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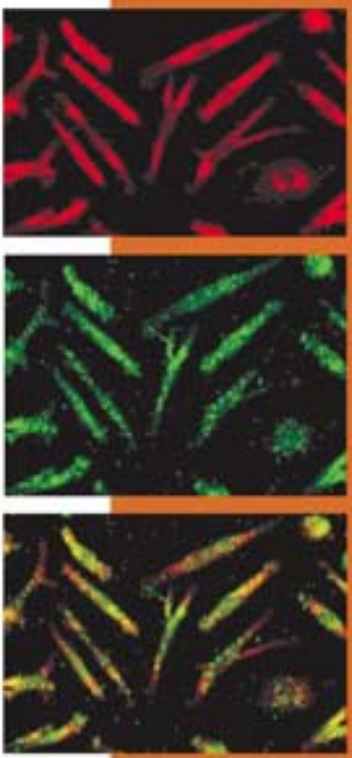

Stem Cell Repair and Regeneration

Volume 2

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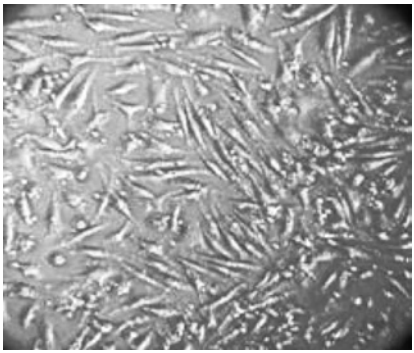

Stem Cell Repair and Regeneration

Volume 2

The Hammersmith Series 2

ICP

Imperial College Press

Published by

Imperial College Press
57 Shelton Street
Covent Garden
London WC2H 9HE

Distributed by

World Scientific Publishing Co. Pte. Ltd.

5 Toh Tuck Link, Singapore 596224

USA office: 27 Warren Street, Suite 401-402, Hackensack, NJ 07601

UK office: 57 Shelton Street, Covent Garden, London WC2H 9HE

British Library Cataloguing-in-Publication Data
A catalogue record for this book is available from the British Library.

STEM CELL REPAIR AND REGENERATION
Volume 2

ISBN-13 978-1-86094-711-7
ISBN-10 1-86094-711-5

Typeset by Stallion Press
Email: enquiries@stallionpress.com

Printed in Singapore.

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Preface

This book is the definitive reference on one of the most exciting areas of life science research — stem cells and their use in repair and regeneration of different organs and tissues. The volume is rounded off by a set of chapters on basic stem cell biology and clinical applications and clinical experiences of stem cell therapy, considering cardiovascular disease, neurological diseases, liver disease and diabetes. These offer a sound and well-balanced view of successes to date and indications for future therapeutic routes. It presents the current state of knowledge in both basic science and clinical practice, and is an essential reference for scientists, students, and clinicians.

The editors would like to thank the authors for their contributions, which have made possible the publication of this volume.

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Defining Progenitors Based on Their Expression of Aldehyde Dehydrogenase

Robert W. Storms

Umbilical Cord Blood Transplantation (UCBT)

Bone marrow transplantation (BMT) provides a profound and rich history for newly emerging cellular therapies. Over the past 30 years one of the most significant advances in BMT has been the use of allogeneic umbilical cord blood (UCB) as an alternative hematopoietic graft.[1–3] Historically, UCB was considered as a discarded by-product from childbirth but now it is routinely banked for transplantation.[4,5] UCB offers multiple practical advantages, which include the following:

(1) an ease of procurement;
(2) no risk to the graft donor;
(3) no donor attrition; and
(4) a reduced likelihood of transmitting infections.

Furthermore, UCBT apparently offers a reduced risk of severe (Grade III/IV) Graft-versus-Host disease; therefore, the criteria for HLA matching

are less stringent.[6–8] This in itself greatly increases the probability of donor–recipient matches. UCBT provides a particularly appealing alternative for recipients who do not have a matched, related or unrelated hematopoietic stem cell (HSC) donor.

In spite of all its advantages, the principal problem with UCBT is that engraftment of all the hematopoietic cell lineages is delayed. This raises central questions surrounding the graft itself. The speed of hematopoietic engraftment correlates most strongly with the total dose of mononuclear UCB cells delivered to the patient. Unfortunately, UCB is collected in a single harvest and there is no opportunity to add to the graft at a later time. More recent studies correlate patient survival with the graft's total content of $CD34^+$ progenitor cells.[8] Thus, increasing the total dose of hematopoietic progenitors in a graft might provide a means to improve clinical outcome. It is well established that HSC can provide long-term hematopoietic recovery;[9,10] however, other progenitors replenish the blood in the period immediately after a transplant.[11,12] Strategies to enhance cord blood grafts would be greatly strengthened by a more complete understanding of the total progenitor content of UCB. Toward that end, over the past several years we have developed alternative strategies to explore the fundamental physiology of the HSC and progenitor compartment. One promising strategy has been to define progenitors based on their expression of aldehyde dehydrogenase (ALDH).

Early hematopoietic development

Based on over 20 years of experimental modeling, normal hematopoietic development has been carefully dissected to define various progenitor compartments (Fig. 1).[9,10] Murine progenitors, in particular, have been rigorously defined based not only on their cell surface antigen expression, but more importantly, also by their function.[9–11] In the broadest terms, hematopoietic progenitors can be classified based on their capacity for self-renewal and their capacity for myeloid and/or lymphoid development. As a therapeutic endpoint, hematopoietic progenitors are also frequently stratified based on their transplantability. HSC are the most primitive hematopoietic cell subset. These are transplantable cells that can repopulate all the blood cell lineages for the lifespan of the recipient and are therefore frequently referred to as long-term HSC (LT-HSC). Their permanence infers that the cells must divide while maintaining their full developmental potential. In experimental animal models, this can be confirmed by performing

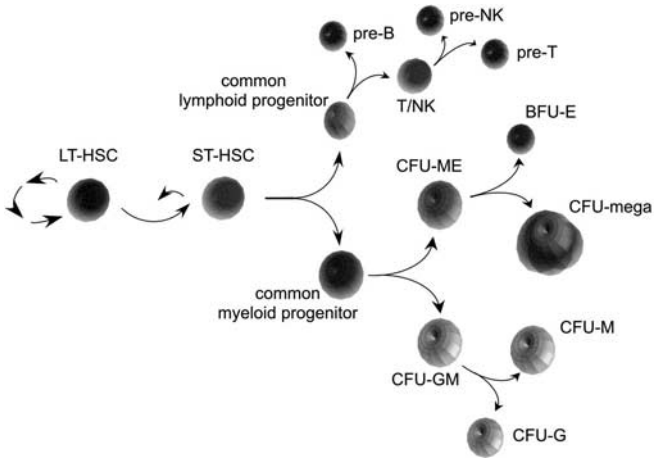

Fig. 1. Hematopoietic development. Stable and permanent hematopoiesis is established from long-term hematopoietic stem cells (LT-HSC). These give rise to short-term (ST)-HSC. Both the LT- and ST-HSC have the capacity to differentiate into all of the hematopoietic cell lineages. The fundamental distinction between the LT- and ST-HSC is that the LT-HSC self-renew while maintaining their entire hematopoietic potential. As their capacity for lineage development becomes more restricted, the ST-HSC give rise to progenitors that specify either myeloid or lymphoid cell lineages.

secondary bone marrow transplants. This inherent capacity for self-renewal distinguishes LT-HSC from all other progenitors. Other transplantable cells establish themselves only transiently.[12,13] Specifically, as LT-HSC differentiate they give rise to short-term HSC (ST-HSC). Similar to LT-HSC, the ST-HSC are transplantable progenitors that engraft to develop into all of the hematopoietic cell lineages; however, unlike LT-HSC, they persist in the transplant recipient for only a limited time. As ST-HSC differentiate they give rise to progenitors that can specify either the entire lymphoid compartment or the entire myeloid compartment.[14,15] These are referred to as the common lymphoid progenitor (CLP) or the common myeloid progenitor (CMP), respectively. The CLP and CMP give rise to progenitors that are increasingly lineage-restricted. This is particularly evident within the myeloid compartment since those progenitor cells form distinctive colonies based on their lineage potential. These clonogenic cells demonstrate that they are active progenitors and not mature cells. Thus, in total, hematopoietic differentiation can be viewed as a process where the stem cells first lose their capacity for self-renewal and then become progressively restricted in their capacity for lineage development.

Early human hematopoietic development has been most extensively defined within the $CD34^+$ cell subset, a cell fraction that is both heterogeneous and diverse.[16–25] Human $CD34^+$ cells clearly contain transplantable cells with a capacity for multilineage development,[16–20] yet also comprise cells with more limited potentials.[21–25] To discriminate cells with distinct functions, $CD34^+$ cells have been fractionated based on their expression of other antigens. As an example, $CD34^+$ $CD38^{neg}$ cells contain the highest frequency of NOD/SCID repopulating cells (SRC) (1 SRC/600 cells), a population of primitive myelo-lymphoid progenitors.[16] As $CD34^+$ cells differentiate they begin to express CD38,[21] however, the expression of CD38 is common to many hematopoietic progenitors and by itself it cannot be used to distinguish between myeloid- and lymphoid-specific progenitors. Therefore, other antigens must be used to separate these two compartments. For example, human $CD34^+$ lymphoid-specific progenitors may express either CD7 or CD10.[22–25]

Physiological Parameters that Define Hematopoietic Progenitors

While CD antigens have been considered as extremely useful tools to fractionate cells, these antigens are frequently not linked with any specific physiological context or consequence. Several years ago we initiated a series of strategies to define hematopoietic progenitors based on their physiology.[26–29] These studies have focused primarily on mechanisms for drug resistance and were based largely on the clinical experience that autologous HSC sometimes survive chemotherapies. Resistance to chemotherapies may be mediated by a variety of mechanisms. In some cases, membrane-associated pumps actively rid cells of toxic agents.[26,30,31] Efflux pumps are not specific for a single drug, but rather recognize classes of drugs that share structure. Their activity can be measured by monitoring the loss of fluorescent dyes. This strategy has gained popularity most recently through a Hoechst 33342 dye efflux assay originally described by Goodell *et al*.[30] However, a wide variety of similar assays have been described previously.[31] Moreover, although these assays rely primarily on efflux, each of these assays may also depend on secondary physiological properties, such as the cell's degree of mitochondrial activation.[31] Thus, characterizing progenitors based on the capacity to efflux dyes is a strategy that should perhaps be explored more deeply.

As part of our studies we have developed and characterized an alternative strategy for identifying hematopoietic progenitors based on their expression of ALDH,[32–34] an intracellular enzyme that specifically confers resistance to the nitrogen mustard cyclophosphamide. In general, this cell isolation strategy relies on the use of non-polar fluorescent aldehyde substrates that can traverse the cell membrane (Fig. 2).[27,35] In the presence of ALDH the aldehydes are oxidized, become polar and are retained by the cell membrane. Thus, fluorescence increases in the cells that express ALDH. The first formal tests for this strategy were performed at the Johns Hopkins University using murine BMT assays.[36] In those studies, hematopoietic progenitors were initially fractionated by counter-current elutriation, a process that enriches small, primitive progenitors (Fr25 cells). Cells that expressed high levels of ALDH were then purified by their conversion of the fluorescent substrate dansyl aminoacetaldehyde. The Fr25 ALDH^{br} cells contained progenitors that were capable of transplanting themselves into secondary hosts, even when as few as ten cells were assayed per transplant. To achieve re-transplantation, the purified cells must have been expanded in the primary transplant recipient while maintaining a capacity to repopulate hematopoiesis. Therefore, these studies provided strong evidence

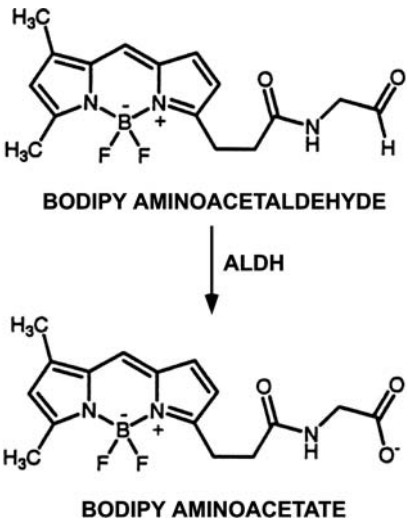

Fig. 2. Fluorescent substrates for ALDH. Fluorescent ALDH substrates (such as BODIPY aminoacetaldehyde) are non-polar and traverse the membrane. Upon exposure to ALDH these dyes acquire charge and become cell impermeant, and thus cell fluorescence increases (modified from Storms *et al.*[27]).

that LT-HSC expressed ALDH. In addition, the Fr25ALDH^{br} cell population was equally notable for the absence of specific progenitors. The Fr25 ALDH^{br} cells did not form foci in the spleen, a measurement for short-term progenitors with a capacity for both myeloid and lymphoid development. Thus, the expression of ALDH discriminated LT-HSC from transplantable short-term progenitors.

The work in murine transplantation model was provocative and exciting; however, the cell purification itself required exposure to a potentially harmful ultraviolet laser. To eliminate exposure to ultraviolet light and as a first step toward transferring this work to a clinical setting, a second-generation aldehyde substrate was synthesized using BODIPY-FL fluorochrome.[27] Using BODIPY aminoacetaldehyde (BAAA; Aldefluor®), cells that comprised slightly less than 1% of the total UCB could be readily identified based on their low orthogonal light scatter and their high ALDH expression (SSC^{lo} ALDH^{br} cells, see Fig. 3).[27,29] These cells did not express

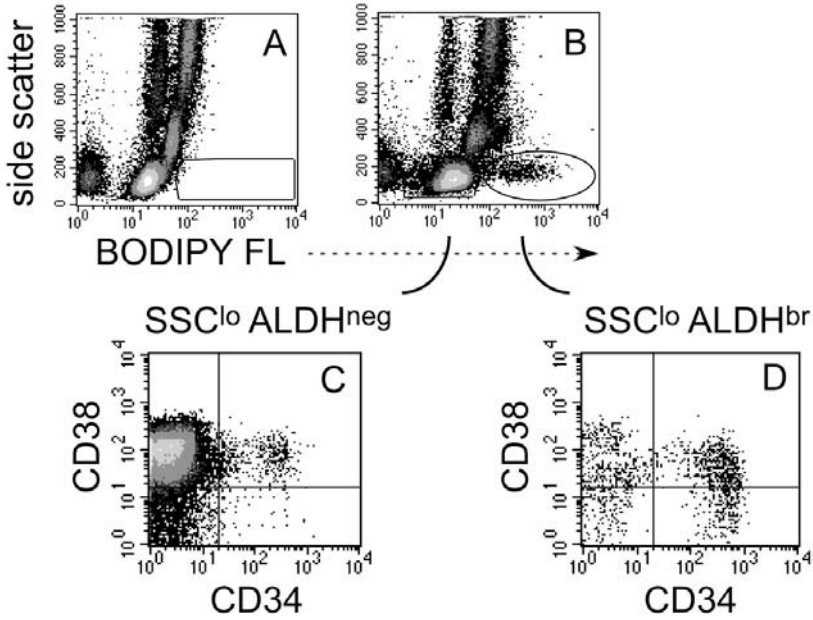

Fig. 3. Defining ALDH^{br} progenitors. For our studies, cells were stained with 1 μM BODIPY aminoacetaldehyde. SSC^{lo} ALDH^{br} and SSC^{lo} ALDH^{neg} cells were defined (B) based on the background fluorescence established using enzyme-inhibited controls (A). On average, approximately 50% of SSC^{lo} ALDH^{br} cells expressed CD34^{+} (D) and these cells were highly enriched with CD34^{+} CD38^{neg} cells (modified from Storms *et al.*[29]).

lineage-specific antigens and, more importantly, they were capable of eliminating the fluorescent dye by a mechanism that apparently involved a verapamil-sensitive efflux pump.[27] From a clinical perspective, this was significant because the dye would not linger inside progenitor cells and any potential cytotoxic effects from foreign agents would decrease. From a biological perspective, this was significant because a single lin^{neg} cell population appeared to simultaneously express two physiological mechanisms for drug resistance. This phenotype — lin^{neg} $efflux^{+}$ $ALDH^{br}$ — strongly suggested a cell with progenitor function. Indeed, when placed into developmental assays, the SSC^{lo} $ALDH^{br}$ cell fraction contained clonogenic myeloid progenitors (CFU) as well as more primitive cells with a capacity to initiate long-term cultures (LTC). Subsequent studies indicated that SSC^{lo} $ALDH^{br}$ UCB cells were also highly enriched with transplantable cells that engraft NOD/SCID mice (SRC).[29,33,37] Therefore, by both phenotype and function, the SSC^{lo} $ALDH^{br}$ cell fraction rapidly enriched progenitors.

In the studies on human UCB, this simple isolation strategy enriched a mixture of progenitors that included clonogenic cells committed to the myeloid lineage as well as transplantable cells with a potential for both myeloid and lymphoid development. This was unlike the $ALDH^{br}$ cells that had been isolated from murine bone marrow, which appeared to represent a nearly pure population of long-term repopulating stem cells. The primary distinction between the two studies resides with the use of counter-current elutriation to carefully fractionate the murine bone marrow based on cell size. Indeed, this fractionation had been used previously to enrich transplantable cells.[34] However, we note that the progenitors that were enriched from SSC^{lo} $ALDH^{br}$ UCB were entirely consistent with bone-marrow-derived progenitors that display resistance to cyclophosphamide.[38–40]

Defining $CD34^{+}$ Progenitors Based on Their Expression of ALDH

Preliminary studies from multiple different groups had all noted that the SSC^{lo} $ALDH^{br}$ cells were enriched with $CD34^{+}$ cells, and that the $ALDH^{br}$ $CD34^{+}$ cells included a high frequency of $CD34^{+}$ $CD38^{neg}$ cells (Fig. 3).[27,28,37] However, while approximately 60% of the SSC^{lo} $CD34^{+}$ lin^{neg} express ALDH, at least some $CD34^{+}$ cells did not. This provided

an opportunity to evaluate the association of ALDH within the context of well-described $CD34^+$ progenitors.

Primitive $CD34^+$ cells express ALDH

The NOD/SCID xenograft model is commonly used to monitor transplantable human progenitors.[16] In those studies, human hematopoietic engraftment to the bone marrow can be readily assayed by the presence of human $CD45^+$ cells. In addition, a progenitor's capacity for multilineage development is typically confirmed by the presence of human B lymphoid cells as well as shorter-lived myeloid cells in the marrow. Finally, in some mice, human $CD34^+$ progenitors persist in the marrow even 18–20 weeks post-transplant.[29] This suggests that human hematopoiesis is still actively evolving, even at relatively late time points.

When $CD34^+$ progenitors were fractionated as $ALDH^{br}$ and $ALDH^{neg}$, the two populations were strikingly dissimilar in their capacity to engraft NOD/SCID marrow.[29] In both short- and long-term transplantation assays, 1000 to 3000 $ALDH^{br}$ $CD34^+$ cells were sufficient to reliably engraft the marrow of NOD/SCID mice. Limiting dilution analyses estimated that approximately 1 in 4700 $ALDH^{br}$ $CD34^+$ cells were SRC (95% confidence interval [CI]: 1/3700 to 1/6900). In contrast, SRC were too rare within the $ALDH^{neg}$ $CD34^+$ cell fraction for their frequency to be reliably estimated. A similar observation was described recently when lin^{neg} $ALDH^{br}$ UCB cells were fractionated based on their expression of CD133.[32] In those studies the highest frequency of SRC was found within cells that co-expressed CD133 and ALDH. Although a subset of these cells was $CD34^{neg}$, it was noteworthy that a majority of $CD133^+$ $ALDH^{br}$ cells also expressed CD34. While it remains to be formally tested, collectively these data suggest that the cells that co-express the constellation of CD34, CD133 and ALDH might be the cells that most efficiently achieve engraftment, at least as monitored in the NOD/SCID transplantation model. At least part of the transplantability of these cells may lie in an inherent capacity for the cells to rapidly establish themselves within the marrow.[32]

Primitive progenitors can also be monitored by their growth in LTC assays. After five weeks in culture, LTC maintain clonogenic myeloid progenitors, committed lymphoid progenitors as well as cells that can re-initiate secondary LTC cultures.[29] Therefore, cells that initiate LTC are myelo-lymphoid progenitors; however, the culture assays themselves do not carry the more stringent requirement for cell homing that is present in

transplantation assays. LTC have been established with ALDH^{br} CD34^{+} and ALDH^{neg} CD34^{+} UCB cells as an independent measurement for primitive progenitors. As had been observed with SRC, the two cell populations were quite dissimilar in their relative content of cells that could initiate secondary LTC.[29] After ten weeks in culture, the ALDH^{br} CD34^{+} cells yielded approximately 100-fold greater CFU when compared with ALDH^{neg} CD34^{+} cells. However, the more critical comparison came from limiting dilution analyses which estimated that 1 in 43 ALDH^{br} CD34^{+} cells would initiate secondary LTC whereas only 1 in 1130 ALDH^{neg} CD34^{+} cells initiated secondary LTC. Therefore, based on their performance in LTC, primitive progenitors were 25-fold more frequent within the ALDH^{br} CD34^{+} cell fraction.

ALDH^{br} and ALDH^{neg} CD34^{+} cells display different responses in short-term culture

Progenitor cells with more limited developmental potentials also contribute to hematopoiesis. Unlike what had been observed in studies on primitive progenitors, the ALDH^{br} and ALDH^{neg} CD34^{+} cell fractions displayed more subtle differences in their contents of short-term progenitors.[29]

Short-term myeloid progenitors are most commonly discriminated by their capacity to form colonies in hematopoietic progenitor colony assays (HPCA). Both ALDH^{br} CD34^{+} and ALDH^{neg} CD34^{+} UCB cell fractions contained clonogenic progenitors; however, myeloid CFU were 2.5-fold more frequent within the ALDH^{br} CD34^{+} subset (Fig. 4). This was consistent with previous observations that the SSC^{lo} ALDH^{br} cell fraction was enriched with myeloid progenitors. In complementary studies, the growth and differentiation of ALDH^{br} and ALDH^{neg} CD34^{+} progenitors were monitored in short-term cultures that contained recombinant human IL-3, IL-7 and IL-15 (Fig. 4). These conditions specifically encourage the rapid and robust development of NK cells. However, even though the ALDH^{br} CD34^{+} cells expanded extensively under these conditions, they predominantly gave rise to CD13^{+} myeloid cells. In paired cultures, the ALDH^{neg} CD34^{+} cells also gave rise to myeloid progeny although to a significantly lesser degree. The total myeloid output was approximately seven-fold greater in cultures initiated with ALDH^{br} CD34^{+} cells. This was presumably measuring a response to IL-3, and was consistent with their clonogenic potential as monitored in HPCA. In total, these data indicated that short-term myeloid progenitors were more frequent among the

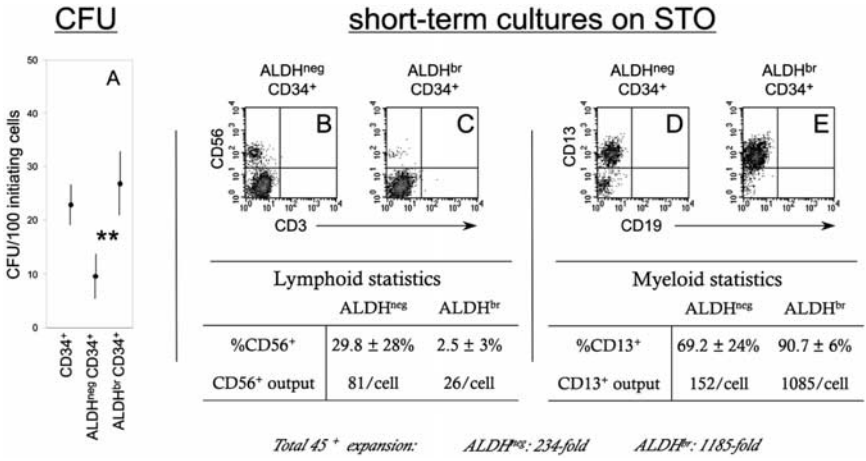

Fig. 4. Defining short-term progenitors. (A) ALDH^{br} CD34^{+} and ALDH^{neg} CD34^{+} cells were monitored for clonogeneic myeloid progenitors and the total number of myeloid colonies were normalized per 100 cells that initiated each culture ($n = 7$). **Statistical comparisons were drawn between ALDH^{br} and ALDH^{neg} cells using paired non-parametric analysis (Wilcoxon signed rank test; $^{**}P = 0.06$). (B–E) Short-term progenitors were cultured on STO fibroblast feeder stroma in the presence of IL-3, IL-7 and IL-15 under the conditions that were designed to support NK development ($n = 10$). The progeny of ALDH^{br} CD34^{+} and ALDH^{neg} CD34^{+} cells were compared their relative expression of the lymphoid antigen CD56 (B and C) or the myeloid antigen CD13 (D and E). Average percentages (± SD) and the total output of (modified from Storms *et al.*[29]).

ALDH^{br} CD34^{+} cells. These data might also suggest that both cell fractions contain cells that are irreversibly committed to myeloid differentiation.

As mentioned above, NK development is rapid and robust in response to IL-3, IL-7 and IL-15. This was most clearly evident in cultures initiated with ALDH^{neg} CD34^{+} cells, which consistently gave rise to high percentages of CD56^{+} lymphoid cells (Fig. 4). In contrast, even with vigorous growth, the ALDH^{br} CD34^{+} cells exhibited only a limited capacity toward NK development. In total, the ALDH^{neg} CD34^{+} cells produced fewer total cells yet gave rise to three-fold more NK progeny than did the ALDH^{br} CD34^{+} cells. These differences are consistent with their expression of the early lymphoid antigenic determinants CD7 and CD10 (Fig. 5). These data suggest that the expression of CD7 was distributed across the ALDH^{neg} and a ALDH^{dim} CD34^{+} cell subset; however, CD34^{+} CD10^{+} cells were nearly exclusively ALDH^{neg}.

To briefly summarize these data, the ALDH^{br} CD34^{+} phenotype was associated with primitive hematopoietic progenitors and with short-term myeloid progenitors. In contrast, the ALDH^{neg} CD34^{+} cells may be a reservoir of committed lymphoid progenitors. Based on data with regard to cell phenotypes, it is tempting to stratify the CD34^{+} cell compartment based on their expression of ALDH and CD38. On going studies are attempting to define whether distinct CD34^{+} developmental compartments can be defined based on three primary phenotypes (Fig. 5):

(1) ALDH^{br} CD34^{+} CD38^{neg} cells should be enriched with primitive cells, such as SRC;
(2) ALDH$^{dim/br}$CD34^{+}CD38$^{dim/br}$ cells should be enriched with myeloid progenitors; and
(3) ALDH^{neg} CD34^{+} CD38^{br} cells should be enriched with lymphoid progenitors.

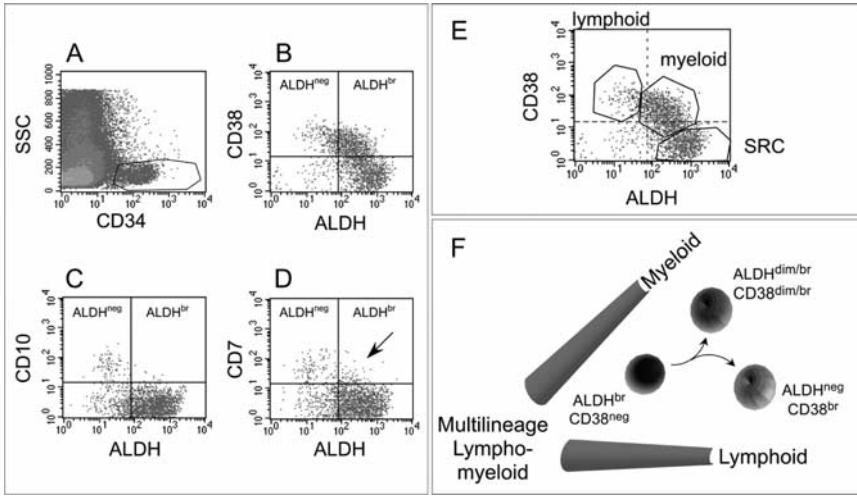

Fig. 5. Defining human hematopoietic progenitors. SSC^{lo} CD34^{+} cells were defined in UCB (A) to examine the expression of CD38, CD10 and CD7 relative to the expression of ALDH (B,C,D). The CD34^{+} cells with the brightest ALDH expression exhibited the lowest CD38 expression (B). In contrast, the expression of CD10 and CD7 were predominantly found within CD34^{+} cells that do not express ALDH (C and D). Some CD7^{+} cells were evident within an ALDH^{dim} cell fraction (indicated by an arrow). Based on the data regarding their developmental potentials, it is tempting to speculate that the relative expression of ALDH, CD34, and CD38 might be used to discriminate functional subsets of CD34^{+} cells (E) A model for lineage-specific development by CD34^{+} cells is presented (F).

Clinical Endpoints

This cell isolation strategy was intended not only to increase our understanding of basic progenitor physiology, but hopefully to also translate that knowledge into the clinic. To date, this simple assay has been used within the context of clinical monitoring. In two studies the expression of ALDH has been used to evaluate the quality of human hematopoietic transplant grafts.[28,41] Both studies suggest that the content of SSC^{lo} $ALDH^{br}$ cells within the graft is a positive clinical indicator for successful engraftment. One study suggests a correlation between these cells and the graft's total content of clonogenic progenitors. CFU content is routinely monitored in clinical transplantation studies as an indicator of the progenitor content of the graft. These data are consistent with our understanding of the total progenitor content on the $ALDH^{br}$ cell fraction.

ALDH in Other Tissues

The expression of ALDH has been most extensively characterized within the context of early hematopoiesis. In addition, although most studies have focused primarily on normal hematopoiesis, recent studies suggest that ALDH may be a part of the phenotypic signature for at least some human leukemic stem cells.[42] One question that remains is whether ALDH participates within the early ontogeny of other cell types. ALDH is highly expressed in neural progenitors identified from the fetal rat brain.[43] Furthermore, when treated with a specific inhibitor of ALDH, zebrafish embryos suffer a total loss of hindbrain development,[44] a phenotype that mimics a known mutant in a retinoic acid receptor. This strong phenotype suggests that ALDH may play a pivotal role during early neurogenesis. Other tissues have not been explored in detail. We have noted subpopulations of adipose-derived mesodermal progenitors that express ALDH;[45] however, a careful examination of the developmental potential of those cells has yet to be performed.

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Fetal Mesenchymal Stem Cells are More Primitive than Adult Mesenchymal Stem Cells

Cecilia Götherström, Pascale V. Guillot and Nicholas M. Fisk

Stem cells are present at various stages of development, from the inner cell mass through fetal and finally, adult sources. Despite a corresponding decline in differentiation potency, and despite having access to pluripotent embryonic stem cells, many laboratories still choose to study adult stem cells. This is not only on ethical grounds, because embryonic stem cells necessitate destruction of a human embryo, but also out of expediency. The main, practical reason is that adult stem cells are relatively easily obtained, at least compared to their competitor sources, and indeed can be purchased commercially. Adult multipotent stem cells, like the mesenchymal stem cell (MSC), not only differentiate into several mesodermal cell types but also produce important cytokines and growth factors, which is the basis for their extensive potential for tissue repair. Considerable debate has focussed on the contrasting merits of embryonic versus adult stem cells. Early fetal tissues also contain a population of human MSC that appear more primitive and with greater multipotentiality than their adult counterparts. Fetal MSC may thus represent an intermediate cell type between embryonic and adult stem cells, and therefore prove advantageous as a source for cellular therapy.

Biological Role of MSC

Multipotent MSC can be isolated from a number of different tissues, but readily accessible postnatal bone marrow is the most common source. Human MSC have also been isolated from several fetal tissues, such as first trimester blood, bone marrow, liver and placenta[1–3] and from second trimester blood, bone marrow, liver, lung, spleen, pancreas, kidney, brain and amniotic fluid.[4–9]

MSC make up only a minor fraction of bone marrow and other tissues. The exact proportion is difficult to establish since the different methods used to collect the bone marrow affect the harvest, but it is estimated that MSC comprise 0.001% to 0.01% of the total adult bone marrow, and are hence about ten-fold less abundant than haematopoietic stem cells (HSC).[10] Furthermore, the prevalence of MSC declines with advancing age: in the marrow of a newborn, one MSC is found among 10,000 nucleated marrow cells, compared to one MSC per 250,000 nucleated cells in adult bone marrow and one per 2×10^6 in that of an 80-year-old.[10] In contrast, the fetus is relatively rich in MSC, first trimester fetal blood containing one MSC among every 3000 nucleated cells (which then decline rapidly with advancing gestation[1] and second trimester fetal bone marrow one MSC among every 400 cells.[5]

Adult MSC represent a fundamental component of the stromal microenvironment that plays an important role in the regulation of haematopoiesis and the homing and engraftment of haematopoietic cells. Adult MSC produce vital haematopoietic cytokines and extracellular matrix (ECM) components, and provide critical cell-cell interactions, which may attract HSC to the marrow.[11–14] MSC have been shown to maintain haematopoiesis in long-term cultures[11,15] and to support expansion of umbilical cord blood (UCB)-derived HSC.[16,17] Furthermore, adult human MSC enhance the engraftment of UCB-derived CD34 positive haematopoietic cells in NOD/SCID mice[18] and fetal sheep.[19,20] The MSC support is not lineage restricted since it involves cells of lymphoid, myeloid and megakaryocytic lineages.[21–23]

The decrease of circulating MSC in fetal blood during the first trimester and the higher frequency of MSC in the second trimester bone marrow are likely to be related to their migration from one haematopoietic site to another in the developing fetus. This additional role is supported by the detection of maximal numbers of fibroblast colony-forming units in murine fetal liver, spleen and bone marrow at the time haematopoiesis begins at

each site, suggesting the existence of a migration of a stromal stem cell population on which HSC are seeded.[24] Fetal MSC have recently been demonstrated to localise within haematopoietic sites throughout ontogeny, consistent with parallel and coordinate development of both haematopoietic and mesenchymal systems.[25] The transfer of haematopoiesis from the fetal liver to the bone marrow may also be result from a decrease in the capacity of the fetal liver to support haematopoiesis. Adhesion of murine fetal liver HSC to bone marrow stromal layers was significantly greater than to fetal liver stromal cells.[12] The affinity of HSC derived from fetal liver for bone marrow stromal layers is consistent with the preferential migration of haematopoietic cells from the fetal liver to the bone marrow. Furthermore, first trimester fetal MSC have a similar pattern of expression of adhesion molecules as first trimester HSC.[26] In accordance with this, it has been determined that fetal MSC maintain haematopoiesis in long-term cultures and support expansion of umbilical cord blood-derived HSC *in vitro*[1,27] and *in vivo* animal studies show that MSC support and enhance HSC engraftment.[27,28] Human fetal bone marrow-derived stromal cells also support commitment and differentiation of human HSC to CD19 positive B-lineage cells.[29] These data indicates that the fetal MSC are implicated in the establishment of haematopoiesis.

MSC are defined as multipotent cells that are capable of differentiating into several lineages of mesenchymal origin including bone, cartilage, tendon, marrow stroma and adipose tissue.[30–32] Given their ability for self-renewal and multi-lineage differentiation, they could be used in repairing and regenerating damaged tissues of mesenchymal origin. A number of studies show the feasibility of MSC-transplantation for various purposes. MSC have been demonstrated to have the ability to repair bone defects in canine femurs,[33] sheep long bones[34] and the murine craniofacial skeleton.[35] Delivery of MSC to large tendon defects in rabbits significantly improved the biomechanics and structure of the tendon,[36] as well as promoting regeneration of cartilage in a rabbit[37] and human study.[38] Osteogenesis Imperfecta (OI) is a rare genetic disorder of type 1 collagen, the major structural protein of the ECM of bone and other connective tissues, which results in generalised osteopenia, bony deformities, and excessive fragility with fracturing and short stature. After infusion of MSC in a mouse model of OI, a small number of donor MSC engrafted and normal collagen was detected in the bone.[39] Furthermore, studies in humans suggest a possible therapeutic effect of MSC in OI. Five affected children were transplanted with allogeneic bone marrow-derived HSC and all had an increase

in total body mineral content, compared to predicted values for healthy children.[40,41] These improvements were associated with increased growth velocity and fewer bone fractures. In a later study, gene-marked allogeneic MSC expanded from the original HSC-donor were infused to the same OI patients.[42] Engraftment of MSC and an acceleration of growth velocity during the six months post transplantation could be demonstrated. Other studies show the ability of MSC to differentiate into cardiomyocytes[43,44] and their capacity to repair infarcted regions in the heart of experimental animals,[45,46] rats[47,48] and possibly humans.[49] Transplanted cells engrafted in infarcted myocardium and formed cardiomyocytes. Sanchez-Ramos[50] evaluated the ability of MSC to differentiate into neural lineages *in vitro* and after transplantation in rodents to conclude that they may eventually have a role in the treatment of stroke, traumatic injury and Parkinson's disease. Furthermore, it has been demonstrated that adult bone marrow cells can enter the human brain and generate neurons after transplantation.[51]

MSC have a poorly understood ability to migrate to sites of tissue injury participate in the healing of tissues. Studies have shown enhanced engraftment of MSC at sites of tissue injury, as in lung tissue following exposure to bleomycin that induces lung injury and inflammation[52] and in areas of induced muscle degeneration, where MSC undergo myogenic differentiation and participate in the regeneration of damaged fibres.[53]

Phenotype

Despite considerable effort in identifying specific surface markers on MSC for definition and identification of the cells *in vivo* and *in vitro*, no unique immunophenotype or set of biological markers has yet been established that define MSC. Notwithstanding this, human fetal and adult MSC expanded *in vitro* are known not to express the haematopoietic or endothelial surface markers CD11b, CD14, CD31, CD34 or CD45 but stain positive for CD29, CD44, CD73, CD90, CD105 and CD166.[1,2,27,32,54,55] MSC also express a variety of cell adhesion molecules as integrins $\alpha 1, \alpha 2, \alpha 3, \alpha 4, \alpha 5, \alpha 6, \alpha v, \beta 1, \beta 3, \beta 4$ and $\beta 5$.[55,56] Further characterisation reveals expression of ligands for surface proteins present on cells of the haematopoietic lineage, including ICAM-1, ICAM-2, VCAM-1, LFA3 and CD72,[32,55–57] molecules important in cell binding and homing interactions. Several ECM molecules, such as collagen, fibronectin, laminin and proteoglycans are also secreted by

MSC, suggesting that they may play a central role in the organisation of the ECM.[1,32,58] In addition, MSC constitutively express mRNA for cytokines including interleukin-6, -7, -8, -11, -12, -14, -15, leukaemia inhibitory factor (LIF), macrophage colony-stimulating factor (M-CSF), stem cell factor (SCF) and *fms*-like tyrosine kinase-3 (Flt-3) ligand, which are important for HSC differentiation and support.[11,31]

Stem Cell Properties

MSC are thus defined as multipotent cells, capable of differentiating into mesodermally-derived tissues such as bone, cartilage, tendon, marrow stroma and adipose tissue (Fig. 1).[30,32] Single-cell clones of fetal and adult MSC retain their multilineage potential[1,32,59] and further verification of their stem cell nature comes from the fact that single-cell colonies of MSC co-express genes characteristic for the osteoblastic, chondrocytic, adipocytic, myoblast, haematopoiesis-supporting stroma, endothelial, epithelial and neuronal lineages,[60,61] indicating that they can give rise to a broad range of cells. Furthermore, animal studies have shown that when infused intravenously, MSC widely engraft in multiple tissues and demonstrate site-specific differentiation.[62,63]

Fetal and adult MSC are both readily transduced with integrating vectors without affecting their stem cell properties of self renewal and multi-lineage differentiation.[64,65] During standard expansion cultures, MSC preserve their karyotype and show no sign of differentiation, but following long-term *in vitro* culture (four to five months), spontaneous transformations appear.[66]

Differences Between Fetal and Adult MSC

Fetal and adult MSC share several characteristics, including their morphology and the expression of common surface molecules, but in other aspects they differ. These dissimilarities are now being more deeply explored and understood.

Greater expansion potential

MSC can be extensively expanded *in vitro* to quantities sufficient for therapeutic application. First trimester fetal MSC have considerably higher

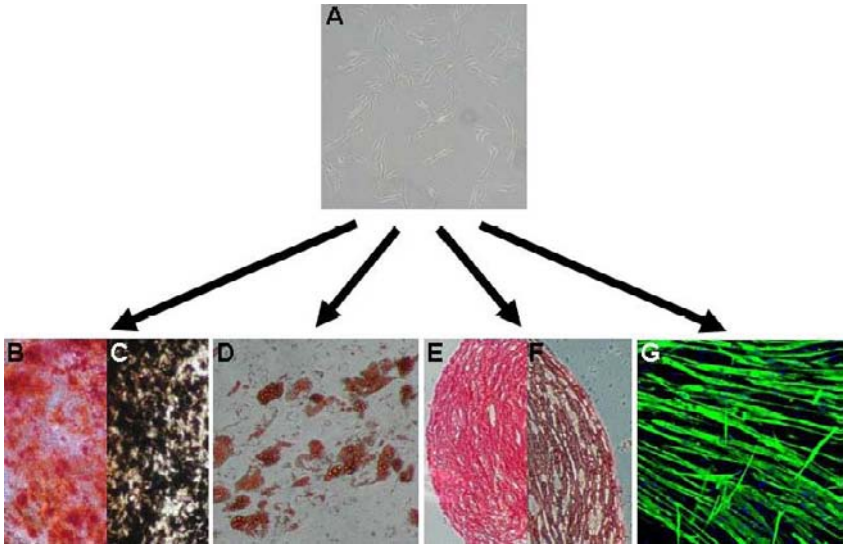

Fig. 1. Human first trimester fetal MSC can differentiate into a variety of cell lineages *in vitro*. Fetal MSC grown in culture are adherent and have a spindle-shaped morphology (A). Fetal MSC induced in culture towards the osteocytic lineage deposit extracellular calcium that stains red with Alizarin Red S (B) and phosphates that stains black with Von Kossa (C). Adipogenic differentiation is indicated by the accumulation of neutral lipid vacuoles that stains with Oil Red O (D). Chondrogenic differentiation is evident by morphological changes and positive red staining for glucosaminoglycans with Safranin O (E) and brown staining for Collagen type I (F). MSC grown in myogenic induction media containing galectin1 fuse to form multinucleated myofibres, which stains positive for Desmin. Magnification ×20. (Courtesy of Dr. J. Chan.)

expansion potential than adult MSC, which typically undergo about 15–40 cell doublings *in vitro* before senescing (Table 1).[67,68] In contrast, fetal MSC can be expanded for up to 70 population doubling without signs of senescence or apoptosis.[69,70] Fetal MSC also cycle faster than adult MSC, with a doubling time of 27.1 ± 5.3 hours compared to 124 ± 20 hours for adult MSC under the same conditions over 10 population doublings in early passages.[70] Consistent with the above, first trimester human fetal liver-derived MSC have increased expression of transcripts implicated in cell cycle promotion, chromatin regulation and DNA repair compared to adult bone marrow MSC analysed with microarrays.[57]

In view of the greater expansion capacity of fetal MSC, we recently analysed telomere length and telomerase activity of fetal and adult MSC, as putative indicators of cellular ageing and senescence. Fetal MSC-derived

Table 1. Overview of the differences between human fetal and adult MSC.

	Fetal MSC	Adult MSC
Prevalence *in vivo*	+++	+
Doubling time	+++	+
Expansion potential	+++	+
Telomere length	+++	+
Telomerase activity	+++	−
Primitive markers	+++	−
Pluripotency markers	+++	−
Differentiation potential	+++	++
Immunological inertness	+++	++
Immunosuppression	+	+++

− Negative, + faint positive, ++ positive, +++ distinctly positive.

from blood, bone marrow and liver had significantly longer telomeres than adult bone marrow MSC. In addition, all sources of fetal MSC exhibited significantly greater telomerase activity and expression of the hTERT gene compared to adult MSC.[71] These data demonstrate that fetal but not adult MSC have a high capacity for self-replication; billions of fetal MSC can be generated from a small amount of starting material. This is of relevance not only to allogeneic expansion for cell banking but also potentially to autologous applications in *ex vivo* gene therapy, where rapid expansion will be required after collection prior to reinfusion.

Primitiveness

Gene array data indicate that fetal MSC are less lineage committed that adult MSC, since they express more primitive transcripts.[57] These involve genes regulating germplasm and limb patterning, brain and early muscle development (Table 1). Furthermore, fetal MSC showed lower expression compared to adult MSC of transcripts, which characterise more mature cell types. Additionally, we have also shown that fetal but not adult MSC express the pluripotency stem cell markers Oct-4, nanog, Rex-1, SSEA-3, SSEA-4, Tra-1-60 and Tra-1-81 (Table 1)[71] suggesting that fetal MSC are more primitive than adult MSC and can maintain this stage for longer.

The differentiative capacity of fetal and adult MSC also seems to differ (Fig. 1, Table 1). D'Ippolito *et al.*[72] examined the osteogenic differentiation of postnatal MSC of different age and found that younger individuals exhibited increased osteogenic potential than older individuals. Furthermore, it

has been shown that first trimester fetal MSC have a higher osteogenic potential than adult MSC, demonstrated by default osteogenesis and basal osteogenic gene expression during *in vitro* and *in vivo* differentiation.[73] Lee *et al.*[74] showed that term UCB-derived MSC can differentiate in addition to the standard bone, cartilage and fat lineages into neuroglial- and hepatocyte-like cells, hence suggesting an ability to differentiate into cell types of all three germ layers. Other studies suggest less lineage restriction in first trimester fetal MSC, which differentiate readily into muscle, oligodendrocytes and haematopoietic cells.[70,75,76]

Immunological differences

The potential to use MSC for cellular therapeutics has driven the investigation of how MSC affect the immune response. Several adhesion molecules expressed by MSC are essential for the interaction with T-cells, like VCAM-1, ICAM-2 and LFA-3, but MSC do not express FAS ligand or the co-stimulatory molecules CD40, CD40L, CD80 or CD86.[56,57,77] First trimester fetal and adult MSC differ in their expression of alloantigens (Table 1).[56,78,79] Both cell types express the major transplantation antigen human leucocyte antigen (HLA) class I on the surface, but fetal MSC have lower expression than adult MSC. However, adult MSC contain intracellular deposits of HLA class II, whose cell surface expression can easily be induced by treating of the cells with INFγ for one day.[78] Fetal MSC on the other hand, have no HLA class II intracellularly and require two days INFγ exposure for *de novo* synthesis and intracellular expression, and seven days for full cell surface expression.[79]

Both fetal and adult MSC seem to escape the immune system *in vitro*, since they are not detected by allogeneic lymphocytes in co-culture experiments[2,77,80,81] and MSC stimulated with interferon γ (IFNγ) still escape recognition of alloreactive T-cells.[78,79] Surprisingly, this immunoprivilege applies equally to MSC after differentiation into adipocytes or osteoblasts. Neither are MSC lysed by natural killer cells mis-matched on the killer immunoglobulin-like receptors and it has been shown that adult MSC escape recognition by cytotoxic T-cells.[82] Animal studies have shown that allogeneic MSC persist when transplanted without immunosuppression into a new host.[62,63] In contrast to these data, MHC mis-matched adult murine MSC were rejected and implants had increased proportion of host-derived lymphoid cells,[83] and in another study host macrophage invasion was observed with the majority of MSC being eliminated within

two weeks.[84] These data demonstrates that MSC may not be intrinsically immunoprivileged cells *in vivo*, which warrants further investigation.

Fetal and adult MSC both inhibit the proliferation of non-specific mitogen-activated HLA mis-matched peripheral blood lymphocytes in co-culture[2,18,80,85] while adult MSC have been shown to inhibit formation of cytotoxic T-cells[82] and suppress monocyte differentiation into dendritic cells.[86] Surprisingly, fetal MSC do not inhibit proliferating allogeneic lymphocytes, unlike adult MSC which do in a dose-dependent manner (Table 1).[77,79,80,85,87] Nevertheless, stimulating fetal MSC with IFNγ for seven days for full surface expression of HLA class II, results in suppression of lymphocyte proliferation at a magnitude similar to that seen with adult MSC.[79] Thus, despite the up-regulation of HLA class II, other concurrent events induced by IFNγ appear to enhance the anti-proliferative effect that fetal MSC exert on lymphocyte proliferation. This characteristic of fetal MSC could be related to the relative immaturity of fetal compared to adult MSC. Few data are available on the immunosuppressive effect of MSC *in vivo*, but in immunocompetent baboons transplantation of allogeneic MSC prolongs third party skin graft survival[88] and murine MSC reduce inflammation in lung tissue of mice challenged with bleomycin,[52] as well ameliorate experimental autoimmune encephalomyelitis in mice.[89] These data are further supported by the infusion of haploidentical adult MSC to treat grade IV acute treatment-resistant graft versus host disease in a patient suffering from acute leukaemia, which reversed with no induction of tolerance.[90]

The fact that MSC are not inherently immunogenic and have the ability to suppress proliferating lymphocytes may make them useful for cell and immune therapy. The exact mechanism for the non-restricted immunomodulatory effect described above is unknown, but numerous mechanisms have been proposed[77,85,91–97] and are most likely to be complex and involve a soluble molecule(s).

To summarise, donor MSC which are incompatible with the recipient at the HLA antigen level, escape the immune system, a property which makes them potentially useful for various transplantation purposes.

Therapeutic applications of fetal MSC

MSC offer many advantages for developing cellular therapies; ease of isolation, expansion potential, stable phenotype, the capacity to differentiate into several mesenchymal tissues and that they escape the immune

system. Prenatal diagnosis and intrauterine transplantation (IUT) presents one opportunity for early treatment of disease. There are several advantages to the IUT approach:

(i) the developing immune system of the fetus may not yet have the capacity to mount graft rejection of foreign tissue,
(ii) the increased cell dosage achievable relative to the size and weight of the fetus,
(iii) the rapid growth of the fetus provides greater opportunity for engraftment and expansion of the donor cells,
(iv) the normal ontological migration of stem cells to different anatomical compartments,
(v) the lack of need for myeloablation,
(vi) early treatment of the disease before irreversible and organ damage and finally,
(vii) the better psychological situation for the parents resulting from the birth of a child who has already been treated.

In humans, successful outcomes after IUT have until recently only been achieved in cases of immunodeficiency,[98–104] while IUT in fetuses suffering from other disorders such as inborn errors of metabolism and haemoglobinopathies have failed,[105] presumably due to immune rejection. It has been proposed that diseases with a defective immunological response might facilitate engraftment of allogeneic donor cells which would then have a selective competitive advantages over host cells.[106,107] Thus, success in engrafting severe combined immune deficiency (SCID) fetuses is not unexpected, since SCID patients have also been transplanted postnatally with minimal or no cytoablation.[108] Successful IUT in non-immune deficient disorders would represent a major step forward in the management of patients with congenital metabolic and haematological disorders. Theoretically, this may be possible because MSC seem largely to escape the immune system and also have immunomodulatory effects. After intrauterine transplantation into pre-immune fetal sheep, human adult MSC engraft albeit at low level in the absence of selective advantage or tissue injury, and demonstrate site-specific differentiation into chondrocytes, adipocytes, myocytes, cardiomyocytes, bone marrow stroma and thymic stroma.[62] It is noteworthy that long-term engraftment occurred with MSC transplanted after the expected development of immunocompetence.

A number of studies now show advantages of fetal over adult human cells in transplantation models. Taylor *et al.*[109] showed a ten-fold competitive engraftment advantage of murine fetal liver cells relative to adult bone marrow cells in an IUT model in SCID mice and fetal liver cells had a reparative advantage over adult bone marrow cells in a neonatal muscle injury model.[110] Furthermore, fetal MSC derived from kidney gave rise to human blood and liver-like cells with broad engraftment within other organs in a xenogeneic fetal sheep model.[4] We also noticed circulating human CD45 negative cells in the progeny after IUT of human fetal MSC into mid-gestation fetal sheep,[76] which was not found after comparable transfusion of adult MSC. On the other hand, Chou *et al.*[111] found that adult MSC transplanted into fetal mice similarly resulted in circulating human CD45 positive cells. In terms of injury models, we have found that fetal MSC transplanted *in utero* into immunodeficient damaged murine muscle participate in regeneration of muscle by forming muscle fibres, although at low levels.[70] Pre-exposing the fetal MSC with galectin-1, a muscle inducing stimulus, four-fold more human muscle fibres were formed by fetal MSC *in vivo* than non-stimulated MSC. Potential *in vivo* engraftment in bone with positive effects has also been shown after infusion of adult MSC in children with osteogenesis imperfecta.[42] Recently, a human fetus with severe osteogenesis imperfecta type III was transplanted *in utero* at 32 weeks with allogeneic first trimester fetal liver-derived MSC, resulting in donor cell engraftment of 0.5%–7.4% in bone at one year with better than expected development, although clinical benefit was difficult to attribute due to confounding affect of biphosphonate treatment.[112] Nevertheless, the data showed that allogeneic HLA-mismatched fetal MSC engraft and differentiate into bone in an immunocompetent fetus.

Conclusion

We conclude that increasing differences are emerging between fetal and adult MSC. These dissimilarities suggest that human first trimester fetal MSC are more primitive and have an increased proliferative and differentiative ability compared to adult bone marrow-derived MSC, characteristics advantageous for use in cell therapy. Whether these findings translate into superior persistence of cells after transplantation and a greater regenerative capacity of fetal MSC in tissue engineering awaits future *in vivo* studies.

Acknowledgements

CG is funded by the Medical Research Council and PG by Action Medical Research. The authors acknowledge other support for their work in this area from Action Medical Research, Wellbeing of Women, the European Union, and the Institute of Obstetrics and Gynaecology Trust.

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3

The Immunoregulatory Role of Mesenchymal Stem Cells

Francesco Dazzi and Jennifer Timoshanko

Recently, several provocative links have been made between stem cells and the induction of specific tolerance. Embryonic, hematopoietic and mesenchymal stem cells (MSC) all display immunoregulatory properties, and have successfully induced tolerance in a variety of rodent and large animal studies. MSCs have been reported to evade the immune system and suppress immune responses to nominal and transplantation antigens.

Evidence for these conclusions has evolved from *in vitro* experiments utilising MLRs that report a hypoimmunogenic nature of MSC, and a remarkable ability of MSC to suppress immune responses. MSC are able to inhibit the proliferative response of CD8+ and CD4+ T cells, B cells, natural killer cells and dendritic cells in a dose dependent manner. Several mechanisms responsible for the inhibitory effect of MSCs have been suggested and include induction of apoptosis, generation of T regulatory cells, and division arrest anergy.

Regardless of which is the most important mechanism, a cross-talk between the MSC and its target cell is critical, and includes direct and indirect pathways. Thus, a greater therapeutic potential for MSCs beyond their traditional accessory function in haematopoiesis has been suggested.

This is strengthened by the facts that MSCs are relatively easy to expand *in vitro* and their use does not involve ethical issues. This chapter discusses the biology of MSCs and examines in depth their various effects on immune cells, including, their potential for therapy of autoimmune diseases.

Definition of "Multipotent Mesenchymal Stem Cell Stromal Cell"

Mesenchymal stem cells (MSCs) are multipotential non-hematopoietic progenitor cells that are capable of differentiating into various lineages of the mesenchyme. MSCs have been isolated and characterised *in vitro*[1–3] based on their property to adhere to plastic.[4,5] Sources of MSCs include trabecular bone, peripheral blood, adipose tissue, synovium, skeletal muscle, lung, deciduous teeth, amniotic fluid, cord blood and various foetal tissues.[6–10] However, most studies typically use bone marrow derived MSC.[11,12] Within the marrow, MSCs comprise a very small proportion (0.001% to 0.1%) of the total cell population of nucleated cells.[13,14] Cultures of MSC have demonstrated an important property, which is, their ability to proliferate and form colonies (colony forming unit-fibroblast assay).[1,5,15] The general approach to culturing MSCs involves the isolation of bone marrow mononuclear cells and seeding these cells on tissue culture plates at a low density in a minimal essential medium base containing foetal bovine serum. After 72 hrs non-adherent cells are removed and adherent cells are cultured to confluency.[16]

Whilst no specific marker exists for MSC identification, considerable progress has been made on the characterisation of MSCs surface phenotype. A pattern of expression and lack of expression for a number of different markers has been generated by Deans and Moseley.[17] Generally MSCs are devoid of haematopoietic (CD45) and endothelial markers (CD31),[14] whilst positive for CD44, CD49e, CD62, MHC I and Sca-1.[11,16,18] The expression of CD90, CD117, SH2, SH3, SH4 and STRO-1 on MSCs is considered selective but it varies, perhaps due to differing tissue and species of origin of MSCs studied.[19,20]

Currently the technique utilised to identify MSC is, by their ability to differentiate *in vitro* into several cell types.[11] The typical default pathway for MSCs is osteogenesis. Although a majority of clonally expanded MSCs can

be induced to form proteoglycan-rich bone nodules,[21] other clones under correct culture conditions produce lipid droplets and differentiate along the adipogeneic pathway,[22] and fewer clones can produce glycosaminoglycan proteins and differentiate into chondrocytes.[5,23] Human MSCs *in vitro* and *in vivo* can differentiate along the osteogenic, chondrogenic, myogenic, endothelial and adipogenic pathways.[11,24] *In vivo* studies using retro-viral gene marking appear to confirm MSC plasticity.[24,25]

To summarise, the definition of MSC centres on the following criteria: plastic adherence, fibroblast-like morphology, CFU-F content, phenotypic pattern of surface markers and tri-differentiation capacity under appropriate conditions.[26] However, there is no unequivocal evidence indicating that MSCs can be directly isolated *ex vivo*, because no specific marker exists. Furthermore, a major difficulty when comparing MSC data arises from the array of MSC populations being studied among laboratories with isolation methods, the way to expand MSC *in vitro*, the best culture conditions, and passage number all debated.

Immunogenicity of MSC

Both rodent and human MSC exhibit cell surface expression of MHC I, while MHC II expression remains controversial. Although some reports have described a low expression of MHC II by MSC, the general consensus is that MSC are MHC II negative[27] and this may be a component contributing to their reduced immunogenicity.[28] When used as stimulators in mixed lymphocyte reactions (MLR), MSCs independent of the species of origin — human,[29] rat, or mouse[30] have been found unable to provoke a proliferative T cell response.[31–33] Studies in animal models of allogeneic transfer have also demonstrated that MSCs are not rejected by the immune system and retain their potential to differentiate.[34] Saito *et al.*[35] have administered xenogeneic MSCs and traced their fate in recipient animals which had undergone coronary artery ligation. Results showed that LacZ labelled mice MSC engrafted bone marrow of immunocompetent recipient rats for at least 13 weeks after transplantation. Furthermore, numerous mice cells were identified in the infarcted myocardium that were absent before coronary ligation. Finally, MSC infused into patients with Hurlers' syndrome did not result in the generation of alloreactive T cells nor graft versus host disease.[36]

MSC mediated suppression of T cell responses

Several *in vitro* studies have demonstrated the ability of MSCs to modulate T-cell-mediated immune responses in an MHC independent manner.[29,33,37–42] Reports concur that despite the use of either autologous, allogeneic, murine or human origin, MSC suppress T cell proliferation stimulated by non-specific mitogens, alloantigens, or CD3/CD28 in a dose dependent manner.[32–34,39,42,43]

Following HSC transplantation MSC engraft the thymus and participate in the positive selection of thymocytes, indicating a role in central tolerance.[44,45] Their potent immunosuppressive activity also suggests that they may be involved in particular circumstances in peripheral tolerance.[31] The potential of MSCs to act as a tolerogenic cell is based on the observation of MHC I and II expression (particularly in inflamed conditions) in the absence of co-stimulatory molecules. To date, no one has recorded the expression of B7-1, B7-2, CD-40 and CD40-L on MSC.[17] Such a dichotomy usually results in T cell anergy, highlighting a possible mechanism of tolerance. However, in a recent study Klyushnenkova *et al.*[29] assessed the involvement of co-stimulatory molecules by transducing MSC with B7.1 and B7.2. This did not enhance the ability of MSC to stimulate alloreactive T cells, indicating that the lack of co-stimulation is not the major mechanism responsible for T cell unresponsiveness.

In this study, they stimulated T cells with irradiated allogenic PBMC and MSCs. The re-stimulation with PBMCs resulted in a greater T cell response, indicating that T cells had been primed by MSCs. MSCs express adhesion molecules such as ICAM-1/LFA-1[11,46] that possess co-stimulatory properties[47] and therefore may participate in T cell priming. This capacity for MSC was recorded by Majumdar *et al.*[46] who demonstrated that, MSCs have the ability to deliver signals in an Ag dependent manner to CD4+ T lymphocytes, resulting in, proliferation and cytokines secretion. A state of "split tolerance" has been suggested by Glennie *et al.* who demonstrated the inability of memory T cells once cultured with MSC to respond to their cognate Ag upon re-stimulation.[33]

Although the immunogenicity of MSC may still be a matter of discussion, their ability to mediate immunosuppression is very consistent. Krampera *et al.*[33] and Beyth *et al.*[31] have highlighted the dependence of cell-to-cell contact for hMSC and mMSC-mediated inhibition. Withdrawal of contact by either replacing MSCs with conditioned media, or when T cells

and MSCs are on opposite sides of a transwell membrane diminishes the MSC suppressive effect. Furthermore, Augello *et al.*[48] demonstrated that direct contact between MSC and T-cells via the inhibitory molecule programmed death 1 (PD-1) to its ligands PD-L1 and PD-L2, results in the inhibition of T cell proliferation.

Other immunosuppressive mechanisms such as induction of T cell apoptosis and the generation of regulatory T cells have also been addressed, with differing conclusions. Plumas *et al.*[40] found that MSC inhibit T-cell proliferation by inducing apoptosis of activated T cells, but have no effect on resting T cells. This finding would be supported by the notion that MSC express indoleamine 2,3-dioxygenase (IDO), which by converting tryptophan into kynurenine induces apoptosis in T cells. The evidence for an involvement of regulatory T cells in MSC induced immunosuppression is very limited. Maccario *et al.*[49] studied the interaction of human MSC with cells involved in alloantigen-specific immune response in mixed lymphocyte culture (MLC) and observed a remarkable increase in the percentage of CD4+CTLA4+ cells and CD4+CD25+CTLA4+ cells in the presence of MSC.

However, the elimination of CD25+ cells at the beginning of the culture does not interfere with the immunosuppressive activity of MSC, thus excluding any significant role.[50]

MSC mediated inhibition of B lymphocytes

MSCs have similar immunosuppressive effects on B cells. Glennie *et al.*[37] first and Augello *et al.*[48] later reported that murine B lymphocyte activation induced by anti-CD40/IL-4 or pokeweed mitogen (PWM), was blocked by the addition of MSC. However, in the human system, Krampera *et al.*[50] reported no suppressive effect of MSC on B cell proliferation stimulated by DSP30F CpG-containing ODN. Recently Corcione *et al.*[51] demonstrated that, at high ratios, MSC do in fact abrogate B cell proliferation upon stimulation with CpG 2006, rCD40L, anti-Ig Abs, Il-2 and IL-4. Following culture with MSC, B cell proliferation and differentiation to IgM, IgG and IgA plasma cells was abrogated and associated with arrest of the cell cycle in G0-G1 phase. As in the case of T cells, the inhibition of B cells by MSC requires soluble factors. Interestingly, the expression on B cells of several chemokine receptors (CXCR4, CXCR5 and CCR7) and consequently B cell chemotaxis is down regulated by MSC.[51]

The effect of MSC on natural killer cytotoxic T cells

Most studies have focused on the impact MSC have in the suppression of CD4+ T cell responses, but there is some evidence indicating that MSC may also suppress CD8+T cell responses and natural killer (NK) cells. Rasmusson *et al.*[41] reported that MSC are not recognised by NK or CD8+ T cells. More importantly, it has been shown that MSCs inhibit the proliferation of CD8+ T and NK cells in a dose dependent manner.[37,49,50,52] MSCs also partially inhibit the ability of NK and CD8+ T cells to lyse their targets[50] but this is not confirmed and it is probably only a minor effect.[41]

MSC affect antigen presenting cell maturation and function

The induction of an immune response relies on the interaction between antigen presenting cells (APC) and T cells. Dendritic cells (DCs) are the most potent APC in mediating cellular and humoral immune responses against self and non-self Ags.[53] Their maturation represents an important checkpoint in mounting an immune response as immature DCs promote T cell tolerance. Thus MSCs may modulate the generation and antigen-presenting capacity of DCs and in turn influence the stimulation of T cells. In fact, a few studies have observed that MSC interfere with reported DC differentiation (CD83, CD40 and CD86 expression), maturation (endocytosis and IL-2 expression), and activation (stimulation of allogeneic T cells).[31,38,54,55] Noteworthy, monocytes exposed to maturing stimuli in the presence of MSC, acquire a tolerogenic phenotype.[31] The DC obtained in these conditions show a significant reduction in IL-12 and TNF production, thus accounting for their ability to inhibit T cell responses.[56]

The role of soluble factors in MSC mediated immunosuppression

Although the role of soluble factors for MSC mediated suppression is negligible in murine models, there is substantial evidence that they are important in human MSC. IFN-γ, PGE2, HGF, IDO and IL-10 have all been implicated. The initial study from Di Nicola *et al.*[32] has shown that

neutralisation of TGF-β and HGF abrogates MSC inhibition. Similarly, MSC has been reported to secrete TGF-β upon activation through monocytes' interaction and play a major role in their immunosuppressive activity. Although MSC produced detectable levels of TGF-β in cultures, their role in MSC induced immunosuppression is unclear. In fact, others have failed to restore T cell responses by the addition in cultures of anti TGF-β antibodies.[33,42]

Inhibition of immune responses can also be mediated through the withdrawal of nutrients in the tissue microenvironment. Such a mechanism is well documented in pregnancy associated tolerance, whereby the creation of "tryptophan desert" at the site of materno-foetal interface induces maternal T cell tolerance to the foetus. The major mediator of this effect is IDO which catalyses the conversion from tryptophan to kynurenine. Further *in vitro* studies showed that monocyte-derived macrophages exposed to macrophage colony-stimulating factor (M-CSF) acquire the capacity to suppress T cell proliferation by arresting T cells in the mid-G1 phase of the cell cycle via up regulation of IDO. The same mechanism of immunosuppression has been suggested to be employed by MSC. Meisel *et al.*[57] showed that, under inflammatory conditions MSC express high level of IDO and addition of tryptophan has the ability to revert MSC immunosuppressive activity. Furthermore, tryptophan degradation products, kynurenine and kynurenic acid are pro-apoptotic molecules, and in some studies MSC induce apoptotic changes in activated T cells.

Although others have not confirmed Meisel's initial observation, the concept that an inflammatory environment could confer on MSC, the ability to become immunosuppressive, has been pursued in other studies. The inhibition of anti IFN-γ receptor completely abolishes the suppressive effect and MSCs cannot inhibit the proliferation of CD4+CRTH2+ T cells, which represent a pure population of Th2 cells incapable of producing IFN-γ. Groh *et al.*[58] recently demonstrated the ability of $CD14^+$ monocytes from blood to activate MSCs. Thus the suppressive activity of MSCs is dependent on crosstalk between MSCs and the target cell.[50]

PGE2 has been proposed as an intriguing candidate for MSC induced immunosuppression. Prostaglandins are small lipid molecules that physiologically act as regulator of kidney function, platelet aggregation and play a major role in inflammation. PGE2 exhibit diverse effects on functions of T cells, B cells and APC. Recently, two studies have demonstrated the potential role of PGE2 as a possible mediator of MSC immunosuppressive activity. Aggarwal and Pittenger[54] have shown that T cell inhibition

induced by MSC could be partially reversible when PGE2 inhibitors were added to cultures. Tse *et al.*[42] observed that despite the reduction of PGE2 by addition of indomethacin the suppressive effect of MSC on PBMC proliferation was unaltered. Perhaps an explanation of these varied results is derived from a study by Rasmusson *et al.*[41] who saw a suppressive role for PGE2 in the mitogen activation of T cells, but not after alloantigen stimulation. Thus, further studies need to assess the involvement of PGE2 as an inhibitory factor. Noteworthy are Aggarwal and Pittenger[54] results which indicate that MSC skew immune responses towards an anti-inflammatory phenotype whereby MSC determines a Th2 cytokine profile. This observation is similar to the T cell phenotype induced by PGE2. Therefore, although the complete restoration of T cell proliferation is not attainable using PGE2 inhibitors, the pattern of immune responses strongly suggest that PGE2 at least in part participate in the MSC induced immunosuppression.

Krampera *et al.*[50] assessed the effect two different competitive inhibitors of the IDO pathway, 1-Methyl Tryptophan and Norharmane would have on the inhibitory activity of MSC T cell-proliferation. Results indicated a partial yet substantial attenuation of MSC inhibition. Accompanied by the observation that PGE2 inhibitors do not completely abolish the MSC immunosuppressive activity, Krampera *et al.*[50] concluded that both pathways are involved, and are mediated via IFN-γ. As inhibition of IFN-γ, lead to a complete abrogation of MSCs suppressive activity.

The effect of MSC *in vivo*

The clinical efficacy of MSC for tissue repair has been documented. The various properties outlined in the previous sections make MSC an extremely attractive therapeutic tool also with a view to reducing unwanted immune responses such as graft-versus-host disease (GVHD), graft rejection, autoimmune diseases and other inflammatory conditions.

In the context of transplantation, Bartholomew *et al.*[43] showed in an animal model that the administration of MSC can significantly prolong allogeneic skin graft survival. The use of MSC in clinical bone marrow transplantation has yielded even more encouraging results, because MSCs have been successfully utilised in a patient with severe acute GVHD of the gut with rapid improvement of the symptoms.[59]

The efficacy of bone marrow transplantation for the treatment of autoimmune diseases is now well known both in experimental systems and in

the clinical setting. A possible critical role for MSC in the therapeutic effect of bone marrow transplant (BMT) has been suggested by Kushida *et al.*[60] who reported that the removal of adherent cells from the bone marrow preparations abolished the beneficial effect of BMT in MRL/lpr mice. More convincing evidence for the great potential of MSC in the treatment of autoimmune disease derives from the EAE model. The administration of MSC at the onset of EAE produced dramatic improvement of the disease, with a decreased mean maximum score, milder neurological impairment, reduced demyelination and cellular infiltrate into the CNS in mice given MSC. However, the efficacy was much reduced when MSC were administered during the chronic phase of the disease.[61] In contrast, with these data are those obtained in the rheumatoid arthritis model,[62] whereby MSC administration to mice with collagen induced arthritis, increased Th1 responses and worsened disease. The discrepancies might be related to a different inflammatory environment which can be deleterious to the ability of MSC to acquire their immunosuppressive properties.

The immunomodulatory activity of MSC appears to affect also the inflammatory process because, at least in some circumstances, the MSC mediated tissue repair is associated with a significant reduction of the inflammatory component. The administration of MSC immediately after bleomycin (BLM) induced lung damage reduced the extent of inflammation.[63] Recently, Togel *et al.*[64] and Lange *et al.*[65] observed that MSC improve renal function following ischemia-reperfusion-induced acute renal failure. They saw significantly improved renal function, higher proliferative and lower apoptotic indexes when MSC where administered either immediately or 24 hrs after ischemia. Similarly, others have reported an attenuation of carbon tetrachloride and dimethylnitrosamine-induced liver fibrosis following MSC administration.[66,67]

Conclusions

MSC have been extensively proven to possess a powerful immunosuppressive effect on virtually all the cellular components of the immune system. Although the mechanisms of this inhibition remain to be fully elucidated, there is consensus that MSC selectively impairs the proliferative ability rather than the effector functions of immune cells. These properties have a great impact on the development of new cellular therapy strategies for the treatment of pathological or unwanted immune responses.

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4

Understanding Cell Migration Through the Paradigm of T-Lymphocyte Homing

Vincenzo Mirenda and Federica M. Marelli-Berg

Introduction

Cell trafficking and localisation at functional sites and microenvironments are the key events in most physiological and pathological processes, including inflammation and immunity, tissue repair and regeneration. Thus, the understanding of the molecular mechanisms and cell–cell interactions underlying these processes is essential for therapeutic strategies involving cell transfer and differentiation, including stem-cell therapy. Based on the multi-step paradigm of leukocyte migration, we present an overview of the current knowledge of the molecular interactions and mechanisms regulating cell trafficking.

Multi-step Paradigm of Leukocyte Migration

It has been widely accepted that leukocyte trafficking is controlled by a sequence of at least four molecular distinct adhesion and signalling events

(Fig. 1A). These adhesion cascades are initiated by a tethering step that allows leukocytes to bind loosely to endothelial cells (Step 1a). Selectin molecule interactions with their ligands are key molecular events in this step, but integrin molecules, such as $\alpha_4\beta_7$ and $\alpha_4\beta_1$, can also mediate this step. The marginated cells are then pushed forward by the blood stream, resulting in slow rolling along the vessels (Step 1b). Subsequently, rolling cells encounter chemotactic stimuli on the endothelium (chemokines) that engage specific receptors on leukocytes (Step 2). Chemoattractant binding induces intracellular signals triggering leukocyte polarisation (i.e. a change in shape and redistribution of surface adhesion receptors that are dependent on cytoskeletal rearrangements), which renders them suitable to spread on the endothelial surface and allows their directional motility (Fig. 1B). Activation-dependent firm adhesion to the endothelium (Step 3) is mediated by the integrin family of adhesion receptors. Finally, a cohort of molecules including integrins, CD31, junctional adhesion molecules and CD99 guide leukocyte diapedesis (Step 4) through the vessel wall.

Tethering/Rolling and Selectins

Tethering and rolling of leukocytes are mediated by the interaction of selectin molecules with specific carbohydrate moieties bound to a protein backbone.[1]

The selectins are a family of three C-type lectins expressed exclusively by bone-marrow-derived cells and endothelial cells. Selectins are identified by uppercase letters, L for leukocyte (L-selectin), E for endothelial cell (E-selectin) and P for platelet and endothelial cell selectin (P-selectin). Their modular structure is represented by an N-terminal lectin domain, one EGF-like domain, several consensus repeats with homology to complement regulatory proteins, a single membrane-spanning domain and a C-terminal cytoplasmic domain.[2–4] The main physiological function of all selectins is in mediating leukocyte adhesion under flow, but both selectins and their ligands also have signalling functions.[5] L-selectin is expressed by all myeloid cells, naive T cells and some activated and memory T cells, whereas P-selectin is found in secretory granules of platelets and is expressed on the platelet surface after activation. E-selectin is expressed by acutely inflamed endothelial cells in most organs and in non-inflamed skin microvessels. P-selectin is also constitutively expressed on the endothelium of lung and

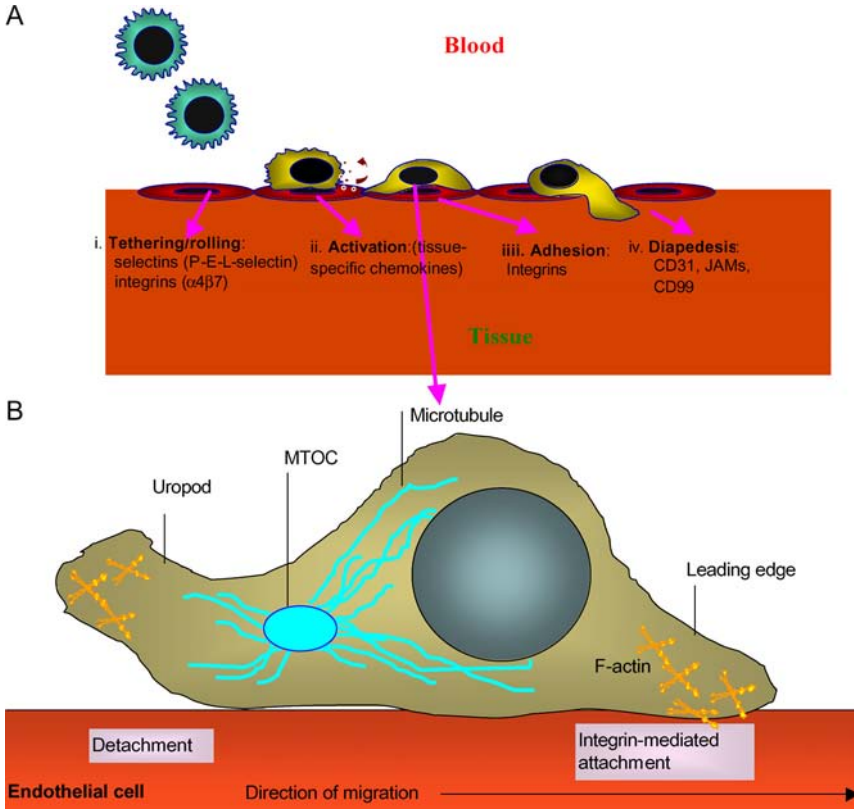

Fig. 1. The regulation of leukocyte migration. (A) Rolling and tethering of leukocytes are induced by the interaction between selectins, expressed at the lymphocyte cell surface, with their cognate sialyl-glycoproteins expressed by the vascular endothelium. Firm adhesion (arrest) follows the activation of LFA1 and α_4-integrins, expressed on rolling lymphocytes, by chemokines associated with the apical surface of the endothelium. After activation, the avidity of these integrins for their ligands, such as ICAMs, MADCAM1 and VCAM1, is increased. Attached lymphocytes migrate along and through the endothelium, usually at intercellular junction. At this level, junctional adhesion molecules, such as CD31, CD99 and JAM1, mediate diapedesis. (B) Schematic representation of a migrating lymphocyte. After adhesion, lymphocytes are polarised and develop an actin-polymer-forming lamellipodia at the leading edge, which is at the front of the cell, and a uropod at the rear end. During migration, cells attach with the leading edge to the endothelial surface and detach with the uropod. The microtubule network extends to the front of the cell from the microtubule-organising centre (MTOC).

choroid plexus microvessels,[6] and can be induced in inflamed endothelial cells in many diseases including atherosclerosis.[2–4]

Selectin ligands are carbohydrate-containing molecules and several glycosyltransferases that have a role in the biosynthesis of selectin ligands have already been identified. The main ligand for L-selectin is glyCAM-1: L-selectin also binds to endothelial ligands, most of which are characterized by their reaction to the MECA-79 monoclonal antibody and are collectively known as peripheral node addressins (PNADs). MECA-79 is a monoclonal antibody that detects sulphated *N*-acetylglucosamine (GlcNAc-6-SO_4). PNADs were expressed by high endothelial venules that are found in lymphoid tissues.

P-selectin glycoprotein ligand 1 (PSGL1) participates in P-selectin binding. PSGL1 is a 240 kDa homodimer that, if properly glycosylated, can bind all three selectins.[7] Although E-selectin can bind to PSGL1, it is not the main E-selectin ligand, and the other E-selectin ligand(s) remain poorly defined. The monoclonal antibody, HECA-452, recognises the T-cell antigen cutaneous lymphocyte antigen (CLA), so called as HECA-452 reactivity is preferentially found in T cells that infiltrate the skin.[8] HECA-452 recognises sLe^x and/or a closely related carbohydrate epitope, whereas HECA-452 reactivity correlates with the ability of T cells to bind to E-selectin, which is constitutively expressed in skin microvessels.[9]

Chemokines: Activation of Adhesion and Cell Polarisation

Chemokines (chemotactic cytokines) are a large family of molecules, including more than 40 members initially recognised for their effects on cell activation, differentiation and trafficking.[10] Chemokines play an important role in many biological processes, including angiogenesis, angiostasis, haematopoiesis, organogenesis, cell proliferation, lymphocyte polarisation, apoptosis, tumour metastasis and host defence.

The superfamily of chemokines can be divided into four subgroups based on the number and spacing of the first two conserved cysteine residues at the N-terminus: CXC family, CC family and CX3C family (where "X" represents an amino acid.[11]

Functionally, chemokines can be divided into inducible or "inflammatory" chemokines and constitutively expressed or "homeostatic"

chemokines.[12] Inflammatory chemokines are critical for attracting a diverse set of effector leukocytes to inflammatory sites including lymphocytes, neutrophils, monocytes/macrophages, dendritic cells and natural killer (NK) cells. Inflammatory chemokines typically bind to more than one chemokine receptor, which suggests that there is considerable redundancy in the inflammatory chemokine network.

Homeostatic chemokines are important for the migration of lymphocytes into the lymph node, where immune surveillance and antigen-induced activation occur, as well as for effector T cells to access and infiltrate target tissue.[13] In contrast to inflammatory chemokines, homeostatic chemokines display a more restricted receptor usage.

Chemokines mediate their biological effects by binding to G-protein-coupled seven-transmembrane receptors, which can activate an array of signalling pathways including phosphatidylinositol 3-kinase, phospholipase C, RAS- and RHO-family small GTPase and mitogen-activated protein kinase signalling cascades.

Chemokines are secreted from the stromal, epithelial and endothelial components of tissues in a site-specific manner, which are later taken up and displayed by the local vasculature. The stimulation of lymphocytes with chemokines generates cell polarity, including the development of a leading edge through actin polymerisation, thereby, inducing the formation of a uropod at the rear end of the cell by actin–myosin-based contraction (Fig. 1B). This leads to an attachment at the front end of the cell and a detachment at the back in an integrin-dependent manner.[14] Induction of T cell polarity is necessary for their directional movement and migration. In parallel, leukocyte exposure to chemokines bound to the endothelial cell surface leads to increased affinity and avidity of the molecular interactions, leading to firm adhesion on the endothelial surface, which are mediated by the integrin molecule superfamily. This process is defined as "inside-out signalling" (see below).

The Integrin Family of Adhesion Molecules

Integrins are cell surface receptors that mediate adhesion to cells, to extracellular matrix (ECM) and binding to the components of plasma. Each integrin consists of an α-subunit and a β-subunit. So far, 18 α-subunits and 8 β-subunits, which give rise to 24 $\alpha\beta$ pairs, have been identified in mammals.[15] Integrin-mediated adhesion has been implicated in a variety

of biological processes, including embryogenesis, angiogenesis, thrombosis, wound healing and cancer spreading. Integrins have essential roles in various processes of the immune system, including leukocyte attachment to endothelial cells and antigen-presenting cells (APCs), cytotoxic killing and extravasation of cells into tissues. In particular, integrins that are expressed by leukocytes, such as the β_2 integrin LFA1 (lymphocyte function-associated antigen 1; $\alpha_L\beta_2$-integrin) and the α_4-integrins VLA4 (very late antigen 4; $\alpha_4\beta_1$-integrin) and $\alpha_4\beta_7$-integrin, are important for immune-cell function, through binding ICAM1 (intracellular adhesion molecule 1) and ICAM2 (for LFA1); and VCAM1 (vascular cell-adhesion molecule 1) and MAdCAM1 (mucosal vascular addressin cell-adhesion 1) (for α_4-integrins).[16]

Unstimulated lymphocytes are non-adherent, and in response to encountering chemokines or antigens they become adherent to other cells and ECM components after a period of less than one second to several minutes. This process is possible owing to the ability of integrins to alter their avidity through an intracellular signalling process, which is known as inside-out signalling.[17] This pathway modulates the affinity of integrins for their ligands, and the extent to which integrins diffuse and cluster on the cell surface. The triggering of inside-out signalling by chemokines increases the avidity — the total adhesive strength of integrins — which results in firm attachment to their ligands. This strong adhesion is followed by leukocyte migration through the endothelium into lymphoid or inflamed tissues.[16] The avidity of integrins can be altered by affinity regulation and valency regulation. The structural basis of the affinity regulation of integrin avidity has been revealed by studies on integrin structure.[18] The α- and β-subunits form a globular, ligand-binding head-piece; this is connected to the plasma membrane by two "stalks", formed by each subunit, and each subunit also has a short cytoplasmic tail (Fig. 1). Changes in affinity are associated with distinct conformational changes in the extracellular domains of integrins. In the low-affinity conformation, the stalk region is acutely bent at the *genu* (knee), with the ligand-binding head-piece in close proximity to the membrane–proximal stalk region. A switch-blade-like extension of the stalk region then shifts the molecule to higher affinity conformations, as represented in Fig. 2. For LFA1, the ligand-binding domain is the I domain.

The process, known as valency regulation of integrin avidity, involves integrin diffusion and clustering in the plasma membrane. This mediates multivalent interactions with ligands. Integrin clustering is observed using

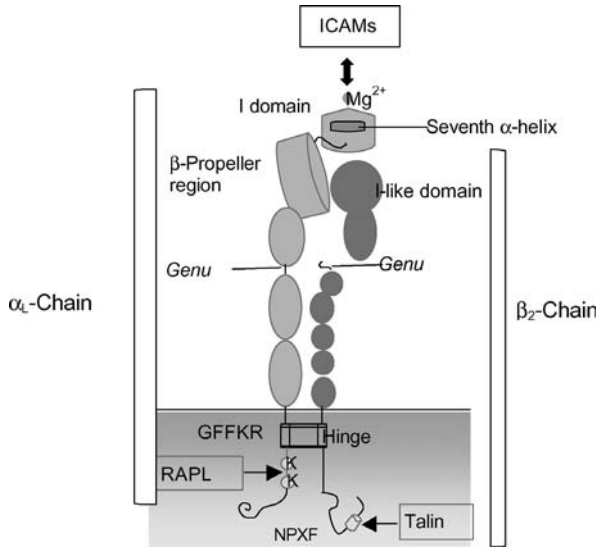

Fig. 2. Structural representation of LFA1. The ligand-binding I domain (inserted domain) of LFA1 binds ICAM1 in a manner that depends on the presence of a magnesium ion in the metal-ion-dependent adhesion site (MIDAS). The seventh α-propeller and the seventh α-helix interacts with the I-like domain of the β_2-chain, and when triggered, the linker "pulls" the seventh α-helix, which induces a change in the conformation of the MIDAS that results in the high-affinity conformation of LFA1.

a microscope; integrin first clusters into *microclusters*, and then may be redistributed to form large, unipolar *patch-like clusters*.[19]

Lymphocyte Homing and the Structural Organisation of the Immune System

In an organised immune system cell migration needs to be directed. Tissue and microenvironment-selective lymphocyte homing is the basis for this organisation. The regulated expression of adhesion molecules and their ligands and of chemokines and their receptors underlie the structural and systemic organisations of the immune system, being essential for leukocyte development, lymphocyte recirculation, immune surveillance, and effector lymphocyte differentiation and targeting. In other words, tissue-specific homing is achieved by a sequence of overlapping steps with combinatorial diversity allowing for an "address" or "code" for leukocyte

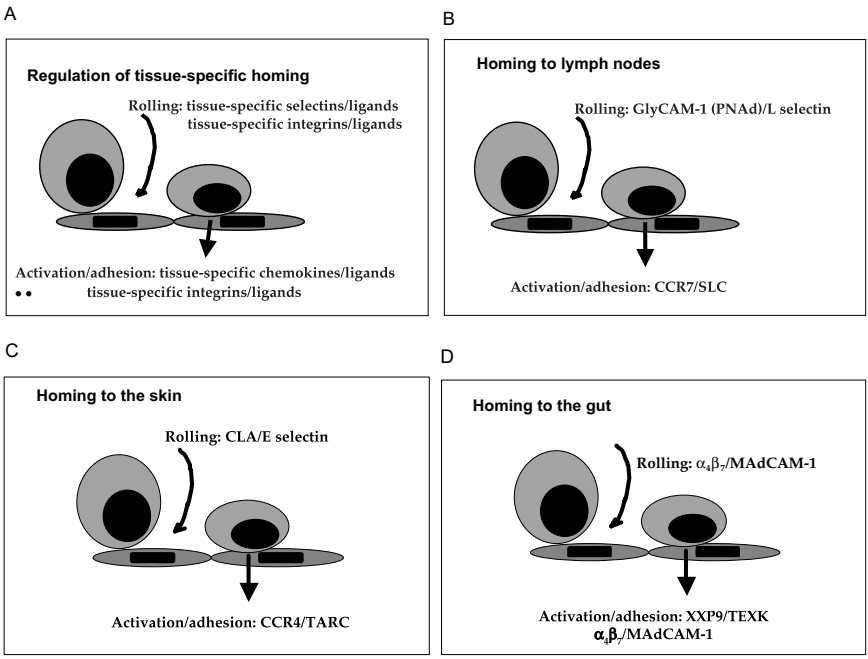

Fig. 3. Tissue-specific T cell homing. Post-capillary venules in different tissues and microenvironments express unique combinations of adhesion molecules and chemokines involved in the recruitment of lymphocytes that express the appropriate counter receptor (A). (B–D) A schematic representation of the molecular interactions mediating T cell migration to lymph nodes, skin and gut, respectively, is provided.

migration (Fig. 3). In this section, we refer to the T cell homing patterns as a reproducible scenario for the regulation of tissue localisation of other migrating cells.

T cells originate from bone marrow precursors that mature into naive T cells in the thymus. Naive T cells traffic to secondary lymphatic organs, including peripheral lymph nodes, Peyer's patches, mesenteric lymph nodes and the spleen, where they might encounter antigen and become polarised into T helper 1 (TH1), TH2 and other effector T cells, which are collected in efferent lymphatics and then enter the circulation via the thoracic duct. Activated T cells traffic to extralymphoid organs, including the non-inflamed lungs, skin, central nervous system and gastrointestinal organs. Many activated T cells ultimately migrate to the liver to undergo apoptosis. Activated T cells can home to almost all inflamed organs and tissues.

Post-capillary venules in different tissues and microenvironments express unique combinations of adhesion molecules and chemokines involved in the recruitment of lymphocytes that express the appropriate counter receptor.[20]

For example, expression of L-selectin by naive T lymphocytes is required for migration through high endothelial venules (expressing its counter-receptor GlyCAM-1) and access to the lymph node parenchyma. Interaction of E-selectin (expressed by inflamed skin endothelium) with its ligand CLA directs T lymphocytes to migrate into the skin. Similarly, LFA1–ICAM interactions mediate lymphocyte trafficking to peripheral lymph nodes and attachment to APCs, whereas the $\alpha_4\beta_7$–integrin–MADCAM1 and VLA-4–VCAM1 interactions have central roles in lymphocyte migration to mucosal lymphoid organs and inflamed tissues, respectively.[20]

Chemokines and their receptors are leading molecules in the regulation of lymphocyte homing. Once displayed on the endothelial surface chemokines can trigger integrin activation leading to leukocyte arrest on the endothelial wall.[21]

Chemokines and their receptors play a key role in programming migrations during lymphocyte development. The response of pro-thymocytes in murine foetal blood to CXCL12 and CCL25 (TECK, thymus expressed chemokine) suggests that they are involved, with their receptors CXCR4 and CCR9, in the colonisation of the thymus.[22] Once CD4 and CD8 single-positive populations of thymocytes have developed, they acquire the expression of the chemokine receptor CCR7 before exiting the thymus.[23] This receptor is important for the exit from the thymus, and also for T cell localisation to secondary lymphoid tissues. Its receptor CCL19 (ELC) is expressed by venular endothelial cells in the thymic medulla, suggesting that it favours T cell emigration.[24]

After the development of lymphocyte in the thymus, lymphocyte migration and activation in secondary lymphoid tissues are regulated by the CCR7 ligands, CCL19 and CCL21 (SLC). Lymphocytes are targeted in the T cell areas of the lymph node by specific chemokines such as CCL21 and CXCL12, whereas migration to B cell follicles depends on CXCL13.[25]

Once naive T cells are activated by antigen encounter, they undergo reprogramming of their homing properties during proliferation, which enables them to retain a “memory” of the site of activation and their target tissue. The induction of differential homing properties is probably

determined by the tissue-derived factors that reach the tissue-draining lymph nodes.[26,27] For example, T cells responding to an antigen in the intestine-associated lymphoid tissue up-regulate the intestine homing receptor $\alpha_4\beta_7$ and respond to the intestinal chemokine CCL25[27] (Fig. 3). Similarly, the population of skin-homing lymphocytes expressing CLA as homing receptor responds to the CCL17 (TARC) chemokine, which is produced by the parenchymal components in the skin.[28]

T Cell Homing as a Paradigm for Understanding (and Directing) Cell Migration

From the concepts discussed here, it emerges that the tissue microenvironment actively defines the specificity of lymphocyte trafficking and the nature of local immune specialisation. This observation has obvious therapeutic implications and needs to be borne in mind during the adoptive transfer of immune competent cells in a clinical setting. Moreover, we believe that it is possible to extrapolate the knowledge of the mechanisms regulating T cell homing for a better understanding of the general mechanisms controlling cell migration, differentiation and localisation in specific tissues.

For instance, all leukocyte lineages are derived from pluripotent haematopoietic stem cells (HSCs) that predominantly localise to the foetal liver and adult bone marrow. These rare cells can be mobilised to the peripheral blood by cytokine treatment, and constitutive physiological recirculation of HSC has been previously demonstrated in mice.[29] It has now been elucidated that the chemokine CXCL12 (SDF-1α) and its receptor CXCR4 play a central role in HSC trafficking to the bone marrow. CXCL12 is constitutively expressed by the stromal cell in the bone marrow and is found on the endothelium of bone marrow microvessels.[30]

It has been shown that antibodies to CXCR4 block engraftment of human HSC in irradiated non-obese/severe combined immunodeficiency disease (SCID) mice.[31]

In summary, applying the rules that regulate T cell homing for targeting other migrating cells to specific organs is the next challenge to achieving a more efficient clinical use of cell-based therapies for tissue repair and regeneration.

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5

Blueprint for the Response of Blood and Bone Marrow-Derived Stem Cells and Their Progeny to Hypoxia

Suzanne M. Watt, Jon Smythe, Andreas Fox, Youyi Zhang, Nita Fisher, Grigorios Tsaknakis, Sinead Forde, Sarah Hale, Dacey Ryan, Emma Frith and Enca Martin-Rendon

Background

The studies presented here derive from the National Blood Service Stem Cell Research and Service Laboratories in Oxford, which are under the umbrella of the Stem Cells and Immunotherapies (SCI) Function of the NHS Blood and Transplant Authority (NHSBT) and a member of the Nuffield Department of Clinical Laboratory Sciences at the University of Oxford. This is one of a network of nine SCI laboratories sited in Oxford, Cambridge, Birmingham, Bristol, Leeds, Sheffield, Southampton, Manchester and Edgware (Fig. 1). The latter is the site of the NHS Cord Blood Bank.[1]

The SCI routine stem cell processing and cryostorage laboratories are located near to patients undergoing relevant treatments. To different extents these undertake the following three key activities:

- Donor recruitment and selection.
- Haemopoietic stem and immune cell collection, processing, manipulation and cryopreservation from bone marrow, mobilised peripheral blood

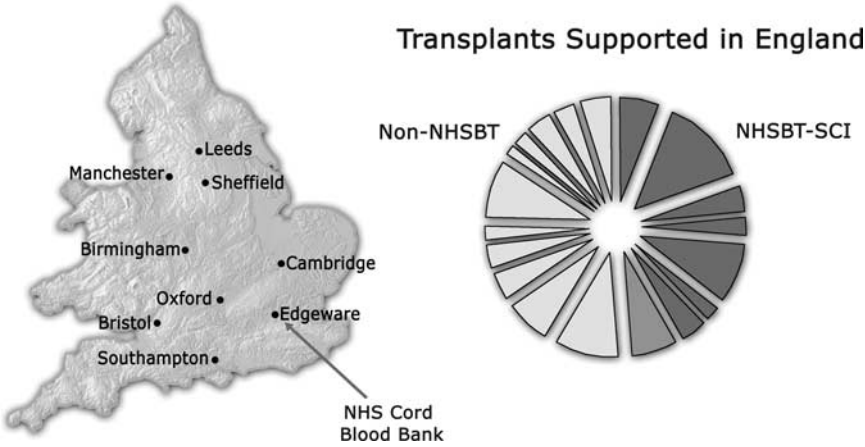

Fig. 1. The network of SCI accredited laboratories in England. The SCI laboratories exist in nine sites throughout England as shown. These are all cGMP grade and MHRA accredited, five have been inspected recently for JACIE accreditation and the NHS Cord Blood Bank is also FACT-Netcord accredited. The laboratory staff collect, process, manipulate and bank haemopoietic stem cells and related products from bone marrow, mobilised peripheral blood and cord blood to support almost 40% of transplants in England or in the order of three to five per day for NHS patients. Cellular therapy support for clinical trials is also provided.

and cord blood in MHRA, JACIE and/or FACT-Netcord accredited GMP processing facilities (Fig. 2).

- Routine service development, together with basic and translational research, with the aim of introducing new treatments into the clinic through participation in clinical trials and improved service provision to critically ill patients in the NHS.

These laboratories process, manipulate and bank haemopoietic stem and other blood and bone marrow cells required for almost 40% of haemopoietic stem cell transplants in England. This represents three to five transplants per day, mostly carried out in regions outside London. SCI has also fostered a close partnership with NHS Trust hospitals and academic university departments and institutes to establish a credible scientific base that will enable the translation of its basic research into clinical practice. The laboratories contribute to and are members of the cancer (NTRAC) and cellular therapies networks in England. An example of this latter successful partnership has been between the CRUK Institute for Cancer Studies and the SCI laboratory in Birmingham with the development of novel immunotherapy

GMP-grade MHRA/JACIE accredited laboratory in SCI Oxford

Fig. 2. cGMP grade facilities and expertise within SCI. A Health Professions Council-registered biomedical scientist processing haemopoietic stem cells for transplantation in a cGMP grade clean room within the Oxford NBS SCI laboratory.

protocols using tetramer selected T cells from haemopoietic stem cell transplant donors to combat CMV infections (Fig. 3).[2]

One key theme of the research carried out in the Stem Cell Research laboratory in Oxford, which is complementary to the transplant and translational research programme, are our studies on the hypoxia code. This research has been brought to fruition through the wealth of collaborations with key groups in and around Oxford. These include those established with Professors Adrian Harris, Enzo Cerundolo and Lars Fugger, CRUK and Molecular Immunology Units at the Weatherall Institute of Molecular Medicine; Professors Kevin Gatter, Alistair Buchan and Fengang Cui, University of Oxford; Mr Mike Tyler, Stoke-Mandeville Burns Hospital; Professor Kay Davies, MRC Centre for Gene Function; and Professor Kieran Clarke, Cardiac Research Centre. Some of these studies are described below, as part of a general review of this subject.

The Importance of the Hypoxic Microenvironment

An important determinant of successful stem cell transplantation is the ability of transplanted stem cells to proliferate, migrate and efficiently repopulate and repair damaged tissues with functional cells. As the number of adult

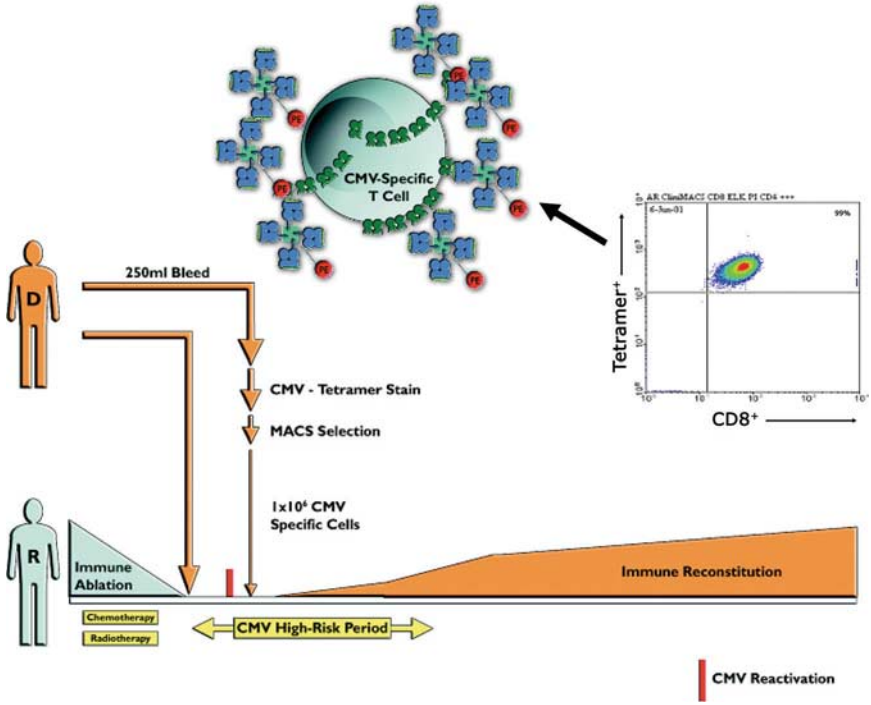

Fig. 3. Novel immunotherapy research feeding into improved patient treatments and outcomes. An example of the development of a novel and successful immunotherapy for treating patients undergoing haemopoietic stem cell transplantation and who have developed a CMV infection. This has been made possible through a partnership between Dr Mark Cobbold, Professor Paul Moss and colleagues at the CRUK Institute of Cancer Studies and Dr Dorothy McDonald's group in the NBS SCI department in Birmingham. CMV-tetramer-positive T cells (99% purity by flow cytometric analysis) purified on the CliniMACS (Fig. 2) were infused into patients who has received an allogeneic transplant and subsequently began to develop a CMV infection. The viral titre fell as the CMV-specific T cells expand, combating the viral infection. Details are provided in Ref. 2.

haemopoietic stem cells for transplantation is usually limited, particularly from umbilical cord blood (UCB), it would be extremely valuable to promote the proliferation and engraftment potential of these cells *ex vivo* prior to transplantation or at the time of transplantation within the hypoxic bone marrow. Hence, understanding the mechanisms by which these cells are able to perpetuate themselves and generate larger number of new functional end cells that engraft efficiently is crucial. A similar scenario is required for other types of stem cells and their progeny entering damaged tissues

(e.g. blood vessel precursors in burns injuries[3] or ischaemic cardiovascular disease,[4–6] mesenchymal stem cells for bone and cartilage repair, and for preventing graft versus host disease in patients undergoing allogeneic haemopoietic stem cell transplants,[1,3,7,8]), and is generally applicable in other situations requiring tissue repair and regeneration. In contrast, infiltration of the bone marrow, particularly in cases of acute leukaemia and myeloma, or of other tissues with malignant cells is very often associated with an increased blood supply to the tumour, with the improved vascularisation promoting survival and growth of the malignant clones.[9,10] Here, understanding the mechanisms by which endothelial stem/progenitor cells perpetuate themselves and contribute to this neovascularisation of tumours would allow the development of improved therapeutics to limit this vascularisation and, hence, the supply of nutrients to the malignant cells. Finally, cancer stem cells can adapt themselves to survive and proliferate under hypoxia.[10,11]

A common theme of these physiological and pathological conditions is that the microenvironments, into which the stem cells or their progeny seed, survive and proliferate, are hypoxic.[12–16] For example

- the bone marrow is naturally hypoxic,
- regions within tumours can be hypoxic,
- ischaemic cardiovascular tissue and areas of burns injuries are hypoxic,
- cardiomyocytes do not survive in hypoxia,
- blood vessels increase in hypoxia,
- the differentiation or proliferation potentials of primitive haemopoietic stem/precursor cells (SRA), chondrocytes, osteoblasts, adipocytes, and neural crest and CNS stem cells are altered in hypoxia.

Thus, for tissue maintenance and repair, it is essential that tissues maintain oxygen homeostasis, the diffusion limit for oxygen being 100–200 μm. They do so in times of hypoxic stress through the induction of glycolytic metabolism in cells, through increased transport of oxygen to tissues by inducing an increase in vascular density and in erythropoiesis from committed precursors or stem cells, and by modulating the cellular programmes that allow stem/progenitor cells to survive, home, engraft and proliferate in low oxygen atmospheres. Our overall hypothesis is therefore that key genes involved in controlling haemopoietic, mesenchymal and endothelial stem/progenitor cell survival, proliferation, migration and engraftment are regulated not only by cytokines, chemokines and other biochemical

mediators in the microenvironment, but also by hypoxia. We also hypothesise that stem/progenitor cells and their regulator microenvironmental cells (stromal, endothelial cells, etc.) respond to hypoxia in two ways: (i) by expressing a shared hypoxia gene code that reflects their common survival mechanisms, and (ii) an hypoxic code that is unique or specific and complementary for each cell type. It is particularly notable in this respect that recent studies[15] have identified a role for the Notch signalling pathway in promoting the maintenance of the neural stem cell in the undifferentiated state under reduced oxygen conditions. Our approach to studying these codes is the subject of this review.

Oxygen Sensing: The Mechanism

The hypoxia-inducible transcription factors (HIFs) include HIF-1, -2 and -3.[17] HIFs are heterodimeric proteins composed of different α subunits and a common β subunit. The β subunit is constitutively expressed, whilst the activity of the α subunits are regulated by hypoxia. HIF-1α has been studied extensively and plays a critical role in cellular responses to hypoxia, representing one of the most well-known oxygen-sensing molecules in metazoans. Only responses involving HIF-1α are described here. Under normal conditions (normoxia), the HIF-1α subunit is proline hydroxylated by the oxygen sensors, propyl hydroxylases, which permits binding to the von Hippel-Lindau (E3 ubiquitin ligase pVHL) protein resulting in rapid ubiquitination and eventual proteosomal degradation. A further degree of HIF-1α regulation occurs in normoxia by asparagine hydroxylation, this modification allows the binding of factor inhibiting HIF-1α (FIH) blocking the interaction of HIF-1α with p300/CBP transcriptional co-activators. In hypoxia, the ubiquitination of HIF-1α is inhibited and consequently HIF-1α is stabilised against degradation. The α subunit then translocates to the nucleus where it binds to the β subunit. The HIF complex then binds to hypoxia-responsive element (HRE) sequences (core sequence: *ACGTG*) present in oxygen-sensing genes, activating their transcription via recruitment of the appropriate co-activators p300 and CBP.[18,19] The mechanism of HIF-1 regulation of gene expression is illustrated in Fig. 4.

HIF-1α regulates a broad spectrum of genes, the main outcome of which is to increase cell survival under conditions where the oxygen homeostasis

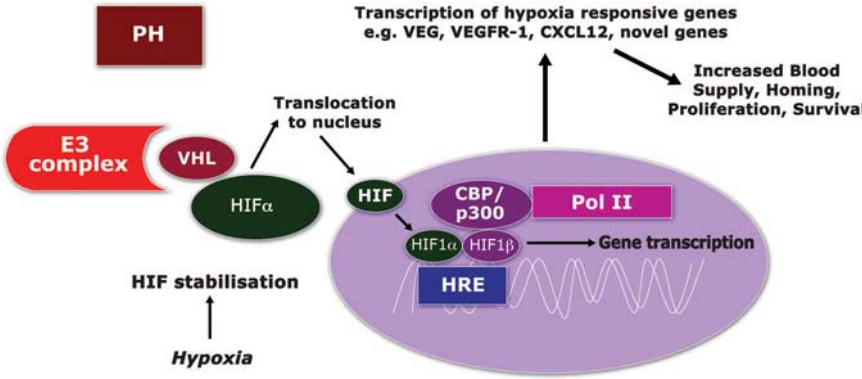

Fig. 4. Regulation of the universal oxygen-sensing HIF-1α molecule. In normoxia, the oxygen sensors or proline hydoxylases hydroxylate proline residues 402 and 564 on HIF-1α. These bind to VHL resulting in HIF-1α ubiquitination and proteosome degradation. Hydroxylation of asparagine residue 803 by FIH reduces interactions of HIF-1α with p300/CBP. Hypoxia inactivates proline hydroxylases in turn stabilising HIF-1α, which translocates to the nucleus and binds to HIF-1β, p300/CBP. This complex binds to HRE elements in the promoter regions of hypoxia-responsive genes such as VEGFA.

in the cells is disrupted. For instance, HIF-1α induces the transcription of genes involved in glucose transport and metabolism, iron metabolism, inflammation, cell proliferation, apoptosis, vasculogenesis, angiogenesis, erythropoiesis and the maintenance of stem cells in an undifferentiated state.[18–29] The majority of HIF-target genes have been identified using a variety of criteria, such as loss of HIF expression in HIF-1α-null cells, overexpression of stable HIF-1α mutants, gain of expression of VHL-null cells and identification of HREs. Many HIF-target genes are induced in a cell-specific manner, which is dependent on the inherent molecular programme of the cell itself.

What are the Effects on Vascular Development of Upregulating Known Hypoxia-Responsive Genes?

The ability to provide an adequate local microcirculation is pivotal not only to wound healing and tissue regeneration processes, but also for tumour growth and dissemination.[3] There is a growing body of evidence supporting the view that (i) circulating endothelial progenitor cells (EPCs) play

an important role in postnatal neovascularisation by the process of vasculogenesis or the *de novo* formation of blood vessels from stem/progenitor cells, (ii) these cells are mobilised into the peripheral blood and seed to sites of active neovascularisation, and (iii) this occurs in response to mediators or mobilising factors released following vascular trauma and the resultant hypoxic or low oxygen conditions that ensue.[3,29–33] Specific features of the response to hypoxia of endothelial cells are as follows: (i) the universal and master regulator of hypoxia, HIF-1α; (ii) cells switch from oxidative phosphorylation to glycolytic metabolism; (iii) the upregulation of vascular endothelial growth factor A (VEGFA), one of the most potent factors for stimulating blood vessel proliferation, formation or function, occurs; and (iv) increased production of VEGFA and CXCL12 from the region of the compromised vasculature or associated cells in the microenvironment attracts endothelial and potentially other stem/progenitor cells to the existing vasculature where they proliferate.

VEGF as a paradigm for the hypoxic response in haematological malignancies

The precise mechanisms regulating EPC recruitment and blood vessel patterning during tumour development remain incompletely understood, but known hypoxia-inducible factors, such as VEGFA and its tyrosine kinase receptors VEGFR-1 and VEGFR-2, as well as stromal-derived factor 1 (CXCL12) and its receptor CXCR4, are considered major players both in mediating the recruitment of endothelial precursor cells to sites of tumour formation and in promoting tumour growth.

The VEGF family, which has been reviewed recently[9] includes VEGFA to VEGFE and placental growth factor (PIGF). There are four main differentially spliced isoforms of VEGFA: $VEGF_{121}$, $VEGF_{165}$, $VEGF_{189}$ and $VEGF_{206}$. All isoforms, except $VEGF_{121}$, bind to heparan sulphate, a mechanism that we have previously demonstrated stabilises and concentrates such growth factors as GM-CSF in the immediate microenvironment.[34] The most abundant isoforms are $VEGF_{121}$, $VEGF_{165}$ and $VEGF_{189}$, with the former two being mitogenic for endothelial cells. Most endothelial cells express both VEGFR-1 and VEGF-2, the exception being vascular endothelial cells in the brain. VEGFR-1, like VEGFA but not VEGFR-2, is upregulated by hypoxia directly via the HIF-1α transcription factor.[35] VEGFA induces the formation of homodimers of each receptor type, as well

as VEGFR-1/VEGFR-2 heterodimers. EPCs express VEGFR-2, whereas haemopoietic progenitor cells functionally express the VEGFR-1 required for the survival and repopulation of these cells via a VEGFA–VEGFR-1 autocrine loop.[36,37]

Upregulation of angiogenesis or vasculogenesis has been demonstrated in a variety of haematological malignancies by many groups including our own.[9,10,38,39] We have studied the expression and cellular localisation of VEGFA in leukaemias and non-neoplastic myeloproliferation syndromes and have also evaluated the microvessel density (MVD) using archival samples of bone marrow.[10] Our studies showed a significant difference between the expression of VEGFA in the bone marrow sections from acute leukaemic (AML, ALL) patients when compared to reactive controls ($p < 0.0001$ for both; Figs. 5A–5C). Stronger expression of VEGFA was also observed in leukaemic blasts in acute myeloid and lymphoid leukaemias (Figs. 5A–5C), when compared with the leukaemic blasts and early myeloid precursors in chronic myeloid leukaemia and non-neoplastic myeloproliferative disorders. In the latter conditions, these precursors were located parallel to the bony trabeculae, the preferred niche for a proportion of haemopoietic stem cells in normal marrow. Endothelial cells were also positive for VEGFA expression in acute and chronic myeloid leukaemias. VEGFA expression correlated statistically with increased MVDs in both AML and ALL (Figs. 5D and 5E; tied p-value < 0.0001). It was particularly striking that, in contrast to other haematological conditions, the majority of AML and ALL sections had higher MVDs (12.9 ± 1.2 and 9.0 ± 0.9 microvessels per cm^2, respectively) than the median value (5.66). As illustrated in Fig. 6, in many of these diseases, VEGFA is secreted by the neoplastic cells and these cells also express VEGF receptors giving rise to an autocrine loop of growth control. In addition, VEGFA and its receptors act in a paracrine manner on endothelial cells or their precursors, resulting in tumour angiogenesis or vasculogenesis (Fig. 6). It is well known that the autocrine VEGF/VEGFR system is not unique to malignant cells, but as indicated above has been noted to be key to the survival and growth of stem cells. The presence of functional VEGFA receptors on leukaemic cells, possessing signalling cascades related to those seen in endothelial cells, renders anti-VEGF and anti-VEGF receptor small molecule inhibitor therapy a potential effective anti-angiogenic, anti-vasculogenic and anti-mitogenic agent in the treatment of these diseases. This subject has been extensively reviewed recently.[9]

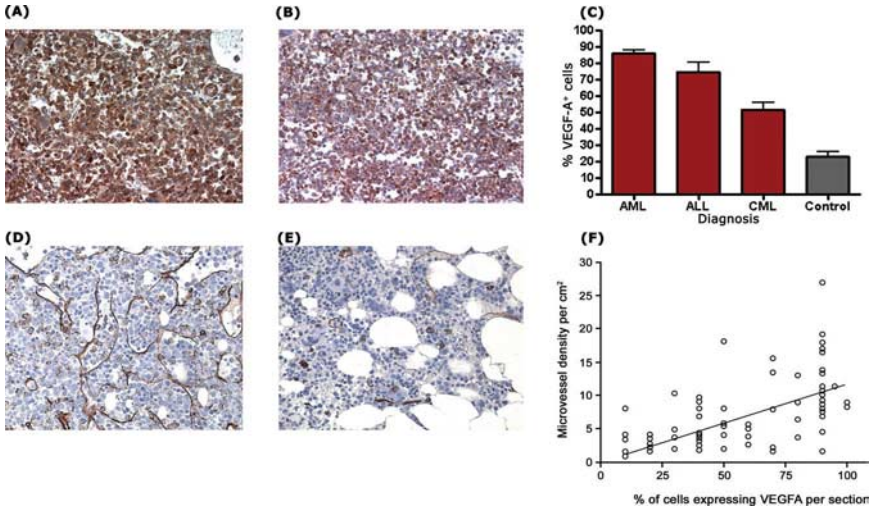

Fig. 5. VEGFA upregulation correlates with increased blood vessel density in haematological malignancies. Paraffin-embedded sections from an AML (A) and ALL (B) bone marrow showing staining of leukaemic blast cells with an anti-VEGFA antibody. (C) The mean ± SEM for the percentage of cells in the bone marrow sections of each diagnostic group of diseases (AML, ALL, CML) expressing the VEGFA compared to control bone marrows are shown. VEGFA level was significantly more highly expressed in AML, ALL and CML bone marrows than in controls ($p < 0.0001$ for all). Immunoperoxidase staining of representative sections for microvessels in AML (D) compared with control (E) bone marrow sections using anti-vWF. (F) For each bone marrow specimen tested from patients with haematological disorders,[10] MVDs per cm^2 were plotted against the per cent cells expressing VEGFA per section. Significance of the regression analysis was calculated by Spearman test. There was a significant correlation ($p < 0.0001$; Spearman coefficient $r = 0.6261$) between these two variables, indicating higher number of microvessels with higher number of cells expressing VEGFA.

VEGF as a paradigm for the hypoxic response in burns injuries

Thermal injury is one of the most physiologically challenging insults to the human body.[3] Ischaemia, as well as vascular injury, are involved in a burn, with resulting hypoxia at the burnt site. In both instances, hypoxia-inducible mobilising factors such as VEGFA and CXCL12 are released and subsequently promote the mobilisation of EPCs into the peripheral circulation.[30] Thus, burns injury represents an acute and novel pathophysiological process in which to study the effects of hypoxia-inducible genes.

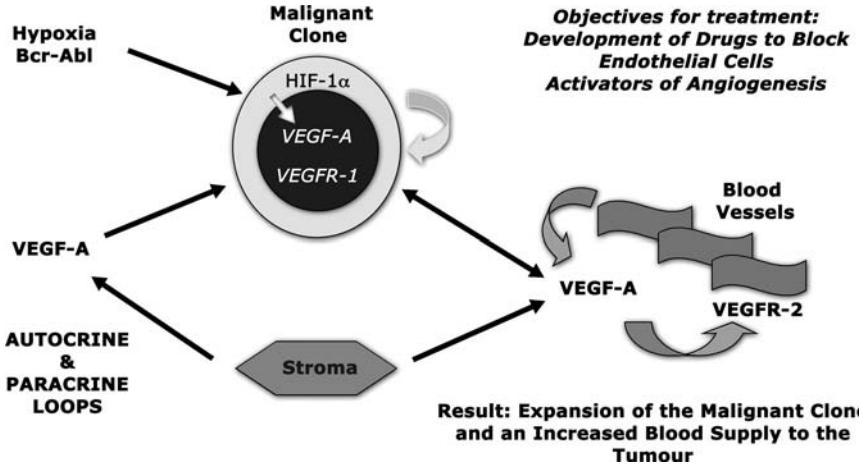

Fig. 6. Schematic representation of the effects of VEGFA upregulation in haematological malignancies. VEGFA expression can be regulated through various pathways, including exposure to hypoxia, cytokines (e.g. IL6, IGF-1, TPO, VEGF) produced from stromal cells, endothelial cells and the malignant clone, adhesion molecule signalling, and oncogenes such as bcr-abl in CML. The production of VEGFA by the leukaemic blasts or endothelial/stromal cells promotes blood vessel growth and survival and proliferation of the malignant clone via autocrine/paracrine pathways.

As there is a dearth of knowledge on this subject, our initial objectives were to analyse VEGFA and CXCL12 levels in the plasma of adult patients who had sustained an acute burn of greater than 5% total body surface area. We have compared these to the levels in the circulation of normal adult blood donors and in cord blood taken at birth, which is used for transplantation. Our results have shown an elevation in plasma VEGFA and CXCL12 in patients with burns.[30] The highest levels of these mobilising factors were seen within the first 24 hours of the burns injury, with patients having mean VEGFA levels six-fold higher than the control population. Similar results were obtained for CXCL12. There was a strong correlation between the size of the burn in terms of surface area and the levels of both mobilising factors ($r = -0.89$, $p < 0.001$; $r =$ Spearman rank correlation).

Following on from these studies, we examined whether the levels of these mobilising factors in plasma correlated with the levels of circulating endothelial cells (CECs) or EPCs in blood. To properly identify CEC and EPC, we have developed an improved multi-parameter flow cytometric assay for their enumeration, based on previous research.[3,40–49] This assay

uses antibodies recognising both CD133 and CD34 and more specific markers to separate EPCs from the more mature CECs. The markers used were as follows: CD45, a pan-leucocyte marker; CD34, a marker for haemopoietic and endothelial precursors and for mature endothelial cells; and CD133, a marker for stem/progenitor cells of the mesenchymal, haemopoietic and endothelial lineages. The more specific markers were CD144 (VE-Cadherin) and VEGFR-2, which are both markers of endothelial cell lineage that may also be present on subsets of other cell types but in combination are thought to be specific for endothelial cells. The CD45–ECD, CD34–RPE, CD144–FITC and VEGFR-2–biotin combination marked the CEC phenotype and the same antibodies with CD133 substituted for CD34 marked the EPC type.

We have used this assay to determine the range of CECs and EPCs in normal blood donors and also in UCB. Peripheral blood was collected into EDTA from 50 normal blood donors with an age range of 26 to 65 years (mean 42 years), equally split into male and female donors. Cord blood was collected from mothers after informed consent. Plasma was collected from the peripheral blood for VEGF and CXCL12 assays. After red cell lysis, the remaining cells were treated with FcR blocking reagent then incubated with labelled specific antibodies or controls as shown in Table 1. Positive events were first identified as mononuclear cells that were CD45 dim/−. Mature CEC or immature EPCs were gated from the CD45 dim/− population as $CD34^+$ or $CD133^+$ cells, respectively, and then further analysed for co-expression of the more specific endothelial cell markers, CD144 and VEGFR-2. Isotype controls in place of the last two markers were used to exclude non-specific antibody binding and numbers were adjusted accordingly. In normal peripheral blood, the mean CEC numbers per ml were 51 $\pm$ 101 ($n = 47$) and mean EPC numbers per ml were 51 $\pm$ 65 ($n = 50$).

Table 1. Antibody markers for flow cytometric analysis of endothelial precursor cells.

Test number	Cell type	Antibody specificity and fluorochrome			
		ECD	RPE	FITC	PC5
1	Control 1	CD45	CD34	IgG	IgG
2	CEC	CD45	CD34	CD144	VEGFR-2
3	Control 2	CD45	CD133	IgG	IgG
4	EPC	CD45	CD133	CD144	VEGFR-2

There was no statistical difference in CEC or EPC levels between male and female subjects. No significant intra-subject variability in EPCs was observed over a three-week time span, suggesting that levels remain constant at least in the short term. Interestingly, some "normal" subjects have consistently higher levels of EPCs than others, but the reason for this was not determined. Relationships between EPC levels and many pathophysiological processes, including burns, diabetes and peripheral vascular disease, may shed some light into the significance of this finding. Higher numbers of both CEC (18-fold) and EPC (six-fold) were found in cord blood ($n = 11$) compared with peripheral blood. In cord blood there were 719 ± 338 CEC per ml and 299 ± 245 EPC per ml. Since cord blood is known to contain a relatively high number of haemopoietic progenitor cells then a higher number of EPCs compared with adult blood is not unexpected. In fact, there were 2.4 times the number of CEC to EPC in cord blood, which was in contrast to almost equal numbers observed on average in adult peripheral blood. These data may also reflect the sloughing of CEC from the high concentration of blood vessels present in both the umbilical cord and placenta.

By following EPC levels in the peripheral blood of burns patients over time, an acute but transient mobilisation of EPCs to the peripheral circulation following burns injury was observed in our studies (manuscript in preparation). This was maximal in the first 24 hours following a burn, with levels returning to those of control subjects by day 3 post-injury. There was a 9-fold increase in the mean levels of EPCs when compared to the control population. Such a significant response in the setting of an acute ischaemic and vascular insult is a novel finding. This mobilisation related both to the surface area as well as depth of injury. In superficial burns (Fig. 7), the severity of burn in terms of total body surface area correlated strongly with the degree of mobilisation. The larger the area burnt, the greater the degree of EPC mobilisation observed. Full thickness burns also resulted in a significant increase in mobilisation of EPCs, although the relationship between size of the area burnt and EPC number was less clear. The data obtained from this study showed no significant difference in the mean levels of EPCs between patients suffering superficial or full thickness injuries. This may suggest that the chemical signals released following superficial or full thickness injuries are similar.

Interestingly, a high positive correlation was found between EPC levels and both VEGFA levels ($r = 0.96$, $p < 0.001$) and CXCL12 levels ($r = 0.90$, $p < 0.001$, $r =$ Spearman's rank correlation). However, we did not find a similar correlation between CEC numbers and either of the two

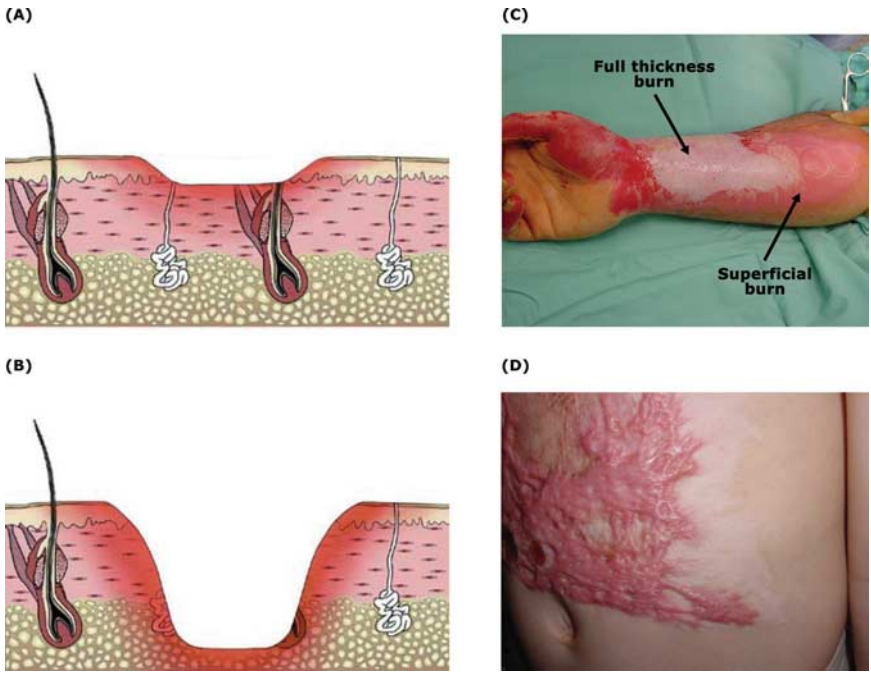

Fig. 7. Patients with burns injuries. Schematic cross-section of skin showing (A) a superficial burn affecting the epidermis and part of the upper dermis and (B) a full thickness burn affecting the entire skin thickness. Patients with (C) both a full thickness and superficial burn. (D) Scarring after burns injuries.

mobilising factors. These results suggest that the levels of EPC in whole blood may be determined by these mobilising factors. The lack of correlation between mobilising factor levels and CEC numbers suggests that these cells are perhaps not mobilised into the circulation in response to the factors, but sloughed from existing vessels as a normal part of vessel function.

Correlation between EPC number and VEGFA/CXCL12 suggests that an acute injury associated with a hypoxic response such as a burn, results in the release of cytokines/chemokines into the circulation which in turn facilitates the release of EPCs into the circulation and their homing to the hypoxic region. This novel finding may be of clinical usefulness in several ways. For example at present, it is difficult to infer the state of the vascular endothelium in a non-invasive manner. Levels of EPCs may act as surrogate markers for vascular injury and can be used in a variety of settings to detect the effectiveness of a therapeutic intervention or disease progression.

If cytokines/chemokines such as VEGFA and CXCL12 correlate strongly with EPC levels then these can in turn be used to monitor the state of the endothelium in pathophysiological processes.

Identifying EPCs and mobilising factors that play an important role in the wound healing process may also provide a future therapeutic target for patients with major burns (>30% surface area). For example, such cells could be extracted from the blood or bone marrow of a patient who has sustained a burn, and expanded *in vitro*. They could then be returned to the patient, at a time when their own bone marrow or blood cannot meet this demand, to facilitate wound healing. The factors responsible for mobilisation of EPCs and even the cells themselves could also be impregnated in dressings and applied to the injured tissue, thus helping to recruit the relevant cell populations to the area of injury where they are most needed.

The Hypoxia Code: Cellular and Molecular Responses to Hypoxia

Although molecules such as VEGFA and CXCL12 and their receptors play significant roles in blood vessel generation in hypoxia-mediated tissue damage, the complete molecular response to hypoxic conditions for stem cells and their progeny and for the stromal/endothelial cells with which they interact in their immediate microenvironment is not understood. Global and more specific gene expression profiling approaches have been used to study differential gene expression under low oxygen tensions, especially in macrophages, endothelial cells and tumour cells. Interestingly, the major conclusion that can be drawn from these studies is that the response to hypoxia is cell type-specific.

The development of research tools for global gene expression analyses has allowed the characterisation of the transcriptomes of different stem/progenitor cell populations. In pursuit of a "stemness" code, the first studies comparing transcriptional profiles of embryonic, neural and haematopoietic stem cells were published in 2002.[50,51] Surprisingly, the two transcription profiles from different stem cells uncovered a minimal overlap between them. These studies have been followed by a stream of reports that yield the same conclusions.[52,53] The minimal overlap found between studies cannot be accounted for by differences in experimental approaches, but rather the absence of a single common genetic programme

controlling the unique character of a stem cell. Instead, a commonly observed feature is that most genes enriched in the stem cell populations tested are not exclusive to these cells. Mikkers and Frisen[54] describe "stemness" as an open mind state where stem/progenitor cells express many genes that are also abundant in cells committed to a certain lineage. Consistent with this observation are the bioinformatics results we have obtained by comparing genes enriched in stem/progenitor cell subsets that occur in the bone marrow and can circulate in the peripheral blood, such as human bone marrow-derived mesenchymal stem cells (MSC)[55] and in $CD34^+$ haemopoietic and endothelial stem/progenitor cells.[56] Figure 8 shows that 12,746 transcripts are enriched in human MSC, whilst approximately 14,246 are in human $CD34^+$ cells.[56,57] Interestingly, more than 72% (10,297) of genes enriched in the individual stem/progenitor cell populations are shared between them.

In order to specifically identify genes (in the human stem/progenitor cells that circulate in the blood and seed, the bone marrow and other tissues, and in their associated stromal cells (BMSC) and endothelial

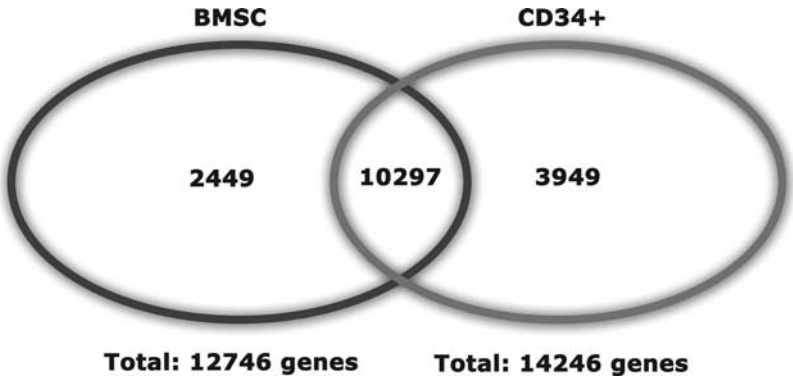

Fig. 8. Common genes enriched in BMSC and $CD34^+$ cells. The gene expression profile of human BMSC[55] and $CD34^+$ cells isolated from bone marrow[59] have been reported previously. Serial analysis of gene expression (SAGE) has been carried out for both stem/progenitor cell populations yielding a total of 34,649 SAGE tags from BMSC and 42,399 unique tags from $CD34^+$ cells. A non-redundant set of gene-oriented clusters have been compiled using UniGene Cluster software. Only 12,746 tags of the 34,649 SAGE tags from BMSC have UniGene Cluster numbers. From the $CD34^+$ SAGE dataset, only 20,725 tags were available in UniGene Cluster number. Removal of repetitive genes yielded a list of 14,246 tags with UniGene Cluster number from $CD34^+$ cells. When combined, these two sets of data show a total of 10,249 of genes (>72%) being enriched in both BMSC and $CD34^+$ cells.

niche cells) which are regulated following exposure to hypoxic microenvironments, we have subjected human BMSC, endothelial cells and UCB CD133$^+$ stem/progenitor cells to two experimental conditions, normoxia (21% oxygen) and hypoxia (1.5% oxygen) and have carried out cDNA

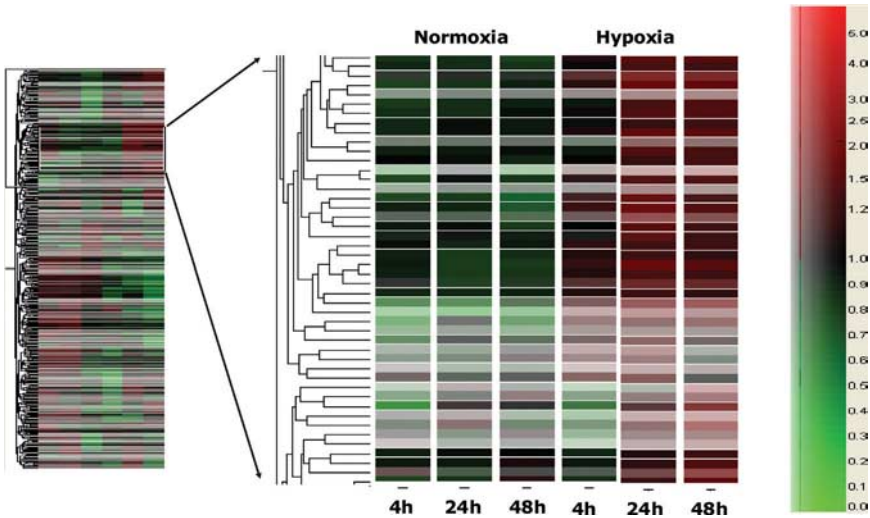

Fig. 9. Gene array analyses. Gene microarrays showing changes in gene expression after BMSCs are exposed to hypoxia (1.5% O_2) for 4, 24 and 48 hours.

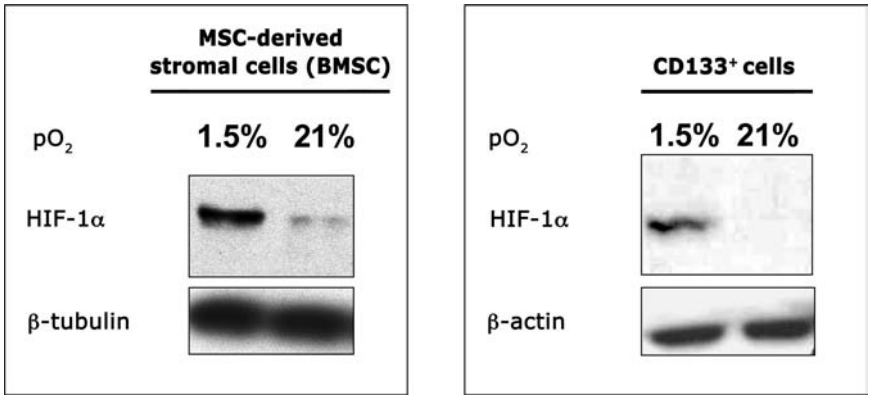

Fig. 10. HIF-1α stabilisation in BMSC and CD133$^+$ UCB cells. Western blots of HIF-1α in BMSCs and CD133$^+$ cells under normoxic (21% O_2) or after 24-hour exposure to hypoxia (1.5% O_2). Tubulin served as the positive loading control.

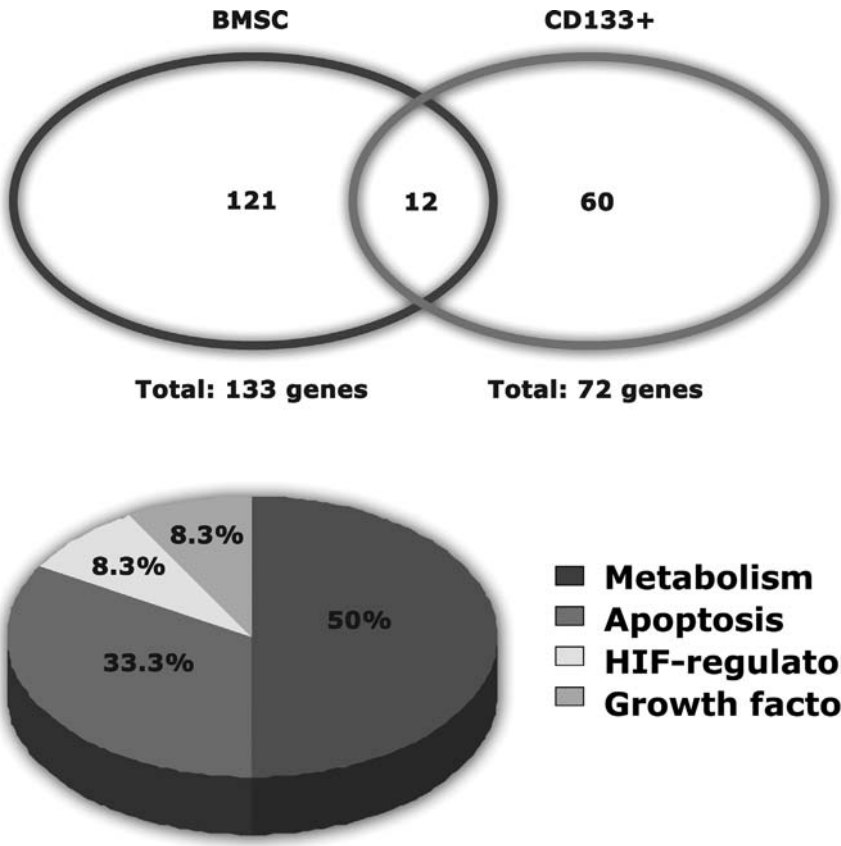

Fig. 11. The HIF-1α response of BMSC and CD133$^+$ cord blood cells after short exposure to hypoxia. BMSC and CD133$^+$ cells isolated from cord blood were subjected to two different conditions. Cells were exposed to normoxia (21% O_2) or hypoxia (1.5% O_2) for 24 hours. Total mRNA was extracted from these cells, labelled and hybridised to high density oligoarrays that contained 22,283 probes. Statistical analysis was carried out using ANOVA to identify those probes for which a significant difference ($p < 0.05$) in mean hybridisation intensity was found between the two conditions at a given incubation time. A threshold change of 1.5-fold was selected on the basis that this cut-off captured many of the genes known to be regulated by hypoxia. A total of 133 genes and 72 genes were regulated by hypoxia in BMSC and CD133$^+$ cells, respectively. We compared these gene lists and found that only 12 of these genes were shared between the two cell populations. Genes in these lists were classified according to Gene Ontology (GO) with roles in biological processes and/or molecular function. Pie chart shows the function of these 12 genes.

microarray hybridisation (Fig. 9) and proteomics analyses on these cell types to compare their molecular and cellular response to hypoxia. All cell subsets were found to respond to hypoxia by stabilising HIF-1α. An example of this after 24-hour exposure to hypoxia is shown in Fig. 10 for BMSC and CD133$^+$ cells.

In our array studies (Fig. 11), we have found that a total of 133 mRNAs in BMSC and 72 mRNAs in UCB CD133$^+$ cells were regulated after 24-hour exposure to hypoxia. Interestingly, over 20% of these genes have unknown functions (Table 2). Only 12 common genes were found to be regulated by hypoxia in both cell types. Six of these 12 genes encode proteins that are involved in metabolism, representing enzymes that catalyse the glycolytic

Table 2. Classification of genes regulated by hypoxia at 24 hours.

Biological process (GO)	Percentage of genes
(A) BMSC	
Unknown	21.6
Cell proliferation/survival	16.4
Signal transduction	11.9
Cell adhesion/motility	11.9
Metabolism	11.2
Protein metabolism	9.7
Regulation of transcription	9.5
Transport	6
Nucleic acid metabolism	3.7
Structural proteins	1.5
Immune response	1.5
Blood coagulation	0.74
Sex determination	0.74
(B) CD133$^+$ cells	
Unknown	22.9
Cell proliferation/survival	14.3
Metabolism	14.3
Transport	12.9
Protein metabolism	10
Signal transduction	10
Regulation of transcription	5.7
Cell adhesion/motility	5.7
Nucleic acid metabolism	2.9
Immune response	1.4

Classification of genes regulated by hypoxia at 24 hours according to biological processes using Gene Ontology (GO) software.

pathways. Four of the common genes encode pro-apoptotic factors. One is an HIF-regulator and the last of these 12 genes encodes VEGF. It would therefore be extremely tempting to speculate that these 12 genes may constitute a "hypoxia code" shared between these cell types. Of further interest was the finding that the transcriptional code of the response to hypoxia of BMSC and UCB $CD133^+$ cells seems to be characterised by (i) the need to generate energy through glycolysis; (ii) the balance between cell survival and cell death, which is reflected by the expression of growth factors and pro-apoptotic factors; and (iii) the negative feedback regulation of HIF1-α.

Conclusions

Hypoxia plays a major role in both physiological and pathological conditions. The healthy bone marrow, which supports haemopoiesis throughout adult life, is one of the most hypoxic organs in the body. Furthermore, the trafficking of circulating stem/progenitor cells to areas of tissue injury, ischaemia or tumour formation is regulated by hypoxic gradients via the induction of HIF1-α and the production of mobilising factors such as VEGF and CXCL12, initially in microenvironmental niche or stromal/endothelial cells. Once within the hypoxic area, these stem/progenitor cells may themselves demonstrate a hypoxic response. As we have found, both the stem/progenitor cells and the niche cells share some common responses to hypoxia, particularly when these regulate their survival. They also show specific responses to hypoxia which allows them to interact with each other and regulate their own and each other's quiescence, proliferation, homing or differentiation via specific receptor–ligand interactions.[58–60] The implications of this work are that these pathways may be part of a global hypoxia code shared with other stem/progenitor cell types and their regulating microenvironmental niche cells, whether during normal stem cell function, or during pathophysiological processes such as inflammation, ischaemic cardiovascular disease, burns injuries and malignancies.

Acknowledgements

The work of these authors benefits from funding received from the NHS R&D Directorate, NTRAC, the Leukaemia Research Fund, the Medical

Research Council, the British Heart Foundation and the Wellcome Trust. A.F. is a Duke of Kent Fellow and receives support from the Royal College of Surgeons (Ed.), the Robert McAlpine Foundation and the Stoke-Mandeville Burns and Reconstructive Surgery Research Trust. Owing to space constraints, only selected reviews and articles have been cited here, but the authors wish to acknowledge all researchers who have contributed extensively to this field. The authors wish in particular to thank Professor Marcela Contreras for her support and Mrs Brenda Cooley for assistance in typing the manuscript.

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6

The Potential of Stem Cells in Tissue Engineering

Nicholas D. Evans and Julia M. Polak

Introduction

A hundred years ago, the controversial French surgeon Alexis Carrel devised a technique for seamlessly stitching arteries together after learning his needlework skills from a Lyonaisse embroiderer. In doing so, he pioneered organ transplantation, later winning the Nobel Prize for Medicine for his efforts. The concept of the body as a machine and the surgeon as its engineer had begun to be realised. Today, transplant surgery is a routine, if oversubscribed, service in 21st century medicine. We regard the scientists and surgeons who made it possible as heroes. In another hundred years, how will people regard the pioneers of tissue engineering as a discipline — and will they even remember them?

Of course, the shortfall of organs and tissues for transplant surgery was the catalyst for the birth of tissue engineering as a discipline, as is exemplified in its oft-quoted definition — "an interdisciplinary field that

applies the principles of engineering and life sciences toward the development of biological substitutes for the repair or regeneration of tissue or organ functions".[1] Tissue engineers are faced with the daunting task of constructing organs or parts of organs from scratch, using cells, scaffolds and matrices as building materials. The challenges are great; but depending on the application, tissue engineering for some tissues is a lot simpler than for others. Take the tissue engineering of skin — a rudimentary form of this tissue has been engineered successfully in the laboratory. One such commercial product, Apligraf (Organogenesis Inc.), which consists of a bilayer of foreskin-derived skin cells combined with sheets of bovine collagen, has received FDA approval in the United States for dressing ulcers.[2] However, for other tissues and organs this kind of tissue approach faces bigger challenges.

Tissues for allografts — the transplantation of a tissue from one individual of a species to another of the same species — may be in short supply or simply unavailable. The tissue may provoke a morbid immune response,[3] which is not the case for Apligraf™ (fibroblasts and keratinocytes, which lack MHC II antigens, appear to be immunoprivileged[4]). Autografting — the transplantation of a tissue from one site of an individual to another site — has been used successfully for applications such as bone grafting and currently 408,000 of these procedures are performed annually in Europe.[5] But these procedures cause damage and pain at the site of removal, the amount of tissue that can be harvested safely is very limited and the quality of the autograft tends to decline with the age of the patient.[5] For other tissue types, the cells required for a potential autograft may be in short supply or may be simply unavailable. And for tissues with complex three-dimensional structures the challenge is vastly greater: size may become a problem, where the nutrient transport becomes a limiting factor; the replacement tissue may need to satisfy certain mechanical needs, such as in bone; and it may be simply very difficult to create the sheer intricacies of the tissue in question.

But tissue engineers are beginning to address these challenges. Better techniques in the growth and differentiation of stem cells, new "smart" biomaterials, organ printing, bioreactor design and an increased understanding of the development of tissues *in vivo* all provide encouragement that tissue engineering will be a successful practical solution to many of the burgeoning degenerative diseases that trouble our ageing 21st century societies.

The Need for Tissue Engineering

Therapeutic applications of tissue engineering include any disease in which tissue is lost or damaged. For example, in type I diabetes the pancreatic islets of Langerhans are selectively attacked and destroyed by the body's own immune system. Recently, Shapiro and co-workers[6] have shown that islet transplantation can, over a short period of time, correct this disease and free its sufferers from reliance on insulin injections and regular glucose checks. A method for generating this structurally simple yet elusive tissue *en masse* in culture would be of massive benefit, both socially and economically — in 1997 it was estimated that over 120 million people have diabetes worldwide, of which 5% to 10% suffer from type 1 diabetes.[7] Other autoimmune diseases, such as rheumatoid arthritis, where the joints are the target of the immune attack, may also find a therapy in tissue engineering.

Changes in lifestyles and the rapidly increasing age of populations in the 21st century are the risk factors leading to a large increase in the number of diseases. Cardiovascular disease, where the tissues of the heart die or are damaged, is a leading cause of death in the developed world. Forty per cent of all deaths in the US each year are due to this disease[8] and, in Europe, the estimated direct annual cost is €473 billion.[9] A wide range of treatment options is available to people who suffer from this disease, with heart transplant being the most extreme, but strategies that aim to repair or replace damaged heart tissue using cellular therapies could have great potential in decreasing the need for transplantation.

Similarly, neurodegenerative diseases impart an increasing burden on health services, with age being the main risk factor. Parkinson's disease alone, with its 100,000 sufferers, has been estimated to cost almost €1 billion in the United Kingdom per year.[10] This disease is caused by the degeneration of the nigrostriatal dopaminergic neurons in the brain, leading to tremors and difficulty in movement.[11] Currently, it can be treated with drugs that are metabolised to dopamine, but these become gradually less potent with continued use, and have unpleasant side effects when administered at too high a dose. The transplantation of cells from aborted foetuses has been shown to have beneficial effects but, besides the ethical concerns it raises, this tissue is too limited and its effect too varied for it to be considered as an option for many.[11] Other neurodegenerative diseases that could be addressed by tissue engineering include stroke, amyotrophic lateral sclerosis and Alzheimer's disease, all of which are due to the loss

of cells in the brain and all of which are on the increase as the population ages.

Bone and cartilage disease, more common in the elderly, is another looming problem in medicine today. Prosthetic joint replacements have been one of the great success stories in medicine in the 20th century with 475,000 hip and knee replacements performed each year in the US at a cost of over €10 billion.[12] These are generally performed to alleviate pain and immobility in the joints, which occur as cartilage and bone degenerates and becomes worn. Unfortunately, although such implants can provide sustained relief, they tend to fail after a period of around 15 years;[13] therefore, more biocompatible implants are urgently needed.

The solution to all of these problems requires a source of replacement cells, but where might a tissue engineer look to find such a source?

Cell Sources

In the best-case scenario, cells could be extracted from the individual, grown *in vitro* to form the desired tissue and re-implanted back into the host. For some tissue types, such as skin and urothelium,[14] this is already possible. But in practice the cell types required are either not available or cannot be expanded in culture very easily. To solve this kind of problem tissue engineers have looked to progenitor cells or stem cells. Stem cells are defined by their ability to self-renew and to differentiate into multiple cell types, and are normally divided into somatic and embryonic stem (ES) cells.

Haematopoietic Stem Cells

Haematologists can probably lay claim as being the pioneers of stem cell science, as Jansen *et al.*[15] note in an excellent review; the concept of a stem cell was first propounded in 1909 by the Russian biologist Alexander Maximow. But it was not until the middle of the 20th century that this concept was found to be true. Firstly, Ford *et al.*[16] found that it was a cellular entity that had the potential to reconstitute the immune system in mice after radiation-induced destruction of the bone marrow. Then Mathé *et al.*[17] found that haematopoietic stem cells (HSCs), as they became known, could replace the blood system and cure leukaemia in humans. These highly proliferative cells, found in the blood and bone marrow, can differentiate into all lineages of the blood system. Even more intriguingly there has been a degree of excitement recently and, inevitably, controversy with the

discovery that these cells or similar but as yet unidentified cells resident in the bone marrow are able to generate cells not only of the blood but also other somatic cells, such as the epithelium.[18,19] Presently, however, the origin of these cells is not precisely known and they cannot be grown in culture, so their relevance to tissue engineering is not yet widely established.

Mesenchymal Stem Cells

Another adult stem cell type, the mesenchymal stem cell (MSC), which was originally identified in the bone marrow, has a more established promise for tissue engineering applications. The presence of these cells, often referred to as marrow stem cells or stromal stem cells, was first hinted at by Friedenstein *et al.*[20] who noticed that some rare cells in aspirates of bone marrow grew to form adherent colonies that could then proliferate *in vitro*. They were initially called CFU-Fs (colony-forming units — fibroblasts) and are now thought to constitute around 0.001% of nucleated cells in bone marrow aspirates,[21] depending on the species.[22] In subsequent studies it was found that colony-forming cells isolated in this way could be cultured with a fibroblast-like morphology for multiple passages — typically between 20 and 40 — before senescence, depending on the age of the donor. In addition, the multipotentiality of these cells has been clearly demonstrated: under the right culture conditions MSCs can be selectively differentiated into cells of the mesoderm lineage — commonly osteoblasts, adipocytes and chondrocytes,[23] and sometimes myocytes.[24] The identity of the MSC has been something of a mystery, however. It was initially suspected that cultures of MSCs derived from bone marrow may comprise many types of progenitor cells, each with different capacities to differentiate into a specific tissue. But Pittenger *et al.*[25] recently provided evidence against this. They found, by FACS analysis of cell-surface markers, that the population was homogeneous and, more importantly, they demonstrated that subcultures derived from single cells could themselves be differentiated into adipocytes, chondrocytes and osteoblasts. Despite this clarification of the identity of the MSC as a tissue culture cell strain, it is likely that many other different progenitor cells with varying degrees of multipotentiality exist within the bone marrow. For instance, Verfaillie and co-workers[26,27] recently unearthed another type of stem cell, named the multipotent adult progenitor cell (MAPC). These cells can differentiate *in vitro* not only into mesoderm cells but also into ectoderm and endoderm. They also found that they could maintain MAPCs in an undifferentiated state for more than

120 population doublings and, most importantly, that single MAPCs, when injected into the blastocyst of a mouse, could contribute to blood, spleen, liver, and lung epithelium. MAPCs are, therefore, an encouraging prospect for tissue engineering applications. Further research is needed in other laboratories before their true potential can be realised, however.

Other sources of adult stem cells

Stem cells are also known to be distributed throughout the body in various other niches. In the 1990s, Reynolds and Weiss[28] and Bartlett and coworkers[29] showed that single cells from the adult forebrain could be grown into an undifferentiated state *in vitro* as neurospheres. Given the correct signals, cells from these neurospheres — neural stem cells — could then be induced to differentiate into neurons, oligodendrocytes and astrocytes. Subsequently, other researchers have shown that these cells have more than just a restricted neuronal repertoire of progeny; they could also be induced to differentiate into pancreatic beta-cell-like cells[30] and blood cells[31] and, when injected into mouse blastocysts they were found to contribute to heart, kidney, muscle, lung,and liver.

Recently, it has been shown that other tissues also have stem cell populations. Most tissues have a population of progenitor cells, which can divide and form nascent tissue. A good example is progenitor cells in the skin, which are situated at the basal layer of the epidermis and divide to form keratinocytes, replacing those lost due to normal wear and tear. But some of these cells are thought be more versatile. For instance, skeletal stem cells can differentiate to form cardiomyocytes,[32] oval cells of the liver to bile duct cells,[33] and skin stem cells have even been shown to differentiate into bone, nerves, and skeletal muscle.[34] While these discoveries challenge our idea of the lineage commitment and potentiality of various cell types in the body, methods for the large-scale culture and manipulation of such tissue-specific stem cells will be needed before we can design therapies based on their use.

Embryonic stem cells

Embryonic stem (ES) cells have attracted a massive amount of public and scientific interest in recent years. This is largely because of their touted potential use for combating a number of diseases but also because of their controversial method of derivation, which currently involves the destruction of human blastocysts. They are usually derived several days post-fertilisation, before the 14th day (after which experimentation is outlawed

in the UK). At this time the future embryo consists of an outer layer of cells (the trophectoderm, which goes on to form the placenta), a fluid-filled interior and a small ball of cells attached to the inner trophectoderm wall called the inner cell mass. These latter cells go on to form the embryo and are the cells that are isolated to create the ES cell line. Following expansion in culture ES cells can go on to form teratomas — tumours composed of tissues of all three germ layers — when implanted into immunodeficient (SCID) mice, defining them as being pluripotent. They are also thought to be immortal and can divide indefinitely in this state in culture, under a specific set of culture conditions.

ES cell research has its roots in the study of teratomas and teratocarcinomas — morbidly fascinating germ line cancers found rarely in people, sometimes resulting in the formation of whole tissue, such as teeth and hair, in the gonads of adults. Cells from these tumours, which retain the capacity to self-renew and to form further teratomas in immunocompromised animals, were subsequently grown *in vitro* by Martin and Evans[35] and were studied as a model system to investigate early mammalian development, something that is extremely difficult *in vivo*. This pioneering work led in 1981 to two reports on the isolation of pluripotent cells from early mouse blastocysts — ES cells.[36,37] In later years, manipulating the genome of these cells allowed the creation of transgenic animals, one of the great tools of developmental biology, used extensively to find out the function of particular genes. In 1998 Thomson *et al*.,[38] after earlier successes with other primates, reported the first derivation of a human ES cell line, creating headline news around the world.

Because of several drawbacks, ES cells have been considered to be a better bet than adult stem cells for cell replacement and tissue engineering strategies. For example, although HSCs have been shown to engraft in other organs *in vivo*, they cannot be expanded *in vitro*, and so they are not currently useable for large-scale tissue engineering applications. MSCs, on the other hand, grow as adherent, homogenous cells *in vitro*. They can be induced to form bone, cartilage and fat with high efficiency *in vitro* and are currently the focus of a great deal of research for bone and cartilage applications.[21,38] But these cells are restricted to applications for these tissues, their ability to replicate declines with age[38,39] — a particular problem because elderly patients are the main source of demand for replacement bone and cartilage — and there is evidence to suggest that they may accumulate genetic abnormalities.[40] On the other hand, ES cells can be grown in potentially unlimited numbers *in vitro* and they are thought to be able to differentiate

into any cell type. In addition, a recent work has demonstrated the possibility of producing patient-specific ES cells by somatic-cell nuclear transfer,[41] with the attractive result of avoiding any problems of host immune rejection. So the biomedical community is encouraged that, if we are able to control the cell types into which they develop, ES cells could provide an ideal source of tissue for transplantation.

The main challenge faced by researchers is how to differentiate ES cells reproducibly and efficiently so that they become sufficiently like the *in vivo* cells that need to be replaced.

The development of a whole organism of differentiated cells from a single, undifferentiated precursor is perhaps best understood in the nematode worm, *Caenorhabditis elegans*. John Sulston and co-workers[42,43] have described the complete cell lineage of this creature and have found that development proceeds from a single cell to a functional organism containing 959 specialised cells. Here, a self-organising and vast array of cascading chemical and physical cues determine the function and spatial arrangement of the mature cells in the organism. It is possible that this process is much more complex in the mammal (a human being is estimated to have 100 trillion mature cells) and it is very unlikely that we will ever be able to design a system so complex as to recapitulate all the signals and events that occur to make mature tissue in the course of human development.

To overcome this problem many strategies have sought to make an artificial analogue of the developing mammalian embryo *in vitro*. This approach is based on the early work by Gail Martin's group[44] on murine embryonic carcinoma cells, where, after transfer to suspension medium, the cells aggregate and form structures called embryoid bodies (EBs). Despite the absence of a body axis, this technique appears to be sufficient to cause the differentiation of both murine and human ES cells into a range of tissues, including those derived from all three germ layers — endoderm, mesoderm and ectoderm — as well as from extra-embryonic tissues.

To induce differentiation in a more precise manner, however, researchers have used several strategies: the addition of soluble growth factors, cytokines and non-proteinaceous compounds to the growth medium; the growth of cells on specific extracellular matrices; co-culture with other cell types or growth in media conditioned by other cell types; genetic manipulation of ES cells; adjusting physical parameters; or a combination of two or more of these strategies. The literature is now huge, with over 1500 papers

on ES cell differentiation published since 1998, and far too many to review in a single article.

In a famous example of an experiment designed to direct ES cell differentiation, Lumelsky *et al.*[45] used a complicated five-step process to promote the differentiation of murine ES cells into pancreatic islet tissue. This involved, firstly, the expansion of ES cells in an undifferentiated state, followed by four days of EB formation in suspension culture. Then these EBs were allowed to adhere to tissue culture plastic and grown in the absence of serum — a strategy designed to select for cells expressing nestin, a putative marker of pancreatic precursors. Next, the remaining cells were expanded for a period of time using the mitogen fibroblast growth factor (FGF). Its removal several days later caused the induction of differentiation.

Growth factors, such as those of the BMP family, can also influence stem cell differentiation. Xu *et al.*[46] and Pera *et al.*[47] showed that, in the absence of EB formation, ES cells differentiate into trophoblast-like cells or extra-embryonic endoderm-like cells when exposed to BMP4 or BMP2, respectively. Blockade of BMP2 signalling in the latter study, using a BMP inhibitor called Noggin, induced differentiation of neural precursor cells. But a transient addition of Noggin prior to EB formation in another study[48] led to an increase in the formation of cardiomyocytes, or the addition of BMP2 during EB formation caused an increase in the formation of adipocytes.[49] These results illustrate the complexity of ES cell differentiation *in vitro* and that a single growth factor may have many effects, depending on the timing of its addition.

Non-proteinaceous factors or cell-specific media have also been shown to affect differentiation *in vitro*. Adding ascorbic acid and β-glycerophosphate to cultures of dissociated EBs[50–52] causes an increase in the degree of ES cell differentiation into osteoblasts while others have shown that growth of ES cells in small airway growth medium promotes the differentiation of ES cells into type II pneumocytes.[53–55] Growth of ES cells in the presence of the soluble products of Bioglass — an inorganic glass ceramic used in bone tissue engineering — could also increase the differentiation of ES cells into osteoblasts.[56] Retinoic acid has a spectrum of effects, inducing differentiation of cells into mesoderm[57] but more commonly to ectoderm derivatives.[58]

Co-culture is another method which is often used to induce differentiation. Kawasaki *et al.*[59] grew ES cells on PA6, a stromal cell line, and showed that this induced neural differentiation; Mummery *et al.*[60] co-cultured ES

cells with a visceral endoderm cell line, END2, and showed cardiac differentiation; while the following works by Rathjen *et al.*[61] and Hwang *et al.*[62] showed that conditioned medium from a similar cell line, HepG2, can induce mesoderm formation. Others have co-cultured primary tissue with ES cells[63,64] or added cellular extracts[65] to direct differentiation to a tissue type of interest.

The effect of cell matrix on ES cell differentiation has been less well investigated; however, in an interesting experiment Coraux *et al.*[66] reported that growth of ES cells on a matrix derived from human fibroblasts could induce the formation of a heterogeneous, layered structure resembling skin, while Gerami-Naini *et al*.[67] showed that EBs embedded in Matrigel, a matrix derived from a sarcoma (mesoderm cancer) cell line, have characteristics of the trophoblast.

Genetic manipulation can be used to induce differentiation. Kim and co-workers[68] showed that ES cell overexpression of *nurr1*, a gene involved in the differentiation of neural precursors into dopamine neurons, and subsequent implantation of these cells into a rat model of Parkinson's disease, could cause an improvement in the condition. Also Tai *et al.*[69,70] have shown that over-expression of Osterix, a bone-specific transcription factor, can induce bone formation by ES cells.

Ironically the very malleability of ES cells, which makes them so attractive for generating many of the tissues that are required for tissue engineering, is also a curse. ES cells have a potentially dangerous tendency to differentiate into cell types other than those required. So it is ultimately likely that cell selection will be necessary to harvest homogenous populations of the correct cell type. Many groups have used fluorescent antibodies, specific to cell-surface markers of interest, and FACS analysis to select certain cell populations. For instance, Yamashita *et al.*[71] selected Flk-1 positive cells, which were then introduced into mouse blastocysts and contributed to the endothelial lineage, demonstrating their potential in vascular tissue engineering. Similarly, the same group used a combination of several antibodies to isolate the various lineages that contribute to the blood–cell system.[72] Where surface markers are not present or are unknown, other groups have made ES cell lines with fluorescent proteins under the control of tissue-specific promoters[73,74] that light up when differentiation is achieved, enabling FACS selection of the fluorescent cells. Alternatively inappropriate cells can be eliminated after differentiation. For example, Klug *et al.*[75] introduced the gene for aminoglycoside phosphotransferase under the control of the cardiomyocyte-specific promoter of the gene for

the alpha-cardiac myosin heavy chain. After EB differentiation, G418 was used to kill all cells in which this gene was not active, producing a fairly pure population of cardiomyoctes.

Because the differentiation of ES cells is so complex — being affected by a multitude of different factors — and because conventional cell culture experimentation cannot interrogate such an array of variables without a large amount of time and expense, some groups have begun to investigate high-throughput methods of probing differentiation. In 2003, Ding *et al.*[76] presented work in which an embryonic carcinoma cell line was screened for neuronal differentiation with over 100,000 small molecules. In this case, the cells were transfected with a luciferase gene under the control of the neural-specific gene, Tα1 tubulin, and were grown in 384-well plates in the presence of different small molecules. The group was able to identify the small molecules that had a positive effect using a luminescence assay, and could then study them in more detail. One of the molecules, TWS119, was found to have an affinity for GSK-3β, a kinase involved in embryonic development with pleiotropic effects but with specific implication in a Wnt-dependent neuronal signalling pathway.

There is interest in scaling down even further. The "genomic age" has seen the birth of high-throughput, data-farming technologies such as DNA and RNA microarrays and also, more recently, the protein array.[77] These new methods are based on microfabrication. In the first report of a microarray designed for studying stem cell differentiation, Robert Langer's group[78] made a matrix-spotted microarray slide. Twenty-five different synthetic matrix compounds were spotted onto the slide in 576 combinations and the effect of the resultant composite biomaterials on the differentiation of human ES cells into epithelial cells was tested. Cells were seeded onto the array, allowed to adhere, cultured for several days and then immunostained for the epithelial-specific marker cytokeratin 7. In doing so, they could identify those composite polymers that supported ES cell growth and which directed cellular differentiation to a desired lineage. Since 20 1728-spot polymer arrays can be synthesised in one day and very little culture medium or growth and analysis reagents are needed, this represents great savings in time and money.

Following this, Flaim *et al.*[79] once again adapted DNA spotting technology such that natural matrix proteins — collagen I, III, IV, laminin and fibronectin — could be spotted onto the slide. In this case, 32 combinations of these proteins were evaluated for their ability to direct murine ES cells to a hepatocyte fate. An important advantage of this technology is the ability

to identify synergy between two or more growth factors, where alone they may have no effect, or even a negative effect.

Factorial analysis methods can be applied in these situations. These statistical tricks can be used to garner the maximum amount of data from a minimum number of experiments. Surprisingly, using this technique, Flaim *et al.*[79] found such information as that while collagen I, III and laminin had negative effects when administered alone, combinations of them gave positive effects. In a similar manner, Chang and Zandstra[80] have found synergistic interactions between glucose, retinoic acid, FGF, EGF, and insulin on the differentiation of murine ES cells into endoderm cells, as measured by immunostaining for CK8 and HNF3β. Finally, Chin *et al.*[77] microfabricated a miniature platform for studying stem cell fates, often at the single cell level. High-throughput strategies such as these will undoubtedly find further use in elucidating the role of the many protagonists in stem cell differentiation.

Scaffolds

The transplantation of cells alone may be sufficient to improve the symptoms of many degenerative diseases. But for other applications in tissue engineering structured implants may be needed, where cells, such as those described above, are grown on scaffolds. Scaffolds provide a three-dimensional environment on which cells can grow, interact with each other and form a microenvironment in which tissue, with its associated extracellular matrix, is formed. Many materials have been used for tissue engineering purposes. Some are synthetic but biodegradable, such as poly(lactic acid) (PLA)[81,82] or poly(glycolic acid) (PGA).[82] Others are bioactive, such as Bioglass,[83] which increases osteoblast proliferation and bone formation *in vitro*.[84,85] Others may be derived naturally, such as alginate,[86] chitosan,[87] or collagen.[88]

In bone and cartilage engineering, MSCs have been used extensively on a multitude of different scaffolds. For example, Uematsu *et al.*[89] recently showed the MSCs cultured on a porous poly-lactic–glycolic acid (PLGA) scaffold could form mature cartilage tissue in an animal defect model. Kim *et al.*,[90] on the other hand, ingeniously designed a PLGA scaffold that slowly exuded ascorbate and β-glycerophosphate, increasing bone differentiation of MSCs. Other scaffolds have been designed for other tissues, such as arteries,[91] liver[92] and even retina.[93]

In contrast, there have been very few publications on the use of ES cells on scaffolds, possibly because the efficient differentiation of ES cells into a specific cell type has not yet been achieved. In some rare examples, Levenberg *et al.*[94] cultured human ES cells and EBs on PLGA porous scaffolds, demonstrating their differentiation into a variety of tissues; Chaudhry *et al.*[95] seeded EB-derived cells on three-dimensional PLA-derived scaffolds and demonstrated bone nodule formation; Gerecht-Nir *et al.*[96] showed that EBs cultured in porous alginate scaffolds have a higher degree of vascularisation than those grown in suspension; and Liu and Roy[97] showed increased haematopoietic precursor cell differentiation when they grew EBs in a proprietary porous scaffold. More recently, Levenberg *et al.*[98] once again demonstrated the differentiation of human ES cells into three-dimensional neural rosette structures when grown on PLA and PLGA scaffolds in the presence of growth factors.

It remains a major problem, though, that the use of ES cells for tissue engineering strategies *in vivo* might suffer from the problems of differentiation to inappropriate types or, even worse, teratoma formation. Levenberg *et al.*[99] suggest that genetically modified ES cells that undergo apoptosis in response to inappropriate differentiation could be used to prevent these problems from happening.

Future Perspectives

Stem cells, particularly ES cells, have huge potential as a source of autologous cells for cell replacement therapy and tissue engineering. But before they can be used for these applications, a number of challenges need to be tackled.

As we have seen, reproducible methods are needed for the efficient differentiation of stem cells into a desired lineage. It appears that some cells are easier to generate than others. For example, we are closer to generating populations of neural cells for the treatment of diseases such as Parkinson's and stroke than we are for generating populations of functional pancreatic beta cells for diabetes. Assuming this is possible, we need culture systems in which large quantities of our desired cell types can be grown, preferably in an automated fashion where the skill of a research scientist is not needed. Bioreactors have been used in many other applications to mass produce cells or the products that they secrete,[100] and some groups are at the early

stages of applying them to stem cell culture.[101] And if stem cells are ever to be used in a clinical setting, their production will have to obey strict quality-control guidelines — the application of stem cells to clinical settings can surely take lessons from clinically approved tissue engineered constructs, such as Apligraf.

Creating structured three-dimensional tissue leads us to a wealth of other challenges. For instance, large grafts are needed for bone tissue engineering. Tissue culture has traditionally been a two-dimensional world, where cells can grow happily in close contact with nutrients and dissolved gases needed for cell survival. But the culture of large chunks of tissue *in vitro* in the absence of a blood supply is limited by the diffusion distances of these nutrients — the centre of these potential grafts quickly become necrotic. Again, advances in bioreactor technology, as well as new technologies such as microfluidics and microfabrication, offer potential solutions to these problems, where multilayered cell/matrix structures infiltrated with growth medium-perfused channels could ensure the viability of deeply embedded cells. Work on these types of scaffolds is already in progress.[102–104] Biocompatibility of potential tissue engineered constructs is also a problem and new biomaterials, which incorporate specific cell-binding peptides, are beginning to address this need.[105]

Summary

"The worst thing about medicine is that one kind makes another necessary", said Elbert Hubbard, the American philosopher. And so it is with tissue engineering. The great successes of medicine during the 20th century mean that people are living longer and their worn-out parts need replacing more often. These problems are unlikely to go away overnight, and so it is likely that moving some of the technologies discussed in this chapter from the bench to the bedside will be critical in 21st century medical care.

Acknowledgements

We thank our funders: the Rosetrees Trust, the Medical Research Council (Co-operative Group G9900355 and Component Grant G0300106), the Wellcome Trust and the Defense Advanced Research Projects Agency

(DARPA). We also thank Mark Placzek, Eileen Gentleman and Anne Bishop for helpful advice.

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Joint Tissue Engineering

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Introduction

Tissue engineering and regenerative medicine are indicated in conditions where tissue loss causes a persistent loss of function and spontaneous regeneration and compensatory mechanisms are either absent or insufficient. For instance, bone has a remarkable repair capacity that guarantees efficient fracture repair. However, fracture repair fails in lesions that exceed a certain size, which can vary depending on the site, age of the patient, and other patient-related circumstances including co-morbidity. Cell-based regenerative strategies aim at restoring tissue/organ integrity and function utilising cells and bioactive molecules to trigger, enhance, support and complement the residual capacity for repair.

Many branches of medicine can benefit from these technologies, and intensive research currently underway, resulting in some cases, in clinical

applications. Notable examples of cell-based repair are in skin,[1] bone,[2,3] articular cartilage,[4] cardiac muscle,[5–8] and Parkinson's disease.[9,10]

Cells are typically manipulated *ex vivo* and administered to patients as living and dynamic biological agents. In this chapter we will focus on the issues that these new therapeutic agents raise and are difficult to address within the paradigms of the traditional pharmacology. They include determination of the mechanism of action, dose, evaluation of potency, safety and toxicity, upscale, delivery, and identification of proper indications. We will use the case of autologous chondrocyte implantation (ACI), a procedure used to repair localised joint surface defects (JSDs), as a prototypic example of advanced tissue engineering technology entering routine clinical application to discuss the important issues and challenges posed by these novel treatments in medicine.

The Indications

In cases of end-stage failure of tissue/organ systems, prosthetic replacement provides immediate, though partial functional restoration in a large number of clinical conditions including large bone defects and advanced osteoarthritis (OA). When there is a severe functional impairment, a rapid, even if partial, recovery is a satisfactory result. When anatomical damage and functional loss are more limited, such as in the case of a localised joint tissue defect, the therapeutic goal is to achieve an optimal functional as well as anatomical restoration and to restore homeostasis, thereby preventing the possible evolution towards end stage OA, ideally with a minimally invasive surgery. This is especially true for particularly demanding patient populations such as young individuals and professional athletes. In these cases repair is sought using biologically active devices, e.g. cells and/or resorbable biomaterials that restore tissue integrity through a process of remodeling.[11] In addition, it is thought, but not proven with clinical trials yet, that these treatments would prevent the evolution of limited joint surface lesions towards OA. Recently, Buckwalter *et al.*[12] reported that post-traumatic OA accounts for 13% of knee OA and 73% of ankle OA and, in a prospective study, Gelber *et al.*[13] observed that traumatic joint lesions in young adults increase the risk of developing knee OA. These studies therefore point to a need to develop/optimise protocols for the biological repair of the joint surface to prevent the establishment of secondary pathological conditions such as OA and evolution towards tissue/organ end-stage failure.

Autologous chondrocyte implantation

The biological repair of the joint surface using cultured autologous cells has been incepted and first explored in the eighties.[14,15] This technology was further developed into a clinical application by Brittberg *et al.*[4] This breakthrough opened the doors of clinical applicability to the field of tissue engineering, until then essentially confined to proof of principle studies in small animal models. Localised joint surface defects were treated by the implantation of autologous, *in vitro*-expanded chondrocytes injected in suspension under a periosteal flap. The chondrocytes were obtained with a cartilage biopsy from an uninvolved area of the same joint. The first, pilot study[4] stirred excitement in the clinical and scientific world showing symptomatic (good to excellent) relief in 14 out of 16 patients with lesions of the femoral condyle at two years follow-up. Subsequently, the same group periodically reported follow-ups up to 11 years[16,17] demonstrating long-term efficacy of this procedure in up to 92% of the patients when lesions were localised to the femoral condyle and lower in other sites. Despite its proven long-term symptomatic efficacy, whether ACI prevents the evolution of these localised lesions towards OA remains to be investigated. In addition, it is not yet clear which lesions may evolve towards OA.[12,13] Likewise, the large variability in terms of quality of the repair tissue and clinical outcome reported in several studies[18,19] raises issues of further standardisation of the procedure as well as definition of proper clinical indications.

Patient-related factors

The outcome of ACI, as well as that of all tissue engineering approaches utilising autologous cell preparations, is influenced by patient selection, not only because the patient is the recipient of the treatment, but also because he/she is the source of the cells used for his/her own therapy.

The patient as the recipient of the cell preparation

As with all treatments, the identification of a proper indication and the selection of patients are crucial for the evaluation of the efficacy. So far, there is a general consensus that the patients eligible for ACI are young-adult individuals with a joint surface lesion of the knee that is full thickness, limited, and persistently symptomatic. Patient-related factors, such as age, body weight, joint congruity and stability, general health status, general and local (articular) co-morbidity, size, depth and site of the lesion are known to influence the outcome. ACI is usually carried out for small lesions when

other techniques have failed[20] and still has a high success rate. Nevertheless, when compared to other biological joint resurfacing techniques in prospective randomised clinical trials, ACI was not superior to microfracture[18] and proved only marginally better than mosaicplasty.[19] One possible interpretation is that we might be missing stringent indication criteria allowing identification at an early stage of those patients who would not respond to other therapies and would indeed benefit from ACI. Tissue damage results from disparate mechanisms of injury combined with the incapacity of the body to compensate with repair. As the reasons for this can be different in individual patients, it is conceivable that there is no "perfect approach" to joint surface tissue repair, but that, as our understanding of damage and repair mechanisms advance, we might be able to tailor appropriate treatment strategies for much better focused indications.

The patient as the donor of the cell preparation

It is a peculiarity of autologous cell-based approaches that the patient is not only the recipient, but also the source of the therapeutic preparation. Consequently, patient-related factors may influence quality and properties of the therapeutic preparation. For instance, the age of the patient can influence the yield of chondrocytes from a cartilage biopsy, their expandability, and their phenotype.[21] Other factors include the size and site of the cartilage biopsy, co-morbidity, and the condition of the cartilage at the time of biopsy. The influence of these factors on the efficacy of cell preparations for ACI has not been investigated thoroughly. Since the cell preparations are derived from individual patients, cell preparations from every single patient have to be considered as "individual batches" and be quality controlled accordingly, even if the entire procedure of cell isolation, expansion and preparation is perfectly standardised.[22] This appears to be a limitation of autologous cell therapies, and the amount and complexity of quality controls make these procedures costly, thereby limiting their routine applicability. Nonetheless, autologous cell therapies offer the advantages of minimal risk of disease transmission and of immunological rejection; hence nowadays in clinical settings they are preferred to allogeneic cells.

The cells

The cells represent the living "active component" of cell-based therapies. Besides the patient-related factors discussed above, any cellular product will be affected also by the preparation technology.

ACI offers the advantage of using cells that are stably committed to the appropriate phenotype and can form *in vivo* stable cartilage tissue, resistant to vascular invasion, mineralisation and ossification[23] and therefore the maintenance of this property is essential. However, chondrocyte expansion in monolayer cultures is known to induce a progressive derangement of the cell phenotype *in vitro* often referred to as dedifferentiation. This results in the loss of the capacity of the expanded chondrocyte populations to form cartilage *in vivo*, a possible source of outcome variability in clinical settings.[23] Indeed, the repair tissue after ACI appears as a hypercellular cartilage sometimes displaying hyaline-like features.[11] In some patients, however, the repair tissue is constituted of poorly differentiated and disorganised fibrocartilage,[11] which could at least partly be due to the partial loss of the phenotypic stability of the expanded chondrocytes throughout *in vitro* culture.[24] It is critical, therefore, to ensure that the phenotypic stability and the capacity of articular chondrocytes to form stable hyaline cartilage *in vivo* are retained throughout cell expansion. The development of a potency assay to measure the *in vivo* phenotypic stability of expanded articular chondrocytes represents an important scientific and regulatory issue. To address this issue, we have standardised an *in vivo* assay in nude mice to measure the potential of adult human articular chondrocytes to generate stable hyaline cartilage resistant to vascular invasion, dystrophic calcification and replacement by bone.[23] We have shown that early-passage *in vitro*-expanded human articular chondrocytes, when injected in suspension into the muscle of nude mice, form within two weeks ectopic stable cartilage, which persists over at least 12 weeks. Importantly, this capacity is progressively lost upon serial passaging. We have then identified molecular markers that predict stable cartilage formation in this assay by *in vitro* culture expanded articular chondrocytes.[23] Cell populations expressing this molecular signature of stable cartilage-forming cells are currently being tested in a prospective, multicentric, randomised clinical trial.[25]

As discussed above, this quality control needs to be applied to each individual cell preparation, thereby adding significantly to the costs of ACI. In addition, the autologous nature of the cell populations does not facilitate an "industrial" upscaling. With this regard, the generation of large batches of certified, quality controlled off-the-shelf expanded cell populations for allogeneic cell transplantation would be more convenient.

Stem cells

Stem cells are clonogenic cells, with high self-renewal capacity, and phenotypically responsive to environmental cues. These properties represent the appeal of stem cells for tissue engineering purposes. The preservation of their differentiation potentials during long-term culture expansion would allow upscaling and generation of large, quality-controlled homogeneous batches, thus circumventing the limitations and patient-to-patient variability of autologous implantations of mature cells such as articular chondrocytes. The use of these cells has been advocated for joint surface repair also because the cartilage biopsy, necessary to obtain chondrocytes for ACI, results in additional damage to the joint surface and, ideally, should be avoided. The rationale for the use of stem cells for cartilage repair is that the damaged environment would be promoting the differentiation towards the osteochondrogenic lineage as required for the repair of the local damage. This concept is supported by the finding that bone marrow-derived cells contribute largely to the repair of joint surface defects in rabbits through a process reminiscent of embryonic skeletogenesis encompassing mesenchymal aggregation and endochondral bone formation.[26]

Mesenchymal stem cells (MSCs) have been isolated from several tissues including bone marrow,[27] synovial membrane,[28,29] periosteum,[30] and articular cartilage.[5,21,31,32] These cells can be extensively expanded in culture and effectively cryopreserved, while maintaining their phenotype and multilineage differentiation potential. The use of MSCs for joint surface repair is therefore intensively sought[33,34] and has been recently explored in humans.[35] These studies have shown promising results, with cartilage formation at early time points and a process of endochondral bone formation progressively restoring the bone front. The main concern, with these cells, is the potential risk of heterotopic tissue formation[36] and excessive advancement of the bone front at the expense of the articular cartilage layer.[37] This phenomenon has not been reported in patients treated with ACI. One possible explanation is that, as discussed above, articular chondrocyte populations retain, at least for a limited number of population duplications, phenotypic stability allowing them to generate hyaline-like cartilage *in vivo*.[23] It is likely that this phenotypic "memory" may contribute to limiting the advancement of the bone front thereby preserving the thickness of the repair cartilage tissue. This is also supported by data showing that the implanted phenotypically stable cells, in a goat model of ACI, persisted long term in the site of implantation retaining a cartilage phenotype as assessed by production of type II collagen.[38] Also, it is possible that MSCs from bone marrow,

which appear to have osteogenesis as a default differentiation pathway and a high frequency of chondrocyte hypertrophy,[39] may not be the ideal chondroprogenitors for the repair of articular cartilage. Indeed a recent report has shown the superiority of MSCs from the synovial membrane (SM-MSCs) for cartilage formation *in vitro* when compared with MSCs from other sources including bone marrow and periosteum.[40] However, it is not known whether this difference is inherent to the cells or due to heterogeneity of the MSC populations with possible contamination by mature or committed cell types (e.g. osteoblasts in bone marrow preparations or chondrocytes in synovial membrane cell preparations). This greater chondrogenic potential could be explained by the different embryonic derivations of these tissues. Indeed, an elegant lineage-tracking study using cre/LoxP technology in mouse[41] has convincingly demonstrated a common embryological origin of the articular cartilage and of the synovial membrane from the gdf5-positive cells populating the joint interzone, a stripe of mesenchymal tissue segmentating the cartilage anlagen in the developing skeleton at the site where the prospective joint will subsequently form.[42,43] It remains to be investigated whether this superiority in cartilage formation of the synovial membrane-derived cells is also observed *in vivo* in appropriate animal models of joint surface repair.

Stem cells are undifferentiated multipotent cells and therefore precommitment *in vitro* towards the chondrogenic lineage has been proposed as a method to reduce the risk of undesired/heterotopic tissue formation by restricting alternate differentiation pathways.[44] Nevertheless, although a stable chondrocyte-like phenotype, as determined by the expression of the molecular signature of stable chondrocytes[23] mentioned above, could be induced by TGF-β1 treatment in micromass cultures of SM-MSCs *in vitro*, this acquired phenotype was not stable *in vivo* in a mouse model of ectopic cartilage formation.[45] Importantly, the molecular markers identified so far as being predictive of the *in vivo* stable-cartilage-forming capacity appear to be specific to articular cartilage derived cell populations[23] and, under these experimental conditions, not directly applicable to other chondrogenic cell populations. Clearly, the culture conditions used, were able to force the cells to acquire a chondrocyte-like phenotype, but not sufficient to reprogram their phenotypic "memory". It is intriguing to argue that, during embryonic development, cells acquire a defined differentiation profile, possibly through epigenetic factors. This emphasises the basic question related to the identification of the signaling pathways that are necessary and sufficient to obtain and maintain the stable-chondrocyte phenotype.[46]

The joint environment itself may be a source of signaling sufficient to prime chondrogenesis in MSCs. This is supported by studies in both animal models and humans[33,35] in which joint surface repair was obtained by implanting undifferentiated MSCs. The outcome in terms of maturation of the repair tissue was variable, ranging from hyaline-like cartilage to fibrous tissue formation. This has prompted attempts to support tissue maturation by using cartilage promoting growth factors,[47,48] which has led not only to an amelioration of tissue maturation, but also to a better patterning and architecture of the repair tissue. As an example, Mason *et al.*[48] have explored the use of periosteum-derived MSCs engineered to stably express human bone morphogenetic protein-7 (BMP-7). The implantation of these cells seeded into polyglycolic acid scaffolds into joint defects in rabbits resulted in repair tissue that was superior to that obtained implanting the scaffold alone (no cells), or the scaffold loaded with cells transduced with empty vector. Importantly, the improvement was not only limited to the repair tissue maturation, but BMP-7 transduced cells also induced a better regeneration of subchondral bone, underlying a layer of hyaline, patterned articular cartilage resembling the original osteochondral structure.[48]

Although these sophisticated and complex systems are promising, the combination of at least four variables (the surgery, the biomaterials, the cells, the morphogens, and when applicable the cell transfection/transduction) make the assessment of efficacy, dose response, toxicity and of the occurrence of undesired events complex and the path from the animal experiments to the application in clinical settings particularly challenging. It is feared that the implanted living cells, especially if manipulated *ex vivo* and genetically modified, may undergo neoplastic transformation or cause ectopic tissue formation. This possibility is difficult to assess because the consequent adverse events may arise many years after implantation, and in anatomical locations different from the implantation site.

In addition, difficult upscaling and high costs limit the clinical applicability of protocols involving autologous cell preparations. The use of large batches of allogeneic stem cells would allow the fabrication of off-the-shelf pre-seeded, validated and quality controlled implants ready for implantation. This approach would be encouraged by the evidence that MSCs appear to be poorly immunogenic and even to induce tolerance under specific conditions.[49–52] Nonetheless, the low immunogenicity and the possible immunomodulatory properties of culture expanded MSCs remain controversial and require further investigations. With this regard, human SM-MSC were readily rejected when implanted into immunocompetent

mice, whereas grafting was successful in immunodeficient nude mice or in pharmacologically immunosuppressed immunocompetent mice.[29] Additionally, an immune response with repair failure has been reported when joint surface tissue repair was attempted using allogeneic bone marrow cells but not when using autologous cells.[34] These discrepancies may depend on specific experimental conditions, tissue/organ systems, animal species/strains, or the specific biological readout. Another important consideration is that the acquired differentiated phenotype of the implanted undifferentiated stem cells may result in the loss of the immunological "privilege", and consequent rejection of the cells.[53]

Another concern for the use of *in vitro* expanded somatic cells is the risk of tumour formation. As opposed to ES cells, which are known to have an intrinsic capacity to form teratoma *in vivo*, somatic primary cells including chondrocytes and MSCs are commonly regarded as non-tumourigenic. However, sporadic neoplastic transformation of primary cells during prolonged *in vitro* culture expansion has been reported with human adipose tissue-derived MSCs[54] and therefore represents an at least theoretical concern. In over 15 years of clinical use of expanded chondrocytes for ACI there has been no report of tumour formation. In addition, several recent clinical trials have reported the safety of adult stem cells.[5–8] However, specific studies are needed to evaluate this particular safety issue.

Embryonic stem (ES) cells are also advocated for tissue engineering applications. The ES cells are pluripotent cells obtained from the inner cell mass of embryos, which can be expanded extensively *in vitro* while maintaining their original undifferentiated phenotype and pluripotent differentiation potential. Due to their pluripotency, they can theoretically be used to repair any tissue of the body. However, undifferentiated ES cells implanted as such tend to form tumours, unless they are pre-differentiated before implantation. Nonetheless, because of the risk of tumour formation, the use of ES cells is currently pursued mainly for severe diseases, in which there is little therapeutic alternative such as advanced Parkinson's disease[10,55] or poorly controlled diabetes mellitus.[56] Another limitation to the use of ES cells for therapeutic applications is the risk of rejection. Two approaches have been proposed to circumvent this problem. The first solution foresees the implementation of cell banks for collections of a great number of clinically graded ES cell lines obtained from "genotyped" embryos. This would ensure, on the long term, the availability of a large array of HLA types to match a wide number of potential recipients. The advantage of this approach over traditional organ transplantation is that, because of the great

expandability of ES cells, every single line would be maintained in the bank and be used for a theoretically unlimited number of recipients, whereas, in traditional organ transplantation, one donor could only help one matched recipient. Another important advantage is that while with organ transplantation the logistics are complex and donor and recipient need to be ready almost simultaneously, cells can be stored and thawed for immediate use in HLA matched recipients. This approach, however, finds limitations in ethical issues for derivation of new ES cell lines. Indeed, at present, human ES cells are derived from human blastocysts and the debate as to whether it is ethical to destroy human blastocysts to obtain ES cells is felt very strongly by the general public as well as by the governments.

The second approach is "therapeutic cloning" in which "autologous" ES cells are obtained implanting the nucleus of a somatic cell of the prospective recipient into an enucleated oocyte.[55,57] This approach is still far from the routine clinical application because of its low efficiency, potential problems related to incomplete nuclear reprogramming, and ethical concerns.[55,57]

Biomaterials

Until a few years ago, biomaterials were used essentially to retain the cells in the site of implantation and to confer specific biomechanical properties to the repair tissue. Preparations of naturally occurring biomaterials, including demineralised bone matrix,[58] hyaluronan,[44,59] collagen gels, and collagen membranes[60] have been extensively popular over the years in a variety of applications. Several more or less resorbable synthetic or semi-synthetic materials have been proposed, with the advantage, over natural products, of a progressively more defined product identity characterisation and batch to batch consistency.[22,60] These biomaterials include porous ceramics, bioactive glass, polyglycolic and polylactic acid, in many variants and composites.[60] Although limited defects cells can be implanted in suspension, in large lesions, scaffolds are necessary to give support to the implanted cells and to keep them in the site desired. This is the case of end stage OA with eburnated condyles, where there is very little cartilage left and the tissue engineer needs therefore to reproduce *ex vivo* the specific architecture of a functional articular cartilage tissue to cover the eburnated condyle. Using acid-soluble collagen as scaffolding material seeded with culture-expanded bone marrow cells, Wakitani *et al.*[35] have successfully repaired eburnated condyles of patients with advanced OA. Not only do

scaffolding materials provide a valid substrate for cell adhesion, proliferation and biomechanical support, but they can, in some cases, influence or even instruct cell differentiation, tissue patterning and maturation.[44,60,61] Already in the sixties Marshall Urist described the potent bone-inducing capacities of demineralised bone matrix (DBM) and postulated the presence in its preparations of soluble factors,[62] which afterwards were cloned and characterised as a large family of morphogens known as bone morphogenetic proteins (BMPs).[63–66] The use of these and analogous secreted factors have been proposed for tissue engineering purposes of bone and cartilage.[22,43,66] Modern biomaterials, therefore, are engineered also as a controlled delivery system of bioactive molecules to achieve a coordinated process of cell differentiation and tissue repair.[67]

One of the major limitations of the first generation bioactive biomaterials such as DBM is that the amount and the kinetics of release of the bioactive molecules are dependent on many variables including the source and the batch preparation. The recent development of "smart" self-assembling polymers that can guarantee a controlled release of bioactive molecules enhance the consistency of the outcome, and most importantly allows upscaling and tailored applications in which specific bioactive substances can be released or presented locally. Self-assembling synthetic peptides can be used to generate 3D scaffolds that polymerise spontaneously *in situ*. One major advantage of this approach is that, by modifying the sequence of the monomeric components, it is possible to include bioactive molecules and achieve high density presentation of such molecules to cells incapsulated within the scaffold microenvironment.[68] As an example, Silva *et al.*[68] have generated a self-assembling peptide carrying the neurite-promoting laminin epitope that, when mixed with a suspension of neural progenitor cells, rapidly assembled itself into nanofibres encapsulating the cells presenting them the bioactive peptide at high density. This resulted in rapid neuronal differentiation of the progenitor cells while preventing development of astrocytes. Similar technologies can be used to achieve a controlled delivery of soluble molecules. As an example, Lutolf *et al.*[69] have manufactured a hydrogel containing pendant oligopeptide ligands to promote cell adhesion and ingrowth, and matrix metalloproteinase cleavage sites as linkers between the individual chains. Such hydrogel, engineered to deliver BMP-2 at the site of critical size cranial defects in rats, was rapidly infiltrated by cells and replaced with bone tissue. These technologies could be used in many contexts to generate microenvironments that promote differentiation of stem cells and preserve the phenotypic stability of the repair tissue.

Mechanisms of Action

It is tempting to assume that the implanted cells, being multipotent, simply replace the missing components of the damaged tissue. However, there is increasing evidence that this may not be the only or even the primary mechanism. In some experimental conditions, paracrine effects and immune regulation of the implanted cell populations play a role in functional restoration that appears to be much greater than their structural contribution to the repair tissue.[70–73] For instance, the transplantation of c-kit positive bone marrow cells in a mouse model of diabetes mellitus reduced hyperglycemia mainly by triggering regeneration of the recipient's own pancreatic cells rather than by a direct contribution of the donor's cells to the regenerated tissue.[70] In another study, the administration of human cord blood-derived CD34 positive cells to immunocompromised mice subjected to experimental stroke 48 hours earlier induced neovascularisation in the ischaemic zone, thereby providing an environment conducive for neuronal regeneration.[72] Therefore, the mechanisms by which transplanted cells restore anatomy and homeostasis are more complex than previously believed. Hence, the understanding of these mechanisms is necessary to develop potency assays, which should address mode of action relevant to specific aspects of the repair process. Unveiling the molecular basis of the paracrine and modulatory effects of cell-based therapies for tissue repair would also open the opportunity to develop "cell-free" approaches in which specific molecules are delivered in a more controlled manner to facilitate endogenous repair.

Indeed, the joint environment is rich of mesenchymal progenitor cells potentially incoming from bone marrow, periosteum, synovial membrane, and articular cartilage itself.[22,24,28,29,31,32,74–77] Therefore, to circumvent the problems related to *ex vivo* manipulation of cell populations, an alternative or complementary approach for joint tissue repair would be to recruit resident MSCs at the site of damage and trigger the subsequent steps of the repair process, such as proliferation, patterning, differentiation and maturation *in vivo* by controlled release of specific morphogens using, for instance, bioactive scaffolds ideally implanted arthroscopically, without the need of an open knee surgery. A crucial step to achieve this is the identification of the appropriate molecular signal(s). Understanding the cellular and molecular mechanisms of tissue repair will be instrumental to building the necessary knowledge to ensure that the appropriate bioactive factor is delivered to the appropriate site at the right time and in the optimal dose to support the local residual mechanisms of repair.

Conclusion

The potential benefits of treating diseases by using cell-based approaches and biomaterial sciences are enormous. In a few years they have moved the boundaries of regenerative medicine going from bench to bedside. Nonetheless, a number of issues need to be solved as these technologies are being translated into routine clinical application. Good clinical practice requires the clinician to question product identity characterisation, definition of appropriate quality controls, potency measurements, reproducibility and consistency, immunogenicity, validation and appropriate regulation of these new treatments.

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Adult Stem Cells as a Treatment for Liver Diseases

Nataša Levičar

Chronic Liver Disease

Liver diseases impose a heavy burden and affect approximately 17% of the population. Cirrhosis, the end result of long-term liver damage, has long been an important cause of death in the UK and showed a large increase in death rates over the past 20 years.[1] Cirrhosis is a progressive liver disease and is marked by the gradual destruction of liver tissue over a period of time. Several liver diseases fall under this category, including fibrosis of the liver, and hepatitis B and C viral infections. At the cirrhotic stage, liver disease is considered irreversible and the only alternative is orthotopic liver transplantation. While orthotopic liver transplantation cures chronic liver disease and a variety of metabolic and genetic deficiency disorders, the increasing shortage of donor organs restricts liver transplantation. The number of patients waiting for orthotopic liver transplantation is far greater than the organ supply, and there is obviously a demand for new strategies to supplement orthotopic liver transplantation.

In many diseases that require liver transplantation, correction of hepatocyte functional deficiency is the prime goal of the procedure. There is growing evidence in support of the role of stem cells as a unique source for cell transplantation and as an attractive alternative for the treatment of liver diseases.

The current concept of liver regeneration can be modelled as at least a three-tier system of cell replacement.[2] First, mature hepatocytes and cholangiocytes contribute to normal cell turnover and responses to certain types of mild injuries. Second, there is an intra-organ stem cell compartment, located mostly in the smallest, most proximal branches of the biliary tree, i.e. the canals of Hering and the intralobular bile ducts.[3] Reconstructive studies on human livers in massive hepatic necrosis demonstrated that the proliferating cells of the ductular reaction differentiate into hepatocytes. The same is true in the case of cirrhosis where activation of an intrabiliary stem cell compartment gives rise to newly formed hepatocytes. The third tier of liver cell replacement consists of stem cells entering from the circulation. This cell compartment, in part of bone marrow origin, enters the liver in a seemingly random distribution, as isolated mature cells, or in a portal and periportal distribution when there is marked injury.[4] In this latter mode, responding to severe injury, they first enter as an intermediate cell population, which then mature into hepatocytes. In search of generating a clinically viable and sustainable source of functional hepatocytes, various strategies are currently being explored. Possibilities include the expansion of existing hepatocytes, differentiation of progenitor/stem cells in the liver, differentiation of embryonic stem cells and extrahepatic adult stem cells, particularly those from the bone marrow.

Cellular Therapeutics

Cell therapy has emerged as a novel approach for the treatment of many human diseases. The aim of cell therapy is to replace, repair or enhance the biological function of damaged tissues or organs, which can be achieved by transplanting cells to a target organ in sufficient numbers so that they can survive long enough to restore the normal function of organs and tissues. It is believed that the organs and tissues treated by this approach can perform their normal function more efficiently than those treated by conventional therapies such as transplantation. Several strategies are currently being employed and evaluated to find the appropriate cell for each specific

disease. Possible candidates include autologous primary cells, cell lines, various stem cells including bone marrow stem cells, cord blood stem cells and embryonic stem cells.[5] In recent years, the use of stem cells for regenerative medicine has been proposed and several studies have shown a great promise that stem cells hold for cell therapy.[6] The ethical and legal issues associated with embryonic stem cells have shifted the focus to adult stem cells and their regenerative potential has been under intense investigation. Adult stem cells usually form only 1% to 2% of the cell population, their main role being the replenishment of the tissue's cells in appropriate proportions and numbers in response to "wear and tear" loss or direct organ damage.[7] Adult stem and progenitor cells possess the capability of self-renewal and differentiation into at least one or more mature cell types. Most adult tissues contain multipotential stem cells, i.e. cells that are capable of lifelong renewal and produce a limited range of differentiated cell lineages appropriate to their location.[8] Such properties make it possible to use adult stem cells to regenerate damaged or senescent cells throughout life.

Adult Haematopoietic Stem Cells for Hepatocyte Generation

Stem cells are undifferentiated and highly clonogenic, with self-renewal capacity and multilineage differentiation potential, and are responsive to environmental cues.[9] These properties make them ideal candidates for stem cell-based therapies and tissue engineering. The most studied adult stem cells are haematopoietic stem cells (HSCs), which sustain the formation of blood and immune system throughout life. The bone marrow compartment is largely made up of committed progenitor cells and non-circulating stromal cells [called mesenchymal stem cells (MSC)] that have the ability to develop into mesenchymal lineages and HSCs.[10–12] It was previously taught that adult stem cells were lineage restricted, but recent studies demonstrated that bone marrow-derived progenitors in addition to haematopoiesis also participate in the regeneration of ischaemic myocardium,[13] damaged skeletal muscle[14] and neurogenesis.[15]

Focusing attention on the liver, several independent reports have demonstrated that adult bone marrow cells can give rise to different hepatic epithelial cell types, including oval cells, hepatocytes and duct epithelium.[4,16–20] These observations have resulted in the hypothesis that bone marrow

resident stem cells, specifically HSC, could be an important source for liver epithelial cell replacement,[21] particularly during chronic injury. Several cytokines and growth factors have been shown to promote hepatic differentiation *in vitro* and in majority of the studies most commonly used are hepatocyte growth factor (HGF), transforming growth factor (TGF) and epidermal growth factor (EGF).[22–24]

In vitro studies showed that rat bone marrow contains a subpopulation of hepatocyte-oriented cells expressing α-fetoprotein (αFP), c-met, CD34 and c-kit.[25,26] Similarly, Okumuto *et al.*[27] observed that those cells cultured in the presence of HGF expressed liver-enriched transcription factor HNF1α and cytokeratin CK8. A subpopulation of murine mononuclear bone marrow cells isolated by chemotaxis to the α-chemokine stromal-derived factor-1 (SDF-1) expressed mRNA for αFP and population enriched for Sca-1 expressed mRNA for αFP, c-met and CK19.[28] Purified murine HSC differentiated into liver cells expressing (αFP, GATA4, HNF4, HNF3β, HNF1α) and mature hepatocyte markers (CK18, albumin, transferrin) when co-cultured with injured liver tissue.[29]

MSC can be isolated as a growing adherent population and can differentiate into osteoblasts, adipocytes and chondrocytes.[10] It has been shown that subpopulation of human MSC, known as multipotent adult progenitor cells (MAPC), can differentiate into hepatocyte-like cells *in vitro* in the presence of HGF and fibroblast growth factor-4 (FGF-4),[30] and shows pluripotency in SCID-recipient mice.[30] However, there is a substantial delay between the time MAPC are isolated and the time they can be shown to differentiate into hepatocyte-like cells, and their use in the clinical applications has been questioned. Lee *et al.*[31] showed hepatic differentiation of MSC *in vitro*, in the presence of HGF and oncostatin M. After a long differentiation period of 4 weeks, cells expressed marker genes specific for liver cells and demonstrated *in vitro* functions characteristic of liver cells, including albumin production, glycogen storage, urea secretion, uptake of low-density lipoprotein and phenobarbital-inducible cytochrome P_{450} activity.

Studies on human bone marrow cells showed that they are also able to differentiate into cells with liver-cell-like characteristics. These cells cultured on a collagen matrix and in the presence of HGF they expressed liver-specific genes, such as albumin and cytokeratin CK19.[32] When selected by SDF-1 chemotaxis they appear to be multipotent and express αFP.[28] Recently, Kucia *et al.*[28] proposed a circulating pool of tissue-committed stem cells, which are stored in the bone marrow and can be mobilised to different tissues when they are needed.

Stem Cell Trafficking in Liver Injury

The exact mechanism of stem cell trafficking in liver injury is still unknown. Tissue repair and regeneration after injury is thought to involve the selective recruitment of circulating or resident stem cell populations.[33] Mobilisation/trafficking/homing of HSC is a multistep process.[34] First, HSCs have to egress from their tissue/organ niche and enter into peripheral blood or lymph. At a certain point of time they sense a chemo-attracting gradient that directs their homing. They attach to the endothelium and subsequently penetrate the vessel wall. At the new site, cells may engraft and find an environmental niche that protects them from apoptosis and allow them to expand and regenerate damaged tissues. Increasing body of evidence suggests that liver injury induces the expression and secretion of various factors, such as SDF-1, interleukin-8 (IL-8), matrix metalloproteases (MMPs), stem cell factor (SCF) and HGF, which facilitate homing and engraftment of HSC to the liver.[35,36] SDF-1 and its receptor CXCR-4 are involved in recruiting inflammatory cells into injured liver[37,38] and are playing a key role in the migration of human progenitor cells to the liver.[33] SDF-1/CXCR-4 axis activates several signalling pathways, which affect cell trafficking and interaction with the intercellular environment. Inhibition of CXCR-4 receptor abolishes homing and engraftment of human $CD34^+$ HSC to the liver of irradiated NOD/SCID mice, whereas local injection of human SDF-1 increases their homing.[33] HGF, which is increased following liver injury,[39] increases the motility of $CD34^+$ cells to SDF-1 signalling by inducing CXCR-4 up-regulation and synergising with SCF. Other factors such as proteolytic enzyme MMP-9, involved in the degradation of extracellular matrix, and IL-8 are thought to be implicated in HSC migration into the liver. IL-8 has the potential to induce the release of HSC into the peripheral blood via the activation of circulating neutrophils and MMP-9.[40] MMP-9 enhances the release of HSC by degrading SDF-1[41] in bone marrow and by facilitating migration of stem cells through basement membrane and therefore help recruiting HSC to the liver.

Adult Haematopoietic Stem Cells for Liver Regeneration

Animal studies

Petersen *et al.*[4] first showed hepatic transdifferentiation in rats, which underwent cross-sex bone marrow transplantation after the hepatic injury

was induced by CCl_4. Examination of the liver revealed that hepatic cells were from donor origin, as detected by FISH and showed the presence of Y-chromosome in female animals. The other demonstration of hepatocyte regeneration from bone marrow cells is the extensive repopulation of damaged livers of FAH mice, an inducible animal model of tyrosinaemia type I,[42] in a lethal hereditary liver disease.[43] Bone marrow cells were transplanted into $FAH^{-/-}$ mice and after the engraftment, liver failure was induced. In this system, the kinetics of liver repopulation by HSC is slow, although significant. The first hepatocyte generated from HSC appeared 7 weeks after the transplantation. Twenty-two weeks after the transplantation, reconstitution from transplanted HSC constituted approximately 30% of the entire liver. The animals have restored liver biochemical functions and normal weights as well. This led to the conclusion that bone marrow stem cells have the capacity to generate hepatocytes albeit in the presence of injury in which the replicative potential of host hepatocytes was impaired. However, some studies have shown that bone marrow stem cells can repopulate liver even in the absence of any liver injury.

Thiese *et al.*[18] transplanted unfractionated male bone marrow or $CD34^{+}lin^{-}$ cells into irradiated female mice. The liver analysis demonstrated significant levels of donor-derived hepatocytes. Krause *et al.*[44] injected single male HSC into irradiated mice and obtained engraftment in several organs. In addition to hepatic engraftment, they have found male cells in the gastrointestinal tract, bronchus and skin of recipient animals. In support, Wang *et al.*[45] found albumin-expressing hepatocyte-like cells in the livers of mice transplanted with highly purified HSC. Moreover, transplantation of bone marrow stem cells reduced CCl_4-induced liver fibrosis.[46] However, Wagers *et al.*[47] failed to observe HSC contribution to liver when GFP-labelled HSCs were transplanted into irradiated mice in the absence of liver damage. Kanazawa and Verma[48] used various liver injury models to assess hepatic regeneration and concluded that there was little or no contribution of bone marrow cells to the replacement of hepatocytes. Similarly Dahlke *et al.*[49] observed the same by using retrorsine/CCl_4 model of acute liver injury.

Nevertheless, several studies have shown that HSC engraft, repopulate and have survival advantage when transplanted into the injured liver. Mallet *et al.*[50] used JO_2 antibody, the murine anti-Fas agonist to induce hepatic apoptosis. Unfractionated bone marrow cells expressing Bcl-2 under the control of a liver-specific promoter were transplanted into normal mice. Some mice received repeated weekly injections of JO_2 antibody to induce

liver injury. Bone marrow-derived hepatocytes expressing Bcl-2 were only seen in the liver of the mice, which received JO_2 antibody injections. In contrast, no Bcl-2 expression could be detected without the selective pressure of JO_2, implying that differentiation is inefficient under physiologic conditions. In a model for liver fibrosis, 26% of the recipient liver was repopulated with GFP-expressing hepatocytes derived from the bone marrow of GFP transgenic mice.[51] Jang *et al.*[29] reported that murine HSCs were converted into viable hepatocytes with increasing liver injury. They have noted that liver function restored 2–7 days after the transplantation of murine $Fr25lin^-$ cells into irradiated and CCl_4-injured murine liver. Although the contribution of HSC to hepatocyte lineages *in vivo* still remains divisive, the differences between the studies may in part reflect the types of cells used, different injury models used and the method used to detect engrafted stem cells.

Transdifferentiation or Fusion

The mechanism by which extrahepatic stem cells regenerate the liver is not yet resolved. Transdifferentiation of stem cells into hepatocytes and fusion of haematopoietic cells with existing hepatocytes have been suggested as explanations. Terada *et al.*[38] showed that bone marrow cells can fuse with embryonic stem cells and adopt a phenotype of recipient cells in cell culture, suggesting that the plasticity of bone marrow cells is a consequence of the fusion of bone marrow-derived cells with endogenous hepatocytes. This conclusion was confirmed by Wang *et al.*[52] and Vassilopoulos *et al.*[53] Whereas Wang *et al.*[52] transplanted female bone marrow cells into male mice with $Fah^{-/-}$ deficiency and found that the repopulating hepatocytes in the liver were heterozygous for alleles unique to the donor marrow. Cytogenetic analysis demonstrated cells with XXXY and XXXXYY karyotypes in male transplanted mice with female bone marrow, indicating the fusion between donor and recipient cells. Similarly, Vassilopoulos *et al.*[53] showed that the regenerating liver nodules in $Fah^{-/-}$ transplant recipients were derived from donor haematopoietic cells that fused with host hepatocytes. However, other studies suggested that HSC form hepatocytes by transdifferentiation.[29,54] Jang *et al.*[29] co-cultured HSCs, injured liver tissue and showed albumin-producing cells as early as 8 hours, suggesting that the hepatic phenotype was the result of transdifferentiation

due to micro-environmental cues instead of cell fusion. Similarly, Harris *et al.*[54] concluded that bone marrow-derived hepatocytes were not the result of cell fusion. Whatever the underlying mechanism by which bone marrow stem cells participate in tissue regeneration, it is clear that they are actively involved in the reparative process in liver injury.

Human studies

Several groups have also demonstrated the presence of cells of bone marrow origin in the human liver. Alison *et al.*[16] investigated whether adult human HSC contributed to the regeneration of hepatocytes in damaged human liver tissue. They tested livers of female patients who had received a bone marrow transplant from a male donor and detected the presence of Y-chromosome-positive hepatocytes, indicating that they originated from the male donor bone marrow. Similar results were found by Thiese *et al.*[17], who investigated livers from recipients of sex-mismatched therapeutic bone marrow transplantation and orthotopic liver transplantations. They have reported 4% to 43% hepatocytes and 4% to 38% cholangiocytes of bone marrow origin. However, less significant number of donor-derived hepatocytes (4% to 7%) were detected in liver biopsies of female patients, who had undergone peripheral blood transplantation from male donors[55] and after orthotopic liver transplantation (1.6%).[56] Two other studies of liver transplant patients did not detect bone marrow-derived hepatocytes.[57,58] Hove *et al.*[59] examined livers of 16 transplanted patients and reported chimerism of endothelial cells in 14 patients, bile duct epithelial cell chimerism in 5 patients and hepatocyte chimerism in only 1 patient.

The difference and non-consistent results of the published studies could be due to the use of different techniques to identify recipient-derived hepatocytes in transplanted patients. Also, various markers can be used for hepatocyte identification and the accuracy of the methods used for identification is variable.

First published clinical study on stem cell therapy in liver disease was performed by the German group.[60] Three patients were infused by autologous $CD133^+$ cells subsequent to portal vein embolisation of right liver segments. Computerised tomography scan volumetry showed 2.5-fold increase in proliferation rate of left lateral segments compared with the control group of three patients treated without the infusion of stem cells, suggesting that cell therapy could enhance and accelerate the hepatic regeneration. The second clinical study using adult HSCs in the treatment of liver diseases

was carried out by our group.[61] We have performed phase I clinical trial and five patients with liver insufficiency were given G-CSF to mobilise their stem cells for collection by leukapheresis. Between 1×10^6 and 2×10^8 $CD34^+$ cells were injected into either portal vein or hepatic artery. Clinically, the procedure was well tolerated with no observed procedure-related complications. Three out of five patients showed improvement in serum bilirubin and four out of five in serum albumin, even though the trial was designed to be a safety and efficacy study. The results clearly suggested that stem cells contribute to liver regeneration.

Conclusions

Cell-based treatments are an attractive clinical application for a range of medical needs and have already been used therapeutically for many years in malignant haematological diseases and skin grafting. However, for liver diseases, stem cell therapy treatment approach is still in its infancy. Despite recent progress in the laboratory and in the clinical trials, there are still many gaps in our understanding of basic stem cell biology that must be addressed before stem cell therapy can be applied to its fullest potential in the clinic. However, a number of clinical trials using different populations of stem cells to treat a growing number of diseases have already started. Clinical trials in which bone marrow stem cells are injected in the cardiac muscle of patients with myocardial infarction are the most numerous and have been performed with some success.[62] For therapeutic stem cell transplantation to occur, many problems remain to be resolved. It must be defined which patient groups are suitable for this therapy and which stem cell types are the most effective given the underlying pathology. The optimum timing and method of delivery need to be determined as they may have a significant influence on the outcome of cell transplantation. Long-term side effects of the treatment are unknown as most of the clinical studies are very recent. In addition, there is a growing evidence that transplanted cells, being multipotent, not only simply replace missing tissue but also trigger local mechanism to initiate a repair response. Paracrine effects and immune regulation of the transplanted cells play a role in functional restoration of the tissue.[63,64] In order to achieve the goal of cell therapy, a number of criteria must be considered. These include (1) source of cells; (2) generation of sufficient numbers of cells; (3) maintenance of the differentiated phenotype; and

(4) engraftment and homing of transplanted in damaged tissue. Ideally, cells for liver therapies should expand extensively *in vitro*, differentiate into mature liver cells, have minimal immunogenicity and should be able to reconstitute liver tissue when transplanted *in vivo*. It is an exciting time for scientists and clinicians in this field and hopefully we will see stem cell therapy in more clinical applications soon.

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9

The Generation of Pancreatic Beta Cells from Stem Cells: Intra- and Extrapancreatic Sources

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Diabetes mellitus is an incurable metabolic disease characterised by hyperglycaemia, which results largely from either the autoimmune destruction of the pancreatic beta cells (Type I diabetes, T1D), or from insufficient production of insulin to meet the increased resistance of peripheral tissues to the action of insulin (Type II diabetes, T2D), both of which may have severe clinical consequences.

Currently, allogeneic islet cell transplantation is hampered by a shortage of donor tissue and the need for lifelong immunosuppressive therapy, and thus, the generation of functional beta cells from stem cells may prove the most effective treatment for diabetes. Current research into the various sources of stem cells providing the most efficient and viable source of beta cells, and the optimal means of inducing these stem cells to differentiate and form functionally competent beta cells, is discussed in detail in this review.

There is evidence both for and against the presence of an indigenous stem cell population within the adult pancreas, and it is possible that adult somatic stem cells, in particular those originating in the bone marrow,

and/or pluripotent embryonic stem cells may be capable of differentiating to form beta cells.

There have been several reports of beta cells derived *in vitro* from both extrapancreatic adult stem cells and embryonic stem cells, and long-term reversal of hyperglycaemia in diabetic mice by implantation of beta cells derived from both embryonic stem cells and bone marrow cells has been shown. However, these results have proved difficult to reproduce, and it is argued that insulin-secreting cells derived from extrapancreatic adult and embryonic stem cells may not be functionally competent.

Although the regeneration of the damaged pancreas using stem cell-derived beta cells looks promising, much progress must be made before this stem cell therapy can be applied clinically.

Introduction

The pancreas

The pancreas is composed of two distinct compartments, the endocrine pancreas and the exocrine pancreas. The exocrine pancreas comprises the bulk of the tissue and is made up of acinar cells that specialise in the secretion of digestive enzymes *via* the epithelial pancreatic ducts. Located throughout the exocrine pancreas are clusters of endocrine cells known as the *islets of Langerhans*, highly vascular, innervated structures composed of 4 distinct lineages; the glucagon-producing α cells, the insulin-producing β cells, the somatostatin-producing δ cells, and the pancreatic polypeptide-producing γ cells, each of which delivers its hormones to the bloodstream. The global prevalence of diabetes was approximately 2.8% in 2000, and it is expected to double by 2030, largely due to an ageing population and a rapid rise in the incidence of obesity.[1]

Diabetes mellitus

Type 1 diabetes

Type 1 diabetes (T1D) accounts for up to 10% of all diabetes, and is usually diagnosed in children and adolescents, although demographics are changing, predominantly due to the emergence of Type 2 diabetes (T2D) in adolescents, most likely as a consequence of the recent epidemic of obesity in this generation.[2] T1D is characterised by abnormally high blood glucose levels following autoimmune β cell destruction, and can lead to serious long-term complications including blindness, kidney failure, stroke, heart and vascular diseases.[3] T1D is currently incurable, and is most commonly

treated by lifelong daily insulin injections, although if inappropriately managed, this carries an increased risk of hypoglycaemia, and the restoration of normoglycaemia does not always alleviate the aforementioned secondary complications, and patients often have a reduced life expectancy.[4]

Type 2 diabetes

T2D is the most common form of diabetes, characterised by insulin resistance and impaired glucose tolerance, often with an associated increase in β cell mass due to over-production of insulin in an attempt to overcome this resistance. T2D is initially managed by diet and oral hypoglycaemic agents, although in approximately 50% of cases, the β cells fail, creating a requirement for insulin injection and often resulting in T1D-associated secondary side-effects. T2D usually occurs in genetically-predisposed people over the age of 40, although it is increasingly reported among younger people.

Islet Replacement Therapy

β cell replacement can restore glycaemic control and provide prolonged independence from exogenous insulin.[5] Over recent years, advances have been made in islet transplantation methods based upon the initial success of the Edmonton Protocol, developed to treat patients with T1D. In the initial Edmonton Protocol in 2000, 100% insulin-independence was observed in 7 patients each of whom received islets isolated from 2 immunologically compatible donor pancreata in conjunction with a glucocorticoid-free immunosuppressive regimen, wherein the transplanted islets were infused *via* the portal vein.[6]

This method resulted in insignificant side-effects, and is favourable to the transplantation of a whole pancreas as it avoids major surgery. There has been a subsequent exponential increase in clinical islet transplantation, although follow-up investigations have revealed a progressive attrition of the grafted islets, with only 50% of patients remaining insulin-free after 5 years, possibly due to a mixture of slow rejection, recurrence of the autoimmune disease or exhaustion of the β cells.[7]

Moreover, the Edmonton Protocol is hindered by a lack of donor tissue, as freshly isolated islets from at least 2 donors are required to achieve sustained insulin independence.[8] It remains to be seen whether the Edmonton

Protocol can be justified against the shortage of donor pancreata and the risks of life-long immunosuppression.[9]

Islet Cell Generation from Stem Cells

In pursuit of generating a clinically viable and sustainable source of functional β cells, various strategies are currently being explored, and possibilities include the expansion of existing β cells, differentiation of progenitor/stem cells in the pancreas, and the differentiation of embryonic stem cells and extrapancreatic adult stem cells, particularly those from the bone marrow and liver, to form β cells. However, as outlined in this review, although much excitement has been generated from initial progress in this emergent field, it is vital to tread with caution as results are currently open to huge misinterpretation and are frequently contradictory or non-reproducible.

Intrapancreatic stem cells

Evidence for self-replication by existing adult β cells

It has not been possible to isolate and characterise a definitive stem cell population within the adult pancreas to date, possibly due to the lack of definitive markers within this postulated primitive cell population. The β cells are the most abundant lineage in the islets, and are generally believed to undergo slow, continual turnover in the adult pancreas. The β cell mass increases during pre- and neonatal life in rodents and humans, but the rate of proliferation declines dramatically with age.[10] Adult rodent β cells proliferate at a rate of 2% to 3% of cells per day,[11–13] which was then correlated with data showing a continual increase in β cell mass throughout adulthood in rats,[14] by implicating β cell proliferation in slow self-replication and expansion of β cell numbers in adulthood. Zajicek *et al.*,[15] proposed that cell migration and differentiation in the pancreas and other organs occurs *via* the "streaming hypothesis". This study utilised DNA incorporation of tritiated thymidine by cycling cells in the male rat pancreas to investigate the route and distance travelled by label-retaining cells at various timepoints cells gradually streamed away from the ducts; the intensity of labelled acinar cells decreased with time, whereas the islet cells showed a labelling peak on day 42, which then declined. It was proposed that acinar cells are "islet cell precursors" which emerge from the ducts and continuously stream to

the islets at a low rate, where they replicate and differentiate to form islet cells.[15] Extraordinary observations have not been confirmed to date.

A more recent study in mice has used a continuous bromodeoxyuridine (BrdU) labelling protocol, which is preferable to the single BrdU injection 6 hours prior to sacrifice used previously,[14] as it allows calculation of β cell proliferation in terms of the entire cell population. In this study, three outcomes of BrdU labelling were predicted.

Firstly, if β cells have a rapid turnover, as formerly suggested,[13] and the period of BrdU administration exceeded β cell lifespan, then all cells would become labelled;

Secondly, if β cells have a slow proliferation rate, then only a limited number of cells would incorporate the BrdU label;

Thirdly, heterogeneity within the islets might result in nonlinear BrdU labelling, wherein a limited population of rapidly dividing but short-lived β cells could coexist with slowly proliferating long-lived β cells.

Results showed that adult β cells have a minimal replication rate, and acquire BrdU label very slowly, with only 1 in 1400 adult β cells proliferating per day.[16] The discrepancy between these results and previous results obtained in rats,[11–13] is unlikely to be due to a species difference, but is more probably a result of the failure of previous studies to specifically detect insulin-positive cells within the islets, whereas double immunostaining for BrdU and insulin was performed in this more recent study.[16]

Studies of β cell proliferation in the human pancreas are often restricted to pancreata from only a few donors, and *in vitro* preparations of human islets are often contaminated by other endocrine lineages, though it is generally accepted that human β cells can undergo proliferation, albeit at a significantly reduced rate in comparison to rodents.[17,18] Human β cell proliferation can be stimulated, albeit modestly, when islets are grafted into insulin-resistant nude mice, but this ability is seen to decrease with increasing donor age.[19]

The subject of islet stem cells was directly addressed by Dor *et al.*,[20] who bred RIP-CreER mice, in which the insulin promoter drove the expression of a tamoxifen-dependent Cre-recombinase enzyme in the β cells, with the Z/AP reporter strain, which constitutively expresses the β-galactosidase (lacZ) gene flanked by two loxP sites, followed downstream by the human alkaline phosphatase protein (HPAP) reporter gene, to form bigenic RIP-CreER;Z/AP mice. Following a "pulse" of tamoxifen to RIP-CreER;Z/AP mice, CreER translocates to the nucleus and allows Cre-mediated removal of the loxP-flanked lacZ STOP sequence, resulting in the permanent,

heritable expression of HPAP, which is detected immunohistochemically in β cells.

Following a defined "chase" period, during which cellular turnover had occurred, all islets contained HPAP-labelled cells, and importantly, the percentage of HPAP- and insulin-positive β cells remained stable for up to 12 months post-tamoxifen, indicating that pre-existing β cells were a self-duplicating population, and did not arise from undifferentiated (stem) cells. The authors then further investigated the possibility that a stem cell population(s) may be employed only during periods of increased cellular turnover in the pancreas, by giving mice a 70% pancreatectomy (Px), 14 days after the tamoxifen pulse, and administering BrdU to label cells in S-phase. Therefore, β cells that were generated after Px would express BrdU and insulin, and would be HPAP-positive or -negative, depending on their respective origin from a pre-existing β cell, or an undifferentiated (insulin-negative) stem cell. 61.5% of BrdU-expressing β cells were HPAP-positive, and 61% of the total cells counted were HPAP-positive, indicating that all newly-generated cells were derived from pre-existing β cells.[20] It is, however, important to state that this study does not rule-out the presence of a pancreatic stem cell population, as the stem cells themselves may express insulin, and it has been argued that the number of islets analysed in this study was inadequate.[21] Furthermore, when a strong promoter is expressed for a prolonged period, recombinase-dependent excision can occur in the absence of tamoxifen.[22]

Islet neogenesis from the ductal epithelium

Pancreatic regeneration has also been proposed to occur by *neogenesis*, i.e. the production of new islet cells by a population of duct-located potential pancreatic stem cells, based partly on the observation that islets can be seen budding from pancreatic ducts during regeneration, with hormone-positive cells being seen to form within the ductal epithelium.[21,23,24]

β cells may be formed by epithelial-mesenchymal-epithelial transition (EMET)

A mechanism of β cell differentiation was suggested from observations *in vitro* that adult pancreatic cells undergo an epithelial-mesenchymal transition (EMT), wherein they initially dedifferentiate to a fibroblast-like state and then redifferentiate to form islet cell clusters, in response to regenerative stimuli. This apparent dedifferentiation of adult pancreatic cells in culture

has been shown in the ductal epithelium of the rat,[21] and in cells of isolated human islets,[25] which has led to the proposal that EMET may account for reports of the apparent generation of pancreatic β cells from other pre-existing pancreatic lineages.[20] However, it is vital to note that insulin production by cells in culture in the aforementioned studies was vastly reduced when compared to normal adult β cells, and therefore it cannot be assumed that this *in vitro* mechanism is a true reflection of how β cells are produced *in situ*. Moreover, distinguishing which cell lineages are actually producing β cells in human pancreatic culture systems is hampered by contamination of ductal, acinar, stromal and endothelial cells lineages in the starting culture, as it is difficult to obtain an 100% pure cell population.

This was recently tackled by Gao *et al.*,[26] who created endocrine cell-depleted cultures by magnetic cell separation. Cells within non-endocrine cultures, although capable of proliferation, did not form β cells. This indicates that the ductal epithelial cells may not comprise a pancreatic progenitor/stem cell lineage, as presumed previously, and further studies of this nature are essential before any conclusion can be drawn.

Possible markers of intrapancreatic stem cells

Nestin does not represent a pancreatic stem cell marker

The neural stem cell marker, nestin, was proposed as a potential marker of β cell progenitor cells, following isolation of nestin-expressing cells from the ductal epithelia of the rat and human pancreas. Nestin-expressing cells proliferate *in vitro* and differentiate to form multiple pancreatic lineages.[27] The specificity of nestin as a pancreatic β cell progenitor cell marker was further investigated using primary cultures of nestin-positive cells from fractionated mouse islets (5% of total cells) and ducts (1% of total cells).

Colony-forming cells were present in a low frequency in both nestin-positive and nestin-negative fractions, and nestin was expressed in most cells in colonies derived from both nestin-positive and nestin-negative fractions, and it therefore appears that nestin is not the most primitive marker for pancreatic stem cells.[28] These data are in concordance with previous suggestions that nestin is not a marker of pancreatic progenitor cells,[29–31] and moreover, the utility of nestin as a stem cell marker is controversial since nestin is also expressed by vimentin-positive mesenchymal cells and cells of the pancreatic vasculature.[32,33]

In the study by Seaberg *et al.*,[28] single colony-forming cells, termed pancreas-derived multipotent precursors (PMPs), differentiated to form

multiple pancreatic lineages *in vitro*. However, the true nature of PMP-derived β cells is questionable because although akin to endogenous pancreatic β cells, PMP-derived cells expressed insulin I, glucokinase, GLUT2, Pax6, Beta2/NeuroD, Hlxb9, Isl-1, Nkx2.2, Nkx6.1 and PDX-1, they lacked expression of insulin II, had reduced insulin I content and insulin secretion, and displayed a limited renewing capacity when compared to true β cells. The value of transplantation of PMP-derived cells for the treatment of diabetes is unknown and must be investigated before these cells are considered to be functional β cells.

c-Met is a possible marker of intrapancreatic stem cells

Suzuki *et al.*[34] isolated cells from neonatal and adult mouse pancreata that expressed the hepatocyte growth factor (HGF) receptor, c-Met, which is involved in pancreatic development[35,36] and has been shown to enhance budding of islet-like structures from ductal epithelial cells *in vitro*.[37] Colonies formed from c-Met-positive pancreatic cells were highly proliferative and underwent multilineage differentiation, forming clonal populations of pancreatic endocrine, acinar and ductal lineages. Therefore, c-Met was proposed as a pancreatic stem cell marker, supported by the presence of c-Met-immunoreactive cells within the pancreatic ducts.

However, cells derived *in vitro* from these c-Met-positive lineages were not fully functionally competent, and moreover, following transplantation of c-Met-positive cells, only very low levels of donor-derived pancreatic endocrine cells were observed and these studies do not rule out the possibility that these cells are a product of fusion between a donor cell and a pre-existing endocrine cell. The results of this study have never been reproduced, and further studies must investigate the validity of c-Met as a pancreatic stem cell marker, including the role of these cells in pancreatic regeneration *via* differentiation to form functional pancreatic lineages, before c-Met can unequivocally be described as a pancreatic stem cell marker.

ABC transporters

Recently, ABC transporters such as ABCG2 and MDR-1 have been proposed as potential stem cell markers for a broad range of adult tissues.[38] Responsible for the so-called *side population* phenotype, ABC transporters (notably ABCG2) have been shown by northern blotting and immunohistochemistry to be expressed in both normal and malignant

pancreatic tissue.[39–41] Lineage-negative side population (SP) cells isolated from human foetal islets have been shown to have high clonogenic and proliferative potential compared to non-SP cells, and can be induced to form lineages which produce glucagon or insulin, Glut2 and PDX-1 *in vitro.*

However, insulin release in response to glucose is reduced compared to primary β cells.[42] ABC transporters are cytoprotective in a broad range of differentiated cell types, and correlation with other markers must be shown before they may be used successfully to delineate potential pancreatic precursor cells.

Extrapancreatic sources of stem cells for β cell generation

Bone marrow stem cells

Adult stem cells are classically defined by their ability for limitless self-renewal, and their capacity for multipotential differentiation, resulting in the formation of the entire specialised cell repertoire within their native tissue. Adult stem cells maintain homeostasis by proliferating and differentiating to form the cells required to regenerate damaged or diseased tissue.[38]

Emerging data regarding the surprisingly flexible potential of stem cells in their differentiation capacities has recently challenged the classical belief that tissue-specific stem cells are restricted to the production of lineages within their tissue of residence. Some stem cells, notably those in the adult bone marrow, can seemingly abandon their intrinsic pattern of cellular differentiation, and can engraft within foreign tissues and contribute to functional adult lineages within these tissues, although the migratory signals and molecular mechanisms that influence the stem cells to differentiate *via* specific pathways are currently not known.[43]

It appears that selection pressure induced by target organ damage can improve the efficacy of this process, which has been shown in various tissues in both mice and humans.[44–50] It is possible that stem cells respond to specific signals from damaged or diseased tissues whereupon they migrate to, and engraft within this tissue, and aid regeneration and remodelling by contributing to differentiated adult cells. Consequent to this discovery, adult stem cells now represent an entire new field of regenerative medicine, and may hold the key to the treatment of a number of diseases such as cancer, cardiovascular disease, neurodegenerative disease and diabetes.[43]

The potential for bone marrow cells to generate immunogenic tolerance and reduce graft rejection episodes has been demonstrated in both[51,52] and clinical[53,54] diabetes, following transplantation of bone marrow cells

in combination with donor bone marrow major histocompatibility complex (MHC) class I-matched islets. However, the apparent capacity of bone marrow cells to differentiate to form cells with a β cell phenotype is a more recent observation.

Evidence for bone marrow-derived β cells

Ianus *et al.*[55] utilised a Cre-loxP system, wherein transgenic mice expressing Cre-recombinase under the control of the rat insulin II promoter (INS2-CRE) were crossed with transgenic mice expressing GFP downstream of the ROSA26 locus, floxed by three STOP codons (ROSA-EGFP). In the INS2*EGFP offspring, Cre-recombinase is activated in insulin-producing cells, and recognises the LoxP sequence of the STOP codons and therefore removes the ROSA26 locus, and cells permanently express GFP. Bone marrow from INS2*EGFP male mice was transplanted into wild type female mice, and therefore only bone marrow-derived β cells in the recipient pancreata would be EGFP-positive.

In a second experiment, bone marrow from INS2-CRE male mice was transplanted into female ROSA-EGFP mice, and therefore, if true bone marrow cell transdifferentiation had occurred, β cells would be of male origin but not express EGFP, and EGFP expression in cells expressing CRE would indicate fusion of a donor bone marrow cell with a pre-existing β cell. The results showed that 4–6 weeks following transplant, bone marrow cells engrafted within the adult pancreas and contributed to approximately 1.7% to 3% of insulin-immunoreactive cells; these cells also expressed β cell-specific transcription factors *in vivo* and mRNA for several β cell-specific genes *in vitro*, as well as releasing insulin in a glucose-dependent manner.[55]

The second experiment revealed no evidence of cell fusion *in vivo*, thereby indicating that adult bone marrow cells can be directed to differentiate to produce functional β cells in pancreatic islets,[55] although the incidence of these cells was low and it would be interesting to investigate means of enhancing the bone marrow cell contribution to the β cell mass, possibly *via* models of damage or disease.

In a mouse model of streptozotocin (STZ)-induced hyperglycaemia, blood glucose and serum insulin levels were restored to almost normal levels within 7 days following transplantation of bone marrow from GFP transgenic mice.[56] Approximately 2.5% of cells in the pancreata of STZ mice co-expressed GFP and insulin, compared to almost no cells in the non-diabetic control mice. However, RT-PCR showed an absence of the

transcription factor PDX-1 in GFP-positive cells, and it is possible that although the cells adopt a β cell phenotype, they are not fully differentiated β cells.

Indeed, the authors state that bone marrow-derived cells do not contribute to insulin production and the restoration of normoglycaemia, as GFP-positive, insulin-positive cells are not present during the initial stages of blood glucose reduction, although the bone marrow cells are believed to induce endogenous islet cells to undergo proliferation and insulin production *via* an unknown mechanism.[56]

Many of the donor-derived cells in the pancreata of STZ-treated mice expressed PECAM-1 and were therefore believed to be endothelial in nature; likewise other investigators have noted a bone marrow contribution to islet vasculature but little or none to the β-cell mass.[57–59] This indicates a potential role for bone marrow cells in pancreatic regeneration, although this is not believed to be *via* neogenesis of pancreatic islets, but rather, bone marrow-derived endothelial cells are proposed to be a vital accessory to stimulate regeneration by indigenous islet cells.[56]

This observation is consistent with previous studies showing a requirement of inductive paracrine endothelial stimulation for pancreatic development,[60] and it is well documented that a population of cells in the adult bone marrow, known as endothelial progenitor cells (EPCs), play a role in vascular repair and remodelling in a number of specific physiologic and pathologic conditions.[45,61–65]

Bone marrow-derived cells may form extrapancreatic insulin-expressing cells

A recent study produced the surprising result that insulin gene expression is upregulated in multiple extrapancreatic tissues in conditions of hyperglycaemia, and bone marrow appears to be the major source of these proinsulin-expressing cells, thus highlighting the potential of extra-pancreatic cell sources in the treatment of diabetes. Following bone marrow transplantation and STZ-induced hyperglycaemia in mice, proinsulin and insulin-positive cells were present in the liver, adipose tissue, spleen, bone marrow and thymus, and many of these cells produced glucagon, somatostatin and C-peptide.[66]

Up to 23% of bone marrow cells in hyperglycaemic mice were immunoreactive for proinsulin, and up to 90% of proinsulin-immunoreactive cells in the liver were of bone marrow origin. This implies that

hyperglycaemia induces proinsulin production in a population of bone marrow cells, which then migrate and home to the liver, although it is also possible that bone marrow cells migrate to the liver in hyperglycaemic conditions, and subsequently acquire the capacity for proinsulin production.

Interestingly, bone marrow-derived, proinsulin-positive cells were observed, albeit rarely, in the acini of the hyperglycaemic pancreas, but were not found in the islets.[66] It is possible that these cells may be the so-called non-haematopoietic tissue committed stem cells (TCSC), identified within the murine bone marrow by Kucia *et al.*,[67] which are released into the peripheral blood following tissue injury and contribute to regeneration in multiple tissues.

The spleen as a potential source of β cells

Transplantation of fresh, unprocessed wild type adult splenocytes into diabetic NOD mice imposes normoglycaemia and induces long-term disease elimination. Donor-derived islet and pancreatic duct cells were observed, and interestingly, no islets composed entirely of cells of host origin were seen, and pancreatic ducts that were purely of host origin were never seen adjacent to islets containing donor cells. The donor-derived cells contained a normal chromosomal complement and were morphologically normal, suggesting that these cells are formed by splenocyte differentiation and not by fusion of a transplanted cell with an indigenous pancreatic cell.[68]

Stem cells in liver with pancreatic potential

The enormous self-renewing capacity of the liver is well documented, for example, following a two-third partial hepatectomy in the rat, the liver mass returns to a normal size within two weeks,[69] and it is generally accepted that the liver contains a highly efficient stem cell(s) population.[70] Transconversion between the cells of the liver and pancreas has been demonstrated *in vitro*,[71–73] possibly related to the common origin of both tissues from the upper primitive foregut endoderm, suggesting a population of pluripotent cells that retain the capacity to differentiate to form both adult liver and pancreatic lineages.[74]

Candidate stem cells were isolated from foetal mouse livers and, following a defined period of growth in culture, these cells were shown to express insulin, glucagon and somatostatin. Following transplantation into the mouse pancreas, these liver-derived stem cells formed pancreatic duct and acinar cells *in vivo*.[34] Other stem cells isolated from the biliary ducts of

the rat liver, namely oval cells, developed islet-like clusters following long-term culture in a high glucose medium, and expressed genes of pancreatic differentiation including insulin, Pdx-1, Pax-4, Pax-6, glucose transporter 2 (GLUT2), glucagon and somatostatin.[75] These cells were immunoreactive for insulin and glucagon, and secreted insulin in response to glucose. Preliminary data indicated that these cells can reverse hyperglycaemia in diabetic mice,[75] although further studies of the production of pancreatic β cells from oval cells *in vivo* are essential.

Over-expression of PDX-1 induces pancreatic differentiation by liver cells

Human foetal hepatic stem cells, genetically modified to over-express *Pdx-1*, can be activated to express multiple β cell genes *in vitro*, and can synthesise, store and secrete insulin in a regulated manner in response to glucose.[76] Moreover, these cells normalised hyperglycaemia for prolonged periods following transplantation into diabetic immunodeficient mice. Therefore, it is proposed that the human foetal liver contains progenitor cells that, when transduced with *Pdx-1*, may be therapeutic in the treatment of diabetes by replacement of β cells.[76]

Sapir and colleagues investigated the potential of adult human liver (AHL) cells to form insulin-producing β cells in response to *PDX-1*. AHL cells were isolated and transfected with an adenovirus encoding *PDX-1* under the control of the heterologous CMV promoter, and GFP under the control of RIP (the rat insulin-1 promoter). Therefore, cells that activate the ectopic insulin promoter in response to PDX-1 are identified by green fluorescence. 10% to 25% of AHL cells were GFP-positive, and therefore *PDX-1* can induce pancreatic endocrine cell differentiation, even in adult liver cells.

However, the high expression of *Ngn3* in these cells implied that they are retained at a relatively immature state of differentiation. 10% to 25% of these cells produced proinsulin, which is stored within granules and released in response to glucose, although the granules do not contain a dense core as in intact pancreatic islets, but instead resemble those present in other β cell lines that contain reduced levels of insulin.[77] When transplanted into diabetic mice, glucose levels decreased but did not reach normal levels, although it is suggested that selection and enrichment of AHL cells demonstrating insulin promoter activation may enhance the therapeutic response,[78] and although these preliminary studies are promising, further

clarification that AHL-derived cells form true functional adult β cells is necessary.

Pdx-1 can also induce β cell differentiation in mouse liver cells;[79] delivery of an adenovirus expressing rat *Pdx-1* to mice resulted in the expression of rat *Pdx-1* and insulin genes by cells in the liver of these animals, although rat *Pdx-1* expression was not observed in the pancreas, kidney or spleen. In 25 of 34 mice, the insulin-2 gene was expressed in the liver, although the insulin-1 gene was only present in the liver of 10 of 34 mice, and liver extracts from these mice contained insulin protein levels that were less than 1% of that in the pancreas. *Pdx-1*-induced hepatic insulin appeared to normalise blood glucose levels in diabetic mice, although due to a lack of evidence of cell transdifferentiation in this study, it has been suggested that this *in vivo* observation may simply be due to a low ectopic activation of the insulin gene, as *Pdx-1* also acts as a specific transcription factor for the insulin gene itself.[80]

Xenopus tadpole embryos that over-expressed an activated form of *Pdx1* in their liver undergo conversion of liver cells to pancreas cells, often with complete organ transformation of the liver to form a whole ectopic pancreas. The newly-formed pancreatic cells are thought to arise *via* transdifferentiation of liver cells, and can produce insulin, glucagon and amylase with a parallel loss of liver gene expression, indicating that the directed over-expression of *Pdx-1* induces differentiation of both endocrine and exocrine pancreas, although it is important to note that hepatic differentiation was not observed in control experiments using unmodified *Pdx1*.[80]

The same authors have subsequently shown pancreatic differentiation in a human hepatoma cell line (HepG2) following transduction of the *Xenopus Pdx-1* transgene driven by a liver-specific promoter. Cells expressed insulin, glucagon, somatostatin and amylase, although did not express pancreatic polypeptide. Hepatic protein expression was lost, indicating that hepatic cells undergo transdifferentiation to form both endocrine and exocrine pancreatic lineages. Although the incidence of transdifferentiated cells was low, newly-formed β cells were shown to be functional and could secrete insulin in response to glucose stimulation.[81]

These results showing the conversion of liver to pancreas *via* over-expression of the pancreas-specific promoter, *PDX-1*, are promising. Future investigations should deduce the specific lineage in the mouse and human liver that responds to *Pdx-1* and undergoes pancreatic differentiation, and means of enhancing the numbers of transdifferentiating cells, e.g. by combining over-expression of *Pdx-1* with specific growth factors and/or other

transcription factors, are important. Present studies in mouse and human do not prove direct transdifferentiation of liver cells to pancreatic lineages, and often cells are not fully mature or functionally competent. The prospect of tissue conversion by specific promoters and transcription factors represents an exciting new area of therapy.

Evidence for embryonic stem cell-derived β cells

Embryonic stem (ES) cells represent undifferentiated, pluripotent cells derived from the inner cell mass (ICM) of blastocysts, capable of both unlimited self-renewal and, when subjected to appropriate stimulatory factors, differentiate to form adult cells derived from all three embryonic germ layers both *in vivo* and *in vitro*.[82] ES cells can readily be genetically manipulated to overcome the problem of immune rejection, thus circumventing the need for life-long immunosuppressive drug therapy currently associated with islet cell transplantation.[83] However, the vast proliferative capacity of ES cells can cause teratoma or teratocarcinoma formation following transplant into syngeneic animals,[84,85] and this possibility needs eliminating before ES-derived β cells can be used in humans.

In the absence of differentiation factors *in vitro*, ES cells rarely spontaneously differentiate to express β cell markers,[86,87] and it appears that modulation of these cells *in vitro* is essential for the production of cells that show functional characteristics of pancreatic β cells, i.e. glucose-dependent insulin release, specific ion channel activity, capacity for normal levels of insulin production and secretion, and normalisation of glycaemia in animal models of diabetes.[88]

Transfection of a mouse ES cell line with a selection vector i.e., a drug resistance gene under control of the human insulin promoter, allowed the induction of insulin production and secretion *in vitro*, similar to that of normal mouse islet cells, although the insulin content of these cells was far lower than in indigenous β cells. These genetically-manipulated cells formed islet-like cell clusters *in vitro*, one of which was capable of normalising glycaemia when implanted into the spleen of an STZ-induced diabetic mice, although this normalisation was subsequently reversed 12 weeks post-implant in 40% of mice.[89]

Pancreatic β cells are derived from the foregut endoderm during embryonic development, although insulin-producing cells can also be detected within ectodermal-derived tissues, e.g. the developing neuroepithelial.[90] It has therefore been suggested that ES-derived cells that are encouraged to

differentiate along an endodermal pathway may form pancreatic β cells that are more similar to indigenous β cells[90] than those observed in past studies wherein ES cells have been selected based upon their expression of ectodermal markers.[89] Chickens and humans have a single insulin gene, although rodents have two genes, insulin I and II.[91] Insulin I is of endodermal origin and is mainly expressed in β cells at high levels, whereas insulin II is expressed in cells of an ectodermal origin during development and is synthesised in far lower amounts and is secreted rapidly in an inactive form.

Future studies aim to manipulate ES-derived cells to differentiate to express markers of endodermal commitment such as alpha foetoprotein (AFP), FoxA2, FoxA3, GATA4, or GATA5, or alternatively, to manipulate ectodermal-derived insulin-expressing cells, which are more easily obtainable *in vitro* than endodermal-derived cells, to enrich their insulin production.[90]

Following culture in serum-free medium, non-manipulated mouse ES-derived cells selected for enriched nestin expression, were induced to differentiate to form cellular aggregates that expressed a number of pancreatic endocrine cell markers, including insulin and glucagon.[92] However, with the induction of insulin expression in these cells was a concomitant inhibition of cell proliferation, and moreover, these cells produced 50-fold less insulin than normal islet cells, although they were capable of appropriate insulin secretion in response to specific physiological stimuli. The ES-derived cell clusters were grafted subcutaneously into diabetic mice, where they formed islet-like structures, although they did not normalise glycaemia, possibly due to their low levels of insulin production.

Human ES cells have also been shown to form proliferative insulin-secreting cells that co-express insulin and C-peptide, or insulin and somatostatin or glucagon, similar to cells in the developing pancreas,[93,94] thus raising doubts that ES-derived cells do not represent mature β cells of the adult pancreas.

As a word of caution, it is worth noting that studies that rely on insulin immunohistochemistry for evidence of insulin production by ES-derived cells can mistakenly ascribe insulin production with what is actually insulin uptake from the culture medium.[95–97] It is therefore essential that other lines of evidence are sought, including appropriate granule ultra structure and the ability of such cells to rescue diabetic animals for a reasonable period of time (e.g. 1 month).

The transcription factors *Pdx-1*, and *Pax-4* are essential for normal pancreatic development during embryogenesis.[98] Mice that do not express

Pdx-1 do not develop any pancreatic islets,[99] and mice lacking *Pax-4* fail to develop any β cells and become diabetic.[100] *Pdx-1* is expressed in the pancreatic endoderm and is essential for early development, and also regulates β cell insulin gene expression in the adult pancreas.[101] Mouse ES cells, genetically manipulated to over-express *Pax-4* showed a significantly enhanced expression of the genes involved in pancreatic development *in vitro*, and positive selection for nestin expression, had up to 5-fold enhanced levels of insulin production.[102] *Pax-4*-positive ES-derived cells contained insulin-positive secretory granules and could normalise and maintain normal blood glucose levels in STZ-induced diabetic mice, although the transplanted mice often developed tumours in their kidneys and spleens. RT-PCR showed that the ES-derived, *Pax-4*-positive cells expressed insulin, islet amyloid polypeptide (IAPP) and Glut-2 mRNA, and often co-expressed insulin and C-peptide mRNA, indicating that these were functional cells.

The validity of nestin as a marker of pancreatic β cell progenitors has recently been questioned, as nestin is not expressed in pancreatic progenitor cells, but is expressed in the mesenchymal cells of the pancreas and progenitor cells of other tissues including the nervous system.[88] Therefore, positive selection for nestin expression in ES cells may result in the selection of cells that are committed to differentiate to form non-pancreatic lineages including neural cells, and it is now claimed that nestin-negative ES cells can form functional β cells.[88] That nestin-negative, ES-derived cells may prove to be more potent β cell progenitors than nestin-positive ES-derived cells, fits nicely with the aforementioned theory of Roche *et al.*,[90] that ES-derived cells that differentiate to form insulin-expressing endodermal lineages are more likely progenitors of pancreatic β cells than insulin-expressing ectodermal-derived lineages, which differentiate *in vivo* to form neuroepithelial lineages, but do not form pancreatic β cells.

Conclusion

The recent discovery of the capability of stem cells to differentiate from the normal lineage boundaries has presented us with an entire new field of regenerative biology, providing much hope for alternative cell therapies for currently incurable disorders, such as diabetes. Unfortunately at present many observations are contradictory or irreproducible, so until a consensus

is reached, the relative merits of adult stem cells versus ES cells for the treatment of diabetes must remain *sub judice*; it would surely be injudicious to ignore the obvious potential of both ES cells *and* adult stem cells for the treatment of diabetes.

With respect to the generation of functional cells for the treatment of diabetes, it is apparent that a potential for differentiation to pancreatic endocrine lineages lies within an as yet undefined indigenous pancreatic cell population, although whether this is a primitive stem cell in the ductal epithelium, or whether the pre-existing adult β cells replicate themselves sufficiently to maintain homeostasis is a matter of some debate, although evidence indicates that both processes may occur.

There is some evidence that adult stem cells, particularly within the bone marrow, have the potential to differentiate and contribute to cells of the pancreatic islets. However, it will be essential to establish a reproducible protocol whereby bone marrow-derived cells that are functional in pancreatic regeneration are produced. It will also be vital to address some important issues that have arisen from preliminary studies of the bone marrow contribution to pancreatic regeneration in order to maximise their therapeutic contribution, namely,

(1) What are the mechanisms by which bone marrow cells form pancreatic lineages i.e., *de novo* formation of pancreatic cells or do the bone marrow stem cells merely fuse with an indigenous β cell to form a heterokaryon and assume a differentiated phenotype?

(2) Do bone marrow cells differentiate to form true β cells, or do bone marrow-derived cells contribute to vasculogenesis by forming vascular lineages, thereby acting as accessory cells, stimulating β cell regeneration *via* paracrine secretion?

(3) Is it possible to enhance bone marrow cell engraftment and differentiation to contribute to pancreatic regeneration by, for example, the introduction of genes such as Pdx1, which regulates insulin gene expression in pancreatic β cells *in vivo*?

Much progress is being made on the capacity of ES cells to differentiate and express markers of β cell differentiation, although again some results have not been confirmed and it is also argued that ES-derived cells are not wholly functional adult β cells, with reduced levels of gene expression and essential hormone production.

However, recent studies have raised a lot of issues that, when addressed, may provide further progress in the engineering of functional β cells from ES cells. For example, the previous use of nestin as a marker for

insulin-producing ES-derived cells may provide an explanation for the low levels of insulin production by ES-derived cells observed to date, as nestin is expressed by cells committed to neural differentiation.

It has been suggested that nestin-negative ES-derived cells are more likely candidates to produce insulin-producing ES-derived cells that are functionally more similar to pancreatic β cells. Ending on a cautionary note, we should also be mindful of potential pitfalls that have trapped the unwary, such as the uptake of exogenous insulin by ES cells being mistaken for insulin production.

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Cytokines and Stem Cell Differentiation into Cardiomyocyte Lineage Cells

Ioannis Dimarakis

Introduction

A novel therapeutic approach to confront ischaemic heart disease currently under investigation is stem cell transplantation. A variety of stem cell types as well as delivery approaches are under scrutiny in both experimental animal and clinical human transplantation protocols, as we have recently summarised.[1] Two stem cell populations appear to attract preponderance of interest owing to their varying degrees of perceived plasticity. These two populations are none other than the totipotent/pluripotent embryonic stem cells and the multipotent adult stem cells.

Milieu-dependent differentiation and cell-to-cell fusion are the two principal theoretical mechanisms to which plasticity of transplanted cells, as demonstrated in *in vivo* studies, has been mainly attributed. Although this may provide researchers with optimism, all underlying mechanisms continue to remain within the realm of medical hypothesis. Development of *in vitro* protocols to direct stem cell differentiation towards the

cardiomyocyte lineage bears a double role; not only to assist with deciphering molecular signalling pathways of differentiation, but also to augment the creation of safer and more efficient stem cell transplantation models.

A plethora of molecular pathways have been implicated in the transition of stem cells into functional myocardial cells once transplanted *in vivo*. Soluble chemokines, cellular surface receptors, extracellular matrix substrata as well as intercellular gap junctions, are all potential co-factors. As cell fusion has also been shown to take place[2] pathways regulating this process also remain to be clarified. Obtaining the molecular differentiation blueprints would translate in being able to initiate and direct the differentiation process in a stepwise fashion; researchers would be able to differentiate cells to certain predefined points prior to clinical transplantation, tailored each time to the respective *in vivo* model.

Post-transplantation survival of stem cell populations may be severely affected by factors including inability of homing and cell death secondary to local inflammation. By definition, stem cells demonstrate the inclination to undergo site-specific differentiation into multiple cell types once transplanted *in vivo*.[3] This may easily lead to suboptimal absolute cell counts effectively regenerating the cell type of interest. Delivery of pre-committed stem cell populations may provide a way to overcome many of these issues. Bittira *et al.*[4] has actually shown that converting scar into myogenic tissue may be augmented by cell pre-programming before implantation.

Amongst the arguments opposing the use of undifferentiated embryonic stem cells in *in vivo* models is their potential to form teratomas once transplanted.[5] Although adult stem cells have not been associated with the development of tumour-like growths, Wang *et al.*[6] demonstrated that bone marrow stromal cells can traffic through the coronary system to the injured heart and form cardiomyocytes or fibroblasts, depending on the specific microenvironment.This may sequentially lead to propagation of the myocardial fibrous scar, an opposite effect from the one desired. In both cases previous *in vitro* instigation of directional differentiation towards the cardiomyocyte lineage would prevent such complications.

Predefined Cell Culture Conditions

In a preclinical setting all human- and/or animal-derived products should ideally be excluded and synthetic recombinant alternatives used instead.

Multiple reasons make such an approach favourable. As serum is chemically ill-defined, a reduction of variability in qualitative and quantitative cell culture medium composition secondary to interbatch differences is expected. This alone will eventually lead to a more robust level of quality control. The risk of prion, viral or zoonose contamination will also be decreased in parallel with the demand on animals for product supply in accordance with the 3Rs principle of Reduction. Serum contains a variety of proteins that may attach to cells in culture and act as antigenic substrates for immunological reactions once transplanted. This has been shown by Selvaggi *et al.*[7] when patients infused with lymphocytes cultured in medium supplemented with fetal calf serum developed arthus-like reactions. The detection of newly formed antibodies to fetal calf serum is an indication that immune complex formation followed cell infusions. In a more relevant clinical study, it has been hypothesised that the contact of skeletal myoblasts with fetal calf serum in culture was the cause of unexplained arrhythmias that lead to significant malignant ventricular arrhythmias and sudden deaths in patients.[8]

Despite the very early recognition and adaptation of this concept in clinical work[9] it has proven extremely difficult to rid culture protocols of feeding layers and/or serum supplements.[10–13] Mesenchymal stem cell research has not been unscathed by this principal; rat mesenchymal stem cells have been shown to retain their proliferation and differentiation potential even when cultured in serum-free medium.[14] Another aspect of the nature of serum is the possible presence of unrecognised inhibitory factors. Cardiomyocyte differentiation of human embryonic stem cells in a coculture protocol was shown to be enhanced 24-fold in the absence of fetal calf serum when compared with the presence of 20% fetal calf serum.[15] The authors justly raise the point of serum-free conditions providing the ideal environment for assessing potential cardiogenic factors without interference from serum-derived modulatory factors. Until a chemically defined serum-free medium is widely available, autologous serum supplementation should not be overlooked. This modality is particularly useful in the adult bone marrow-derived clinical setting on a single patient basis and aids overcoming the above and many ethical considerations. Autologous serum has been shown not only to be indistinguishable from fetal calf serum with regard to both isolation and expansion of human mesenchymal stem cells, but also has proved to be superior with respect to osteogenic differentiation.[16] In another study autologous human serum provided sufficient *ex vivo* expansion of human bone marrow-derived mesenchymal

stem cells while maintaining higher cell motility compared to fetal calf serum.[17] In addition switching to autologous human serum terminated the side-effects associated with immune reactions in the aforementioned studies.[7,8]

Cytokines and Growth Factors

Cardiogenesis in embryonic life constitutes cellular proliferation, differentiation and three-dimensional structural organisation. Within the core of this process is the activation of certain transcription factors (examples include FOG-2, members of the GATA family, Nkx2.5, etc.) via morphogenetic proteins (e.g. members of the bone morphogenetic protein family). The range of molecules and pathways involved in cardiogenesis is actively widening to incorporate more transcriptional activators such serum response factor[18] and morphogenesis-controlling pathways such as the *wnt* pathway.[19,20] A plethora of growth factors has been shown to initiate this process via a variety of pathways (Table 1). A valid approach in the attempt to generate optimal conditions for directional differentiation of stem cells is the *in vitro* manipulation of these already identified signalling pathways. This is mainly achieved via supplementation of the culture medium with soluble regulator molecules trying to promote phenotypic commitment via known as well as unknown (awaiting discovery) signalling cascades.

Table 1. Principal growth factor–mediated signalling pathways

Growth factor	Main signalling pathway
Transforming growth factor-β superfamily (BMP, TGF β, activin)[49–52]	Smad/p38/ TAK1/ERK /JNK/MAPK
Insulin-like growth factor-I[53]	ERK/JAK-STAT/PI3K
Fibroblast growth factor[54]	PLCγ/Ras/MAPK
Leukaemia inhibitory factor/ Cardiotrophin-1[55]	JAK-STAT/ MAPK
Platelet-derived growth factor[56,57]	JAK/PI3K/ MAPK
Erythropoietin[58]	JAK-STAT/Akt

Akt, Protein Kinase B; ERK, Extracellular Signal-Regulated Kinase; JAK, Janus Kinase; JNK, c-Jun N-Terminal Kinase; MAPK, Mitogen Activated Protein Kinase; PI3K, PhosphatidylInositol 3-Kinase; PLCγ, PhosphoLipase C-γ; STAT, Signal Transducer and Activator of Transcription; TAK1, Tyrosine Kinase 1.

Bone morphogenetic proteins

Zhang and Bradley[21] demonstrated the central role of bone morphogenetic protein-2 (BMP2) in mammalian cardiogenesis. In a murine embryonic model the generation of a homozygous null mutation for BMP2 led to abnormalities in cardiac development. Full cardiac differentiation of non-cardiogenic tissue by administration of soluble BMP2 or BMP4 to explant cultures was shown by Schultheiss *et al.*[22] Data has been produced for the discreet roles of BMP2 and BMP4 in the developing mouse heart. It appears that the formation of the atrioventricular junction and valves is linked to BMP2, with BMP4 involved in the development of the outflow tract myocardium and of the endocardial cushion.[23] Furthermore the exposure of precardiac mesoderm to Noggin, a known BMP antagonist, resulted in inhibition of cardiac differentiation.[22] Even though BMPs play a crucial role in both processes of mesodermal induction[24] and cardiomyocyte differentiation,[21] there seems to be a transient but essential inhibitory phase of BMP signalling between them.[25]

Transforming growth factors

The transforming growth factor-β (TGFβ) superfamily consists of a large and divergent group of polypeptide morphogens including besides the TGFβs themselves, members such as the activins and the aforementioned BMPs.[26] Mice deficient for TGFβ2 demonstrate high perinatal mortality along with a wide range of congenital cardiovascular anomalies comparable to malformations seen in humans.[27] Even though all three recognised TGFβ isoforms (β1, β2, β3) are expressed within the developing murine heart,[28] each isoform appears to exert specific functions the other two cannot compensate for.[29] When undifferentiated murine embryonic stem cells were exposed to the combined effect *in vitro* of TGFβ and BMP2, up-regulation of both mesodermal and cardiac specific transcription factors were noted; likewise, *in vivo* transplantation lead to cardiac differentiation only in animals with an intact TGFβ/BMP2 signalling pathway.[30] The individual effects of each one of the three isoforms on cardiomyocyte differentiation from embryonic stem cells were compared by Kumar and Sun.[31] A significant increase in embryoid body proliferation as well as spontaneous beating was noticed only in the TGFβ2 subgroup. Finally, in a murine myocardial infarction model, transplantation of TGFβ pretreated bone marrow-derived CD117+ cells revealed superior functional improvement compared to untreated cell transplantation.[32] Most importantly, newly

formed myocardial tissue was seen on histology only in the TGF pretreated group.

Activins

The activins are another member of the TGFβ superfamily that has been studied less extensively. Similarly to TGFβ, activin has been shown to promote cardiac myogenesis in avian embryonic cells not of cardiac fate.[33] Authors submit evidence of activin/TGFβ signalling lying upstream of BMP signalling in the cardiac myogenesis pathway; the hypothesis is raised for TGFβ acting via up-regulation of activin expression. Human amniotic mesenchymal cells stimulated with activin A were shown to express a variety of cardiomyocyte-specific genes including Nkx2.5, ANP, and α-MHC.[34] Samples without stimulation on the contrary did not express any of the marker genes mentioned above.

Insulin and insulin-like growth factors

The gene expression of insulin receptors and insulin-like growth factors (IGFs) with their respective receptors has been documented from the early phases of murine embryonic life.[35] IGF1 is essential for normal embryonic growth and development in mice,[36] with severe deficiency affecting the functional maturation of the cardiovascular system.[37] Antin *et al.*[38] have suggested that insulin and IGFs may promote avian cardiac development *in vivo* by both autocrine and paracrine mechanisms. Pre-transplantation treatment of a murine embryonic stem cell suspension with recombinant IGF1 was associated with increased *in vivo* expression of cardiomyocyte phenotype and functional improvement.[39] So besides their well described role involving linear growth, glucose metabolism and organ homeostasis, these pleiotropic hormones appear to be involved in early phases of cardiogenesis.

Fibroblast growth factors

When examined if BMP2 alone is capable of inducing cardiac differentiation in chicken embryo precardiac and non-precardiac mesoderm, the results were discouraging.[40] Based on their previous work involving the effects of the fibroblast growth factor (FGF) family on cardiogenesis,[41,42] inclusion of FGF4 in the predefined medium by Lough *et al.*[43] produced dramatic results. Cardiac differentiation was documented by the formation of contractile multicellular vesicles that stained positive for the sarcomeric

marker α-actin, indicating a synergistic function between these two growth factors in order to induce cardiogenesis. The same group has moved on to produce exciting data in regards to which members of the BMP and FGF families are crucial in this process, the exact concentrations required for *in vitro* studies along with a precise timetable of growth factor supplementation for maximal results. The importance of optimised growth factor concentrations in relation to time in growth medium supplementation has been further demonstrated in murine embryonic stem cells.[44] Cardiogenic differentiation was achieved only when FGF2 and/or BMP2 were added at precise concentrations for the first three days of cell culture. The FGF family consists of a large and diverse group of small polypeptide growth factors. Differentiation of a murine cardiac resident precursor cell population *in vitro* and *in vivo* also seems to be controlled by FGF2.[45] Even though FGF2 and FGF4 appear to be the most promising members in the process of cardiogenesis, other members have been shown to be implicated.[46,47] A vastly diverse set of receptors mediates the effects of the fibroblast family of growth factors; the importance of these receptors has been recognised for *in vitro* cardiomyocyte development from murine embryonic stem cells.[48]

Leukaemia inhibitory factor and Cardiotrophin-1

Apart from a well described growth factor for haematopoiesis, bone and lipid metabolism and neuronal tissue development, leukaemia inhibitory factor (LIF) has also been shown to exercise differentiation factor properties.[59] This pleiotropic cytokine, essential for the survival of the normal pool of stem cells,[60] is known to inhibit differentiation of embryonic stem cells *in vitro*.[61] LIF has been demonstrated to induce cardiac hypertrophy by stimulating the JAK-STAT pathway in cardiomyocytes.[62] Two forms of this cytokine have been recognised, one diffusible and one bound to the extracellular matrix.[63] Commitment to a cardiomyocyte phenotype of differentiating embryonic bodies requires interestingly complete absence of all forms of this cytokine.[64] Minimal concentrations of diffusible LIF are necessary for induction of differentiation, but following the inductive event, LIF attenuates cardiomyocyte differentiation in a dose-dependent fashion. Once differentiation is complete though, LIF promotes proliferation and longevity. Intramuscular injection of LIF not only caused local regeneration but also enhanced mobilisation of bone marrow cells to the heart and their differentiation into cardiomyocytes in a murine myocardial infarction

model.[65] Cardiotrophin-1 (CT-1)[66] is another cytokine with the ability to stimulate cardiac hypertrophy *in vivo*,[66–68] while exerting various cardioprotective functions.[69–73] Stimulation of cardiomyogenesis with increased nuclear expression of the proliferation marker Ki-67 in pluripotent murine embryonic stem cells has also been attributed to CT-1.[74]

Both of the aforementioned cytokines belong to the interleukin-6 cytokine superfamily with their respective functional receptor complexes sharing the low-affinity LIF receptor (LIF-R) and the gp130 signal transducing component;[75,76] a possible reason for any functional overlap observed in the above studies. Knockout models of both components of the LIFR-gp130 heterodimer proved incompatible with life, with additional evidence of hypoplastic ventricular myocardium 16.5 days postcoitum and later in embryos homozygous for the gp130 mutation.[77,78] These results highlight the necessity of investigating genetic manipulation/targeting of the LIF-R/gp 130 receptor complex in order to promote stem cell differentiation down a cardiomyocyte lineage.

Platelet-derived growth factor

Platelet-derived growth factor (PDGF) consists of two structurally alike A and B polypeptide chains which in turn combine forming homodimers (AA,BB) as well as heterodimers (AB). This connective tissue cell mitogen that shares topological similarities with TGFβ[79] has been associated with the myogenic differentiation process in human myoblast clones more than a decade ago.[80] Treatment of rodent bone marrow derived stem cells with PDGF-AB enhanced cardiomyocyte phenotype generation *in vitro* and when co-delivered with bone marrow cells in a post-infarction myocardial ligation model promoted the formation of independent cardiac myocyte islands.[81] An increase in the expression of cardiac specific myosin heavy chain alpha and beta in embryoid bodies when stimulated with PDGF with a parallel increase in the number of observed beating embryoid bodies has also been reported.[82]

Erythropoietin

Besides being the main regulatory cytokine of erythropoiesis, erythropoietin (EPO) has shown evidence of cardioprotective properties.[83] On binding with the specific erythropoietin receptor (EPO-R) an array of signalling pathways may be initiated (as seen in Table 1), leading to the activation of various genetic transcripts responsible for cardiomyocyte

proliferation/differentiation. Both EPO-/- and EPOR-/- knockout mice are embryonic lethal and die *in uteri*;[84] ventricular hypoplasia and intraventricular septum defects were some of the defects observed. Endothelial progenitor cells from congestive heart failure patients treated with EPO demonstrated enhanced proliferation rates along with a trend to adhere to cultured endothelial cells.[85] From the same work, cultivated human endothelial progenitor cells *in vitro* with EPO exerted a dose-dependent increase in proliferation and adhesion to fibronectin, cultured endothelial cells, and cardiomyocytes. EPO obviously influences cardiogenesis and may prove useful in "preconditioning" stem cells prior to delivery within the injured myocardium to increase engraftment and post-transplantation survival.

Other cytokines

Many other cytokines have also been suggested to underlie the route of the cardiogenesis process. More specifically, treatment of murine P19 embryonic stem cells with oxytocin promoted the formation of rhythmically beating cells resembling primary cardiomyocytes; an effect not seen in the presence of an oxytocin inhibitor.[86] Finally, the vascular cytokine endothelin-1 was reported to shift differentiation of embryonic stem cell-derived cardiomyocytes toward pacemaker cells in an endothelin receptor-dependent manner without affecting electrophysiological properties.[87] Such findings are extremely important as they shed light on specialised cardiomyocyte-targeted differentiation.

Conclusion

Phenotypic differentiation is the end result of an array of interactions mainly between cells, cytokines/growth factors, extracellular matrix and physical stimuli. All cytokines examined are naturally occurring substances with identified receptors and subsequent signalling pathways. In addition, the majority of stimuli reviewed herein appear to act in a time- and dose-dependent manner; the application of a culture process similar to combinatorial chemistry may prove extremely useful in identifying these very fine relations. As pre-transplantation targeted differentiation may move cardiac regenerative medicine forwards, further basic research prior to commencing clinical trials is advocated.

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Regulatory Networks Controlling Neural Stem Cell Self-renewal and Differentiation

Yanhong Shi

Stem cells have two fundamental properties: self-renewal and multipotency. Stem cell self-renewal and differentiation are defined by the dynamic interplay between transcription factors, epigenetic control, post-transcriptional regulators such as miRNAs, and the microenvironment — or "niche" — in which stem cells reside.

Unraveling the mechanisms by which neural stem cells renew themselves and have the ability to generate distinct neuronal and glial cell types remains a challenge in central nervous system biology. The intrinsic state of stem cells depends on their spatial and temporal history and affects their responsiveness to extrinsic signals from the microenvironment.

Recent progress in defining specific roles for intrinsic and extrinsic factors regulating the maintenance of self-renewal and the capacity for differentiation provides the first glimpse of the multilayered networks involved. It promises to be a productive and active area of future research. This review summarizes our current state of knowledge and attempts to integrate the various levels of regulation in an overall context.

Neural Stem Cells

Depending on their origin, stem cells have the ability to differentiate into multiple but restricted cell types or any cell type in the body. Progression from stem cell to differentiated cell is characterized by striking morphological and functional changes at each stage of the lineage and by sequential expression of transcription factors and other signaling molecules.[1] Neural stem cells are defined as a subset of undifferentiated precursors that retain the ability to proliferate and self-renew, and have the capacity to give rise to both neuronal and glial lineages.[2–5] Since the early 1900s, it has been accepted that the regenerative capacity of the central nervous system (CNS) of adult mammals is very limited.[6] However, almost four decades ago, pioneering work by Altman and Das[7] suggested that neurogenesis continues throughout adulthood in mammalian brains.

Neurogenic stem cells in the adult mammalian brain are concentrated in the subventricular zone (SVZ) of the lateral ventricle wall and the dentate gyrus subgranular zone (SGZ) of the hippocampus.[8] Throughout adulthood, cells born in the SVZ traverse a long distance anteriorly through the rostral migratory stream into the olfactory bulb (OB), where they differentiate into interneurons. Neurons in the dentate gyrus are born locally in the underlying SGZ and migrate a short distance to integrate in the dentate gyrus.[9] A complete understanding of neural stem cells and neurogenesis requires the identification of molecules that determine the self-renewal and multipotent character of these cells. These molecules likely include one or more transcription factors acting to maintain the stem cell state by controlling the activity of a network of downstream target genes.

Transcriptional Regulation

Batteries of transcription factors have been proposed to control stem cell self-renewal and lineage progression (Fig. 1) by eliciting cascades of gene expression.[10] Orphan nuclear receptor TLX is an essential transcriptional regulator of neural stem cell maintenance and self-renewal in the adult brain.[11] TLX maintains adult neural stem cells in an undifferentiated, proliferative state. The TLX-expressing cells isolated from adult TLX-heterozygote brains can proliferate, self-renew and differentiate into all neural cell types *in vitro*. By contrast, TLX-null cells isolated from the

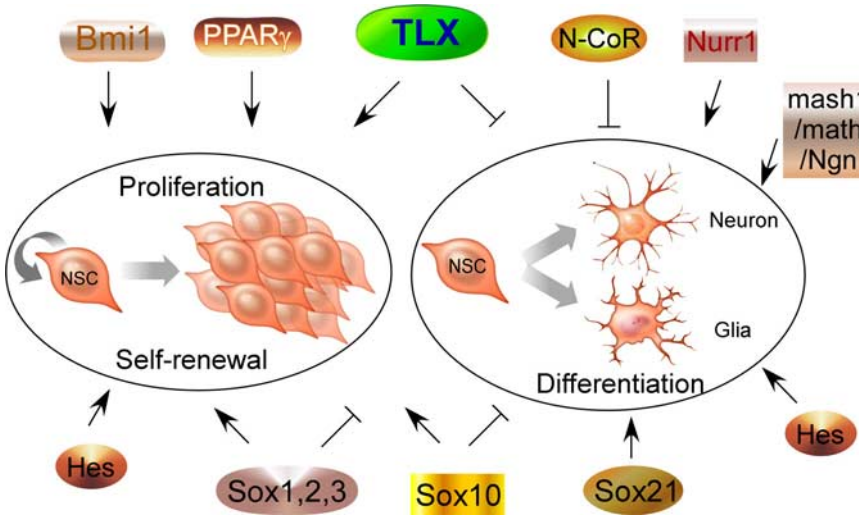

Fig. 1. Transcriptional regulators of neural stem cell self-renewal and differentiation. Transcription factors that are expressed in neural stem cells can potentiate cell proliferation and repress cell differentiation to maintain the undifferentiated and self-renewable state of neural stem cells. On the other hand, transcription factors are also responsible for induction of neuronal and glial differentiation. Due to space limitations, not all related transcription factors are listed here.

brains of adult TLX-mutant mice fail to proliferate. Reintroducing TLX into TLX-null cells rescues their ability to proliferate and to self-renew.[11] TLX could be key regulator acting by controlling the expression of a network of target genes to establish the undifferentiated and self-renewable state of neural stem cells. Indeed, TLX has been shown to silence glia-specific expression of an astrocyte marker, GFAP, in neural stem cells, suggesting that transcriptional repression may be crucial in maintaining the undifferentiated state of these cells.[11] The TLX gene has also been shown to regulate the timing of neurogenesis in the cortex[12] and control patterning of lateral telencephalic progenitor domains.[13]

Recently, other nuclear receptors such as estrogen receptors (ER), thyroid hormone receptors (TR), and peroxisome proliferator-activated receptor (PPAR) γ, have also been shown to regulate neural stem cell proliferation and differentiation.[14–19] Nuclear receptor related-1 (Nurr1), an orphan nuclear receptor, is essential for activation of tyrosine hydroxylase and induction of dopaminergic neuronal differentiation in CNS neural progenitor cells.[20–22] Deficiency in N-CoR, a nuclear receptor co-repressor,

also leads to reduced neural stem cell self-renewal and premature differentiation into astrocytes.[23]

Bmi1 is a polycomb family transcriptional repressor that has been shown to be required for postnatal maintenance of neural stem cells, in both central nervous system (CNS) and peripheral nervous system (PNS). Bmi1-null mice exhibit a postnatal self-renewal defect that leads to the depletion of stem cells by early adulthood.[24] One way in which Bmi1 promotes the maintenance of adult stem cells is by repressing $p16^{Ink4a}$ and $p19^{Arf}$ pathway.[25,26]

The Sox family of high-mobility-group (HMG) DNA binding proteins also plays a role in maintaining the undifferentiated state of stem cells, but this appears to be in a context-dependent manner. Sox2 is necessary for the maintenance of both embryonic neural stem cells and neural stem cells in adult neurogenic areas.[27–31] Sox10 maintains the multipotency of neural crest stem cells by promoting the expression of genes that confer competency for neuronal and glial differentiation but inhibiting their overt differentiation.[32]

In the CNS, Sox10 is required for terminal differentiation of oligodendrocyte progenitors,[33] whereas Sox8 and Sox9, two other members of the SoxE group, are involved in altering the potential of neural stem cells from neurogenic to gliogenic.[34] Sox21 and Sox14, the SoxB2 group, are closely related to SoxB1 proteins (Sox1-3), but have a repression domain instead of an activation domain at the carboxyl-terminus. As a consequence, they promote progression of neurogenesis through interfering with SoxB1-mediated activation.[35] Sox1 has also been shown to regulate neurogenesis by promoting differentiation.[36]

In addition, multiple basic helix-loop-helix (bHLH) genes play a critical role in regulation of neural stem cell maintenance and differentiation.[37–39] Hes genes, homologs of Drosophila hairy and enhancer of split, are repressor-type bHLH genes. Activated by Notch signaling, Hes maintains neural stem cell state but also promotes gliogenesis. On the other hand, the activator-type bHLH genes, such as Mash1, Math, and Neurogenin (Ngn), promote neurogenesis.[39] Recently, Stem cell leukaemia (Scl), another bHLH transcription factor, has been shown to regulate neuronal and glial cell fate acquisition in a region-restricted manner.[40]

Runt/Runx is a family of transcription factors important for cell proliferation and differentiation.[41,42] Recent studies demonstrated that mouse Runx1 is expressed in restricted types of postmitotic motor and sensory neurons and is critical for the differentiation of these neuronal cells.[43] Also,

Runx1 is expressed in particular populations of neural progenitors and plays an important role in coordinating their transition from proliferation to differentiation through transcriptional repression mechanisms.[44]

Together, these data suggest that intrinsic transcription factors have a specific role in neural stem cell regulation. These transcription factors may work together to coordinate neural stem cell maintenance, self-renewal and fate determination.

Epigenetic Control

The transition of stem cells from self-renewal to differentiated state is accompanied by global changes in gene expression. Genes active in proliferative stem/progenitor cells are silenced, whereas cell type-specific genes are turned on. This progression is the result of selective expression of transcription factors in concert with chromatin remodeling and epigenetic modifications, including covalent histone modification and DNA methylation of CpG dinucleotides.

Transcription in eukaryotic cells is influenced by epigenetic modification, particularly the covalent modifications of histones.[45] The amino-terminal tails of the histones are subject to post-translational modifications by acetylation (lysine), methylation (lysine and arginine), phosphorylation (serine) and ubiquitination (lysine).[46–49] Acetylation is one of the most well-characterized histone modifications, mediated by histone acetyltransferases (HATs). Acetylation of the conserved amino-terminal histone tail results in relaxation of the nucleosomes, which facilitates access to transcriptional activators and allows gene activation. Histone deacetylases (HDACs) catalyze the reverse reaction. In the deacetylated state, histones package DNA into condensed chromatin, which in turn prevents access to transcriptional activators to their target sites, to result in transcriptional repression.

We have shown that TLX interacts with a set of histone deacetylases (HDACs) in neural stem cells and association of HDACs with TLX leads to transcriptional repression of TLX downstream target gene (Fig. 2; Shi *et al.*, unpublished results). Inhibiting HDAC activity with HDAC inhibitors Trichostatin A (TSA) and valproic acid (VPA) led to reduced proliferation of neural stem cells and induction of cyclin-dependent kinase inhibitor $p21^{CIP1/WAF1}$ expression. siRNA-mediated knockdown of HDAC expression in neural stem cells also led to reduced cell proliferation,

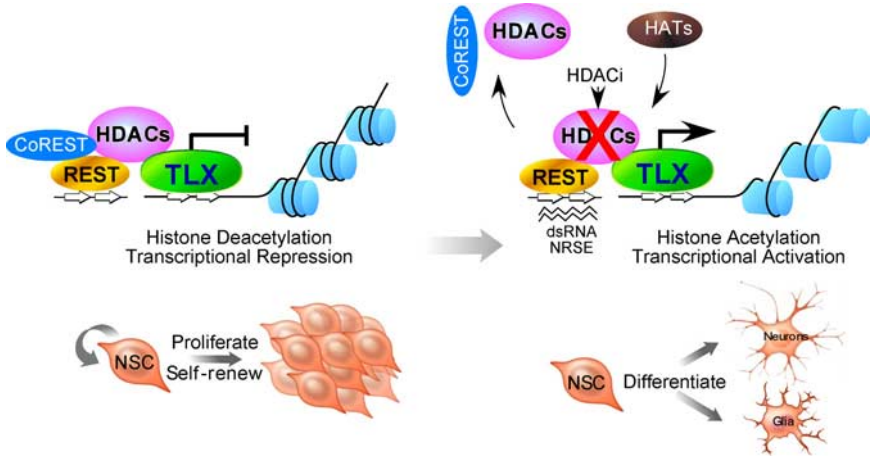

Fig. 2. An example of epigenetic control of neural stem cell self-renewal and differentiation. Transcriptional silencing mediated by epigenetic regulators such as histone deacetylase (HDACs) leads to the undifferentiated and self-renewable state of neural stem cells. The repression can be relieved by treatment of HDAC inhibitors (HDACi), release of HDACs and their associated co-repressors, and/or recruitment of histone acetyltransferase (HAT) co-activator complexes, which in turn activates growth arrest and neural differentiation. Double stranded small RNAs could also regulate neuronal differentiation by targeting neuron-specific repressor REST and its associated HDAC complexes.

reinforcing a role of HDACs in neural stem cell self-renewal. In addition to cell proliferation, HDACs also regulate cell differentiation. Blocking global HDAC activity inhibits the differentiation of embryonic stem cells.[50] Treatment of adult neural stem cells with HDAC inhibitors induced neuronal differentiation,[51] which could be due in part to upregulating REST (RE1 silencing transcription factor, or NRSF)-regulated neuronal-specific genes (Fig. 2). REST is a key transcriptional regulator of many neuronal genes through binding to a conserved 21 bp RE1 binding site.[52,53] In non-neuronal cells, REST interacts with its co-factors, including Co-REST, N-CoR, and mSin3A, which then recruit HDAC complexes to repress neuronal gene expression through epigenetic regulation.[52,54,55]

Recent evidence indicates that transcriptional and epigenetic dysregulation may contribute to molecular pathogenesis of neurodegenerative diseases such as Huntington's disease. HDAC inhibitors have proven to be neuroprotective in Huntington's mouse models, presumably by increasing histone acetylation and transcriptional activation.[56,57] Therapies using

HDAC inhibitors may, therefore, be clinically beneficial to neurodegenerative patients. HDAC inhibitors have also been shown to induce growth arrest, differentiation, and/or apoptosis in cancer cells and to have antitumor activity in clinical trials.[58]

More recently, histone methylation has gained attention as an epigenetic marker.[47,59] Unlike histone acetylation, which only occurs on lysine (K) residues and is generally related with active transcription, methylation was detected on both lysine and arginine (R) residues and is linked to both transcriptional activation and repression.[47] For instance, histone H3 K9 methylation is associated with transcriptional silencing. In contrast, methylation of histone H3 K4 and arginine residues of H3 and H4 leads to transcriptional activation.[60] While histone acetylation is dynamically regulated by HATs and HDACs, histone methylation has been considered a "permanent" modification by histone methyltransferases until recent identification of LSD1 as a histone demethylase.[61] This finding indicates that similar to histone acetylation, histone methylation is also a dynamic process subject to regulation by both methylases and demethylases. The degree of lysine methylation (mono-, di-, or trimethyl histones), as well as the residues modified, is tightly associated with neural and muscle cell differentiation.[62] For example, histones H3 trimethyl K9 and H4 monomethyl K20 were detected in proliferating neural cells, whereas histone H4 trimethyl K20 was enriched in differentiating neurons.

The epigenetic state can also be regulated at the DNA level. The gene silencing effect of DNA methylation is mediated by a family of methylcytosine-binding proteins including MeCP2, which is abundantly expressed in the central nervous system.[63,64] Mutations in the *Mecp2* gene have been linked to a neurodevelopmental disorder, Rett syndrome,[65] suggesting that MeCP2 may play a role in regulating neuronal function. MeCP2 can interact not only with HDACs but also with the histone H3 lysine (K) 9 methyltransferase, SUV39H1, to trigger dimethylation of histone H3 on K9 and provide a histone code indicative of inactive chromatin structure.[54,66–68] DNA methylation and its related chromatin remodeling play critical roles in regulating gene transcription in response to neuronal activity.[69]

Methylation of the signal transducer and activator of transcription 3 (STAT3) binding site in GFAP promoter renders neural stem cells refractory to GFAP activation and astrocyte differentiation during early brain development. Demethylation of this STAT3 binding element leads to activation of GFAP promoter and gliogenesis at later developmental stages.[70]

A similar alteration in methylation pattern also occurs in the promoter of S100β, a calcium binding protein expressed in astrocytes.[71]

Multiple layers of epigenetic modifications, therefore, regulate key transitions in the temporal development of stem cells and their differentiation. This results in expression of unique repertoires of transcription factors at each stage of development and in different lineages.

Small RNA Regulators

While transcription factors are essential players in stem cell self-renewal and differentiation,[11,24,38,72] post-transcriptional gene regulation is emerging as another essential regulator of development. Many different classes of small non-coding RNAs are present in the brain, with diverse roles including RNA modification and chromatin remodeling.[73] Small double-stranded modulatory RNAs have been proposed to regulate the generation of neurons from adult neural stem cells by binding to REST.[74] The sequence of this small noncoding RNA matched the DNA binding site for REST, NRSE. The NRSE dsRNA interactes with REST and converts it from a transcriptional repressor to an activator, which in turn lead to activation of neuronal-specific genes and neuronal differentiation (Fig. 2).

MicroRNA (miRNA) is another recently identified large family of small non-coding RNAs, which are likely key post-transcriptional players in stem cell self-renewal and differentiation (Fig. 3). miRNAs are short 20–22 nucleotide RNA molecules that are expressed in a tissue-specific and developmentally-regulated manner and function as negative regulators of gene expression in a variety of eukaryotic organisms. miRNAs are involved in numerous cellular processes including development, proliferation, differentiation, apoptosis, and the stress response.[75,76]

miRNA genes belong to class II genes, which are transcribed by RNA polymerase II. A majority of miRNA loci are found in intronic regions of protein-coding or non-coding transcription units; others are found in exonic regions of non-coding transcription units.[77] These primary transcripts are processed in the nucleus by RNase III endonuclease Drosha, into 60 to 75 nt hairpin-like precursors (pre-miRNAs), which are subsequently exported to the cytoplasm by exportin-5, a member of the Ran-dependent nuclear transport receptor family. The hairpin precursors are then cleaved into mature miRNAs by Dicer, the cytoplasmic RNase III-type protein. Mature miRNAs

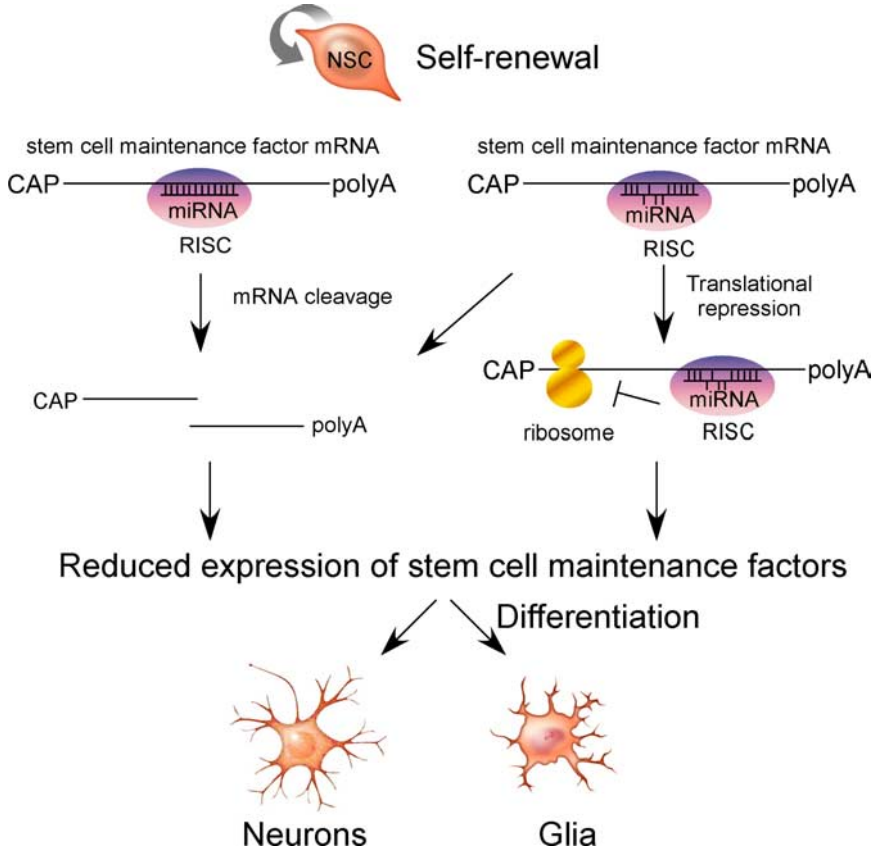

Fig. 3. A model for miRNA action in neural stem cells. Molecules that are expressed in neural stem cells to maintain their self-renewable state are collectively referred to as stem cell maintenance factors. At the onset of differentiation, miRNAs complementary to these factors can direct the cleavage of their mRNAs or inhibit their translation by pairing to their mRNA targets, thus preventing the expression of these stem cell maintenance factors and enabling rapid neural differentiation.

bind to effector complex RISCs that direct the miRNAs to their target transcripts.[78] By base pairing with the target mRNA, miRNA functions as a guide molecule in post-transcriptional gene silencing, leading to mRNA cleavage or translational repression (Fig. 3).

miRNAs are especially attractive candidates for regulating stem cell self-renewal and cell fate decisions because their ability to simultaneously regulate many targets provides a means for coordinated control of concerted

gene action. Although direct evidence for a functional role of miRNAs in stem cell biology is just now emerging, tantalizing hints regarding their involvement based on expression patterns, predicted targets, and overexpression studies, suggest that they will be key regulators. Loss of Dicer1 causes embryonic lethality and loss of stem cell populations.[79,80] Argonaute family members, key components of RISC complexes, are required for maintaining germline stem cells in various species.[81] As stem cells differentiate, they downregulate stem cell maintenance genes and activate lineage-specific genes. These transitions require a rapid switch in gene expression profiles. Although the transcription factor pool is replaced, remaining transcripts that were highly expressed in the previous stage need to be silenced. miRNAs are uniquely poised to rapidly effect such changes through simultaneous repression of many targets of any remaining transcripts.

miRNAs likely play important roles in maintaining mature cell function, as has been described in fat and insulin metabolism.[82,83] miRNAs are especially abundant in the adult brain, suggesting a key role for them in neuronal function and plasticity.[84] Expression profiling of mammalian microRNAs uncovered a set of brain-specific microRNAs with possible roles in neuronal differentiation.[85–88] Study of a set of highly expressed neural miRNA during mouse brain development revealed significant differences in the onset and magnitude of induction for individual miRNAs and marked lineage specificity of the miRNAs.[89] For example, miR-124 and miR-128 were preferentially expressed in neurons, whereas miR-23 was restricted to astrocytes. miR-26 and miR-29 were more strongly expressed in astrocytes than neurons, whereas miR-9 and miR-125 were fairly evenly distributed. A key role for miRNAs in brain formation has been shown by the rescue of brain morphogenesis in maternal-zygotic dicer zebrafish mutants by injection of miR-430.[90] This demonstrates that an individual miRNA can trigger large-scale changes in development, likely through global changes in the transcriptome.[91]

According to bioinformatics target predictions, a single miRNA can target hundreds of mRNAs and about a third of the human genome is targeted by miRNAs.[92] If miRNAs target broad regulatory networks, then a complete list of miRNA targets should not only describe specific targets, but also how the entire collection of targets regulates certain cellular function. The global effect of a miRNA on a set of targets may be to help establish a transcriptional profile characteristic of the cell in which the miRNA is normally expressed. For example, expression of the neuronal-specific miRNA, miR-124, in a heterologous cell shifted the entire transcriptional

profile toward that of a neuron.[91] Identification of comprehensive miRNA targets in the neural system remains an important and non-trivial task that will help us to better understand the regulation of neural stem cell self-renewal and differentiation.

Niche

Stem cell self-renewal and differentiation is regulated by the specialized microenvironment — or "niche" — in which these cells reside.[9,93–95] Direct physical interactions between stem cells and their neighbors are critical in keeping stem cells in their specialized niche and in maintaining stem cell character. Such niches are composed of soluble factors as well as membrane bound molecules and extracellular matrix. Remarkably, classical developmental signals and morphogens such as Wnt, Notch, Shh, BMPs, and Noggin seem to play important roles in maintaining adult neurogenic niches.[9]

Wnts are globally involved in cell fate specification during embryogenesis, and have recently been implicated in governing both proliferation and cell fate specification of somatic stem cells in adults.[93] Genetic studies have implicated the Wnt/β-catenin pathway in neural stem cell self-renewal. In mice that express a stabilized β-catenin, the CNS is greatly enlarged. Progenitors exit the cell cycle less frequently and continue to proliferate in the nervous system.[96] In contrast, ablation of β-catenin results in a marked decrease of the overall size of the nervous system.[97] Mice with null alleles of LRP6, a required co-receptor for Wnt signaling, also showed reduced dentate granule cell production and failed expansion of a defined dentate granule precursor cell pool.[98] These studies indicate that the Wnt/β-catenin signaling plays an important role in the proliferation and self-renewal of neural precursors. On the other hand, Wnt proteins have been shown to promote neuronal differentiation in neural stem cell culture and in adult hippocampus,[99–101] suggesting that signaling by the canonical Wnt pathway has multiple functions in stem cells. How specific stem cells will respond to Wnts is likely to depend not only upon their specialized microenvironment and who delivers the signal, but also on the cell-intrinsic properties.[93] Thus, Wnt pathways are poised at a critical crossroads in balancing neural stem cell self-renewal versus differentiation.

The Notch family of receptors participates in many developmental cell fate decisions and in certain contexts promotes an undifferentiated,

precursor cell state. Notch1 and its cognate ligand, Jagged, are expressed in both the SVZ and SGZ.[102] Notch may participate in suppressing neuronal differentiation and maintaining precursor cell properties. Accumulation of neuroblasts expressing Notch ligands activates Notch signaling in stem cells, thereby suppressing neurogenesis. This could be a niche mechanism for feedback regulation of neuronal differentiation of stem cells or homeostasis. However, Notch signaling is context-dependent: Notch can promote either terminal glial differentiation[103] or a precursor state in adult germinal regions.

Sonic hedgehog (Shh) is an important morphogen in development and has been shown to regulate both SVZ and SGZ neural stem cells. Over-expression of Shh near the dentate gyrus increases proliferation and neurogenesis of SGZ cells. *In vitro*, Shh maintains proliferation of adult hippocampal neuronal progenitors[104] and increases SVZ cell proliferation and the number of cells with stem cell properties.[105] Although the notion of Shh as a stem cell "maintenance" factor is intriguing, another explanation is that Shh may promote proliferation or support survival of the transit-amplifying cells in the SVZ.

Another group of early neural morphogens, the bone morphogenetic proteins (BMPs), also play an important role in adult brain germinal niches. Interestingly, in the adult SVZ, both BMP and Shh signaling are intermixed within the same region. Adult SVZ cells themselves produce BMPs and their receptors. Noggin, a secreted BMP antagonist, is strongly expressed in ependymal cells. This locally-derived BMP antagonist is thought to contribute to the neurogenic niche for SVZ stem cells because it promotes neurogenesis both *in vitro* and in ectopic locations *in vivo*.[106]

Thus, major developmental signaling pathways including Wnt, Notch, Shh and BMPs are retained in adult germinal niches, where they appear to regulate important aspects of proliferation and differentiation. In the early neural tube, neuroepithelial cells are the only cell type and, therefore, serve as stem cells as well as "niche cells" to provide signals critical for stem cell function. Ongoing cell production in the adult mammalian brain depends on astrocytes that reside in special niches within the SVZ and the SGZ of the hippocampus.[95,107] Signaling molecules secreted by endothelial cells could push neural stem cells toward self-renewal while simultaneously priming them for the production of neurons.[108] Moreover, neural stem cells in the adult brains are likely to be influenced by a convergence of signals from many neighboring cell types, including astrocytes, neuroblasts, ependymal cells, endothelial cells, and a factor-rich basal lamina. Understanding more

about the niches may help reveal how these remarkably small, simple units influence tissue development, growth, repair and aging.[109]

Conclusion

A new integrated global regulatory network controlling stem cell self-renewal and differentiation is now emerging and is defined by the interplay of transcription factors, chromatin remodeling components, small RNA regulators, and extracellular signals from stem cell niches. These mechanisms synergize to choreograph stem cell self-renewal and differentiation into various cell lineages. The coordinated regulation of genes specific for stem cells and their differentiated progenies likely relies on transcriptional regulation and epigenetic controls for initial expression in response to developmental cues and extracellular signals from the niche, and are fine-tuned by miRNAs. Understanding how individual signaling cascades integrate into global regulatory networks will be essential to fully appreciate stem cell biology. Armed with this knowledge, researchers will be able to develop new, targeted therapies for a host of neurological disorders, including brain injuries, brain tumors, and neurodegenerative diseases such as Huntington's, Alzheimer's and Parkinson diseases.

Acknowledgments

I apologize to colleagues whose work could not be cited due to space limitations. I thank Dr. Paul Salvaterra for critical comments and Dr. Kristine Justus for editing and proof reading this manuscript. This work was supported by the Rockefeller Brothers Fund and Goldman Philanthropic Partnerships, Stop Cancer Career Development Award, and Whitehall Foundation.

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12

Demyelination as a Therapeutic Target in Spinal Cord Injury

Jill R. Faulkner and Hans S. Keirstead

Stem Cells for Cellular Replacement

Stem cells have proven to be a highly successful research tool and excitement for their use in treatment strategies has grown over the past decade. Stem cells are self-renewing, pluripotent or multipotent cells. Their human therapeutic advantage lies in their high proliferative ability. Some stem cell types offer a seemingly infinite cell supply that can be manipulated *in vitro* to provide a myriad of differentiated cell types that have potential for use in cellular replacement strategies for a number of human diseases. The most suitable cell type is dictated by the characteristics of each disease state.

Embryonic stem cells (ESCs) are a particularly appealing stem cell population due to their ability to provide essentially unlimited stem cell numbers *in vitro*, their amenability to genetic engineering, and their broad developmental capacity. ESCs derive from the inner cell mass of pre-implantation embryos and can proliferate *in vitro* almost indefinitely. These cells are particularly amenable to human cellular replacement

because they can be amplified in fully-defined rodent free media and can be stably stored, and maintain a normal karyotype and differentiation potential for years.[1,2] Pre-implantation or blastocyst-stage embryos can be obtained from supernumerary embryos during i*n vitro* fertilization procedures, by somatic cell nuclear transfer or parthenogenetic activation of the egg.[3,4] Currently, ESCs are the most vigorously studied of all stem cell types. Techniques for directing their differentiation are well advanced. A number of studies have demonstrated that rodent ESCs can be differentiated into neuronal[5–8] or glial fates.[9–12] Human ESCs have been differentiated into multipotent neural precursors,[13,14] low purity motor neurons[15] and recently into high purity oligodendrocyte progenitors.[16,17]

Remyelination by Endogenous Cells

Remyelination is a component of human and rodent demyelinating diseases and insults, and is recognized by its thin and short myelin sheaths. Newly generated myelin is likely formed in a process similar to myelination during development, as evidenced by the re-expression of developmentally regulated genes during the process.[18,19] Remyelination by endogenous cells has been observed in the brains of MS patients within shadow plaques, which are partially remyelinated lesions.[20]

The cell type responsible for remyelination in the adult central nervous system (CNS) is currently under investigation. Several studies indicate that cell division is a necessary pre-requisite for remyelination,[21] which suggests a role for progenitors. MOG-positive oligodendrocytes have been suggested to play a role in the process, as they are present within demyelinated MS plaques and may have the capacity to form new myelin sheaths.[22] However, little evidence exists to suggest that differentiated oligodendrocytes are able to revert to a proliferating state. The bulk of evidence in the literature suggests that remyelination is accomplished by NG2 positive and/or platelet-derived growth factor expressing adult progenitor cells.[21,23–26] Endogenous[27] or transplanted mature oligodendrocytes[28] are not capable of remyelination in experimental models of demyelination.

Unfortunately, remyelination failure is more prevalent than its success. Remyelination failure is best illustrated by lesions in the later stages of MS, which are characterized by demyelination and oligodendrocyte loss, with remyelination limited to the borders of inactive plaques.[29] Remyelination has been shown to be less successful in older animals.[30] Animal

studies suggest that endogenous progenitor cells are depleted by recurring demyelinating episodes. However, oligodendrocyte progenitor cells (OPCs) are present even in chronic MS lesions.[20,31] However, adult OPCs, which have poor migratory ability,[32] and require extended growth factor exposure to rapidly proliferate,[33] may not have the regenerative capacity necessary for extensive remyelination in the adult CNS. Remyelination failure may also be a result of astrogliosis, which may ensheath demyelinated axons and thereby prevent OPC access to demyelinated axons,[16] or by expression of molecules that inhibit OPC maturation or oligodendrocyte myelination. For example, astrocytes within demyelinated lesions express Jagged1, the ligand for oligodendroglial Notch1 receptors, which inhibits oligodendrocyte differentiation and process outgrowth when bound; notably, these studies found that Jagged1 expression was negligible within remyelinated lesions.[34] Current research suggests that the failure of remyelination is likely a combination of environmental factors at the site of the lesion and the relatively dormant nature of endogenous adult OPCs.

Demyelination as a Cellular Replacement Target

Loss of myelin is prevalent in a number of CNS disorders such as spinal cord injury, brain injury and stroke, and affects a large number of patients.[35–37] Recently, demyelination has been associated with functional deterioration related to Alzheimer's disease,[38] normal aging,[39,40] and psychiatric disorders such as schizophrenia.[41,42] Overlapping pathology associated with most CNS insults make it difficult to assess the functional deficits attributable to demyelination. However, remyelination has been shown improve locomotion in animal models[43,44] and to restore saltatory conduction in axons.[45–47] Remyelination of demyelinated axons may also decrease axonal degeneration and transection.[48–50] The success of remyelination during acute stages of human demyelinating insults, and in experimental demyelination, which usually models acute injury, suggests that the therapeutic window of opportunity is narrow. This period of time represents an opportunity for transplantation approaches to repair.

In a recent report, we documented the extent of demyelination and remyelination from 1 to 450 days following contusion SCI. Microscopic analysis 1 week after contusion injury indicated widespread tissue loss and demyelination within the spinal cord (Fig. 1a). At this time, very little

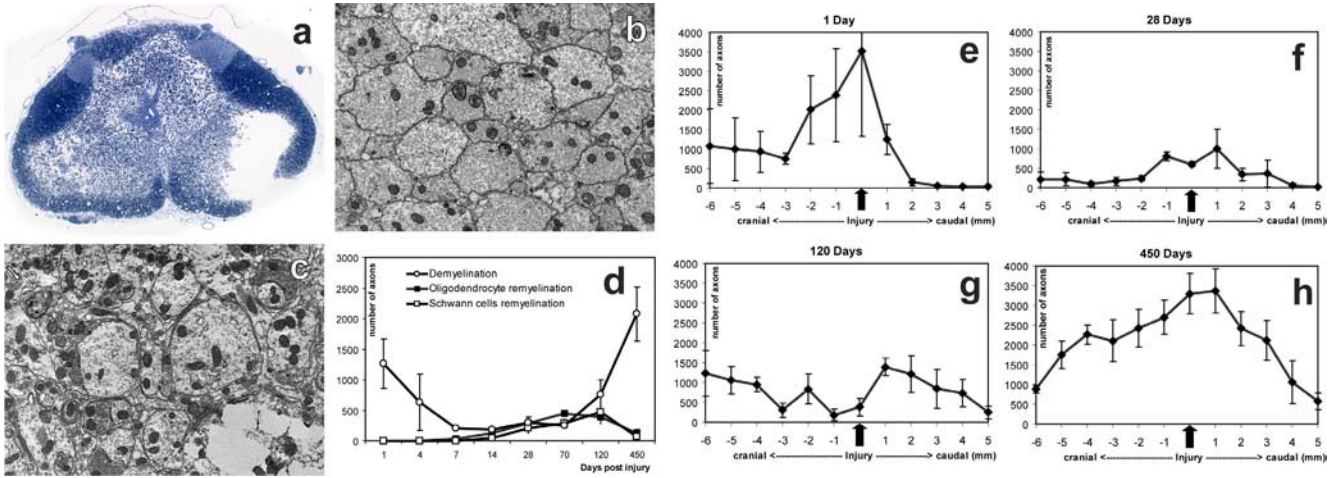


Fig. 1. Spinal cord contusion injury resulted in widespread demyelination at early and late time points post-injury. (a) Spinal cord contusion injury resulted in a loss of tissue structure within the central region of the spinal cord by 1 week post-injury. This image is from a tissue section at the injury epicenter. (b) Demyelinated axons were consistently found in intimate juxtaposition to other demyelinated axons throughout the dorsal, lateral, and ventral columns at 14 days post-injury. (c) Demyelinated axons were most often separated from one another and surrounded by thick fibrous astrocytic processes (AP) at 120 days post-injury. (d) Quantification of demyelinated and remyelinated axons at different time points post-injury. The number of axons at the six cranial and six caudal levels examined for all animals within a group were averaged to generate each point on the graph. The number of demyelinated axons was high at 1 day post-injury and decreased substantially by 7 days post-injury. Thereafter, demyelination was a chronic and progressive phenomenon. Both oligodendrocyte and Schwann cell remyelination was present at all subsequent time points post-injury. (e–h) Quantification of demyelinated axons throughout the craniocaudal axis of the spinal cord at 1 (e), 28 (f), 120 (g), and 450 (h) days post-injury. Demyelinated axons were concentrated around the injury site except for 120 days post-injury, when the craniocaudal pattern of demyelination reversed, being lowest around the injury site and relatively higher cranial and caudal to the injury site. Demyelination increased in a statistically significant manner from 70 to 450 days post-injury, indicating that demyelination was a chronic and progressive phenomenon. 4× for (a); 33,000× for (b) and (c).

grey and white matter remained at the lesion epicenter, which was surrounded by inflammatory cells, cavitations and an increased extracellular space. Demyelinated axons were evident among lymphocytes and debris-laden macrophages (Fig. 1b). By 70 days post-injury this area was predominantly scar tissue. Microscopic analysis revealed demyelinated axons present among very few lymphocytes and surrounded by a dense astrocytic scar and fibrous matrix (Fig. 1c). Normally myelinated axons were present at all time points in the area of surviving tissue (data not shown).

Quantification of the extent of demyelination revealed a high number of demyelinated axons on day 1 at the injury epicenter (Figs. 1d and 1e). This was probably the result of the initial mechanical insult to the spinal cord, resulting in myelin loss around many axons that would go on to die. The total number of demyelinated axons decreased substantially by seven days post-injury (Fig. 1d), and was maintained (Fig. 1f). The number of demyelinated axons markedly increased at 120 and 450 days post-injury (Figs. 1g and 1h). This increase in demyelination at later time points suggests that demyelination is a chronic and progressive event. This evidence demonstrates that oligodendrocyte death and myelin loss is not a static phenomenon after an initial insult. Demyelination increases up to at least 450 days post-injury. This study and others support the idea that demyelination after SCI is a chronic and progressive phenomenon.

Remyelination as a Therapeutic Strategy

Demyelination following spinal cord injury is a therapeutic target for cell replacement strategies. A remyelinating population of transplanted cells will be required to perform their function at or very near the site of implantation and they will not be required to extend processes to a distant target. The myelinogenic potential of rodent[9,12,51] and human[14,16,17] ESC derivatives has been well documented following transplantation into a variety of animal models of demyelination and dysmyelination.

Remyelination has been shown to result from the transplantation of oligodendroglial lineage cells,[16,46,52–55] Schwann cells,[56–60] olfactory ensheathing cells[61–64] and various types of stem cells.[9,10,16,17,65–68]

These cells are strongly influenced by the environment of the host. To be successful, cell replacement strategies will need to address this issue. Therefore, it is necessary better understand what types of environments support

transplant-mediated remyelination so as to determine whether transplantation represents a viable treatment strategy for demyelination.

We investigated the ability of the complex and reactive disease state of the chronic demyelinating MHV model of MS to support transplant-mediated remyelination. This model provides an environment of ongoing demyelinating pathogenesis, and is thus distinct from gliotoxin lesions. Striatal neural precursors derived from postnatal day 1 mice were committed to a glial cell lineage and labeled with bromodioxyuridine (BrdU) as previously described.[55,69]

After 7 days on an adherent substrate, differentiated stem cells were identified by their morphology and immunolabelling. Quantification of the transplant population indicated that 85% ± 10.7% (range = 64–96, median = 80) of the total cell population was BrdU immunoreactive following an overnight pulse (Fig. 2c), and that the differentiation protocol yielded 67.4% ± 4.4% GalC-positive oligodendrocytes (range = 60–72, median = 66) (Fig. 2a), 26% ± 7.4% GFAP-positive astrocytes (range = 20–40, median = 30) (Figs. 2b and 2c) and 6.6% ± 6.2% other cell types (range = 1–13, median = 7), which included NeuN + neurons, CD11b + microglia, and other Hoechst-positive cells not identified by the immunostains tested.

Twelve days after MHV intracerebral injection, animals received a single injection of 240,000 stem cell-derived glial precursors. The animals survived for 3 weeks following transplantation during which behavioral analysis was performed. Transplanted glial-committed progenitor cells survived, integrated into the spinal cord tissue and differentiated into mature oligodendrocytes. Immunohistochemistry of BrdU pre-labeled cells indicated that transplanted cells survived and spread throughout the mediolateral extent of the spinal cord, were present in highest concentration at the sight of implantation and were detected 12 mm cranial to 10 mm caudal.

Transplantation of glial committed progenitors resulted in widespread remyelination (Figs. 2f and 2g) ranging from 54% to 67% of the axons extending 12 mm cranial and 10 mm caudal to the implantation site. The number (Fig. 2f) and percentage (Fig. 2g) of remyelinated axons in non-transplanted animals was significantly lower ($p < 0.01$) than in transplanted animals, and composed less than 10% of the total number of axons counted. This remyelination was accompanied by a significantly higher total number of axons in transplanted animals. These data suggest that remyelination resulting from transplantation of glial-committed precursors protects axons from degeneration/transection.

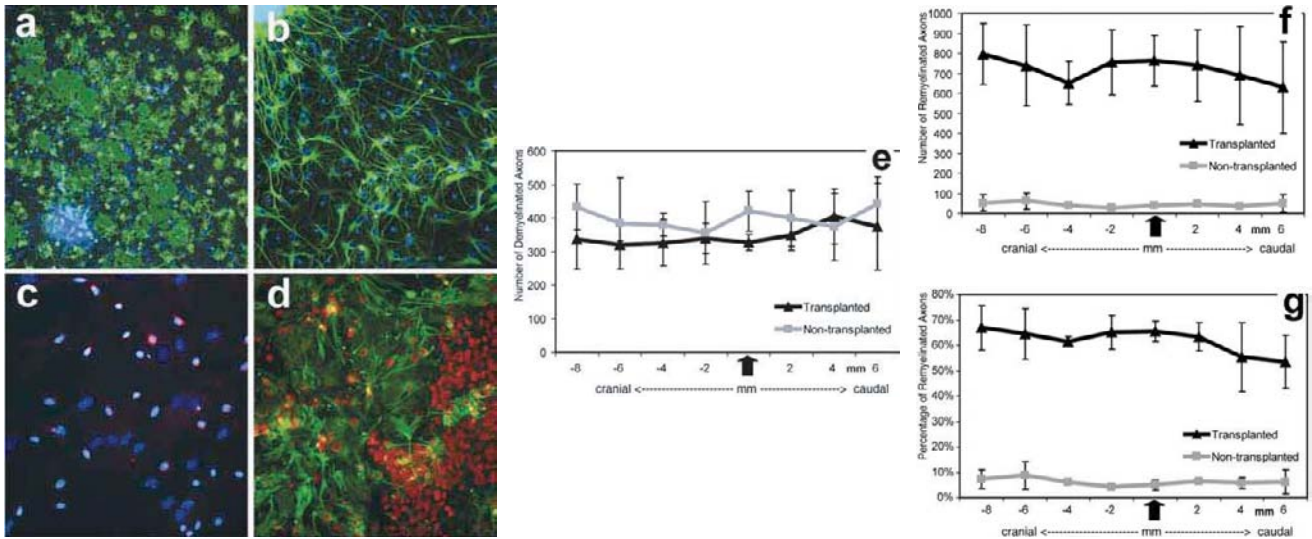


Fig. 2. (a) Multipolar GalC-positive (green) oligodendrocytes were abundant after 7 days of growth on adherent substrate in the absence of growth factors (Hoechst-positive nuclei are blue). (b) GFAP-positive (green) astrocytes were also abundant after 7 days of growth on adherent substrate in the absence of growth factors (Hoechst positive nuclei are blue). (c) The majority of cells (85% +/− 10.7%) were BrdU-positive. This field illustrates BrdU-positive (red) GFAP-positive (green) astrocytes. (d) Electron photomicrograph of spinal cord white matter from an MHV-infected mouse 33 days after induction of disease illustrating a demyelinated axon (arrow) and a normally myelinated axon (arrowhead). (e) Demyelinated axons were present in transplanted and non-transplanted animals; their numbers were not statistically different ($p > 0.05$), reflecting ongoing pathology. (f) Remyelination extended 8 mm cranial and 6 mm caudal to the implantation site (arrow) in transplanted animals (the extent of tissue examined) and was significantly greater than the degree of remyelination in non-transplanted animals at every point examined ($p < 0.01$). (g) Throughout this region, 54% to 67% of the total number of axons in the ventral and lateral columns of transplanted animals was remyelinated. Remyelination in non-transplanted animals was significantly less at every point examined ($p < 0.01$). 100× for (a) and (b); 200× for (c); 39000× for (d).

In addition, transplantation significantly improved locomotor function. MHV animals transplanted with glial-committed progenitors demonstrated weight-supported walking with partial hindlimb weakness, whereas non-transplanted animals remained paralyzed. This evidence demonstrates that transplantation of glial-committed progenitors into an area of demyelination results in remyelination and recovery of function. Although remyelination was likely carried out by the transplanted cells, we cannot rule out the possibility that remyelination was carried out by endogenous cells that that may have been activated as a result of trophic support by the transplant population.

The survival and migration of our gliogenic cell population in complex environmental conditions does not indicate that they will respond similarly in a normal adult CNS. Studies have shown that glial progenitor cells transplanted into an intact adult CNS fail to survive or migrate.[70,71] However, when transplanted onto a region of active demyelination, where survival factors are elevated, glial progenitors survive and enter the lesion.[72] These findings suggest that the survival and migration of cell in our study was partially due in the continuity of pathology throughout the region of migration.

The Need to Control Stem Cell Differentiation

The use of stem cells for the treatment of myelin loss required that several technical challenges be overcome. Stem cells are easily influenced by environmental factors and cellular deficiencies operating at the site of implantation, which are difficult to control. Pre-differentiation of expanded stem cell populations prior to transplantation is necessary to harness the power of stem cells while circumventing the risk of undesired differentiation. This is of particular concern in human neurodegenerative disease states or injuries, which are much more complex than "physiologically recipient" experimental models of acute demyelination. Cell-cell contact, genetic modulation, and diffusible factors such as bone morphogenic protein, sonic hedgehog, and tissue specific growth factors, all contribute to the complex regulation of cell differentiation and pattern formation *in vivo*. Transplantation of multipotent cell populations into adult CNS injury sites often results in extensive migration from the site of implantation, and the generation of large numbers of astrocytes.[73,74] Pre-differentiation of stem cells prior to transplantation is a means to direct their post-implantation fate.[16,75]

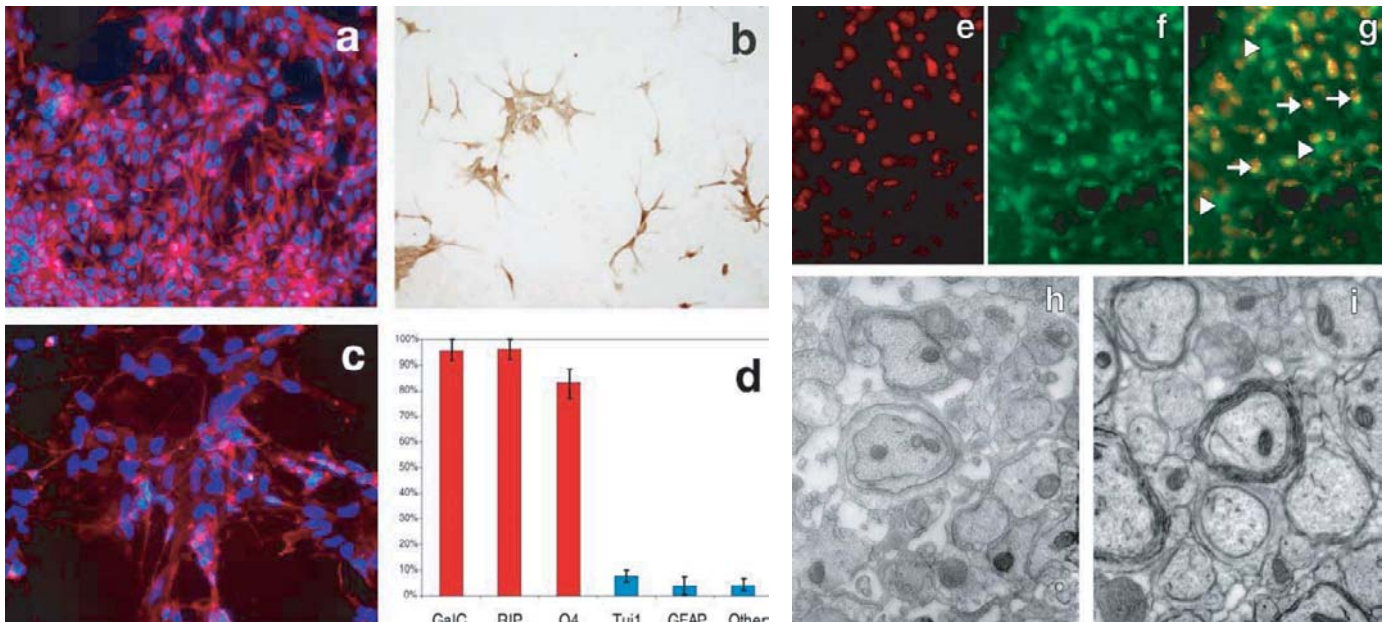
Tumor formation is another significant risk for transplant strategies involving stem cells, which increases inversely with the developmental age of the transplant population. Transplanted ESCs by definition form teratomas, which consist of endodermal, mesodermal and ectodermal lineages.[14] Pre-differentiation of ESCs prior to transplantation has been demonstrated to be a beneficial therapeutic regime that does not result in tumor formation.[9,16]

Oligodendrocyte Progenitor Derivation from hESCs

To address demyelination in human disease and injury states, we need human oligodendrocytes in high purity, and in large numbers. ESCs are the one tissue source that is capable of generating large amounts of tissue. One of the greatest challenges to the ESC field is the derivation of high purity cell populations. Our laboratory has recently demonstrated the ability to derive a high purity population of oligodendrocyte progenitor cells (OPCs) from human embryonic stem cells (hESCs).[17] Benefiting from studies of rodent and human stem cells over the past 20 years,[76] we developed a protocol for differentiating human oligodendrocytes and their progenitors using federally approved hESCs. Our protocol resulted in a high yield of high- purity OPCs.[16,17]

Briefly, one week after plating the spheres at low density in the absence of growth factors, greater than 95% of the cells labeled with oligodendroglial markers GalC, RIP, and O4 (Figs. 3a–3d). Less than 5% of the cells labeled for non-oligodendrocyte markers GFAP or TUJ1 (Fig. 3d). The hESC-derived OPCs also expressed morphology characteristic of oligodendroglial cells.[16,17]

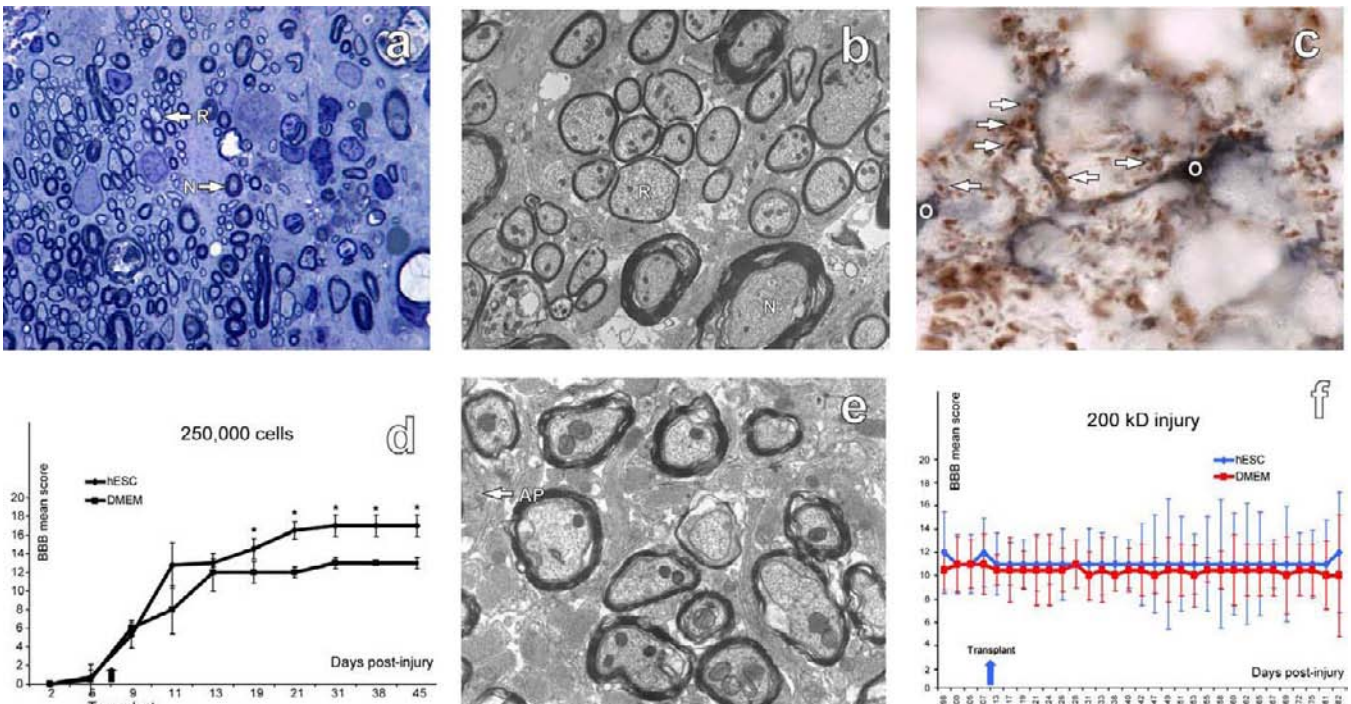
After derivation of high-purity OPCs, we then demonstrated their ability to integrate and display a functional phenotype *in vivo*. We utilized the spinal cords of the *shiverer* mouse model of dysmyelination in order to demonstrate that OPCs are capable of generating compact myelin. *Shiverer* mice are homozygous for a mutation in the myelin basic protein (MBP) gene located on chromosome 18 (*Mbpshi/Mbpshi)*, causing severe myelin deficiency throughout the CNS.[77] Such mice do not produce MBP which is essential for myelin compaction, and contain axons that are devoid of myelin or are surrounded by one or two uncompacted wraps of myelin.[66,78] Because *shiverer* mice do not contain compact



myelin or MBP, detection of multilayered compact myelin and MBP immunostaining following transplantation into *shiverer* mice has been used as the gold standard for demonstrating myelination from transplanted oligodendrocytes.[10,53,66,78–82]

Six weeks after transplantation, spinal cord tissue was collected for immunohistochemical analysis to determine cell survival and function in the host. BrdU pre-labeling was used to determine cell survival and migration. Immunohistochemical analysis of BrdU clearly demonstrated that the transplanted cells survived and integrated into the white matter (Fig. 3e). BrdU-labeled cells were present in transplanted animals 5mm rostral to 5 mm caudal to the implantation site (the entire length of tissue examined). In transplanted animals, BrdU and CC-1 double labeling was evident throughout the white matter, indicating that the hESC-derived OPCs differentiated into mature oligodendrocytes *in vivo* (Figs. 3e–3g). Electron microscopic analysis demonstrated that non-transplanted *shiverer* mice did not have multilayered compact myelin (Fig. 3h) or evidence of MBP positive staining. In contrast, *shiverer* mice transplanted with hESC-derived OPCs contained multilayered compact myelin in patches throughout the white matter (Fig. 3i) as well as patches of MBP positive staining in the white matter.

Fig. 3. Terminal differentiation of human embryonic stem cell (hESC)- derived oligodendrocytes evidenced by immunocytochemical staining, 1 week after plating at low density in the absence of growth factors. Greater than 95% of cells labeled with the oligodendroglial markers GalC (a), RIP (b), and 04 (c). (d) Quantitation of immunolabeling. Error bars illustrate standard deviation. (e–g) hESC-derived oligodendrocytes integrate, differentiate and display a functional myelinating phenotype following transplantation into the *shiverer* mutant mouse. (e) BrdU immunostaining illustrating the presence of transplanted BrdU pre-labeled cells within the spinal cord white matter. BrdU pre-labeled cells were found almost exclusively within spinal cord white matter. (f) CC-1 immunostaining on the same section as (e) illustrating oligodendrocytes. (g) Composite of BrdU and CC-1 double immunostaining, illustrating that transplanted BrdU+ cells adopted the oligodendroglial marker CC-1. Arrows point to double labeled cells; arrowheads point to BrdU-CC-1+ cell (endogenous *shiverer* oligodendrocytes). These panels indicate that transplanted BrdU pre-labeled cells survived and integrated within the spinal cord white matter, and became oligodendrocytes. (h) Electron micrograph illustrating that axons of *shiverer* mice are devoid of myelin or are surrounded by one or two uncompacted wraps of myelin. (i) Electron micrograph of the dorsal column white matter of a *shiverer* mouse 6 weeks after transplantation of hESC-derived oligodendrocyte progenitors, illustrating multilayered compact myelin 200× for (a); 400× for (b); 100× for (c); 400× for (e–g); 33,000× for (h) and (i).



These findings demonstrate the ability to derive a high purity population of OPCs from hESCs and that transplantation of these cells into an environment of demyelination results in integration, differentiation into oligodendrocytes and expression of a functional phenotype, thereby confirming oligodendrogliogenesis.

Transplantation of hESC-Derived OPCs into SCI

To assess the viability of hESCs as a cell replacement therapy for demyelination and restoration of function, we transplanted hESC-derived OPCs into a rodent model of SCI. We transplanted these cells into rat spinal cords 7 days (acute) and 10 months (chronic) after contusion injury.

OPCs transplanted 7 days following SCI survived, migrated 11 mm rostral and 3 mm caudal to the injury site and double labeled for BrdU and CC1[16] suggesting that the transplanted OPC differentiated into mature oligodendrocytes *in vivo*. Electron microscopic analysis demonstrated that acute transplantation of hESC-derived OPCs resulted in a significant increase in the density of oligodendrocyte myelination compared to human fibroblast (hFb) transplanted or DMEM controls (Figs. 4a–4c). We assessed the tissue for the extent of normally myelinated, demyelinated, oligodendrocyte or Schwann cell remyelinated axons around the injury

Fig. 4. Toluidine blue-stained transverse section (a) and electron micrograph (b) illustrating robust oligodendrocyte remyelination (labeled R, with characteristically thin myelin sheaths) amongst few normally-myelinated axons (labeled N). (c) Anti-GFP and anti-neurofilament double immunostains illustrating highly branched GFP + OPCs (labeled O) extending processes that ensheath nearby neurofilament + axons (arrows), confirming that remyelination was carried out by eGFP-labeled transplanted cells. (d) From approximately 3 weeks post-implantation for the duration of testing, animals that received 250,000 hESC-derived OPCs consistently demonstrated significantly greater locomotor capabilities ($p < 0.01$) as compared to controls, as determined using the BBB locomotor rating scale. (e) Electron micrograph of the transplant environment at 10 months post-injury, illustrating axons surrounded by enlarged intermediate filament-rich astrocytic processes, which occupied virtually all of the extracellular space. (f) The degree of locomotor recovery in animals that received 1.5 million cell-transplants was not significantly ($p > 0.1$) different from those that received vehicle-only injections, regardless of the severity of injury. 400× for (a); 3000× for (b); 600× for (c); 3000× for (e).

site. Quantification indicated that the density of oligodendrocyte remyelinated axons was significantly ($p < 0.01$) higher in OPC transplanted animals as compared to hFb-transplanted or DMEM injected control animals. OPC transplanted animals also contained significantly ($p < 0.01$) lower densities of demyelinated and Schwann cell remyelinated axons when compared to hFb-transplanted or DMEM injected control animals.[16] These findings indicate that transplanted hESC-derived OPCs effected robust remyelination, and suggest that the transplanted cells out-competed endogenous Schwann cells in the remyelination of axons around the injury site.

Behavioral analysis of animals transplanted at 7 days post-injury demonstrated a significant improvement in locomotor function (Fig. 4d). Animals transplanted with hESC-derived OPCs at 7 days performed significantly better on the BBB locomotor test than those transplanted with hFb or DMEM. Furthermore, animals that received OPCs at 7 days also exhibited improved motor scores using a four-parameter kinematic analysis when compared to controls. Taken together these data illustrate that transplantation of high-purity hESC-derived OPCs into SCI results in migration from the implantation site, integration into the white matter, remyelination of spared axons and subsequent recovery of function. This suggests that acute SCI may be a viable target for hESC-derived OPCs.

When OPC were transplanted at 10 months after SCI the cells again survived, migrated 11 mm rostral and 1 mm caudal to the injury, and integrated into the host tissue. Immunohistochemical analysis revealed that the transplant population double labeled for BrdU and CC1, indicating that they differentiated into oligodendrocytes.

Although hESC-derived OPCs transplanted into a chronic spinal cord injury survived and integrated into the host tissue, the cells failed to remyelinate spared axons. Normally myelinated and demyelinated axons were present; however, they were surrounded by thick astrocytic processes (Fig. 4e). Histological analysis illustrated that almost all of the extracellular space was occupied by astrocytic processes (Fig. 4e). Quantitative analysis demonstrated no significant difference in oligodendrocyte remyelinated axons in OPC-transplanted animals compared with controls. As expected based on histological evidence, animals transplanted with OPCs at 10 months did not show any locomotor improvement on the BBB compared to control animals (Fig. 4f). These data indicate that there is a therapeutic window for the functional benefits of OPC transplantation. OPCs transplanted into a chronic SCI did not remyelinate or contribute to locomotor improvement. This is likely due to widespread astrocytic scarring at

the injury site. The demyelinated axons were completely surrounded by astrocytic processes and the transplanted OPCs were unable to remyelinate them. Furthermore, these studies suggest that there is a therapeutic window of opportunity for remyelination and functional locomotor improvement following cell transplantation into SCI.[16]

Challenges for hESCs as a Clinical Cellular Replacement Strategy

The ability to derive myelinogenic transplant populations from stem cells and their success in mediating remyelination in experimental models does not guarantee their success in human demyelinating diseases. The complex nature of the lesioned human CNS presents several challenges that must be well understood and overcome before cellular transplant therapy will be successful.

Firstly, transplanted cells may be exposed to the same innate environmental conditions that led to the progression of the initial pathology. In immune-mediated demyelinating disorders such as MS, it is conceivable that the underlying inflammation may compromise the transplant, and the transplant could trigger autoaggressive inflammation that exacerbates disease. Long-term studies must be undertaken to understand the survival of transplanted cells in the chronic disease state. Secondly, the inability of glial progenitors to migrate through normal tissue[71] renders them less than ideal for remyelinating disparate foci of demyelination as seen in clinical disease. Although intracerebroventricular administration of myelinogenic transplant populations results in widespread dissemination and myelination in multiple animal models,[83] biodistribution outside of the targeted disease sites increases risk in the clinical setting. Thirdly, the glial scarring that is present in chronic lesions may interfere with the ability of a transplant population to extensively remyelinate. Fourthly, our inability to control inflammation within demyelinating lesions is likely to influence remyelination. Although inflammation may exacerbate rejection of the transplant, it has been shown to enhance migration and remyelination by transplanted cells.[83,84] This is especially problematic for immune-mediated disorders such as MS, where patients are treated with immunomodulatory agents. Lastly, preventing rejection of transplanted cells will likely require life-long immunosuppression, if methods to induce tolerance in transplant recipients[85] or generate

stable transplant populations with the genetic make-up of the donor[86] are not successful or commercially viable.

Summary

Recent research on human embryonic stem cells has demonstrated their ability to perform multiple beneficial functions following transplantation and their exciting therapeutic potential, but this is not without caution. These cells have enormous capacity for self-renewal, which is advantageous for human cellular replacement strategies, but have the potential to lead to teratomas. Pre-differentiation of hESCs is necessary to reduce the likelihood of tumor growth and increase the likelihood that the transplant population will survive and maintain their desired phenotype in the host. These cells can be driven to yield a high purity population of a desired lineage for a specific cell replacement strategy, reducing the influence of environmental signals on the transplant population. Although hESCs may be ideally suited for transplant-mediated remyelination, the environment of human demyelinating pathologies is not. The complex nature of human demyelinating diseases presents significant challenges that must be overcome before cellular replacement strategies become a clinical reality.

Acknowledgments

The authors thank Oswald Steward, Tom Lane, Jane Lebkowski, Catherine Priest and Jerrod Denham for discussion and advice. We thank Gabriel Nistor, Minodora Totoiu, Frank Cloutier, Rafael Gonzalez, Kelli Sharp, Charlie Mendoza, Julio Espinosa, and DoQuyen Huynh for intellectual input, assistance with animal surgeries and tissue processing.

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Microchimeric Foetal Stem Cells and Non-Invasive Prenatal Genetic Diagnosis

Magued Adel Aziz Mikhail

Introduction

Stem cells are a subject of great research interest and may in the near future provide the basis of new therapies for damaged or lacking cells, tissues or even organs. Cell-based therapy, often referred to as regenerative medicine, is a promising field; thus, encouraging the scientists to invest time and effort. The eventual isolation of stem cells and the studies on their biology have led to new important insights into biology, development, tissue regeneration, pathological conditions and cancer. All of these came into existence by applying "stem cell thinking" to already known processes. More medical applications of stem cells are expected in the future, especially if all the aspects of stem cell research are pursued in a safe, harmless and regulated manner.

Non-invasive prenatal genetic diagnosis could be one of those fields that could also benefit from the clinical application of stem cells. Foetal

and prenatal medicine is a rapidly expanding field and the development of methods for isolation and detection of foetal cells from maternal blood has evolved into an important research area, which shows promise for future applications in non-invasive prenatal diagnosis.[1] Early prenatal determination of foetal gender is important and is an advantage to parents who are known to be at risk of X-linked genetic disorders or conditions associated with ambiguous development of external genitalia.[2]

The development of a low-cost, low-risk, reproducible, reliable and non-invasive method based on the retrieval of rare foetal cells from peripheral venous maternal blood will render testing practical for the general population. If chromosome and single-gene analysis could be accomplished non-invasively, prenatal diagnosis could then be offered to all pregnant women. Reliable prenatal genetic diagnosis currently involves the analysis of foetal cells cultured after recovery by using invasive procedures, such as chorionic villus sampling (CVS), amniocentesis and umbilical cord sampling.

These procedures are costly and require special skills and each procedure is a significant risk to the mother and foetus. Invasive procedures are generally not offered to all pregnant women but only to those who are at high risk: 35 years and older, women with abnormal maternal serum marker detected by screening, ultrasound detection of foetal abnormalities and increased likelihood of a single-gene disorder.

Amniocentesis

Amniocentesis is the most common procedure at present. It is normally carried out in the second trimester between the 15th and 18th week of pregnancy, this timing is determined since there is a need for the test to be carried out late such that it would result in a consistently successful outcome. We need 2–3 weeks for the cells to be grown before the results are available. Decisions have to be made quickly, especially if a termination is indicated. The risks associated with this procedure include a 0.5% to 1% foetal loss rate and a rare chance of either sepsis in the mother or needle puncture in the foetus.[3] A slight increase in postural deformities (Talipes) and respiratory difficulties in the neonate have been reported. Beside its known hazards, amniocentesis should not be used if there is an active vaginal infection, uterine abnormalities or fibroids.

Chorionic Villi Sampling

CVS allows for the karyotyping of the foetus during the first trimester of pregnancy. The test is performed at 10–13 weeks by taking a small sample of the chorion using a needle directed by ultrasound. This can be done either by a trans-cervical or by a trans-abdominal approach. The trans-cervical route is associated with a greater risk of infection, ruptured membranes and abortion.[4] This test allows for an earlier diagnosis of abnormality than mid-term amniocentesis but there is a slightly weaker correlation between the results obtained by this method and those obtained by amniocentesis because of the mosaicism confined to the placenta (the placenta may have cells with an abnormal complement of chromosomes even though the foetal karyotype is normal). The foetal loss rate with this technique is about 3% to 4%.[4]

Also CVS should not be performed if neural tube defects are suspected or if the placenta is abnormally positioned.

Foetal Blood Sampling, Also Called Cordocentesis

Cordocentesis allows a blood sample to be taken from the umbilical vein, where it is attached to the placenta, allowing for a number of tests to be performed including PCR and karyotyping. The major risks of this technique are foetal exsanguinations, haematoma of the cord, chorioamnionitis and about 6% above the normal foetal loss rate.[3]

Maternal Serum Screening

In maternal serum screening, specific proteins produced by the foetus or placenta are measured and compared with population standards. The ability to detect abnormal levels of hormones and other proteins in the maternal blood has long been the goal of clinicians. Probably, the best known test is the triple screen designed to assess the risk of a mother carrying a foetus suffering from trisomy 21 (Down's syndrome), neural tube defects, trisomy 18, trisomy 13 and sex chromosome aneuploidy.

Prenatal Imaging

Ultrasound, normally used to screen pregnancies, can also be used as a diagnostic tool for structural abnormalities that are known to be found within

a family but have no known chromosomal or molecular defects. Many ultrasonographic markers have been associated with trisomy 21, including nuchal fold thickening, cardiac defects and shortening of the long bones.[5] Targeted foetal ultrasonographic assessment performed in referral centres identifies 40% to 60% of foetuses with trisomy 21.

Foetal Cells in the Maternal Circulation

Over the past 20 years there has been much interest in the development of non-invasive techniques. Of these, isolation of foetal cells from the maternal circulation early in pregnancy could replace existing methods since it should allow exact genetic diagnosis without risks to the foetus. Despite the variety of foetal cells recognised in the maternal circulation, including trophoblast cells, nucleated erythrocytes, leucocytes, platelets and progenitors, non-invasive prenatal diagnosis has been hampered by technical challenges in enrichment, identification and diagnosis. In particular, current strategies for prenatal diagnosis on foetal cells in the maternal circulation are limited due to the lack of a cell type present only in foetal blood and by the low frequency of the trafficking foetal cells.[6–8]

Although more than a century has elapsed since Schmorl[9] demonstrated the presence of trophoblasts in the pulmonary circulation of pregnant women who had died of complications of eclampsia. Douglas *et al.*[10] identified circulating trophoblasts in 1959. Foetal cells in maternal blood were not considered as a material for prenatal genetic diagnosis until Walknowska and Grumbach[11] observed male cells among cultured lymphocytes obtained from 21 pregnant women, 19 of whom subsequently delivered male infants. These findings were later validated by other investigators, who reported the presence of cells with Y-chromatin in the circulation of women pregnant with male foetuses.[11]

Herzenberg and co-workers[12,13] used fluorescence-activated cell sorting (FACS) to detect and isolate foetal cells in the late 1970s and early 1980s. Convincing evidence with regard to the existence of foetal cells in maternal blood came in 1990 with the application of molecular methods to detect unique amplified foetal DNA sequences from cellular components of the blood of pregnant women.[14,15] Bianchi *et al.*[16] found foetal erythroblasts identified by Y chromosome sequences in a small series of male pregnancies using FACS for enrichment of foetal erythroblasts from the maternal blood.[16] This apparent ability to select foetal nucleated red blood

cells (FNRBC) with FACS led Bianchi and others to couple it with fluorescence *in situ* hybridisation (FISH) with chromosome-specific probes to identify foetal aneuploidies.[17–21]

Methods of Foetal Cell Isolation

Since the examination of millions of nucleated cells present in a small volume of blood is time- and labour-intensive, attempts at foetal cell isolation and analysis have used some processes intended to separate foetal from maternal cells, or to enrich the cell sample population for foetal cell percentage presence. Enrichment by FACS or magnetic-activated cell sorting (MACS) is used to separate one type of cell from another. FACS uses physical parameters, such as cell size, in addition to antibody recognition of single cells to mechanically direct targeted cells into a collection vessel. MACS traps the cell in an iron–antibody complex and attracts it to a magnetised surface from which it can subsequently be freed and collected.[22]

To test the approaches for using foetal cell properties, many researchers constructed model systems using cord or foetal blood cells, or CVS cells seeded in adult blood to mimic the behaviour of foetal cells in the maternal circulation.[23–26] Usually, there are initial steps to deplete or eliminate unwanted cells beginning with the mature, enucleate red cells and then progressing towards unwanted maternal nucleated cells. This may or may not be followed by some step intended to "select" or deliberately sort out foetal cells from the remaining background of maternal cells. Initial steps have included bulk separation, lysis and various forms of selective centrifugation. Many protocols begin with some form of density gradient centrifugation intended to concentrate mononuclear cells (including erythroblasts) in a discrete "band" of cells at a specific density controlled by the composition of the gradient.

Numerous methods for isolating foetal cells from maternal peripheral blood have been developed, including density gradient centrifugation,[27,28] MACS of FNRBC[29–31] or trophoblast,[32–34] flow cytometry,[16,17,35–37] micromanipulation of individual cells,[38–40] selective lysis[41] and charge flow separation.[42] These methods required high levels of experience and when successful, yielded only a few foetal cells.

Trophoblasts are interesting cells because they are specific to pregnancy and are unique to the embryo; however, their large size and being multinucleated or anucleated,[43] and not being consistently detected in the maternal

circulation and difficult to separate from maternal circulation[44] make them problematic to study.

After Bianchi's success, most people ignored the trophoblast in favour of the erythroblast.[22]

Many researchers have selected nucleated red blood cells (NRBC) as the target cells for the development of non-invasive prenatal diagnosis in maternal blood as it is the most common foetal blood cell.[22]

Foetal NRBC are commonly targeted with anti-CD71 antibody to the transferrin receptor, but this labels other foetal nucleated cells and adult cells as well.[22] Erythroblasts have several advantages, such as their relative abundance in the early foetal circulation, the expression of antigens that allows their enrichment and identification, and their short life span, which precludes the isolation of foetal cells from previous pregnancies.[45–47]

The disadvantage of NRBC is that they cannot be expanded in culture, thereby ruling out metaphase chromosome analysis, an essential component of prenatal diagnosis. Moreover, a recent report presenting the summary of first five years of NIFTY study (National Institute of Child Health and Human Development Foetal Cell Study) revealed that the foetal male NRBC could be detected in only 48% of the samples in which MACS was employed as the enrichment.[48]

Leucocytes are long-lived cells and may potentially cause culture contamination with foetal cells from previous pregnancies.[24]

Foetal Cell Microchimerism

Foetal cell microchimerism, originally described in mice, is defined as the presence of a small number of foetal cells in the maternal blood and tissues without any apparent graft versus host reaction or graft rejection.[49] Foetal cells can persist in the maternal blood or tissues for decades, creating a state of physiologic microchimerism in the parous woman.

Foetal cells enter the maternal circulation during all pregnancies[50] but are very rare. The number of foetal cells entering into the maternal circulation may be influenced by foeto–maternal histocompatibility.[51] Near term, at 36 weeks of gestation, 100% of pregnant women have foetal cells in their circulation.[50]

The amount of foetal cell transfer to the maternal circulation is also affected by foetal and placental abnormalities.

There is an increased foeto–maternal cell transfer in cases of foetal aneuploidy,[52] maternal pre-eclampsia, toxaemia of pregnancy[53] or following terminations of pregnancy.[54] The latter was shown by meta-analysis where a reproductive history that includes an elective termination or an early foetal loss is associated with a higher incidence of microchimerism in maternal tissues probably due to increased transfusion at the time of loss or to the transfer of a cell at an early developmental stage that is more likely to engraft in the mother.[51] After delivery this fraction rapidly decreases. In as many as 90% of healthy postpartum women, foetal cells were detected by examining peripheral blood mononuclear cells.[55]

Although difficult to detect, foetal cell microchimerism is a widespread phenomenon and it is now accepted that foetal cells are transferred to the maternal circulation during all human pregnancies and persist for decades after delivery.[51]

Foetal cells may play a role both in the mother's peripheral circulation and in her tissues during gestation. During pregnancy, foetal cells have been shown to reflect the biology of pregnancy by being associated with dermatologic disorders, such as polymorphic eruption of pregnancy[56] and systemic disorders, such as pre-eclampsia.[57] Foetal microchimeric cells were also present in higher numbers in women with some autoimmune diseases, such as systemic sclerosis, than in control groups[58–62] and non-autoimmune disorders, such as hepatitis C[63] and cervical cancer.[64] Thus, foetal cells were associated with the maternal response to injury as opposed to causing disease. A non-invasive procedure based on the retrieval of microchimeric foetal cells circulating within the maternal blood for antenatal genetic analysis would enable foetal testing without risking the pregnancy. Male and female foetal cells cross the placenta in equal numbers. However, it is quicker and easier to detect the presence of chimeric male cells due to the universal nature of the Y chromosome, which is uniquely present in foetal cells and absent in maternal cells (Fig. 1).

Foetal Cells and Non-invasive Prenatal Genetic Diagnosis

Foetal cells enter the maternal circulation during all pregnancies[50,65] and have long been considered the Holy Grail of non-invasive prenatal diagnosis.[6] Molecular technology has provided definitive evidence of foetal

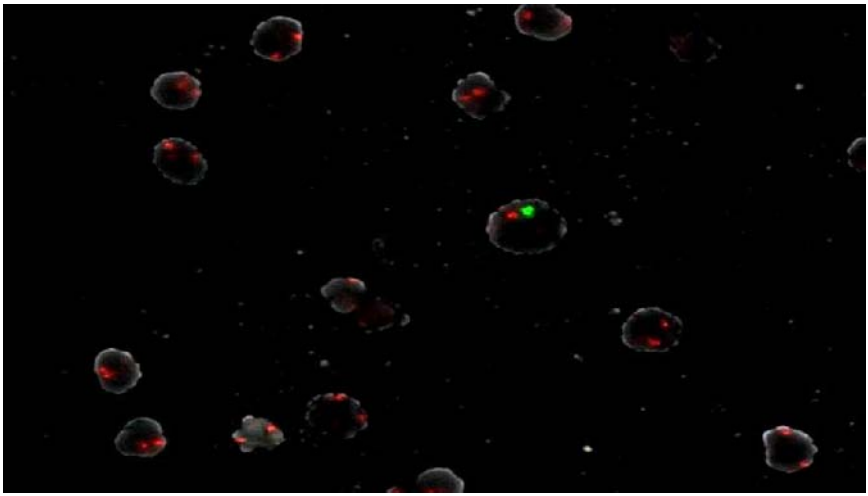

Fig. 1. Normal male cell among female cells identified by the FISH technique: the red spot marks the X chromosome and the green spot the Y chromosome.

nucleated cells in the circulation of pregnant women,[14,66] from as early as 4–6 weeks' gestation.[67] Using foetal cells isolated from the maternal peripheral blood might allow prenatal genetic diagnosis to be performed earlier than currently possible.

Different types of foetal cells were found to be present in the maternal circulation during pregnancy offering a promising approach for non-invasive prenatal diagnosis and including haematopoietic progenitors and stem cells,[68,69] nucleated red blood cells (NRBC or erythroblasts),[16,70,71] leucocytes[72] and trophoblasts.[32,40,44]

The Challenges of Isolating Foetal Cells from Maternal Peripheral Blood

Although the presence of foetal cells in the maternal circulation has been proven beyond any doubt, the clinical application of the techniques developed so far is not yet feasible.[1] Targeting the foetal cells has proved to be technically challenging because of three main problems impeding the isolation of foetal cells from maternal blood. The main problem is their scarcity and low frequency in the maternal blood. The frequency of foetal cells detected in the blood of pregnant women increases with the gestational age[73] occurring at a frequency of 1 foetal cell/ml of maternal peripheral

venous blood in the normal second trimester of pregnancy as determined when the total number of foetal cells was studied by quantitative PCR.[74]

The absence of suitable foetal cell markers and corresponding monoclonal antibodies is another serious challenge[24,75,76] impeding the development of a method for non-invasive foetal cell sampling for prenatal genetic diagnosis.

A third problem in foetal cell analysis is the persistence of foetal cells in the maternal circulation after delivery. The presence of residual foetal cells from previous pregnancies in the maternal circulation can interfere with the diagnosis of subsequent pregnancies. The ones most likely to persist are the less differentiated cells.[24,77]

Overcoming the Challenges

To be useful in prenatal diagnosis, foetal cells must not only be separated and distinguished from a vast majority of maternal cells but also must be enriched to an acceptable level of purity and unequivocally identified as cells of foetal origin.[36] Culture expansion of candidate cells could potentially aid in overcoming some of the current difficulties by providing a larger number of cells for molecular and cytogenetic analysis including dividing cells for the metaphase karyotype analysis. Analysis of almost all the recovered cells is also needed and this could be achieved by using automated image cytometry.[78]

The detection and analysis of foetal cells is currently performed by the FISH technique using chromosome-specific probes and PCR.

Foetal Stem Cells: The Solution?

A true stem cell can be defined as a primitive undifferentiated cell with high capacity for self-renewal which can give rise to one or more specialised cell types with specific functions in the body.[79–81]

Stem cells have three unique properties: they do not have any tissue-specific structures that allow them to perform specialised functions (unspecialised); they are capable of dividing and renewing themselves for long indefinite periods without a change in phenotype; and they can give rise to specialised cell types under certain physiologic or experimental conditions.[82,83] Some stem cells are characterised by the expression of the $CD34^+$ cell-surface antigen.[84–86] Some studies have demonstrated that

the foetal blood presents a higher frequency of haematopoietic progenitors than adult peripheral blood.[85] The foetal microchimeric cell population must contain stem cells that can proliferate because it is difficult to imagine how fully differentiated foetal cells that have a short half-life without a self-renewal capacity could regularly appear in the maternal blood, and tissue decades after delivery[51,87] also showed that the uterus is a dynamic organ permeable to foetal stem cells capable of trans-differentiation. Taylor[88] further revealed that the uterus is an end organ in which adult bone marrow stem cells may find a home and differentiate. Thus, the uterus can now be thought of as a bi-directional conduit for stem cell transfer.[89]

The disparate ranges of foetal cell types that traffic into the maternal bloodstream include haemopoietic stem cells (HSC) and non-haemopoietic mesenchymal stem cells (MSC). Foetal haemopoietic progenitors have been found in the maternal circulation from early gestation onwards. Foetal MSC, which circulate in first trimester foetal blood, have been proposed as an alternative target cell for non-invasive prenatal diagnosis because they appear to have no counterpart in adult blood and can be clonally expanded into a pure source of foetal cells. However, although foetal MSC are likely to cross the placenta based both on the theoretical considerations and on findings, foetal MSC are detectable in a small proportion of maternal blood samples, and they appear to circulate at very low numbers, making any application in the field of non-invasive prenatal diagnosis unlikely.[90]

Nevertheless, following pregnancy, male foetal cells have been demonstrated in the $CD34^+$ compartment.[77,91,92] They have also been found in various sorted subsets of maternal peripheral mononuclear blood cells,[55,93] suggesting that foetal microchimeric cells may be capable of engraftment and differentiation along the haematopoietic pathway.

The haematopoietic progenitor $CD34^+$ cell is an attractive candidate cell and, because in all human pregnancies foetal progenitor cells that express CD34 and foetal haematopoietic stem cells are transferred into the maternal circulation,[77] they have the potential to allow early prenatal diagnosis without having to invade the gravid uterus.

The other advantage over other types of foetal cells is that $CD34^+$ haematopoietic stem cells in maternal blood are clonogenic; hence, they can be cultured and amplified to proliferate *in vitro*.[36,68,94–96] Under appropriate conditions these cells proliferate in culture even more rapidly than those of adult origin.[97] Targeting a cell capable of proliferation in culture partly alleviates the difficulties arising from the scarcity of foetal cells in maternal blood. Enrichment of $CD34^+$ stem cells from the peripheral

mononuclear cells can be performed using MACS before FISH analysis is performed. Target chromosomes can be identified using fluorescent probes. Therefore, cells bearing the Y chromosome identified among maternal cells can demonstrate foetal cell migration to maternal blood during pregnancy.[98] Foetal-specific markers for $CD34^+$ cells have not been identified. Gender-based detection with FISH or PCR is possible in the case of male foetus. However, this approach does not provide an adequate solution because female foetal $CD34^+$ cells cannot be distinguished from maternal $CD34^+$ cells.[99] Female foetal cells can be detected using family-specific DNA polymorphisms or uniquely paternally inherited genes.[100]

The concern has been raised that foetal $CD34^+$ cells can persist in the maternal circulation after delivery and potentially interfere with the interpretation of results derived from circulating foetal cells in consecutive pregnancies.[24] Using either culture or analysis by FISH and PCR, Coata *et al.*[69] proved that foetal $CD34^+$ stem cells in maternal circulation do not affect diagnosis in later pregnancies by enriching $CD34^+$ stem cells from the peripheral blood of pregnant women including eight with male foetuses, non-pregnant women who have given birth to male offsprings, and women who have never been pregnant (Fig. 2). The analysis of cultured $CD34^+$ stem cells from mothers with positive Y PCR did not detect any male cells in any sample. Coata *et al.*[69] concluded that foetal stem cells do not persist in the maternal blood and also explained the presence of chimeric stem cells as long as 27 years postpartum by probably being of a paternal origin since fallopian tubes are open to the peritoneal cavity and cellular microchimerism may be induced by the sexual activities. The other hypothesis is that foetal cells in maternal blood have been demonstrated to

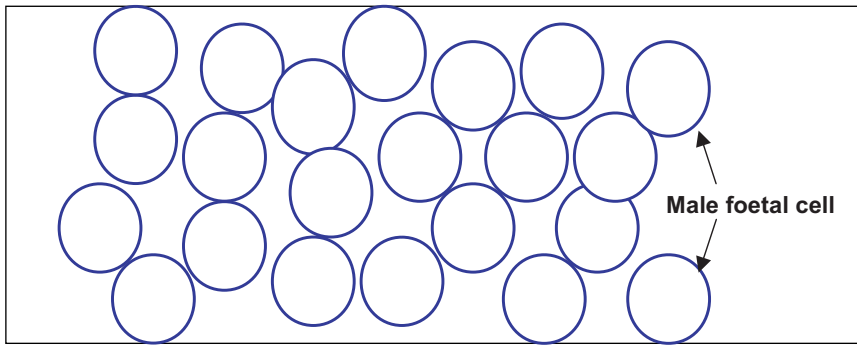

Fig. 2. Illustration of foetal cells in peripheral maternal blood.

have a mean half-life of 16.3 minutes and they are rapidly cleared from the maternal circulation.[101,102]

Conclusion

During pregnancy, the foetal cells that enter the maternal circulation are predominantly of haematopoietic origin, such as NRBC, lymphocytes or haematopoietic stem cells.[6,103] Trophoblasts and MSC also circulate within maternal blood.[104,105]

Non-invasive prenatal diagnosis through isolation and diagnosis of rare foetal cells present in the maternal circulation has been successfully demonstrated in numerous experimental systems. However, a reliable reproducible protocol that would enable application in a clinical-diagnostic setting is not yet available.[77]

The rarity of foetal cells in the maternal circulation and the lack of specific foetal cell markers is the reason for the most challenging difficulties to isolate, identify and examine these cells. Sensitivity and recovery of foetal cells is jeopardised by the minute number of circulating foetal cells and their loss during the enrichment procedure. The clonogenic stem cells can overcome the current challenging difficulties of the rarity of foetal cells and is giving hope for the possibility of an accurate, non-invasive, reliable and cheap prenatal diagnostic tool.

Foetal stem cells cross into maternal blood during pregnancy and thus represent a potential non-invasive source of foetal genetic material for prenatal diagnosis.[22] Targeting a cell type capable of proliferation in culture would partly alleviate the difficulties arising from the scarcity of foetal cells in maternal blood. Along these lines, the possibility of targeting foetal $CD34^+$ cells, since they proliferate in culture, have been identified in the maternal circulation[77] and is recommended.

Similar to many expanding fields of scientific inquiry, stem cells raise questions and hopes as rapidly as generating new discoveries. Further research is needed to explore the phenomenon of bi-directional foeto–maternal cell trafficking.

The future studies will show whether the foetal stem cells could be used to diagnose, as well as to increase our understanding of diseases that occur in the mother during and after pregnancy. A reliable method to detect the foetal gender and the presence of numerical or structural chromosomal abnormalities is a possibility that will hopefully be achieved one day by the non-invasive isolation and the direct examination of the foetal stem cells.

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14

The Role of Stem Cells in Liver and Gastrointestinal Cancer

Malcolm R. Alison, Nicholas A. Wright and Simon Leedham

Introduction

Numerous studies point to the fact that many tumours are derived from single cells (monoclonal), but the important question is, which cell? Stem cell biology and cancer are inextricably linked. In continually renewing tissues such as the intestinal mucosa and epidermis, where a steady flux of cells occurs from the stem cell zone to the terminally differentiated cells that are imminently to be lost, it is widely accepted that cancer is a disease of stem cells, since these are the only cells that persist in the tissue for a sufficient length of time to acquire the requisite number of genetic changes for neoplastic development. In the liver the identity of founder cells for the two major primary tumours, hepatocellular carcinoma (HCC) and cholangiocarcinoma (CC), is more problematic. The reason for this is that no such obvious unidirectional flux occurs in the liver, although it is held that the centrilobular hepatocytes may be more differentiated (polyploid) and closer to cell senescence than those cells

closest to the portal areas. Moreover the existence of bipotential hepatic progenitor cells (HPCs), along with hepatocytes endowed with longevity and long-term repopulating potential suggests that there may be more than one type of carcinogen target cell. Irrespective of which target cell is involved, cell proliferation at the time of carcinogen exposure is pivotal for the "fixation" of genotoxic injury into a heritable form. Taking this view, any proliferative cell in the liver can be susceptible to neoplastic transformation, whereas throughout the gastrointestinal tract the respective stem cell zones are the likely sites of transformation. An in-depth discussion on the causes of cancer in the gut and liver are beyond the scope of this chapter, but infectious agents such as *Helicobacter pylori* and hepatotropic viruses, by virtue of causing chronic inflammation, play a major role. Elevated epithelial proliferation in a milieu rich in inflammatory cells, growth factors and DNA-damaging agents (reactive oxygen species and nitrogen species produced to fight infection) will lead to permanent genetic changes in proliferating cells. The up-regulation of the transcription factor NF-κB in transformed epithelial cells may be critical for tumour progression given the mitogenic and anti-apoptotic properties of proteins encoded by many of NF-κB's target genes.

Liver and Gut Cancer

Two major primary cancers of the liver are HCC and CC. HCC is the fifth most common cancer worldwide and the third most common cause of cancer death. HCC is defined by the World Health Organization as a malignant tumour composed of cells resembling hepatocytes but abnormal in appearance; a plate-like organisation around sinusoids is common and is nearly always present somewhere in a tumour. Most HCCs (80%) arise in a cirrhotic liver, i.e. a situation where there has been long-standing hepatocyte damage and chronic inflammation leading to fibrosis. There are huge geographical variations in the incidence of HCC, with the highest incidence in areas such as Eastern Asia and sub-Saharan Africa where chronic hepatitis B virus (HBV) infection is a major risk factor.[1] In Europe and the United States, the incidence of HCC is low but slowly increasing, probably due to the rise in people infected with hepatitis C virus. Apart from hepatotropic viruses, the other major risk factors for HCC are those that led to cirrhosis such as alcohol abuse and metabolic

liver disease, mutagens such as aflatoxins, and toxic metabolites of the food mould *Aspergillus* sp.

CCs are believed to arise from biliary epithelium that is either within the liver (intrahepatic) or extrahepatic. The tumour is much less common than HCC, but its incidence and associated mortality has been increasing steadily over the past two to three decades, with most tumours arising in persons over 50 years, suggesting that carcinogenesis is a protracted and (possibly) multi-step process.[2] Injury to the biliary epithelium with chronic inflammation, together with impedance of bile flow, are the common factors in high-risk conditions for CC such as primary sclerosing cholangitis, hepatolithiasis (gall stones) and liver fluke infestation by *Opistorchis viverrini* and *Clonorchis sinensis*. Thus, chronic inflammation features prominently in the histogenesis of both HCC and CC, where the coordinated actions of the likes of iNOS and cytochrome *c* oxidase-2 (COX-2) will lead to oxidative DNA damage, cell proliferation and a suppression of apoptosis.[3]

The cells that line the gastrointestinal tract are amongst the most rapidly proliferating cells in the body with differentiated cells undergoing continual replacement. They are also exposed to a hostile environment as they come into close contact with numerous toxins and carcinogens contained in digested food. Thus it is of little surprise that cancer of the digestive system is common, with 255,640 new cases in the US alone in 2004.[4] Partly because of its rapid cell turnover and high cancer prevalence the gastrointestinal epithelium is an important tissue in the understanding of cancer biology. Colonic polyposis syndromes were first recognised 200 years ago, and it has been 100 years since inflammatory and adenomatous polyps were characterised.[5] The observation of familial cancer syndromes led to the establishment of polyposis registries, with one of the largest starting at St Marks Hospital in London in 1925. Work on the familial colonic cancer syndromes, including familial adenomatous polyposis (FAP), has led to a number of advances in the understanding of intestinal tumour initiation including the recognition that many colonic adenocarcinomas arise from adenomas.[6] The adenoma–carcinoma sequence has subsequently been established as a stepwise pattern of mutational activation of oncogenes and inactivation of tumour suppressor genes that result in cancer.[7] Malignant cells share a number of characteristics with stem cells, such as the ability to self-replicate and proliferate, and it is widely believed that the gastrointestinal stem cell is the target for mutational changes.

Stem Cells of the Liver and Gut

If we believe that tumours have their origins in normal stem cells, or at least in cells with sufficient longevity to acquire multiple genetic mutations, then we need to appreciate normal cell turnover in the liver and intestine.

Hepatic stem cells

Foetal liver is a source of bipotential progenitor cells (hepatoblasts) as seen by their extensive colonisation of the diseased livers of rats after transplantation.[8] In postnatal animals, hepatocytes are highly differentiated cells with multiple synthetic and metabolic functions; they are also the functional stem cells in the liver under most circumstances. In health, individual hepatocytes have a life expectancy of over a year. Therefore in the normal adult liver, there is little cell proliferation detectable with only 0.01% of hepatocytes in the cell cycle at any one time. However, in response to parenchymal cell loss, hepatocytes restore the liver mass by self-replication. This is a very efficient system and in rodents, when two-thirds of the liver is resected (partial hepatectomy, PH), the remaining remnant can re-grow to the original liver size in approximately ten days. This model has been intensively studied and has provided much data on the mechanisms controlling liver regeneration.[9,10] In response to this stimulus, normally quiescent hepatocytes leave G_0 to enter the cell cycle under the influence of many growth factors. Hepatocyte proliferation begins in the periportal region of the liver and spreads to the centrilobular region. This regeneration requires each hepatocyte to undergo, on average, less than two rounds of replication to restore the liver to its original size. This does not however mean that hepatocytes have a limited replication potential. Hepatocyte transplantation models in mice have shown that hepatocytes are capable of significant clonal expansion within the diseased livers of experimental animals. In the fumarylacetoacetate hydrolase (FAH)-deficient mouse, a model of hereditary type 1 tyrosinemia, there is strong positive selection pressure exerted on transplanted wild-type hepatocytes as host hepatocytes readily undergo cell death due to the cytoplasmic accumulation of fumarylacetoacetate (FAA). Without transplantation, the FAH null genotype is lethal unless the mice are protected by 2-(2-nitro-4-trifluoro-methylbenzoyl)-1,3-cyclohexanedione (NTBC), a compound that prevents the accumulation of cytotoxic FAA. When 10^4 normal hepatocytes from congenic male wild-type mice are intrasplenically injected into mutant female mice and the NTBC treatment is

withdrawn, these cells colonise the mutant liver efficiently.[11] Moreover, serial transplantations from these colonised livers to other mutant livers indicated that at least 69 hepatocyte doublings can occur, thereby confirming the clonogenic potential of hepatocytes, meeting one of the main criteria that defines stem cells.

The clonogenic ability of human hepatocytes in chronic hepatitis can be indirectly estimated. Using mathematical modelling of viral kinetics it has been estimated that in chronic HBV infection, between 0.3% and 3% of all hepatocytes are killed daily and therefore replaced to maintain a stable liver cell mass (this approximates to 10^9 of the liver's 2×10^{11} hepatocytes).[12] This is in accordance with the hepatocyte proliferation levels in chronic hepatitis B and C, where proliferating cell nuclear antigen (PCNA) indices of 0.1% to 3.6% are found, and Ki-67 labelling indices in hepatitis C of 1% to 14%.[13,14] In chronic hepatitis the parenchymal mass can therefore be maintained through prolonged hepatocyte self-replication, and such cells could be the target for DNA-damaging agents and thus initiation events.

The hepatocyte proliferation rate increases in hepatitis C with increasing histological damage until cirrhosis is reached when the proliferation rate falls.[15] This fall in hepatocyte proliferation rate may represent hepatocytes coming to the end of their division potential and undergoing *replicative senescence*,[16] although other factors such as distortion of blood flow through the liver are also likely to play a part. Whatever the reason, the reduction in hepatocyte proliferation indices in chronic hepatitis occurs concurrently with the activation of a potential stem cell compartment located within the smallest branches of the intrahepatic biliary tree. This so-called ductular reaction in human liver is equivalent to the oval cell reaction seen in many rodent models of hepatocarcinogenesis. The development of an oval cell reaction in response to hepatocyte replicative senescence has been demonstrated in a transgenic mouse model of fatty liver and DNA damage. In this model, the mice developed fatty livers and a large number of senescent hepatocytes. A striking oval cell response developed in these mice which related to the number of senescent mature hepatocytes.[17]

Following extensive liver damage or in situations where hepatocyte regeneration after damage is compromised, a potential stem cell compartment located within the smallest branches of the intrahepatic biliary tree is activated. This "oval cell" or "ductular reaction" amplifies a biliary population of transit-amplifying cells that are at least bipotential, capable of differentiating into either hepatocytes or cholangiocytes. Most rodent models of oval cell activation have employed potential carcinogens to inhibit

hepatocyte replication in the face of a regenerative stimulus. For example in the rat, protocols have included administering 2-acetylaminofluorene (2-AAF) to inhibit hepatocyte proliferation before creating a demand for hepatocyte proliferation by PH or a necrogenic dose of carbon tetrachloride.[18] The need to maintain parenchymal cell mass results in the development of an oval cell response in the liver that spreads from the edge of the portal tract to deep into the parenchyma (Fig. 1A). Oval cells are small cells with a large nuclear to cytoplasmic ratio, in which the nucleus has a distinctive ovoid shape. Because oval cells express some of the antigens traditionally associated with haematopoietic cells (c-kit, flt-3, Thy-1 and CD34), there was speculation that hepatic oval cells were directly derived from bone marrow precursor cells. However, most studies now concur that the location of a stem cell niche for oval cells is in the canals of Hering, which is

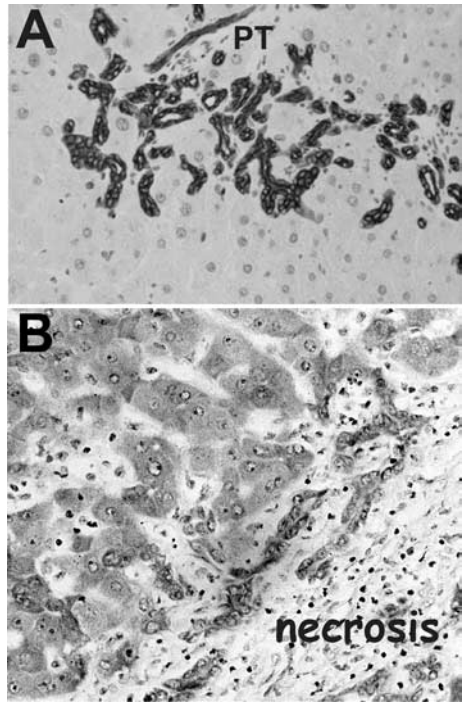

Fig. 1. (A) An oval cell reaction in the rat liver. Oval cells, highlighted by cytokeratin 19 immunoexpression, branch out from the portal tract (PT). (B) An extensive ductular reaction in a human liver in response to parenchymal necrosis. Both hepatocytes and ductular cells express cytokeratin 18, but the strongest expression is in the ductular cells.

a transitional zone between the periportal hepatocytes and the biliary cells lining the smallest terminal bile ducts.

The human counterparts to the oval cells described in rodent models are often referred to as HPCs. These have been observed after severe hepatocellular necrosis, chronic viral hepatitis, alcoholic liver disease and non-alcoholic fatty liver disease. It is thought that activation of the potential stem cell compartment leads to the formation of reactive ductules, anastomosing cords of immature biliary cells with an oval nucleus and a small rim of cytoplasm (Fig.1B).

Differentiation towards the hepatocyte lineage occurs via intermediate hepatocytes, these are polygonal cells with a size and phenotype intermediate between progenitor cells and hepatocytes (Fig. 2).[19] After submassive liver cell necrosis, reactive ductules, in continuity with intermediate hepatocytes, are seen at the periphery of the necrotic areas. In patients studied with sequential liver biopsies, intermediate hepatocytes become more numerous with time and extend further into the liver lobule. This sequence of changes suggests the gradual differentiation of human progenitor cells into intermediate hepatocytes, analogous with what is seen in rat models of chemical injury associated with impaired hepatocyte replication. Elegant three-dimensional reconstructions of serial sections of human liver immunostained for cytokeratin-19 have shown that the smallest biliary ducts, the canals of Hering, normally extend into the proximate third of the lobule (unlike those in rodents), and it is envisaged that these canals react to massive liver damage (akin to a trip-wire), proliferation and then differentiation into hepatocytes.[20] Oval cell numbers in human liver rise with increasing severity of liver disease.[21]

There is also the possibility that some oval cells/hepatocytes could be derived from circulating bone marrow cells (BMCs), although the results to date have been highly variable ranging from non-existent in some studies of long-term liver allografts to over 40% of hepatocytes being derived from bone marrow. One suspects that this variation is partly due to differences in the severity of parenchymal damage, but equally well may be a reflection of the ability (or lack of it) of the intrahepatic stem/progenitor cells to mount an effective regenerative response to damage.[8]

The “proof of principle” demonstration that bone marrow could cure mice with a potentially fatal metabolic liver disease, hereditary tyrosinemia type-1, was a landmark publication.[22] In the setting of liver failure, wild-type bone marrow could apparently switch lineage determination and differentiate into hepatocytes expressing the enzyme “fumarylacetoacetate

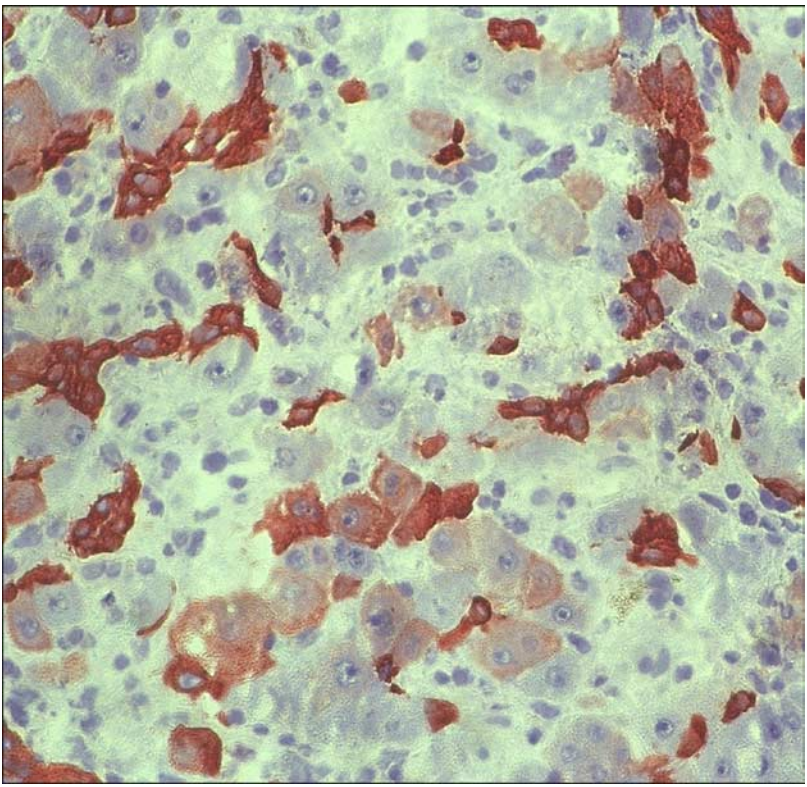

Fig. 2. Ductular reaction in a case of massive hepatic necrosis due to acetaminophen toxicity, immunohistochemically stained for CK7. Note many intermediate cells are still with a biliary-type staining pattern ($CK7^+$), but with a morphology midway between cholangiocytes and hepatocytes (courtesy of Professor Tania Roskams with permission from John Wiley Publishers).

hydrolase" (Fah), the component of the tyrosine catabolic pathway absent in tyrosinemic ($fah^{-/-}$) animals. Such lineage switching has been called *transdifferentiation* or *plasticity*. However, it is now clear that the *new* functioning liver cells in the $fah^{-/-}$ mouse are each the result of *cell fusion* between a donor bone marrow-derived macrophage and an $fah^{-/-}$ hepatocyte nucleus.[23] From an oncology point of view, the interest in such heterokaryons is that many of these cells appeared to be genomically unstable, seemingly shedding chromosomes at random to become aneuploid.[24] Although the exact significance of bone marrow-derived cells to liver disease is far from fully elucidated, the fact that damaged hepatocytes can alter

the lineage commitment of haematopoietic stem cells (HSCs) towards that of hepatocytes without the occurrence of cell fusion[25] and that a number of studies now report on the ability of human cord blood mononuclear cells to give rise to hepatocytes in the liver of NOD/SCID mice (reviewed in Alison *et al.*[8]), the possibility that primary liver tumours could be initiated in bone marrow-derived cells cannot be discounted.

Intestinal stem cells

The immature, relatively undifferentiated nature of gastrointestinal epithelial stem cells means that they are not directly identifiable and researchers in this field in the past have had to rely on ingenious indirect methods to identify their position and track their progeny.[26] The putative stem cell compartment position varies according to the location in the digestive tract. Throughout the small and large intestine the luminal surface is clothed by a simple columnar epithelial cell layer, with glandular invaginations called crypts. Several of these crypts contribute epithelium to finger-like projections called villi in the small bowel. The cells of the intestinal epithelium are arranged hierarchically, becoming progressively more differentiated as they age and pass along the crypt-to-villus axis. The stem cell compartment is believed to be at the origin of this axis, the base of the colonic crypt and at cell positions 4 to 5 in the small bowel (reviewed by Brittan and Wright[27]). The number of stem cells within each crypt is debated but is generally believed to be between 4 and 6.[28,29] Stem cells themselves divide infrequently and it is the first few generations of stem cell daughters, known as transit-amplifying cells, that proliferate in the lower part of the crypt.[30] Stem cells reside within a stem cell compartment or "niche". This is a group of epithelial and mesenchymal cells and extracellular substrates, which provide an optimal microenvironment for stem cells to give rise to their differentiated progeny. In the intestinal crypts, this is formed by a fenestrated sheath of surrounding mesenchymal cells which regulate stem cell behaviour through paracrine secretion of growth factors and cytokines.[31] Functionally, a niche is characterised by its persistence on the removal of stem cells and the cessation of stem cell potential when cells are removed from this niche.[32] The rapid turnover of the gastrointestinal epithelium means that differentiating cells are shed into the lumen and replaced every few days; thus, they do not have a sufficient lifespan to gather the multiple genetic defects required for malignant transformation. Therefore, the

perpetual stem cell has long been considered the target of carcinogenic mutations.[30,33,34]

Where Do the Cancers Arise?

Liver cancer

The assessment of DNA alterations in tumour cells allows a precise determination of their clonality. Where HBV is involved, there is no doubt that the integration of HBV–DNA into the hepatic genome is a significant event in hepatocarcinogenesis.[1,35,36] Moreover inspection of viral integration sites amongst tumour cells clearly indicates that each tumour is monoclonal, i.e. derived from a single cell.[37–40] Likewise, studies of HCC clonality based on restriction fragment length polymorphisms of X-linked genes such as the androgen receptor gene (HUMARA) come to the same conclusion.[41] The important question is, which cell is involved in cancer initiation? As discussed above, in the liver there are many cells endowed with longevity and long-term repopulating potential, suggesting that there may be more than one type of carcinogen target cell. Irrespective of which target cell is involved, what is clear is that cell proliferation at the time of carcinogen exposure is pivotal for "fixation" of the genotoxic injury into a heritable form.

Animal models

Many models of liver cancer utilise a brief exposure to an initiating carcinogen at a time when the liver is in a proliferative state, either during the period of postnatal growth or shortly after a PH or necrogenic insult. For example, Craddock[42] clearly demonstrated the carcinogenic effects of dimethylnitrosamine (DMN) on the rat liver when it was administered one day after a PH (when some 30% to 40% of hepatocytes would be in S phase), whereas the same compound, at the same dose, was not carcinogenic to normal adult rats.

Taking this view, Sell[43–45] has opined that in models of experimental hepatocarcinognesis as a whole, there may be at least four distinct cell lineages susceptible to neoplastic transformation (see Fig. 3). This supposition is based on the fact that there is considerable heterogeneity in the proliferative responses that ensue after injury in the many different models of hepatocarcinogenesis. Thus, hepatocytes are implicated in some models of

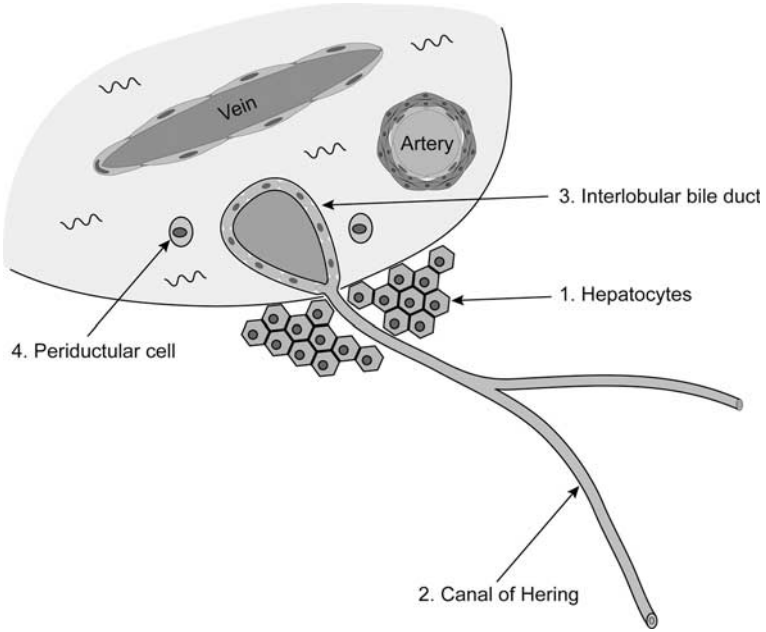

Fig. 3. Schematic diagram of various lineages that respond to specific cell damaging insults and therefore are likely founder cells for the tumours that develop subsequently. (1) The cells that normally respond to hepatocyte loss are the hepatocytes themselves; (2) potential stem cells may reside in the canal of Hering and they or their progeny (oval cells/HPCs) may give rise to most HCCs; (3) the interlobular bile duct epithelia may give rise to CCs associated with fluke infection; and (4) periductular cells are associated with experimental hepatocarcinogenesis associated with ethionine and a choline-deficient diet. Largely based on an idea by Stewart Sell.

HCC, direct injury to the biliary epithelium implicates essentially unipotent cholangiocytes in some models of CC, whereas HPC/oval cell activation accompanies many instances of liver damage irrespective of aetiology, making such cells most likely to be carcinogen targets. A fourth cell type that might be susceptible to neoplastic transformation is the so-called non-descript periductular cell that responds to periportal injury; the suggestion that such a cell may be of bone marrow origin would be experimentally verifiable in the context of a sex-mismatch bone marrow transplantation (see above) and the appropriate carcinogenic regimen.

So, for example, after diethylnitrosamine (DEN) exposure, there is little oval cell proliferation but the emergence of α-fetoprotein (AFP)-positive hepatocytes, followed by AFP^+ foci and eventually AFP^+ HCC, suggests

that HCC develop from hepatocytes in this model.[45] On the other hand, direct injury to the bile ducts induced by furan leads to bile duct hyperplasia and intestinal metaplasia, and prolonged furan exposure results in CCs with a smaller number of HCCs, observations consistent with a bile duct cell origin of these tumours. Likewise, fluke infection can lead to marked bile duct hyperplasia and subsequent exposure to DMN leads to the rapid development of CC. Many models of hepatocarcinogenesis are characterised by a striking proliferation of oval cells, particularly the so-called "Solt-Farber" model, where a single exposure to DEN is followed by a course of 2-AAF designed to block the regenerative ability of normal hepatocytes; thus, when a PH is performed only those cells "initiated" and therefore resistant to the antiproliferative effects of 2-AAF can respond — hence the "resistant hepatocyte" model of carcinogenesis (reviewed in Allison *et al.*[46]). Despite the name of the model, Sell concludes that the sequence of hepatocyte foci, to nodules of increasing size to HCC is most likely to have its origins in bipotential oval cells. In animal models of metabolic liver disease such as Wilson's disease, hepatocyte destruction and inflammation is accompanied by a prominent oval cell reaction (Fig. 4), and, perhaps not surprisingly, a high incidence of both HCC and CC. A fourth type of cell that may be involved in carcinogenesis is the periductular oval cell[47] that proliferates and expresses AFP in response to carcinogens such as ethionine in a choline-deficient diet (CDE diet).

The direct involvement of hepatocytes in hepatocarcinogenesis has been clearly established in rats. Gournay *et al.*[48] found that some preneoplastic foci (expressing gamma glutamyl transpeptidase and the placental form of glutathione *S*-transferase) were directly descended from hepatocytes. This was achieved by stably labelling hepatocytes at one day after a 2/3 PH with β-galactosidase using a recombinant retroviral vector containing the β-galactosidase gene; subsequent feeding with 2-AAF lead to foci, some of which were composed of β-galactosidase-expressing cells. Using the same labelling protocol, Bralet *et al.*[49] found that 18% of hepatocytes expressed β-galactosidase at the completion of regeneration after a 2/3 PH; subsequent chronic treatment with DEN resulted in many HCCs, 17.7% of which also expressed β-galactosidase, leading to the conclusion that a random clonal origin of HCC from mature hepatocytes was operative in the model.

Extensive polyploidy is associated with terminal differentiation and cell senescence, and in many models of chronic injury precedes overt oval cell development. Moreover, the carcinogenic process in animals and man is associated with the presence of more diploid cells — consistent with an

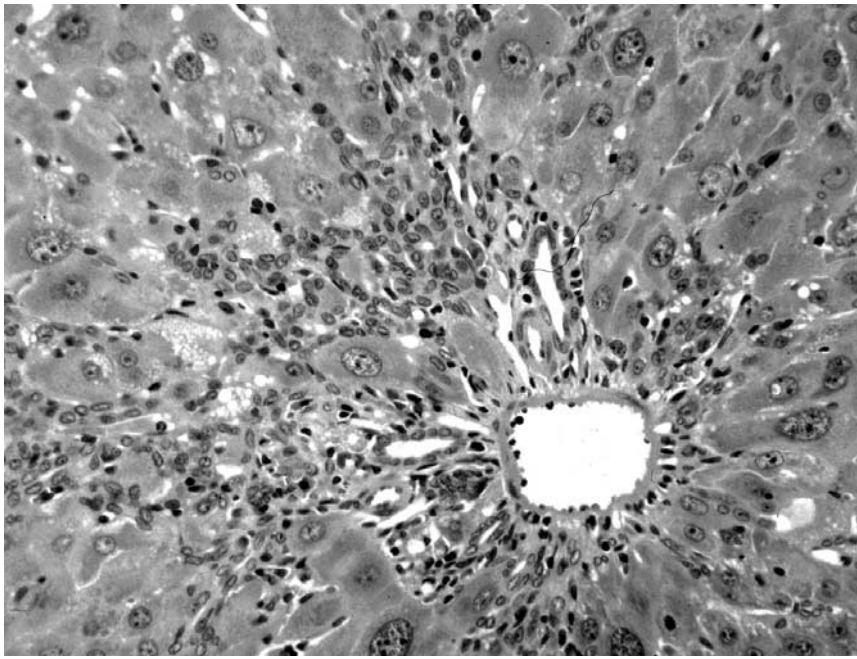

Fig. 4. An oval cell reaction in a Long Evans Cinnamon (LEC) rat. These animals develop HCC and CC with a high frequency.

expansion of oval/HPCs during ongoing liver injury, cells that eventually give rise to so-called small cell dysplasia, a likely precursor lesion for HCC (see below). If tumours do arise from oval/HPCs, then this would suggest a block in oval cell differentiation, a process called stem cell maturation arrest by Sell and Pierce.[50] Direct evidence for the involvement of oval cells in the histogenesis of HCC was provided by Dumble *et al.*[51] who isolated oval cells from p53 null mice; when these cells were transplanted into athymic nude mice they produced HCCs.

Human studies

As in rodents, HCC appears to evolve from focal precursor lesions that reflect the stages of multi-step carcinogenesis. Usually in a setting of chronic inflammation with liver cell damage and concurrent regeneration, activation of HPCs invariably follows,[15,19,21,52] and the first lesions are thought to be either foci of small cell dysplasia or low-grade dysplastic nodules.[53] Further rounds of mutation and clonal expansion eventually led to HCC (Fig. 5).

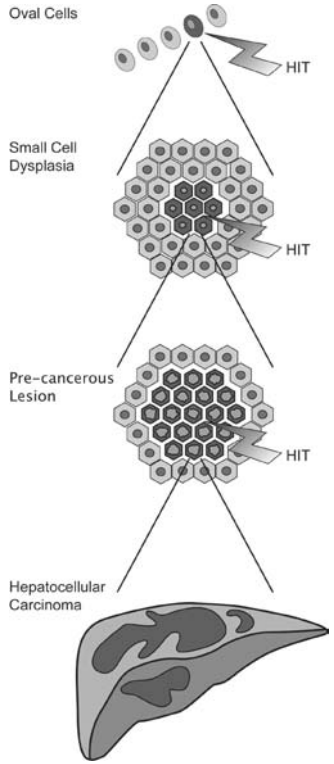

Fig. 5. A multi-step model for the progression to HCC in human liver. Oval cells (HPCs) may be involved in the histogenesis of many HCCs and initially give rise to foci of small cell dysplasia. Further rounds of mutation and clonal expansion may give rise to other pre-cancerous lesions such as low-grade dysplastic nodules and high-grade dysplastic nodules before HCC develops.

It seems highly likely that mature polyploid hepatocytes are not the cells of origin of most HCCs, but rather that most HCCs have their origin in HPCs.

An origin of HCC from HPCs is often inferred from the fact that many tumours contain an admixture of mature cells and cells phenotypically similar to HPCs. This would include small oval-shaped cells expressing OV-6, CK7 and CK19, and chromogranin-A, along with cells with a phenotype intermediate between HPCs and the more mature malignant hepatocytes[54] (Fig. 6). Cells resembling HPCs (OV.1$^+$ or OV-6$^+$) have also been noted in hepatoblastoma;[52] this tumour, the most common liver tumour in childhood is widely believed to be stem cell-derived given that there can be

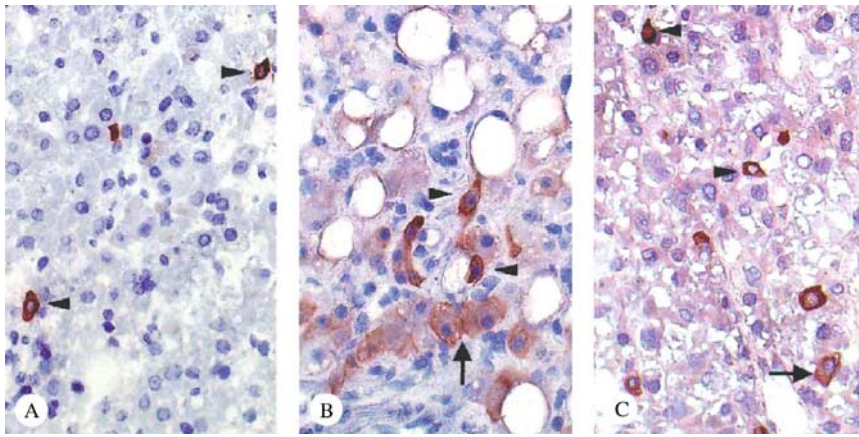

Fig. 6. HPCs (arrowheads) in a hepatic adenoma immunoreactive for CK19 (A), OV-6 (B), and chromogranin-A (C). Intermediate hepatocyte-like cells (arrows) positive for OV-6 and chromogranin-A surround the HPCs (courtesy of Professor Tania Roskams).

both epithelial and mesenchymal tissue components. Cells with an HPC phenotype have also been noted in a relatively rare subset of hepatic malignancies where there are clearly two major components, an HCC component and a CC component, again suggestive of an origin from a bipotential progenitor.[55]

Colon cancer

Numerous steps are involved in the progression of normal tissue through dysplasia to malignancy, and based on the observation that the accumulation of molecular alterations seemed to parallel the clinical progression of tumours, Vogelstein *et al.*[7] proposed a stepwise model of colorectal tumourigenesis. The molecular pathogenesis of FAP has shed much light on the initial mutations required in this step-like progression. FAP results in the formation of multiple bowel adenomas in the second and third decades of life. Colonic cancer is inevitable in these patients who therefore require prophylactic colectomy. The heritable nature of FAP was first recognised at the end of the 19th century; however, it was not until 1986 that an interstitial deletion of chromosome 5q was observed in an FAP patient.[56] This prompted linkage analysis studies which co-demonstrated tight linkage of the condition to markers on chromosome 5q21.[57] The gene responsible was Adenomatous Polyposis Coli (*APC*),[58] which encodes a large (approximately 2800 amino acids), multi-functional cytoplasmic protein. APC binds

and down-regulates β-catenin and is vital in control of Wnt signalling. Mutations in *APC* are also found in 63% of sporadic adenomas[59] and up to 80% of sporadic colorectal cancers.[60] Mutations in *APC* are present in very early adenomas[59] and are sufficient to promote small adenoma growth in the absence of microsatellite instability, K-ras or β-catenin mutation or allelic loss of 1p.[61] Thus, APC inactivation provides a stem cell with a selective growth advantage by allowing unregulated activation of Wnt signalling. Mutations in β-catenin, preventing its breakdown can also promote adenoma initiation; however, small adenomas with β-catenin mutations alone do not progress to larger adenomas or carcinomas as frequently as adenomas with *APC* mutations.[62] Therefore, although the role of APC in the regulation of Wnt signalling is most important in the prevention of tumour initiation, its involvement in apoptosis and chromosomal stability also have an effect on the progression of the adenoma growth (reviewed in Fodde *et al.*[63]).

Clonal expansion of mutated cells — niche succession and crypt fission

There are three possibilities that can result from a stem cell division:

- The production of one stem cell and one daughter cell — asymmetric division.
- Symmetric division with self-replication, where two stem cells are produced.
- Symmetric division with stem cell loss, where both daughter cells go on to differentiate.

The majority of divisions are thought to be asymmetric and there is some evidence supporting the retention of template DNA strand within the stem cells located in the niche — the so-called immortal strand hypothesis, ensuring any DNA replication errors are passed on to the differentiating, short-lived daughter cells, affording a mechanism of stem cell genome protection.[64] Park *et al.*[65] used ethyl nitrosurea (ENU) to induce mutations in the X-linked gene for glucose-6-phosphate dehydrogenase (G6PD) to demonstrate the expansion of a mutated clone within the crypt. *G6PD* gene mutation resulted in loss of staining in affected cells. After ENU treatment, they initially observed crypts that were only partially stained for G6PD, which eventually disappeared with the contemporaneous emergence of fully mutated crypts (monoclonal conversion or crypt purification). These eventually gave rise to patches of crypts that failed to stain with

G6PD. More recently, Taylor *et al.*[66] used mitochondrial DNA mutations in colonic crypt cells to demonstrate the presence of partially mutated crypts in the human colon. They observed that human colonic crypt cells accumulate sufficient mitochondrial DNA (mtDNA) mutations with age to cause a biochemical defect in the mtDNA-coded subunits of COX. This defect can also be stained for with immunohistochemistry. Normal colonic tissue shows numerous completely COX-deficient crypts, but also a few partially stained crypts. Serial sections of these partial crypts allowed them to reconstruct three-dimensional images of the crypt revealing a ribbon of COX negative cells extending from the base of the crypt to the top. The ribbon of mutated, COX negative cells appear to be the progeny of one of the small number of stem cells in the niche, and that the partially negative crypts are likely to be intermediate steps in the expansion of the mutated clone with eventual formation of a completely clonal COX-deficient crypt.

Yatabe *et al.*[67] used CpG-methylation patterns in three non-expressed genes to study the dynamics of the stem cells within the niche, and proposed the niche succession model. They showed that the differences in methylation tag sequence between cells in adjacent crypts (intercrypt variation) were more pronounced than the tag variation seen between cells in the same crypt (intracrypt variation). They proposed that intracrypt variation was a consequence of multiple, yet related stem cells within each crypt. Stochastic extinction or amplification of one stem cell line by occasional symmetrical division results in a "bottleneck" effect, wherein all cells in the crypt are descended from an original stem cell. Mathematical modelling suggested that this bottleneck occurs once every 8.2 years in the normal human colon. In normal appearing crypts from FAP patients; however, there was a greater intracrypt variation in methylation tags suggesting slower niche succession, probably as a consequence of enhanced stem cell survival. The longevity of $APC^{+/-}$ stem cells in FAP patients increases the chances of receiving or selecting for a second hit in the gene.[68] Once APC protein function is impaired, a growth advantage is bestowed on the cell and clonal expansion would then occur much more quickly.

Niche succession is a way by which a single stem cell line can "hitchhike" its way to clonal dominance of a single crypt,[69] but then how does a stem cell line expand into adjacent tissue? As described above, clonality experiments in both mice and humans have shown clustering of mutated, phenotypically similar crypts together in patches.[65,66] It is thought that a process called *crypt fission*, whereby crypts undergo basal bifurcation followed by longitudinal division, with the ultimate formation of two daughter

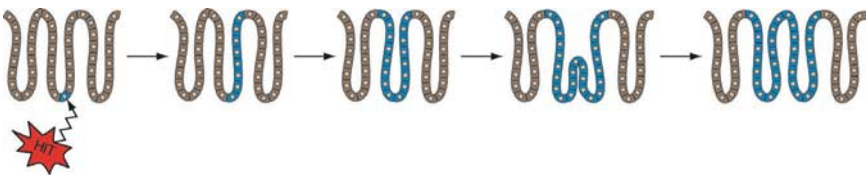

Fig. 7. A model for the development of early colorectal cancer (CRC). Mutation occurs in a stem cell located near the base of the crypt and mutated cell progeny occupy part of the crypt. Through a stochastic process (called niche succession or monoclonal conversion) the affected crypt becomes wholly occupied by dysplastic cells — the monocryptal adenoma. Further expansion can occur by the dysplastic crypt undergoing crypt fission and budding leading to an oligocryptal adenoma (aberrant crypt focus). Key: normal colonocytes are brown and mutated colonocytes are blue.

crypts, is responsible for the clustering of apparently related crypts. This process is central in the massive increase in crypt number (in both the small and large intestine) in the postnatal period and in the regenerative phase following radiation. The crypt cycle — crypts born by crypt fission gradually increasing in size until they, themselves, divide by crypt fission — takes approximately 108 days in the mouse jejunum and 9–18 years in the human large intestine. It was originally suggested that a crypt would be prompted to go into fission once it had reached a threshold size; however, attention has now focused on the stem cell number being the important factor. The rate of crypt fission is increased in many pathological conditions and is undoubtedly the mechanism by which dysplastic crypts multiply to form microadenomas or dysplastic aberrant crypt foci (ACF)[70] (see Fig. 7). ACF are morphologically and genetically distinct lesions that are the precursors of adenomas and cancers.[71]

Top-down or bottom-up?

Studies have shown that dysplastic ACF are clonal populations,[72] and expand by crypt fission;[73] however, the expansion of a mutated clone from a single cell to form small adenomas is contentious, with two main theories — the top-down and bottom-up models (Fig. 8).

The top-down model is based upon the frequent observation of dysplastic cells solely at the luminal surface of the crypts,[74] along with apparent retrograde migration of adenomatous cells from the surface to the base of the crypt. Shih *et al.*[74] examined the morphology and molecular characteristics of small (1–3 mm), well-orientated specimens from sporadic adenomas. Using digital single nucleotide polymorphism (SNP) analysis

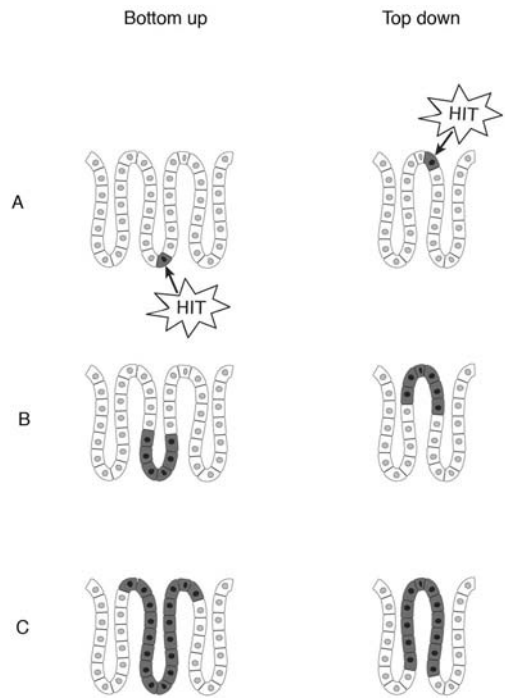

Fig. 8. Top-down or bottom-up growth of colorectal adenomas? Bottom-up: the stem cell, located in the crypt base undergoes *APC* mutation (A). The mutated cell proliferates (B) and spreads to the top of a crypt to form a monocryptal adenoma (C). Initial further expansion is by crypt fission (based on Preston *et al.*[79]). Top-down: the initial transformation event occurs in a cell in the intracryptal zone (A) and then spreads laterally and downwards (B) eventually filling the whole crypt (C) (adapted from Shih *et al.*[74]).

of four SNPs within the *APC* gene, they assessed for LOH of *APC* in cells in the upper portion of the crypts, most of which had truncating *APC* mutations on nucleotide sequence analysis. These were not seen in the histologically normal crypt bases. Only these upper crypt cells showed prominent proliferative activity and nuclear localisation of β-catenin. These observations were not easily reconciled with the conventional view of the stem cell origin of cancer, and the authors proposed two possible explanations to explain their findings. First, they considered a relocation of the stem cell area to the intracryptal zone, and second they suggested that a mutated stem cell migrates from the base of the crypt to the luminal surface before expanding laterally and downwards. Lamprecht and Lipkin[75] adjusted the latter model slightly to suggest that *APC* mutations occur within

a transit-amplifying cell, preventing it from terminally differentiating and altering the migration dynamics of the cell, and allowing it to remain in the mucosa as an incipient aberrant clone. The bottom-up model involves the recognition of the earliest lesion in tumour development, the *monocryptal adenoma*, where the dysplastic cells occupy an entire single crypt. These lesions are common in FAP,[76] and although rare in non-FAP cases, have been described previously.[77] Clonality studies in the XO/XY FAP patient have shown that monocryptal adenomas are clonal populations.[78] Analysis of tiny (<3 mm) adenomas in FAP patients showed increased proliferative activity and nuclear β-catenin translocation in morphologically dysplastic cells from the crypt base to the luminal surface. Additionally, there was a sharp cut-off between the dysplastic surface epithelium with nuclear β-catenin activity, and the normal mucosa in a neighbouring unaffected crypt. The observation of an increased, asymmetrical crypt fission index in adenomatous tissue led the researchers to propose the bottom-up model — an abnormal stem cell clone with a growth advantage expands from the stem cell niche at the crypt base to fill an entire crypt. Thereafter, initial spread is by crypt fission to form ACF, with top-down spread undoubtedly occurring in slightly larger lesions.[79]

Bone Marrow and Liver and Gut Cancer

The possibility that BMCs could be involved in carcinogenesis has been explored: regarding the liver, female mice were transplanted with male BMCs from β-galactosidase transgenic mice and HCCs were induced by DEN; however, none of the tumours was male or X-gal positive.[80] However, more extensive studies would be needed to rule out such a possibility, and indeed such an association has been claimed for *Helicobacter felis*-related murine gastric cancer, where ablation of the indigenous stem cell compartment because of protracted tissue damage (gastritis), has resulted in gastric adenocarcinoma being derived from bone marrow,[81,82] possibly mesenchymal stem cells rather than HSCs (Fig. 9).

BMCs may indirectly influence tumour behaviour through a contribution to the desmoplastic response and to the tumour vasculature (Fig. 10). Endothelial progenitor cells (EPCs) constitute a unique population of peripheral blood mononuclear cells derived from bone marrow that are involved in postnatal angiogenesis during wound healing, limb ischaemia, post-myocardial infarction, atherosclerosis and tumour

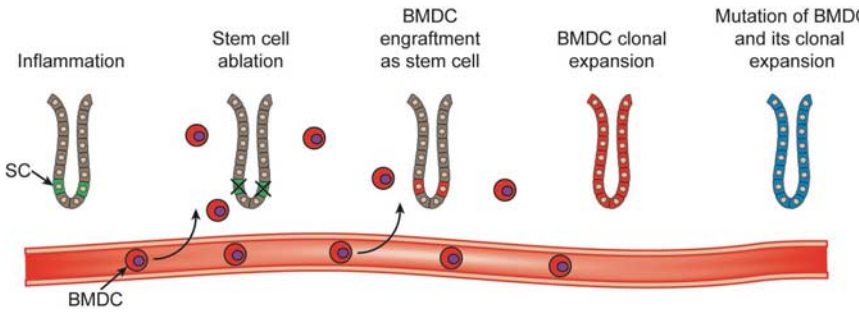

Fig. 9. A new paradigm of epithelial cancer development. Continued tissue damage leads to loss of the indigenous stem cell compartment and its replacement by bone marrow-derived cells (BMDCs), whose progeny subsequently repopulate the whole crypt. Mutation in a BMDC engrafted as a stem cell can then lead to adenoma formation as described in Fig. 7. Key: Indigenous normal epithelial cells are brown, indigenous stem cells are green, BMDCs are red and mutated BMDCs are blue.

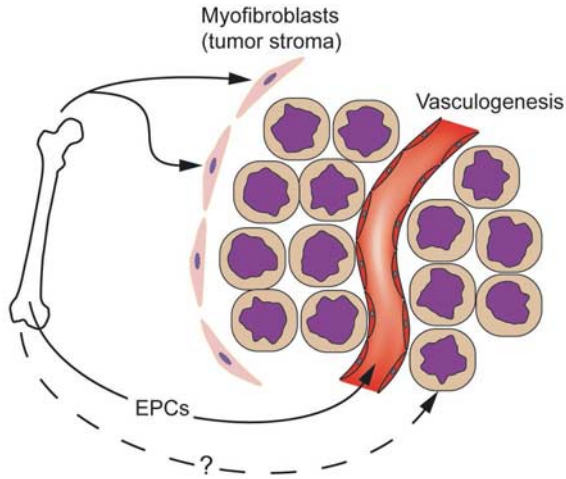

Fig. 10. In the context of tumours, the myofibroblasts and fibroblasts comprising the tumour stroma can be derived from the bone marrow as can many of the endothelial cells of the neovasculature (vasculogenesis). Gastric carcinomas in mice have been found to arise from BMCs engrafted in the gastric epithelium. Whether many human carcinomas have a similar origin is unknown, but it will be difficult to ascertain.

vascularisation. Circulating EPCs are mobilised endogenously in response to tissue ischaemia or exogenously by cytokine therapy to augment neovascularisation. Davidoff *et al.*[83] convincingly demonstrated the therapeutic potential of BMCs by transducing them with a retroviral vector encoding a soluble, truncated form of VEGF-2 (Flk-1; tsFlk-1); when transplanted into mice bearing neuroblastomas they were found to engraft into the neovasculature and very significantly impede tumour growth. In transgenic mice engineered to develop mammary tumours, Dwenger *et al.*[84] observed that BMCs preferentially home to the blood vessels of the tumour. In humans too, BMCs contribute to tumour vasculature: Peters *et al.*[85] observed that up to 12% of endothelial cells can be from the bone marrow. All these observations indicate that BMCs can be important delivery agents for anti-tumour gene therapies as well as for conventional anti-angiogenic drugs.

Myofibroblasts are cells with features of both smooth muscle cells and fibroblasts. They are widely distributed, having roles in growth and differentiation as well as in the inflammatory response. They are also important in injury and contribute to the processes of fibrosis and scarring, where they produce extracellular matrix proteins such as the interstitial collagens. BMCs contribute to myofibroblast populations throughout the body,[86] including the liver[87] and the gastrointestinal tract.[88] More significantly, BMCs also contribute to tumour stromal myofibroblasts.[89,90] The significance of such observations is that it is becoming increasingly evident that stromal myofibroblasts are key players in the control of tumour cell behaviour,[91] and will surely be exploited in the development of many new anti-cancer strategies.

Conclusions

In this chapter we have tried to summarise the role of several cell lineages that might be involved in neoplastic development in the liver and gut. In the gut, the founder cells of carcinoma intuitively seem to be the mucosal stem cells, and examination of the growth patterns of early lesions would support such a proposal. In the liver, the ability of hepatocytes, the unipotent cholangiocytes and the bipotent oval/HPCs to contribute to liver regeneration is not in doubt, although the identity of the cells within the parenchymal mass and biliary tree deserving of the appellation of "stem cells" is still unclear: all such cells could be carcinogen targets. Tumour stromal myofibroblasts

and vascular cells may be bone marrow-derived, illustrating the pivotal role of BMCs in supporting tumour development; a direct involvement of BMCs in carcinoma formation has been found in one case.

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Embryonic Stem Cells: Innovation in Predictive Toxicology

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Predictive Toxicology and the Promise of Embryonic Stem Cells

Predictive toxicology focuses on the evaluation of chemicals that can result in adverse effects on humans. Hundreds of chemicals, namely enzymes, hormones, and neurotransmitters are synthesized endogenously in mammalian organisms and are critical to cellular physiology and homeostasis. Predictive toxicology addresses largely synthetic chemicals that are not produced by target mammalian organisms, or xenobiotics, such as therapeutic small molecules, and environmental toxicants.

Adverse chemical effects on biological systems range from genome-wide nuclei acid mutagenesis, or genotoxicity, to tissue-specific alterations, such as liver failure and cardiac arrhythmia. Importantly, chemical exposure during critical periods of development and/or adulthood may be an underlying cause of several life-threatening and debilitating diseases such as Parkinson's disease,[1] autism[2] and cancer.[3] Strikingly, certain alterations

from toxic response can be transmitted through the germ line and may affect as much as three generations from the exposed individual.[4,5] The effects of environmental chemicals on subsequent generations is considerable, since inheritable changes are not restricted to a few individuals, but may affect the majority of the offsprings, as suggested by recent findings in epigenetic changes in sperm.[5]

This discovery urges the development and implementation of robust screening systems to prevent both inheritable and non-heritable abnormalities following human exposure to harmful chemicals.

Animal models have been invaluable for risk assessment of chemical compound safety; however, critical limitations persist for robust prediction of certain toxic outcomes in humans. Toxicity to tissues such as the hematopoetic and gastrointestinal systems has overall similar outcomes between animal models and humans.[6] In other organs, such as the liver, where xenobiotic-induced toxicity accounts for approximately 50% of acute liver failure in humans,[7] the value of animal models is very limited.[8] A favorable prognosis to acute liver failure is conferred upon liver transplantation, which is a challenge in the face of persistent organ shortening. The restriction of animal models to accurately reflect human response is also true for other areas in toxicology.

Abnormal development of human fetuses following *in utero* chemical exposure may arise despite opposite predictions from animal studies, since the sensitivity of dose exposure can be higher in humans than that detected in animals.[9] Indeed, no animal species has been viewed as ideal for developmental toxicity,[10] where the majority of studies are conducted on rabbits and rats.

Moreover, decision-making findings from regulatory studies in animals may take up to two years, as indicated by standard carcinogenicity studies in rats.[11] In the European Member States, approximately 1 million animals are sacrificed per year for toxicology studies.[12] In addition to significant costs and ethical factors associated with the use of experimental animals, the implementation of initiatives such as the REACH program (Registration, Evaluation and Authorization of new and existing Chemicals) by the European Parliament[13] strengthens the urgent need of alternative, cost-effective and time-saving models to test for the adverse effects of thousands of chemicals in humans. The goal of REACH is to consolidate full risk assessment of all chemicals marketed at more than one ton within the next 10 to 15 years, which translates into a pressing need for toxicology assays that are more efficient. The opportunity for alternative toxicology

models is favored by REACH guidelines, which strongly support the use of *in vitro* testing for the first phase of chemical analysis. Alternative toxicology models will not replace experimental animals altogether, since certain *in vivo* systemic interactions that are relevant for toxic outcomes cannot be anticipated *in vitro*. Nonetheless, alternative *in vitro* testing may serve as a better predictor for human response in certain cell types and may consequently contribute to refine and reduce the use of animals in research.

In vitro differentiation of embryonic stem (ES) cells provides a renewable source of multiple cell types that are important targets of adverse chemical effects.[14,15] The successful isolation and establishment of human embryonic stem cell (hESC, Fig. 1) lines[16] enabled striking opportunities for predictive toxicology studies in genetically stable, non-immortalized human cells that can be translated to chemical safety assessments in both embryonic and somatic cell types. Functional neurons[17] and cardiomyocytes,[18] which are key targets of toxicology effects, have been generated following *in vitro* differentiation of human embryonic stem cells. hESC-neurons and cardiomyocytes exhibited lineage-specific transcripts

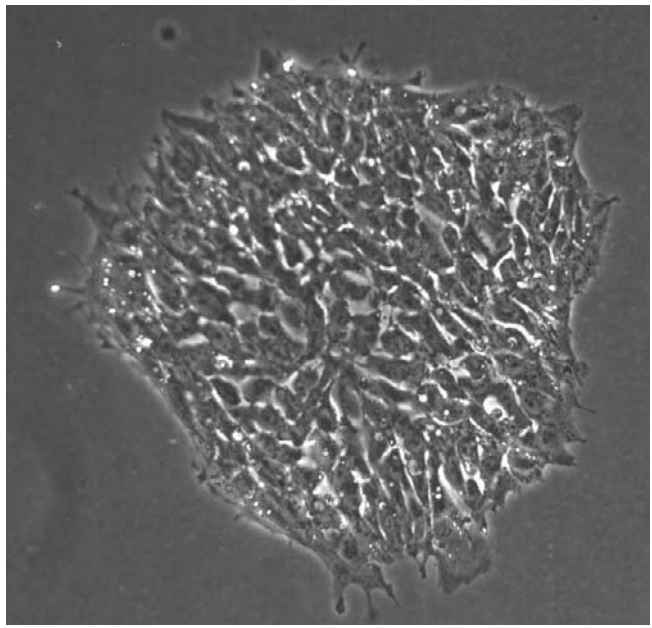

Fig. 1. An undifferentiated colony of H1 human embryonic stem cells grown in the absence of feeder cells.

and structural proteins but most importantly, demonstrated functional phenotypes with release of neurotransmitters and intercellular conduction of electric stimuli, respectively. The finding that certain human embryonic stem cell-derived cell types exhibit functional properties of *in vivo* counterparts increases the accuracy in which toxic effects can be detected. Cellular alterations in response to chemical insult can now be identified and interpreted in multiple levels, from changes in gene expression to cellular physiology and metabolism.[19,20] In summary, generation of functional differentiated cells from human embryonic stem cells supports systems toxicology studies which provide sensitive detection of chemical safety risks.

The liver is the major organ for metabolism and transformation of xenobiotics, where hepatocytes, the primary cells of the liver, perform core functions, including metabolism of diverse dietary molecules and chemicals and detoxification of compounds. Therefore, the availability of large numbers of human hepatocytes is a constant critical demand in toxicology and chemical risk assessment.[21] Discarded livers which cannot be used for organ transplantation are the limited source for primary human hepatocytes, which in turn exhibit very limited *in vitro* proliferation ability.[22] Initial progress has been achieved to generate hepatocyte-like cells from human embryonic stem cells following extensive research efforts.[23,24] Recent studies revealed that differentiation of the endoderm, the liver precursor lineage, evolves from human embryonic stem cells in a more intricate manner than ectoderm and mesoderm lineages.[25] For proper differentiation to ensue, an apparent epithelial-to-mesenchymal transition is required.[25] This study has also determined *in vitro* morphogens and culture conditions that favor definitive endoderm, which should facilitate further progress to obtain functional hepatocytes from hESC for toxicity and transplantation applications.

In summary, development of alternative toxicology systems based upon embryonic stem cells have promising opportunities in multiple areas, such as developmental, cardiac and hepatic toxicity. While *in vitro* assays lack systemic effects that may be involved in toxic response, a humanized model offers a valuable opportunity to replace, reduce and refine (3Rs of animal research) the use of experimental animals. Additionally, the use of human embryonic stem cells to understand signaling pathways involved in toxic response with the translation of these findings to epidemiology and preventative medicine may be a shorter term benefit of this technology towards human health in comparison to longer term clinical applications.

Embryonic Stem Cell Opportunities in Developmental Toxicity and Cognitive Disorders

Developmental toxicity comprehends alterations in mammalian development and postnatal physiology that result from chemical exposure during pregnancy. For regulatory and scientific purposes, developmental toxicity is classified in four categories:

(1) Embryonic lethality,
(2) Dysmorphogenesis or structural abnormalities,
(3) Intra-uterine growth restriction, and
(4) Functional toxicities.

Functional toxicities relate to alterations that are detected after birth, and may be persistent throughout an individual's lifetime. Postnatal development alterations are exemplified by neurobehavioral abnormalities and changes in cognitive function, among others. The predictive ability of animal models for functional toxicity is questioned since neurobehavioral and cognitive function may be significantly affected by cross-species differences.[26] The eye blink conditioning task in one of the few phenotypes in rats which provide information of human learning patterns.[27] Developmental anomalies affect 2% to 3% of all babies born in the United States.[28] Birth defects are the main cause for infant mortality as reported in a study across 36 countries.[29] Approximately 65% of birth defects have unknown causes.[30] Therefore, analysis of teratogenic effects of chemicals on established pregnancies are of utmost importance towards development of robust predictive toxicology models. Concern exists that environmental exposures may be playing a significant role. This concern has been difficult to evaluate without a model for testing developmental toxicity of more than 80,000 chemicals in the market, 2000 are newly introduced annually.[31] Fewer than 5% have been tested for reproductive outcomes and even fewer for developmental toxicity.[32]

In vitro differentiation of human embryonic stem cells recapitulates mammalian organogenesis.[33–36] This property may render hESC technology as an ideal screening system to unravel developmental pathways that are subject to changes and alterations following chemical exposure. Most importantly, functional toxicity in postnatal life can be predicted using hESC technology since differentiated cells with critical *in vivo* properties can be generated *in vitro*.

We are currently studying the effects of multiple known human teratogens to devise a robust hESC-based assay for regulatory categories (2) dysmorphogenesis and (4) functional impairment. The value of embryonic stem cells in developmental toxicity studies has been validated by initiates such as ECVAM (European Center for Validation of Alternative Methods) and the EST test (embryonic stem cell test).[37] Here, embryoid bodies generated from mouse embryonic stem cells were subject to a battery of 20 chemicals with known teratogenic effects in a standardized double-blind multi-center trial. The outcomes were highly promising for inclusion of embryonic stem cells in alternative, or *in vitro*, toxicity testing. The EST test determines teratogenesis by inhibition of differentiation of cardiomyocytes from embryoid bodies following exposure to chemical compounds. Overall, this assay showed a 78% statistically significant correlation to *in vivo* studies and was able to separate strong teratogens from moderate/weak and non-embryotoxic compounds. Strong embryotoxicants were actually predicted with 100% accuracy.

Since developmental toxicity is a complex process which affects multiple organs and tissues, it is likely that further assay development should focus on specialized cell types in addition to embryoid bodies, which contain all three primordial mammalian germ layers. Embryonic stem cells may also answer critical questions in regards to developmental windows for chemical insult. Pellizzer *et al.*[38] proposed gene expression analysis of cardiac and skeletal markers as a means to identify when alterations occur that could trigger downstream phenotypic abnormalities. Interestingly, this approach was also predictive of results obtained from *in vivo* studies. Therefore, multiple studies using endpoints such as differentiation and gene expression changes serve to reiterate the usefulness of embryonic stem cells to predict teratogenicity of chemicals.

The nervous system is particularly sensitive to detrimental effects of chemical exposure and it remains to be elucidated to what degree resulting neurotoxicity is involved in determination of diseases such as Parkinson's disease[1] and autism.[39] Genetic mutations have been identified across a subset of genes for both diseases, yet disease mechanisms are complex, and a potential interplay between genetic and environmental factors as causal effects are yet to be determined. Autism is a syndrome characterized by alterations in social interaction, language development, repetitive movements and patterns of behavior. Its prevalence may affect 1 in 150 US children.[40] No single region of the brain or pathophysiological mechanism has yet been identified as the major determinant for autism.[41]

Genetic causes account for approximately 10% of autism cases with multiple candidate target genes identified, such as the serotonin transporter gene, *DBH*, *GABRB3*, *UBE3A*, *RAY1/ST7*, *WNT2*, *RELN*, *SPCH1/FOXP2* and *GRIK2*.[40–43] Environmental effects are strongly suggested to contribute to disease etiology. There is substantial evidence from animal models and human epidemiology studies[44] that environmental (mercury/thimerosal, a preservative used in vaccines and organophoshates) and therapeutic chemicals (thalidomide, valproic acid) exert specific teratogenic effects during neurodevelopment which lead to altered neuronal migration and function and autistic syndrome.[39,45] Damaging neuronal and behavioral patterns in autistic children may also result from an indirect rather than direct environmental effect. In mice, repeated exposure to thimerosal leads to neurobehavioral deteriorations, increased oxidative stress and decreased intracellular levels of glutathione, a major antioxidative and detoxifying agent.[46] Therefore, autism may result from reduced detoxifying abilities. Other epidemiological studies failed to correlate mercury and thimerosal exposure to the risk of autism.[47,48]

Controversial findings, therefore, populate the field and call for models with human cells to delineate the direct and indirect effects of chemical exposure during development on the pathogenesis of autism. Limitations of animal models to determine mechanistic factors in autism may result from the fact that this is a disease with pronounced changes in human behavior and personality traits.

Functional impairment of hindbrain serotonergic (5HT) neurons, serotonine neurotransmission and the serotonine transporter has been observed in children with autism.[49] Therapeutic intervention with selective serotonin reuptake inhibitors, which specifically target the serotonin transporter, alleviates symptoms of autism[50] which strengthens the likely role of 5HT neurons in disease mechanism. Serotonergic neurons are generated following *in vitro* differentiation of mouse and primate embryonic stem cells.[51,52]

Early studies confirmed the presence of serotonin positive neurons in approximately 15% of mouse ES-derived neurons[51] as a result of Sonic hedgehog activity. Therefore, a striking opportunity exists to investigate critical pathways of environmental toxicants on the etiology of a very complex cognitive disorder using embryonic stem cells. Proof of this concept was achieved with an elegant model using mouse ES cells to investigate the role of known teratogens (valproic acid) on the development of autism.[2] Here, *in vitro* treatment of neural precursors with valproic acid disrupted functional development of serotonergic neurons. Strikingly, ES-cell based

models also support reversal of disease phenotype studies following toxic injury. The addition of sonic hedgehog, a known inducer of serotonergic neurons[53] was able to partially prevent valproic acid-induced alterations to ES-derived serotonergic neurons.[2] These findings were correlated to *in vivo* parallel studies in rats.

Exposure of pregnant rats to valproic acid, a drug with bipolar disorder indications, resulted in changes in the allocation and function of serotonergic neurons that were similar to disease phenotype in patients.[2] We are currently undertaking pioneer studies using chemical biology associated with hESC technology to investigate abnormal neuronal development and functional impairments that result from chemical insult and potentially underlie autism.

Cardiotoxicity: An Embryonic Stem Cell-Based Assay for a Serious Side Effect

Cardiotoxicity is the major cause of compound attrition in drug industries, followed immediately by hepatotoxicity.[14,54] Compound attrition is the progressive loss of chemical targets throughout drug discovery and development, and is influenced by two major mechanisms: safety and efficacy.[55]

Cross-species variability between animals and humans, and an inherent inaccuracy of current animal models to predict human response is a considerable determinant of compound attrition.[6] QT prolongation, a syndrome that leads to delayed ventricular repolarization and cardiac arrhythmia, is induced by a vast range of chemical entities for multiple therapeutic areas. This syndrome accounts for as much as 80% of cardiac side effects of drugs.[54]

As a result, fatal episodes following ventricular fibrillation have been reported in otherwise healthy individuals following administration of drugs.[56] Similarly, QT prolongation has resulted in the removal of marketed compounds following the average industry timeline of 15 years of research and development and US$800 million of investments per drug.[57] Cardiac arrhythmias and cardiotoxic episodes arise from interactions of therapeutic drugs with cardiac ion channels. Specifically, the action potential duration (APD), which is an *in vitro* measurement that reflects *in vivo* cell surface electrocardiogram outputs, depends upon multiple ion channels, such as sodium (Nav 1.5), L-type calcium and the delayed inward cardiac rectifier

potassium channel, known as hERG.[58] Although hERG compound inhibition is the major component of cardiotoxicity screening;[59] there have been many reports of cardiotoxic episodes associated with other channels, such as the L-type calcium channel.[60,61] Strikingly, despite extensive research, there is no direct mechanistic association between hERG and arrythmogenesis, which is the principal symptom of cardiac toxic events.[54,62]

Initial studies have shown that cardiomyocytes derived from human embryonic stem cells exhibit functional properties that are necessary for their use in predictive toxicology studies.[18] Immunostaining of hESC-derived cardiomyocytes identified connexin 43 (Cx43) and connexin 45 (Cx45), indicating the presence of gap junctions which infer cardiac maturation. In addition, cardiomyocytes developed a functional syncytium with synchronized action potential propagation.[18] The presence of cardiac conduction in ES-derived cardiomyocytes enables *in vitro* models focused on stimulus propagation in human cardiac tissue and changes that arise in response to chemical exposure.

We have recently validated an embryonic stem cell-based assay that targets a cardiac ion channel implicated in chemically induced cardiotoxicity, the L-type calcium channel.[63] This study utilized the calcium transient to infer a cardiac lineage (Fig. 2). Cardiomyocytes derived from mouse ES cells exhibited a functional response to chemical modulators of L-type calcium channel activity, accurately reflecting *in vivo* cardiac reaction. In this study, treatment of ES-derived cardiomyocytes with agonists and antagonists of the L-type calcium channel induced intracellular increases and decreases in calcium transients respectively, which emulated an *in vivo*

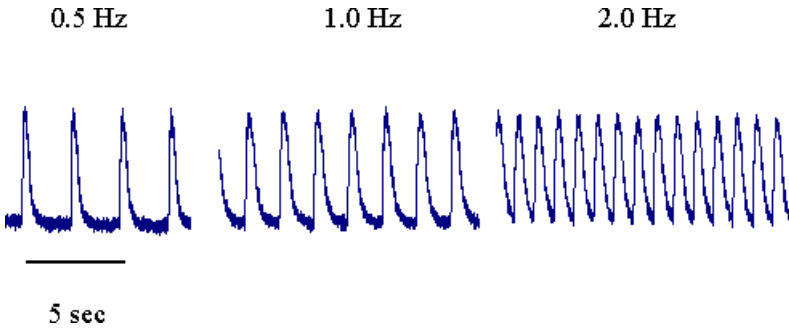

Fig. 2. Frequency dependence of calcium transients in ES-derived cardiomyocytes. Representative trace of an electrically induced calcium transient, following rhythmic pacing at multiple frequencies by field stimulation.[63]

cardiac response. ES-derived cardiomyocytes were comparable to *in vivo* cardiac cells in regards to specific gene transcripts and cardiac proteins.

The expression of cardiac specific transcription factors (Nkx 2.5, MLC-2V) and cardiac-specific functional genes (cardiac ryanodine receptor, phospholamban, SERCA2a, calsequestrin, connexins 45 and 43, Cav1.2a, Kv 1.5 and mERG) was confirmed in ES-derived cardiomyocytes in comparison to multiple sources of cardiac and non-cardiac myocytes, such as *in vivo* cardiomyocytes, the immortalized atrial cardiomyocyte line HL-5 and immortalized skeletal myocytes C2C12. Nonetheless, ES-derived cardiomyocytes are immature in direct molecular comparison to the adult heart, with persistent expression of mRNA for early development genes (alpha-fetoprotein, glypican 3, homeobox X, tenascin-C).

This is consistent with previous studies in mouse ES cells, where ES-derived cardiomyocytes differed from the adult cells.[64] Maltsev *et al.*[64] identified changes in voltage half-inactivation of I_{tO}, expression of I_K, response to hormonal regulation and gene expression when comparing early stage ES-derived cardiomyocytes to terminally differentiated cardiomyocytes. Studies in hESC have also shown that excitation-contraction coupling properties in hESC-derived cardiomyocytes differ from the adult myocardium, possibly due to immature sarcoplasmic reticulum function.[65] Although this aspect may not be detrimental to predictive toxicology, it highlights the importance of scientific investigation into the definition of toxic endpoints.

The ES-cell based assay developed in our study[63] was enabled by an innovative approach to isolate ES-derived differentiated cells. The use of laser capture microdissection and pressure catapulting (LMPC) enabled enrichment of ES-derived cardiomyocytes from heterogeneous populations of ES-derived cell types. This technology platform may be applicable to other cell types derived from embryonic stem cells for *in vitro* screening purposes and exploratory clinical applications.

LMPC uses a high precision laser micromanipulation system (cutting size: less than 1μm) that is able to yield enriched cell populations from live or fixed tissue.[66] This photonics tool is based upon a 337 nm nitrogen laser that operates in the UV-A range, allowing the surrounding tissue to remain intact. The laser-excised cells are removed by a phenomenon known as "cold ablation", in which the targeted cells are excised, catapulted upward, and captured in a media filled cap in a contact free manner. ES-derived beating foci often retained contractility during LMPC, or regained it following 6 to 12 hours of *in vitro* culture.[63]

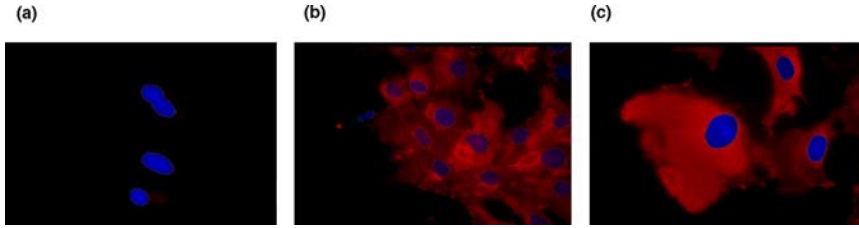

Fig. 3. Immunohistochemistry of ES-derived cardiomyocytes isolated with laser capture micro dissection: structural cardiac proteins were detected in cells of beating foci. (a) Control IgG, (b) anti-cardiac actin, and (c) anti-cardiac myosin.[63]

As reported above, functional and molecular characterization following LMPC demonstrated that cell viability was preserved as well as cell-cell interactions that sustain the pacemaker-driven contractility of cardiomyocyte aggregates. Enrichment of ES-derived cardiomyocytes by laser capture micro dissection was also confirmed by immunohistochemistry analysis of excised foci with antibodies directed against cardiac proteins such as cardiac alpha-actin and cardiac myosin (Fig. 3).

Importantly, beating foci were amenable to predictive safety investigation in 96-well format, which greatly streamline testing in a multiple chemical compound setting. In summary, the presence of multiple components of the cardiac phenotype in ES-derived cardiomyocytes, such as transcripts, proteins, electrophysiology and most importantly, functional pharmacology, bear enormous opportunities for alternative testing in cardiotoxicity studies of therapeutic compounds.

Conclusions

The main goal of our current scientific endeavor in stem cell biology is to evaluate embryonic stem cells, particularly hESC, as a valuable resource for predictive toxicology studies. The benefits of hESC to understand mechanisms associated with toxicity-induced adverse reactions and diseases are numerous.

Robustness is a hallmark of this technology, in comparison to standard assays which rely on animals or immortalized cells. hESC can be scaled by indefinite *in vitro* culture, providing cellular substrates for screening of thousands of chemicals without the variability or limited number of cells detected in other screening systems, such as primary cellular explants.[22]

Cryopreservation of human embryonic stem cells greatly streamlines assay development and harmonization across multiple testing sites and conditions.

Risk assessment studies and international regulatory initiatives such as REACH, will require interlaboratory standardization and validation and distribution which are applicable to cryopreserved cells. The development of *in vitro* testing systems is in alternative for the experimental use of animals. The complexity of toxic response, however, cannot be predicted in its entirety without *in vivo* systemic interactions. Therefore, alternative hESC-based predictive toxicology assays serve an important role as we strive to exercise the 3Rs theme initially proposed by Russel and Burch[67] to refine, reduce and replace the use of experimental animals.

The field of developmental toxicology may be particularly awarded with extraordinary achievements with the advent of hESC technology. Genetic and epigenetic effects of chemical exposure on human development may be unraveled for multiple birth defects and consequent pediatric disorders through *in vitro* organogenesis modeling. In essence, we strongly believe that the opportunity to generate human neurons, hepatocytes and cardiomyocytes may significantly impact our understanding of disease mechanisms and the role of environmental toxicants and xenobiotics on disease etiology.

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