*** (NB: DNase treatment is much less effective at uncovering the PCNA epitope than when used for BrdU labeling).

3. Inadequate staining of microtubules or membrane proteins.

Solution: use EGS/Sulfo-EGS fixation instead of paraformaldehyde.

i. Rinse cells with prewarmed (37°C) PBS.

ii. Decant 90% PBS.

iii. Working quickly, add appropriate volume of EGS diluted in PBS to final concentration of 10 mM (some researchers would recommend using the Sulfo-EGS form because it is water-soluble, but not cell-permeant, thus it will only fix antigens on the cell surface. However, it can be much more expensive).

iv. Incubate (covered) at 37°C for 10 min.

v. Rinse three times with PBS-glycine or TBS.

vi. Permeabilize with TBST for 5 min at room temperature.
vii. Rinse and proceed with staining protocol.

3.4.3. FACS Problems

1. BrdU seems to have worked, but FACS counts show profound skewing of data toward unlabeled or singly labeled particles, if using two antibodies.
   Reason: HCl denaturation can make cells quite susceptible to mechanical damage.
   Solution: use DNase protocol.
2. Cells disintegrate during centrifugation, vortexing or sorting even in the absence of HCl treatment.
   Reason: detergents such as Triton X-100 dissolve cell membranes, making cells more fragile.
   Solution: use milder, reversible detergent such as saponin.
3. FACS count produces predominantly doublets, rather than single particle counts.
   Reason: Although many histological treatments and conditions can induce cell clumping, two of the biggest offenders include use of alcohol-based fixatives, and incomplete tissue digestion/dissociation.
   Solutions: three solutions follow:
   i. Add ethanol dropwise while swirling cells or switch to 2% PFA fixative.
   ii. Use fresh enzyme, incubate at 37°C, ensure cofactor requirements are met (e.g., some enzymes require cysteine or magnesium as cofactors) and use a calcium- and magnesium-free medium to aid in dissociations.
   iii. Use filter FACS tubes BD 352235 12 × 75 5-ml tube with 70 μM filter cap, nonsterile.
4. No signal in APC channel.
   Reason: ethanol destroys APC signal.
   Solution: Fix with PFA 1–2%.

3.4.4. Loss of Fluorescence

1. Quantum dots (e.g., QDots, Invitrogen) lose fluorescence after several days.
   Reason: QDot's metallic salt core is covered by a polymer coating that is dissolved by many types of mounting media (e.g., Gelvatol). Once dissolved, the fluorescence is lost.
   Solution: mount in plain glycerol/PBS (1:1) and seal with nail polish.
2. Bright, beautiful looking cells are there one minute and gone the next.
   Reason: photobleaching.
   Solution 1: turn down the laser power, if using a confocal, or insert a neutral density filter into the illumination path, if using a standard fluorescent microscope.
   Solution 2: use a more stable fluorophore (e.g., Alexa 488 bleaches slower than fluorescein).

Solution 3: use a mounting medium that contains an antifade compound. There are many to choose from, but Prolong Antifade is the best when using Alexa-conjugated secondaries, but it is the most expensive as well. Less expensive alternatives include homemade PVA-DABCO, PVA-NPG, or glycerol-NPG (*see* **Note 45**).

4. Notes

1. For cytoskeleton and microtubules, you can fix in either PEM or CB buffer if having problems with plain PBS (+ Mg^{2+}/Ca^{2+}).
2. There is lore that when dissolving PFA, you should not heat the solution >60°C, because the formaldehyde will rapidly convert formic acid. Although a small amount of acid is produced, it is not significant, fortunately, for everyone who forgot to stick the thermometer in the PFA while it was stirring on the hot-plate ***(1)***.
3. Make these solutions no more than 24 h before perfusion and keep ice cold. Although older solutions may be fine for fixing cultured cells, these aged PFA solutions contain increasing amounts of formic acid that impair fixation and may elevate autofluorescence. In addition, many epitopes are sensitive to modification during initial perfusion (e.g., phosphoproteins may dephosphorylate when ATP levels drop). These changes can be minimized by working quickly and efficiently initiating perfusion with ice-cold phosphate-based buffers.
4. Never use Tris or other amine-containing buffers, because they bind up aldehydes, thereby inactivating the fixative.
5. PFA is toxic and volatile. For safety, perform all operations in a fume hood (not a tissue culture hood) to avoid breathing in either the PFA dust or fumes.
6. Buffer your sample according to the optimal pH range for your specific fluorophore.
7. A few general notes on this antibody list: first, the antibody supplier can make a big difference in specificity, affinity, working dilution, and so on, even if both antibodies were generated against the same peptide epitope in the same animal. Second, no single marker is pathognomonic for any immature cell type, which is why you need to use a panel of markers. Third, antibodies that recognize epitopes across species are not necessarily labeling the same cells, in the same developmental stage. Fourth, generally, you can use a lower concentration of antibody than stated when working with gently fixed cultured cells.
8. PCNA is expressed during most of the cell cycle: G1-S-G2/M, not G0. This antibody can be tricky to work with (*see* **Subheading 3.4.2.2.**).
9. HH3 expression is limited to phase G2/M of the cell cycle.
10. MCM2 is expressed throughout the cell, but even in G0 there may be some limited expression (not usually significant).
11. Ki-67 begins to be expressed in late G1, and then it remains high throughout the rest of the cell cycle, but it is almost undetectable 2 h into G0.

12. The antibody is specific for human cells, but not entirely sensitive, sometime labeling only 50% of cells in culture.
13. These dyes are more resistant to bleaching than the Cy2, -3, -5, -7 dyes, FITC, or rhodamine, and they are relatively insensitive to pH changes. However, Alexa 350 still bleaches rather quickly, although not as fast as 7-amino-4-methylcoumarin-3-acetic acid.
14. SSEA2/3 do not recognize murine embryonic stem (ES) cells.
15. SSEA1 is only NSC-specific antibody in human cells; in mouse, it is expressed by ES cells, but it is downregulated upon differentiation.
16. Developed as proprietary antibody by Stem Cells Inc., but it may be obtainable from the Wiessman Laboratory at Stanford University (Stanford, CA).
17. Classically, PDGFRα was considered a specific marker of multipotent glial progenitors (O-2A cells). However, more recent evidence suggests that PDGFRα-positive cells in SVZ of rodents can form neurons as well as glia ***(12)***. Whether this is the case for human progenitors is yet to be established.
18. You want the farthest red-emitting antibody to label the antigen with the greatest density.
19. This cell suspension also may be derived from acutely dissociated human/mouse brain. In such case, you can refer to Dr. Rao's chapter in the volume, detailing the isolation of stem cells from fetal tissue.
20. NSCs are surprisingly sensitive to growth factor withdrawal (e.g., 15 min in insulin-free medium reduces glycogen synthase kinase-3β activation by >75%. Therefore, I try to always keep NSCs in medium that contains at least insulin and the least transfers.
21. Count viable cells by using a vital dye such as trypan blue.
22. If using acutely dissociated human tissue, you may not have a full million cells. I have found yields of 3–5 × 10^5 cells are perfectly usable, it will just take longer to sort.
23. If your FACS machine does not handle 96-well plates, staining can be carried out in polypropylene tubes.
24. Half of what the manufacturers recommend is a good starting point.
25. If you are using an antibody that reacts with insulin, transferrin, or a growth factor that may be adsorbed to the BSA in BIT9500, then you should substitute plain fraction V BSA (Sigma-Aldrich) at 5–10 mg/ml.
26. I have observed that some neuronal lineages do not fare well when incubated on ice (e.g., retinal ganglion cells). In such cases, try incubating cells at room temperature. The main drawback is the risk of receptor internalization.
27. If you intend to modify this protocol for use in cell counting, you will need to prepare single labeled controls in parallel. These controls will allow the technician to compensate for bleed-through fluorescence, ensuring more accurate counts. These steps have been left out of the sort protocol, because fresh NSCs are often very limited in number.
28. An extensive set of protocols also are available from the Herzenberg Laboratory at Stanford (http://herzenberg.stanford.edu/Protocols/default.htm).

29. The LZG line is based on the original 293 GPG cells, which were generated from HEK-293 (not 293T) cells in Richard Mulligan's laboratory ***(14)***. The 293GPG line produces high-titer amphotropic, VSV-G pseudotyped retrovirus in the absence of tetracycline. My laboratory has infected the 293 GPG cells with the LZRS-CAG-mut4GFP, a replication competent retrovirus ***(13)***. Clones were grown out and selected for those producing the highest titers when tetracycline is withdrawn.
30. Many cows are fed tetracycline; thus, their calves' serum contains high tetracycline levels. Therefore, it is safest to use an FCS that has been screened for the absence of tetracycline (*see* **Subheading 2.**).
31. Tet only has an effective half-life of about 24 h.
32. Make sure to freeze down a few dozen vials of freshly selected cells at every couple of passages. In addition, when freezing use DMEM + %10 FCS + %10 DMSO + 2 µg/ml tetracycline, but not Pen/Strept/Fungizone/G-418/puromycin).
33. Either concentrated or unconcentrated virus can be stored at 4°C for short-term use. When frozen at –80°C, the virus will last indefinitely; however, titer drops by one half for each freeze-thaw cycle. Virus can be stored in any buffered medium containing Polybrene.
34. The reaction can be halted by adding 50 µl of 1 N HCl, for endpoint assays. The yellow end product can then be read at 450 nm and it is stable for $\geq$1 h.
35. Surface tension frequently rips unfixed cells off the coverslips during the initial wash. Fixing cells before the first wash will not affect specificity of intracellular antibody labeling, but it will save you from loosing all your cells.
36. Choosing a fixative (crosslinking versus precipitating):

    a. Cross-linking fixatives include glutaraldehyde, formaldehyde, and formalin. (NB: PFA is actually polymerized formaldehyde, and it requires depolymerization in buffer to be active. Formalin is buffered formaldehyde that has methanol added to prevent repolymerization.) The primary advantage of cross-linking fixatives is that they preserve the three-dimensional architecture of cells and tissue. Amongst cross-linkers, paraformaldehyde is generally preferred over glutaraldehyde, because its small molecular weight allows it to permeate tissue more quickly, while more "gentle" cross-linking reduces background fluorescence. Glutaraldehyde is used in applications such as immunoelectron microscopy that require a more powerful and irreversible cross-linker. The two major drawbacks to aldehydes are the generation of free aldehyde groups that can autofluorescence and the potential for covalently modifying target proteins, thereby destroying the antibody recognition site.
    b. Fixation by precipitation (e.g., –20°C methanol or acetone) has the following advantages: (i) Fixation occurs more quickly; however, given the short fixation time for cultured cells, speed is rarely an issue. (ii) Because precipitation does not covalently modify target protein, solvent fixation rarely destroys epitope (presuming the protein was not washed away by the solvents). (iii) Solvents such as methanol simultaneously permeabilize cellular membranes, which may eliminate the need for detergent

permeabilization. (iv) Precipitation does not introduce autofluorescence into cells/tissue. However, fixation with organic solvents has its drawbacks, including it solublizes and removes membrane bound antigens, and it poorly preserves three-dimensional structure. This is less of an issue when studying NSCs in vitro, but it can make tissue unsuitable for confocal microscopy. In addition, methanol-fixed cultured cells can shrink to half their size. (NB: thicker specimens may actually look better after shrinkage, because a greater proportion with be within the very limited depth of focus found on high numerical aperture objective).

37. Methanol fixation protocol: immerse coverslips in 100% methanol at –20°C for 5 min and rehydrate coverslips in TBS or TBSS for 5 min at 4°C.
38. Choosing a permeabilization method:

    a. Saponin is a relatively mild detergent that solvates cholesterol present in the plasma membrane. At low concentrations, internal membranes remain intact. The detergent saponin has been shown to intercalate in the membranes to replace cholesterol and to permeabilize cells in a reversible way, maintaining much of the morphology of the membrane structure of the cell. It is useful for labeling smaller molecules that exist in a soluble state within the cytoplasm. It should be prepared as a stock in DMSO, and it is typically used at 0.5 to 1 mg/ml.
    b. Triton X-100 is probably the most commonly used permeabilization agent for immunofluorescent staining. This detergent efficiently solvates cellular membranes without disturbing protein–protein interactions. This produces irreversible permeabilization of cell membrane

39. Saponin permeabilization is reversible, unlike that with Triton X-100. Therefore, if saponin is used, it must be included in all steps, including all washes. Otherwise, the membrane will reseal, barring entry of the antibodies into the cell.
40. HCL denaturation of BrdU epitope: DNase treatment is usually sufficient to unmask this epitope. However, if BrdU staining looks erratic or absent, then this more time-consuming protocol can be used as an alternative. These steps should be performed after all other antibody labeling is completed, because HCl destroys many epitopes:

    a. Stains sections with primary and secondary antibodies.
    b. Rinse three times in PBS.
    c. Postfix cells/sections with either 2 or 4% PFA, respectively (my laboratory uses frozen PFA). This ensures that the primary–secondary labels stay bound to cells.
    d. Rinse in 0.9% saline two times.
    e. Incubate in 1 M HCl for 30 min (cells) or 1 M HCl (if new bottle, otherwise use 2–3 M) for 1 h (tissue) at 37°C (*see* **Note 41**). If staining tissues on slides, immerse slide in prewarmed HCl for 30 min.

f. Depending on amount of tissue processed, replenish with fresh HCl (unnecessary for cultured cells). Use 2 ml of fresh 1 M HCl per every 10–20 sections.
g. Acid is aspirated from the wells and the acid is neutralized by washing three times in borate buffer, pH 8.5 (or just use regular buffer and do many more washes).
h. Quick rinse in TBS.
i. Block for 30 min in TBS++ (TBS + 3% NDS or BSA + 0.3%Triton-X).
j. 48 h in anti-BrdU (1:500 in TBS++ with NDS reduced to 1%).
k. Rinse three times in TBS.
l. Overnight in secondary antibody (TBS++ with NDS reduced to 1% different fluorophore conjugate than used for other secondaries).
m. Rinse three times in TBS (add TOPRO-3 or DAPI to final rinse).
n. Mount and coverslip with antifade agent.

41. IMPORTANT: HCl solution must be made fresh each time. HCl will degrade in the stock bottle, and if the bottle does not aggressively fume when opened, increase concentration to 2 or 3 M if initial staining fails to detect BrdU. NB:?? 1 M (if new bottle) HCl in H_2O = 8.4 ml conc. HCl + 91.6 ml ddH_2O.
42. Most common antigen retrieval protocols call for heating section in an acidic buffer (e.g., 20 mM citrate, pH 6.0), and in many cases, heat denaturing the protein and then allowing it to cool at an acidic pH provides better antibody access than if the tissue is allowed to cool at neutral pH. However, the literature consistently shows that heating in an alkaline buffer (pH 8–9) produces superior staining for most antigens encountered in NSC research ***(15–17)***.
43. Most common antigen retrieval protocols call for heating section in an acidic buffer (e.g., 20 mM citrate, pH 6.0, and in many cases, heat denaturing the protein and then allowing it to cool at an acidic pH provide better antibody access than if the tissue is allowed to cool at neutral pH. However, the literature consistently shows that heating in an alkaline buffer (pH 8–9) produces superior staining for most antigens encountered in NSC research ***(15–17)***.
44. Do not forget the possibility that the reason you are not seeing anything is because nothing is there. Everybody, at one time or another, is convinced that their marker is staining weakly, but specifically. This leads to an endless tweaking of the microscope, antibody concentrations. Be careful not to deceive yourself into seeing what you want to see. Here, are a few tips that may help:

    a. If you adjust the confocal settings long enough (e.g., laser power, gain, pinhole), everything will look labeled.
    b. Every fluorescent marker can bleed through into higher wavelength channels.
    c. All tissue has some autofluorescence when illuminated at 488 nm, especially neurospheres.

45. Avoid P-phenylendiamine-based media, because it discolors over time more than *n*-propyl gallate ***(22)***.

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III

In Vitro Studies: B. Molecular Techniques

21

Differential Gene Expression in ES-Derived Neural Stem Cells by Using RT-PCR

Nicole Slawny, Crystal Pacut, and Theresa E. Gratsch

Summary

Embryonic stem (ES) cells hold promise to treat a variety of disease. The major obstacle is to determine the requirements that will drive these cells to a particular lineage. Two approaches to examine lineage commitment are the addition of growth factors or directed differentiation of ES cells. Although many neural genes have been identified, the cascade of gene expression that directs neural differentiation is not well understood. Today, with microarray technology, large data sets of differential gene expression patterns are used to identify genes that may be used as indicators of a particular cell lineage or tissue type. Semiquantitative polymerase chain reaction (PCR) can be carried out to verify the expression of individual genes, followed by quantitative PCR to precisely determine the level of mRNA expression. However, functional analysis of potential neurogenic genes must be done to identify those genes that play a critical role in neural lineage commitment.

Key Words: ES cells; differential gene expression; neural lineage commitment; RT-PCR; qPCR; gradient PCR; SYBR Green.

1. Introduction

Reverse transcription and the polymerase chain reaction (RT-PCR) provides a very sensitive method to identify known genes that are both upregulated and downregulated during neuronal differentiation. More recently, quantitative PCR (qPCR) has been developed to precisely determine the changes in the level of gene expression between two or more cell or tissue populations. Genes that are differentially expressed suggest potential candidates that may play a role in commitment to a particular lineage. The single most critical factor to any of

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these assays is the quality and integrity of the RNA, because this is the template for all subsequent reactions. Once high-quality RNA has been isolated, first strand cDNAs are generated in the initial reaction with reverse transcriptase (RT) in the presence of random nonamers or oligo(dT)-primed RNAs. The resultant cDNAs provide the templates for amplification of gene-specific targets with the appropriately designed primers. The presence of chromosomal DNA in the RNA preparation will influence the reliability of both RNA concentration and the PCR products generated from these RNA templates in the reverse transcription reactions. Many parameters in PCR have to be optimized for each gene-specific primer pair and template; they include primer and $MgCl_2$ concentrations, primer annealing temperatures, and the number of PCR cycles ***(1–3)***.

There is a huge difference in the costs of semiquantitative and qPCR reagents, so we initially screen candidate genes by semiquantitative PCR, and those positive for differential expression are then subjected to qPCR to precisely determine -fold changes in expression. The requirements for template concentration, primer design, and optimization are more stringent for qPCR ***(4)***. These include gradient PCR, melt curve, and standard curve analysis. Generally, primers designed for qPCR also can be used in semiquantitative PCR, but the opposite is not the case. Each qPCR template and primer are run in triplicate and compared back to a reference gene (i.e., β-actin) to determine differences in the level of expression.

Once potential candidate neural genes have been identified, the next step is to determine their functional involvement in neural differentiation. Functional analysis is initially carried out by either overexpression or knockdown of a particular gene. *In Situ* hybridization also may be done to determine the temporal and spacial expression pattern for the selected gene. A valuable technique for all of these studies involves the purification and cloning of RT-PCR products. We present an efficient TA cloning method to directly clone RT-PCR products.

2. Materials

2.1. RNA Extraction and Purification

1. TRIzol Reagent (Invitrogen, Carlsbad, CA).
2. Chloroform.
3. Isopropanol alcohol.
4. 75% Ethanol in diethyl pyrocarbonate (DEPC) H_2O (*see* **Note 1**).
5. DNase I [1 U/µl] 10× buffer and 50 mM EDTA (Sigma-Aldrich, St. Louis, MO).
6. 100% ethanol.
7. DEPC H_2O/RNase-free H_2O.

8. 1.5-ml microtubes.
9. Agarose.
10. 10× Tris borate-EDTA (TBE): 108 g of Tris base, 55 g of boric acid, 0.5 M EDTA, pH 8, 40 ml to 1 liter with double-distilled H_2O (ddH_2O).
11. Ethidium bromide (EtBr) (10 mg/ml).
12. RNA loading buffer: 90% deionized formamide, 10 mM NaOH, 1 mM EDTA, 0.1% bromphenol blue, and xylene cyanol.
13. TE buffer: 10 mM Tris-Cl and 1 mM EDTA.
14. UV spectrophotometer.

2.2. Reverse Transcription

1. Total RNA (100 ng–1 µg) per reaction.
2. DEPC H_2O.
3. Oligo$(dT)_{12-18}$ primer (0.5 µg/ml) (Invitrogen).
4. Random nonamers, 250 mM (Illumina, San Diego, CA).
5. 0.1 M dithiothreitol (DTT; Clontech, Mountain View, CA).
6. 10 mM dNTP mix, 10 mM each of dATP, dCTP, dGTP, dTTP (Invitrogen).
7. PowerScript Reverse Transcriptase and 5× First strand buffer (Clontech).

2.3. Polymerase Chain Reaction

1. RT template (*see* **Subheading 2.2.1.**).
2. 200-µl thin-walled PCR tubes.
3. Taq DNA polymerase [5 U/µl] and 10× Taq DNA polymerase (Pol) buffer, (Invitrogen).
4. 50 mM $MgCl_2$ (Invitrogen).
5. 10 mM dNTP mix (same as **item 6** under **Subheading 2.2.**).
6. Gene specific primers, 10–20 pmol/reaction.
7. Dimethyl sulfoxide (DMSO) (Sigma-Aldrich).
8. 5 M betaine (Sigma-Aldrich).
9. Thermocycler (MJ Research Watertown, MA, MJR PTC100).
10. 25-base pair (bp) or 100-bp DNA ladders (New England Biolabs, Natick, MA and Invitrogen).
11. DNA gel loading buffer: 40% glycerol, 0.1 M EDTA, pH 8.0, and 0.5% sodium dodecyl sulfate and bromophenol blue (Sigma).

2.4. Quantitative PCR

1. RT template (*see* **Subheading 2.2.1.**).
2. DMSO (Sigma-Aldrich).
3. 5 M betaine (Sigma-Aldrich).
4. Q SYBR-Green Supermix (Bio-Rad, Hercules, CA).
5. Gene-specific primers (Illumina and Invitrogen), 5–10 pmol/reaction.
6. 25-bp and 100-bp DNA Ladders (Invitrogen and New England Biolabs).

7. Agarose.
8. DNA gel loading buffer (*see* **Subheading 2.3.**, **item 11**).
9. 200-µl thin-walled PCR tubes and optically clear PCR tubes.
10. Bio-Rad I-Cycler.
11. Gradient thermocycler (Eppendorf, Hamburg, Germany, Mastercycler 5331).
12. Software: iCycler iQ version 3.0 (Bio-Rad).

2.5. Purification and Cloning of PCR Products

1. DNA gel extraction/purification kit (QIAGEN, Valencia, CA).
2. pBS SK cloning vector (Stratagene, La Jolla, CA).
3. EcoRV 10 U/µl (New England Biolabs).
4. 10 mM dTTP (Invitrogen).
5. T4 DNA ligase [1 U/µl] and 5× T4 DNA ligase buffer (Invitrogen).
6. DH5 α *Escheerichia coli* competent cells (Invitrogen).
7. Super Optimal Catabolite [S.O.C] medium (Invitrogen).
8. LB agar and LB broth.
9. Ampicillin (20 mg/ml).
10. Colony lysis buffer: TE buffer with 0.1% Tween.
11. T3 and T7 primers.
12. Standard PCR reagents (*see* **Subheading 2.3.**).
13. Plasmid prep kit (QIAGEN).

3. Methods

3.1. RNA Extraction and Purification

This procedure involves the extraction and purification of total RNA from undifferentiated and neural differentiated mouse or human embryonic stem (ES) cells. Precautions must always be taken when working with RNA because of the abundance of RNases in the environment (*see* **Note 1**). The presence of genomic DNA in the RNA preparation will interfere with the PCR and can yield products independent of mRNA expression. The RNA is treated with RNase-free DNase I to remove chromosomal DNA. Gel electrophoresis and spectrophotometry verify the integrity and purity of the RNA sample. There are a variety of reagents and kits available for the isolation of total RNA; we prefer the TRIzol Reagent and follow the manufacturer's protocol.

1. To harvest ES cells for RNA, remove the culture medium and discard. Add 500 µl of TRIzol to the cells in a 35-mm dish (2–5 × 10^5 cells); generally, we use six-well plates, or 35-mm dishes. Gently rock the plate so the TRIzol covers the cell monolayers, with a pipette transfer the cell lysates to 1.5-ml tubes and place on ice immediately. Cells can be scraped as well and then transferred to a 1.5-ml tube. If there are more plates to harvest, be sure to keep all cell lysates on ice

until all plates have been harvested. At this point, the samples can be stored at –80°C for at least 1 month. To finish the extractions at a later time, thaw tubes on ice and then proceed to step 2.

2. Incubate tubes at room temperature (rt) for 5 min. Then, add 100 µl of chloroform to each tube, shake tubes by hand for 15 s, and incubate samples at rt for 3 min. Centrifuge samples at 12,500 rpm at 4°C for 10 min.
3. Carefully pipette off the upper, aqueous phase (60% of TRIzol vol or 300 µl) and put in a fresh 1.5-ml tube (*see* **Note 2**). Add 250 µl of isopropanol to each tube, mix by inversion, and incubate rt for 10 min.
4. Centrifuge 4°C for 10 min. Pour off the isopropanol from the side opposite the pellet (the pellet will be clear and gel-like, but usually visible). To wash the pellet, add 500 µl of 75% ethanol ETOH (in DEPC H_2O) and centrifuge 4°C for 10 min.
5. Remove the ETOH gently with a pipette and discard. Recentrifuge the samples for 1 m. Carefully pipette off the residual ETOH (~100 µl); do not disturb the pellet. Do not let the pellet dry completely; it will be difficult to resuspend.
6. RNA pellets are resuspended in 10–100 µl of DEPC H_2O, dependent on cell number, and keep on ice. For 5×10^5 cells (1–35-mm dish), resuspend the pellet in 10–20 µl with yields of 0.5–1 µg/µl RNA. Determine the concentration of RNA by UV spectrophotometry (*see* **Note 3**).
7. Electrophorese 2 µl of RNA in a 1% agarose gel with 0.3 µg of EtBr/ml and 1× TBE (standard agarose gel). To each 2 µl RNA sample, add 2 µl of DEPC H_2O and 1 µl of RNA loading buffer and heat denature at 65°C for 10 min. Put tubes on ice and load directly. Run gel for 2 h at 100 V. Examine the gel on an UV transilluminator and photograph (*see* **Fig. 1**).
8. Treat 1 µg of the RNA with DNase I amplification grade (Sigma-Aldrich), 1 µl of 10× DNase I buffer, 1 µl of DNase I (1 U/µl); bring volume to 10 µl with DEPC H_2O. Let reaction incubate at rt for 15 min; stop the reaction with 1 µl of 25 mM EDTA, heat inactivate DNase I, 65°C/10 min (*see* **Note 4**).
9. β-Actin PCR (before RT reaction) ensures that the DNase I removed the genomic DNA. Prepare a master mix (*see* **Note 5**), and set up the following reaction for each RNA template: 39.5 µl of H_2O, 5 µl of 10× Taq polymerase. buffer, 2.5 µl of 50 mM $MgCl_2$, 0.5 µl of 10 mM dNTPs, 1 µl (10 pmol)/each primer, 0.25 µl of Taq polymerase, and 0.25 µl of RNA. PCR conditions: 94 °C/3 min (94 °C/30 s, 55° C/30 s, 72°C/1 min) × 25–30 cycles, 72°C/7 min. Be sure to include a positive control template. Analyze PCR products in 1% agarose gel; DNased RNAs should not yield a PCR product (*see* **Note 4**).

3.2. Reverse Transcription

RT will use the purified RNA as a template to synthesize a first strand cDNA in the presence of random nonamers or oligo(dT) primers (*see* **Note 6**). The result is thousands of cDNA copies of the cellular mRNAs, which provide templates for semiquantitative and quantitative PCR analysis. The amount of

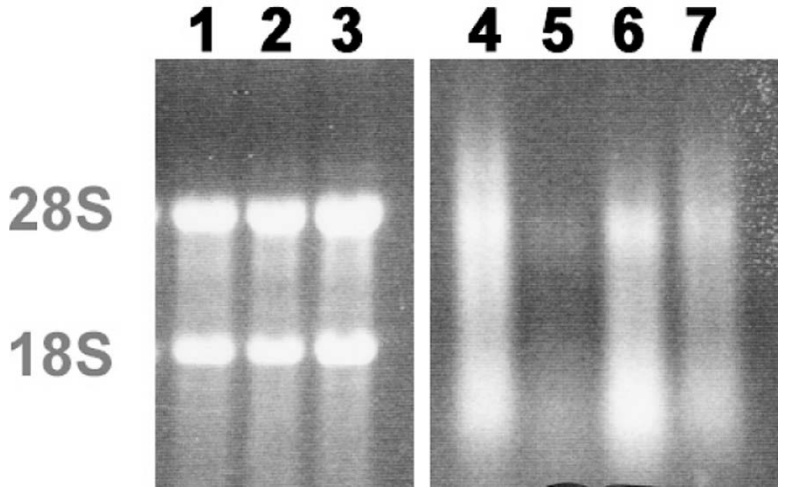

Fig. 1. Agarose gel of purified total RNA. A good RNA prep will have two distinct bands at 4.6 and 2.1 kb, representing the 28S and 18S ribosomal bands, lanes 1–3. Degraded RNA has a smeary appearance and no distinct bands, lanes 4–7.

total RNA used in RT reactions can range from 100 ng–1 µg, but equivalent amounts of RNA must be used if gene expression patterns are to be compared.

1. Denature the RNA template as follows: combine RNA (1 µg = 11 µl of DNased RNA; see **Subheading 3.1.8.**), 0.5 µl of oligo(dT) (0.5 µg/µl) or 1 µl of 250 µM random nonamers in a 0.6-ml PCR tube to a total volume of 11.5–12 µl. Incubate tubes at 70°C for 2 min.
2. To the denatured RNAs add 1.5 µl of 0.1 M DTT, 4 µl of 5× first strand buffer, 2 µl of 10 mM dNTP mix and 1 µl of PowerScript RT. Master mixes should be made as much as possible to decrease pipetting errors (*see* **Note 5**). Incubate at 48°C for 1 h and 30 min and heat inactivate RT at 70°C/10 min (*see* **Note 7**). The RT templates are ready to use in PCR.
3. **Steps 1** and **2** can all be done in a thermocycler, H_2O bath, or heat block. We find using the thermocycler gives the most consistent results experiment to experiment.

3.3. Polymerase Chain Reaction

The RT reactions serve as templates with specific primers to amplify selected gene targets in cells or tissues by PCR. We use semiquantitative PCR routinely to characterize mouse embryonic stem cells or human embryonic stem cells transfected with either overexpression or knockdown plasmids (short hairpin RNA) that contain or target potential neurogenic genes *(**5,6**)*. We have designed mouse and human primers to identify stem cells (Nanog, Oct-4, Sox2), neural progenitors (Nestin, Musashi 1, Sox3), pan neuronal (Neuronal

tubulin, Sox1, NeuroD1, -2, -3), epiderm (Keratin 14, BMP2, -4), mesoderm (Brachyury), endoderm (Sox17, Gata 4), and a positive control gene (β-actin). The positive control primers represent a gene that is expressed at the same level by all cells, typically β-actin or glyceraldehyde-3-phosphate dehydrogenase (GAPDH). The primers selected for PCR can be customized for individual projects. Recently, large scale gene expression patterns from different cell populations can be generated by approaches such as microarrays, differential display, or serial analysis of gene expression. RT-PCR is used to confirm differentially expressed genes of interest identified in these gene screens. We also use semiquantitative RT-PCR as a platform to select candidates for quantitative PCR assays in which the -fold changes in expression is determined between cell populations.

Many parameters in PCR have to be determined for each gene-specific primer pair; they include primer and $MgCl_2$ concentrations, primer annealing temperatures, template concentration, and cycle number (*see* **Note 8**). PCR products also can be generated if there is a contaminant present; any reagent in the reaction could be contaminated with a DNA template. This is caused by nonsterile techniques such as not changing pipette tips between tubes. Therefore, it is essential to have a reagent only (no RT template) control with each PCR.

All reactions are set up in 200- or 500-µl RNase/DNase-free, thin-walled PCR tubes.

1. A master mix (*see* **Note 6**) is made for each primer pair to be tested. For each 50-µl reaction, combine 1 µl of the total RT reaction, 5 µl of 10× Taq DNA polymerase buffer, 1.25–2.50 µl (1.5–2.5 mM) 50 mM $MgCl_2$, 0.5 µl 10 mM dNTP mix, 1 µl (10 pmol) of each primer (i.e., β-actin forward and β-actin reverse), 0.25 µl of Taq DNA polymerase (5 U/µl), and bring total volume to 50 µl with RNase/DNase-free H_2O. Make sure tops are closed tightly (to prevent evaporation) and place tubes in thermocycler.
2. Always test the "new" RT templates first with the + control primer, β-actin before other gene specific primers. Always include a positive template control (this can be any previously synthesized RT template) for the primer pair and a reagent only (no template) control. Standard PCR conditions are: 94 °C/3 m (initial denaturation), 94 °C/30 s (denaturation/cycle), 50–64° C/30 s-1 m (annealing, primer dependent), 72° C/1–2 m (extension, products synthesized 500bp/30s) for 20–35 cycles, 72° C/10 m (final extension).
3. Electrophorese the β-actin PCR products in a 1% agarose gel with 1× TBE and EtBr. Combine 1 µl of 10× DNA loading buffer and one fifth of the total reaction (10 µl). Also include a 100-bp DNA ladder and 1× DNA loading buffer in a total volume of 10 µl, so that the size of the PCR product(s) can be determined. The gel is run at 100 V for 1–2 h.

4. Examine the gel on an UV transilluminator and photograph. The positive control should yield a PCR product of the expected size and same intensity in all reactions except the reagent control. The ES cell and neural stem cell markers will vary in intensity between the experimental templates (*see* **Fig. 2** and **Note 9**).

3.4. Quantitative RT-PCR

qPCR or real-time PCR is a method of simultaneous DNA quantification and amplification, capable of detecting and quantifying very small amounts of DNA, or cDNA in a sample. Common methods of quantification include the use of fluorescent dyes that intercalate with double-stranded (ds)DNA and

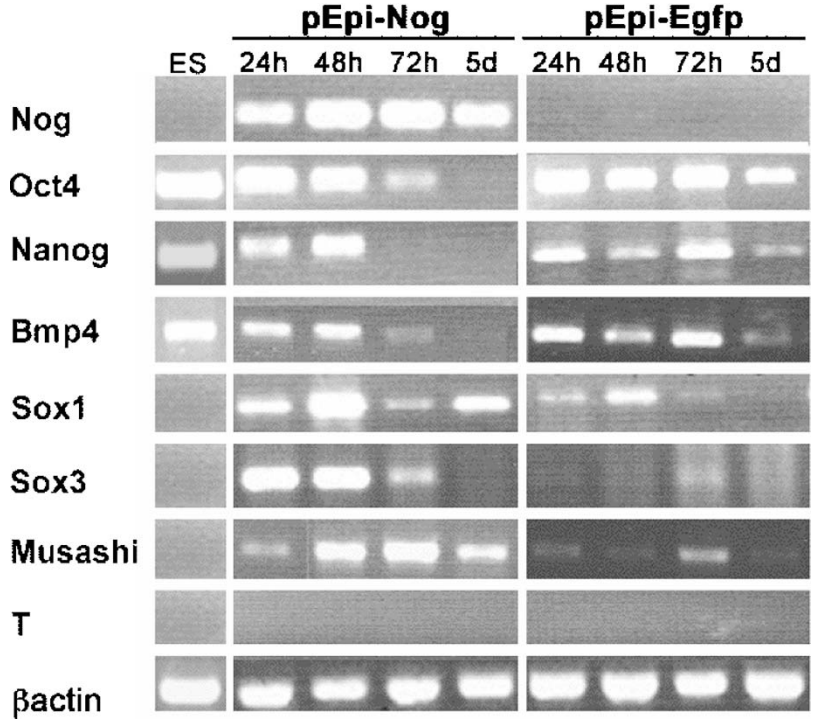

Fig. 2. Gene expression. RT-PCR analysis of noggin and control (*Egfp*)-transfected ES cells. All cells were maintained in neuronal differentiation medium and RNA extracted at 24 h, 48 h, 72 h, and 5 days. The pluripotent ES markers Nanog and Oct4, as well as the noggin antagonist Bmp4 are downregulated with noggin expression, whereas expression is maintained in the controls. Neural progentior markers Sox3 and Musashi 1, and pan neuronal Sox1, are upregulated in the noggin-transfected cells compared to controls. An ES cell control is included to show the endogenous expression of each gene.

modified DNA oligonucleotides/probes that fluoresce when hybridized with a complementary DNA. SYBR Green I is a dye that binds to dsDNA and produces a fluorescent signal. The dye allows for analysis of many different targets without generating target-specific–labeled probes. The intensity of the signal is comparative to the amount of dsDNA (PCR product) present in the reaction. At each step of the PCR reaction, the signal intensity increases as the amount of gene-specific product increases; this provides a very simple and reliable method to monitor the generation of product in real time. Another advantage of the SYBR Green technique is that unmodified oligonucleotide primers are required that make primer synthesis easier and less expensive. Primer design is critical; qPCR is very sensitive to primer dimers and secondary structure, so analysis must be done to select primers that work together efficiently. The size of the PCR amplicon should be 80–200 bp; there are many primer design programs available to help select possible primer sequences (*see* **Note 10**). The specificity of each primer nucleotide sequence can be determined by BLAST analysis (National Center for Biotechnology Information [NCBI], Bethesda, MD); the primer(s) with the greatest degree of specificity only identify the gene of interest. If a gene is a member of a family, be sure that the primers do not recognize a conserved region. A primer design program such as LaserGene Primer Select (DNAstar, Madison, WI) is used to compute the possibilities of complementary sequence within and between primers to form dimers, along with secondary structure formations. The primer/primer interactions are measured as a ΔG value, an indicator of primer quality. A more positive value for ΔG indicates a better primer, because there is less of a chance to form dimers. The same qPCR primers also can be used for semiquantitative analyses, this allows for a single primer pair to be designed for each gene of interest. Once the primers are in hand, the optimal reaction conditions for each primer pair is established. Gradient PCR is used to determine the optimal annealing temperature for each primer pair. Other factors that affect optimization include primer and template concentration and the addition of denaturants such as DMSO or betaine.

3.4.1. Gradient PCR

Gradient PCR is done to determine the optimal annealing temperature for a primer pair to generate a single PCR product of the correct size without forming secondary products. Betaine or DMSO can be added to the reaction to eliminate secondary structure of template or primers. We test all new primers +/–DMSO or betaine. An example of a typical gradient program: 95°C/5 min (95°C/30 s, 57.5°C/30 s [gradient 57.5 ± 7.5°C), 72°C/1 min], 30–35 cycles, 72°C/5 min.

1. Dilute RT template 1:2 (this should equal ~25 ng/µl); the template concentration must be the same in all reactions.
2. Make a master PCR mix and add the template directly into the mix. Determine how many temperatures you want to test and set up the same number of tubes. Twelve different temperatures can be tested per run. For each reaction, use 8 µl of H_2O, 12.5 µl of 2× iQ SYBR-Green Supermix, 0.5 µl (5 pmol) of each forward and reverse primer, 2.5 µl of DMSO or betaine, and 1 µl of diluted RT template (*see* **Note 11**). Add 25 µl of the master mix (including template) into 200-µl PCR tubes.
3. Load tubes into the appropriate well in the gradient block. PCR program: 95°C/5 min (95°C/30 s, 57.5°C/30 s [gradient 57.5 ± 7.5°C), 72°C/1 min], 30–35 cycles, 72°C/5 min.
4. Analyze 10–15 µl of the samples in a 1% agarose gel along with a 25- or 100-bp DNA ladder (dependent on product size) at 100 V for ~1 h. Determine the size of the product and if there are additional products (*see* **Fig. 3** and **Note 12**).

3.4.2. Optimization of qPCR

qPCR allows a researcher to verify the presence of the gene of interest through the copy number, which is calculated from the -fold changes. Once the annealing temperature is established in gradient PCR, the primers are further optimized for efficiency and reproducibility. Primer dimers and nonspecific PCR products also will contribute to the fluorescent signal and result in an

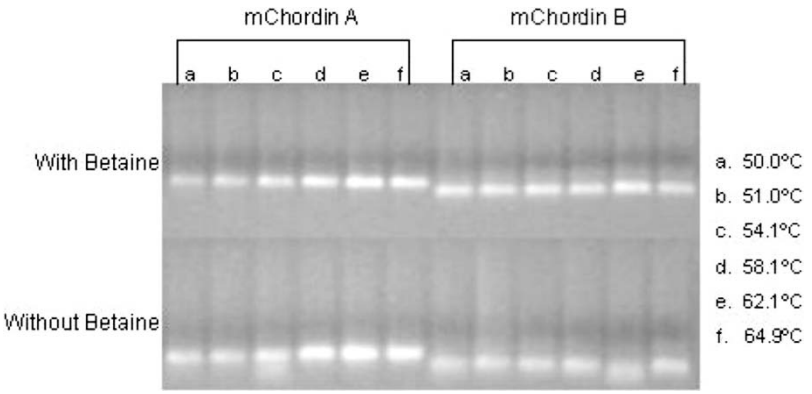

Fig. 3. This gel is a representation of a gradient PCR run on mChordin. Six different temperatures were tested with and without betaine. Notice how the Betaine bands are much sharper than the panel without betaine. Also, both the primer sets seem to amplify the gene better at the higher temperatures (shown by the amount of DNA visually present).

errant quantitation of the target gene. The following is the procedure that is used to determine whether the primers will be reliable in qPCR.

1. Standard curve.
   a. Dilute test RT template 1:4 (this should equal ~12.5 ng/µl). This can be any previous/leftover RT sample that would express the gene of interest.
   b. A 5-fold serial dilution of the template is used to determine accuracy of the primer pairs and the smallest amount of template necessary for detection of the gene of interest (*see* **Fig. 4**).
   c. Make a master PCR mix for the reaction. For each reaction, use 8 µl of H_2O, 12.5 µl of 2× iQ SYBR-Green Supermix, 0.5 µl (5 pmol) of each forward and reverse primer, and 2.5 µl of DMSO or betaine (if required).
   d. Add 24 µl of the master mix to a 200-µl optically clear PCR tube; each tube contains 1 µl of the appropriate diluted RT template (*see* **Note 13**).
   e. The PCR conditions are established in the previous gradient PCR (*see* **Subheading 3.4.1.**).
   f. Always include a housekeeping gene (β-actin or GAPDH) as a control to ensure that the PCR reagents work. In most assays, target gene amplification is compared with the housekeeping or reference gene when doing calculations to determine -fold changes in expression between different cell populations (*see* **Fig. 5A, B**).
2. Melt curve

   A melt curve should be run on the samples to ensure that there is a single product in your reaction. If there is more than one sharp peak on the melt curve this indicates the formation of a secondary PCR product. The iCycler can be programmed to couple the standard curve and melt curve in the same run (*see* **Fig. 5C**).

   a. qPCR program: 95°C/1:30 min, 95°C/30 s × °C/30 s l 40 cycles 72°C/30 s × °C/1:00 min × °C/10 s 80 cycles melt curve R = 0.5°C/s.

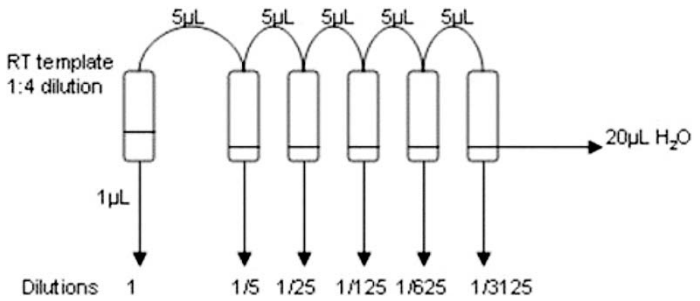

Fig. 4. Serial dilution schematic. Starting with a one-fourth dilution of the RT template, pipette 5 µl into 20 µl of ddH_2O for a fivefold serial dilution to obtain the standard curve.

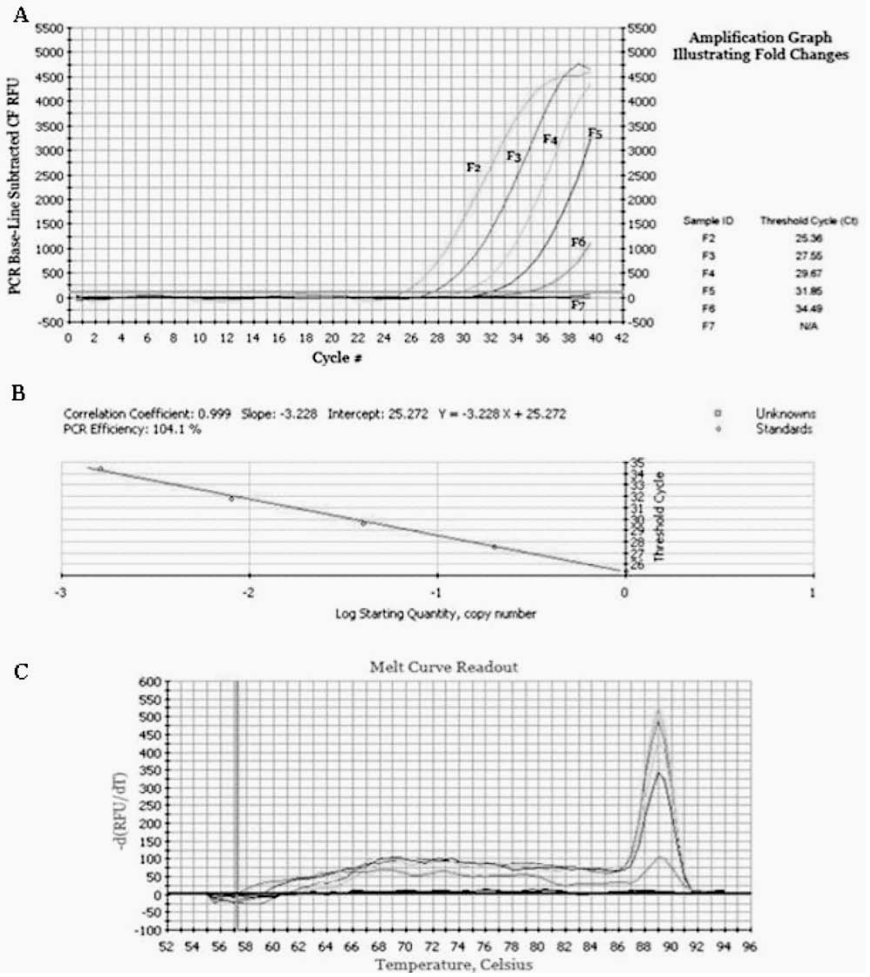

Fig. 5. **(A)** Amplification curves. -Fold changes are illustrated by a dilution series where F2–F6 represents a series of fivefold dilutions and F7 is 0 (water only). Notice how the curves of the graph are spaced about two- to threefold apart, indicating good pipetting and good amplification. **(B)** Primer efficiency. The efficiency of this primer is 104%, and the correlation coefficient (R^2) is 0.999. Both sets of values fall within the preset parameters. **(C)** Melt curve. This graph shows the melting peaks of all the products in the reaction. Usually one sharp peak is observed; however, if multiple peaks are present, then this is indicative of either a reaction contamination or an amplification of secondary products (*see* **Note 15**).

3. Repeat standard curve in triplicate
   Once the primers have passed the standard curve and melt curve analysis, repeat the assay with each sample in triplicate. This will ensure the amplification is reproducible and the final stage of primer optimization.
4. qPCR with gene-specific primers.
   a. Run the assay by using the experimental RT templates diluted 1:4. To each tube add 1 µl of diluted RT templates and 24 µl of qPCR master mix (*see* **Subheading 3.4.2.**). A dilution series is not necessary.
   b. Always run your samples in triplicate and include a housekeeping gene, for your reference sample.
   c. PCR conditions are established in gradient PCR under **Subheading 3.4.1.**
5. qPCR template considerations and analysis.
   a. Template considerations
      One of the key factors to successful two-step qPCR is the addition of cDNA at the right concentration per reaction. Complete removal of genomic DNA is critical to proper quantification of gene expression, because the reaction products are typically so small (<200 bp) that they do not cross an intron/exon boundary. Even very small amounts of genomic DNA contamination can cause a dramatic overestimation of the amount of target gene present in a given sample. Too high a template concentration can have two effects on the target gene quantification. First, very high concentrations of the target gene cDNA can lead to a saturation effect, causing a premature plateau of the amplification curve and a relatively small amplification peak (*see* **Fig. 6** and **Note 16**). When the amplification curve crosses the threshold too early, the qPCR machine improperly sets the baseline cycles. The overall affect is usually a peculiar-looking amplification curve and an underestimation of the amount of target gene present in the sample. This happens most frequently in our hands with reference genes used for relative gene quantification (see below). We have the greatest success using 1 µl of a 1:2 to 1:4 template dilution in a 25-µl reaction. Also, the buffer used in the RT reaction can be inhibitory to the QPCR. The amount of RT added to any given qPCR should always be <10% of the final volume to prevent an underestimation of target gene expression (*see* **Note 16**).

Finally, all samples should be run in triplicate, because pipetting errors cause large fluctuations in threshold cycles *(7)*. Performing all calculations (described below) with triplicate results reduces well-to-well variation as well as making dramatic outliers more obvious.

3.4.3. qPCR Analysis

qPCR results are analyzed by the relative method instead of the absolute method, because it requires far fewer time-consuming and expensive reactions

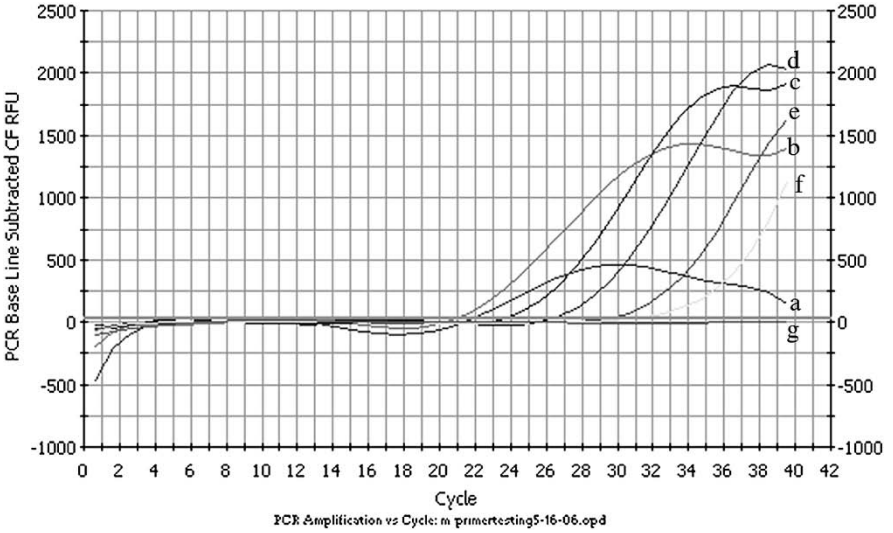

Fig. 6. Increased cDNA template in qPCR leads to a poor amplification curve. β-Actin qPCR with 1 µl of undiluted template (*a*) resulted in a curve that crossed the detection threshold late and peaked too early. The remaining lines are template dilutions: 1:5 (*b*), 1:25 (*c*), 1:125 (*d*), 1:625 (*e*), and 1:3125 (*f*). *g* is a no template control (*see* **Note 14**).

to generate reliable data. The relative method is frequently described as the $\Delta\ \Delta C_T$ method ***(8)***. There are several important considerations when using this technique. The threshold cycle of the reference gene must not vary significantly between experimental conditions/samples or a new reference gene must be chosen. The efficiency of the target gene PCR must be very close to that of the reference gene PCR or any resulting calculations based on comparison between the two will not be accurate. We have chosen to design all primers used within the laboratory (reference as well as target genes) to have an efficiency between 90 and 110%, with efficiency = $10^{(-1/s)}$ –1 (s is slope of a standard curve made from template dilutions). Many qPCR machines will calculate the efficiency of a given primer set just by running a dilution series experimental run. You must choose a system to compare your samples in a pairwise manner to demonstrate differences between target gene expression in different samples/conditions. One sample must be designated as the calibrator or the reference to which the other sample/sample will be compared one at a time (*see* **Fig. 7** and **Note 17**).

The calculations for the relative method are as follows:

$$\Delta\ C_T(\text{sample}) = C_T \text{ target gene} - C_T \text{ reference gene}$$

$$\Delta\ C_T(\text{calibrator}) = C_T \text{ target gene} - C_T \text{ reference gene}$$

Oct 4 24 hours

Samples in Triplicate	Oct4 CT	Actb CT	Delta CT		Delta2 CT	Fold Change with SD		
pEpi	27.2	21.7	5.5					
pEpi	28.6	22	6.6	**Standard**			**Standard**	
pEpi	27.9	22.3	5.6	**Deviation**		0.66	**Deviation**	
			5.9	0.61	0.00	**1.00**	**Fold Change**	
						1.52	0.44	
pNanogi	29.3	22.7	6.6					
pNanogi	31.9	23.6	8.3	**Standard**			**DOWN**	**Standard**
pNanogi	30.5	22.9	7.6	**Deviation**		0.15	6.74	**Deviation**
								Fold
			7.5	1.20	1.55	**0.34**	**2.93**	**Change**
						0.79	1.27	2.80
pNanog	27.1	21.7	5.4					
pNanog	27.2	22.2	5.0	**Standard**			Standard	
pNanog	27.4	20.4	7.0	**Deviation**		0.51	**Deviation**	
			5.8	1.06	-0.10	**1.07**	**Fold Change**	
						2.23	0.88	

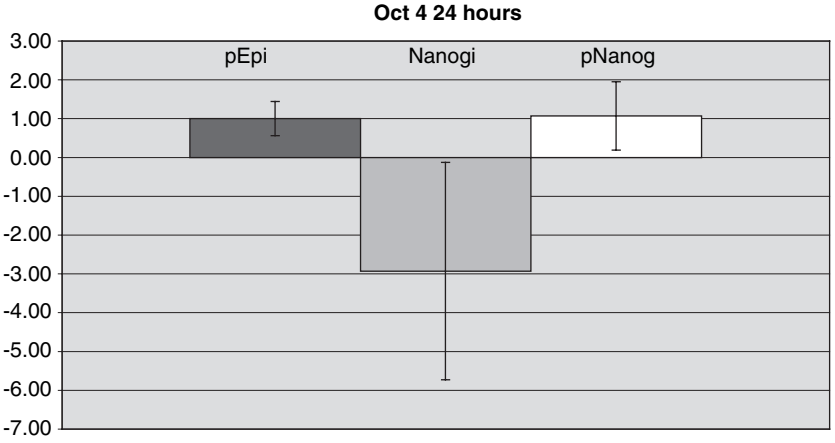

Fig. 7. Mouse ES cells were transfected with plasmids either for Nanog overexpression or RNA interference and cultured for 24 or 72 h before RNA harvest and analysis by qPCR. The pEpi control plasmid data served as the calibrator sample for these experiments; therefore, its Δ ΔCT was equal to 1. *Nanogi*, Nanog RNAi plasmid; *pNanog*, Nanog overexpression plasmid.

$$\Delta\Delta\ C_T = \Delta C_T(\text{Sample}) - \Delta C_T(\text{Calibrator})$$

$$\text{Normalized target gene expression level} = 2^{\Delta\Delta CT}$$

One of the most difficult data sets to describe graphically is a set where the target gene expression increases in some sample and decreases in other

samples. We have found the most logical way to illustrate these results on one graph is to designate the decreased samples as $1/\Delta\Delta\ C^T$ (*see* **Fig. 7**).

3.5. Purification and Cloning of RT-PCR Products

The ability to amplify and clone gene-specific PCR products is a useful technique. The cloned cDNAs provide a stable source of template for the synthesis of gene-specific probes for *in situ*, Northern, and Southern hybridizations; these assays identify where and when a gene is expressed but not its function. Typically, gene function is studied by overexpression or knockdown expression of the gene of interest. Full-length cDNAs of genes identified in microarrays or other gene screen methods can be generated in RT-PCR, with gene-specific primers that encompass the entire open reading frame. Initially, we clone and sequence the PCR products to confirm amplification of the expected gene. The PCR product from each optimized primer pair is cloned into pBS SK, by using a simple TA cloning approach. There are several commercial TA cloning vectors available, but this is a low-cost, efficient method to clone an infinite number of cDNAs in a very short time. The PCR product does not have to be modified (i.e., restriction enzyme digestion) before cloning; only gel purification is required to clean up the PCR product.

1. To gel purify the cDNAs there are several kits available. We prefer to use the DNA gel extraction kit from QIAGEN, a quick protocol that yields great recovery of the cDNAs. Pour a standard 1% agarose gel in TBE and use a comb with wells that will be able to hold 20–40 µl. Combine 20–40 µl PCR product with 2–4 µl of 10× DNA loading buffer. Load the entire sample and electrophorese at 100 V for 1–2 h. Examine the gel on an UV transilluminator. Remove the gel from the tray and excise the bands of the correct size cDNAs, then place each in a 1.5-ml tube. Extract and purify the DNA from the gel slice according to the manufacturer's protocol. A decrease in the elution volume on the column to 30–35 µl of H_2O, increases concentration of the gel-purified cDNA.
2. Check the recovery of the cDNAs, run 4 µl (~100–200 ng) from a total volume of 50 µl of each in a 1% agarose gel (*see* **Note 18**).
3. Prepare pBS SK plasmid for TA cloning of PCR products. The pBS SK (3 µg) is linearized in the MCS at the EcoRV site. Reaction: 3 µg of pBS SK (or a similar vector), 3 µl of 10× NEB buffer 3, 1.5 µl of bovine serum albumin, 2 µl of EcoRV (NEB 10 U/µl), bring total volume to 30 µl with ddH_2O. Incubate overnight at 37°C. Gel purify the linear pBS SK (3 kilobases [kb]) (*see* **Subheading 3.5.1.**). Elute DNA from the columns with 35 µl of H_2O.
4. Addition of dTTP at the blunt-ended EcoRV site with Taq Pol and 10 mM dTTP. Reaction: 32 µl of gel-purified vector, 5 µl of 10× Taq DNA polymerase buffer, 1.5 µl of 50 mM $MgCl_2$, 10 µl of 10 mM dTTP, 1.5 µl of Taq DNA polymerase (5 U/µl), total volume to 50 µl with ddH_2O. The reaction is incubated at 70°C for 2 h. Gel purify the T-tailed vector as described in 3.5.1. After purification,

run 4 µl of the vector in a 0.8% agarose gel to estimate the quantity of DNA (*see* **Note 18**). Dilute the sample to ~5 ng/µl and store the vector in 10-µl aliquots at –20°C, avoid freeze-thaw of the vector once it is T-tailed.

5. To ligate the purified PCR product into pSK TA set up ligations (L). L1: 0.5 µl of pSK TA, 6.5 µl of cDNA, 2 µl of 5× T4 DNA ligase buffer, 1 µl of T4 DNA ligase (1 U/µl), TV = 10 µl. L2: 0.5 µl of pSK, 2.5 µl of cDNA, 1 µl of 5× T4 DNA ligase buffer, 1 µl of T4 DNA ligase, TV = 5 µl. LO: 0.5 µl of pSK, 6.5 of µl ddH_2O, 2 µl of 5× T4 DNA ligase buffer, 1 µl of T4 DNA ligase, TV = 10 µl. The LO reaction is a negative control for self-ligation of the vector alone. Incubate the ligations in a water bath or thermocycler at 14°C o/n (*see* **Note 19**).
6. Transformation: thaw DH5 α *E. coli*-competent cells on ice. The total volume of the ligation is brought to 50 µl with ddH_2O. Place four tubes on ice, one for each ligation and a positive control. Aliquot 50 µl of competent cells in each tube, and then add 5 µl of each ligation to the appropriate tube; the remainder of the ligations can be stored at –20°C and used for up to 1 week. For the positive control, add 2 ng of pSK to 25 µl of competent cells. Keep the tubes on ice for 30 min, heat shock cells at 42°C/45 s (critical step), and then return them to ice for 2 min. Add 700 µl of SOC (warmed to rt) to each tube and place tubes in a shaker incubator, at 37° C for 1 h, at 220 rpm. Plate the cells on LB agar with 40 µg/ml ampicillin. For L1 and L2, add 100 µl and 400 µl/plate for each. To the LO, add 100 µl/plate and for pSK add 10 µl/plate. Let the plates air dry about 30 min, and then place inverted in a 37°C incubator overnight (*see* **Note 19**).
7. Set up replica plates of single, isolated colonies from the L1 and L2 plates Incubate plates inverted at 37°C overnight (*see* **Note 20**).
8. Colony lysis PCR is a procedure to quickly screen colonies for positive ligation event. This takes advantage of the T3 and T7 primer binding sites, which flank the multiple cloning site in p BS SK. The cDNA insert is amplified in PCR with the T3 and T7 primers. Colony lysis buffer (50 µl) is added to a 1.5-ml tube for each colony to be analyzed. A sterile pipette tip is used to pick each colony (a small amount of bacteria just visible on the end of the tip is more than enough). Make sure the bacteria are mixed well with the colony lysis buffer. Incubate the tubes in boiling H_2O for 10 min. Centrifuge at rt for 10 min and transfer the clear supernatant to a fresh tube; this is the PCR template. For each reaction, combine the following in a 200-µl tube: 7.3 µl of H_2O, 2 µl of DMSO, 2 µl of 10× Taq DNA polymerase buffer, 1.5 µl of 50 mM $MgCl_2$, 1 µl of 10 µM dNTP mix, 2 µl (10 pmol) of the T3 and T7 primer, 2 µl of colony lysate, and 0.2 µl of Taq DNA polymerase (5 U/µl), TV = 20 µl. To decrease pipetting errors, make a master mix that includes all of the reagents except the templates. The PCR conditions are 94°C/3 min, 94°C/30 s, 55°C/1 min, 72°C/1 min for 35 cycles, 72°C/7 min. Analyze 20 µl of each in a 1% agarose gel, at 100 V for 1–2 h. The PCR product will be 120 bp (this represents the pSK MCS) if there is no insert. When an insert is present, the product size is 120 bp larger than the original PCR product (i.e., original β-actin product is 500 bp, the product on this gel will be 620 bp). Those

colonies that contain inserts are selected for DNA plasmid column preps. DNA column preps are done using the QIAGEN column preps and protocol. The final DNA plasmids (i.e., pSK/PCR product X.) are sequenced to verify the correct cDNA. DNA plasmids can be stored indefinitely at –20°C.

4. Notes

1. The precautions for working with RNA are listed in many references ***(2,5)***. Be sure to always wear gloves and use only sterilized tips, tubes, and glassware at minimum. It is also a good idea to have reagents that are only used with RNA. In all RNA protocols, DEPC H_2O is always used in reagents, reactions, and always to resuspend RNAs.
2. After removal of the aqueous layer, a second extraction is done by adding 100 µl of TE to the organic phase, mixing, and centrifuging at 4°C for 5 min. This will improve recovery of RNA; this step is especially important if the quantity of RNA is limited.
3. UV spectrophotometry is done to determine the concentration of RNA or DNA. Make a 1:100 dilution of each sample in TE and obtain A_{260} and $A_{260/280}$ ratio. The A_{260} is used to determine concentration, for RNA ($A_{260} \times 40$ µg/ml × dilution factor), and DNA ($A_{260} \times 50$ µg/ml × dilution factor). The $A_{260/280}$ ratio indicates the purity of the sample, and it should be in the range of 1.7–2.0. A ratio less than 1.7 indicates that the preparation contains contaminants that could interfere with the RT-PCR.
4. All RNase-free DNase I enzymes retain some RNase activity, so over time RNA stored for long periods after the DNase I reaction undergo some degree of degradation. DNase I treat RNA only as needed for RT reactions. After the DNase I treatment the RNA (0.5 µl) serves as the template in PCR with β-actin primers, include a positive template control (*see* **Subheading 3.1.9.**). The PCR product is electrophoresed in a 1% agarose gel. If all genomic DNA is degraded, then there should be no PCR products; the positive control is the only template that should yield a product. If there is a product with any of the Dnased RNAs, then genomic DNA is still present, add another 1µl of DNase I to the RNAs and repeat the reaction. This does not occur frequently, but it does happen once in a while.
5. A master mix decreases the number of possible pipetting errors. The mix contains all the common reagents (usually not templates or primers) for a particular reaction. It is a good rule to make enough of the master mix for one additional reaction (i.e., for nine reactions, the master mix is made for 10 reactions). Be sure to mix well (vortex) before use. The last of the master mix can be used as the reagent control in PCR, to identify any contaminant.
6. The ability to detect a particular mRNA in a cell population is dependent on primer location and priming during the RT reaction. Random nonamers will prime the RT reaction along the entire mRNA, but oligo(dT) is more likely to prime the 3′ end of the mRNAs. For example, a PCR product for gene X may not be detected because the RNA was primed with oligo(dT) and the primers

for gene X sit in the 5′ end of the mRNA. We now use random nonamers to prime all RNA for both semiquantitative and qPCR. We have found the results to be more reproducible for all primer sets regardless of there location on the mRNA.

7. Some RNAs have a lot of secondary structure that is inhibitory during the RT reaction. A couple of suggestions to minimize the effects of secondary structure include increasing the temperature of the RT reaction (most RTs are active up to 55°C) and adding 5% DMSO.
8. Gene-specific primer design and PCR optimization have been covered extensively ***(1**,**2**,**5)***. Primer design and analysis websites:
   a. GeneFisher Primer program, http://bibiserv.techfak.uni-bielefeld.de.genefisher/.
   b. Primer3, http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi.
   c. NCBI: www.ncbi.nlm.nih.gov/. This site, nucleotide sequences can be obtained from GenBank and BLAST analysis can be done to determine primer specificity. Primers from published papers should be screened by BLAST analysis before ordering. We have found sequences to be incorrect or identity to a different gene than stated.
9. A PCR product with the DNased RNA alone (no RT reaction) indicates that there is still some chromosomal DNA present in the preparation. When a product of the same size is amplified with all of the templates, including the PCR reagent control, it indicates that some component in the reaction is contaminated with an extraneous DNA template. The best way to resolve the contamination problem is to discard the reagents that were used and start with fresh reagents. If more than one PCR product is present, then the reaction conditions need to be optimized for the primer pair (i.e., alter annealing temperature [primer], $MgCl_2$). If none of the templates yield PCR products with the control primer pair (i.e., β-actin), it is possible one of the reagents could have been left out of either the RT reaction or PCR (i.e., RT, Taq DNA polymerase, dNTPs). Templates that are very G:C rich can sometimes be difficult to amplify, in this case add DMSO (10%) or 5 M betaine to the PCR. Another possibility is that the RNA sample could be degraded. To troubleshoot, repeat the PCR with only a couple of the templates. If a product is obtained, then some component of the PCR was not present in the initial PCR. If there still is no product, electrophorese 2 µl of the RNAs in a 1% agarose gel to check for RNA degradation (*see* **Fig. 1**). If the RNA is not degraded, repeat both the RT reaction and PCR (other remedies may be to increase the amount of RNA in the RT reaction or the amount of template in the PCR). However, if the RNA is degraded, it will be necessary to obtain new RNA samples. Templates that are very G:C rich can sometimes be difficult to amplify, in this case add DMSO (10%) or 5 M betaine to the PCR.
10. qPCR primer websites
    a. http://www.gene-quantification.info/.
    b. http://pathmicro.med.sc.edu/pcr/realtime-home.htm.

c. http://web.ncifcrf.gov/rtp/gel/primerdb/.
d. http://medgen.ugent.be/rtprimerdb/.
e. www.realtimeprimers.org/.

11. Bio-Rad iQ Supermix (2×): 100 mM KCl, 40 mM Tris-HCl, pH 8.4, 0.4 mM dNTPS, 50 U/ml iTaq (Hot start Taq polymerase), 6 mM $MgCl_2$, and proprietary stabilizers. Although gradient PCR is not quantitative, these are the same reagents that are used in the final quantitative reactions.
12. Two different primer pairs, mChordin A and B, were tested in gradient PCR over six different temperatures (lanes a–f), with and without betaine. Both primer sets yield a single PCR product of the correct size at all temperatures tested; the primers will amplify at annealing temperatures of 50 to 65°C. The addition of betaine results in sharper bands/products on the gel. Primers that amplify multiple products at all temperatures, need to be redesigned, some primers may only amplify at a single temperature in the gradient. We usually design at least 2-primer pairs/gene of interest, for optimization.
13. Precautions to take when setting up qPCR. A designated clean space or sterile cabinet should be used for setting up qPCR. We also have a set of pipettes that are only used for qPCR, and for all PCR only use filter pipette tips.
14. When setting up the actual QPCR, templates need to be more dilute than in gradient or semiquantitative PCR, because the assay is far more sensitive. The RT template is diluted 1:4 and use 1 µl per qPCR; too much template can be inhibitory (*see* **Fig. 6**).
15. To analyze the peaks in the melt curve data, peaks present at lower temperatures indicate the amplification of a second product; peaks at higher temperatures are a sign of contamination.
16. QIAGEN website for qPCR manual: http://www1.qiagen.com/literature/brochures/pcr/QT/1037490_AG_PCR_0206_Int_lr.pdf. This is a very useful overview of qPCR to aid in experimental design for successful qPCR.
17. Microsoft Excel (Microsoft, Redmond, WA) spreadsheets are an excellent way to handle the large number of repetitive calculations necessary for this analysis as well as making graphical representations of the results easy.
18. The concentration of gel-purified products cannot be determined accurately in spectrophotometry. Serial dilutions with a plasmid of known concentration are run on the same gel as the purified DNA to estimate concentration.
19. The general rule for ligation reactions is that the insert (cDNA) is always present at a higher concentration than the vector (pSK). The recommended molar ratio is 3:1 (insert:vector) ***(2)***.
20. Record the number of colonies on each plate if feasible, some plates may be covered with colonies (to numerous to count). The L0 plate should have far fewer colonies than the L1 and L2 plates. If colonies are only on the positive control plate, and there are no colonies on the L1 and L2 plates, the transformation protocol and reagents worked fine, but there is a problem with the ligations. Repeat the ligation reaction and transformation.

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Microfluidic Devices for High-Throughput Gene Expression Profiling of Single hESC-Derived Neural Stem Cells

Yan Chen and Jiang F. Zhong

Summary

Isolating pure stem cell populations is one of the major obstacles in stem cell gene expression profiling due to the lack of stem cell markers. Many results of gene expression profiling studies are difficult to interpret because of the heterogeneous cell populations used in these studies. Single-cell gene expression profiling is perhaps the most attractive gene expression profiling method for studying stem cell gene regulation, because isolating pure stem cell population is not needed. However, current single-cell gene expression profiling methods such as laser capture microdissection (LCM) and patch-clamp analysis lack the high-throughput ability in sample processing. For better understanding of the gene regulation networks during cellular events, a large number of gene expression profiles are required. Therefore, we developed inexpensive microfluidic devices for high-throughput single-cell gene expression profiling. With our devices, cDNA could be obtained from 50 individual cells within 3 hours. This approach can be applied to neural stem cells, and other cell types.

Key Words: Single-cells analysis; high throughput; gene expression; microfluidics, gene expression; human embryonic stem cell; mRNA extraction; multiplex PCR.

1. Introduction

The fundamental challenge for studying stem cell gene expression is that stem cells are rare, and no definitive markers ensure isolation of a homogenous stem cell population. Many stem cell gene expression profiling studies have to use heterogeneous population of stem cells and progenitor cells. Therefore,

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the expression profiles obtained only reflect the sum of all the subpopulations in the culture. Without knowing the percentages of stem cells and progenitor cells in the interrogated population, these gene expression profiles that are the sum of all expression profiles from various cell types, are very difficult to interpret and they can give only limited information. Single-cell gene expression profiling combined with bioinformatic analysis can overcome this challenge without the need of homogeneous stem cell populations. After obtaining gene expression profiles from individual cells at various maturation stages, bioinformatic analysis such as hierarchical clustering can sort similar profiles into groups and calculate the correlation coefficient. Based on correlation coefficient, single-cell gene expression profiles can be clustered into a dendrogram to show the differentiation/maturation order of the interrogated cells. The rationale is that differentiation/maturation of cells is orchestrated by sequentially expressing a series of genes. Therefore, gene expression profiles from consecutive maturation stages are more similar than those from other stages. Clustered consecutive single-cell gene expression profiles can reveal and distinguish molecular differences among different differentiation stages. The expression timing of genes also could implicate the regulatory relationship. Most importantly, this approach does not require homogeneous stem cell populations, because a single cell is perhaps the purest population by itself. However, this approach is very difficult to perform with current single-cell gene expression profiling methods, because these methods are deficient in high-throughput sample processing.

Multiple single-cell gene expression profiling studies with laser capture microdissection (LCM) ***(1–10)***, patch-clamp analysis ***(11–13)***, and *in situ* mRNA amplification ***(12,14)*** are currently in use. Single-cell whole genome microarray gene expression screening ***(2,3,15)*** and single-cell cDNA library construction ***(16–18)*** also have been conducted. Single-cell gene expression profiling from early embryos showed transient expression of critical regulatory genes, and it underscores the importance of systematic single-cell expression profiling ***(11,17)***. Although these studies demonstrated the value of single-cell gene expression profiling, they also showed the limitation of these methods in processing a large number of samples. To produce consecutive gene expression profiles from target cell populations, a large number of cells must be profiled for a particular experiment to cover all the significant cellular stages. The LCM and single-cell reverse transcription-polymerase chain reaction (RT-PCR) are inadequate for large scale single-cell analysis, because these methods are difficult to perform in a high throughput manner. A single mammalian cell contains 20–40 pg of total RNA ***(19,20)*** and only 0.5–1.0 pg of mRNA (10^5–10^6 mRNA molecules) ***(21)***. Any attempt for large scale single-cell gene expression profiles must be able to manipulate nanoliters of

material in a high-throughput manner without significant material loss. The microfluidic system reported here has high-throughput ability. Our microfluidic devices can simultaneously process 50 individual cells into cDNA within 3 hours.

2. Materials

2.1. Stem Cell Culture

1. Human embryonic stem cell (hESC) H9 lines (WiCell Research Institute, Madison, WI).
2. Mouse embryonic fibroblasts (MEFs) (Cell Biolabs, Inc., San Diego, CA).
3. hESC medium: DMEM/Ham's F-12 (Invitrogen, Carlsbad, CA), 20% knockout serum (Invitrogen), 0.1 mM nonessential amino acids (Invitrogen), 1 mM L-glutamine (Invitrogen), 0.1 mM 2-mercaptoethanol (Sigma-Aldrich, St. Louis, MO), and 4 ng/ml basic fibroblast growth factor (bFGF; Invitrogen).
4. Neural differentiation medium: DMEM/Ham's F-12 (Invitrogen), N2 supplement (Invitrogen), 0.1 mM nonessential amino acids (Invitrogen), 1 mg/ml heparin (Sigma-Aldrich), 10 ng/ml bFGF (R&D Systems, Minneapolis, MN), 20 ng/ml epidermal growth factor (EGF); (R&D Systems).
5. Monoclonal mouse anti-human Nestin (Chemicon International, Temecula, CA)
6. Fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse IgG (Chemicon International).

2.2. Device Fabrication

2.2.1. Mold Fabrication

1. 7.6-cm (3-in.) silicon wafer (Silicon Quest International, Santa Clara, CA).
2. SU-8 photoresist (Microchem, Newton, MA).
3. SPR photoresist (Shipley Marlborough, MA).
4. AZ photoresist (Clariant, Somerville, NJ).
5. Spin processor (Laurell Technology Corporation, North Wales, PA).
6. Hotplate (VWR, West Chester, PA).
7. MJB3 mask aligner (Karl Suss, Waterbury Center, VT).

2.2.2. Chip Fabrication

1. RTV (General Electric, Fairfield, CT).
2. Hybrid mixer (Keyence, Woodcliff Lake, NJ).
3. Spincoater (Specialty Coating System, Indianapolis, IN).
4. Oven (Fisher Scientific, Pittsburgh, PA).

2.3. Device Operation

1. Pressure source (Fluidigm, South San Francisco, CA).
2. 23-gauge pins (New England Small Tube, Litchfield, NH).

3. Tygon tubing (VWR).
4. NI-DAQ card (National Instruments, Austin, TX).
5. Labview software (National Instruments).

2.4. RNA Capture and First Strand cDNA Synthesis

1. Dynabeads mRNA Direct kit (Invitrogen).
2. RNasin Plus RNase Inhibitor (Promega, Madison, WI).
3. Sensiscript reverse transcriptase (RT) kit (QIAGEN, Valencia, WI).
4. QuantiTect Multiplex PCR NoRox Kit (QIAGEN).

3. Methods

3.1. Device Fabrication Processes

3.1.1. Mold Fabrication

A. Control Mold

1. Spin Su8-2025 at 3,000 rpm for 45 s.
2. Soft bake mold for 2 min/5 min at 65°C/95°C.
3. Expose mold under a transparency mask with the fluidic design for 1.2 min on MJB mask aligner.
4. Bake mold postexposure for 2 min/5 min at 65°C /95°C.
5. Develop in SU8 nano developer.
6. Once developed, bake mold at 95°C for 45 s to evaporate excess solvent.

B. Flow Mold

1. Spin Su8-2025 at 3,000 rpm for 45 s.
2. Soft bake mold for 1 min/3 min at 65°C/95°C.
3. Expose mold 50 s under a transparency mask with the fluidic design on MJB mask aligner (7 mW/cm^2).
4. Bake mold postexposure for 1 min/3 min at 65°C/95°C.
5. Develop in Su8 nano developer.
6. Hard bake mold at 150°C for 2 h for the formation of the 10-μm-high flow channels.
7. Expose mold to HMDS vapor for 90 s.
8. Spin Spr220-7 at 1,500 rpm for 1 min.
9. Soft bake mold for 90 s at 105°C.
10. Expose mold for 3.2 min on MJB mask aligner.
11. Develop mold in MF-319 developer and rinse under a stream of H_2O.
12. Hard bake 2 h at 200°C for the formation of the 15-μm-high flow channels.
13. Expose mold to HMDS vapor for 90 s.
14. Spin AZ-50 at 1,600 rpm for 1 min.
15. Soft bake mold for 1 min/5 min/1 min at 65°C/115°C/65°C, respectively.
16. Expose mold for 4 min on MJB mask aligner.

17. Develop mold in 3:1 H_2O:2401 developer. Rinse mold under a stream of H_2O.
18. Hard bake 3 h at 200°C for the formation of the 40-μm-high flow channels.

3.1.2. Chip Fabrication

1. Prepare 5:1 GE RTV A:RTV B (mix 1 min, de-foam 5 min).
2. Expose flow mold to trimethylchlorosilane (TMCS) vapor for 2 min.
3. Pour 30 g of 5:1 GE RTV A:RTV B on respective flow mold.
4. De-gas flow mold under vacuum.
5. Bake flow mold 45 min at 80°C.
6. Prepare 20:1 GE RTV A:RTV B.
7. Expose control mold to TMCS vapor for 2 min.
8. Spin 20:1 RTV mix at 1,800 rpm for 60 s (15-s ramp).
9. Bake control mold 30 min at 80°C.
10. Cut devices out of flow mold and punch holes with 650-μm-diameter punch tool.
11. Clean the flow device and align to control mold.
12. Bake the resulting two-layer device for 45 min at 80°C.
13. Prepare 20:1 GE RTV A:RTV B.
14. Expose blank wafer to TMCS vapor for 2 min.
15. Spin 20:1 RTV mix on blank wafer at 1,600 rpm for 60 s (15-s ramp).
16. Bake blank wafer for 30 min at 80°C.
17. Cut out the two-layer device from control mold and punch holes with 650-μm-diameter punch tool.
18. Clean the device and mount on a blank wafer. Check for debris and collapsed valves.
19. Bake three-layer RTV device for 3 h at 80°C.
20. Cut three-layer device out and mount on a glass slide.
21. Bake finished device overnight at 80°C.

A completed device is illustrated in **Fig. 1**. We are willing to provide advice on the construction of mold and chip.

3.2. Neural Stem Cell Preparation

1. MEFs were irradiated (5,500 rads) and plated at 56,000 cells/cm^2 at tissue culture plates with hESC medium.
2. hECSs were maintained on MEF with hESC medium.
3. Nestin+ neural stem cells were differentiated from hESC by replacing hESC medium with differentiation medium (*see* **Fig. 2**).
4. Single Nestin+ cells were isolated from trypsinized differentiated hESCs by fluorescence-activated cell sorting (FACS) after labeling cells with monoclonal mouse anti-human Nestin (Chemicon International) and FITC-conjugated goat anti-mouse IgG antibodies (*see* **Note 1**).

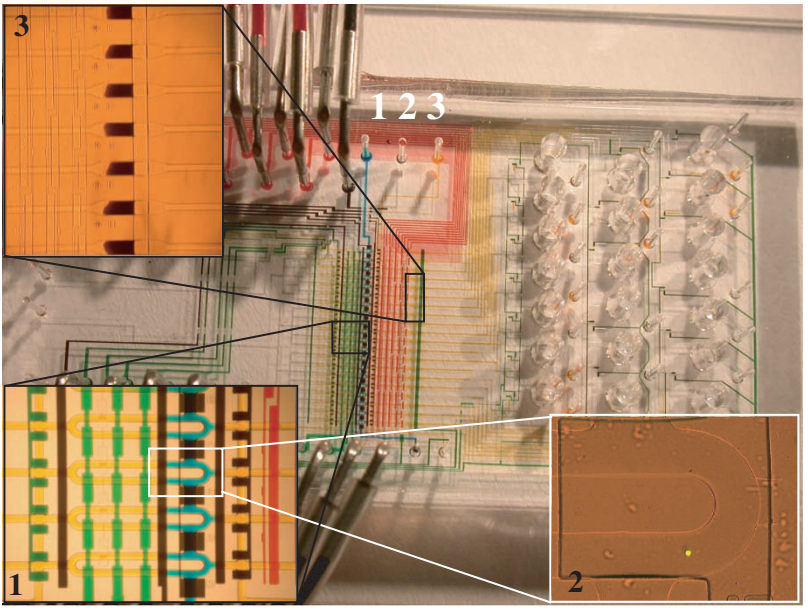

Fig. 1. Microfluidic device filled with food dye for illustration. All flow channels are filled with yellow dye, multiplexer control channels are filled with red dye, and waste and collection control channels are filled with blue dye. (**Insert**) Cell lysis module. Cells are portioned in the flow channels filled with blue dye, lysis buffer is represented with yellow dye, and the pump valves are in green. Individual modules are separated by fluidic valves (*black squares*). (**Insert**) A single neural stem cell (green) captured in the channel before lysis. (**Insert**) Seven stacked bead columns. After cell lysis, the lysates from individual cells are pushed through the oligo(dT) bead columns. The single-cell mRNAs were captured by the oligo(dT) beads of these columns.

3.3. Device Operation Processes

3.3.1. Microfluidic Chip Control

The on-off valves in the microfluidic chip are controlled by individual pressure sources via 23-gauge pins and Tygon tubing. An NI-DAQ card is used through a Labview interface to actuate the pressure sources. A microfluidic station is shown in **Fig. 3** (*see* **Note 2**).

3.3.2. Column Construction

1. Add 1 μl (40 units) of RNase inhibitor to 99 μl of lysis buffer from Dynabeads mRNA Direct kit. Load the resulting lysis buffer with RNase inhibitor into the flow channel from inlet 2 until it reaches the waste outlets (*see* **Fig. 1**).

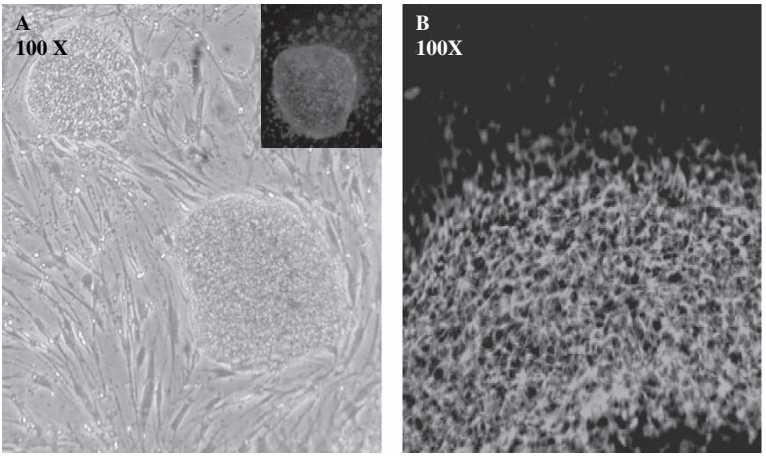

Fig. 2. Undifferentiated hESC and hESC-derived neural stem cells. Nuclei were labeled with 4,6-diamidino-2-phenylindole (blue). **(A)** Pluripotent hESC colonies on MEFs (insert shows red Oct-3/4 staining as pluripotent marker). **(B)** After neural differentiation, the majority of cells became Nestin+ (green) neural stem cells. These cells were trypsinized into single cells and isolated by FACS.

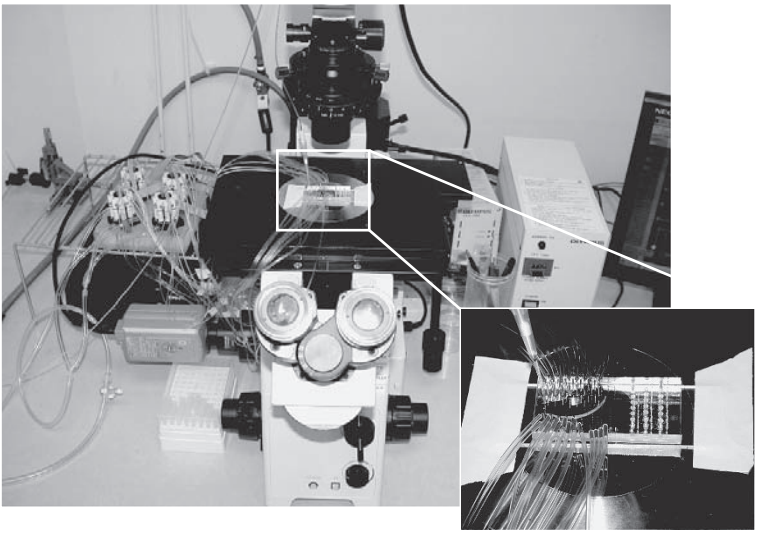

Fig. 3. Microfluidic chip control station. The microfluidic chip on the microscope thermal stage is controlled by individual pressure sources. **(Insert)** Pressure is introduced to the microfluidic chip via 23-gauge pins and Tygon tubing. Biochemical reagents are loaded to the chip by using a pipette tip.

2. Pellet 40 μl of beads form Dynabeads mRNA Direct kit with centrifugation and resuspend beads in 40 μl of lysis buffer. Pellet the beads again and reduce the lysis buffer to 20 μl. Vortex to resuspend the beads. It is important to resuspend the beads before loading into the microfluidic device from inlet 3 (*see* **Fig. 1**, **inset 3**) (*see* **Note 3**).
3. The multiplexer opens the individual flow line when it is addressed, allowing the bead columns to be stacked in a serial manner. The sieve valves are actuated when the beads flow in the channels. The sieve valves allow the fluid but not the beads to pass. Once the columns are built, excess beads in the flow channels are pushed into the column with lysis buffer to stack into the column (*see* **Note 4**).
4. Pellet single cells isolated by FACS with centrifugation and resuspend cells in 100 μl of phosphate-buffered saline. Pipette up and down gently before loading cells into microfluidic devices through inlet 1. Cells are contained in the right part of the rings (*see* **Fig. 1**, **inset 2**). Lysis buffer is then loaded into the left part of the rings. Cells are lysed chemically by mixing cells with lysis buffer in the ring. Mixing occurs by executing a peristaltic pump (*see* **Fig. 1, inset 1**) sequentially (*see* **Note 1**).

3.3.3. Capturing mRNA, Synthesizing First Strand cDNA, and Recovery of cDNA

1. Prepare 90 μl of RT buffer from Sensiscript RT kit: 9 μl of 10× buffer, 9 μl of 5 mM dNTP, 4.5 μl of RT, 2.25 μl of RNase inhibitor, and 65.25 μl of H_2O.
2. Cell lysates are pushed through the oligo$(dT)_{25}$ columns via pneumatic pressure, and the mRNAs are captured by attaching to the oligo$(dT)_{25}$ sequences on the beads. Then, the columns are washed by first strand cDNA synthesis buffer. The first strand synthesis is carried out by heating the device to 40°C on a thermal microscope stage. The reaction mixture is flown over the columns for 45 min. The reaction is completed by heating the device to 70°C for 15 min (*see* **Note 5**).
3. The polydimethylsiloxane (PDMS) microfluidic device is peeled off from the supporting glass slide. Individual collection wells are cut off from the devices and placed in micro-centrifuge tube with open end face down. Beads are collected by centrifugation.

3.4. Analysis of Single-Cell cDNA

Beads with attached cDNA from single cells were subject to multiplex quantitative PCR. A typical triplex quantitative PCR performed with Bio-Rad IQ5 (Hercules, CA) is shown in **Fig. 4**. Absolute number of mRNA molecules in individual cells can be calculated from a standard curve generated with known amounts of DNA corresponding to the target genes. The efficiency of the cDNA synthesis in microfluidic devices can be calculated by loading known amounts of standard artificial mRNA (such as GeneChip Poly-A RNA control,

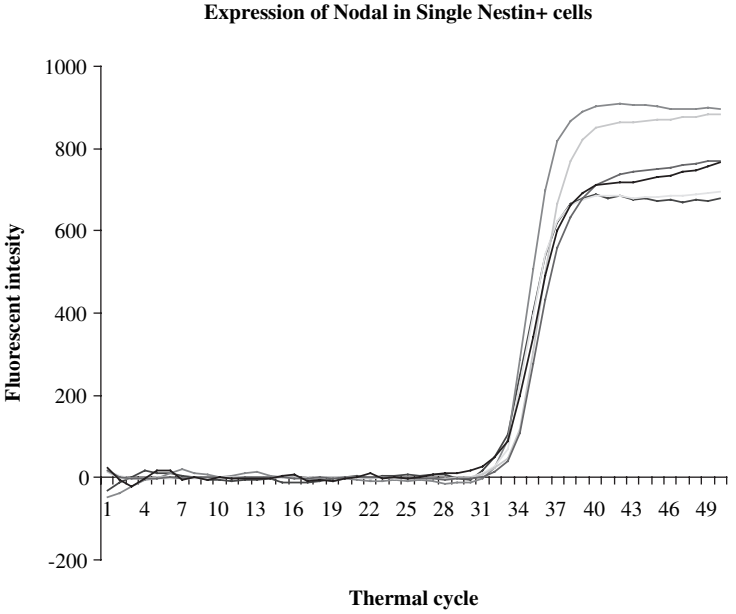

Fig. 4. Expression of Nodal in single hESC-derived neural stem cell. The representative quantitative PCR amplification curves showed the expression of Nodal in six individual neural stem cells.

Affymetrix, Santa Clara, CA) to the device. Because artificial mRNA does not exist in eukaryotic cells, it can be spiked into the lysis buffer. The volume of lysis buffer used for each cell lysis module is known, and the spike-in mRNA can be used to calculate the efficiency of cDNA synthesis for each processed cell. Besides being used for multiplex quantitative PCR, the cDNA obtained also can be amplified and used for whole genome microarray analysis.

4. Notes

1. It is critical to obtain single-cell suspension by FACS. Clumps of cells or debris could clot the flow channels. Due to the small size of the channels, the clot is very difficult to clear, and it often makes the chip useless. Therefore, the FACS procedure is not only for isolating desired cells but also for obtaining single-cell suspensions.
2. Appropriate pressure should be used to control the microfluidic chip. The control channel is normally working well under a pressure of 18–22 psi in our laboratory; use the pressure that can completely close the valves, but do not let the pressure go too high.

3. Lysis buffer and beads can be loaded to the flow channels at 2 psi, but cells should be loaded in the channel at lower pressure 0.3 psi to control the flow rate.
4. During mixing of the cells and lysis buffer, the pump valves should be operated at lower pressure 16 psi (not fully closing the valve 18–22 psi) to enable efficient pumping.
5. In the 45-min first-strand cDNA synthesis process, always make sure there is enough cDNA synthesis buffer flowing in the channel so that the bead column does not dry up.

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23

Electroporation Strategies for Genetic Manipulation and Cell Labeling

Terence J. Van Raay, Rhonda T. Lassiter, and Michael R. Stark

Summary

Electroporation has emerged as an effective method for cell labeling and manipulation of gene expression. In the past decade, electroporation applications have expanded to include in vivo chick, mouse, *Xenopus*, and zebrafish techniques, along with numerous in vitro strategies for cell and tissue culture. We focus on applications relevant to neural stem cell research, providing detailed protocols for in ovo chick electroporation and in vitro targeting of neuroepithelial precursor cells. Electroporation descriptions and related figures identify the tools and reagents needed to carry out targeting of the neuroepithelium. Various applications of the electroporation technique in neural stem cell research are highlighted, along with corresponding publications.

Key Words: In ovo chick electroporation; cell labeling; targeted gene misexpression.

1. Introduction

During the last decade, significant advances in stem cell research and developmental neuroscience have led to the development of creative applications in genetic manipulation and cell labeling. Because transgenic model systems for evaluating gene function are not feasible for many research paradigms, scientists have developed alternative methods of gene transfer and cell labeling that allow a wider range of research applications. In the past several years, scientists have developed, modified, and effectively applied the technique of electroporation to introduce gene expression plasmids into embryonic tissue and

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cultured cells. The first broadly applied applications were in ovo chick electroporation strategies ***(1–13)***; however, mouse electroporation strategies also were described several years ago ***(13–15)***. Today, the technique has been adapted for several different applications, including chick, zebrafish, and *Xenopus* embryo electroporation, in utero (exo utero) mouse electroporation, and in vitro cell

Table 1
Examples of electroporation techniques and applications

Model	Reference	Technique and application
In vivo		
Chick	20	In ovo electroporation techniques article: describes applications for overexpression, RNA interference (RNAi), morpholino
	26	In ovo chick RNAi techniques
	29	Basic/helix-loop-helix protein interactions studied by electroporation and fluorescence resonance energy transfer
Zebrafish	16	DNA, RNA, and morpholino electroporation
	28	GFP targeting of neural tube
Xenopus	18	Method to study neuronal properties from single cells to the entire brain
Newborn rodent	22	Retinal labeling and effective RNAi gene knockdown
In utero		
Mouse	25	Long-term glial cell lineage tracing; GFP-labeled myelinating oligodendrocytes originate from ventral forebrain
	17	In utero labeling and subsequent imaging of proliferative zone neural precursor cells
	19	Labeling and visualization of precerebellar neurons: their developmental migration and contribution to brain nuclei
	24	Mouse hippocampal neurogenesis study
In vitro		
Stem cells	21	Transfection of primary central and peripheral nervous system neurons
	23	Human embryonic stem cell transfection techniques
	27	Electroporation-based gene transfer into neural precursor cells
Tissue explants	30	Labeled chick neural tissue explanted to collagen gel matrix
	22	Rat and mouse retinal tissue explant electroporation

culture electroporation. Applications related to nervous system development and neuronal stem cells are prevalent. Electroporation is now a widely accepted technique, because it has proven to be an extremely effective method of targeting cells for gene misexpression, and for labeling cells (typically with a marker such as green fluorescent protein [GFP]) in developing embryos and in tissue culture.

The purpose of this chapter is to highlight the basic technique of in vivo chick electroporation and to describe a method of targeting embryonic neuroepithelial precursor cells in vitro. However, because electroporation has been used in a variety of applications, we also include a table (**Table 1**) highlighting descriptive and translational publications relevant to this technique in neural stem cell research ***(16–30)***. This table is not meant to be comprehensive, but instead it provides additional references and examples of specific applications.

2. Materials

2.1. Electroporation Apparatus

For the experiments described here, we used a BTX T820 ElectroSquarePorator (Genetronics, San Diego, CA, model ECM 830) in combination with BTX Genetrode 508 electrodes or fine tungsten electrodes. Other equipment commonly used is the Protech CUY-21 electroporator and the Intracel TSS20 Ovodyne electroporator ***(20)***.

2.2. Plasmid Preparation

1. Eukaryotic expression vector (2 µg/µl minimum concentration; 8 µg/µl preferred concentration) containing the gene of interest (in this case GFP) under the control of a strong promoter, such as cytomegalovirus (CMV), Rous sarcoma virus (RSV), or simian virus (SV) 40. The high DNA concentration improves gene transfer efficiency.
2. 5% Fast green (Fisher Scientific, Pittsburgh, PA, cat. no. F-99) solution in H_2O.
3. Sterile 15% sucrose solution.

2.3. In Ovo Chick Embryo Electroporation

1. Fertile chicken eggs.
2. Humidified incubator, 38°C (G.Q.F. Manufacturing, Savannah, GA, model 1550E).
3. Sterile phosphate-buffered saline (PBS).
4. Penicillin/streptomycin (Pen/Strep; Invitrogen, Carlsbad, CA, cat. no. 15070-063).
5. Pelikan Fount India ink (available at many art supply stores, including www.misterart.com).

6. Syringes: 10 and 1 ml.
7. Hypodermic needles: 1-in. 18-gauge, 5/8 in. 22-gauge.
8. Transparent sealing tape (e.g., Scotch tape).
9. Small surgery scissors.
10. Picospritzer injector (General Valve Corp., Fairfield, NJ) with 1.5-mm injection handle.
11. 1.5-mm fine-drawn glass pipettes.
12. 10-µl Wiretrol calibrated micropipets (Drummond Scientific, Broomall, PA, Fisher cat. no. 21-175B).
13. Fine forceps.
14. Micromanipulator(s).
15. Black electrical tape.
16. Banana plug fittings and attachment cords.
17. Tungsten wire (Goodfellow cat. no. W005137), sharpened by etching ***(31)***. Briefly, wire sharpening can be achieved by repeatedly dipping the tip of a tungsten wire into hot fused sodium nitrite. Care should be taken to perform sharpening in a fume hood while continually heating the sodium nitrite to keep it from solidifying. Alternatively, etching can be performed using a 12-V or variable transformer to pass a current through a saturated sodium nitrite or sodium nitrite/potassium hydroxide solution (71 g/l $NaNO_2$, 34 g/l KOH in H_2O). Briefly, a carbon rod is attached to one lead (preferably the red anode), and it is partially submersed into the sodium nitrite solution. The tungsten wire is attached to the other lead, and it is dipped repeatedly into the solution, creating a current and resulting in rapid etching of the wire. Before or after sharpening, the tungsten wire is used to make the tungsten electrode with a 1-ml syringe and a 22-gauge needle (*see* **Fig. 1A, C**). To make the electrode, carefully thread the tungsten wire through the needle to the desired length (approx 0.6 cm [1/4 in.]), leaving about 10 cm of wire available to run through the 1-ml syringe and attach to the banana plug. Attach banana plug to syringe using electrical tape. Carefully paint the needle and part of the tungsten wire with fingernail polish and wrap with electrical tape. Assemble the electrode to resemble that shown in **Fig. 1**, ensuring a good connection is made between the wire and the banana plug. Other types of electrodes can be purchased or made depending on the desired application.

2.4. In vitro Embryonic Neural Tube Electroporation

1. Embryonic (E) day 9.5 mouse embryos (*see* **Note 1**).
2. Sterile PBS.
3. Collagenase/dispase solution (10 ml; make fresh): 1.0 mg/ml collagenase type I (Worthington Biochemicals, Freehold, NJ, cat. no. S6M622), 2.0 mg/ml dispase II (Roche Diagnostics, Indianapolis, IN, cat. no. 165 859), in 1× Hanks' balanced salt solution (Invitrogen, cat. no. 14170) in sterile water. Filter through a 0.22-µm syringe filter, dispense into 500-µl aliquots, and store on ice.

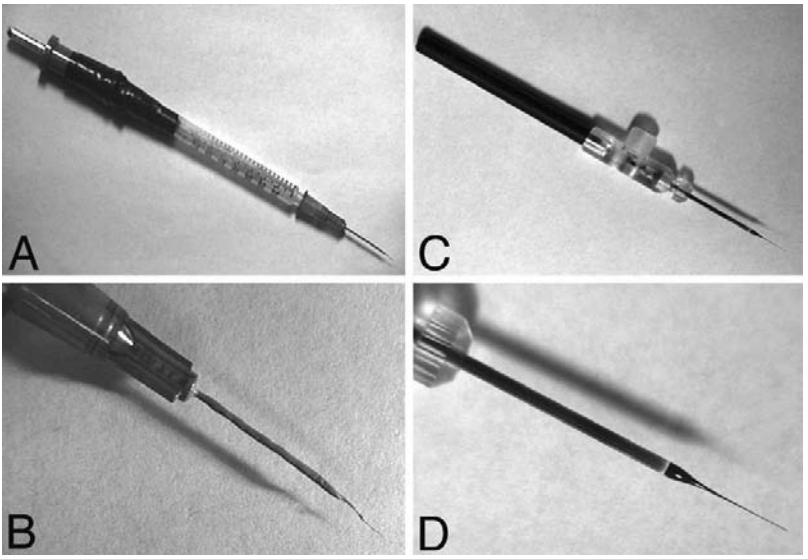

Fig. 1. Electrode and injection needles. **(A)** The negative electrode contains a sharpened tungsten wire passed through a 1-ml syringe and a 5/8-in. 22-gauge needle (see item 17 in section 2.3 for details). **(B)** Higher magnification of electrode showing sharpened tungsten wire. **(C,D)** A 1.5-mm fine-drawn glass pipette is back-filled with appropriate DNA/dye solution using a Wiretrol calibrated micropipet. **(D)** Higher magnification of the fine-drawn end of the glass pipette shown in **C**.

4. Trypsin-EDTA.
5. Chick embryo extract (CEE; good for 1–2 years at –80°C).
   a. Incubate chicken eggs at 37°C in humidified incubator for 11 days.
   b. Rinse eggs with 70% ethanol, dissect out embryos, and place in sterile modified Eagle's medium with glutamine and Earle's salts (MEM 2×; Invitrogen, cat. no. 11935) at 4°C.
   c. Macerate approximately 10 embryos at a time by passing them through a 30-ml syringe into a 50-ml Falcon tube. This produces approximately 25 ml.
   d. Add an equal volume of MEM and shake at 4°C for 45 min.
   e. Add 100 µl of 10 mg/ml sterile hyaluronidase (Sigma-Aldrich, St. Louis, MO, cat. no. H3884) per 50 ml of embyro/MEM mix.
   f. Centrifuge for 180,000 × *g*·hours (e.g., 30,000 *g* × 6 h).
   g. Filter supernatant through a 0.45-µm filter and then through a 0.22-µm filter.
   h. Aliquot and store at –80°C.
6. NEP basal media (90 ml; store at 4°C, good for 1–2 weeks): 86 ml of Dulbecco's modified Eagle's medium/Ham's F-12 (Invitrogen, cat. no. 11320), 1 ml of N2

supplement (Invitrogen, cat. no. 17502), 2 ml of B27 supplement (Invitrogen, cat. no. 17504), 1 ml of Pen/Strep (Invitrogen, cat. no. 15070).
7. NEP complete media (make fresh): 90 % NEP basal medium, 10 % CEE, 35 µg/ml human basic fibroblast growth factor (PeproTech, Rocky Hill, NJ, cat. no. 100-18B).
8. Six-well tissue culture dishes.
9. 0.1% fibronectin (Sigma-Aldrich, cat. no. F-1141).
10. Sterile dissecting hood.
11. Fine dissecting tools.
12. CO_2 incubator.
13. Nine-well spot plate (Fisher Scientific, Pittsburgh, PA, cat. no. 13-748B).
14. Sterile glass pipette.
15. 15-ml Falcon tubes (Falcon; BD Biosciences Discovery Labware, Bedford, MA, cat. no. 352096).

3. Methods

3.1. DNA Injection and Electroporation Apparatus

The methodology described here is similar to what others have reported for in ovo electroporation (***4,5,20***). It is important to prepare a pure solution of the desired gene expression construct, and resuspend at a minimum concentration of 2 µg/µl in PBS or water. Subsequently, adding fast green (up to 0.5% final concentration) will allow for easy visualization of the DNA solution upon injection. Addition of sucrose to the DNA tends to slow the dispersion rate and to cause the solution to settle. The electroporation parameters described here have proven most appropriate for this application; however, these parameters may need to be adjusted for individual applications. Conditions range as follows: pulse length, 10–20 ms; voltage, 15–50 V; and number of pulses, 5. It is important to set up the electroporation and injection station before any planned experiment to ensure that the necessary equipment is in place. In addition, we found that having various electrode types available provides versatility during experimentation.

3.2. In Ovo *Chick Embryo Electroporation*

In most areas, fertile White Leghorn chicken eggs can be obtained locally. Alternatively, specific-pathogen-free avian supply eggs (Charles River Spafas, Inc., North Franklin, CT) can be obtained commercially, but at greater expense. Any standard humidified egg incubator should suffice; however, cleaning and sterilization of the incubator will help viability.

1. Incubate fertile chicken eggs horizontally at 38°C for 48 h (or to desired developmental stage) in a humidified incubator.

2. Remove eggs, and sterilize outer shell by wiping gently with 70% ethanol.
3. Carefully poke a small hole in the narrow end of the egg with an 18-gauge needle attached to a 10-ml syringe and remove 3 ml of albumin. If yolk is accidentally withdrawn into the syringe, discard the egg, because the embryo may not develop properly.
4. Seal the needle hole with clear tape, and apply additional tape to the top-most surface of the eggshell, two to three tape strips wide with a little overlap between them. With fine dissecting scissors, carefully poke through the top-most surface of the eggshell (covered with tape), and cut a circle approximately 1 cm in diameter. Usually the embryo will be revealed on the yolk surface near where the eggshell was cut. A larger hole may be needed to accommodate embryo manipulation and electrode placement.
5. To prevent drying and infection, apply a few drops of sterile PBS containing Pen/Strep (1%, v/v) to the surface of the embryonic area. To clearly visualize the embryo, inject a small amount of 10% India ink in PBS into the sub-blastodermal cavity under the embryo by using a 1-ml syringe and a 22-gauge needle (bent in the middle to 90°C) poked into the yolk lateral to the embryo.
6. Carefully remove the vitelline membrane over the area to be electroporated using a fine glass needle or fine forceps.
7. If micro-electroporation is planned using a tungsten electrode as the driving electrode (black cathode), place the reference electrode (attached to the positive pole) near the embryo by using a micromanipulator. It is important to note that gene transfer will occur directionally toward the positive pole. We use one of the two Genetrode electrodes (BTX) for this purpose. Alternatively, for broader electroporation, both Genetrode electrodes can be positioned using a micromanipulator. In this case, one electrode should be placed on either side of the embryo.
8. Carefully inject the DNA into or near the area to be targeted using a pulled glass pipette and a Picospritzer microinjector (*see* **Figs. 2A and 3A,B**). The glass pipette can be back-filled with DNA by using Wiretrol-calibrated micropipets or a Hamilton syringe (Hamilton, Reno, NV).
9. By hand, quickly place the tungsten electrode near the target tissue and activate the electroporator before the DNA can disperse. Optimal conditions used here were as follows: pulse length, 20 ms; voltage, 15–30 V; and number of pulses, 5. Alternatively, the tungsten electrode can be placed before DNA delivery using a micromanipulator. Tissue survival and overall development are enhanced significantly if the tissue near both electrodes is completely submersed in liquid during electroporation. Small bubbles should be produced from the electrodes, indicating a current was generated. In addition, a slight darkening of the fast green may occur.
10. Remove the electrodes and completely seal the eggshell window with clear tape. Return the egg(s) to the incubator and allow development to proceed 1 to 3 days.

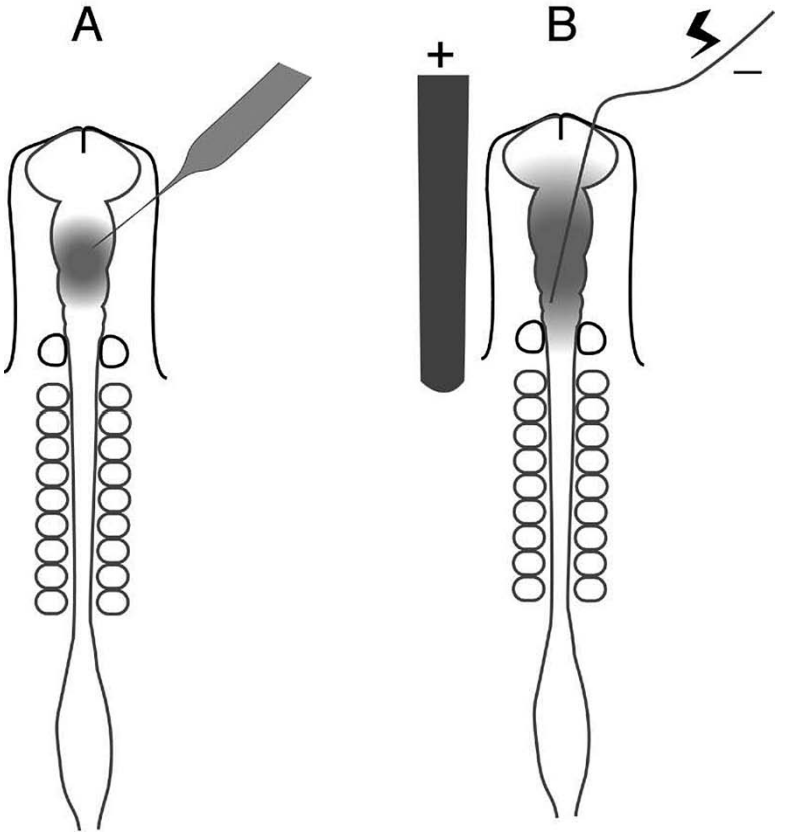

Fig. 2. Electroporation Procedure. **(A)** The DNA/dye solution is injected into the neural tube at desired stage and position (Hamburger and Hamilton ***(32)*** stage 10; hindbrain region shown). **(B)** The positive electrode is placed adjacent to the region of interest, whereas the sharpened tungsten wire (negative electrode) is inserted directly into the neural tube. The electroporation current is initiated (see text).

11. GFP expression can be observed immediately by whole-mount fluorescent microscopy. After confirming expression, embryos can be fixed in 3% formaldehyde and prepared for *in situ* hybridization or for sectioning, as needed.

Several variations can be used for electrode placement and type of electrode. For example, another strategy for directional targeting is dorsal-ventral positioning of the electrodes, where one electrode is placed immediately below the embryo in the sub-blastodermal space (where the India ink was injected), the other electrode is placed dorsal to the embryo. Ventral or dorsal neural tube tissue can be effectively targeted using this strategy. Also, morpholino inhibitory molecules can be electroporated, but we and others ***(33)***

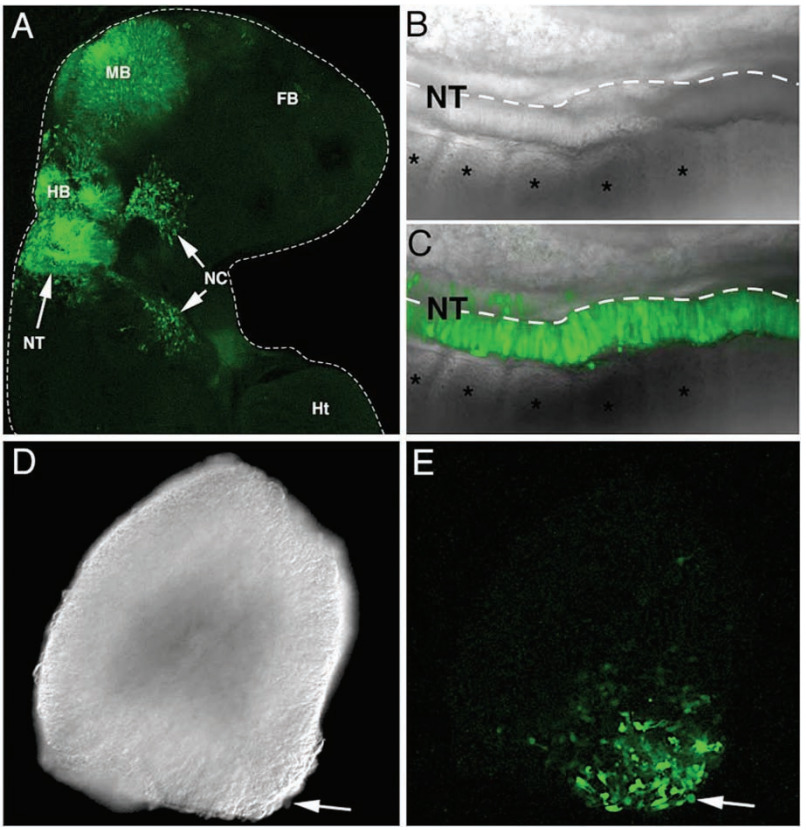

Fig. 3. Electroporation results. **(A)** We injected 3 µg/µl of a DNA vector containing GFP driven by a CMV promoter into the hindbrain region of an HH stage 10 embryo. Electroporation was carried out with five pulses of 10 ms each at 15 V. Twenty-four hours postelectroporation, the embryo was harvested, fixed, and observed under a fluorescent microscope. GFP-labeled cells can be observed throughout the midbrain (*MB*) and hindbrain (*HB*) regions of the neural tube (*NT*). In addition, GFP-labeled cells are also observed in migrating neural crest cells (*NC*). *FB*, forebrain; *Ht*, heart. **(B)** Dorsal view of a brightfield image of a neural tube electroporated with a GFP construct as described in **A**. **(C)** Fluorescent image overlaid on the brightfield image. GFP-positive cells are observed predominantly in only one half of the neural tube. The asterisks label the somites. **(D)** Brightfield image of a mouse E9.5 neural tube 24 h after electroporation. Electroporation was carried out with five pulses of 10 ms each at 20 V. During the 18-h period in suspension culture, the neural tube becomes spherical. **(E)** Confocal fluorescent image of neural tube shown in **D**. Numerous GFP-labeled cells can be observed in the area of electroporation (*arrows*).

have observed that fluorescein-labeled morpholinos sometimes do not follow the predicted targeting path within the electroporation current, but instead incorporate into cells away from the anode (positive) electrode, meaning these molecules migrate in the opposite direction from standard DNA expression reagents.

3.3. In vitro Neuroepithelial Precursor Cell Electroporation

1. Approximately 24 h before experiment, coat tissue culture dishes with 0.002% fibronectin and incubate overnight at 4°C. Remove and recycle fibronectin (can be reused up to four times), carefully rinse with sterile PBS, and store at 4°C in PBS until ready to use.
2. Make up DNA solution to 1–4 μg/μl in 0.5% fast green and 5% sucrose and store on ice.
3. Harvest E9.5 embryos, leaving all extraembryonic membranes intact, from timed pregnant mice in sterile PBS. From this point, all work should be done in a sterile dissecting hood.
4. Using a dissecting microscope, remove all extraembryonic membranes as well as the amnion and move the embryo to clean dish of sterile PBS. Repeat for all embryos.
5. Remove the head just caudal to the otic vesicles and remove as much of the soft tissue as possible. Clip the very end of the tail. This procedure removes as much of the non-neural tissue as possible as well as the cephalic regions, which are not to be included as neuroepithelial precursor cells. Clipping the tail allows for more complete movement of the digestive enzymes. Do this for all the embryos before proceeding to **step 6**.
6. Put approximately three to four embryos into 500 μl of the collagenase/dispase solution and store on ice.
7. Once all of the embryos are in the collagenase/dispase solution, put the tubes at 37°C for 10 min (*see* **Note 2**).
8. Remove embryos from collagenase/dispase solution, quickly rinse in PBS, and place in NEP complete media in nine-well spot plate, mix, and move to fresh NEP complete in nine-well spot plate. This is to dilute out the collagenase/dispase solution.
9. Working with a single neural tube at a time, triturate the embryo with a sterile glass pipette several times. The neural tube should easily separate away from the surrounding somites. Using fine forceps, carefully remove any remaining somites and place the purified neural tube into fresh NEP complete media. Repeat for all embryos.
10. Place approximately three neural tubes per well in a six-well tissue culture dish containing 2 ml of sterile PBS (*see* **Note 3**).
11. Pipette 10 μl of 3 μg/μl DNA/0.01% fast green/5% sucrose solution directly over the neural tube. The sucrose keeps the solution concentrated around the neural tube.

12. Place electrodes on either side of neural tube and electroporate as follows: pulse length, 10 ms; voltage, 20 V; and number of pulses, 5.
13. Allow neural tubes to recover for approximately 10 min and then transfer to clean tissue culture dish with fresh NEP complete media and incubate at 37°C, 5% CO_2 for 4–18 h. The neural tubes become spherical from growing in suspension, and initial gene expression can be observed approximately 8 h after electroporation.
14. Remove neural tubes and place them in a 15-ml sterile Falcon tube, remove media, and add 500 µl of trypsin-EDTA. Carefully watch as neural tubes start to dissociate. Neutralize trypsin by adding 1.5 ml of CEE. Spin down cells at 250 × *g* for 5 min at room temperature. Remove supernatant and add NEP complete media. Resuspend and triturate cells to completely dissociate them. Plate on fibronectin coated tissue culture dishes at desired density. Incubate at 37°C, 5% CO_2. After confirming expression, cells can be fixed in 3% formaldehyde and prepared for histological observation as desired (*see* **Note 4**).

4. Notes

1. If E8.5 embryos are desired, they can be isolated in a similar way, but they are much more sensitive to mechanical manipulation. In addition, trypsin should not be used to dissociate the cells. Instead, the neural tubes should be triturated using a pipette to obtain single-cell suspensions. For older embryos (>E9.5), the collagenase/dispase step will not work. Instead, the neural tube can be isolated mechanically by first separating the neural tube, with some attached mesenchyme, from the embryo. There is a tough meninges layer tightly associated with the neural tube that needs to be removed. Starting from the rostral end, this can be peeled back caudally, removing the mesenchyme tissue with it, and leaving a very clean neural tube.
2. The exact time for this incubation needs to be determined for every lot of collagenase and dispase. In general, test an embryo after 5, 7, and 10 min at 37°C. Longer incubation times may be necessary. The neural tubes are fairly resistant to the digestive enzymes, compared with the surrounding tissue. Beware of prolonged incubations, because they have detrimental effects on the quality of the neural tissue.
3. Electroporation works equally well in NEP complete media if this is desired.
4. Fixation of tissue or cells with MeOH ablates or severely reduces GFP visibility. In this case, antibodies against GFP (Invitrogen, cat. no. A6455) can be used to identify targeted cells.

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24

Novel and Immortalization-Based Protocols for the Generation of Neural CNS Stem Cell Lines for Gene Therapy Approaches

Luciano Conti and Elena Cattaneo

Summary

Transplantation of neural cells engineered to produce growth factors or molecules with antitumor effects have the potential of grafted cells to be used as vectors for protein delivery in animal models of diseases. In this context, neural stem cells (NSCs), since their identification, have been considered an attractive subject for therapeutic applications to the damaged brain. NSCs have been shown to include attributes important for potential successful ex vivo gene therapy approaches: they show extensive in vitro expansion and, in some cases, a particular tropism toward pathological brain areas. Clearly, the challenges for future clinical development of this approach are in the definition of the most appropriate stem cells for a given application, what genes or chemicals can be delivered, and what diseases are suitable targets. Ideally, NSC lines should be homogeneous and well characterized in terms of their in vitro stability and grafting capacity. We discuss two possible approaches to produce homogeneous and stable progenitor and NSC lines that exploit an oncogene-based immortalization, or, in the second case, a novel protocol for growth factor expansion of stem cells with radial glia-like features. Furthermore, we describe the use of retroviral particles for genetic engineering.

Key Words: Neural stem cells; NS cells; gene therapy; immortalization; retroviral vectors; neurodegenerative diseases; stem cell transplantation.

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1. Introduction

Stem cell-based gene therapy approaches have provided the basis for the development of potentially powerful new therapeutic strategies for a broad spectrum of brain diseases. The rationale of this therapeutic approach relies on the transplantation to the central nervous system (CNS) of cells that are able to deliver beneficial molecules and replace lost elements, thereby restoring normal brain function ***(1–3)***.

Neural stem cells (NSCs) are cells that can continuously self-renew and that have the potential to generate intermediate and mature cells of both neuronal and glial lineages ***(4)***. Large-scale sources of NSCs are crucial for both basic research and novel approaches toward treatment of neurological disorders. Indeed, the large-scale expansion of neural stem (NS) and progenitor cells in vitro would make available a large population of progenitors that, besides offering a suitable tool to study the fundamental biology of tissue-specific stem/progenitors and their molecular and biochemical features, would have significant potential in the therapy of a wide range of clinical conditions. NSCs have features that make them appropriate as means of expression and delivery of molecules of therapeutic interest for CNS disorders and that differentiate them from other peripheral cell types. The grafted cells must integrate without inducing further damage to the recipient brain and have no apparent systemic or local side effects, such as uncontrolled growth, production/release of harmful compounds, or activation of an inflammatory response ***(2)***. NSCs have the potential to integrate into the host brain apparently without causing major damage, in contrast to fibroblasts, which have been successfully used as suitable vehicles in other organs. In only one case, in vitro expanded putative NSCs have acquired the capacity to tumorigenic properties ***(5)***. Importantly, NSCs can differentiate into glia or neurons by default, but they cannot give rise to extra neural tissue. Furthermore, besides the capacity of grafted cells to properly integrate in the host tissue, the capability to achieve long-term delivery of proteins of therapeutic interest in the brain represents an important requirement for obtaining effective advances in ex vivo gene therapies. NSCs perfectly suit this point, because they can be expanded ex vivo for extensive periods, and they are hence amenable to the techniques required for genetic manipulation ***(1)***. However, for any possible clinical application, evidence that the gene transfer is completely innocuous and safe for the recipient must be obtained. In this regard, special precautions must be taken to not interfere with other neuronal functions apart from the function to be targeted.

We present methods for generating NSC lines by exposure to specific growth factors or through immortalization procedures and their engineering for gene therapy purposes.

In the natural setting of the nervous system, stem cell division is tightly regulated. Indeed, in vivo, NSCs reside in specific cellular microenvironments, named "niches," that constitute an appropriate milieu for supporting self-renewal and regulating the balance between symmetrical self-renewal and commitment divisions, or an asymmetric mode of stem cell division ***(6)***. Even in the early embryonic nervous system, purely exponential cell division occurs only during a brief time window ***(4)***. Therefore, for a stem cell to become a clonal progenitor of a cell line, the intrinsic barriers to continued cell division must be bypassed ***(7)***. All cell lines overcome these constraints and exhibit continuous cell division plausibly involving symmetrical self-renewal. However, with the remarkable exception of embryonic stem (ES) cells ***(8)***, it has proven very complicated to propagate homogenous cultures of tissue-specific stem cells ex vivo.

Historically, normal cells had to be immortalized to suitably expand particular cell types and to make their characterization possible. The rational foundation behind immortalization is to block the progression of a developmental program by forcing the cells to remain in continuous cell cycle. Immortalization can be achieved by a number of manipulations, although the most common is the introduction of exogenous cDNA coding for oncogenic proteins ***(7)***. Importantly, a common property of murine and human NSC lines to date generated via retroviral transduction of immortalizing oncogenes is that they behave like established lines but they show no signs of transformation either in vitro or in vivo ***(9–12)***. Oncogenes such as *myc* and Large-T Antigen have been shown to exhibit immortalizing abilities without fully transforming the cells ***(12)***. The discovery of mutant alleles of particular oncogenes ***(13)*** and strategies for a pharmacologically regulated expression of oncoproteins also have been used to generate safe cell lines for gene therapy purposes ***(14–16)***. Besides the use of temperature-sensitive oncogenes, new strategies such as the use of the CRE-loxP reversible immortalization procedure or the tetracycline-regulated expression have now begun. This may avoid the presence of an immortalizing oncogene in the grafted cell lines that will be safest for use in experimental trials ***(17)***.

In the past 15 years, protocols based on the use of growth factors for NSC expansion have been developed, leading to their growth via propagation of floating cell clusters termed "neurospheres" ***(18–21)***. Neurospheres consist predominantly of committed progenitors mixed with differentiated astrocytes and neurons. This artificial, mixed cellular environment may provide a microenvironment that permits the renewal of only a sparse number of stem cells ***(22,23)***. Thus, an important goal has always been the development of alternative and innovative systems to propagate NSCs clonally in vitro, thereby generating more homogenous nonimmortalized cultures than are available at present.

We have challenged this idea, and we have recently optimized a novel and powerful strategy for lineage-restricted, tissue-specific stem cell line derivation and stable long-term propagation from mouse ES cells ***(24)***. We found that neural precursor cells, normally fated to rapidly differentiate to neurons and glia, can be readily expanded as adherent clonal stem cell lines by using the growth factors epidermal growth factor (EGF) and fibroblast growth factor (FGF)-2, and, importantly, undergo symmetrical stem cell divisions without accompanying differentiation ***(24,25)***.

Immunocytochemical analysis of these cultures reveals that almost all cells express Nestin and Sox2, but they lack the expression of glial fibrillary acidic protein and other neuronal antigens. Shifting the cultures to neuronal differentiation conditions reveals that these cells can efficiently generate mature neurons, astrocytes, and oligodendrocytes, indicating their NS identity. These properties, together with single-cell clonal analysis, are suggestive for the generation of self-renewing NSC cultures that we named NS cells ***(24)***. Together with the aforementioned characteristics, that NS cells are highly amenable to genetic manipulation by normal transfection procedures or by viral transduction and that they can reliably be recovered from standard cryopreservation and retain diploid chromosome content at late passages, indicate that NS cells can be considered stable established cell lines ***(3,24,26,27)***. Interestingly, NS cells exhibit morphological hallmarks of radial glia cells, such as elongated bipolar morphology, lamellate extensions, end-feet, and oval nuclei. Analysis of NS cells revealed that these cells are homogenously immunopositive for Nestin, SSEA1/Lex1, Pax6, prominin, RC2, vimentin, 3CB2, Glast, and BLBP, a set of markers diagnostic for neurogenic radial glia (RG) ***(24,26,27)***. This evidence suggests that NS cells are closely related to an RG lineage. Remarkably, NS cells keep their neurogenic potential after expansion for >150 passages, yet they retain the capability to produce a large proportion of β-III-tubulin–positive neurons ***(25)***. This indicates that the NS cells undergo sustained symmetrical self-renewal divisions with complete suppression of differentiation in response to FGF-2 and EGF. These results indicate that the acquisition of radial glial properties endows the cells with a "niche" that traps them in a state of symmetric cell division.

2. Materials

2.1. Generation of NS Cells from Murine Fetal Telencephalic Tissue

1. Ordinary tissue culture equipment.
2. Phosphate-buffered saline (PBS) solution (Sigma-Aldrich, Milan, Italy, cat no. D8537).
3. 0.1% Gelatin (Sigma-Aldrich, cat. no. G1890) sterile solution prepared in PBS.

4. Accutase solution (Sigma-Aldrich, cat. No. A3317).
5. Protease (Sigma-Aldrich, cat. no. P3417).
6. Enzyme inhibitor: trypsin inhibitor (1 mg/ml; Invitrogen, Milan, Italy, cat. no. 170075) and bovine serum albumin (1 mg/mL; Sigma-Aldrich, cat. no. A9647).
7. Euromed-N (EuroN) medium (Euroclone, Celbio, Milan, Italy, cat. no. ECM0833LD).
8. N2 supplement (Invitrogen, cat. no. 17502).
9. Growth factors containing EuroN medium (GF-EuroN): EuroN, 2 mM L-glutamine (Invitrogen, cat. no. 25030), 50 units/ml penicillin/streptomycin (Invitrogen, cat. no. 15140), N2 supplement, 10–20 ng/ml EGF (PeproTech, Tebu-Bio, cat. no. 016100-15D), 10–20 ng/mL Fibroblast Growth Factor-2 (FGF-2) (PeproTech, Le Perray-en-Yvelines, France, cat # 016100-18D).
10. Corning tissue culture plastic dishes and flasks.

2.2. Growth and Transfection of Packaging Cell Line

1. Ordinary tissue culture equipment.
2. Dulbecco's modified Eagle's medium (DMEM; Invitrogen, cat. no. 41966) containing 0.11 g/l sodium pyruvate (Invitrogen, cat. no. 11840), 2 mM L-glutamine (Invitrogen, cat. no. 25030), 3.7 g/l sodium bicarbonate (Invitrogen, cat. no. 11810) and 50 units/ml penicillin/streptomycin (Invitrogen, cat. no. 15140).
3. Fetal bovine serum (FBS; Invitrogen, cat. no. 10108).
4. 15 ml of conical tubes.
5. 0.22- and 0.45-μm filters.
6. DNA retroviral vector carrying an immortalizing oncogene together to a selection cassette.
7. Chloroquine (Sigma-Aldrich, cat. no. C6628). Chloroquine stock is 50 mM; for 7 ml of media + 3 ml of DNA, add 5μl.
8. 2 M $CaC1_2$ (Sigma-Aldrich, cat. no. 4160).
9. Stock solution of Na_2HPO_4 dibasic (5.25 g in 500 ml of water).
10. 2× HBS: 8.0 g of NaCl, 6.5 g of HEPES (sodium salt; Sigma-Aldrich, cat. no. H-7006), 10 ml of Na_2HPO_4 stock solution, pH to 7.0 using NaOH or HCl. Bring volume up to 500 ml and sterilize by filtering. Check pH again. The pH is very important; it must be exactly 7.0. Because pH is so important, make three batches: pH 6.95, pH 7.00, and pH 7.05. Test each solution and use the solution that yields the best precipitate. All reagents should be at room temperature before use.

2.3. Dissociation of Human Fetal-Derived Telencephalic Tissue Specimen

1. Ordinary tissue culture equipment.
2. Hanks' balanced salt solution with no calcium and magnesium (HBSS) (Invitrogen, cat. no. 14170).

3. Protease (Sigma-Aldrich, cat. no. P3417).
4. Enzyme inhibitor: trypsin inhibitor (1 mg/ml; Invitrogen, cat. no. 170075) and bovine serum albumin (1 mg/ml; Sigma-Aldrich, cat. no. A9647).
5. Poly-D-lysine (Sigma-Aldrich, cat. no. P7280).
6. DMEM/Ham's F-12 (1:1) (Invitrogen, cat. no. 11039).
7. N2 supplement (Invitrogen, cat. no. 17502).
8. Growth factors containing serum-free medium (GF-SFM): DMEM/Ham's F-12 (1:1), N2 supplement, 20 ng/ml EGF (PeproTech, Tebu-Bio, cat. no. 016100-15D), 10–20 ng/ml FGF-2 (PeproTech, Tebu-Bio, cat. no. 016100-18D), and 1% bovine serum albumin.
9. Fetal bovine serum (Invitrogen, cat. no. 10108).
10. Bovine serum albumin (Sigma-Aldrich, cat. no. A9647).

2.4. Infection of CNS Stem Cells

1. Normal tissue culture equipment.
2. Poly-D-lysine (Sigma-Aldrich, cat. no. P7280).
3. Frozen or fresh retroviral vector supernatant.
4. Accutase solution (Sigma-Aldrich, cat. no. A3317).
5. Protease (Sigma, cat. no. P3417).
6. Enzyme inhibitor: trypsin inhibitor (1 mg/ml; Invitrogen, cat. no. 170075) and bovine serum albumin (1 mg/ml; Sigma-Aldrich, cat. no. A9647).
7. EuroN medium (Euroclone, Celbio, cat. no. ECM0833LD).
8. N2 supplement (Invitrogen, cat. no. 17502).
9. GF-EuroN: EuroN, 2 mM L-glutamine (Invitrogen, cat. no. 25030), 50 units/ml penicillin/streptomycin (Invitrogen, cat. no. 15140), N2 supplement, 10–20 ng/ml EGF (PeproTech, Tebu-Bio, cat. no. 016100-15D), 10–20 ng/ml FGF-2 (PeproTech, Tebu-Bio, cat. no. 016100-18D).
10. Neomycin analog Gemeticin (G-418, Invitrogen, cat. no. 11811).
11. Corning tissue culture plastic dishes and flasks.
12. Polybrene (Sigma-Aldrich, cat. no. H9268). Polybrene is prepared as stock solution at 5 mg/ml and stored at –20°C.

2.5. Infection of Immortalized CNS Stem Cells

1. Normal tissue culture equipment.
2. Frozen or fresh retroviral vector supernatant.
3. DMEM (Invitrogen, cat. no. 41966) containing 0.11 g/l sodium pyruvate (Invitrogen, cat. no. 11840), 2 mM L-glutamine (Invitrogen, cat. no. 25030), 3.7 g/l sodium bicarbonate (Invitrogen, cat. no. 11810), and 50 units/ml penicillin/streptomycin (Invitrogen, cat. no. 15140).
4. Fetal bovine serum (Invitrogen, cat. no. 10108).
5. Corning tissue culture plastic dishes and flasks.
6. Polybrene (Sigma-Aldrich, cat. no. H9268). Polybrene is prepared as stock solution at 5 mg/ml and stored at –20°C.

3. Methods

3.1. Generation and Expansion of NS Cells from Murine Fetal Telencephalic Tissue

Below, we describe procedures to generate, expand, and cryopreserve NS cell lines from fetal mouse (embryonic day [E]11.5–18.5) brain tissue.

3.1.1. Generation of NS Cells from Mouse Fetal Cerebral Tissue

1. Dissect the area of interest from mouse fetal brain by standard dissection procedures.
2. Perform an enzymatic dissociation of the tissue for 6 min at 37°C with gentle agitation in PBS containing 3 mg/ml protease.
3. Wash the tissue with warm PBS, and then rinse with warm enzyme inhibitor. If working with E11.5–14.5 brain tissue, the enzymatic dissociation can be avoided. The mechanical dissociation described below is sufficient.
4. Triturate brain tissue fragments approximately 15 times with a 5-ml pipette, and then collect them by centrifugation at 800 × *g* for 3 min.
5. Aspire the supernatant and leave the tissue on ice for 10 min.
6. Resuspend the pellet in 1 ml of EuroN and triturate with pipettes of decreasing bore size, to yield a single-cell suspension.
7. Plate the cells on uncoated tissue culture plastic in GF-EuroN medium, at a density of 3×10^6 cells/T25 flask and grow for 3–4 days at 37°C.
8. Cell clusters will form in suspension. These primary aggregates can be readily converted into adherent NS cell lines by plating onto gelatin-coated substrate in GF-EuroN medium. To promote attachment, it is important that debris/dead cells are first effectively removed by sedimentation and that medium is exchanged completely.
9. Cell aggregates will attach and outgrow over 2–5 days. Outgrowing cells can be dissociated to single cells with Accutase solution treatment, replated, and propagated in GF-EuroN medium. During the early few passages, the derived NS lines have an inclination to aggregate and detach from the flask, mainly if the cell density becomes high. Thus, cultures should be passaged at or below 60% confluence. The tendency to spontaneously aggregate is variable depending on the area of origin, but it is generally reduced upon further passaging and when the optimal passaging density has been determined.

3.1.2. Passage and Expansion of NS Cells

Once established, NS cells are propagated in GF-EuroN medium. NS cells are grown on gelatin-coated plates/flasks, and they are routinely split 1 in 2 to 1 in 5. NS cells have a doubling time of approximately 25 h. Cells are passaged using Accutase solution or through incubation with PBS. For establishment of clonal lines, single cells can be deposited in gelatin-coated microwells in

expansion medium. Less rigorously, cells can be plated at very low density, 1,000 cells/10-cm dish. Colonies form within 2 weeks, and they can be picked and expanded.

3.1.3. Cryopreservation and Recovery of NS Cells

NS cells can be readily recovered after freezing/thawing procedures. Routinely, a 60–90% confluent T75 flask can be treated with Accutase solution, and the pellet can be resuspended into 1.5 ml of EuroN medium plus 10% dimethyl sulfoxide (DMSO). This is then split into 3 × 1 ml cryotubes (Corning, Celbio) and stored at –80°C. NS cells are recoverable after >6 months storage in these conditions. For long-term storage, frozen vials are transferred to liquid nitrogen. NS cells are thawed by rapidly bringing the vial to 37°C followed by transfer to 10 ml of prewarmed EuroN medium. Cells are pelleted and then resuspended in fresh GF-EuroN medium to remove DMSO. Cell recovery after cryopreservation is >95% for NS cells.

3.2. Growth and Transfection of Packaging Cell Lines

When passaging packaging cell lines, it is important to never let cells reach confluence. This will reduce transfection efficiency in the short term. For maximally healthy cells, a split of 1:4 or 1:5 of a 70–80% confluent 10-cm plate into a new plate every 2–3 days should provide optimal cell conditions.

Three million cells on a 10-cm plate is a good starting point for seeding cells before transfection. It is important to titer slightly up and slightly down to maximize transfection efficiencies. Efficiencies of 50–60% of the cells should be achieved. The highest transfection efficiencies are obtained with cells that are 70–80% confluent at the time of transfection.

IMPORTANT NOTE: Viral supernatants produced by these methods contain potentially hazardous recombinant virus. Exercise due caution in the production, use, and storage of recombinant retroviral virions. During infection, all liquids and items in contact with virus are treated as biohazardous, treated with bleach, and autoclaved (*see* **Notes 1** and **2**).

1. Eighteen to 24 hours before transfection, plate 3 million cells/10-cm plate in 7 ml of DMEM/10% FBS. Gently shake forward and backward, and then side to side, three to four times to distribute cells evenly on the plate. Allow cells to attach. At two-thirds confluence, a 10-cm plate should provide 5 million cells. It is at this subconfluent stage that cells are most transfectable and can survive the rigors of transfection best, giving the highest titer virus possible.
2. About 5 min before transfection, add chloroquine to each plate (25μM final concentration). Chloroquine acts to inhibit lysosomal DNases by neutralizing

vesicle pH. DNA delivered by Ca_2PO_4 transfection is thought to transit through lysosomes.

3. To a 15-ml tube, add (per 10-cm plate, with all reagents at room temperature):
   a. 15–30 µg of DNA (DNA is added in a drop to side of tube).
   b. 1340 µl of double-distilled H_2O (wash the DNA to bottom of tube with water).
   c. 150 µl of 2 M $CaC1_2$.
   d. Mix thoroughly with finger tapping (1,500 µl total volume); scale volume and DNA/reagent amounts.
   e. Add 1.5 ml of 2× HBS quickly, and then bubble vigorously with automatic pipettor (keep eject button depressed) for 3–15 s (actual length of bubbling time depends on each batch of 2× HBS).
4. Add HBS/DNA solution dropwise onto media (gently and quickly) by spreading across cells in media. Observe the cells under a microscope; you should observe evenly distributed small dark particles.
5. Gently shake plates a few times to distribute Ca_2PO_4/DNA complexes.
6. Put plates in 37°C incubator and let the cells grow for 24 h.
7. Change media to 5 ml of fresh DMEM/10% FBS and leave for further 24 h. Virus is more stable if incubation is carried out at 33°C, although 37°C is fine as well. Do not leave chloroquine on cells >24 h. because conditions become toxic.
8. Pipette supernatant from transfected cells into-15 ml tubes and centrifuge at 800 × *g* for 5 min to pellet cell debris. Filtering through 0.45-µm filter removes cells as well. Supernatant can be frozen at –80°C for later infection, although titer drops by one-half for each freeze-thaw cycle.

If the transfected DNA carried a reporter gene, transfection efficiency can be eventually monitored.

The transfected cells can be selected by adding the specific agent to obtain a stable producer cell line. This will avoid the transfection procedure having to be performed every time, and it will ensure a ready-to-use high-titer retroviral supernatant.

3.3. Retroviral-Mediated Immortalization of CNS Stem Cells

1. To immortalize primary CNS stem cells (*see* **Notes 1** and **2**), perform an enzymatic dissociation of the tissue for 6 min at 37°C with gentle agitation in HBSS containing 3 mg/ml protease.
2. Wash the tissue with warm HBSS, and then wash with warm enzyme inhibitor. If working with E12.5–16.5 murine brain tissue, the enzymatic dissociation can be avoided. The mechanical dissociation described below is enough.
3. Triturate the brain tissue fragments approximately 15 times with a 10-ml pipette, and then collect them by centrifugation at 800 × *g* for 3 min.
4. Aspire supernatant and leave the tissue on ice for 10 min.

5. Resuspend the pellet in 1 ml of DMEM/Ham's F-12 (1:1)/10% FBS and triturate with pipettes of decreasing bore size, to yield a single-cell suspension.
6. Plate the cells on poly-D-lysine–coated tissue culture plastic in DMEM/Ham's F-12 (1:1)/10% FBS, at a density of 8×10^6 cells/100-mm dish.
7. After 12 or 16 h, replace the medium with GF-SFM and grow for 4 days at 37°C.
8. Infect the cultures by incubation for 24 h at 33°C with a mixture of 1 vol of retroviral vector supernatant (DMEM/10% FBS; 4×10^5 colony-forming units [cfu]/ml) and 2 vol of GF-SFM to which 5μg/ml Polybrene has been added.
9. Replace medium with GF-SFM and incubate at 37°C or at 33°C if temperature-sensitive oncogene has been used.
10. Two days after infection, start G-418 selection. We recommend a starting dilution of 200μg/ml and changing weekly two thirds of the medium with fresh GF-SFM containing 250μg/ml G-418. During the first week of G-418 selection, a consistent cell death will occur. Colonies should appear 2–3 weeks after infection.
11. Clones can be isolated, expanded as single-cell clones in coated or uncoated plasticware using GF-SFM or serum-supplemented medium (requirement for growth factors should be assayed), and passaged using trypsin-EDTA, depending on the replication time of the specific clone. Expanded cells can be cryopreserved in a freezing solution consisting of FBS and 10% dimethyl sulfoxide (if the cells are of human origin, use a freezing solution consisting of 50% FBS, 40% GF-SFM, and 10% dimethyl sulfoxide). Expression of the immortalizing oncogene's mRNA and/or oncoprotein should be confirmed by reverse transcription-polymerase chain reaction (RT-PCR) amplification and/or Western blot/immunocytochemistry, respectively. Monoclonality should be confirmed by identification of a single and identical genomic insertion site by Southern blot analysis.

3.4. Engineering of CNS Stem Cells to Express Therapeutical Genes

Procedures to deliver therapeutical genes in growth factor-expanded and immortalized CNS stem cells by retroviral transduction procedures are described here (*see* **Note 2**). Because, in most cases, the therapeutical gene consists of a secreted molecule, it is important to precisely evaluate the amount of transgene produced by using, whenever possible, an enzyme-linked immunosorbent assay (ELISA) assay (*see* **Notes 3** and **4**).

3.4.1. Engineering of NS Cells

1. The day before the infection, dissociate NS cell cultures by Accutase solution treatment and plate at a final density of 40,000/cm^2 in nontreated 25-cm^2 flasks in 10 ml of GF-EuroN medium.

2. The following day, change the media with 15 ml of retroviral vector supernatant (DMEM/10% FBS; 4×10^5 cfu/ml) supplemented with Polybrene (8μg/ml).
3. Centrifuge cells at 1,000 × *g* at room temperature for 60 min and incubate at 33°C for 2 h.
4. Collect the cells by centrifugation (900 × *g* for 10 min) and resuspend in GF-EuroN medium.
5. Incubate for 24 h at 37°C.
6. Repeat **steps 2–4** and incubate at 37°C.
7. Two days after infection, start selection with the appropriate drug. If using G-418, a starting dilution of 150μg/ml is recommended. Replace weekly two thirds of the medium with fresh GF-SFM containing 200μg/ml G-418. Resistant cells should appear 1 week postinfection.
8. Cells can be expanded and cryopreserved as described under **Subheading 3.2.**.
9. Expression of the delivered gene's mRNA and/or protein should be confirmed by RT-PCR amplification and/or Western blot/immunocytochemistry, respectively. If the delivered gene codes for a secreted protein, quantitative analysis of the synthesized protein should be evaluated by ELISA assay.

3.4.2. Engineering of Immortalized CNS Stem Cell Lines

Immortalized rodent CNS stem cell lines are different from human stem cell lines, and they have been shown to be cultured in presence of serum, without the requirement of EGF or FGF-2. Here, we refer to the former; however, the same procedure can be used for cell lines of human origin, simply by using growth factor-supplemented serum-free medium.

1. The day before infection, plate the cells on poly-D-lysine–coated tissue culture plastic in DMEM/10% FBS, at a density of 5×10^5 cells/10-cm dish.
2. Infect the cultures by incubation for 24 h at 33°C with 10 ml of retroviral vector supernatant (DMEM/10% FBS; 4×10^5 cfu/ml) in the absence of Polybrene.
3. Replace medium with fresh retroviral vector supernatant and incubate at 33°C for 24 h.
4. Repeat **step 3**.
5. Plate the cells at a very low density (about 20 cells for a 10-cm plate) in DMEM/10% FBS and start selection with the appropriate drug. Colonies should appear after 10 days.
6. Clones can be isolated, expanded, and assayed for transgene expression as described in the previous section.

4. Notes

1. Immortalization of CNS stem cells of human origin can be achieved by using the same retroviral transduction procedure described for rodent CNS stem cells (*see* **Subheading 3.2.**). In this case, to produce retroviral particles able to infect cells of

human origin, amphotropic packaging cell lines, such as theGP+envAM12 must be used. When using amphotropic (infective but replication-defective) retroviral vector to generate the immortalized cell lines, there is the remote possibility that the cells themselves could become producers of amphotropic retroviral particles vehicling potentially oncogenic products. Thus, it is of primary importance to assay supernatant of immortalized cell lines for the presence of these particles. The absence of such biohazardous agents could be directly tested by incubating other human cells in culture (such as HeLa cells) with the supernatant from immortalized cells and starting selection with the selective agent. No clones should be obtained from these cultures.

2. Different oncogenes have been used to immortalize murine CNS stem/progenitor cells from diverse brain regions and donor ages, v-*myc* and the temperature-sensitive allele of the large-T Antigen (tsA58U19) being the most extensively used oncogenes. In this respect, human neural material seems to differ from that of rodent origin. In particular, large-T Antigen seems not successful in inducing immortalization of human CNS stem cells ***(28,29)***, probably due to a more stringent control of cell cycle progression in these cells compared with the murine ones. On the contrary, *myc* acts via stimulation of growth factor regulated genes, overcoming the tight cell cycle regulation. Notably, the solely two cell lines of human origin reported to date ***(15,28)*** have been generated with the retroviral transduction of the v-*myc* (*gag-myc* fusion) oncogene. In this respect, it is also worthy to note that division of immortalized human CNS stem cell lines seems to be more strictly dependent on growth factors and that it is fully reversible upon cessation of mitogen stimulation. Moreover, as observed in grafting experiments performed in rodent brain, integrated v-*myc*–transduced human CNS stem cells downregulate by themselves (the exact mechanisms are not yet addressed), their v-*myc* expression stops dividing (due to the limited presence of EGF/FGF-2 in the normal brain), and they show no sign of transformation, overgrowth, disruption, or tumorigenesis.
3. To obtain a finer control of the oncoprotein presence both in vitro and in vivo for the cell line generated by Sah and colleagues ***(15)***, the v-*myc* expression was driven by a modified, Tet-off regulatable cytomegalovirus promoter. Upon addition of tetracycline to the culture medium, production of the immortalizing protein in this system is pharmacologically shut off. This, besides the "natural" tendency of cells to downregulate v-*myc* upon growth factor withdrawal, constitutes a further safe mechanism for a more stately use of these cell lines in therapeutic protocols.
4. Genetically modified CNS stem cells to produce neurotrophic factors have been largely used and described as a useful therapeutic approach to neurodegenerative disease ***(30,31)***. More generally, it is difficult to imagine that the introduction into the CNS of a constitutively produced compound that was not part of normal CNS function would be completely harmless. To this respect, once again the Tet systems represent an efficacious therapeutic tool for pharmacologically controlled gene expression.

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IV

Studies for Transplantation

25

Identification of Newborn Cells by BrdU Labeling and Immunocytochemistry In Vivo

Sanjay S. Magavi and Jeffrey D. Macklis

Summary

Bromodeoxyuridine, variously abbreviated as BrdU, BudR, and BrdUrd, is a halogenated thymidine analog that is permanently integrated into the DNA of dividing cells during DNA synthesis in S phase. BrdU can be immunocytochemically detected in vitro and in vivo, allowing the identification of cells that were dividing the period of BrdU exposure. In vivo, it has been used to identify the "birthdate" of cells during development, to examine the fate of postnatally generated cells, and to label cells before transplantation, for subsequent identification.

Key Words: BrdU labeling; birthdating; neurogenesis; gliogenesis; double labeling.

1. Introduction

Bromodeoxyuridine (BrdU) can be used in situations where tritiated thymidine would otherwise be used. BrdU is preferable for some applications and limited in others, compared with tritiated thymidine. BrdU can be very quickly detected; BrdU immunocytochemistry is highly reproducible, and it takes about a day, whereas tritiated thymidine autoradiography is more variable and typically requires at least weeks of exposure. In addition, fluorescent BrdU labeling can be imaged on confocal microscopes, allowing positive identification of multilabeled cells. BrdU labeling reveals details about nuclear morphology that tritiated thymidine autoradiography does not. In addition, BrdU can be detected in 40–50-μm sections, unlike tritiated thymidine, which can only detect labeled cells in the most superficial 3–5 μm of sections. In

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contrast, counting grains on tritiated thymidine autoradiographs can allow the tracing of the number of divisions a cell has gone through since the tritiated thymidine incorporation. Differences in the degree of immunocytochemical BrdU labeling, although sometimes apparent, are not reliable markers of the number of divisions a cell has gone through.

Iododeoxyuridine (IrdU) is a halogenated thymidine analog similar to BrdU that is less commonly used in neuroscience research. The majority of antibodies detect both BrdU and IrdU, but using antibodies that can distinguish between the two can enable measurement of cells' cycle times or identification of cells born at different times ***(1)***.

Due to its bromine side group, BrdU is potentially more toxic to experimental cells and animals than tritiated thymidine, a factor that should be taken into consideration when administering BrdU. Negative effects of BrdU on the survival of labeled cells have been reported ***(2)***, and there are reports that embryonic BrdU treatment can yield reduced brain and body weight ***(3)***. However, BrdU-labeled cells can undergo apparently normal division, differentiation, tangential and radial migration, and process integration both in developmental studies ***(4)*** and in studies of adult neurogenesis ***(5–7)***. With these potential caveats in mind, you should minimize the amount of BrdU used, to reduce cell toxicity and experimental artifacts.

In vitro, there is evidence that BrdU can integrate into apoptotic cells, presumably as a result of DNA damage and repair. The appearance of nuclear BrdU labeling in dying cells in these in vitro studies is typically that of minor nuclear speckling rather than the diffuse labeling of the entire nucleus seen in mitotic cells. However, these in vitro data call attention to the need for caution with BrdU.

In vivo, there is recent evidence that BrdU can integrate into adult cells undergoing abortive cell cycle entry after ischemic injury, or, rarely, in sporadic neurodegenerative disease, although these neurons then go on to die and disappear within a few weeks ***(15,16)***. However, this seems not to be the case in neurons undergoing either excitotoxic or cell intrinsically induced targeted apoptotic death, or in early postnatal neurons undergoing apoptotic death (Magavi and Macklis, unpubl. obs.). In the first of these in vivo experiments, e.g., adult mice received intracortical ibotenic acid injections, inducing the apparently necrotic death of cortical neurons and glia. BrdU was injected at equal intervals and sections were examined at 3, 6, and 24 h after induction of necrosis. Sections were processed for BrdU immunocytochemistry and stained with the nuclear dye Hoechst 33258, which reveals condensed nuclei of dying cells. Daily BrdU doses ranging from 1,000 to 3,200 mg/kg revealed no dying cells that integrated detectable levels of BrdU ($n \approx 10{,}000$ cells observed). Postnatal day 0 mice, which normally contain many neurons undergoing

developmental apoptosis, were similarly treated with 350 mg/kg BrdU every 1.5 h for 6 h (1,400 mg/kg total dose) and labeled for both BrdU and cleaved caspase-3, a protein involved in apoptosis that labels apoptotic cells. None of the apoptotic cells were BrdU-labeled (n ≈ 1,000 cells observed). Thus, it is highly unlikely that apoptotic cells would be mistaken for newly born cells in vivo by using rigorous BrdU labeling criteria.

BrdU labeling can be used to assess proliferation of a population of cells or to assess the survival of labeled cells. When combined with phenotypic markers, it can be used to establish the fate of cells labeled during the period of BrdU administration. To specifically assess effects on proliferation, animals are commonly injected with BrdU intraperitoneally and sacrificed 2 h later ***(6)***. After longer periods, cells may die or migrate away from the region of interest, affecting cell counts. Survival of BrdU-labeled cells can be assessed analyzing tissue after longer periods; in such experiments, the possible effects of migration also must be considered.

2. Materials

2.1. BrdU Administration

1. 5-BrdU (Sigma-Aldrich, St. Louis, MO, cat. no. B5002).
2. Acrodisc syringe filter (Gelman Instrument Co., Ann Arbor, MI, cat. no. 4192).
3. Sterile saline, pH 7.35.
4. Double-distilled H_2O) (ddH_2O).

2.2. BrdU Staining

1. Antibody to BrdU, no. MAS250p, Harlan-Ser Lab, Loughborough, UK; and clone BU1/75 rat anti-BrdU antibody, Accurate Chemical & Scientific, Westbury, NY. Rat-derived antibodies are relatively uncommon, making it easier to perform double or triple labeling by using antibodies derived from other species easier.
2. Fluorescent secondary antibody, Alexa 488 goat anti-rat antibody, Invitrogen, Carlsbad, CA, cat. no. A11006.
3. Phosphate-buffered saline (PBS): 2.625 g/l monobasic sodium phosphate (anhydrous), Sigma-Aldrich, St. Louis, MO, cat. no. S0751; 11.5 g/l dibasic sodium phosphate (anhydrous), Sigma-Aldrich, cat. no. S0876; and 9 g/l sodium chloride, Sigma-Aldrich, cat. no. S9888.
4. Dissolve compounds in ddH_2O and adjust pH to 7.35. PBS is stable indefinitely.
5. Heparin in PBS: dissolve heparin, sodium salt (Sigma-Aldrich cat. no. H3393) in PBS to make a 10 unit/ml solution.
6. 4% Paraformaldehyde in PBS: make an 8% solution of paraformaldehyde prills (Electron Microscopy Sciences, Hatfield, PA, cat. no. 19202) in ddH_2O. Paraformaldehyde does not enter solution easily. To dissolve it more quickly, add

4 ml of 1 M NaOH for each 100 ml of solution, and heat the solution to 50°C, while stirring constantly. Add an equal volume of 2× PBS to make a 4% solution of paraformaldehyde in PBS. Adjust the pH to 7.35 and filter the solution through Fisherbrand Qualitative P8 filter paper, Fisher Scientific, Pittsburg, PA, cat. no. 09-795C. Paraformaldehyde is stable for several weeks. Under this protocol, BrdU staining is incompatible with glutaraldehyde fixation.

7. Blocking solution (*see* **Note 2**): 8% goat serum, Invitrogen, cat. no. 16210-072; 0.3% bovine serum albumin, Sigma-Aldrich, cat. no. A6793; and 0.3% Triton X-100, Sigma-Aldrich, cat. no. T9284. Dissolve serum and Triton in PBS.
8. Netwells, optional: Costar Netwells, VWR, West Chester, PA, cat. no. 29442-138, can be used to conveniently move sections quickly from one solution to another. We use six-well Netwell plates when moving sections through inexpensive solutions such as PBS, blocking solution, and Hoechst 33258 dye.

3. Methods

3.1. BrdU Administration

BrdU can be administered via intraperitoneal (ip) injection, intraperitoneal infusion, oral ingestion, or intraventricular infusion. The particular characteristics of each technique are described below.

3.1.1. Intraperitoneal Injection

The most common method of BrdU administration is through ip injection. The ip injection is technically simple and easily temporally controlled. A single BrdU injection labels cells for approximately 5–6 hours ***(8)***, although this may vary depending on the initial dose given. Thus, labeling cells over an extended period requires repeated injections, a potentially time-consuming and invasive procedure. BrdU doses ranging from 50 mg/kg up to 200 mg/kg are common for rodents. BrdU injected into pregnant dams will label dividing cells in the developing embryos.

To prepare a BrdU solution for injection, dissolve BrdU in saline and adjust pH to 7.35. Filter sterilize through a 0.2-μm filter and store in a sterile vial suitable for storing injectable solutions. When stored at 4°C, BrdU is a stable solution for months.

3.1.2. Intraperitoneal Infusion

Osmotic minipumps can be inserted either subcutaneously or intraperitoneally for a longer term, more stable dosage of BrdU. Although we have not used this technique, it seems likely that a dose of 150 mg/kg per day in saline solution would be appropriate.

3.1.3. Oral Ingestion

Animals can be provided BrdU in their drinking water. This technique of BrdU administration is completely noninvasive and requires no animal handling, making it especially useful for extended BrdU administration. However, it does not allow precise control over the exact time and level of BrdU administration, and it may introduce some variability into experiments. Animals drink different amounts of water depending on ambient temperature, and animals also may drink only at particular times of day, affecting the number of cells that are labeled. In addition, long-term BrdU exposure may lead to metabolic changes that reduce BrdU absorption in a species- and strain-specific manner ***(9)***.

In our experience, dividing cells in the brains of C57/Bl6 mice can be labeled reliably for at least several weeks. Mice drink relatively consistently throughout the day, in a manner roughly proportional to their body weight and room temperature. We recommend a dose of 225 mg/kg per day, calculated using actual tracking of water consumption volumes per mouse. Mice ingesting approximately 300 mg BrdU/kg per day display as many or more labeled, dividing cells than mice injected twice daily with 120 mg/kg BrdU. Mice ingesting approximately 150 mg/kg BrdU per day, display similar numbers of BrdU+ cells; however, labeled cells are less intensely stained, and they are sometimes difficult to detect. Mice ingesting doses of BrdU above 300 mg BrdU/kg per day had similar numbers of BrdU-labeled cells, but these cells were even more brightly labeled. It seems likely that BrdU delivered orally yields lower plasma levels of BrdU than the same amount of BrdU delivered intraperitoneally. BrdU is easily dissolved in drinking water and is stable for at least a week at room temperature.

3.1.4. Intraventricular Infusion

It is possible to examine to label dividing cells near the ventricles by using BrdU administered through an intraventricular cannula. A BrdU solution can be combined with other reagents of interest, such as growth factors, and infused directly into the ventricles. This method delivers BrdU to a limited region of the brain. Cells near the boundary of the ventricles or in the anterior hippocampus are preferentially labeled using this technique. Dividing cells in regions of the brain further from the ventricles are not labeled.

Rats receiving an intraventricular infusion of 120 μg of BrdU per day have significant numbers of BrdU-positive cells in the ipsilateral ventricle ***(10)***, and we find that a significant number of cells are labeled in the mouse at a dose of 100 μg of BrdU per day. We find strong BrdU labeling in the ipsilateral ventricle and anterior hippocampus and less labeling in the contralateral ventricle and

posterior hippocampus. There is little labeling of dividing cells not directly adjacent to the ventricles.

3.2. Staining Techniques

1. BrdU staining is relatively simple and reproducible for those comfortable with immunocytochemistry. BrdU can be stained under a variety of conditions. However, it is necessary to denature nuclear DNA into single-stranded form so antibodies can bind the BrdU. DNA can be denatured using either acid, microwave treatment ***(11)***, rapid heating ***(12)***, or DNase treatment ***(13)***. Treating floating sections with 2 M HCl for 2 h at room temperature is a simple and consistent method for preparing sections for BrdU treatment. The HCl treatment used in this protocol destroys some antigens, in particular, cell surface markers (*see* **Note 1**).
2. Transcardially perfuse with 10 unit/ml heparin followed by 4% paraformaldehyde/PBS. Under this protocol, BrdU staining is incompatible with glutaraldehyde fixation (*see* **Note 3**).
3. Postfix whole brains in paraformaldehyde solution overnight. Cut 40-μm sections by using a vibratome. Store sections in PBS; the BrdU antigen will remain stable for several months. Storing sections in a 0.01% NaN_3/PBS solution will enhance their stability.
4. Wash floating sections in PBS for 3 min.
5. Dip sections in ddH_2O 1 × 3 s. This removes the excess PBS, which could buffer the HCl.
6. Incubate sections in 2 M HCl at room temperature for 2 h on a rocker. Be sure that sections are not floating on top of the solution (*see* **Note 4**). HCl exposure denatures DNA, exposing the BrdU. BrdU antibodies only recognize BrdU in single-stranded DNA; attempting to stain for BrdU without denaturing the DNA will not work. Other protocols use microwave, heat, or DNase antigen exposure to denature the DNA, although HCl exposure has worked most consistently in our hands. Sections will shrink and become whitish and then regain their normal appearance after washing in PBS and blocking solution. We find that sections are actually easier to handle after HCl exposure.
7. Wash sections 4 × 8 min in PBS at room temperature on a rocker to re-equilibrate the sections to pH 7.35. Sections will lose their whitish, shrunken appearance and regain their normal appearance.
8. Wash sections in blocking solution for 1 h at 4°C on a rocker.
9. Incubate sections in anti-BrdU antibody dissolved in blocking solution diluted at 1:400 overnight at 4°C. Approximately 70–80 μl of solution per section is sufficient, although using less may be possible. We incubate sections in 24-well plates, typically placing four or five sections in 400 μl of antibody in a single well. Plates need not be sterile for antibody staining and can be reused if well cleaned.
10. Wash sections 3 × 5 min in PBS.

11. Incubate sections in Alexa 488 anti-rat secondary antibody at 1:250 in blocking solution for 2 h.
12. Wash sections 3 × 5 min in PBS.
13. Briefly dip sections (approximately 1 s) in ddH_2O to remove excess PBS before mounting.
14. Mount sections on gelatin-coated slides and coverslip with Flouromount or PBS. Fluoromount flattens sections, producing crisp pictures, and it stabilizes fluorescence well. Refrigerated Fluoromount-mounted sections kept in the dark will retain their fluorescence for months, if not years.

Mounting sections in PBS with minimal drying preserves the three-dimensional structure of tissue and makes it much easier to distinguish the depth of particular features. This is important when performing multiple labeling with cytoplasmic markers and nuclear markers such as BrdU, because nuclei of glia of other cells can be closely opposed to other cells and mistaken for their nuclei. PBS often yields less crisp-looking images, because more of the section is out of the objectives depth of focus. Coverslips can be removed from PBS-mounted sections, allowing the sections to be stained for additional markers, if the need arises. To mount sections in PBS, place a drop of PBS on the sections, gently place the coverslip, and then remove excess PBS using bibulous paper. The coverslip will stop sliding easily when you have removed enough PBS. Use nail polish to seal the edges and prevent the PBS from evaporating. PBS-mounted sections can later be dehydrated and mounted in Fluoromount.

BrdU-positive cells will have nuclear labeling but no cytoplasmic labeling. Brain sections containing the hippocampus or subventricular zone of BrdU-treated animals can be used as positive controls, because these portions of the brain contain populations of constituitively dividing cells.

4. Notes

1. HCl exposure is compatible with many immunocytochemical analyses, including those for neuronal nuclei, Hu, Doublecortin, TOAD-64, microtubule-associated protein-2, glial fibrillary acidic protein, insulin-like growth factor-I receptor, and RIP antigens. HCl exposure destroys other antigens, such as MBP, A2B5, and O4. Some secondary antibodies, including molecular probes Alexa 546 secondary antibodies, are stable through HCl exposure. This allows complete staining for a sensitive antigen and then exposes the sections to HCl for BrdU staining, to double label sections. BrdU has been reported to quench Hoechst staining ***(14)***, but this quenching seems undetectable in tissue sections under the staining protocol that follows.
2. Because blocking solution contains serum, it can be contaminated. Blocking solution is stable for a week at 4°C and for several months frozen. If the solution becomes cloudy, it has become contaminated and should not be used. Adding

0.025% sodium azide, a bacteriostatic preservative, can reduce the risk of contamination, but it may interfere with certain types of staining.

3. Improperly perfused animals will have high levels of background staining, and they may be difficult to section. Often, commonly used secondary antibodies against IgG groups will bind to blood or immune cells, staining the vasculature in the brain. Perfusing with more heparinized PBS solution can reduce background due to vasculature.
4. Often, "floating" sections (a misnomer, because it is necessary that they sink) will float on the surface of the solution they are in, exposing only one side of the section to the solution and leading to inconsistent staining. This is easily avoided by gently shaking the solution until the sections sink, or simply dunking them using a fine paintbrush.

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26

Immunocytochemical Analysis of Neuronal Differentiation

Sanjay S. Magavi and Jeffrey D. Macklis

Summary

Fully understanding the phenotype of neurons in vivo involves examining their morphology, immunocytochemically analyzing their protein expression, examining their afferent and efferent integration into neuronal microcircuitry, and functionally examining their activity. This task is significantly more difficult when you are attempting to determine whether multipotent precursor cells, often referred to as stem cells, differentiate into neurons in vivo. Transplanted or endogenous precursor cells often produce relatively small numbers of new neurons in the adult brain, making electron microscopy or electrophysiological analysis extremely challenging, and functional analysis difficult. Studying such cells usually depends heavily on immunocytochemical approaches. We review a range of immunocytochemical techniques for identifying whether transplanted or endogenous neural precursors have differentiated into mature neurons. We provide immunocytochemical protocols for the migratory neuronal marker Doublecortin (Dcx), the early expressed marker Hu, and mature neuronal marker NeuN. In Chapters 25 and 27 of Part IV, we provide protocols for identifying newborn cells by using the mitotic label bromodeoxyuridine and for examining axonal projections by using the retrograde label FluoroGold.

Key Words: Immunocytochemistry; neuronal differentiation; neuronal markers; phenotype analysis.

1. Introduction

Fully understanding the phenotype of neurons in vivo involves examining their morphology, immunocytochemically analyzing their protein expression, examining their afferent and efferent integration into neuronal microcircuitry,

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and functionally examining their activity. This task is significantly more difficult when you are attempting to determine whether multipotent precursor cells, often referred to as stem cells, differentiate into neurons in vivo. Transplanted or endogenous precursor cells often produce relatively small numbers of new neurons in the adult brain, making electron microscopy or electrophysiological analysis extremely challenging, and functional analysis difficult. Studying such cells usually depends heavily on immunocytochemical approaches. We review a range of immunocytochemical techniques for identifying whether transplanted or endogenous neural precursors have differentiated into mature neurons. We provide immunocytochemical protocols for the migratory neuronal marker Doublecortin (Dcx), the early expressed marker Hu, and mature neuronal marker neuronal nuclei (NeuN). In Chapters 1 and 3 of Part IV, we provide protocols for identifying newborn cells by using the mitotic label bromodeoxyuridine (BrdU) and for examining axonal projections by using the retrograde label FluoroGold.

The underlying assumption behind immunocytochemical cell identification approaches is that particular cell types uniquely express particular proteins. Thus, because antibodies can be produced that specifically recognize such proteins, we can identify the phenotype of cells by assessing whether they express particular proteins. The first issue in neuronal phenotype analysis to consider is the choice of antibodies. It is important to examine the literature and choose antibodies that specifically recognize proteins expressed only by neurons. Sometimes, it is only discovered after some time that a particular antibody is not specific for a particular phenotype. For example, neuron-specific enolase (NSE) can label glia in addition to neurons. Therefore, despite its name, NSE is no longer an appropriate marker to use for neuronal identification. It is necessary to thoroughly review the relevant literature when choosing markers for neuronal identification.

Even if you are using antibodies that are normally specific to proteins expressed by neurons, it is still theoretically possible that multipotent cells under analysis could pathologically misexpress both neuronal and non-neuronal proteins during the process of differentiation. Thus, it is necessary to assess whether the cells of interest both ***(1)*** express more than one neuronal marker and ***(2)*** do not express markers of potential alternative cell phenotypes, such as astroglia and oligodendroglia. Only cells that express multiple neuronal markers and do not express glial markers can be considered neurons (or positive and negative markers of other cell types, as appropriate for the desired cell type identification).

A further understanding of the development and phenotype of neurons formed from transplanted or endogenous precursors can be gained by using neuronal markers whose expression is temporally regulated. By showing the

development of new neurons, you also can exclude the possibility that preexisting neurons were artifactually labeled with markers intended to identify transplanted or endogenous precursors. For example, we used such an approach of progressive developmental markers to examine induced cortical neurogenesis using immunocytochemical markers Dcx, Hu, and NeuN ***(1,8)***. The Dcx enabled us to identify that at least some of the newborn cells arose from the subventricular zone and migrated into neocortex, whereas Hu and NeuN allowed us to demonstrate that they survived for many months and differentiated into mature neurons.

In addition to the broader issues concerning antibody labeling, there are a number of technical issues to consider. The first is simplest; it is necessary to confirm that there is not any artifactual cross-reactivity between the antibodies used and other proteins. Secondary antibodies used to visualize labeling can nonspecifically bind to brain tissue, although generally at low levels and in a fairly reproducible manner. It is thus important to perform relevant control experiments, such as omission of the primary antibody, to clearly understand the level and appearance of background staining. Background staining in vivo can sometimes resemble specific labeling, and it may take substantial experience to differentiate between the two. Background staining can be reduced by incubating the tissue in the serum of the species in which the secondary antibodies are derived ("blocking"). Blocking tissue sections in 5–10% serum or 0.3–0.5% serum albumin solution for an hour is often sufficient to substantially reduce background staining. Background staining can be further reduced by using 5–10% serum or 0.3–0.5% serum albumin in the primary and secondary antibody solutions.

When using multiple immunocytochemical labels simultaneously to double or triple label sections, it is necessary to ensure that the secondary antibodies used do not cross-react with unintended primary antibodies. This is best achieved by using (1) primary antibodies developed in different species and (2) highly cross-adsorbed secondary antibodies that are species specific. Highly cross-adsorbed secondary antibodies are generally most specific for their antigens, but even these need antibodies to be tested to ensure their specificity. For example, if you were labeling sections with both a mouse-derived NeuN antibody and a rat-derived BrdU antibody, it would be important to ensure that the anti-mouse secondary antibody intended to bind the NeuN primary antibody does not bind to the rat-derived BrdU primary antibody.

Another issue arises when attempting to identify the phenotype of cells labeled with a marker that does not indicate the morphology of the cell. Cells labeled with BrdU, which only labels the nucleus, or fluorescence *in situ* hybridization, which produces punctate nuclear staining, can be confused with cells located immediately above or below, as reported and described as a

concern previously ***(1,2,8)***. Drying and mounting tissue can cause the tissue to shrink to as little as one quarter its original thickness, making it difficult to identify the limits of a cell along the *z*-axis. This difficulty can be overcome using confocal microscopy, which provides a very narrow focal depth, and enables very high-resolution images along the *z*-axis of tissue. Examining images obtained at various depths through the tissue can enable confirmation that both the cell nucleus and phenotypic markers are colocalized. By using confocal microscopy to analyze serial sections through a cell and produce three-dimensional reconstructions of cells, it is possible to unequivocally demonstrate or rule out that two labels are colocalized.

You also can increase resolution in the *z*-axis by mounting tissue in PBS without drying it significantly. Tissue that has not been dehydrated does not shrink substantially, making it much easier to resolve the depth of individual cells by routine microscopy with high numerical aperture, narrow depth-of-focus objectives. Colocalization must still be confirmed by confocal microscopy, but the phosphate-buffered saline (PBS) mounting technique can greatly reduce the number of false positives taken in for confocal examination.

Counterstaining with the nuclear dye Hoechst 33258, which labels all DNA, or an equivalent nuclear marker can assist in confirming that particular nuclei belong to particular cells. This technique also can assist in identifying the morphology of neuronal versus non-neuronal nuclei. Neurons generally have much larger, rounder nuclei than glial cells, with dispersed heterochromatin, giving insight into their identity. However, nuclear characteristics alone are not sufficient to identify neurons, because dividing or recently mitotic cells also can have large nuclei. A protocol for Hoechst staining follows the immunocytochemistry protocol.

2. Materials

2.1. Immunocytochemistry Protocol

The majority of the following protocol is general for Dcx, Hu, and NeuN, as examples. The only differences are in the concentrations of the primary antibodies used and the type of secondary antibodies used to label the primary antibodies. Other markers can be used by extension and generalization.

2.2. Immunocytochemical Cell Identification

Dcx is a microtubule-associated protein that is expressed exclusively in migrating and differentiating neurons. It is downregulated with maturation. Dcx is expressed in the soma and leading processes of migrating neurons, and in the axons of differentiating neurons ***(3,4)***. Hu is an RNA-binding protein that

begins to be expressed soon after neuronal differentiation and continues to be expressed through adulthood. It, too, is expressed in the nucleus and soma of neurons *(5,6)*. NeuN is expressed exclusively in mature neurons *(7)*. It is highly expressed in the nucleus of neurons and more weakly in the cytoplasm of the soma. Long axonal or dendritic processes are not visible with this marker.

1. Antibody to Dcx: J. Gleeson, University of California–San Diego, La Jolla. CA, or C. Walsh, Harvard Medical School, Boston, MA, a rabbit IgG antibody.
2. Antibody to Hu: monoclonal antibody (mAb) facility, University of Oregon, Eugene, OR. mAb 16a11; a mouse IgG antibody.
3. Antibody to NeuN: Chemicon International, Temecula, CA, cat. no. MAB377; a mouse IgG antibody.
4. Fluorescent secondary antibodies: Alexa 546 goat anti-mouse IgG (H+L), highly cross-adsorbed antibody, Invitrogen, Carlsbad, CA, cat. no. A11030 and Alexa 546 goat anti-rabbit IgG (H+L), highly cross-adsorbed antibody, Invitrogen, cat. no. A11035.
5. PBS: 2.625 g/l monobasic sodium phosphate (anhydrous) (Sigma-Aldrich, St. Louis, MO, cat. no. S0751), 11.5 g/l dibasic sodium phosphate (anhydrous) (Sigma-Aldrich, cat. no. S0876), and 9 g/l sodium chloride (Sigma-Aldrich, cat. no. S9888). Dissolve compounds in double-distilled water (ddH_2O) and adjust pH to 7.35. PBS is stable indefinitely.
6. Heparin in PBS: dissolve heparin, sodium salt (Sigma-Aldrich, cat. no. H3393) in PBS to make a 10 unit/ml solution. Store at 4°C. Heparin solution is stable for at least 6 months.
7. 4% paraformaldehyde in PBS: make an 8% solution of paraformaldehyde prills (Electron Microscopy Sciences, Hatfield, PA, cat. no. 19202) in ddH_2O. Paraformaldehyde does not enter solution easily. To dissolve it more quickly, add 4 ml of 1 M NaOH for each 100 ml of solution, and heat the solution to 50°C, while stirring constantly. Add an equal volume of 2× PBS to make a 4% solution of paraformaldehyde in PBS. Adjust the pH to 7.35 and filter the solution through Fisherbrand Qualitative P8 filter paper, Fisher Scientific, Pittsburgh, PA, cat. no. 09-795C. Paraformaldehyde is stable for several weeks.
8. Blocking solution: 8% goat serum (Invitrogen, cat. no. 16210-072), 0.3% bovine serum albumin (Sigma-Aldrich, cat. no. A6793), 0.3% Triton X-100 (Sigma-Aldrich, cat. no. T9284). Dissolve serum and Triton X-100 in PBS (*see* **Note 1**).
9. Netwells, optional: Costar Netwells, VWR, West Chester, PA, cat. no. 29442-138, can be used to conveniently move sections quickly from one solution to another. We use six-well Netwell plates when moving sections through inexpensive solutions such as PBS, blocking solution, and Hoechst 33258 dye.
10. Fluoromount: BDH Laboratory Supplies, Poole, UK, product no. 360982B.

2.3. Hoechst Dye Staining

1. PBS (*see* **Subheading 2.2., item 5**).
2. Hoechst 33258, also known as bisbenzimide, Sigma-Aldrich, cat. no. B2883.

3. Methods

3.1. Immunocytochemistry

1. Transcardially perfuse with 10 units/ml heparin followed by 4% paraformaldehyde/PBS. High-quality perfusion will reduce background staining (*see* **Note 2**).
2. Postfix whole brains in paraformaldehyde solution overnight. Cut 40-µm sections by using a vibratome. Store sections in PBS: the NeuN and Hu antigens will remain stable for several months. Storing sections in a 0.01% NaN_3/PBS solution will enhance the stability of sections.
3. Wash floating sections in PBS for 3 min.
4. Incubate sections in blocking solution for 1 h at 4°C on a rocker. Be sure that the sections are not floating on the surface of any of the solutions throughout this protocol (*see* **Note 3**).
5. Incubate sections in blocking solution containing either anti-Dcx antibody at 1:100, anti-Hu antibody at 2 µg/ml, or anti-NeuN antibody at 1:400.
6. Incubate sections overnight at 4°C with rocking; 70–80 µl of solution per section is sufficient, although using less may be possible.
7. We incubate the sections in 24-well plates, usually putting four or five sections in 400 µl of antibody solution in a single well. The plates need not be sterile for immunostaining and can be reused if cleaned well.
8. During incubation, the wells should be covered with parafilm to prevent evaporation. Antibody concentrations should always be titrated for the particular experiment in question (*see* **Note 4**).
9. Wash sections 3 × 5 min in PBS.
10. Incubate sections in secondary antibody at 1:750 in blocking solution for 2 h.
    a. for Dcx: Alexa 546 anti-rabbit IgG.
    b. for Hu: Alexa 546 anti-mouse IgG.
    c. for NeuN: Alexa 546 anti-mouse IgG.
11. Wash sections 3 × 5 min in PBS.
12. Briefly dip sections (approximately 1 s) in ddH_2O to remove excess PBS before mounting.
13. Mount sections on gelatin-coated slides and coverslip with Flouromount or PBS. Fluoromount clears and flattens the sections, producing crisp images, and it stabilizes the fluorescence well for long periods. Refrigerated, Fluoromount-mounted sections kept in the dark will retain their fluorescence for months, if not years. Alternatively, mounting sections in PBS, without drying them excessively, preserves their three-dimensional structure. Evaporation of the PBS can be prevented by sealing the edges of the coverslip with nail polish (*see* **Note 5**).

3.2. Hoechst Dye Staining

Hoechst staining allows you to identify the nuclei of cells, making it a useful fluorescent counterstain. Hoechst 33258, also known as bisbenzimide,

intercalcates into A-T–rich regions of DNA. It looks blue-white under fluorescence microscopy.

1. Perfuse animals, postfix brains, and cut brains as described in **steps 1–3**.
2. Incubate floating sections in a 2.5 μg/ml solution of Hoechst in PBS at room temperature for 4.5 min with rocking.
3. Wash sections 3 × 5 min in PBS.
4. Mount sections as described in **step 13**.

4. Notes

1. Because blocking solution contains serum, it can be contaminated. Blocking solution is stable for a week at 4°C and for several months frozen. If the solution becomes cloudy, it has become contaminated and should not be used. Adding 0.025% sodium azide, a bacteriostatic preservative, can reduce the risk of contamination, but it may interfere with certain types of staining.
2. Improperly perfused animals will have high levels of background staining and may be difficult to section. Often, commonly used secondary antibodies against IgG groups will bind to blood or immune cells, staining the vasculature in the brain. Perfusing with more heparinized PBS solution can reduce background due to vasculature.
3. Often, "floating" sections (a misnomer, because it is necessary that they sink) will float on the surface of the solution they are in, exposing only one side of the section to the solution and leading to inconsistent staining. This is easily avoided by gently shaking the solution until the sections sink, or simply dunking them using a fine paintbrush.
4. Titrate primary and secondary antibody concentrations to obtain the best possible staining. It is important to titrate antibodies so as to obtain bright staining without producing undue background or wasting costly antibodies. Laboratory-to-laboratory variations, such as the intensity of fluorescence illumination or specific filter set on microscopes, can make identically stained sections seem either overexposed or dim.
5. Mounting sections in PBS with minimal drying preserves the three-dimensional structure of tissue and makes it much easier to distinguish the depth of particular features. This is important when performing multiple labeling with cytoplasmic markers and nuclear markers such as BrdU, because nuclei of glia of other cells can be closely opposed to other cells and mistaken for their nuclei. PBS often yields less crisp-looking images, because more of the section is out of the objective's depth of focus. Coverslips can be removed from PBS-mounted sections, allowing the sections to be stained for additional markers, if the need arises. To mount sections in PBS, place a drop of PBS on the sections, gently place the coverslip, and then remove excess PBS by using bibulous paper. The coverslip will stop sliding easily when you have removed enough PBS. Use nail polish to seal the edges and prevent the PBS from evaporating. PBS-mounted sections can later be dehydrated and mounted in Fluoromount.

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27

Neuroanatomical Tracing of Neuronal Projections with Fluoro-Gold

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Summary

The study of neuronal connectivity requires the ability to trace axons from the neuronal cell body to its axon terminal (anterograde tracing) and from the terminal back to the soma (retrograde tracing). Such neuroanatomical tracing is frequently used to identify neurons on the basis of their pre- or postsynaptic connections. Neuroanatomical tracing has become particularly important in nervous system regeneration and repair, allowing investigators to follow the axon projections of newly born, transplanted, or axotomized neurons in lesioned or neurodegenerative environments. To allow further study of neurons identified and labeled in this way, it is particularly important that tracers are compatible with other tissue processing such as immunocytochemistry. Fluoro-Gold (Fluorochrome Inc., Denver CO) is one such highly flexible fluorescent retrograde marker commonly used for neuronal labeling and neuroanatomical tracing.

Key Words: Retrograde labeling; axonal tracing; neuronal projection tracing; Fluoro-Gold; connectivity analysis.

1. Introduction

Like other retrograde markers, Fluoro-Gold (FG; Fluorochrome Inc., Denver CO) is taken up by axon terminals, or through injured axons, and retrogradely transported to neuronal cell bodies, thereby specifically labeling neurons that project to the region of application. The main advantages of FG, compared with

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other retrograde markers, are (1) its ability to be visualized without additional processing, (2) its extensive labeling of distal dendrites, and (3) its stability for relatively long periods after application and under a variety of fixation and immunocytochemical conditions.

FG is taken up by intact axon terminals and injured axons but not by intact axons at nonterminal sites ("axons of passage") ***(1)***. Intact axonal uptake occurs via endocytosis at nerve terminals ***(2)***. After retrograde transport, FG labels neuronal soma and dendrites. In the soma, FG is associated with vesicles in the cytoplasm, the plasma membrane, and the nucleolus. It does not diffuse from labeled neurons, and it is not transported transsynaptically.

Because of its stability, FG may be applied to axon terminals or cut nerves by a relatively wide variety of methods. FG may be administered directly to the target region via pressure injection (typically <1 μl), iontophoresed (+5 to +10 μA/10 min) from small-tipped pipettes that can be used to record physiological activity from cells before labeling ***(3)***, or placed in the target region in crystalline form. FG also may be applied indirectly (and nonspecifically) to axon terminals by intraperitoneal (ip) or intravenous (iv) injection ***(4)***. FG widely labels axons terminals in the peripheral nervous system after ip or iv injections. However, it does not cross the blood-brain barrier (BBB); therefore, it does not label neurons that project to areas within the central nervous system protected by the BBB.

A significant limitation of FG as a marker of specific projection neurons is its tendency to diffuse from the site of injection in neural or muscle tissue. This diffusion can lead to nonspecific labeling of neurons that project to regions neighboring the intended target region ***(5)***.

FG is delivered to the cell body by fast axonal transport ***(6)***. The time required for retrograde labeling depends on distance and on degree of dendritic filling desired. FG labeling of cell bodies has been detected as soon as 2 h after injection ***(7)***. Typically, minimum survival time for axonal and cytoplasmic labeling is 1–2 days. Longer survival periods result in more complete labeling of high order neurites, up to hundreds of microns beyond the soma ***(8)***. FG labeling is stable for weeks after injection, and it persists up to 2 months in some reports.

FG is functional at a wide range of concentrations (1–10%, w/v). Lower concentrations (2–4%) are recommended for most procedures. Higher concentrations and larger injection volumes cause more intense retrograde labeling, but they also induce more necrotic damage at the injection site. FG labeling in the neuronal cell body is nontoxic at recommended concentrations.

FG has been reported to locally label non-neuronal cells in vivo. Almost all cells at the injection site become labeled. Rinamin et al. ***(9)*** found phagocytic microglia and other macrophages containing FG after degeneration of

retrogradely labeled axotomized neurons, presumably due to ingestion of dying FG-labeled neurons. Similarly, Streit et al. ***(10)*** reported FG-containing glia after toxic injury to motor neurons but not in control (nontoxic) conditions. Injection of large volumes of FG into the lateral ventricle in rats labels periventricular astrocytes and ependymal cells.

FG can be visualized directly by fluorescence microscopy by using an ultraviolet filter (excitation, 323 nm; emission, 408 nm). Color varies slightly with changes in pH from gold (neutral and basic pH) to blue (acidic pH). The ability to detect FG without additional histochemical processing allows for identification and dissection of FG-labeled regions under UV light ***(11)***. Visualization by fluorescence microscopy is limited by FG's tendency to photobleach, although its photostability far exceeds commonly used chromophores such as aminomethylcoumarin acetate (Invitrogen, Carlsbad, CA) or Cy3. Ju and Han ***(12)*** suggest that photobleaching is largely due to water in tissue sections and that it may be avoided or minimized by the use of alternative mounting media.

Several methods have been developed to convert FG's fluorescence into more stable, electron-dense products for electron microscopy (EM). FG can be converted directly to an electron-dense diaminobenzidine (DAB) reaction product by photo-oxidation to allow for EM visualization ***(13)***. The development of antisera against FG has also allowed FG to be detected with DAB or immunogold-silver or using immunofluoresence ***(14)***. In these protocols, FG is detected not only in lysosomes but also dispersed throughout the cytoplasm and distal dendrites, two to three branch points beyond the soma. FG is reported to be stable in a variety of fixatives, or in the absence of fixation, and it is not affected by most standard immunocytochemical procedures.

We have used FG to label a variety of cortical projection neuron populations in vivo in adult and neonatal mouse neocortex ***(15–19)***; Fricker-Gates et al., ***(20)***. The following protocol outlines our methods for labeling cortico-thalamic neurons in adult mice, and it can be adapted to retrogradely label different populations of projection neurons.

2. Materials

2.1. Fluoro-Gold

1. Fluorochrome Inc.

2.2. Fluoro-Gold Solution

1. Make a 2% (w/v) solution of FG in double distilled water (*see* **Note 1**).

2.3. Nanoject Variable

1. The Nanoject Variable (Drummond Scientific, Broomall, PA) is a digitally controlled oocyte pressure injector that is especially useful for injecting nanoliter volumes of solutions extracellularly into the brain, via small-diameter pulled glass micropipettes.

3. Methods

In this protocol, we inject FG into the thalamus by using a posterior approach, inserting the glass micropipette at a 30° angle from the vertical axis to avoid injury to more anterior cortex, which was important for the specific experiments we were conducting.

1. Anesthetize and prepare the mouse for surgery in compliance with institutional and National Institutes of Health guidelines.
2. Make a midline incision in the scalp from 2 mm posterior to the interaural line to 1 mm posterior to the eyes. Expose the lambda cranial suture and regions up to 5 mm lateral to it.
3. Mount the mouse in a stereotactic frame. It is critical that the skull is mounted in a stable horizontal position. The orientation of the skull can be confirmed by measuring the height of the lambda and bregma sutures and confirming that they are equal.
4. Make a small craniotomy with a scalpel (a drill may be used for animals with thicker skulls, such as rats) by using the following coordinates: medial boundary, 0.5 mm lateral to midline; lateral boundary, 2 mm lateral to midline; posterior boundary, 1 mm anterior to lambda; and anterior boundary, 2 mm anterior to lambda. Be careful to avoid damaging vasculature to minimize bleeding. It is not necessary to replace the removed bone fragment after surgery with such small craniotomies (*see* **Note 2**).
5. Make three injections into the thalamus. Insert pulled glass micropipette at a 30° angle from the vertical axis at the following coordinates:

	1	2	3
Anterior to lambda	1.5 mm	1.5 mm	1.5 mm
Lateral to midline	0.8 mm	1.2 mm	1.6 m
Depth	3.6 mm	3.7 mm	3.7 m

These coordinates place the pipette tip in the following locations, targeting the thalamic nuclei VLc, VPLo, Area X, and vLo, which receive projections from motor cortex:

Anterior to lambda	3.24 m
Posterior to bregma	0.97 m
Lateral to midline	0.9, 1.2, 1.6 m
Depth	3.04 m

6. Inject 32 nl of FG solution and wait 1 min. Retract the glass micropipette 100 μm, inject another 32 nl, and wait an additional minute. Retract the pipette slowly. Waiting for the FG to diffuse, and withdrawing the pipette slowly, minimizes the amount of FG that potentially effluxes along the micropipette's path. Repeat at each injection site (*see* **Note 3**).
7. Suture the incision area and allow the mouse to recover. We allow 7 days for retrograde transport of FG to neuronal cell bodies in adult animals (*see* **Note 4**).
8. Transcardially perfuse with 10 units/ml heparin/phosphate-buffered saline (PBS) solution followed by 4% paraformaldehyde in PBS solution. Postfix brains overnight in 4% paraformaldehyde at 4°C. Section brains at 40 μm by using a vibrating microtome; mount sections on gelatin-coated slides using Fluoromount (BDH); and examine by fluorescence microscopy under a filter with a 350-380 nm excitation spectrum.

4. Notes

1. FG is soluble in saline solution as well, but it precipitates out of PBS, forming a suspension. FG solution stored in the dark at 4°C is stable in solution for 6 months. FG crystals are stable for years when protected from light and stored at 4°C.
2. Cranial bleeding can usually be stopped using gel-foam absorbable gelatin sponges (Harvard Apparatus Inc., Holliston, MA, cat. no. 59-9863).
3. The inner diameter of the glass micropipettes should be as small as possible; an outer diameter of 40 μm is sufficient. It is also possible to inject FG by using a Hamilton syringe (Hamilton, Reno, NV). However, the large bore of the metal needle is likely to injure a much larger region of the brain, and it is likely to result in additional efflux along the injection path. In addition, it is difficult to accurately inject the small volumes required to target single areas of the rodent brain by using a syringe and needle.
4. FG may transport in shorter periods of time. Allowing longer times for transport can result in brighter nuclear labeling and increased labeling of dendrites.

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Labeling Stem Cells In Vitro for Identification of their Differentiated Phenotypes after Grafting into the CNS

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Summary

Grafting neural stem cells is a widely used experimental approach to central nervous system (CNS) repair after trauma or neurodegeneration. It is likely to be a realistic clinical therapy for human CNS disorders in the near future. One of the challenges of this approach is the ability to identify both the survival and differentiated phenotype of various stem cell populations after engraftment into the CNS. There is no single protocol that will work for all cell types and all applications. Labeling stem cells for CNS grafting is an empirical process. The type of stem cell, its fate after engraftment, and the context in which it is anatomically and histologically evaluated all contribute to a decision as to the best approach to take. We have provided the range of conditions under which various labels have been successfully used in CNS grafting studies and delineated the parameters that have to be empirically established. Given a clear understanding of the limitations of the respective labels and the expected outcome of the grafting experiment, these labeling guidelines should enable any investigator to develop a successful approach. Our own personal bias is to use labels that cannot be transferred to host cells. Initially, we preferred 5-bromo-2'-deoxyuridine, or retrovirally delivered enhanced green fluorescent protein or *lacZ*. More recently, we have found syngeneic grafts of human placental alkaline phosphatase stem cells to work very well. However, each investigator will have to decide what is optimal for his or her cell population and experimental design. We summarize the various approaches to labeling and identifying stem cells, pointing out both the limitations and strengths of the various approaches delineated.

Key Words: Stem cell grafting; fluorescent labels; genetic markers; differentiated neural phenotype.

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1. Introduction

Stem cells have unlimited therapeutic potential for restoring central nervous system (CNS) function lost secondary to trauma or degenerative disease. To date, stem cell grafts have, only in a few instances, partially ameliorated functional deficits in experimental models ***(1–6)***, and substantial preclinical studies remain to be undertaken before clinical application can be considered. Regardless of the injury model investigated, successful use of stem cell grafts requires a functional change after grafting. However, unequivocal demonstration that the engrafted stem cells have survived, integrated into the host CNS, and differentiated with a phenotype consistent with those functional changes is of paramount importance. This latter issue is not a trivial issue and must be approached very carefully. We summarize the various methods that can be used to label stem cells in vitro before grafting, delineate the advantages and problems with each approach, and discuss the most appropriate ways to document their differentiated phenotype(s) in vivo. Unfortunately, there is no single method that works best for all cell types. Optimal labeling methods have to be empirically determined for each cell type and application. What we present here are the critical variables that have to be established. Excellent comprehensive reviews and comparative experimental studies examining this issue have been previously published ***(7–9)***, and we are here delineating where the field presently stands.

2. Materials

2.1. Choosing an Appropriate Label Depends on Several Variables

1) Is a nuclear, membrane, or cytoplasmic label desired?
2) Are the engrafted cells expected to continue dividing, and if so, for how long?
3) Is cell lineage-specific expression of the label desired?
4) How long will the cells be required to express the label in vivo?
5) Does the label alter the physiology of the cells?
6) What detection strategy will be used for both the label and any cell-specific antigen that might be concurrently used?
7) What are the technical limitations of the investigator?

The answers to these questions will dictate the type(s) of labels that can and cannot be used for a given application. Table 1 lists various types of labels that have been used for CNS grafting. All are suitable for stem cell grafting, but each has advantages and disadvantages. In general, the membrane, nuclear, and cytoplasmic labels listed in Table 1 are very easy to use, but they are neither as definitive nor long-lasting as reporter genes. Conversely, reporter genes require expertise with viral vectors and a higher biosafety level of containment for cells derived from transgenic animals. Most of the membrane, nuclear, and

Table 1
Approaches to label stem cells for CNS grafting

Labeled compartment	Label	Conc	Labeling conditions	Detection
Nuclear	Bisbenzimide[a]	10 μg/ml	3 min–1 h	UV fluorescence
	BrdU	0.5–10 μM	4 h–overnight	Immuohistochemistry[b]
	[^{3}H]Thymidine	1 μCi/ml	48 h	Autoradiography
Cytoplasmic	Fluorescent beads	0.1–5.0%	5 min–6 h	Fluorescence[b]
	Fast Blue/True Blue	20 μg/ml	15 min–3 h	UV fluorescence
	Fluoro-Gold	1–10 mg/ml	5 min–2 h	UV fluorescence/ immunohistochemistry[b]
	Gold particles	0.01–2.5%	1 h	Silver precipitation/ electron microscopy
	RDA[c]	50 μg/ml	30 min	Rhodamine fluorescence[d]
	CFSE	0.05–5.0 μM	30 min	FITC fluorescence
	CellTracker Orange	50%	15 min	Rhodamine fluorescence[d]
	Iron particles[e]	2 μg–2 mg/ml	1–48 h	MRI
Membrane	DiI	25–40 μg/ml	10–40 min	Rhodamine fluorescence[d]
	PKH26	1 μM	3–8 min	Texas Red fluorescence[d]
	PHA-L[f]	0.2–20 mg/ml	10–40 min	Immunohistochemistry[c]
Reporter genes	lacZ[g]			Enzyme histochemistry/ immunohistochemistry
	EGFP			FITC fluorescence/ immunohistochemistry
	Alkaline phosphatase			Enzyme histochemistry
	luciferase			Enzyme assay/ immunohistochemistry

[a]Also called Hoechst 33342.
[b] The filter set used depends on which fluor is chosen for use.
[c]Rhodamine dextran amine.
[d]Optimal filters for rhodamine, Texas Red, or Cy3 have similar excitation and emission spectra, and they can be used for all three fluors.
[e]Supraparamagnetic iron oxide (SIO) contrast agent Feridex (25 μg/ml–1 h; ***(13)*** or dextran-coated SIO (2 mg/ml–48 h; ***(14)***.
[f]*Phaseolus vulgaris* leukoagglutinin.
[g]*E. coli* β-galactosidase.

cytoplasmic labels listed in Table 1 can be added directly to the culture medium of growing stem cells, and the concentrations and labeling parameters are indicated. All of these labels will be diluted out as the cells divide in vitro, so it is imperative to label the cells as close to the time of engraftment as possible. As is discussed subsequently, this is not the case with the reporter genes.

The advantage of using carboxyfluoroscein succinimidyl ester (CFSE) and CellTracker™ Orange is that these labels are cell membrane permeable, but they are modified intracellularly to a membrane-impermeant form. Thus, in principal, these labels cannot be transferred to host cells. The problem with CFSE is that it bleaches very rapidly upon exposure to light. However, immunostaining with a specific antibody can overcome this problem ***(10)***. CellTracker™ Orange seems to label cells up to 4 months postengraftment ***(11)***. If it is essential that cell morphology be identified in vivo in the absence of additional antibody staining to delineate cell phenotype, then either cytoplasmic or membrane labels must be used. Although the membrane labels 1,1'dioctadecyl-3,3,3',3'-tetramethyllindocarbocyanine perchlorate (DiI) and PKH26 are very bright fluorescent labels, membrane does turn over, and with extended times both in vitro and in vivo, these labels tend to end up in lysosomal vesicles and cell morphology becomes difficult to distinguish. Nuclear labels obviously will not provide any information about cell shape. Excellent results have been obtained for all of these labels in grafting experiments in which the cells do not remain *in situ* for more than a month. However, with the exception of 5-bromo-2'-deoxyuridine (BrdU) and [^{3}H]thymidine, none of these labels can be used for chronic grafting experiments, because the labels tend to leak out of the cells. Recently, it has been shown that thymidine analogs can be transferred from grafted to host cell after CNS transplantation ***(12)***. Thus, if the grafts are expected to remain in vivo for longer than 6 weeks before analysis, BrdU or one of the reporter genes should be used. Otherwise it becomes extremely difficult to unequivocally state that a label-expressing cell is of graft origin. For example, **Fig. 1A,C** shows BrdU-labeled cortical stem cells 2 months after engraftment into the adult rat spinal cord. Note the clear nuclear labeling of the engrafted stem cells. For both BrdU and [^{3}H]thymidine, the cells have to be actively dividing, because these labels incorporate into the DNA of dividing cells. Slowly proliferating populations of stem cells may have to be labeled for extended periods if a high percentage of cells are not labeled with the standard overnight incubation. BrdU can be detected with an antibody, but [^{3}H]thymidine requires autoradiographic detection, and as such, it is technically more difficult to work with. Most recently, iron particles have been used to label engrafted cells that can subsequently be identified using magnetic resonance imaging (MRI) ***(13–15)***. The obvious advantage to this approach is that the cells can be detected in live animals at sequential time points. The disadvantages are

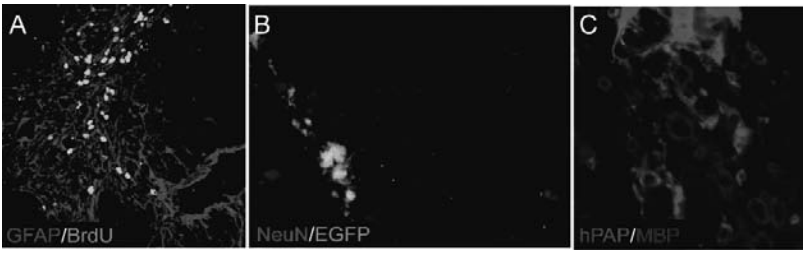

Fig. 1. Detection of BrdU-, EGFP-, hPAP-labeled rat stem cells after engraftment into the adult rat CNS. **(A)** BrdU-labeled fetal cortical stem cells double-stained with glial fibrillary acidic protein (*GFAP*) showing extensive astrocytic differentiation 2 months after engraftment into the ventral white matter of the adult rat spinal cord. **(B)** Enhanced green fluorescent protein (*EGFP*)-labeled fetal spinal cord neuronal-restricted precursor cells 1 month after engraftment into the CA1 region of the adult hippocampus and double stained for the mature neuronal nuclear marker *NeuN*. Note the extensive axonal outgrowth that is evident from the cytoplasmic EGFP staining. **(C)** Adult oligodendrocyte precursor cells isolated from a rat that constitutively expresses hPAP 2 months after engraftment-labeled stem cells double-stained with the neuronal nuclear marker *NeuN*, showing extensive neuronal differentation 2 months after engraftment into the ventrolateral funiculus of the contused thoracic adult rat spinal cord. Note that many of the $hPAP^+$ cells have formed myelin basic protein (*MBP*)-positive rings, likely ensheathing previously demyelinated axons.

that the resolution is not good, and access to an animal MRI facility is needed. However, this approach holds great promise for following grafted cell fate over time.

3. Methods

3.1. Establishing Nontoxic Concentrations of Labels

All cell types are differentially sensitive to the various labels. Moreover, a stem cell may be more or less sensitive to a particular label while proliferating than after differentiation, and this has to be empirically determined. When evaluating a label, concentrations bracketing those that have been used by other investigators should be evaluated with the percentage of labeled cells and the survival of those proliferating cells as the crucial variables. This can easily be done by counting the cells in a hemocytometer with trypan blue. In the case of stem cells that will differentiate in vivo, it is imperative that the stability of the label and the toxicity be established in differentiated cells in vitro before undertaking grafting studies. Differentiated cells cannot be counted in a hemocytometer, but they must be immunostained with appropriate

cell-specific antigens and the number of specific cell types counted. This is essential because one differentiated cell type may be differentially sensitive to a particular label. For example, BrdU labeling does not affect the number of neurons, astrocytes, or oligodendrocytes observed after differentiation of embryonic day (E)14 cortical stem cells in vitro ***(16)***, whereas fluorescent microspheres are tolerated by proliferating RN33B cells, but they are toxic to differentiated cells ***(6)***.

In addition, because all of these labels have the potential to be transferred to endogenous cells (e.g., **ref.** ***8***), it is essential that a lysed cell control graft is performed. Cells can be freeze-thawed twice at the identical concentration to that used for grafting, checked with a hemocytometer and trypan blue to ensure that all the cells are dead, and identically engrafted. Note also that if a functional outcome measure is to be used to evaluate the effectiveness of a particular grafted cell population, an additional graft control must be run in which a cell type that does not differentiate into a predicted cell type is used. This is necessary because grafted cells can have significant influence on host tissues ***(17)***. For example, a fibroblast would be a good graft control for neuronal or oligodendrocytic differentiation of engrafted cells.

3.2. Labeling with Reporter Genes

Using reporter genes requires the investigator to have some expertise with molecular biology, cell culture experience, or both with various virus vectors. Thus, this approach is technically more challenging. The advantage of using these reporter genes is that they can be stably integrated into the stem cell genome so that they cannot be transferred to endogenous cells. That, combined with their continued expression for long periods, makes them an excellent choice of label for chronic grafting experiments. Several reporter genes have been successfully used to label grafted cells. The two most commonly used are *Escherichia coli* β-galactosidase (*lacZ*) and enhanced green fluorescent protein (EGFP) (or a variety of other color fluorescent proteins, e.g., Clontech Living Colors Fluorescent Proteins, www.clontech.com), but both alkaline phosphatase and luciferase also have been used. The advantage of EGFP over *lacZ* is that it is constitutively fluorescent, and it can be seen without additional identification steps. In practice, however, it is much better to use antibody detection of EGFP expression in grafts, because the immunostaining markedly amplifies the fluorescent signal and enables better detection of the signal. This is especially true when trying to identify axons and dendrites of differentiated neurons in vivo. Although *lacZ* requires additional detection methods, its advantage is that either histochemical or immunohistochemistry can be used. The latter is more sensitive, but histochemical detection can be seen at the

light and electron microscopy (EM) level, and it does not require fluorescence microscopy. *LacZ* also can be detected by EM immunohistochemistry ***(18)***. The one concern with histochemical detection of the *lacZ* reporter gene is that there is endogenous β-galactosidase activity in the mammalian CNS. The pH of the histochemical detection solutions must be very carefully controlled to eliminate that endogenous activity ***(19)***. Alkaline phosphatase shares many of the properties of β-galactosidase as a reporter gene, and it is also optimally detected with antibodies. Luciferase is a much less widely used reporter gene for grafting studies. Its main advantage would be in quantifying graft survival by enzyme assay in unfixed tissue homogenates, because the assay is extremely sensitive; however, it also can be immunohistochemically detected. All of these reporter genes show cell morphology, because they are cytoplasmic markers, unless a nuclear localization signal is included in the reported gene construct.

There are several ways to deliver reporter genes to stem cells. They can be cloned into eukaryotic expression vectors and transfected into the proliferating stem cells. This is relatively easy, but the frequency of transfection is often quite low. However, inclusion of a selectable marker, such as the neomycin or hygromycin resistance genes, allows selection of expressing cells. Alternatively, the reporter gene can be delivered by viral infection, either with adenovirus, adeno-associated virus (AAV), retrovirus, or lentivirus. Both retroviral and lentiviral-delivered reporter genes will be stably integrated into the host genome and passed on to all cell progeny as they divide. Thus, labeled cells can be expanded to high numbers, and if EGFP or *lacZ* is used as the reporter gene, enrichment by fluorescent activated cell sorting is possible. **Figure 1B,D** shows E14 rat cortical stem cells, labeled with an EGFP retrovirus 2 months after engraftment into the adult spinal cord (**Fig. 1B**) or hippocampus (**Fig. 1D**). Note that EGFP is expressed in the cytoplasm and delineates cell morphology. The advantage of lentiviral vectors is that they infect nondividing cells; the disadvantage is that some Institutional Biosafety Committees require biosafety level 3 containment for their construction. Both adenovirus and AAV very efficiently infect dividing or nondividing cells, but both remain episomal; therefore, infected cells cannot be passaged and expanded before grafting. Moreover, adenovirus is highly cytotoxic, and optimal infection parameters need to be carefully established. The growth of these viruses is now routine in many laboratories, and most of these reporter gene constructs are readily available commercially.

3.3. Alternative Labeling Strategies

There are several alternative ways to label engrafted cells. Again, all have their advantages and disadvantages.

1. Gender-specific grafts. Embryonic male tissues have been grafted into female CNS and the grafted cells detected using Y chromosome-specific DNA probes ***(20–23)***. The problem with this approach is that it requires *in situ* hybridization to detect the engrafted cells, a technique that gives no indication of cell morphology and that is also very difficult to use in conjunction with immunohistochemistry to determine the differentiated phenotype of the cells.
2. Transgenic animals. Transgenic mice expressing EGFP ***(24)*** and rats expressing human placental alkaline phosphatase (hPAP) ***(25)*** have been developed, and numerous investigators have used stem cells derived from these animals for CNS grafting experiments ***(25–30)***. The advantage of the hPAP-grafted cells is that human-specific hPAP antibodies exist, making detection of engrafted cells very easy. Additional transgenic animals expressing EGFP and other reporter genes under the control of constitutive or cell-specific promoters have been and will continue to be developed and can be used in a variety of grafting paradigms. Note that if these cells are delivered as xenografts, immunosuppression regimens must be used.
3. Allogeneic grafts. Allogeneic grafts are grafts between different strains of the same species for which strain-specific antibodies exist. For example, embryonic CNS from mice that express the Thy-1.1 allele of the CNS glycoprotein Thy-1 have been grafted into the CNS of Thy-1.2–expressing hosts, and the grafted cells detected with Thy-1.1–specific antibodies ***(31)***. In principle, strains that differ at class I or II major histocompatibility antigen loci could be used to identify grafted stem cells. However, CNS neurons do not express these antigens, and astrocytes and oligodendrocytes require induction for expression, greatly restricting its utility for CNS grafts. The concern with allografts is that despite the perceived immunological privilege of the CNS, allogeneic grafts can be rejected. We have found lower survival of engrafted stem cells with allogeneic stem cell grafts, and whenever possible, we use syngeneic stem cells and graft recipients.
4. Xenografts. Numerous investigators have grafted CNS stem cells across species, especially human tissues into adult rodent recipients ***(32–42)***. Such grafts can be detected with species-specific antibodies. This also can be done with mouse cells grafted into rat host CNS ***(43)***. Although this is essential to evaluate the potential of the human cells to differentiate after CNS grafting, all xenografts are problematic, because they require immunosuppression to block rejection or the use of immunocompromised hosts. Immunocompromised animals are expensive to buy and house, and it is difficult to maintain laboratory animals long term on immunosuppressive regimens, but it can certainly be done. This is not a recommended approach for graft detection unless there are no other alternatives.

3.4. Identifying Grafted Stem Cell Phenotype In Vivo

Although it is essential to be able to identify surviving grafted stem cells, it is also usually necessary to identify the type of cell into which those grafted stem cells have differentiated. This requires a labeling approach where the

marker for labeling the cells, as well as a cell-specific antibody and indirect immunofluorescence, are used to determine the lineage fate of the engrafted cells. This is most easily done immunohistochemically using two antibodies that are made in distinct species followed by detection with species-specific secondary antibodies that are conjugated to different wavelength fluors. Table 2 lists commonly used antibodies for identifying undifferentiated stem cells, neurons, astrocytes, and oligodendrocytes. We have indicated at least one

Table 2
Antibodies used for detection of differentiated CNS cells

Cell types	Antibodies (species made in)	Source[a]
Neural stem cells	Nestin (mouse)	Development Studies Hybridoma Bank (DSHB; University of Iowa, Iowa City, IA)
Neurons	β-III-Tubulin (rabbit)	Sigma-Aldrich (St. Louis, MO)
	Microtubule-associated protein-2a,b (mouse)	Sigma-Aldrich
	67-kDa neurofilament protein (NFL; mouse)	Sigma-Aldrich
	110-kDa neurofilament protein (NFM; mouse)	Chemicon International (Temecula, CA)
	210-kDa neurofilament protein (NFH; mouse)	Sigma-Aldrich
	Neuron-specific enolase (NSE; rabbit)	Chemicon International
	NeuN (mouse)	Chemicon International
Oligodendrocytes	Rabbit anti-NG2	Chemicon International
	O1 (mouse)[b]	DSHB
	O4 (mouse)[b]	DSHB
	Galactocerebrosidase (mouse)[b]	DSHB
	Rip (mouse)[b]	Chemicon International
	APC (mouse)	Calbiochem (San Diego, CA)
	Myelin basic protein (mouse)	Chemicon International
	mAb328 (mouse)	Chemicon International
Astrocytes	GFAP (rabbit of mouse)	Chemicon International

[a]These vendors are vendors that we have used routinely. However, other vendors make similar antibodies that may work equally well or better.

[b]These antibodies have proven problematic in combination with BrdU immunohistochemistry.

supplier of each antibody, suppliers that we have used ourselves, but multiple companies make these antibodies, and they can be purchased from any of these vendors. Note that immunohistochemical detection of BrdU-labeled stem cells requires 2 N HCl treatment of the tissue sections. Many antigens do not survive this treatment, and if BrdU is the label chosen, the investigator should be aware of this concern. In our experience, those antibodies marked with an asterisk are problematic after the harsh acid treatment needed to remove the chromatin and expose the BrdU in the DNA.

We strongly recommend using Fab' fragments for secondary antibodies as intact IgG or IgM antibodies bind nonspecifically to activated microglia and macrophages, which are always found around grafted cells, especially in allografts or xenografts.

3.4.1. Protocol Optimization for Antibody Detection

All antibodies have different conditions under which they optimally detect antigens in fixed tissues. Conditions such as the fixative used, the thickness of the section, whether paraffin or cryosections are being used, and antibody concentration all markedly affect the strength of the signal and the signal-to-background ratio. As with much of this chapter, we cannot go through specific protocols, because they will be different for each antibody and they must be empirically determined. Rather, we present the variables that have to be optimized and the steps to be taken to ensure appropriate specificity.

1. After choosing an antibody, look at the manufacturer's specification sheet to make sure that it detects the species with which you are working and that it works with immunohistochemistry. The best way to ensure this is to find a reference in the literature that uses that specific antibody. All of the antibodies listed in Table 2 are appropriate for immunohistochemical detection in rat CNS cryosections, although we cannot attest to their suitability in paraffin sections.
2. Run a dilution series of the primary antibody on control tissue to establish optimal dilutions. This is readily done for the cell-specific markers listed in Table 2. We routinely run dilution series of 1:50, 1:100, 1:200, and 1:400. A second narrower dilution series might have to be run to determine the optimal concentration. The dilution of secondary antibody to use in this optimization depends on the antibody used. For 7-amino-4-methylcoumarin-3-acetic acid and fluorescein isothiocyanate (FITC)-conjugated secondary antibodies, we routinely use a 1:100 dilution, 1:200 Texas Red, and 1:400 for Cy3 and Cy5.
3. Once a primary dilution is determined, repeat the dilution series with the secondary antibody with the starting and ending concentrations bracketing the initial concentration. The reason for this step is to lower the background as much as possible while retaining a strong signal.
4. This process should be repeated for all antibodies, although if BrdU is used to label engrafted cells, immunohistochemical parameters will have to be optimized

on grafted animals. For these studies, which can be done while optimizing graft parameters, a 1- or 2-week survival time is sufficient.

5. Not all antibodies work well with identical fixatives. For new antibodies, one also must optimize fixative conditions. We initially cut cryosections from fresh frozen, unfixed tissue; adhere them to microscope slides; and then fix on the slides with a variety of fixatives to see which works optimally. We routinely run low-stringency fixes for 10 min of ice-cold 4% paraformaldehyde in phosphate buffer, ice-cold methanol, and ice-cold acetone. We have often additionally evaluated Karnovsky's or picric acid based fixatives. Once an optimal fixation protocol has been identified, one can then use it for perfusion fixation. We begin with low-volume fixation (30 ml for the mouse and 100 ml for the rat) with no postfixation.
6. For visualization of double-labeled cells, an initial screen with fluorescence microscopy can give an indication of whether the cells express specific markers. However, for unequivocal documentation of grafted cells exhibiting a specific phenotype, confocal microscopy, showing both XZ and YZ planes is essential.

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29

Optimizing Stem Cell Grafting into the CNS

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Summary

Grafting neural stem cell to facilitate repair after central nervous system (CNS) injury is being used in many laboratories. The technical challenges of this approach include the ability to maintain the viability of the cells before grafting, to be minimally invasive with the grafting method so as to not do further damage to the host CNS, and to maintain optimal viability of the cells during the grafting process. We outline an approach to CNS stem cell grafting that has evolved in our laboratories over the past decade ***(1–7)***. The best approach to graft a given stem cell population is empirical, but we provide parameters with which to quickly delineate that approach.

Key Words: Stem cell grafting; micropipette.

1. Introduction

The potential of stem cells to be used for repair of the injured or degenerating central nervous system (CNS) is unlimited because of their capacity to differentiate into neurons, astrocytes, and oligodendrocytes. In addition, that stem cells can be expanded in vitro before engrafting means that large numbers of clonally derived cells can be obtained. Finally, proliferating stem cells readily incorporate exogenous genes after transfection with eukaryotic expression vectors or infection with viral vectors enabling their use for ex vivo gene delivery. However, the very process of engrafting into the CNS itself creates damage that can be detrimental to the survival of the engrafted cells. This is especially true when grafting into very small structures such as the

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spinal cord. Thus, the potential therapeutic efficacy of stem cell grafts depends on grafting methods that optimize graft survival and minimize the extent of the graft-induced lesion. Both of these variables are dependent on the type of system used to deliver the cells. We have compared several different configurations and sizes of micropipettes for grafting into the adult rat spinal cord, and we discuss our approaches to optimize the survival and integration of these grafts.

2. Materials

1. 0.4% trypan blue in 0.85% NaCl, sterile.
2. Hank's balanced salt solution (HBSS), sterile hemocytometer.
3. Beckman GRP (Beckman Coulter, Fullerton, CA) or equivalent refrigerated centrifuge.
4. TW150-4 glass pipettes (WPI, Sarasota, FL) or its equivalent.
5. Flaming Brown micropipette puller (Sutter Instrument Company, Novato, CA, model P-80 PC) or its equivalent.
6. Model 1300M micropipette beveler (WPI) or its equivalent.
7. Diamond lapping film (WPI) or its equivalent.
8. Bransonic B-2200R-1 sonicator (Branson Ultrasonics Corporation, Danbury, CT) or its equivalent.
9. Picospritzer® II (Parker Instrumentation, Fairfield, NJ) or its equivalent.

3. Methods

3.1. Micropipette Size and Configuration

Although using smaller micropipettes should cause less damage to host tissues, it was not until 1994 that this hypothesis was experimentally demonstrated ***(8)***. However, the choice of an appropriately sized micropipette also depends on the size of the stem cells to be engrafted. For example, a single-cell suspension would readily flow through a 40-µm outer diameter (o.d.) micropipette, whereas a suspension of neurospheres would require a much larger tip diameter if the spheres were to be engrafted intact. We compared various o.d. tips as well as the configuration of the tip (e.g., broken, beveled, fire polished; see **Fig. 1A–C** on the deformation of the spinal cord before the tip penetrated the cord parenchyma, an indication of the degree of damage to host tissue. We also examined the effects of grafting with a 10-µl Hamilton syringe, because many investigators use these for grafting into the CNS. Note that the aperture in the Hamilton syringe is not at the bottom of the syringe (*see* **Fig. 1E**, arrowhead) necessitating an extra 300-µm penetration before cells can be engrafted. The data in **Table 1** demonstrate that ***(1)*** Hamilton syringes are

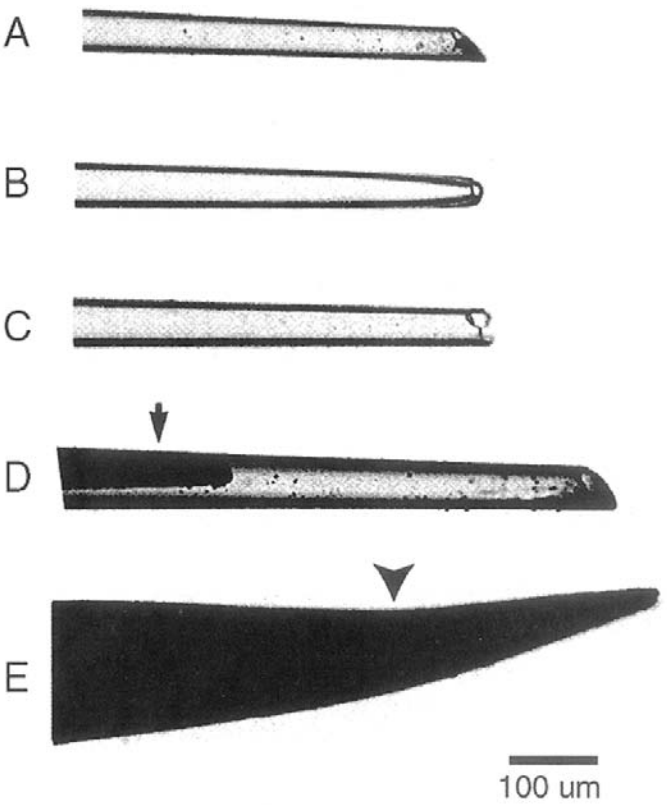

Fig. 1. Micropipette tip configurations. Beveled **(A)**, fire polished **(B)**, broken **(C)**, and beveled **(D)** tips with tungsten filament partially lowered (*arrow*). **(E)** Tip of 10-µm Hamilton syringe. The *arrowhead* indicates the center of the opening in the syringe. Note that it is located 300 µm from the end of syringe tip.

not recommended for use because they cause substantial deformation of the CNS (we also observed a 10% lower cell viability of the injected cells with Hamilton syringes), ***(2)*** beveled tips are better than broken or fire-polished tips, and ***(3)*** the smallest pipette tip appropriate for the size of the stem cells that are to be engrafted should be used. We routinely use 50-µm o.d. beveled pipettes for grafting single stem cell suspensions (*see* Chapter 30). If intact spheres of stem cells are to be grafted, the size of the micropipette needs to be empirically determined, with the cell viability after ejection from the micropipette being the crucial variable for determining tip diameter (*see* **Subheading 3.4.**). Whatever the ultimate tip diameter used, beveled micropipettes should always be used.

Table 1
Micropipette parameters

Pipette size (μm o.d.)	Tip type	Spinal cord deformation (mm)
25	Beveled	0.24±0.02
40	Broken	0.65±0.03
40	Beveled	0.35±0.03
90	Broken	0.93±0.04
90	Beveled	0.72±0.04
90	Fire polished	1.08±0.03
Hamilton syringe		>1.00[a]

[a]Unlike with the pulled glass pipettes, the spinal cord remained deformed after the syringe tip entered the spinal cord.

3.2. Pulling, Beveling, and Calibrating Micropipettes

There are several manufacturers of thin-walled borosilicate capillary tubes, pipette pullers, and pipette bevelers. The aforementioned materials that we use are good options, but any equivalent can be used. The parameters for pulling appropriately sized pipettes vary with both the type of tube and the puller used.

1. Pull the pipettes, put them under on a microscope equipped with a micrometer, and break the tips at the appropriate size using no. 5 forceps.
2. Grind the tip to 55E on diamond lapping film and check its size under the microscope with the micrometer. This process usually takes approximately 2 min for 40-μm and 10 min for 90-μm micropipettes.
3. Clean the tip by dipping in a sonicator containing 100% ethanol for 10–20 s.
4. Use a fine-tipped permanent marker to mark the capillary tubes at 1-mm intervals. These marks will subsequently be used to calibrate the injection system.

3.3. Injection System

There are both manual and electronic means by which to transplant cells into the CNS. Many investigators use a Hamilton syringe or glass micropipettes attached to a Hamilton syringe and manually dispense the desired volumes. For reasons discussed in **Subheading 3.1** above, we do not recommend grafting with Hamilton syringes. It is also very difficult to deliver precise small volumes manually, and the Hamilton syringe/glass micropipette should only be used when large volumes are to be engrafted. Other systems involve motorized syringes connected to micropipettes that can be precisely driven. The problem with this delivery system is that it relies only on pressure for delivery, and if the resistance of either the delivery micropipette or the host tissue changes, the volume of cells engrafted cannot be accurately controlled. A more accurate

way to engraft cells is to use a Picospritzer. Although this approach is more expensive, it delivers a fixed volume in a preset time, and then the pressure returns to ambient. It, too, suffers from the problem of the injection volume being influenced by changes in the tip and tissue resistances, but this can be controlled (*see* **Subheading 3.5.**).

We do all of our grafts using a Picospritzer. Regardless of the injection system, it should be mounted in a three-dimensional micromanipulator, and the head or spinal cord should be rigidly fixed in position to allow accurate and reproducible graft placement.

3.3.1. Modified injection system

One of the complications of using small o.d. pipettes is that they have the potential to become clogged as the cells settle. To obviate this problem, we have fabricated a simple device that prevents cell clogging. Inside of the glass micropipette is a ferromagnetic stainless steel sliding rod (6 mm in length, 0.8 mm in diameter) to which a 25-mm-long, 20-µm-diameter tungsten filament is attached (*see* **Fig. 1D**). It is moved up and down by means of a U-shaped magnet attached to a one-dimension micromanipulator that is parallel to the glass micropipette (*see* **Fig. 2**). When the filament is lowered, it occupies the opening of the micropipette and prevents cells from clogging the tip. When the filament is raised just before engraftment, it not only clears the tip opening but also loosens any aggregated cells that may have packed in the tip. The inclusion of the tungsten filament does not alter the viability of the injected cells.

3.4. Quantifying Cell Number and Viability

Before grafting a suspension of cells, both the number and percentage of viable cells need to be determined. This is easily done for single-cell suspensions, but it is more difficult to determine for cells grown and engrafted as spheres. For single-cell suspensions, cell number and viability are counted in a hemocytometer by using trypan blue. Live cells exclude the dye and look phase bright, whereas dead cells take up the dye and look dark blue.

1. Remove the stem cells from the tissue culture plates or flasks (*see* Chapters 5 and 6), place in a sterile 15-ml conical tube, and pellet in a refrigerated centrifuge (500 × *g* for 2 min at 4°C). The final desired cell density 50,000–100,000 cells/µl (*see* **Subheading 3.5.**) is too concentrated to count with a hemocytometer.
2. Resuspend the cells in HBSS to approximately 2×10^4 cells/µl for counting and viability assessment with a hemocytometer. Once enough preparations have been done, a good estimate of total cell number can be made. We routinely obtain approximately 10^6 viable cells from each 100-mm plate of stem cells at a viability of 85%.

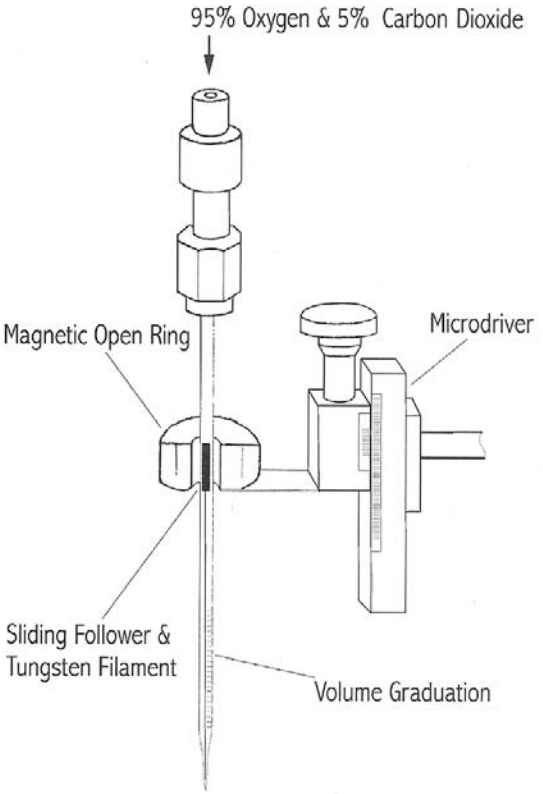

Fig. 2. Tungsten filament to prevent micropipette clogging. An additional microdriver is attached to the micromanipulator that controls the micropipette movement. Note that the micropipette and the microdriver should move in identical directions at all times. Attached to the microdriver is an magnetic open ring that controls the vertical displacement of the tungsten filament that is placed within the micropipette. When the filament is lowered, it prevents cells from aggregating at the narrow beveled opening of the micropipette. When the filament is raised, stem cells can be ejected.

3. Recentrifuge the cells as described in **step 1** and suspend in ice-cold HBSS to desired final concentration. Store on ice.
4. After all engrafting procedures are completed, a second check of cell viability should be done. We typically observe a 30% drop in stem cell viability over a 6-h grafting session, even when the cells are stored on ice. More significant decreases in cell viability would suggest that the later occurring grafts in those experiments will likely be suboptimal.

If you are engrafting intact spheres, there is no good method to get an accurate count of the number of cells. Dissociating intact spheres into single

cells often results in the loss of many cells. However, once a population of spheres has been characterized for its average diameter, the spheres should be dissociated and counted with a hemocytometer to obtain an approximate cell number; both live and dead cells should be counted. The spheres can be pelleted by centrifugation (500 × *g* for 2 min at 4°C), resuspended in a small volume of HBSS, and counted in a hemocytometer. These spheres will be of varied sizes. However, using a microscope micrometer, you can get a sense of the range and median diameters of the spheres and use that median to characterize the cultures. Variables such as the optimal number and size of the spheres for engraftment and the size of the micropipette used for grafting will have to be empirically determined. The latter two parameters must be established first and can be done in vitro with the viability of the cells after injection the critical outcome measure; this can be measured by trypan blue exclusion. Once these have been established, the sphere density that is optimal for grafting can be again empirically established. It may be that despite good viability in vitro, very large spheres do not survive well after engraftment. The preliminary grafting experiments should address this issue.

3.5. Engraftment Parameters

Three parameters need to be established for optimal stem cell grafting: cell density, the volume to be grafted, and the number of grafts to be made. Our experience is that cell densities of 50,000–100,000 cells/µl are optimal for single-cell suspensions. Higher densities are very viscous, and they have the potential to clog the micropipette. Lower densities do not provide an adequate number of cells for grafting. Nikkhah et al. ***(1)*** showed that cell survival and integration are better with multiple grafts of lower volume than single large grafts. Such an engraftment strategy also enables more area to be covered by the grafted cells. However, single injections of volumes as large as 10 µl into the intact adult rat spinal cord are not detrimental to locomotor function. Fluids readily diffuse throughout the spinal cord parenchyma. A 1-µl injection of India ink into the central gray matter of the adult rat spinal cord can be detected 2.9 ± 0.3 mm from the injection site in the parenchyma and >7 mm away in the central canal. A similar injection of 10-µm-diameter fluorescent beads (which approximates the size of a stem cell) is found only within 0.9 mm of the injection site. Therefore, grafting into a large structure necessitates multiple cell grafts. We routinely graft 0.5–1.0 µl/graft site unless the grafts are made into a lesion cavity, in which case volumes up to 10 µl can be accommodated. The number of grafts to be made and their spatial arrangement are determined by the target site.

The volume delivered by any injection system is a function of tip and host tissue resistance. Therefore, it is imperative to use the 1-mm markings on the micropipette (*see* **Subheading 3.2.**) to calibrate the engrafted volume. Because the internal diameter of the micropipette is known, the actual volume injected using a given set of parameters can be precisely determined. If that volume is less or more than desired, the injection parameters can be adjusted accordingly.

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30

Routes of Stem Cell Administration in the Adult Rodent

Alison E. Willing, Svitlana Garbuzova-Davis, Paul R. Sanberg, and Samuel Saporta

Summary

Stem cell transplantation to replace damaged tissue or correct metabolic disease holds the promise of helping a myriad of human afflictions. Although a great deal of attention has focused on pluripotent stem cells derived from embryos, adult stem cells have been described in a variety of tissues, and they likely will prove to be as beneficial as embryonic stem cells in cell replacement therapy and control of inbred errors of metabolism. We describe methods by which stem cells can be introduced into the nervous system, although the techniques are applicable to any portion of the body to be targeted or any cell that may be used for cell therapy. The first and most straight-forward method is introduction of stem cells directly into the brain parenchyma. The second, which in our hands has proven to be superior in some instances, is introduction of the stem cells into the circulatory system.

Key Words: Carotid artery; femoral vein; intravenous; jugular vein; neural stem cell; penile vein; stem cell; stereotaxic; tail vein; transplantation; umbilical cord blood.

1. Introduction

Arguably, one of the most exciting developments in science in the last decade is the discovery and isolation of neural stem cells (NSCs), not only from embryonic ***(1,2)*** but also from adult human brain ***(3,4)***. With the ability of a NSC to proliferate, self-renew, and generate a large number of clonally related progeny of a neuronal, astrocytic, or oligodendrocytic lineage, these cells promise to revolutionize the treatment of neurological disease. The potential of these cells to correct genetic diseases, such as those resulting from inborn

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errors of metabolism, is staggering. For example, studies in myelin-deficient rat ***(5)*** and *shiverer* mouse ***(6)***, show that it is possible to correct a myelin deficiency. Direct transplantation of NSCs into the spinal cord in the first study resulted in remyelination of the cord for up to 3 mm around the implant site. In the second study, the NSCs that were implanted intracerebroventricularly integrated throughout the brain, predominantly differentiating into oligodendrocytes, which elaborate myelin. Therefore, the cells are able to react to environmental cues within the host environment, and differentiate accordingly.

This also suggests that NSCs are able to migrate to the damaged region of the host brain. The specific nature of these migratory cues may change throughout development of the injury or disease state. In the genetic disorders, the optimal outcomes occur with the delivery of the stem cell therapy early in development. If the diagnosis of a leukodystrophy can be made before birth or even shortly after, then the migration and cell development signals may still be in place and can direct the NSCs to the appropriate destination and phenotype. Studies such as these not only demonstrate the utility of these cells for treatment of developmental disorders but also the knowledge gleaned helps us to better understand the normal development of the nervous system.

The utility of NSC extends beyond the treatment of developmental or genetic diseases. It is possible that these cells could provide an unlimited supply of human neural cells for the treatment of diseases of the adult or aging brain as well. However, the environment of the adult brain is not the same as that of the neonate. Aside from specific regions such as the subventricular zone or the dentate gyrus, in which neurogenesis continues in the adult, the neural circuitry in the remainder of the brain has long been established. Important developmental/environmental cues that guide the fate of NSCs in the young animal may not be present in the older animal. In initial studies of mouse NSC transplantation into the injured brain, some of the cells developed into neurons, suggesting that instructive cues are present in the injured or diseased brain that allow the cells to develop along the appropriate phenotypic lines ***(7)***. Thus, it may be possible for the stem cells to differentiate into the needed cell population in the adult brain. Aside from cues that direct differentiation of the NSCs, all the environmental cues that direct cellular migration in the adult or aging brain may not be similar to those present in the developing brain. For example, although stromal derived factor (SDF)-1 is associated with neural development and migration of newly born neurons to their final destinations within nuclei of the brain ***(8)*** during development, migration occurs against a background of high trophic/growth factor levels. There is evidence that trophic support in the brain decreases with age ***(9,10)***. In the adult, chemokines, including SDF-1, are generally associated with inflammatory and immune responses. After a stroke, expression of SDF-1 and other chemokines increases in the injured

brain ***(11)***, resulting in infiltration and activation of peripheral immune cells. Although NSCs also can respond to these signals, and they may migrate to the site of injury ***(12,13)***, they arrive in a hostile environment with altered trophic support and ongoing inflammation that has been shown to inhibit neurogenesis ***(14)***.

There have been several recent reports demonstrating that stem cells capable of differentiating into neural cells exist outside the central nervous system (CNS). For example, two groups recently reported the production of multipotential neural stem cells from skin ***(15)***. It was recently shown that NSCs produce non-neural hematopoietic cells ***(16)***. Furthermore, after systemic delivery of nonhematopoietic stem cells from bone marrow (or bone marrow stromal cells), these cells have been found in the brain ***(17)*** where they have been found to differentiate into astrocytes ***(17–20)***. Two recent articles reported that transplants of whole bone marrow produced cells expressing neuronal markers ***(17–22)***. Furthermore, when these cells are transplanted into a rat model of stroke ***(23)*** or traumatic brain injury ***(24)***, they ameliorate behavioral deficits, and they may promote the proliferation of endogenous stem cells within the subventricular zone ***(25)***.

In our laboratory, we have been examining the ability of cells derived from non-neural sources such as umbilical cord blood, to ameliorate deficits in rodent models of neural injury, stroke, and spinal cord contusion, and diseases such as amyotrophic lateral sclerosis and Sanfilippo Syndrome type B ***(26)***. The first article ***(27)***, a collaborative effort between research groups at University of South Florida and Henry Ford Hospital in Detroit, shows significant improvements in motor function after intravenous (iv) administration of cord blood cells that is maintained 30 days after transplantation in rats with temporary occlusion of the middle cerebral artery. Part of our program has been to compare whether differences in the ability of these cells to provide functional benefits occur with intraparenchymal versus vascular delivery (iv) ***(28)***. Surprisingly, cord blood cells produced longer lasting recovery when delivered iv than they did when directly implanted into the lesioned striatum. Therefore, the decision regarding which delivery route to use will be based, in part, upon the proposed mechanism of recovery. For example, we found that the most likely mechanism by which the cord blood cells produce recovery in stroke is by inhibiting the inflammation that occurs after the insult ***(29)*** and not through cellular replacement. Furthermore, the injected cord blood cells migrate not only to the infarcted side of the brain but also to the spleen ***(30)***, where they alter splenic function ***(31)***. Therefore, even though the site of injury after the stroke is within the brain, there is an interaction with the peripheral immune system that may be critical not only for the mediation of brain damage but also for the therapeutic effects of the cell therapy. This is the case for leukodystrophies

such as X-linked adrenoleukodystrophy and Krabbe disease. Although there is extensive neurological involvement in these diseases, they are systemic diseases that in clinical studies have been shown to respond to hematopoietic cell therapy given iv if it is administered at an early age ***(32–34)***. Therefore, it is critical to have a clear understanding of the mechanism by which the cell is working in a model or disease state to choose between potential routes of administration.

Also in this chapter, we describe the surgical procedures for cell implantation and the issues involved in the transplantation procedures themselves.

2. Materials

2.1. Cell Preparation

1. Stem cells.
2. Medium for cell suspension.
3. 1,1'Dioctadecyl-3,3,3',3'-tetramethyllindocarbocyanine perchlorate (DiI; Invitrogen, Carlsbad, CA; CellTracker CM-DiI cat no. C-7000).
4. Dimethyl sulfoxide (Sigma-Aldrich, St. Louis, cat. no. D-2650).
5. Cholera toxin subunit B (fluorescein isothiocyanate conjugated, Invitrogen).
6. Microcentrifuge tubes.
7. 15-ml conical tubes.
8. 0.22-μm nonpyrogenic filter (Corning Live Sciences, Acton, MA, cat. no. 8110).
9. Syringes.
10. Pipetter and tips.
11. 0.4% trypan blue (Sigma-Aldrich, cat. no. T-8154 or Invitrogen, cat. no. 15250-061).
12. Distilled water.
13. Hemocytometer.
14. Inverted microscope.
15. Water bath.
16. Centrifuge.

2.2. Transplantation

1. Sterile physiological saline (0.9%).
2. Syringes (1, 3, and 10 ml).
3. Betadine.
4. Equithesin or isoflurane.
5. Kopf stereotaxic instrument (David Kopf Instruments, Tujunga, CA).
6. Cotton swabs and gauze.
7. Nylon suture 3.0 and 5.0.
8. Gelfoam.
9. Lidocaine.

2.2.1. Surgical Instruments

1. Scalpel, scissors, needle, retractors, forceps, hemostats, needle drivers, drill bit, spatula, dental pick, and rongeurs.
2. Dental drill.
3. Absorbent table cover.
4. Ethyl alcohol.
5. Vetbond.
6. Hydrogen peroxide.

2.3. Post-Operative Requirements

1. Immunosuppressant.
2. Antibiotic.
3. Acetaminophen.
4. Heating pad.
5. Nest building material for mice.

3. Methods

3.1. Cell Preparation

Detailed descriptions of the cell preparation (isolation, propagation, or differentiation) have been thoroughly discussed in other chapters.

3.2. Transplantation

3.2.1. Brain

3.2.1.1. Striatum

Transplanting cells into the striatum is the most straightforward of those protocols in which cells are transplanted directly into the parenchyma. The only technical difficulties that are likely to arise are related to the extent of damage that may occur with various lesioning procedures used to damage the striatum before transplantation. It is not uncommon for striatal atrophy to occur after excitotoxic or ischemic injury, which may decrease striatal volume.

The general procedure for stereotaxic surgery is similar regardless of the region in which the cells are implanted and is as follows:

1. Anesthetize the animal with Equithesin (*see* **Notes 1–3**), shave the top of the head, place in the stereotaxic instrument, and apply Betadine to the shaved area.
2. Make an incision along the midline of the cranium, clean connective tissue from the top of the head, and locate bregma.
3. Using bregma as a landmark, drill a hole in the cranium at the appropriate anterior/posterior, medial lateral location. Check the burr hole to ensure there

are no bone fragments or spurs that may interfere with the needle trajectory through the hole into the brain.
4. Load the cells into a 10-µl Hamilton syringe (Hamilton, Reno, NV) fitted with a 26-gauge needle.
5. Make a small hole in the dura for the needle to pass through with a dental pick; this decreases the damage that can result from compression of the brain.
6. Lower this needle through the hole until the tip is touching dura. This is considered the zero point in the dorsoventral direction.
7. Slowly lower the needle into position, wait for 2 min to allow the tissue to regain its normal orientation, then deliver the cell suspension at a rate of 0.5 µl/min (*see* **Note 4**).
8. Leave the needle in place for 5 min.
9. If a second cell deposit is to be made, reposition the needle at an adjacent site and the repeat the process.
10. Leave the needle in place 5 min, and then slowly withdraw.
11. Place Gelfoam in the hole and close the incision with wound clips or sutures.

3.2.1.2. Hippocampus

There are more technical difficulties in placing transplants directly into the hippocampus than into the striatum. First, there are many blood vessels on the top of the brain that interfere with access to the hippocampus. To address this issue, a 2- × 4-mm rectangular bone flap is carefully removed by drilling away the perimeter of the bone flap with a dental burr until the majority of the flap is free, and then gently elevating the flap with forceps. We have found it useful to make this craniotomy large enough to obtain good visualization of the cortical surface. This allows us to localize the veins, make minor adjustments in needle placement, if necessary, or gently push a vessel out of the way during needle insertion. However, care must be taken while removing this flap not to disrupt any of these veins. In the event that bleeding does occur, a small piece of Gelfoam or a cotton swab soaked in saline can be applied briefly to the craniotomy to stop bleeding from both emissary veins and the cranium. Once the edges of the craniotomy are cleaned with fine ronguers, an "x" is cut in the dura by using the tip of a number 11 scalpel to create a larger hole in dura. This minimizes any resistance to the needle's passage.

A second issue is that the hippocampus is not attached to the overlying cortex or the thalamus beneath. In addition, the thin layer of the alveus on the ventricular surface of the hippocampus can make transplantation problematic, with the needle either slipping off of the hippocampus or simply compressing it. In either case, the result is a ventricular transplant. To address this issue, we use a sharper beveled needle than is used for the striatal transplant. The tip of the needle is sharpened to a point with a fine whetting stone under a dissecting microscope so that the needle can penetrate the tissue more easily.

3.2.1.3. Cortex

Transplantation into the neocortex of the rat presents several technical difficulties. The cerebral cortex of the rat is approximately 1 mm thick throughout. Insertion of a 30-gauge transplant needle perpendicular to the cortical surface may seem the most direct and expedient method to adopt, but neural tracing studies indicate that the only way to accurately place the tracer in layer 3 or 4 of somatosensory cortex without significant solution reflux is with ionotophoretic injection through glass micropipettes with a tip diameter of 10–20 µm ***(37–40)***.

Transplantation of cells within the neocortex usually cannot be accomplished with such small volumes or with drawn glass tips of such small diameter. Nor is the approach described for striatal injection (a 10-µl microsyringe fitted with a 26-gauge thin wall needle) satisfactory. In an effort to improve this method, without resorting to drawn glass pipettes, we have adapted the striatal delivery system to making cortical transplantation by inserting the needle at an angle, rather than perpendicular, to the cortical surface (*see* **Fig. 1**). This adaptation results in accurate placement of the transplant within the cortex (*see* **Note 5**). The forces on the cortical tissue during tissue penetration result in little tissue deformation. Additionally, some protection of the transplanted tissue is provided because the transplant is placed in a "pocket" within the cortex that is covered with at least a half thickness of intact cortical tissue.

1. Anesthetize the rat and place in a stereotaxic apparatus capable of tilting the injection apparatus to an angle of 20–45°.
2. Prepare a bone flap as described for hippocampal transplants. Clean the edges of the craniotomy with fine ronguers.
3. Load the microsyringe with cells for transplantation and mark the site where the injection needle contacts the surface of the brain. If the target is the motor cortex of the rat, care must be taken that the injection clears the sagittal sinus (*see* **Fig. 1**).
4. Nick the dural surface at the site where the injection needle will enter the cortex with a 26-gauge hypodermic needle.

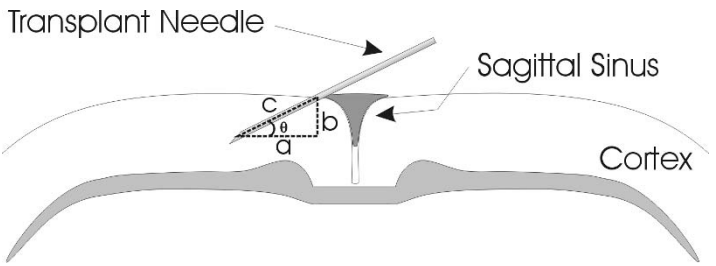

Fig. 1. Needle trajectory for a cortical transplant.

5. Slowly advance the injection needle to the precalculated coordinates, making sure that it is free of the edges of the craniotomy at all times.
6. Leave the needle in place for 5 min to allow the cortical tissue to adjust
7. Inject the cells at a rate of 0.01–0.1 µl/min.
8. Leave the needle in place for an additional 5 min.
9. Withdraw the needle slowly.
10. We routinely place a piece of Gelfoam soaked in saline over the craniotomy.
11. Close the scalp with discontinuous sutures or with Michel clips.

3.2.2. Spinal Cord

Transplants into the spinal cord are performed in a two-step procedure. The first day we gain access to the spinal cord by drilling small holes in the vertebra (*see* **Note 6**). We then wait 24–48 h and evaluate the animals to make sure that they still have intact motor function. On the second surgery day, the cells are implanted directly into the spinal cord.

3.2.2.1. Preparing the Transplant Site

1. Anesthetize the animals with equithesin (3.5 ml/kg intraperitoneal [ip]; *see* **Note 2**).
2. Immobilize the anesthetized animal on a small platform.
3. Make an incision in the skin over the vertebra. In both our rat contusion model and the SOD1 mouse model of amyotrophic lateral sclerosis (ALS), the transplants are placed in the lumbar spinal cord.
4. Remove the erector spinae muscle from the dorsum of the vertebra.
5. Make a hole by gently twirling a 0.8-mm Dremel engraving bit between two fingers. The holes are made 0.5–0.7 mm from the vertebral midline at the level of L_4–L_5.
6. Suture the incision.
7. Monitor the animals to ensure that no damage has been inflicted by these procedures (*see* **Note 7**).

3.2.2.2. Implanting the Cells

One to 2 days after the burr holes have been drilled or the laminectomy performed, the animals are transplanted.

1. Anesthetize the animals with equithesin (3.5 ml/kg ip).
2. Immobilize the anesthetized animal on a small platform.
3. Remove the sutures from the earlier incision and open the incision.
4. Make a hole in the dura mater through which the needle is to pass.
5. Load the cells into a 10-µl Hamilton syringe with a 31-gauge needle.
6. Mount the syringe in a stereotaxic frame to ensure a steady, straight angle of entry.

7. Lower the needle through the prepared site into the ventral horn (1–1.3 mm below the dura).
8. After waiting for 2 min, inject 1 µl of the cell suspension or media into the transplant site over a 5-min period.
9. Wait 5 min.
10. Slowly withdraw the needle.
11. If a laminectomy was performed, cover the spinal cord with Gelfoam (absorbent gelatin).
12. Close the incision. In mice, we do this with Vetbond (tissue adhesive).

3.2.3. Vascular

Introduction of stem cells through the vascular system is a novel, and potentially important, method of allowing the stem cells access to organs of the entire body. Stem cells, regardless of origin, seem to preferentially target areas of damage and enter these sites ***(6,19,20,27,41)***. The carotid artery or femoral, jugular, and tail veins are all potential sites to introduce stem cells into the vascular system (*see* **Fig. 2A**). The choice of vascular route is based more on technical or practical issues than efficacy (*see* **Note 8**). Once that choice has been made, all the vascular surgeries are performed using blunt dissection techniques in order to minimize trauma to the vessels and surrounding tissue (*see* **Note 9**).

3.2.3.1. Tail Vein

Perhaps the most convenient and least traumatic route of vascular access is through the tail vein. The blood to the rat's tail is supplied through bilateral dorsal arteries and is returned by bilateral tail veins (*see* **Fig. 2B**). The tail vein lies laterally, just beneath the surface of the rather thick tail skin and inferior to the small transverse processes of the caudal vertebrae.

1. Load a 1-ml syringe with the cells to be transplanted (a total volume of 0.25 ml works well for us) and attach a 25-gauge need to the syringe. A larger volume of cells is loaded to allow us to fill the syringe and clear it of air (*see* **Note 10**).
2. Place the rat in a rat restrainer.
3. Hold the rat's tail under warm, flowing water (approximately 42°C) for 2 min. The tail vein on both sides will dilate and should be clearly visible beneath the skin on the lateral surface of the tail. Water should flow over the entire tail to dilate as much of the tail vein as possible.
4. Dry the tail and wipe with alcohol.
5. Hold the syringe so that the bevel of the syringe needle is facing up.
6. Insert the needle into the skin, about 6 cm from the base of the tail (*see* **Note 11**). The needle is initially directed at a 30° angle into the skin, and then threaded a short distance into the vein by decreasing the angle in relation to the tail.

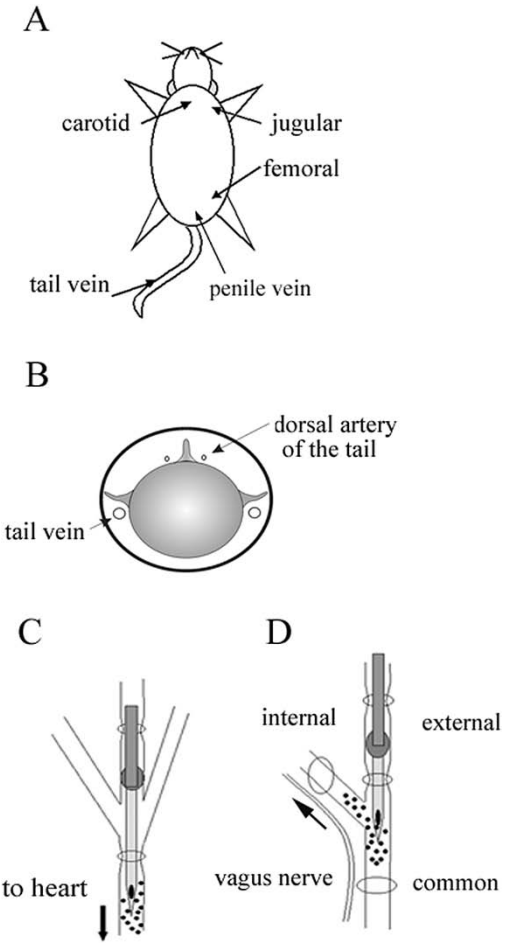

Fig. 2. Vascular routes of cell delivery. **(A)** Location of common vascular delivery sites: common carotid artery, jugular, femoral, penile, and tail veins. **(B)** Schematic diagram illustrating the location of the tail vein. **(C)** Illustration of the cannulation of the jugular vein. **(D)** Cannulation of the carotid artery.

7. During this procedure, gently pull the plunger back just a bit. When the needle enters the tail vein, venous blood will enter the hub of the needle and also will be visible in the syringe.
8. Without moving the syringe, depress the plunger to deliver the contents of the syringe into the tail vein (*see* **Note 12**).
9. Once the cells have been delivered, remove the needle and quickly place pressure over the site of skin penetration to stop the flow of blood. Apply pressure until hemostasis is achieved, usually about 30 s.
10. Release the animal from restraint and place it back in its cage.

3.2.3.2. Penile Vein

In the aging animal, access to the tail vein is much more difficult as the skin toughens with age. An alternative nonsurgical approach, at least in males, is to deliver the cells through the penile vein.

1. Anesthetize the rat, preferably with a short-acting inhaled anesthetic such as isoflurane (*see* **Note 12**).
2. Place the rat in a supine position.
3. Withdraw the penis from within its protective sheath, exposing the vein that runs centrally on the top.
4. Wipe with alcohol.
5. Hold the syringe so that the bevel of the syringe needle is facing up.
6. Insert the needle directly into the vessel, just proximal to where the vessel disappears beneath the skin sheath.
7. During this procedure, gently pull the plunger back just a bit. When the needle enters the penile vein, venous blood will enter the hub of the needle and also will be visible in the syringe.
8. Without moving the syringe, depress the plunger to deliver the contents of the syringe into the vein as described for the tail vein delivery.
9. Remove gas anesthesia and place the animal back in its cage.

3.2.3.3. Jugular Vein

1. Isolate and ligate one branch of the external jugular vein above the junction of the anterior jugular, cephalic, and acromiodeltoid veins (*see* **Fig. 2C**) and **Note 13**).
2. Place a second suture on the vessel past where the hole is to be made and tie it loosely.
3. Puncture the vessel by using a 25-gauge needle.
4. Insert a 31-gauge needle attached to a Hamilton syringe with the cell suspension already loaded through the hole and into the lumen of the vessel past the second suture.
5. Tighten the suture over the needle.
6. Deliver the cells delivered into the vessel over 2 min.
7. While maintaining tension on the suture, withdraw the needle and permanently tie the suture.
8. Close the incision.

3.2.3.4. Femoral Vein

The procedure for the femoral vein cannulation is much the same as for the jugular vein.

1. The animal is placed on its back and an incision is made in the skin to expose the muscles of the medial aspect of the thigh.
2. The femoral vein lies together with the femoral artery superficially overlying boundary between the adductor and extensor muscles and passing under the inguinal ligament to become the external iliac vein of the pelvis.

3. Using blunt dissection, the femoral artery and vein are isolated from each other and the surrounding tissue.
4. The vein is then prepared in the same way as the jugular vein.

3.2.3.5. Arterial

For this procedure, the cells are delivered retrogradely through the external carotid artery into the common carotid artery (*see* **Fig. 2D**).

1. Anesthetize the animal.
2. Make an incision over the sternohyoideus muscles.
3. Using blunt dissection, isolate the common, external and internal carotid arteries from the vagus nerve and surrounding tissues in the neck.
4. Place 2 sutures (5.0 silk) on the external carotid, and permanently tie the one closest to the base of the skull.
5. Place the second tie next to the junction with the common carotid. Do not tie.
6. Place temporary sutures or vascular clamps on the common carotid and the internal carotid to prevent blood loss once a hole is made in the external carotid.
7. Make a hole in the external carotid by using a 25-gauge needle.
8. Insert a 31-gauge delivery needle into the lumen of the common carotid.
9. Tie a half knot around the needle with the suture at the junction of the external and common carotid arteries.
10. Remove the clamps on the common and internal carotid.
11. Inject the cells.
12. Leave the needle in place for 2 min, and then remove the delivery needle.
13. Completely tie the suture at the junction of the external and common carotid.
14. Close the incision.

3.3. Postoperative Care

For the most part, the postoperative care of the mice or rats should be fairly standard. One of the most critical concerns after surgery is body temperature; anesthesia and the incision both may contribute to lowering body temperature. After surgery, the first priority should be to place the animal on a heating pad and monitor it frequently. Mice in particular are very sensitive to temperature changes, and they are subject to both hypo- and hyperthermia. Nest building material is provided to assist mice in maintaining their body temperature and increase their sense of security. In addition, the animals are placed on a prophylactic antibiotic therapy for 5 days postsurgery to minimize the risk of surgery-related infections. Acetaminophen also is placed in the water for 1 week. Finally, the animals should be monitored for dehydration (e.g., dry excrement, nonelastic skin). This can be remedied either by twice daily oral administration of water (1 ml/time), substitution of a soft food, or subcutaneous injections of physiological saline.

In addition to standard postsurgical care, transplanted animals in our care also receive immunosuppressant drugs to minimize graft rejection (*see* **Note 14**). We have used cyclosporine A (10 mg/kg/day ip for rats and mice or in some cases 25 mg/kg/day orally for the mice ***(42)***). Recent evidence would suggest that this may not be necessary; mesenchymal cells, for example, elicited a blunted response in a mixed lymphocyte reaction ***(43)***, inducing a T-helper 2 or anti-inflammatory response ***(44)***. This may explain the observations made by Pan and colleagues ***(45)*** that survival of cord blood cells implanted in the brain was similar regardless of whether the animals were immunosuppressed with cyclosporine. It is not yet clear whether this is necessary. It can be argued that embryonic stem cells or stem cells from umbilical cord blood are nonimmunogenic, because they have relatively few surface markers, but it has yet to be shown that these grafts can be maintained in vivo long-term without immunosuppression.

4. Notes

1. After administration of equithesin (3.5 ml/kg ip), a rat or mouse remains anesthetized for approximately 1–1.5 h. This anesthetic is generally well tolerated by rats in particular and to a lesser extent by mice. Should it be necessary to supplement the initial dose, administer 0.05 ml to the rat or 0.001–0.002 ml for a 20–23-g mouse. After 3–5 min, depth of anesthesia should have increased again.
2. For short-term surgeries that do not require a stereotaxic instrument, the gas anesthetic isoflurane may be used. The depth of anesthesia is easily maintained and the animals recover from anesthesia faster.
3. Respiratory problems can occur under both equithesin and isoflurane anesthesia. These are more easily rectified with gas anesthesia by simply decreasing the amount of anesthetic being delivered. Should problems persist with gas anesthesia or in the case of injectable anesthetic, a number of alternatives exist. The simplest method is to stimulate the animal's breathing center with smelling salts (ammonia inhalant of 15% ammonia and 35% alcohol). Three to five ammonia inhalations (each separated by 15–20 s) should be accompanied by gentle compression of the rib cage. Usually the animal is revived quickly, appearing to sneeze. If there is no response, it may be necessary to administer a respiratory stimulator, such as doxapram hydrochloride (1–2 drops under the tongue followed by 0.1 ml intramuscular). If these measures fail, it may be necessary to perform mouth-to-mouth resuscitation on the animal by using a small tube to blow through. Gentle chest compression also may help.
4. For all surgeries in which the cells are implanted directly into either the brain or spinal cord, there is an issue of seepage of the cell suspension occurring along the needle at the site of penetration. This is usually a function of the cells being injected too quickly and can be easily remedied by injecting the cells

more slowly. Once the entire volume of cells has been administered, the needle should also be left in place longer than with striatal transplants (up to 10 min) before being withdrawn.

5. For the cortical transplants, some basic geometry is required to calculate the insertion point and depth of insertion to reach the desired placement of the needle tip (*see* **Fig. 1**). The formula to determine the depth of transplantation, b, from the cortical surface, and for a given angle Θ is

$$c(\cos\Theta) = b$$

Similarly, the formula to determine the lateral extent of the transplant needle tip from the cortical insertion point is

$$c(\sin\Theta) = a$$

At times, it may be easier to find the transplant target site, determine a preferred course for the needle and calculate the angle of insertion, Θ, with the formula

$$\sin\Theta = a/c.$$

6. When single bilateral injections are made in the mouse spinal cord, we prefer to hand drill burr holes through the vertebra. In our hands, we get a lower incidence of hemorrhaging. If multiple injections are done or we are transplanting in the rat, then we perform a laminectomy. The procedure is similar through the removal of the erector spinae muscle. After that, the spinous process is cut, the lamina is cut on each side and the top of the vertebra is removed.
7. If hemorrhaging occurs as a result of cutting the dura and pia mater, the extent of damage should be determined with a microscope or other optic magnification. If the hemorrhage is extensive, surgery should be stopped, and the animal must be euthanized or sacrificed. In the case of minimal damage, surgery can be continued. The animal should be monitored extensively post-surgery to ensure that there is no surgery-related decrement in motor performance.
8. In the mouse, the femoral and tail veins are too small to practically inject the cells. In the rat, it may be more appropriate to transplant an animal that has had a middle cerebral artery occlusion (MCAO) to induce stroke, not through the jugular vein but through either the femoral or tail vein. The reason for this is that 24 h after MCAO the neck region may be swollen, and the other blood vessels around the carotid may be more reactive than they would be in a naïve animal. Similarly in an animal with a spinal cord injury that is dragging its hind limbs around the cage, a femoral artery injection may increase the risk of infection around the incision site.
9. The procedure for cannulating both veins and arteries is much the same in the rat and the mouse. The main difference beyond the obvious size distinction is the consistency (strength, elasticity) of the tissue; mouse tissue is not as sturdy as rat tissue and requires a more delicate touch.

10. One of the biggest concerns with vascular administration of the cells is that of introducing an air embolus into the bloodstream. Extra care must be taken in loading the syringe and needle to ensure there are no air bubbles and further when the needle is placed in the vein or artery, the tip of the needle should be placed in the vessel so that the surgeon can see the suspension as it leaves the needle tip. If in spite of all precautions an air bubble is injected, if the surgeon is able to see the tip and is delivering the suspension slowly, then it is possible to draw the air bubble back into the syringe. In most cases, a small air bubble can be tolerated in the rat without complications, but this situation is more critical in the mouse.
11. The tail vein is largest at the base of the tail and smallest at the tip of the tail. However, we choose our initial entry site (6 cm from the base) so that we can make a second or even third (rarely) attempt to enter the tail vein more rostrally should the first attempt fail. Should these three attempts fail, the tail vein on the other side of the tail is also available.
12. There should be no resistance felt when depressing the plunger to deliver the cells into the tail or penile veins. A small swelling may be visible where the needle lies in the vein as the bolus is delivered, usually indicating that the injection is proceeding faster than the capacity of the vein. However, if a prominent swelling is seen as the injection is being made, or if resistance to depressing the plunger is felt, the needle is no longer in the vein and the injection is being made in the substance of the tail or penis.
13. The arteries are stronger and more elastic than the veins and maintain their size and shape even with extensive working. The veins, in contrast, will collapse with extensive manipulation. The best approach to isolating the vessels is to manipulate them only as much as is necessary to isolate them from surrounding nerves or connective tissue. Even so, removing excess connective tissue from top of the vessel is desirable since it will make it much easier to make a clean hole in the vessel and place the needle directly into the lumen. If this is not done, the needle can end up in the connective tissue or in the wall of the vessel. If, upon delivery of the cell suspension, the vessel/connective tissue bubbles up or the delivery is not smooth and easy, then the needle is not in the lumen of the vessel. It may be possible to minimize this constrictive effect by applying a few drops of lidocaine before the vessel is isolated from the surrounding tissue.
14. One of the most troublesome postoperative issues involves the long-term use of cyclosporin. Some of the most common side effects of this treatment include dental problems that interfere with normal feeding, higher risk of opportunistic infections, and gastrointestinal problems. These latter problems can range from diarrhea to bloating and constipation and can occur in both rats and mice. However, we have found that in the SOD1 mouse in particular, the gas and constipation can be lethal within 3–5 days of onset. During this time, animals are anorexic, their fur becomes disheveled, and their abdomen bloated. Some researchers have reported delayed gastric emptying and delayed colonic transit time in ALS patients ***(46)***. There are also reports of small bowel bacterial

overgrowth ***(47)*** that could contribute to bloating. We have found some efficacy in reversing this problem through the administration of red wine (3–5 drops twice daily on the tongue of the affected mice). The mechanism underlying this effect is not yet clear, but it may be related to antioxidant properties of the wine ***(48)***, modulation of intestinal motility ***(49)***, or its antibacterial activities ***(50)***. We also are exploring other pharmacological methods to speed up the transit of nutrients through the gastrointestinal tract.

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