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Novel Concepts and Prospects for Tumor Therapy

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Editors: O. A. Wiestler | B. Haendler | D. Mumberg

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Preface

The concept that cancer stem cells play an important role in malignant tumors is gaining more and more support due to recent advances in the field. The latest progress in this area is increasingly reported and commented on in the lay press, documenting the high excitement and hopes it generates.

An intricate relationship between stem cells and cancer cells was originally identified in leukemia, and evidence has now accumulated that cancer stem cells are also found in many solid tumor types including breast, prostate, and colon carcinomas. They are closely related to normal stem cells, a very small number of which are found in most tissues. Normal stem cells divide asymmetrically and have the unique capacity to produce an identical daughter cell and one that can differentiate. These progenitor cells have the ability to give rise to all the specialized cells of a given tissue. This process is strictly controlled by the niche microenvironment in which these cells reside, so that the number of stem cells remains constant. Mutations in normal stem cells and failure of the control mechanisms provided by the niche may both play essential roles in the formation of cancer stem cells and in the development of tumors.

The exciting new findings in this field were made possible mainly through the development of novel techniques such as high-speed cell sorting and by the identification of specific cell surface markers. This allowed the purification of the very rare cancer stem cells present in

tumors (often less than 1%). Some of the markers are shared by cancer stem cells of various origins, but others are specific for the tumor type. The establishment of culture conditions appropriate for maintaining the stem cell phenotype will be instrumental in the study of cancer stem cells.

Importantly, cancer stem cells have properties very different from those of the rest of the tumor cell population. They divide much more slowly, which allows them to escape from traditional radio- and chemotherapies that hit fast-multiplying cells. Also, they have very efficient systems to pump out drugs, making them highly resistant to most conventional therapies. Current treatments might therefore only hit the bulk of the tumor but spare cancer stem cells, thus leading to recurrence and metastasis.

Both the origin and the precise impact of cancer stem cells on tumor pathogenesis are still debated. Normal stem cells may over time accumulate genetic and epigenetic changes that disturb the control of self-renewal. An arrest of progenitor cell differentiation and the recovery of unlimited proliferation properties may also be responsible for the initiation and progress of cancer.

On the other hand, some experts in the field consider cancer stem cells to represent the bad seeds of tumors and suggest that eliminating them has the potential to eradicate the disease. The targeting of cancer stem cells, however, represents a shift in focus and will probably require the identification of novel drugs. This is an enormous challenge because of the paucity of cancer stem cells, the technical difficulties of keeping them in culture, and their unusual drug resistance. However, the identification of proliferation and differentiation pathways that are active in cancer stem cells but not in normal, differentiated cells may offer interesting new opportunities for selective therapies.

The Cancer Stem Cell Workshop was organized to discuss recent advances and controversies in this fast-moving research area. We have tried to bring together many of the internationally highly recognized experts in this relatively young field who, through a variety of approaches, have made seminal contributions, thus leading to major strides forward. We are grateful to all of them for their excellent presentations and lively discussions, and also for their contributions to this book. We are convinced that the proceedings of the workshop will allow a better understanding

of the important role that cancer stem cells play in tumors and help in the future development of more effective and selective cancer treatments.

Finally, we would like to express our gratitude to the Ernst Schering Foundation for its generous support and perfect organization, which allowed us to hold this workshop under the best possible conditions. Our grateful thanks to the Berlin-Brandenburg Academy of Sciences and Humanities and to Prof. G. Stock for hosting the meeting on their premises.

Berlin, March 2007

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Niche for Normal and Cancer Stem Cells

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Abstract. An important issue in cancer therapy is the presence of a population that is resistant to anticancer treatment. This resistance has been partly ascribed to the presence of quiescent stem cells in a cancer population. However, how the quiescent state is induced in a proliferating cancer population is totally obscure. We think that our study on the stem cell system of pigment cells will provide some insight into the molecular basis for cancer stem cells, because the quiescent melanocyte stem cell would be the ideal model for understanding the process generating quiescent stem cells. In this article, we review our latest understanding of the quiescent stem cells of the melanocyte lineage by referring to some related topics of cancer stem cells.

1 Introduction

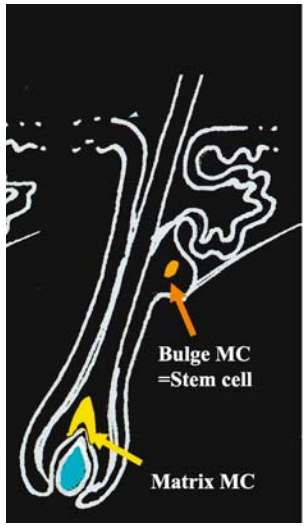
The main purpose of this symposium is to discuss cancer stem cells (Clarke et al. 2006). While the cancer stem cell is not the direct subject of our study, we agree that the cancer stem cell is a notion that may have the potential to radically change our strategy of cancer treatment. The underlying idea is to regard cancer as a hierarchical entity comprising cell populations of distinct characteristics. However, this recognition is not novel. Indeed, there may be no researchers who believe that cancer consists of a homogeneous population. In most cases, in fact, a simple histological examination is enough to discern the heterogeneity of cancer. However, the molecular mechanisms underlying the generation of hierarchical diversity in cancer cells and the functional significance of the diversity have been left largely uninvestigated until recently. For decades, cancer research was concerned with how genetic alterations that accumulate in the genome lead to uncontrolled growth of cells (Vogelstein and Kinzler 2004). This direction of study thus assumed that a particular cancer population is represented by the most malignant population that is expected to dominate the population, and the cells that can grow less are regarded as those disappearing over time. The notion of the cancer stem cell brought this simple competition rule into question by showing that even a slowly cycling population can be the most malignant population. Hence, treatment of cancer needs to take into consideration not only the mechanisms driving cancer cells to proliferate, but also those conferring stem cell features. In this respect, it is interesting that the cancer stem cell has been defined by two features. One is proliferative ability in secondary recipients (Bonnet and Dick 1997), and the other is resistance to anticancer treatment, or in other words, quiescence (Dean et al. 2005). The two features appear contradictory. This may be due either to coexistence of two types of stem cells in one cancer or to coexpression of two contradictory features in the same stem cells. Nonetheless, this question can only be answered by defining the molecular mechanisms underlying the formation of cancer stem cell populations.

Indeed, this question remains unresolved even in the study of normal stem cell systems. In this symposium, we review our study of normal stem cells with these questions about cancer stem cells in mind.

2 The Melanocyte Stem Cell Serves as an Ideal Model to Understand Quiescence of Stem Cells

Hair pigmentation is mediated by the melanocyte (MC) stem cell (SC) system residing in each individual hair follicle (HF). The melanocyte stem cell (MSC) system represents a regenerative type SC system that undergoes a regeneration cycle repeating quiescent and activated states. Concerning the MSC system, the regeneration cycle is regulated coordinately with that of follicular keratinocytes (hair cycle). We have shown previously that MSCs are distinguished from other compartments of MCs by a number of features (Fig. 1) (Nishimura et al. 2002). First, MSCs localize in the vicinity of the bulge region of the HF, whereas other compartments are located in the hair matrix at the lower part of the HF. Second, MSCs are quiescent until being reactivated when the new hair regeneration cycle is initiated. Third, MSCs are resistant to block of the c-kit signal, whereas the c-kit signal is essential for survival of other MC compartments. In fact, when an antagonistic mAb to c-kit is injected in neonatal mice, all mice become depigmented because of depletion of mature MCs. However, MSCs are resistant to this treatment and are able to completely replenish the MC system in the next hair regeneration cycle. Using these phenomenological features, we next attempted to distinguish MSCs in molecular term.

During the course of the characterization of MSCs, we were intrigued by the parallelism between the resistance of MSCs to antagonistic anti-c-kit mAb and the resistance of cancer SCs to therapy. The most striking example is the gastrointestinal stromal tumor (GIST) that is caused by gain-of-function mutations of the c-kit gene. Like melanocyte SCs, a small fraction of the GIST resists treatment with imatinib, which inhibits c-Kit function. While the imatinib treatment suppresses the growth of the GIST tumor, thereby bringing complete remission to the patient, the tumor recurs in all cases in which treatment is stopped. Moreover, even with continuous treatment, GIST eventually recurs because of the accumulation of additional mutations that render the tumor cells resistant to imatinib treatment (van der Zwan and DeMatteo 2005). Although it is not clear whether the imatinib-resistant fraction of GIST corresponds to the stem cells that we defined in the MC system, this result encourages us to think that investigation of the mechanisms un-



-Immature and undifferentiated.
-Resting state.
-Can give rise to the matrix melanocytes.
-Can reconstitute melanocyte system in the hair follicle after transplantation.

Fig. 1. Characteristics of the stem cell compartment of melanocyte in hair follicles. Melanocytes present in a special region of the upper part of hair follicles, which is designated the bulge region, were proven to be stem cells with a number of characteristics listed in the figure

derlying the maintenance of MSCs in the bulge region would provide some clues for understanding the resistance of tumor SCs to treatment. Nonetheless, the most important issue in this respect is to define SCs in molecular term.

3 Definition of MSC

How to define SCs is the first issue for the investigation of any SC systems. In the MC system, the most reliable means to distinguish SCs from other compartments is by its localization. As shown in Fig. 1, MSCs localize in the upper area of HFs, whereas other compartments are in the lower part that is called the hair matrix. Using transgenic mice that are engineered to express GFP specifically in the MC lineage, we isolated individual MSCs that are defined as GFP^+ cells in the upper

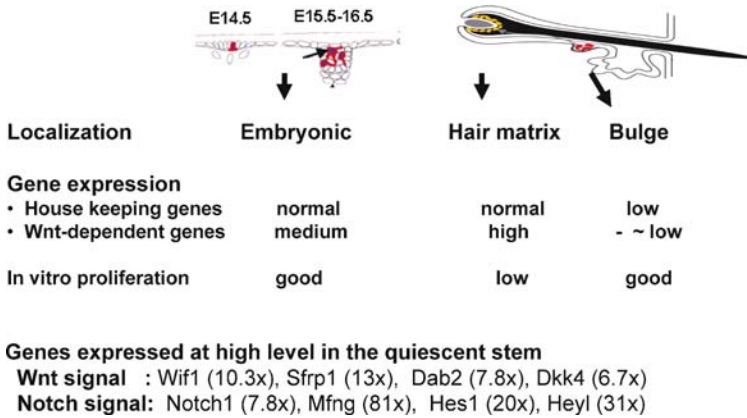


Fig. 2. Characterization of melanocytes purified from different regions of skin. Melanocytes were prepared from embryonic skin or hair follicles of neonatal mice. Hair follicle melanocytes were further divided into two populations in terms of their localization. Results from DNA chip analyses and in vitro proliferation assay using XB2 cell line are summarized. Also listed are genes whose expression in the quiescent stem cells is higher

part of HFs, prepared cDNA by RT-PCR, and analyzed gene expression at the single-cell level and compared it with that from cDNAs from MCs in the embryonic epidermis and from MCs in the hair matrix of adult HFs (Osawa et al. 2005).

Figure 2 presents characteristics of gene expression profiles in these three MC populations. While gene expression profiles of three populations differ from each other to varying extents, MSCs are special in two features, one being the low level of housekeeping gene expression and the other being the suppressed expression of melanocyte-specific genes.

4 Isolation of MSCs En Masse

An important purpose for defining MSCs in molecular terms is to develop a method for purifying MSCs en masse, rather than at a single cell level. Our finding that the expression level of various housekeeping genes is low in MSCs appeared to be applicable for this purpose. Indeed,

the transgenic mouse that we used for isolating single MSCs expresses GFP under the control of the chicken actin (CAG) promoter, a typical housekeeping gene. Hence, we investigated whether or not MSCs are distinguished from other compartments in terms of the expression of the CAG-driven GFP. When skin of P10 neonates was dissociated and analyzed by FACS, we were able to detect both GFP^{high} and GFP^{low} populations (Osawa et al. 2005). In order to confirm that the GFP^{low} population indeed represents MSCs, we analyzed the skin of mice that were injected with Ack2, an antagonistic anti-c-kit mAb, at the neonatal stage, because this treatment depleted all MCs except MSCs in the bulge region. As expected, Ack2 treatment depleted the GFP^{high} population, whereas some of the GFP^{low} population remained unaffected. By FACS sorting, we were able to confirm that the GFP^{low} cells share many characteristics of MSCs, such as downregulation of a group of MC-specific genes. Because FACS sorting allows us to obtain a sufficient number of cells, quantitative analysis of gene expression is easier with sorted cell populations than with manually isolated single cells. By the use of a new series of experiments with FACS-sorted MSCs, we could define other features of MSCs that are also listed in Fig. 2.

At present, the molecular mechanisms underlying the low housekeeping gene expression in MSCs are not clear, and we are currently investigating these mechanisms. Moreover, it would also be interesting to know whether or not there are other stem cells that share the same feature.

5 Evaluation of Gene Function by MC-Specific Gene Manipulation

Despite the global suppression of transcription, we could find genes that are expressed at higher levels in MSCs. The function of such a molecule can be assessed by using MC-specific gene knockout (KO). The Notch signaling pathway is an example that we have investigated to a considerable extent (Moriyama et al. 2006). Comparing gene expression of FACS-sorted MSCs and other compartments by DNA microarray, we found that Notch 1 and its downstream molecule Hes1 are expressed severalfold higher in MSCs than other compartments. In order to in-

vestigate the role of the Notch signaling pathway, we exploited the MC-specific, conditional KO of RBPJκ, which is in the downstream of Notch signaling pathway, with Cre recombinase driven by the *tyrosinase* promoter. All mice bearing the genotype of conditional KO are born depigmented to variable degrees. Thus Notch signaling is involved in the embryonic development of MCs. Selecting mice in which some pigmentation remained, we next examined whether or not MSCs are maintained in such pigmented follicles and we found that all remaining pigmentation disappeared in the next hair cycle. These results indicate that the Notch signal is required both in embryonic development of MCs and in maintenance of MSCs in the HF. Because pigmentation is not required in the life of the mouse, the MC stem cell system provides an ideal model for analyzing the function of molecules in vivo.

6 Induction of Quiescent MSCs Requires Multiple Steps

Another important molecular signature of MSCs is the low expression of genes that are essential for development of MC. This group of genes includes the most upstream genes, such as Sox10 and Mitf, and genes that are regulated by these upstream genes, although there are genes such as Pax3 whose expression is also maintained in MSCs. Recent studies suggested the involvement of the Wnt signal in the activation of Mitf and Sox10 and therefore their downstream genes(Lang et al. 2005). During migration of MCs into developing HFs, those migrating to the hair matrix maintain expression of these genes, whereas those trapped in the bulge region become negative in this expression. This result strongly suggests that the downregulation of these genes is an active process involving extrinsic signals. Because the expression of these genes has been implicated to be regulated by the Wnt signal, it is likely that the Wnt signal is somehow suppressed in MSCs. Consistent with this, it was demonstrated that a high level of Wnt inhibitors, such as Dkk3, Sfrp1, and Dab2, are expressed in the bulge region of HFs (Morris et al. 2004; Tumbar et al. 2004). Moreover, MSCs themselves expressed a higher level of Wnt inhibitors, such as Wif1, Sfrp1, Dab2, and Dkk4(Osawa et al. 2005). From these results, we speculated that inhibition of the Wnt signal is an essential process for the induction of

quiescent MSCs, which takes place during the MC migration into developing HFs. Indeed, histological study showed that Sox10 expression in MCs is downregulated at the upper area of developing HFs, while those reached in the lower part of HFs express Sox10 (Osawa et al. 2005).

However, it is clear that Wnt inhibition may not be sufficient for inducing MCs to become quiescent MSCs. For instance, the downregulation of a set of housekeeping genes is another important feature of MSCs, but it is unlikely that expression of housekeeping genes is regulated by the Wnt signal. More strikingly, we have found that MSCs freshly isolated from the bulge region of HFs are able to survive for more than a week in a simple medium without exogenous growth factors (our unpublished observation). This suggests that in the final stage of MSC differentiation, MSCs have acquired an autonomous ability to survive in the absence of a supporting microenvironment. Of interest in this context are previous studies showing that epigenetic silencing of Wnt inhibitors is found in colorectal cancer (Suzuki et al. 2004). Thus Wnt inhibitors play a role in controlling the tumor cells. If quiescent stem cells exist in such cancers, it would be interesting to know whether or not they inactivate the Wnt pathway again to gain quiescence.

7 A Working Hypothesis for the Induction of Quiescent MSCs

Figure 3 summarizes our working hypothesis about the process by which proliferating MCs generated through embryonic development are induced to become quiescent MSCs. In this model, at least three distinct processes are supposed to be completed in order to induce the quiescent MSC: (1) downregulation of housekeeping genes by a mechanism that suppresses transcription in a global manner, (2) downregulation of MC-specific molecules by inhibiting the Wnt signal, and (3) acquisition of a cell-autonomous survival ability in the absence of a niche. We are speculating that all three processes are regulated extrinsically by the adjacent environment. However, we are also proposing that once quiescent MSCs are induced, the role of a niche may be limited, because MSCs can survive autonomously.

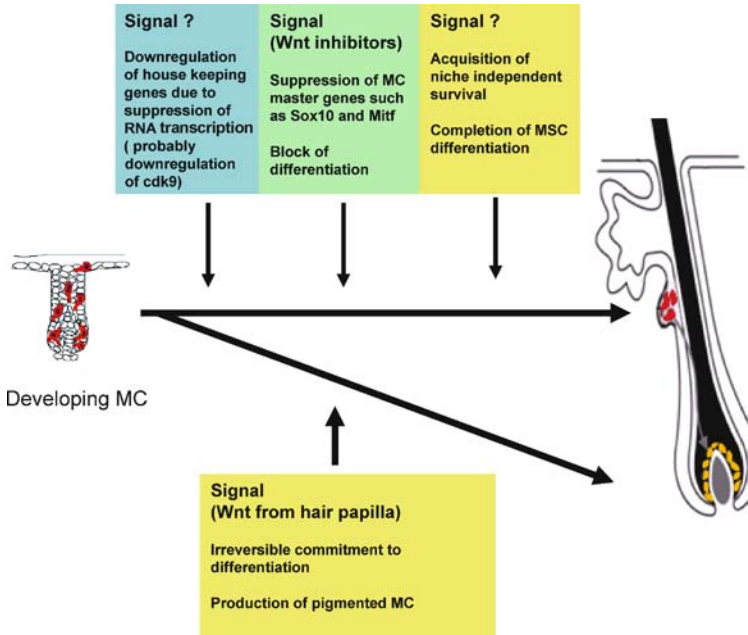


Fig. 3. Three steps during the induction of the quiescent stem cell, which is regulated extrinsically by the microenvironment. During neonatal development, melanocyte differentiation diverges into two pathways. One is to undergo irreversible commitment to mature melanocytes with pigmentation, which takes place in the population migrating to the hair matrix. The second pathway is possible only for the melanocytes remaining in the upper part of the developing hair follicles. From gene expression analysis, three distinct steps are supposed to be required for inducing quiescent stem cells. At the end of these processes, the stem cell acquires a cell-autonomous survival ability

How is transcription of housekeeping genes is suppressed? What is the actual order of the three processes? How is the cell-autonomous survival ability induced in MSCs? All these questions remain for future study. Our preliminary data suggested that the proportion of RNA polymerase II (Pol II) whose serine 2 residue is phosphorylated is significantly low in MSCs, although it is found abundantly in most cells. These

data suggest that the global transcriptional suppression is due to inefficient elongation that requires serine 2 phosphorylation of Pol II. Interestingly, low serine 2 phosphorylation is observed before downregulation of Sox10 (Freter et al., unpublished data). This result suggests that global suppression, Wnt inhibition, and induction of cell-autonomous ability for survival would be the actual order of the three processes, although further studies are required to determine the actual order.

8 Induction of Quiescent MSCs In Vitro: Future Direction

As described previously, there is an established method for MC-specific gene manipulation. Thus evaluation of the functional role of any molecules in the induction of the quiescent MSC may not be difficult. Moreover, we have a definite prediction about the phenotype due to MSC-specific failure. Because MSC differentiation is completed later than MC colonization into hair matrix, defects specifically affecting the MSC should be expressed as a mouse that is born pigmented, but is loosely pigmented in the next hair cycle.

On the other hand, evaluation of an extrinsic signal involved in the induction of quiescent MSCs may not be easy. There is no established method to manipulate genes in the microenvironment adjacent to MSCs. In fact, nearly nothing is known concerning the cellular nature of the functional microenvironment for MSCs. We think that in vitro culture of embryonic MCs may solve this problem, because in vitro culture provides an easier assessment of extrinsic molecules on MCs. The goal of the in vitro study is thus to define the extrinsic condition that induces the quiescent MSC from the proliferating MC.

While a number of culture conditions have been reported for MCs, we found that none of the previous methods is suited for culturing purified MCs. Probably this is because previous culture conditions are optimized for cell mixtures containing MCs in the presence of other skin components. Hence, we explored the culture conditions that can support proliferation and differentiation of MCs that are freshly purified from skin and found that a keratinocyte cell line, XB2 (Rheinwald and Green 1975), is able to support clonogenic proliferation of purified MCs (Yonetani et al., in press). The proliferation of MCs under these culture

conditions is c-Kit dependent, and its differentiation is promoted by endothelin. More importantly, MCs in the culture can reconstitute the whole MC stem cell system in the in vivo assay of hair reconstitution from dissociated keratinocytes. This indicates that MCs proliferating in culture are functional also in vivo and can give rise to MSCs upon encountering the in vivo microenvironment. Hence, it is likely that the new culture conditions recapitulate the actual processes in the mouse skin.

With this new tool, we are currently trying to identify molecules that can induce the quiescent MSCs from proliferating MCs. To date, what we have discovered in the in vitro system is that bFGF can support proliferation of MCs while suppressing further differentiation and also that bFGF can generate a population that can survive in the absence of c-Kit. Clearly, more studies are needed to attain our goal of inducing the quiescent MSC in this culture system. Nonetheless, we are confident that all necessary tools are ready for studying quiescence in the MC stem cell system.

Coming back to the questions of whether the two definitions of cancer stem cells, resistance to cancer treatment and proliferative activity in the secondary recipient, represent different features of the same cancer stem cell or two different cancer stem cells, our preliminary result in MSCs would be interesting. That is, when we compared the proliferation of MCs from the hair matrix and quiescent MSCs in the upper part of HFs in the new culture system, we unexpectedly found that only the quiescent MSCs undergo sustained proliferation (Fig. 2). This indicates that although MCs in the hair matrix are proliferating in vivo, they are already committed to the differentiation pathway. Nonetheless, in the MC stem cell system, proliferative ability in the culture and quiescence are two features of the same MSC. Hence, a similar situation is also likely to be present in some cancer. In this context, we are interested in the fact that chronic myelocytic leukemia and GIST are malignancies that share some common features with the MC stem cell system. Both are derived from cells that are c-Kit dependent, and stem cells can be defined in terms of resistance to treatments that inhibit proliferative signals. Hence, our study on the molecular mechanisms underlying induction of quiescent MSCs by extrinsic factors will provide an important clue for understanding those cancer stem cells and help in the development of new therapies.

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c-Myc and Activated Ras During Skin Tumorigenesis: Cooperation at the Cancer Stem Cell Level?

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Abstract. Mutations leading to overexpression and activation of the oncogenes Myc and Ras are among the most frequent lesions known to occur in human and murine cancers. These genes are also the pioneering example for oncogene cooperation during tumorigenesis, whereby the anticancer effects of Myc deregulation (apoptosis) and oncogenic Ras (senescence) are antagonized and therefore canceled out by each other. Here I review the role of endogenous and overexpressed c-Myc in murine skin, focusing primarily on epidermal stem cells. In addition, recent data suggesting an essential role for the endogenous c-Myc-$p21^{CIP1}$ pathway in Ras-driven skin tumorigenesis are discussed.

1 The Myc Proto-oncogenes

Human c-Myc was the second proto-oncogene identified and encodes a short-lived basic-helix-loop-helix-leucine zipper protein localized in the nucleus. Overexpression of Myc family members has been detected in a large variety of human cancers including Burkitt lymphoma (c-Myc), neuroblastoma (N-Myc), and small cell lung cancer (L-Myc) (Grandori et al. 2000). However, despite its early recognition as a crucial gene promoting tumorigenesis, the molecular and cellular functions of Myc proteins, particularly their physiological roles in vivo, remain surprisingly enigmatic. Myc proteins dimerize with Max, and Myc-Max heterodimers can activate or repress two large, independent sets of target genes (Fernandez et al. 2003; Adhikary and Eilers 2005; Dang et al. 2006; Guccione et al. 2006). In addition, recent data suggest a possible novel function for Myc activity in controlling global chromatin structure (Knoepfler et al. 2006). The biological roles of Myc activity are manifold and include promotion of proliferation, angiogenesis, cellular growth, and apoptosis, as well as inhibition of terminal differentiation. In contrast to L- *myc*, which is a nonessential gene, both c-Myc and N-Myc are required for embryonic development, and knockout embryos die at E10.5 and E11.5, respectively, making it necessary to employ conditional approaches to study these genes in the adult organism (Trumpp et al. 2001; Knoepfler et al. 2002; Murphy et al. 2005). Recent studies have uncovered an unexpected novel role for Myc activity in the differentiation of blood stem cells. Here c-Myc appears to control the balance of hematopoietic stem cell (HSC) self-renewal and differentiation by controlling their entry and exit from the stem cell niche (Wilson et al. 2004; Murphy et al. 2005). In addition, gain- and loss-of-function studies of c-Myc in the skin epidermis and the intestine indicate a role for this gene in stem/progenitor function (Arnold and Watt 2001; Waikel et al. 2001; Benitah et al. 2005; Bettess et al. 2005; Murphy et al. 2005; Zanet et al. 2005; Muncan et al. 2006; Oskarsson et al. 2006). Moreover, c-Myc is one of only four genes (together with KLF4, Oct3/4, and Sox2) sufficient for the generation of embryonic stem cell-like cells directly from adult tail fibroblasts (Takahashi and Yamanaka 2006), further implicating Myc activity in the regulation of stem cell function.

2 Distinct Consequences of c-Myc Overexpression in Epidermal Cell Types

A number of transgenic mouse models have been used to study the in vivo effects of c-Myc ectopic or overexpression in murine skin. For example, human c-Myc (Waikel et al. 1999) or 4-OH tamoxifen-inducible, human c-MycER (Pelengaris et al. 1999; Flores et al. 2004) were targeted to the postmitotic (endogenously c-Myc^{neg}) suprabasal compartment of the skin with the loricrin or involucrin promoters, respectively. In both cases, the mice developed epidermal hyperplasia and papillomas as a result of differentiated keratinocytes re-entering the cell cycle while subsequent terminal differentiation was inhibited. Interestingly, Loricrin-Myc mice exhibit reduced sensitivity to UV-B-induced apoptosis, suggesting that Myc may possess unexpected antiapoptotic functions, at least in some cell types (Waikel et al. 1999). Similar tumors were obtained by overexpressing mouse c-Myc within the basal layer of the epidermis, hair follicle outer root sheath, sebaceous glands, and hair follicle bulge region with the keratin 5 (K5) promoter (Rounbehler et al. 2001). In contrast to the loricrin-Myc mouse model, p53-dependent apoptosis was substantially increased in K5-Myc mice, indicating that Myc-mediated apoptosis/survival might be cell type specific and/or stress dependent. Interestingly, K5-Myc skin showed enhanced sensitivity to DMBA/TPA two-stage skin carcinogenesis because papillomas appeared earlier and more frequently and were significantly larger in size compared to controls. In addition, high c-Myc activity was observed to promote conversion from benign papillomas to malignant carcinomas (Rounbehler et al. 2001).

Finally, two mouse models have been produced that use the keratin 14 (K14) promoter to overexpress human c-Myc or c-MycER in the same regions as K5-Myc. Both of these mouse models develop sebaceous and epidermal hyperplasia at the expense of hair follicle development, suggesting a role for c-Myc in early cell fate decisions (Arnold and Watt 2001; Waikel et al. 2001; Owens and Watt 2003). In addition, the migration of keratinocytes was impaired both in vitro and in vivo, and a large number of genes involved in adhesion and migration, such as β_1-integrin, were downregulated, linking c-Myc activity to a possi-

ble role in controlling cell adhesion and migration (Frye et al. 2003; Gebhardt et al. 2006).

Most interestingly, K14-Myc mice also present with a complete loss of epidermal tissue in areas associated with physical stress, such as around the head and neck, due to scratching. This observation raised the hypothesis that sustained elevated levels of c-Myc activity prevent the maintenance of epidermal stem cells (ESCs) in areas of high tissue renewal and repair. This theory is supported by the observation that putative epidermal stem cells, identified as BrdU "label-retaining cells", are severely depleted within K14-Myc hypoplastic epidermis (Waikel et al. 2001). Epidermal loss was also observed in mice lacking Rac1, a small GTP-binding protein of the Rho family that controls cell adhesion and cytoskeletal architecture, in the epidermis (Benitah et al. 2005). Similar to the K14-Myc mice, elimination of Rac1 in the epidermis caused a transient hyperplasia followed by epidermal thinning and finally loss of normal skin function, suggesting ESC depletion. Most strikingly, this phenotype was associated with increased c-Myc expression, and the authors demonstrated that Rac1 negatively regulates c-Myc expression through p21-activated kinase 2 (PAK2) phosphorylation (Benitah et al. 2005). Although the exact mechanism for the loss of ESCs in these models still remains to be elucidated, one plausible explanation is the premature differentiation of ESCs, similar to what has been observed in HSCs overexpressing c-Myc (Wilson et al. 2004; Wilson and Trumpp 2006). The mechanism by which c-Myc activity is thought to regulate the stem cell populations of both skin and blood is mediated by influencing the interaction of stem cells with the specialized stem cell microenvironment, known as the stem cell niche. Myc overexpression downregulates the expression of a number of critical cell adhesion molecules on stem cells, promoting their exit from the niche, which induces differentiation and subsequent loss of multipotent stem cell activity (Frye et al. 2003; Wilson et al. 2004; Murphy et al. 2005; Wilson and Trumpp 2006). At first glance this may appear counterintuitive, considering the substantial tumor-promoting activity of c-Myc. However, it is important to note that, in addition to its role in driving proliferation of progenitor cells by inhibiting cell cycle exit, c-Myc also blocks terminal differentiation. Therefore, c-Myc expression strongly increases the usually very low self-renewal activity of

progenitor populations, causing the typical hyperplastic phenotypes frequently associated with high Myc activity in preneoplastic lesions (Cory et al. 1999; Grandori et al. 2000). In summary, the effects of ectopic c-Myc expression in the skin are dependent on the differentiation status of the targeted cell type. c-Myc overexpression in postmitotic cells causes cell cycle re-entry and therefore a switch back to a progenitor fate. In proliferative progenitors, high Myc activity inhibits cell cycle exit and terminal differentiation, thus promoting self-renewal. In contrast, c-Myc expression in ESCs and HSCs may cause their irreversible loss due to niche-dependent differentiation into progenitors, which in turn may result in increased self-renewal activity should high c-Myc expression be maintained.

3 The Role of Endogenous c-Myc in the Epidermis

c-Myc is expressed in the basal layer of the interfollicular epidermis and in the bulge region of the hair follicle (Hurlin et al. 1995; Bull et al. 2001), regions known to harbor ESCs (Blanpain and Fuchs 2006), suggesting an important role for c-Myc during skin homeostasis. It is therefore surprising that conditional elimination of a *c-myc* ^{flox} allele in the adult skin with a tamoxifen-inducible K5-CreER^{T} allele revealed no obvious skin abnormalities (Oskarsson et al. 2006). Epidermal proliferation, differentiation, and hair follicle maintenance remained intact, suggesting that c-Myc is dispensable during normal skin homeostasis. Moreover, treatment of the epidermis with a tumorpromoting agent known to induce chronic hyperplasia, 12-*O*-tetradecanoylphorbol-13-acetate (TPA), was not attenuated in the absence of c-Myc (Oskarsson et al. 2006). This was particularly surprising given that endogenous epidermal c-Myc expression has been shown to be rapidly induced by TPA (Rodriguez-Puebla et al. 1998). These unexpected genetic data suggest that epidermal homeostasis and TPA-induced hyperplasia are either controlled by other Myc family members, such as N-myc or L-myc, or alternatively, regulated by a program that is completely Myc independent.

4 The Role of Endogenous c-Myc in Ras-Mediated Skin Tumorigenesis

The two-stage carcinogenesis assay has been used for many decades as a framework to study the multistage nature of cancer development (DiGiovanni 1992). This assay is based on the exposure of shaved mouse skin to the mutagen DMBA (7,12-dimethylbenz[*a*]anthracene) (tumor initiation), followed by repeated TPA treatment (tumor promotion). After the latency of several weeks, a large number of benign papillomas develop, of which about 10% progress to malignant squamous cell carcinomas. Curiously, almost all of the tumors that arise from this protocol carry the same initiating mutation at codon 61 in the Hras gene ($HRas^{mu}$), leading to dominant activation of the Raf-MAPK pathway (Quintanilla et al. 1986). Ras is a signaling molecule regulating multiple signaling pathways affecting cellular proliferation, apoptosis, differentiation, migration, cytoskeletal organization, adhesion, and senescence (Campbell et al. 1998).

Unexpectedly, c-Myc mutants show resistance to DMBA/TPA-induced tumorigenesis, with no papilloma formation evident in the absence of c-Myc. In this respect, it is interesting to note that Ras and Myc have a long, well-characterized history in cancer biology. This gene pair was the first for which oncogene cooperation has been directly demonstrated, since only simultaneous overexpression or activation of both genes was sufficient to transform primary rat embryonic fibroblasts, whereas neither gene alone was able to do so (Land et al. 1986; Henriksson and Luscher 1996). The mechanism for this effect is still under investigation, but it is generally thought that the anticancer effects of Myc deregulation (apoptosis) and oncogenic Ras (senescence) are antagonized and therefore canceled out by the other cooperating oncogene (Kauffmann-Zeh et al. 1997; Serrano et al. 1997). Oncogene-induced senescence has recently been recognized as a barrier to early tumor development, and expression of senescence markers has been documented in a number of preneoplastic lesions (reviewed in Sharpless and DePinho 2005; Braig and Schmitt 2006). Oncogenic Ras provokes cellular senescence by Raf-MAPK-mediated accumulation of $p16^{INK4a}$ and $p21^{CIP1}$ (Serrano et al. 1997; Carnero and Beach 2004). Interestingly, $p21^{CIP1}$ is a very robust target gene directly re-

pressed by c-Myc in a number of cell types (Blanco and Trumpp unpublished; Herold et al. 2002; van de Wetering et al. 2002). Moreover, Ras signaling itself has been shown to mediate stabilization of the normally unstable c-Myc protein through phosphorylation and subsequent prevention of ubiquitin-mediated degradation (Sears 2004). How do these elements now come together to provide an explanation for the observed resistance of c-Myc-deficient epidermis to Ras-mediated tumorigenesis? It has been shown that the induced ablation of c-Myc function in established tumors (but not in TPA-induced hyperplastic skin) results in a massive upregulation of p21 ^{CIP1} (Fig. 1) (Oskarsson et al. 2006). This observation leads to two conclusions; first, oncogenic Ras drives high p21 ^{CIP1}expression, likely through the p19 ^{ARF}-p53 pathway known to be upregulated in DMBA/TPA-induced papillomas (Kelly-Spratt et al. 2004), and second, the observed low p21 ^{CIP1} expression in tumors is due to c-Myc-mediated repression. These observations raised the interesting possibility that c-Myc-driven inhibition of oncogenic Ras-driven p21 ^{CIP1} could be a crucial mechanism for the initiation of tumor development. This hypothesis was confirmed by demonstrating that epidermis lacking both c-Myc and p21 ^{CIP1} regained its normal capacity to form papillomas (Fig. 1) (Oskarsson et al. 2006). Taken together, these experiments demonstrate a single key role for endogenous c-Myc activity during Ras^{Mu}-mediated skin tumorigenesis, namely the repression of p21^{CIP1}.

5 c-Myc and Ras^{Mu} Function in Epidermal Cancer Initiating Cells

Recent models suggest that many, if not most cancers, are driven by cancer stem cells (CSCs) (Wang and Dick 2005; Polyak and Hahn 2006). CSCs comprise a small subpopulation of cells present within the tumor that have long-term self-renewal activity and are the only cells capable of regenerating the complex disease after transplantation into a suitable recipient. Such CSCs may be directly derived from normal tissue stem cells or may develop from progenitor cell types. In the latter case, the genetic lesions that occur must re-establish a self-renewal program similar to that which operates in normal stem cells (Krivtsov et al. 2006).

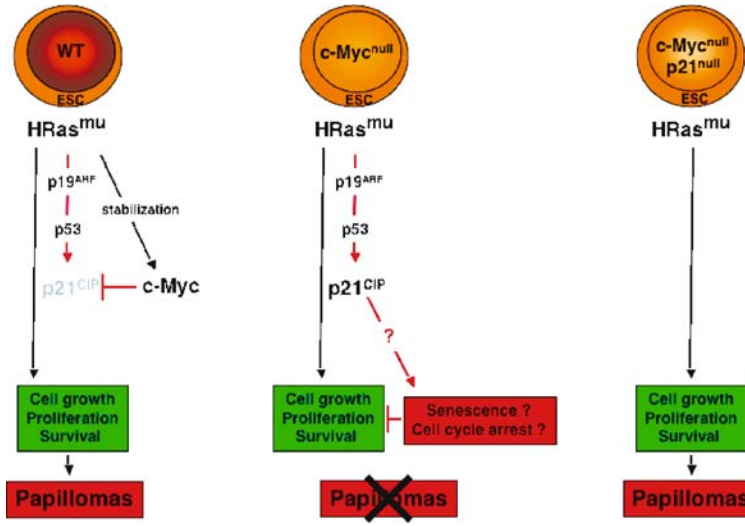

Fig. 1. Model showing the differential response of DMBA/TPA-treated wild-type (WT, *left*), c-Myc^{null} (*middle*) and c-Myc^{null}, p21^{null} double mutant (*right*) epidermal stem cells (ESCs) to develop papillomas. In WT mice, activated HRas (HRas^{mu}) increases p21 ^{CIP1} expression but also stabilizes endogenous c-Myc, which in turn represses p21 ^{CIP1} expression. The pro-oncogenic activities of HRas^{mu} include the promotion of cellular growth, proliferation, and survival, which lead to the formation of papillomas (*left*). In a c-Myc-deficient situation (*middle*), p21^{CIP1} expression is not repressed, causing the activation of a cell cycle arrest and senescence program, which prevents the development of papillomas (*middle*). ESCs lacking both c-Myc and p21^{CIP1} are able to develop papillomas, showing that the single main function of c-Myc during HRas^{mu}-driven papillomagenesis is to repress p21^{CIP1}

Although it has not been rigorously investigated whether skin tumors generated by the DMBA/TPA protocol are driven by CSCs, there is substantial evidence suggesting that the mutagen DMBA may target an epidermal stem cell located either in the bulge region or in the basal layer of the interfollicular epidermis and that these are then turned into putative CSCs (reviewed in Perez-Losada and Balmain 2003; Morris et al. 2004). Most strikingly, TPA treatment immediately following the

initial DMBA exposure, or delayed for as long as 1 year after, generates the same number of papillomas with similar latency (Van Duuren et al. 1975). These data suggest that the target cells that carry oncogenic H-Ras have the ability to remain dormant over long periods of time, a feature only attributable to long-lived normal ESCs. In addition, studies using the two-stage carcinogenesis protocol in which the epidermis, but not the hair follicles, has been removed by a specific abrasion technique suggest that the tumor target cells are located in the follicular region (Morris et al. 2000). This region contains the follicular bulge region, which has recently been shown to house multipotent epidermal stem cells (Morris et al. 2004; Tumbar et al. 2004; Blanpain and Fuchs 2006), again indicating that stem cells are the most likely targets for DMBA/TPA (and therefore activated Ras)-driven skin tumorigenesis (Owens and Watt 2003; Perez-Losada and Balmain 2003).

With respect to the resistance of c-Myc mutants to Ras-driven tumorigenesis, it remains to be determined whether c-Myc activity is required in $HRas^{mu}$ skin stem cells or in $HRas^{mu}$ progenitors. Nevertheless, it is tempting to speculate that c-Myc-deficient skin fails to develop papillomas because of senescence, and consequently, premature loss of self-renewal activity within the tumor-initiating stem cell. This effect could be induced by the high $p21^{CIP1}$ levels driven by oncogenic $HRas^{Mu}$. In support of this hypothesis, primary keratinocytes in which c-Myc has been acutely deleted turn on senescence-associated β-galactosidase activity, similar to what has recently been observed in fibroblasts, suggesting that c-Myc is critical for preventing the activation of a senescence program (Guney and Sedivy 2006; Oskarsson et al. 2006). Future experiments will need to address whether the abovementioned and other senescence-associated events are induced in both normal and c-Myc-deficient, DMBA/TPA-treated skin *in vivo*, and whether this occurs at the stem or progenitor cell level.

In summary, these data show that c-Myc/Ras oncogene cooperation occurs during skin tumorigenesis *in vivo* and suggest that endogenous c-Myc is essential for $HRas^{Mu}$-driven tumorigenesis. Surprisingly, this response appears to be mediated by a single gene, $p21^{CIP1}$, which needs to be controlled/repressed by c-Myc in this scenario. Thus, while oncogenic Ras drives the senescence-promoting activity of $p21^{CIP1}$, it simultaneously eliminates this antioncogenic effect by stabilizing the $p21^{CIP1}$

repressor c-Myc (Fig. 1). This again points toward c-Myc as a putative drug target. In the past, anti-Myc therapy has usually been discarded as a therapeutic regime, given the belief that c-Myc function is essential for the proliferation of many, if not all, cells in our body. Although this assumption is true for most hematopoietic cell types, which indeed require c-Myc function to maintain their proliferative capacity (Wilson et al. 2004), c-Myc appears to be dispensable for normal skin homeostasis (Oskarsson et al. 2006). This raises the possibility that ectopically applied anti-Myc therapy might be a useful strategy to treat Ras-driven skin tumors, a hypothesis testable in preclinical mouse models.

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Wnt Signaling in Stem Cells and Lung Cancer

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Abstract. The Wnt signal transduction pathway plays important roles during embryo development, regulating cell proliferation and survival of immature cells. However, its improper function can lead to harmful consequences for humans, such as aberrant cell proliferation and, therefore, cancer. Increasing evidence suggests that stem cells may be the source of mutant cells that cause cancers to develop and proliferate. Wnt signaling has been shown to promote self-renewal in both gut epithelial and hematopoietic stem cells (HSCs) and to

trigger critical pathways in carcinogenesis. Although the function of stem cells in solid tumor development is unclear, the Wnt pathway's role in determining the fate and self-renewal potential of cancer stem cells suggests a critical role in carcinogenesis. The development of new inhibitors, such as antibodies or small molecules, to inhibit this pathway may be of great therapeutic utility against cancer.

1 Overview of Wnt Signaling

The Wnt signal transduction pathway was named after the *wingless* gene, the Drosophila homologous gene of the first mammalian Wnt gene characterized, *int-1* (Rijsewijk et al. 1987). Secreted Wnt ligands have been shown to activate signal transduction pathways and trigger changes in gene expression, cell behavior, adhesion, and polarity. In mammalian species, Wnt proteins comprise a family of 19 highly conserved signaling molecules. Wnt signaling has been described in at least three pathways (Widelitz 2005), with the best-understood canonical pathway, in which Wnt ligands bind to two distinct families of cell surface receptors, the Frizzled (Fz) receptor family and the LDL receptor-related protein (LRP) family, and activate target genes through stabilization of β-catenin in the nucleus (Akiyama 2000). Wnt proteins can also signal by activating calmodulin kinase II and protein kinase C (known as the Wnt/Ca^{2+} pathway), which involves an increase in intracellular Ca^{2+}, or Jun N-terminal kinase (JNK) (known as the planar cell polarity pathway), which controls cytoskeletal rearrangements and cell polarity (Veeman MT et al. 2003).

1.1 Wnt Ligands

Wnt proteins are secreted glycoproteins of around 40 kDa, with a large number of conserved cysteine residues. They are produced by different cell types, and in humans 19 Wnt proteins currently have been identified (Miller 2002). It was found that cysteine palmitoylation is essential for the function of Wnt proteins (Willert et al. 2003). Hofmann (2000) reported that Porcupine (Porc), required in Wnt-secreting cells, shows homology to acyltransferases in the endoplasmic reticulum (ER). Taken together, it appears that Porc may be the enzyme responsible for cys-

teine palmitoylation of the Wnt proteins (Zhai et al. 2004). In addition, studies in Drosophila revealed that the seven-transmembrane proteins Wntless (Wls) and Evenness interrupted (Evi) are essential for Wnt secretion (Banziger et al. 2006; Bartscherer et al. 2006). In the absence of Wls/Evi, primarily residing in the Golgi apparatus, Wnts are retained inside the Wnt-producing cells. Furthermore, extracellular heparan sulfate proteoglycans (HSPGs) may also play a role in the transport or stabilization of Wnt proteins. (Lin 2004).

1.2 Wnt Receptors and Antagonists

Reception and transduction of Wnt signals involve interaction of Wnt proteins with members of two distinct families of cell surface receptors, the Frizzled (Fz) gene family and the LDL receptor-related protein (LRP) family. Fz proteins bind Wnts through an extracellular N-terminal cysteine-rich domain (CRD), and most Wnt proteins can bind to multiple Fz receptors and vice versa. (Bhanot et al. 1996). Ten human Fz proteins have been identified so far, and their general structure is similar to that of seven-transmembrane G protein-coupled receptors, suggesting that Fz proteins may use heterotrimeric G proteins to transduce Wnt signals (Liu et al. 2001). A single-pass transmembrane molecule of the LRP family, identified as LRP5 or 6, is also required for the signaling (Tamai et al. 2000). It appears that surface expression of both receptor families is required to initiate the Wnt signal, although formation of trimeric complexes involving Wnt molecules with Fz and LRP5/6 has yet to be validated. In addition, two tyrosine kinase receptors, Derailed and Ror2, have been shown to bind Wnts. Derailed binds Wnts through its extracellular WIF (Wnt inhibitory factor) domain, and Ror2 binds Wnts through a Wnt binding CRD motif. Signaling events downstream of these alternative Wnt receptors remain largely unclear (Lu et al. 2004; Mikels and Nusse 2006).

Secreted inhibitory proteins can sequester Wnt ligands from their receptors. Among these are the secreted Frizzled-related proteins (SFRPs) and the Wnt inhibitory factor-1 (WIF-1) (Hsieh et al. 1999; Jones and Jomary 2002). The human SFRP family consists of five members, each containing a CRD domain. The biology of SFRPs is, however, complex, and in some cases, they may act as Wnt agonists (Uren et al.

2000). WIF-1 does not have any sequence homology with SFRPs but contains a unique evolutionarily conserved WIF domain and five epidermal growth factor (EGF)-like repeats. A third class of extracellular Wnt inhibitor is represented by the Dickkopf (Dkk) family, which antagonizes Wnt signaling pathway through inactivation of the surface receptors LRP5/6 (Fedi et al. 1999).

1.3 Canonical Wnt Signaling: Cytosolic and Nuclear Components

When Wnt signaling is in the "off state" (Fig. 1), cytosolic β-catenin is phosphorylated by the serine/threonine kinases casein kinase I (CKI) and GSK3β at four N-terminal residues (Amit et al. 2002). The scaffolding proteins Axin and APC mediate the interaction between the kinases and β-catenin (Hart et al. 1998). These proteins form a β-catenin degradation complex that allows phosphorylated β-catenin to be recognized by β-TrCP and subsequently targeted for ubiquitination and proteasome degradation (Aberle et al. 1997). In the nucleus, the TCF/DNA-binding proteins form a complex with Groucho and act as repressors of Wnt target genes when the Wnt signal is absent (Cavallo et al. 1998). Groucho can interact with histone deacetylases, which makes the DNA refractive to transcriptional activation (Chen et al. 1999). Upon interaction of the Wnt ligands with their receptors, the Fz/LRP coreceptor complex activates the canonical signaling pathway (the "on state" of Wnt signaling) (Fig. 2). Fz can physically interact with Dishevelled (Dvl), a cytosolic protein that functions upstream of β-catenin and the kinase GSK3β. Then the scaffold protein axin translocates to the membrane, where it interacts with either the intracellular tail of LRP or with Fz-bound Dvl (Cliffe et al. 2003). Removing axin from the destruction complex promotes β-catenin stabilization. The "on" and "off" states of Wnt signaling control phosphorylation status of Dvl protein (Wallingford and Habas 2005). It remains unclear, however, whether the binding of Wnt to Fz regulates a direct Fz-Dvl interaction and how phosphorylated Dvl functions during Wnt signal transduction. With the help of BCL9 (Kramps et al. 2002; Krieghoff 2005; Sampietro 2006), stabilized β-catenin enters the nucleus and competes with Groucho for binding to TCF/LEF, recruits Pygopus, and converts the TCF repres-

sor complex into a transcriptional activator complex. A large number of Wnt signaling target genes, including c-Myc, cyclin D1, MMP-7, and WISP, have been identified (a list of the Wnt target genes can be found at http://www.stanford.edu/~rnusse/wntwindow.html). The Wnt signaling pathway plays an important role in cell differentiation and proliferation, and when aberrantly activated, it contributes to most of the features that characterize malignant tumors, including evasion of apoptosis, tissue invasion and metastasis, self-sufficiency of growth signals, insensitivity to growth inhibitors, and sustained angiogenesis (Ilyas 2005).

2 Stem Cells, Cancer Stem Cells, and Lung Cancer

Stem cells are distinguished by their ability to self-renew and differentiate into other cell types and are found in embryonic and adult tissues (Preston et al. 2003). Embryonic stem cells (ES) have the ability, known as pluripotency, to develop into any type of cell required for mammalian development. While it has been proposed that all adult tissues derive from tissue-specific stem cells, that link has been demonstrated in only a limited number of cell types. Examples include hematopoietic stem cells (HSCs) from which all red and white blood cells develop (Morrison and Weissman 1994; Baum et al. 1992; Spangrude et al. 1988) and mesenchymal stem cells (MSCs), present in the bone marrow for later differentiation into bone, cartilage, adipose tissue, and muscle (Zimmermann et al. 2003; Simonsen et al. 2002). In addition, it has been demonstrated that "local" stem cell populations in brain and muscle can repopulate the bone marrow of a radiation-ablated or immune-deficient mouse (Otto 2002).

Although the role of stem cells in cancinogenesis is not well characterized, emerging evidence is providing new insight into this process (Preston et al. 2003; Marx 2003). Although stem cells are rare within any given cell population, several factors make them a likely culprit in the development of cancers. First is their capacity for self-renewal and replication. Second is their long-lived potential as undifferentiated cells, creating a larger window of opportunity for molecular alterations that accumulate over time. Thus, it is not surprising that the Hedgehog (Hh) and Wnt pathways, regulatory mechanisms for stem cell renewal,

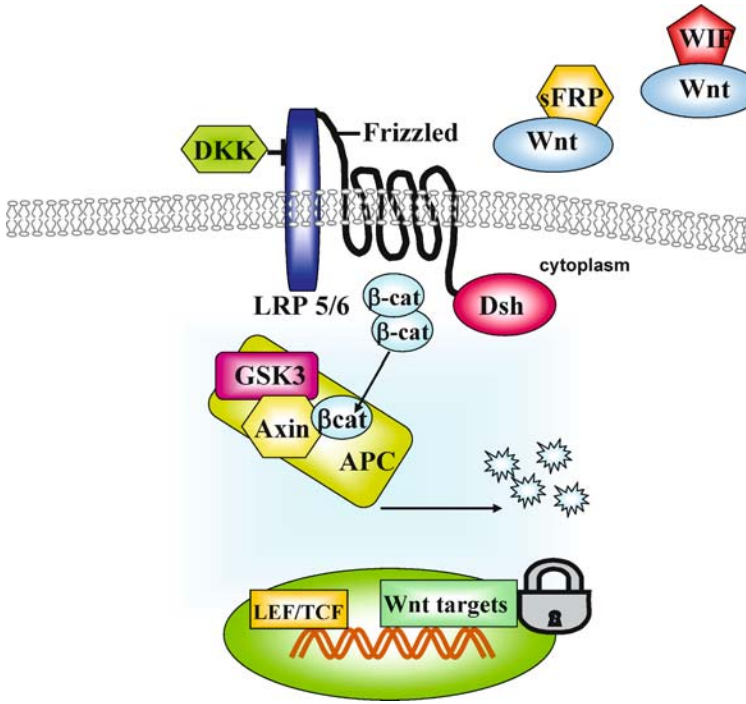

Fig. 1. The inactive canonical Wnt signaling pathway. In the absence of factors that activate Wnt signaling, the complex APC-Axin and GSK-3 binds to β-catenin with subsequent β-catenin phosphorylation, ubiquination, and degradation by proteasomes. TCF/LEF proteins repress target genes through a direct association with co-repressors

tissue repair, and tissue regeneration, are implicated in the development of cancers when aberrantly activated.

Cancer has long been thought to originate and develop from cancer stem cells. Although constituting only a fraction of the cells within the tumor, cancer stem cells are nonetheless critical for its propagation (Park et al. 1971). The concept of cancer stem cells originated from findings in the hematopoietic system (Till 1961) and acute myelogenous leukemia (AML) (Till 1961; Hope et al. 2004; Bonnet and Dick 1997).

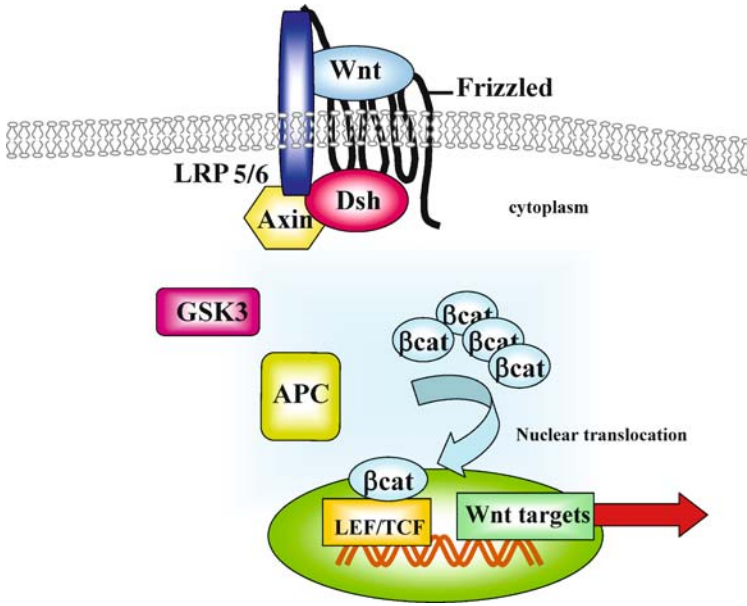

Fig. 2. The active canonical Wnt signaling pathway. When Wnt ligands activate Wnt signaling, Dvl binds to Axin and inhibits the degradation complex APC-Axin-GSK-3. Therefore, β-catenin cannot be phosphorylated and its level increases in the cytoplasm, allowing its translocation to the nucleus, where it converts TCF/LEF factors into transcriptional activators of Wnt target genes, such as c-myc, and cyclin D1, etc.

Cancer stem cells in tumorigenesis have been demonstrated in several cancer types. An important criterion for cancer stem cells is that they enable serial propagation of tumors that retain the often diverse marker profile of the primary tumor (Singh et al. 2004). Cancer stem cells and normal stem cells share numerous properties, including the expression of common cell surface markers, the capacity to self-renew, and unlimited replication potential and the quality of being long-lived, allowing the accrual of multiple mutations over time, increasing the rate of cell proliferation and producing clinically significant cancer.

Lung cancer is the leading cause of cancer mortality in the USA. Approximately 173,770 new cases of lung cancer were diagnosed in 2004, 13% of all new cancer cases, and an estimated 160,440 Americans died from the disease, accounting for 28% of all cancer deaths.

There are two major pathological groups in lung cancer: non-small cell lung cancer (NSCLC), comprising 80% of the total, and small cell lung cancer (SCLC) comprising the remaining 20%. Increasing evidence shows that lung cancer occurs through a multistep oncogenic process. Bronchioalveolar carcinoma (BAC) and atypical adenomatous hyperplasia (AAH), a premalignant lesion believed to be a precursor to BAC, are often present near areas of invasive adenocarcinoma (Yoshida et al. 2005; Saad et al. 2004; Kitamura et al. 1999; Okubo et al. 1999). Both tumor suppressor genes and oncogenes play important roles in the development of lung cancer (Bishop 1991; Weinberg 1991). For example, single allele mutation in a proto-oncogene is often sufficient for aberrant transformation with crucial effects on signal transduction and transcription processes. Amplification, translocation, rearrangement, and point mutations in dominant oncogenes lead to aberrant transformation. Homozygous loss of function in tumor suppressor genes by genetic, epigenetic, or both events leads to abnormal regulation of transcription. Conventional treatments, including chemotherapy and radiation and, in earlier-stage cases, surgery, have succeeded only in slowing the inexorable march of the disease, and overall five-year survival has remained more or less constant at 15% for over a decade. If cancer stem cells are the driving force for cancer formation, then traditional therapeutic interventions that target the main tumor mass, but not the cancer stem cells, are not likely to succeed.

Recently agents targeting the underlying molecular signaling pathways in lung cancer have entered clinical trials with more encouraging results (Smith and Khuri 2004). Therefore better understanding the molecular mechanisms of lung cancer development should improve the diagnosis and treatment of this deadly disease. Identification and characterization of cancer stem cells should accelerate the development of therapeutic drugs that target them. In fact, a recent study in a mouse model by Kim et al. (2005) discovered that some cells at the bronchioalveolar duct junction exhibit features of stem cells. They discovered that cells at this junction carrying Clara cell and alveolar cell mark-

ers ("bronchioalveolar stem cells") appear refractory to naphthalene treatment and start to divide after naphthalene-induced damage, resulting in repair of damaged lung epithelial tissue. Furthermore, their data suggest that these double-positive cells with their stem cell-like features may play an important role in the transformation of normal lung epithelia into adenocarcinomas (Kim et al. 2005). However, there are questions that still need to be answered, such as whether these bronchioalveolar stem cells give rise to lung cancer stem cells, and if so, what events are required to achieve this transformation.

The activation of Hh and Wnt pathways in promoting stem cell renewal, transiently in normal tissues and aberrantly in carcinogenesis, has attracted great scientific interest. Hh pathway activity was initially linked to cancer through identification of mutations in a negative regulator of the Hh receptor, Patched (Ptch) (Wechsler-Reya and Scott 2001; Taipale and Beachy 2001). Recent studies employing Hh-blocking antibodies and a specific Smoothened (Smo) inhibitor, cyclopamine, have demonstrated that Hh pathway activity, which requires ligand activation, is important in the growth of many lethal cancers including SCLC (Thayer et al. 2003; Berman et al. 2003; Watkins et al. 2003). Here we discuss the role the Wnt pathway plays in the maintenance of normal stem cells and the development of lung cancer.

3 Wnt Signaling in Stem Cell Maintenance and Regeneration

Wnt signaling has many functions in animal development including a crucial role in the morphogenesis of the gastrointestinal tract (Korinek et al. 1998), mammary glands (Brennan and Brown 2004), cardiovascular system (Pandur et al. 2002), and bone marrow (Reya et al. 2003). Recent study data also highlight its developmental role in embryogenesis and in the adult lung. For example, Wnt signaling regulates important aspects of epithelial and mesenchymal development during gestation (Morrisey 2003).

More specifically, studies of knockout mice demonstrated the importance of Wnt-2, Wnt-5a, and Wnt-7b in lung maturation (Li et al. 2002; Shu et al. 2002; Weidenfeld et al. 2002; Yamaguchi et al. 1999). Using

oligonucleotide arrays, Bonner et al. demonstrated the contribution of the Wnt pathway to various stages of murine lung development (Bonner et al. 2003). Finally, the Wnt/β-catenin pathway has been shown to be activated in lung inflammatory processes, including idiopathic pulmonary fibrosis (Chilosi et al. 2003).

In addition to its traditional role in embryogenesis, the Wnt pathway has recently been implicated in the maintenance of stem and progenitor cells in adult tissues of the skin, blood, gut, prostate, muscle, and nervous system (Bhardwaj et al. 2001; Karhadkar et al. 2004; Korinek et al. 1998; Lai et al. 2003; Machold et al. 2003; Owens and Watt 2003; Pinto et al. 2003; Perez-Losada and Balmain 2003; Polesskaya et al. 2003; Ramalho-Santos et al. 2000; Reya et al. 2003; Zhang and Kalderon 2001). For example, studies of transgenic mice suggest that activation of the Wnt pathway in epidermal stem cells may lead to epithelial cancers (Gat et al. 1998). It was found consistently that gene expression patterns in colon cancer cells and colon stem cells resembled one another (van de Wetering et al. 2002).

Other evidence points as well to Wnt pathway involvement in stem cell development. Treatment of isolated HSCs with Wnt proteins in vitro increases their proliferative capacity and improves their ability to form colonies, both in vitro and in vivo (Reya et al. 2003). While inhibiting Wnt pathway activation in the intestine fails to prevent the development, initially, of normal epithelial architecture, its inactivation eventually results in a progressive degradation of epithelial structure. This effect is associated with a loss of proliferative activity in the crypts, where stem cells reside (Korinek et al. 1998; Pinto et al. 2003).

Reports have also suggested a role for the Wnt pathway in regenerative responses. For example, its activation has been closely associated with regeneration of muscle (Polesskaya et al. 2003), bile duct (Shackel et al. 2001), kidney (Surendran and Simon 2003), and liver (Monga et al. 2001) after injuries. In mice, it has been demonstrated that Wnt pathway activation enriches the population of mammary progenitors through increased levels of Wnt ligand or stabilized β-catenin (Liu et al. 2004). Moreover, it has been shown that muscle regeneration is inhibited by Wnt antagonists, such as secreted Frizzled-related proteins (SFRPs) (Polesskaya et al. 2003).

Wnt pathway activity has been associated with both chronic tissue injury and carcinogenesis (Dvorak 1986; Coussens and Werb 2002). Indeed, both processes may be related. First, genetic and/or epigenetic events lead to aberrant Wnt pathway activity, preventing activated stem or progenitor cells, once tissue regeneration is complete, from returning to a normal quiescent state, in effect a condition of unregulated tissue repair. In this way, conversion of a normal stem cell into a cancerous one may lock the cell in an active state of renewal. For example, when the lung or skin is continuously exposed to environmental insults, they may shift into a constant renewal state and ultimately become the sites of new cancers.

4 Wnt Signaling in Lung Cancer

In addition to its role in stem cell self-renewal, tissue regeneration, and lung development, Wnt signaling is also intimately involved in tumorigenesis and cancer progression (Polakis 2000; Bienz and Clevers 2000). For example, the organs where Wnt signaling influences stem cell self-renewal are the same organs where those Wnt-pathway-dependent cancers originate. Numerous reports have demonstrated aberrant Wnt activation in many human cancers, including colorectal (Morin et al. 1997; Korinek et al. 1997), head and neck (Rhee et al. 2002), melanoma (Weeraratna et al. 2002), and leukemia (Lu 2004). This activation can be caused by mutations and/or deregulation of many different Wnt signaling components. Mutations in Wnt pathway components are rarely found in lung cancer. Instead, nongenetic events appear to be the major cause of aberrant activation of Wnt signaling in lung cancer.

4.1 Wnt Ligands

Wnt-1 was first identified from retroviral integration that caused mammary tumors in mice (Nusse and Varmus 1982) and was found to be upregulated in a number of human cancers (Katoh 2003; Wong et al. 2002). Moreover, cancer cells expressing Wnt-1 are resistant to therapies that mediate apoptosis (Chen et al. 2001). Overexpression of Wnt-1 has been demonstrated in NSCLC cell lines and primary cancer tissues (He et al. 2004). Blockade of Wnt-1 signaling induces apoptosis in vitro

and suppresses tumor growth in vivo (He et al. 2004). Similar results were observed in head and neck squamous cell carcinoma (Rhee et al. 2002), suggesting that Wnt-1 signaling is a key mediator of apoptosis in epithelial cancers.

The human Wnt-2 gene, located on chromosome 7q31.3, is highly expressed in fetal lung and weakly expressed in placenta (Katoh 2001a). The link between Wnt-2 and tumorigenesis was first proposed after data indicated that Wnt-2 was amplified in human cancers (Yoshida et al. 1988). Similarly, Wnt-2 has been implicated in mouse mammary tumorigenesis through gene amplification (Roelink et al. 1992). Wnt-2 was later shown to be upregulated in gastric cancers (Nessling et al. 1998; Katoh 2001b), colorectal cancers (Vider et al. 1996; Holcombe et al. 2002; Katoh 2001c), and melanoma (Pham et al. 2003).

Recently, we demonstrated overexpression of Wnt-2 in NSCLC (You et al. 2004). Following our same study design in Wnt-1 (He et al. 2004), we demonstrated that inhibition of Wnt-2-mediated signaling by small interfering RNA (siRNA) or a monoclonal antibody induced apoptosis in NSCLC cells (You et al. 2004). Moreover, we most recently found that the anti-Wnt-2 antibody has sufficient potency to inhibit growth in primary human NSCLC tissue cultures (unpublished data).

Recently there has been a suggested role for Wnt-7a in lung cancer. It has been reported that expression of Wnt-7a is downregulated in most lung cancer cell lines and tumor samples (Calvo et al. 2000; Winn et al. 2005). It has been further hypothesized that Wnt-7a upregulates E-cadherin expression in lung cancer cells (Ohira et al. 2003). Interestingly, Wnt-7a functions through a β-catenin-independent pathway during limb development (Kengaku et al. 1998) and through the canonical pathway in lung cancer, as has been suggested, even though TCF/LEF transcriptional activity is not directly targeted (Winn et al. 2005). Further studies from the same group (Winn et al. 2006) indicate that combined expression of Wnt-7a and Frizzled-9 (Fz-9) in NSCLC cell lines inhibits transformed growth and that this antitumorigenic effect of Wnt-7a and Fz-9 is mediated through ERK5-dependent activation of PPARγ.

Wnt-5a, which is known to activate the Wnt/Ca^{2+} noncanonical pathway during development (Moon et al. 1997), has a controversial role in carcinogenesis. Wnt-5a is upregulated in some cancers (Saitoh et al. 2002) and can increase invasion of metastatic melanoma in a β-catenin-

independent manner (Weeraratna et al. 2002). Yet, Wnt-5a also behaves as a tumor suppressor gene in hematopoietic malignancies (Liang et al. 2003). The role of Wnt-5a in human sarcoma and in the development of lung metastases (Nakano et al. 2003) raises interest in its role in primary lung cancer. Indeed, a recent study in 123 patients with NSCLC (Huang et al. 2005) found that Wnt-5a expression in squamous cell carcinoma was significantly higher than that in adenocarcinoma. Furthermore, this study revealed that Wnt-5a overexpression could produce more aggressive NSCLC, especially in squamous cell carcinomas, during tumor progression.

4.2 Wnt Antagonists

There are two groups of Wnt antagonists. The first includes the SFRP family, WIF-1 and Cerberus. They inhibit Wnt signaling by directly binding to Wnt molecules. The second group, which includes the Dickkopf (DKK) family, inhibits Wnt signaling by binding to the LRP5/LRP6 component of the Wnt receptor complex (Kawano and Kypta 2003). These inhibitors have been extensively studied in developmental experiments, with demonstration of a role in oncogenesis, specifically in cervical (Ko et al. 2002), breast (Ugolini et al. 2001), gastric (To et al. 2001), and colorectal cancers (Suzuki et al. 2002; Suzuki et al. 2004; Caldwell et al. 2004).

The role of Wnt antagonists in lung carcinogenesis has recently been described. *WIF-1* was first identified from the human retina and is a highly conserved gene. Overexpression of WIF-1 in Xenopus embryos blocks the Wnt-8 pathway and induces abnormal somitogenesis (Hsieh et al. 1999). Recently, Wissman et al. reported the downregulation of WIF-1 in several cancer types including lung cancer by using a chip hybridization assay and immunohistochemistry (Wissmann et al. 2003). We recently demonstrated, by using methylation-specific PCR (MSP) and sequence analysis after bisulfite treatment, that frequent hypermethylation of CpG islands in the functional *WIF-1* promoter region correlated with its transcriptional silencing in human lung cancer cell lines (Mazieres et al. 2004). We also studied WIF-1 expression in freshly resected lung cancers and showed a downregulation in 83% of cases. This silencing also correlates with *WIF-1* promoter methylation

(Mazieres et al. 2004). We thus propose that methylation silencing of *WIF-1* is a common and likely important mechanism for aberrant activation of Wnt signaling in lung cancer pathogenesis.

SFRP proteins are endogenous modulators of Wnt signaling that compete with Wnt ligands in binding to the frizzled receptors. Previous studies have shown SFRP downregulation in colorectal cancer (Suzuki et al. 2002, 2004; Caldwell et al. 2004), gastric cancer (To et al. 2001), and invasive breast tumors (Wong et al. 2002). Interestingly, Suzuki et al. demonstrated that restoration of SFRP function in colorectal cancer cells attenuates Wnt signaling, even in the presence of β-catenin mutation, and may complement downstream mutations in the development of colorectal cancer (Suzuki et al. 2002). Although somatic mutations were not detected in the SFRP1 coding sequence, a loss of heterozygosity (LOH) analysis found that 38% of informative surgical specimens had LOH in the SFRP1 gene locus (Fukui et al. 2005). It was also found that SFRP was frequently downregulated in NSCLC and mesothelioma cell lines (Lee et al. 2004). Moreover, the SFRP gene promoter was hypermethylated in more than 80% of mesothelioma primary tissues (Lee et al. 2004) and in approximately 55% of primary lung tumors. More recently, it was found that promoter hypermethylation of the APC, CDH1, SFRP1, and WIF-1 genes may be able to discriminate lung primary adenocarcinomas from colorectal metastasis to the lung (Tang et al. 2006).

DKK proteins are a group of secreted glycoproteins that have the ability to antagonize Wnt-mediated signals (Fedi et al. 1999). The DKK-3 gene has been found to be downregulated in many cancer cell lines, including NSCLC (Tsuji et al. 2000), and in 63% of freshly resected NSCLC tissues (Nozaki et al. 2001). It has also been shown that forced expression of DKK-3 can inhibit cell growth (Tsuji et al. 2001). Moreover, no mutation was found within the DKK-3 gene. The gene is instead silenced by promoter hypermethylation in a high proportion of lung cancers (Kobayashi et al. 2002).

4.3 Dishevelled (Dvl)

Dvl proteins are positive mediators of Wnt signaling located downstream of the frizzled receptors and upstream of β-catenin. Three Dishevelled genes have thus far been characterized, Dvl-1 to Dvl-3. Di-

shevelled proteins possess three conserved domains, an N-terminal DIX domain that binds to Axin (Zeng et al. 1997), a central PDZ domain involved in protein-protein interactions (Ponting et al. 1997), and a C-terminal DEP domain found in proteins that regulate Rho GTPases (Ponting and Bork 1996). We showed that Dvl-3 was overexpressed in 75% of fresh microdissected NSCLC samples compared to autologous matched normal tissues (Uematsu et al. 2003a). Moreover, targeted inhibition of Dvl-1, -2 and -3 decreased β-catenin expression and TCF-dependent transcription and inhibited cell growth in human NSCLC cell lines (Uematsu et al. 2003a). We also demonstrated that a PDZ domain deletion mutant of Dvl could suppress tumorigenesis in pleural malignant mesothelioma (Uematsu et al. 2003b). Collectively, we believe these data support the novel hypothesis that Wnt signaling is activated through Dvl overexpression in thoracic malignancies. Many proteins including Daam-1, casein kinases 1 and 2, Notch, and β-arrestin have been shown to interact with Dvl (Wharton Jr 2003). While their role in carcinogenesis has been shown, their specific function in activating Dvl proteins in lung cancer remains largely unknown.

4.4 APC

APC has been identified as a tumor suppressor gene and is mutated in both sporadic and hereditary colorectal tumorigenesis. The allelic loss of the APC gene on chromosome 5q21 is a frequent genetic alteration occurring in 40% of NSCLC (Cooper et al. 1996). Nevertheless, no mutation was found in the APC gene, by single-strand conformational polymorphism analysis, in the 32 tumors described in the study above (Cooper et al. 1996). Other experiments similarly failed to find APC mutations in 55 lung cancer samples analyzed by an RNA protection assay (Horii et al. 1992) and in 29 NSCLC samples analyzed by yeast-based assay (Furuuchi et al. 2000). However, a recent study found 2 cases of APC mutation among 44 squamous cell carcinomas and 1 case in 32 SCLC samples, suggesting that APC mutations may be involved, albeit infrequently, in the pathogenesis of a small subset of lung carcinomas (Ohgaki et al. 2004).

4.5 β-Catenin

β-catenin plays key roles in the Wnt pathway, first by transmitting Wnt signals to the nucleus and second in tumorigenesis, through regulation of oncogenes (including cyclin D1 and c-myc) or by its own sporadic mutations. Overexpression of β-catenin has been reported in lung cancer. It has been reported that increased expression of β-catenin is associated with a high proliferative index, but paradoxically a favorable prognosis (Hommura et al. 2002). Supporting this, two studies reported independently that reduced expression of β-catenin might be associated with a poor prognosis in adenocarcinoma (Retera et al. 1998; Kase et al. 2000). The real significance of β-catenin expression in lung cancer has yet to be elucidated. Its controversial role might be linked to its complex effects since β-catenin also functions as a cadherin-mediated cell adhesion component (Barker et al. 2000). Moreover, mutations in the β-catenin gene appear uncommon in lung cancer. Sunaga et al. screened 46 lung cancer cell lines and 47 primary tissues and found mutations in the β-catenin gene, CTNNB1, in only 1 cell line and 2 surgical specimens, all adenocarcinomas, suggesting that genetic alteration of CTNNB1 is rare in lung cancer and restricted to the adenocarcinoma subtype (Sunaga et al. 2001). Similarly, only 1 adenocarcinoma of 90 primary lung cancers and 3 of 76 lung cancer cell lines studied to date have been reported to have a mutation (Shigemitsu et al. 2001). In another study, none of the 93 lung cancer cells analyzed displayed the mutations (Ueda et al. 2001). Only fetal-type adenocarcinomas, a rare subtype of lung cancer, appear to more consistently possess the activating β-catenin mutations (Nakatani et al. 2002). Based on these studies, it appears that β-catenin, in and of itself, plays less of a role in lung carcinogenesis than in other malignancies, particularly colorectal cancer.

4.6 Non-canonical Pathway

The non-canonical β-catenin-independent Wnt pathways have recently been described for their role in cancer (Veeman et al. 2003). Mechanisms of non-canonical pathways are varied, including signaling through calcium flux, JNK, and heterotrimeric G proteins. The planar cell po-

larity (PCP) pathway is known to act through small GTPases. For example, Rho, Rac, and Cdc 42 have been implicated in vertebrate non-canonical Wnt signaling (Habas et al. 2001; Choi and Han 2002; Penzo-Mendez et al. 2003). The link between Rho proteins and Wnt signaling has been recently reinforced by showing the interactions of Tiam1 (a Wnt-responsive gene) and Rac (Hordijk et al. 1997), WISP-1 and Rac (Soon et al. 2003), and Wrch-1 and Cdc42 (Tao et al. 2001). Additionally, Wnt/Fz signaling has been shown to activate Rac and Rho through Dvl proteins (Habas et al. 2003). Interestingly, many of these small GTPases including RhoA (Allal et al. 2000), RhoB (Mazieres et al. 2004), RhoC (Ikoma et al. 2004), Rac (del Peso et al. 1997), and Cdc 42 (Yao et al. 2002) are known to be involved in lung carcinogenesis, but it is unclear how they contribute to cancer development. We can thus hypothesize that cross talk between Rho and Wnt signaling may contribute in some unspecified manner to lung tumorigenesis, particularly through the mechanisms of cell adhesion and migration (Malliri and Collard 2003). Another putative mediator of non-canonical Wnt signaling is the JNK pathway (Veeman et al. 2003). The role of JNK in Wnt-mediated signaling is unclear. It has been shown that Dvl induces JNK activity through its DEP domain in planar cell polarity (Boutros et al. 1998) and this activation may involve some Rho GTPases (Moriguchi et al. 1999). We recently provided new insights into the field by showing that Wnt-1 blockade by either siRNA or a monoclonal antibody induces apoptosis in β-catenin-deficient mesothelioma cell lines, underscoring the role of the Wnt non-canonical pathway(s) in this process. Interestingly, JNK was found to be upregulated in these cell lines after blockade of Wnt-1 signaling. Moreover, treatment of these cell lines with a selective JNK inhibitor, SP600125, significantly inhibits apoptosis induced by Wnt-1 blockade. These data suggest that both canonical and non-canonical pathway(s) may be involved in Wnt-1-mediated apoptosis (You et al. 2004).

5 Potential Therapeutic Approaches Targeting the Wnt Pathway

The implication of the Wnt signaling pathway in human tumorigenesis holds promise for the development of new anticancer drugs targeting this pathway. Current strategies exploring how to inhibit this pathway are summarized in Table 1.

Inhibiting the Wnt pathway at the cell surface level, such as blocking the binding of Wnts to their Frizzled receptors, seems to be an attractive approach. For example, inhibition of Wnt-1 by either siRNA or a specific monoclonal antibody induced apoptosis-suppressed tumor growth in human cancer cells overexpressing Wnt-1 (He et al. 2004, 2005a; Mikami et al. 2005; Rhee et al. 2002; You et al. 2004b). Similarly, inhibition of Wnt-2-mediated signaling by siRNA or a monoclonal antibody induced apoptosis and inhibited tumor growth in Wnt-2-expressing cancer cells (Mazieres et al. 2005; You et al. 2004a). In addition, Weeraratna et al. demonstrated that blocking this pathway by using antibodies to FzD5, a receptor for Wnt-5a, inhibited PKC activity and cellular invasion (Weeraratna et al. 2002).

The link between downregulation of Wnt antagonists and constitutive activation of the pathway has been documented in cancers (He et al.

Table 1 Summary of current approaches for targeting Wnt signaling pathway for cancer therapy

Targets	Expression change	Approaches
Wnt-1	Elevated	siRNA; antibody
Wnt-2	Elevated	siRNA; antibody
Wnt-7a	Reduced	Forced expression
SFRP	Reduced	Forced expression
WIF-1	Reduced	Forced expression
DKK	Reduced	Forced expression
Axin	Reduced	Forced expression
Dvl	Elevated	siRNA; small-molecule inhibitors
β-Catenin	Elevated	Antisense; siRNA; small-molecule inhibitors

2005b; Lee et al. 2004; Taniguchi et al. 2005). Therefore, forced expression of Wnt antagonists or recombinant antagonist proteins may be a promising therapeutic strategy. For example, transfection of SFRP gene constructs into various types of cancer cell lines lacking SFRP expression induced apoptosis and cell growth suppression (He et al. 2005b; Horvath et al. 2004; Lee et al. 2004; Roth et al. 2000; Suzuki et al. 2004; Zi et al. 2005). In addition, restored WIF-1 expression also led to decreased tumor cell proliferation in different cancer cell lines (He et al. 2005b; Lin et al. 2006; Taniguchi et al. 2005). Interestingly, we observed that restoration of functional WIF-1 could sensitize tumor cells to treatments with chemotherapeutic drugs (unpublished data). The Dkk proteins have also received attention as potential anticancer targets. It has been shown that forced expression of Dkk3 can inhibit cancer cell growth (Kawano et al. 2006; Tsuji et al. 2001) as well as decrease cancer cell migration (Kuphal et al. 2006). Moreover, expression of Dkk3 resulted in inhibition of invasion and motility of cancer cells by modulating the Wnt/β-catenin pathway (Hoang et al. 2004). Similarly, restoration of Dkk1 function in cancer cells lacking Dkk1 expression also reduced colony formation and caused tumor growth inhibition (Aguilera et al. 2006; Lee et al. 2004).

Evidence suggests that intracellular components of the Wnt signaling may also have therapeutic roles. For example, targeted inhibition of Dvl by siRNA decreased β-catenin expression and TCF-dependent transcription and inhibited tumor cell growth of non-small cell lung cancer cells (Uematsu et al. 2003a). More recently, we found that blocking interaction between the PDZ domain of Dvl and the intracellular domain of Fz receptors by small molecule mimetics also suppressed cancer cell growth by attenuating the canonical Wnt signaling pathway (unpublished data). Axin, a negative regulator of the pathway, is mutated in several types of human cancers. Satoh et al. demonstrated that adenovirus-mediated gene transfer of wild-type Axin1 induced apoptosis in hepatocellular and colorectal cancer cells (Satoh et al. 2000). β-Catenin represents another attractive target for the development of anticancer drugs, since specific inhibition of activated β-catenin might reverse the tumorigenic properties of human cancer cells (Kim et al. 2002). Both β-catenin antisense and siRNA showed promising growth suppression results in colon cancer cell lines (Green et al. 2001; Verma

et al. 2003). Another approach is to induce the degradation process of pathogenic β-catenin by redirecting the ubiquitin-conjugation complex machinery to β-catenin, independently of its phosphorylation status (Cong et al. 2003; Liu et al. 2004; Su et al. 2003). Remarkably, this protein knockdown strategy reduced only the cytosolic, but not the membrane-associated, subpopulation of β-catenin, which prevents unwanted side effects. Small-molecule compounds have also been used to block protein-protein interactions between β-catenin and TCF4 (Lepourcelet et al. 2004). The compounds identified potently antagonized cellular effects of β-catenin-dependent activities, including reporter gene activation, transcription of β-catenin/TCF4 target genes, cell proliferation, etc (Lepourcelet et al. 2004). Similarly, a small molecule was identified to downregulate β-catenin/TCF signaling by binding to cyclic AMP response element-binding protein (CBP) (Emami et al. 2004). It selectively induced apoptosis in colon cancer cells, but not in normal colonic epithelial cells.

6 Summary

The Wnt signaling pathway has clearly emerged as a critical pathway in stem cell renewal and lung carcinogenesis, similar to its role in other cancers (Fig. 3). The components of the Wnt pathway have been sufficiently characterized to suggest they may be attractive targets for potential therapeutic agents. Although there are no specific in vivo trials with treatments targeting cancer stem cells, one could postulate that blocking components of the Wnt pathway could be an important way to inhibit the signaling required for cancer stem cells. In addition, it may be possible to manipulate other signaling pathways to inhibit Wnt signaling. It may also be possible to discriminate between levels of risk of tumor development and to develop predictive screening strategies. Therefore, further understanding of Wnt signaling will aid the battle against lung cancer.

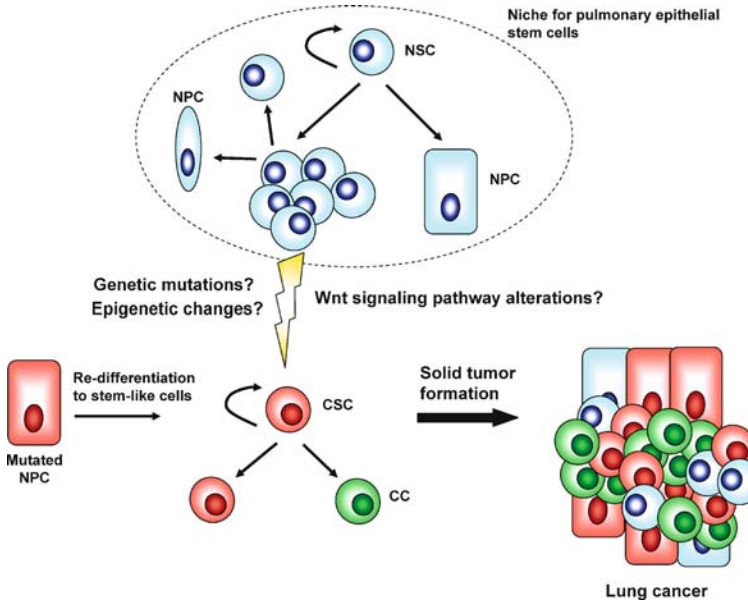

Fig. 3. Stem cells and lung cancer

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Bone Morphogenetic Proteins Regulate Tumorigenicity in Human Glioblastoma Stem Cells

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Abstract. Human glioblastomas appear to be established and expanded by cancer stem cells, which are endowed with tumour-initiating and perpetuating ability. We report that bone morphogenetic proteins (BMPs), amongst which BMP4 elicits the strongest effect, activate their cognate receptors (BMPRs) and trigger the Smad but not the MAP38 kinase signalling cascade in cells isolated from human glioblastomas (GBMs). This is followed by a reduction in proliferation and increased expression of differentiated neural markers, without affecting cell viability. The concomitant reduction in the clonogenic ability, both in the size of the $CD133^+$ side population and in the growth kinetics of GBM cells, indicates that BMP4 triggers a reduction in the *in vitro* cancer stem cell (CSC) pool. Accordingly, transient *ex vivo* exposure to BMP4 abolishes the capacity of transplanted GBM cells to establish intracerebral GBMs. Most important,

in vivo delivery of BMP4 effectively blocks the tumour growth and associated mortality which occur in 100% of control mice in less than 12 weeks, following intracerebral grafting of human GBM cells. These findings show that the BMP-BMPR signalling system, which controls the activity of normal brain stem cells, may also act as a key inhibitory regulator of cancer-initiating, GBM stem-like cells and identifies BMP4 as a novel, non-cytotoxic therapeutic effector, which may be used to prevent growth and recurrence of GBMs in humans.

Significant theoretical and medical implications underlie the hypothesis that transformed precursors, which are endowed with the cardinal properties of stem cells, may be the cellular origin of tumours (Sell 2004). If such cancer stem cells (CSCs) are one of the major culprits in tumour establishment and growth, conventional approaches, which target the heterogeneous body of cells in a neoplasia in a relatively non-specific fashion, may spare CSCs because of their peculiar properties (Reya et al. 2001).

The idea that CSCs may initiate tumours received its initial confirmation in the 1990s, based on studies of acute myeloid leukemia (Lapidot et al. 1994; Bonnet and Dick 1997), and has since been strengthened by findings related to breast cancer (Al-Hajj et al. 2003). A similar involvement of CSCs in brain cancer was also suggested by the fact that neural stem cells are nestin- and glial fibrillary acidic protein (GFAP)-positive precursors (Doetsch et al. 1999) and the discovery that, when targeted to nestin- or GFAP-positive cells, alterations of critical G1 arrest regulatory pathways cause the onset of high-grade gliomas (Holland et al. 1998). The ensuing view that CSCs underpin the development of brain cancer has been confirmed in the last years by their identification in tumours of the central nervous system (Ignatova et al. 2002; Singh et al. 2003, 2004; Hemmati et al. 2003; Galli et al. 2004; Yuan et al. 2004; Taylor et al. 2005).

Somehow similar to stem cells in normal neural tissue, CSCs in GBMs represent a small fraction of the total cell mass (Galli et al. 2004), belong to a $CD133^{+}$-positive precursor pool (Singh et al. 2004) and display the critical neural stem cell features of extensive self-renewal *in vitro*, generation of a large number of progeny and multi-lineage differentiation potential (Potten and Loeffler 1990; Weiss et al. 1996). Yet

they have the capacity to establish GBM tumours at the clonal level and to perpetuate across serial transplantation in immunodeficient mice and thus are defined as GBM tumour-initiating cells (Galli et al. 2004; Singh et al. 2004).

Bone morphogenetic proteins (BMPs) elicit a plethora of actions throughout development and adult life (Chen et al. 2004). These may include pro-survival, pro-apoptotic or pro-differentiation effects, depending on the cell type being examined (Shah et al. 1996; Liem et al. 1997; Sela-Donenfeld and Kalcheim 1999; Panchision et al. 2001; Graham et al. 1996; Furuta et al. 1997; Li et al. 1998; Hall and Miller 2004). Notably, in the adult brain, BMPs play an instructive role in the stem cell niche, where the interaction between these proteins and their inhibitor Noggin regulates the acquisition of an astroglial phenotype in the stem cell progeny (Lim et al. 2000; Panchision and McKay 2002). Given the involvement of stem-like neural precursors in brain cancers and the regulatory effect of BMPs on neural stem cells and their progeny, we sought to examine the role these molecules play in the development of solid central nervous system tumours, in particular of GBM multiforme.

We began by demonstrating the presence of BMP receptor (BMPR) transcripts and their cognate proteins in the CD133-immunoreactive ($CD133^+$) prospective CSCs from primary human GBM tissue and in the progeny that they generate upon mitogenic stimulation in culture (Galli et al. 2004; Singh et al. 2004). The data presented here concern cells isolated from five human GBM specimens, all yielding overlapping findings, cultured for less than five passages, unsorted or sorted with the CD133 antibody and cultured as described previously (Galli et al. 2004; Singh et al. 2004). In all cell preparations, we found transcripts for all three BMPR subtypes (BMPR1A, -1B, -2) in conjunction with other BMPs (not shown), amongst which BMP4 was most abundant (Fig. 1a). All of the cognate receptor proteins were present in primary and cultured GBM cells (Fig. 1b). Thus BMPRs and their ligand(s) can be found in primary and CSC-enriched, cultured GBM cells.

While transcript levels were low in comparison to normal human neural stem cells (not shown), BMPRs in cultured GBM cells were functional. In fact, addition of exogenous BMP4 triggered activation of the receptor-regulated Smads (Fig. 1h–j) pathway, with a two-fold

Fig. 1a–k. Expression and activation of BMP receptors in cells from adult human GBMs. **a** RT-PCR showing the presence of the mRNA transcripts for BMPR1A (top row) BMPR1B (second row from top) and BMPR2 receptors (third row from top) and BMP4 (lower row) in $CD133^+$ GBM cells, soon after surgical specimen dissociation (*lane 2*) and after culturing with mitogens (*lane 3*; cultures enriched in CSCs). *Lane 1*; negative control. *Lane 4* MCF7 cells (positive control). **b–g** Immunofluorescence showing BMPR1A (**b,c**), BMPR1B (**d,e**) and BMPR2 (**f,g**) receptor proteins in acutely dissociated (**b,d** and **f**) and mitogen-cultured GBM cells (**c,e** and **g**). **h–j** Progressive phosphorylation and nuclear translocation of receptor-activated Smad proteins (the antibody recognizes Smads 1, 5 and 8) upon addition of exogenous BMP4 to GBM cultures in the continuous presence of mitogens. **h**, 30 min; **i**, 1 h; **j**, 1.5 h. **c–j** Scale 15 μm, bar in **j**. **k** Cytofluorimetric analysis showing the phosphorylation and nuclear translocation of Smad 1,5,8 as elicited by exogenous BMP4 (green, 93.13±.95%) as compared to cells exposed to mitogens alone (red, 40.56±.08%, *p*lt;.01, *n*, two-tailed Student's *t*-test) in GBM cultures (1.5 h, *top panel*); no activation of the p38 MAPK pathway could be detected (*lower panel*) at different times (5 min to 2 h). Dotted line, isotype controls

increase in the levels of phosphorylated Smad 1, 5, 8 (Fig. 1k, top). However, the p38 MAPK signalling cascade did not appear to be activated (Fig. 1k, bottom).

Given the activation of its receptor signalling pathway, we next sought to determine the effect of adding exogenous BMP4 to GBM cells *in vitro*. Although BMPs elicit pro-apoptotic effects in medulloblastoma cell lines (Hallahan et al. 2003) and in other systems (Graham et al. 1996; Zuzarte-Luis and Hurle 2005; Hruska et al. 2005), flow cytometric analysis showed that neither cell viability nor apoptosis (Fig. 2a, top panels) was altered by BMP4. However, a reduction in the proliferation index was revealed by a significant decrease ($p<0.01$) in the number of cells expressing detectable levels of the cell cycle-related antigen Ki67 (Fig. 2a, lower panel). A similar, though reduced effect was significantly elicited by BMP2, -5, -6, -7, and -8b but not BMP1, -3 and -3b (not shown). The effect of BMP4 on cell proliferation was further confirmed by cell cycle analysis which revealed a significant ($p<0.05$) increase in

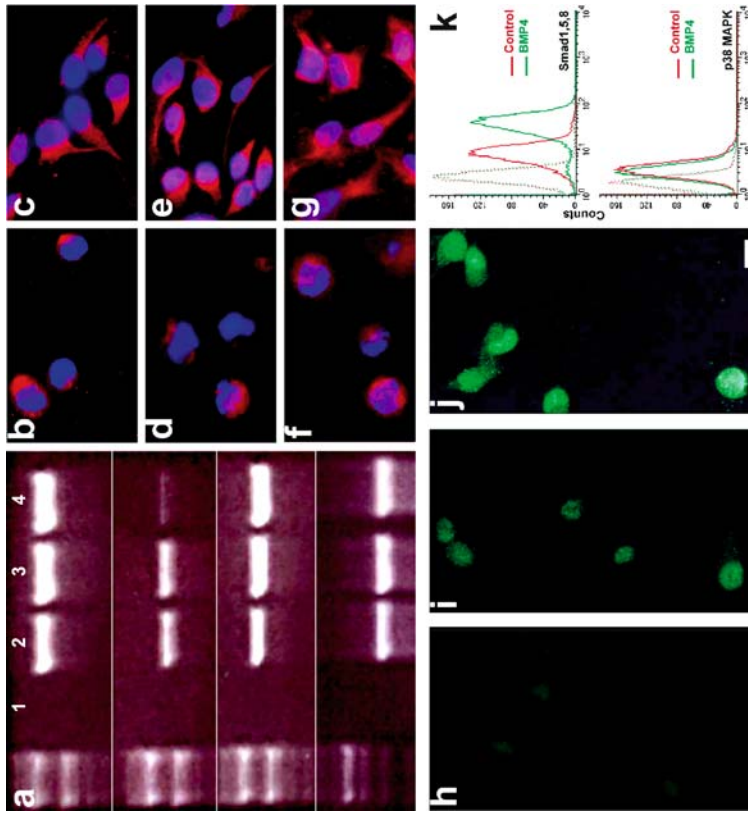
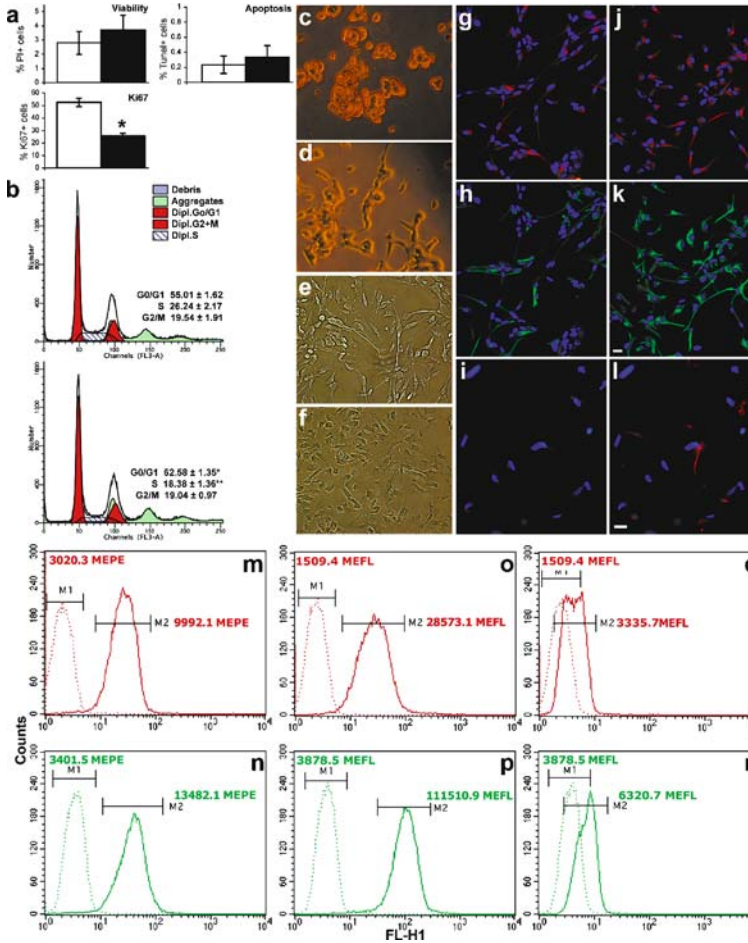


Fig. 2a–r. BMP4 elicits a pro-differentiation effect on cultured GBM cells. Data were obtained 48 h after addition of BMP4 to cultures containing mitogens, established from the $CD133^+$ GBM cell fraction. **a** Addition of BMP4 does not alter cell viability (*top left*) or apoptosis (*top right*) but considerably decreases cell proliferation as shown by Ki67 immunofluorescence assay (*lower panel*), (control, empty columns, BMP4, solid columns). BMP4 also causes a significant increase in the G_0/G_1 cell fraction and a concomitant significant decrease in the S phase fraction (**b**, *lower panel*) as compared to control cultures (**b**, *upper panel*, i.e. mitogens alone). **p*lt;.05; ***p*lt;.001, *n*=3, two-tailed Student's *t*-test. BMP4-treated vs. untreated cultures. GBM cells, which are proliferating in response to mitogens (**c**), become more differentiated (i.e. adherent and phase dark, **d**) upon exposure to BMP4 and display a much more complex, flat morphology upon plating onto Matrigel (**e**, control; **f**, BMP4). Consistent with the idea that BMP4 induces a more differentiated phenotype, increased expression of neuronal (**g**, βIII-tubulin), astroglial (**h**, GFAP) and oligodendroglial (**i**, galC) markers was already apparent 48 h after addition of BMP4 to proliferating GBM cells (**j**, βIII-tubulin; **k**, GFAP; **l**, galC). **c–k,** Scale 20 m, bar in **k**; **i** and **l**, scale 20 m, bar in **l**. Because of their aberrant nature, expression of mature neural markers was found to occur in most of the undifferentiated cells in GBM cultures, making detection of changes in expression of neuronal antigens difficult by direct counting of immunoreactive cells (**g–l**). Hence, a quantitative assay was performed by cytofluorimetric analysis, using calibration curves established by rainbow beads as a reference (Rainbow calibration particles 8 peaks, 3.0–3.4 m Technical Data Sheet, BD Biosciences). By this method, molecules of equivalent phycoerythrin (MEPE) or molecules of equivalent fluorescein (MEFL) provide a value of the intensity of the fluorescence signal for each antigen. *Dotted lines*, isotype control. *Red*, control GBM cultures; *green*, BMP4. BMP4 caused a 43% increase in the levels of βIII tubulin (**n**), 4.1-fold increase in GFAP expression (**p**) and 34% increase in galC levels (**r**) as compared to control cultures (**m**, βIII-tubulin; **o**, GFAP; **q**, galC). **m–r** Data from one representative experiment out of three experiments with triplicate samples giving similar results

the number of cells in G_0/G_1 and a corresponding decrease ($p<0.01$) in the number of cells in S phase as compared to controls (Fig. 2b).

The addition of BMP4 to actively proliferating GBM cells also caused morphological changes, which was more evident when they were plated on Matrigel-coated substrate (Fig. 2c–f). Furthermore, immuno-



fluorescence labelling showed that BMP4 significantly increased the expression of neuronal and glial differentiation antigens, particularly astroglia (Fig. 2j–l). This phenomenon was analysed quantitatively by flow cytometry, demonstrating a 43% increase in βIII-tubulin immunoreactivity (Fig. 2m, n), a 4.1-fold rise in GFAP immunoreactivity (Fig. 2o, p) and a 34% increase in GC immunoreactivity (Fig. 2q, r) in BMP4-treated cells. Together, these findings demonstrate that exposure of undifferentiated and actively proliferating GBM cell cultures to BMP4 causes the acquisition of a more mature phenotype, characterised by the loss of cell proliferation ability and increased expression of neural differentiation markers.

Human GBMs contain a population of $CD133^+$ CSCs which establish *bona fide* GBM-like tumours through intracerebral transplantation (Galli et al. 2004; Singh et al. 2004). As the CSC population is responsible for cell expansion in our GBM cultures (Galli et al. 2004), we hypothesised that this population may be one of the main targets for the BMP4 effects which we observed.

To test this hypothesis we exposed cells from GBMs to BMP4, both after isolation and upon culturing, and subjected them to a combined series of assays which provide an assessment of the CSC pool size. In particular, we studied the effects of BMP4 on (a) the clonogenic index, which provides an estimation of the stem cell population residing in cell cultures, (b) the CSC frequency as derived from cell growth kinetics (Gritti et al. 2002; Galli et al. 2002; Reynolds and Rietze 2005), (c) the size of the $CD133^+$ population embodying the CSC pool (Singh et al. 2004) and (d) the magnitude of the side population, which is highly enriched for CSCs (Kondo et al. 2004; Patrawala et al. 2005).

A 48-h exposure to BMP4 produced a five- ($p<0.05$) or ten-fold ($p<0.05$) reduction in the clonogenic frequency of $CD133^+$ enriched cells from either freshly isolated or cultured GBM cells, respectively (Fig. 3a). This was indicative of a decrease of the CSC frequency and was confirmed by data on growth kinetics. We have previously shown that the slope of the growth curve provides a meaningful estimate of stem cell frequency under the same conditions used here (Galli et al. 2002; Reynolds and Rietze 2005). BMP4 caused a significant ($p<0.001$) reduction in the slope of the growth curve for proliferating GBM cells (Fig. 3b) and abolished the expansion capacity of freshly isolated GBM

cells (Fig. 3b). A similar effect was observed on normal human foetal neural stem cells, while U87 human glioma cell lines (which do not bear BMPRs; Fig. 1a) were unaffected by BMP4. Accordingly, BMP4 produced a three-fold reduction in the size of the $CD133^+$ population (Fig. 3c) and a nearly complete depletion of the SP pool (Fig. 3d)—populations that are both highly enriched in CSCs (Kondo et al. 2004; Singh et al. 2004; Patrawala et al. 2005). These observations suggest that BMP4 targets the CSC pool in human GBMs, causing a notable reduction in its proliferation kinetics and overall size.

We next sought to test the hypothesis that depleting the CSC population *in vitro* would cause a corresponding reduction in the tumour-initiating ability of GBM cells. We exposed our cultured GBM cells to BMP4 for 48 h prior to unilateral, intracranial injection of 3×10^5 viable cells into the right striatum of immunodeficient *scid* mice. Animals were assessed for tumour formation and expansion by histological and immunohistochemical analyses and compared to control animals receiving GBM cells from untreated cultures. Five weeks after the implantation, all animals receiving untreated GBM cells developed well-established tumour masses on the injected side (haematoxylin and eosin in Fig. 4). As shown previously (Galli et al. 2004), these masses demonstrated characteristic glioblastoma features, including marked nuclear atypia, expression of aberrant glial elements, extensive neovascularisation, high mitotic activity and infiltration capability of the brain parenchyma (Fig. 4d, j). Tumour development was such that, at times, cells invaded the adjacent lateral ventricle (Fig. 4k, m) and the third and fourth ventricles (not shown). Conversely, cells treated for 48h with BMP4 did not form invasive tumours after injection (Fig. 4). Rather, small, delimited lesions developed, which were confined to the site of injection (haematoxylin and eosin in Fig. 4b), had a low mitotic index (Fig. 4d, k) and showed no ventricular invasion (Fig. 4l, n, p). Three months post-injection, all control animals had died, whereas all animals receiving BMP4 pre-treated cells survived for at least 5 months and displayed no overt signs of behavioural abnormalities (data not shown). Finally, while we could re-derive tumour-initiating GBM cells throughout serial transplantation from the brains of mice receiving untreated cells (Galli et al. 2004), this was never possible when using animals transplanted with BMP4-treated cells (data not included). Altogether,

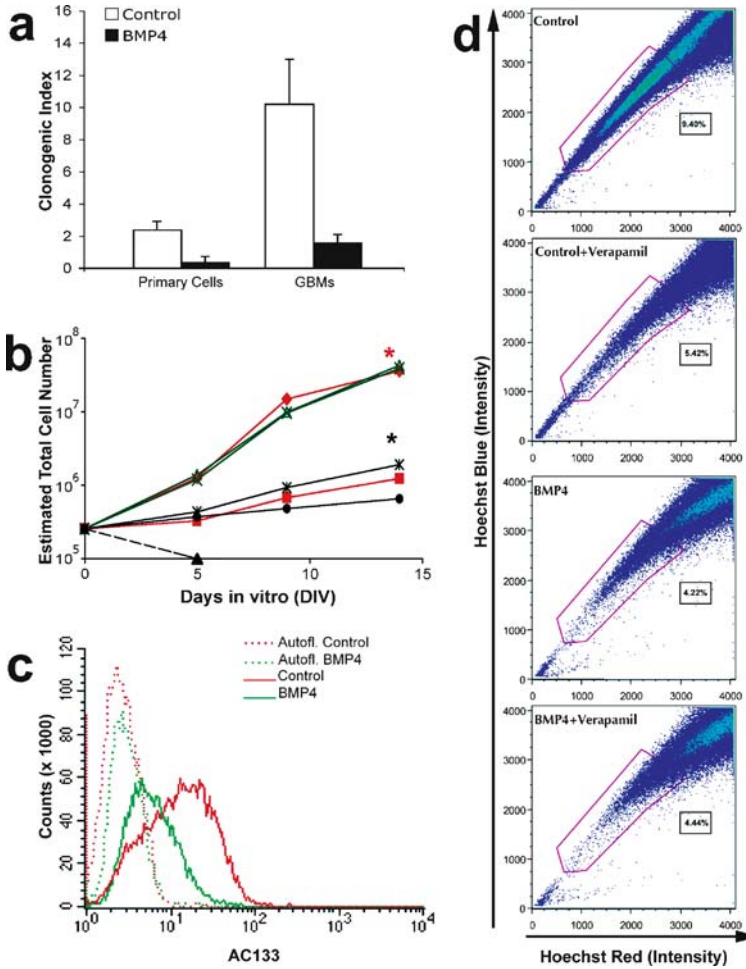


Fig. 3a–d. BMP4 depletes the self-renewing CSC population in GBM cultures. **a** Addition of BMP4 to $CD133^+$ cells obtained from freshly isolated GBMs tumours (primary cells) reduced the formation of clones in response to growth factors by four-fold (2.4±.5 control, empty column; 0.4±.2 BMP4, solid column; *p*lt;.05, *n*, two-tailed Student's *t*-test). An even greater effect (ten-fold decrease in clonogenic index) was seen when BMP4 was added to cultured GBM cells (10.2±.7 control, 1.6±.5 BMP4, *p*lt;.05, *n*=3, two-tailed Student's *t*-test). **b** Accordingly, when GBM cells were propagated *in vitro* with the Neurosphere Assay system–in which the slope of the growth curve depends on the stem cell pool expansion at each passage–cells from primary GBM tissue could not even be serially subcultured if BMP4 was added with mitogens (*black dotted line*, *triangles*). The addition of BMP to those GBM cells that were already expanding in the sole presence of mitogens (*red rhombuses*) resulted in a significant reduction in the slope of the growth curve (*red squares*). While a similar phenomenon was observed in cultured normal human foetal neural stem cells (*black stars*, control versus *black circles*, BMP4), BMP4 was ineffective on U87 human glioma cell lines (*green triangles*, control versus *green stars*, BMP4). * *p*lt;.01 BMP4 versus control in matching cell types. **c** The size of the $CD133^+$ GBM cell population–previously shown to comprise CSCs (Singh et al. 2004)–was reduced by two-thirds after addition of BMP4 to the growth medium (57.62±.34% vs. 18.34±.90% of the total population; *p*lt;.005, *n*=3, two-tailed Student's *t*-test). **d** Cytofluorimetric analysis in GBM cultures enriched for CSCs (Singh et al. 2004) showed that the side cell population–whose size is determined by subtracting the Hoechst 33342 labelling values (*boxed numbers*; percentage of the total cell number) obtained by incubating cells in the absence or presence of 50 M verapamil–in control cells (*first and second panels from top*) was depleted after a 24-h treatment with BMP4 (*third and fourth panels from top*)

these findings demonstrate that, for human GBMs, reduction of the CSC population *in vitro*, with a brief exposure to BMP4, produces a marked decrease in GBM's tumour-initiating ability *in vivo*.

The observation that BMP4 inhibits the proliferation and expansion of CSCs and the fact that these cells are seen as a likely culprit in GBM development beg the following question: Can direct delivery of BMP4 prevent tumour establishment and growth within the brain? To address this, we transplanted control GBM cells from our cultures into

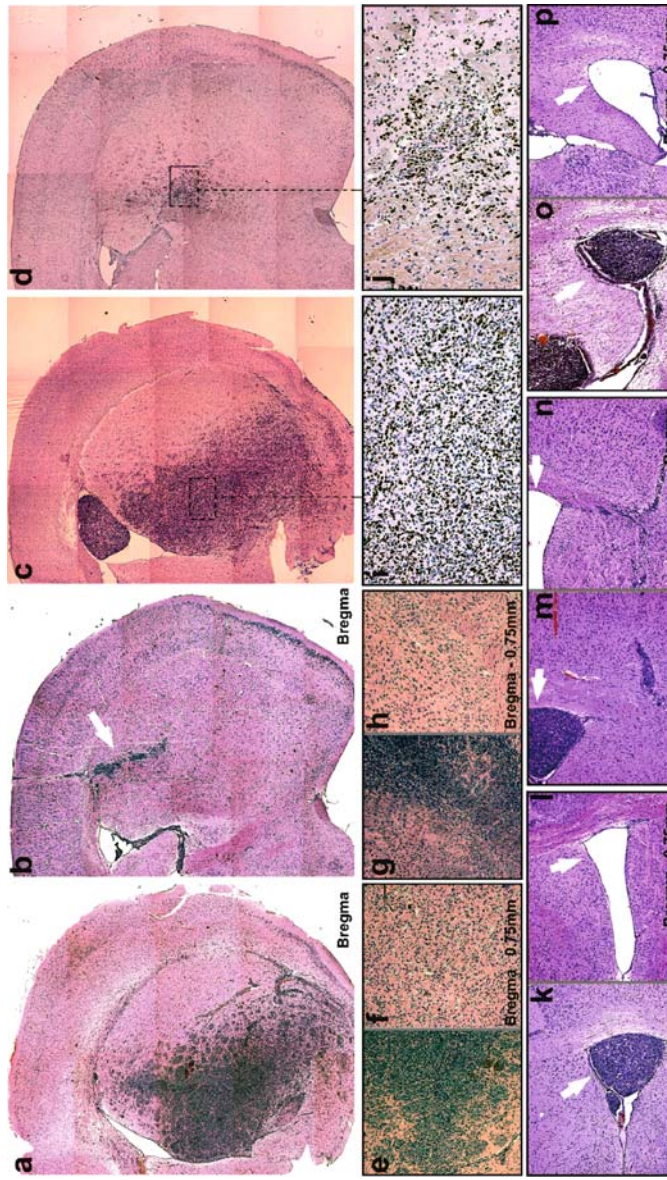
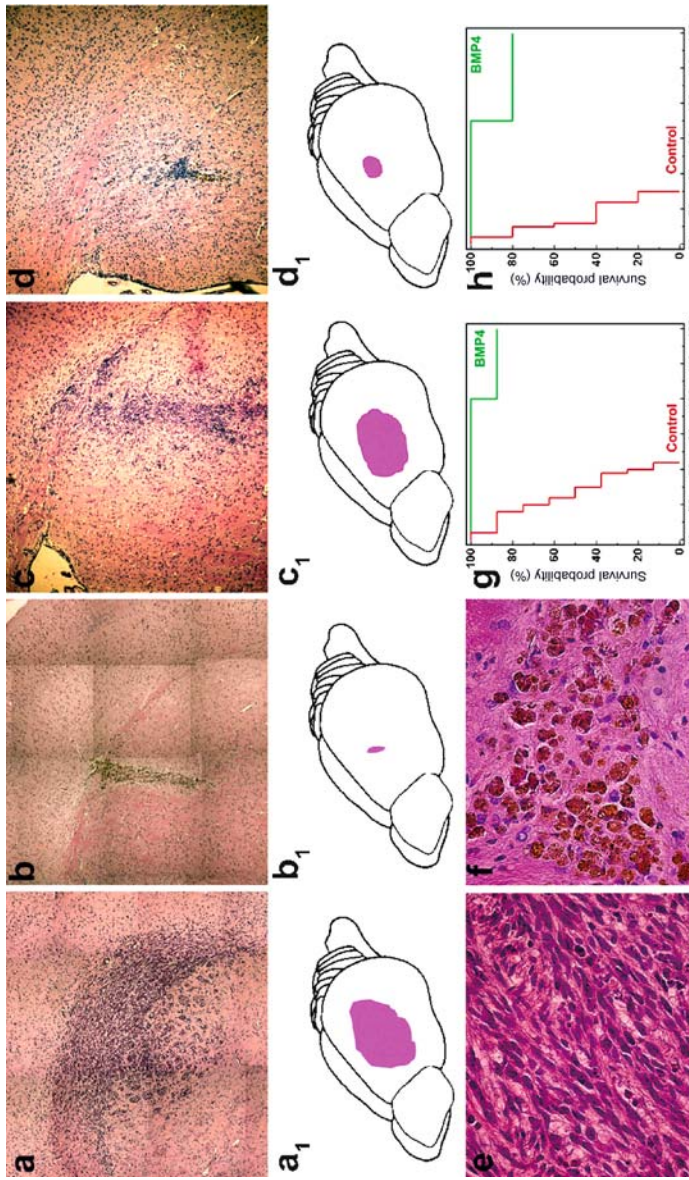


Fig. 4. a–p BMP4 inhibits the tumorigenicity of GBM cells. Transient exposure to BMP4 *in vitro* decreases the size of the CSC population in cells from human GBMs. Since these are tumour-founding cells, we investigated whether this resulted in a reduced ability of BMP4-treated cells to establish GBMs in the mouse brain. GBM cultures were exposed to BMP4 for 48h (while in the continuous presence of growth factors) prior to monolateral injection into the right nucleus striatum of immunodeficient mice (300,000 viable cells/animal). Animals (*n*) were sacrificed 5 weeks post-injection and compared to mice receiving cells expanded under standard conditions (control). Haematoxylin and eosin staining showed typical glioblastoma masses after injection of control cells (**a**, **e**, **g**), whereas BMP4-treated cells generated very small tumours (**b**, *arrow*). Tumours established from control cells displayed a much higher mitotic index as shown by immunocytochemistry for Ki67 (**c**, high power in **i**) than BMP4-treated cells (**d**, high power in **j**) (control 4.28±.28%, BMP4 0.76±.48%; *p*lt;.05, Student's *t*-test, *n*). **k–p** Haematoxylin and eosin staining showing invasion of the lumen of the right lateral ventricle at various antero-posterior levels from the bregma point (**k**,**m**,**o,** *arrows*) at later stages after implantation of control cells. This phenomenon was never observed with BMP4-pretreated GBM cells (**l**,**n**,**p,** *arrows*). Magnification: **a–h** and **k–p**, ×5; **i** and **j**, 10×

the lateral striatum of scid mice, which were also implanted with BMP4-saturated or control (incubated with the vehicle buffer) polyacrylic beads, at the same site. These beads can release BMP4 for over 1 week (S.G.M. Piccirillo, unpublished) and were implanted either at the time of cell transplantation (co-treatment) or, alternatively, at a time when a sizeable tumour had already developed, i.e. 10 days later after injection of 3×10^5 viable cells (post-treatment). In both experimental settings, animals receiving control beads developed large, malignant tumours (Fig. 5a, c, e), causing overt behavioural abnormalities (ptosis, hindlimb hunching, arched back and ipsilateral turning), and soon died (Fig. 5g, h), whereas mice implanted with BMP4-releasing beads displayed small, confined lesions (Fig. 5b, d, f), and survived significantly longer (Fig. 5g, h). They never showed signs of behavioural abnormality. Thus BMP4 inhibits the growth of brain tumours generated by human GBM CSCs *in vivo*.

Fig. 5a–h. BMP4 inhibits the intracerebral growth of tumors established by GBM cells. Cells isolated from human GBM specimens were enriched in CSCs as described previously (Singh et al. 2004) and injected into the right nucleus striatum of immunodeficient mice (300,000 viable cells/animal). In the first set of experiments, animals also received a co-injection of polyacrylic beads, either pre-adsorbed with PBS alone (**a**, control; *n*4) or containing BMP4 (**b**, BMP4; *n*4). Four weeks post-injection, control animals showed well-developed, typical GBM-like masses, which infiltrated the corpus callosum (**a**), whereas those receiving BMP4-infused beads displayed extremely small lesions restricted to the injection site (**b**, *arrow*). Maximum average extension in control mice was coronal 3.00 ± 0.31 mm; antero-posterior 2.06 ± 0.47 mm, which was significantly larger than in BMP4-treated animals: coronal 0.39 ± 0.05 mm, antero-posterior 0.29 ± 0.07 mm (*n*, *p*lt;.01, two-tailed Student's *t*-test). Alternatively, GBM cells were transplanted and allowed to establish the tumour for 10 days. Then, either control or BMP4-infused beads were injected in the proximity of the transplantation site. Histological analysis 20 days later revealed that, while tumours had expanded considerably in control animals (*n*) (**c**), their growth had been inhibited in mice receiving BMP4-beads (*n*) (**d**). Maximum average extension in control mice was coronal 2.50 ± 0.24 mm; antero-posterior 1.76 ± 0.36 mm. This was significantly larger than in BMP4-treated animals: coronal 0.48 ± 0.05 mm, antero-posterior 0.24 ± 0.06 mm (n, $p < 0.01$, two-tailed Student's *t*-test). **e–h** depict the approximate tumour mass based on histological reconstruction for control (**e**) and BMP4-treated animals (**f**) co-transplanted with the beads or receiving control (**g**) or BMP4-infused beads (**h**) 10 days after cell implantation. In both experiments, tumours in control mice were composed of pleiomorphic, highly neoplastic elements, with reactive chromatin and embodied highly malignant, infiltrating cells (**i**). Conversely, BMP4-treated animals displayed lesions nearly devoid of neoplastic cells and contained highly differentiated elements and numerous macrophages (**j**). The mitotic index was always significantly higher in controls with respect to BMP4-treated animals in co-transplantation experiments (3.68 ± 0.17 versus 0.20 ± 0.11, n, $p < 0.01$, two-tailed Student's *t*-test) and after delayed bead injection. Survival of BMP4-treated animals was dramatically enhanced either when beads were co-injected with GBM cells (**g**) or after the 10-day-delayed bead injection (**h**)

GBM is the most common adult malignant brain tumour, with a median survival time of 9–12 months. Survival rates have changed very little in over 30 years and conventional therapy (such as chemother-



apy and radiotherapy) provides only short-term benefits. After these treatments, recurrences are very common (Barker et al. 1998; Castro et al. 2003). The discovery of CSCs in brain tumours changes our understanding of cancer initiation and development, suggesting that our failure to eradicate neoplasias such as glioblastomas may hinge on the misidentification of the cell type(s) that we select as the primary target for therapy. CSCs possess many of the properties of normal stem cells, such as longevity, self-renewal, ability to generate large number of progeny, infrequent cycling nature *in vivo* (Potten and Loeffler 1990) and high expression levels of the drug-resistance ATP-binding cassette (ABC) transporter proteins (Zhou et al. 2001). These features, which are also present in a small subpopulation of GBM-derived cells (Reynolds, unpublished observations; Reya et al. 2001; Dean et al. 2005), may render them resistant to standard cancer therapies and explain their ability to re-establish tumour masses when treatment is suspended (Reya et al. 2001; Pardal et al. 2003). This emphasizes the need for discovering shared regulatory mechanisms between normal neural stem cells and CSCs, which may identify novel therapeutic targets in GBMs.

Here we show that BMP4 influences the fate of adult human GBM cells through a pro-differentiation action, depletes the GBM CSC pool and dramatically inhibits the tumorigenicity of GBM cells. Altogether, these findings demonstrate that BMP4 is a key inhibitor of the CSC population in human GBM cells.

Several mechanisms may explain this phenomenon. For example, one can envision that BMP4 may reduce the frequency of CSCs by decreasing the probability of symmetric stem cell divisions that generate two daughter CSCs. Alternatively, BMP4 may induce the differentiation of a subpopulation of CSCs, thereby forcing acquisition of a more mature phenotype, or simply block the proliferation of the CSCs and their progeny. Either of these scenarios, while not mutually exclusive, would effectively diminish the CSC population.

The results of this study also illustrate that a critical regulatory pathway which controls the fate of normal neural stem cells (Lim et al. 2000; Rajan et al. 2003; Gross et al. 1996) also operates in cancer-initiating stem-like cells from human GBMs. This supports the concept that uncovering the mechanisms underlying basic stem cell biology will help us understand the contribution of stem-like cells to tumour forma-

tion, expansion and invasion. It also unveils a previously undocumented role for BMPs in tumour biology, in their ability to promote differentiation of GBM cells without killing them and to deplete the pool of cancer stem cells residing in human GBMs. Furthermore, BMPs, their cognate receptors and their associated intracellular signalling transduction mechanisms, such as the Smad pathway, may be promising targets for therapeutic development aimed at the cells responsible for the GBM tumour establishment and expansion.

The implications of this phenomenon may be of clinical relevance. The ability of *in vivo* delivered BMP4 to effectively block tumour formation when co-implanted and in pre-established tumours raises the potential to do so in patients after surgical de-bulking of the tumour mass. Furthermore, these experiments support a novel approach to tumour treatment that could be summarised in two concepts: targeting of the tumour-initiating pool residing in human GBM (specificity of treatment) and inhibition of their proliferation-promoting differentiation without killing them (strategy of treatment). This approach could also be used in combination with conventional and radiation therapy to increase the probability of a favourable clinical outcome. Thus there is great hope that the delivery of a pro-differentiating, non-cytotoxic agent such as BMPs or BMP-mimetic drugs may limit the frequency and size of lethal GBM recurrences in human patients.

1 Materials and Methods

1.1 Samples

The data presented in this work are from cells isolated from five different adult human GBM specimens, obtained from standard operating surgical procedures for tumour removal and classified according to the guidelines of the World Health Organisation.

1.2 Primary Culture, Culture Propagation, Cloning and Cell Line Establishment

GBM cells were obtained by processing tumour samples as described by Galli et al. 2004. Primary cells were sorted for their immunoreac-

tivity to CD133 (see below) and plated in 25cm^2 tissue culture flasks at a final density of 2,500 cells/cm^2 in NeuroCult® NS-A serum-free medium (Stem Cell Technologies) containing 2 mM L-glutamine, 0.6% glucose, 9.6 g/ml putrescine, 6.3 ng/ml progesterone, 5.2 ng/ml sodium selenite, 0.025 mg/ml insulin and 0.1 mg/ml transferrin in the presence of 20 ng/ml of both EGF and FGF2 (control medium). Culture propagation, clonogenic assay and population analysis were performed with the same conditions described previously (Galli et al. 2002).

1.3 Immunocytochemistry

GBM cells were plated at a density of 2.5x10^4 cells/cm^2 onto Matrigel-coated glass coverslips (12mm diameter) in the continuous presence of FGF2/EGF and treated with BMP4 (100 ng/ml) for 48h. After treatment, cells were washed and fixed in 4% paraformaldehyde for 10 min. Multiple immunofluorescence assays for neural antigens (GFAP from Dako Corporation, Tuj1 from Babco, Galc from Chemicon) were performed as described by (Galli et al. 2004). Ki67 staining (1:1,000, Novo-Castra, Newcastle, UK) was used to detect actively proliferating cells.

After fixation in 4% paraformaldehyde, immunostaining for BMPR-1A, -1B and -2 was carried out according to the manufacturer's instructions (1:50, R&D Systems). When staining for phospho-Smad 1 (1:100, Cell Signaling, Beverly, MA) cells were treated with BMP4 (100 ng/ml) at different times (from 5 min to 2 h). Appropriate isotypic or negative controls were always included throughout these procedures. Apoptotic cells were detected with a digoxigenin-based modification of the original TUNEL method introduced by (Gavrieli et al. 1992), using the fluorescein-dUTP TUNEL assay (*In Situ* Cell Death Detection Kit, Fluorescein, Roche Applied Science). Briefly, cells grown on 12mm coverslips were fixed in 4% paraformaldehyde for 10 min at room temperature and then rinsed in PBS. Cells were then permeabilised for 2 min on ice before labelling with 50 µl of TUNEL reaction mixture and incubating at 37°C for 1 h in a humidified chamber under parafilm coverslips. After washing with PBS, slides were mounted in DAPI-containing Vectashield^{TM} and examined by fluorescence microscopy. For propidium iodide (PI) staining, cells were fixed in 4% paraformaldehyde for 10 min at room temperature, rinsed in PBS and incubated with

PI (1 μg/ml) for 5 min at room temperature. After washing, coverslips were mounted in DAPI-containing Vectashield™ and analysed by fluorescence microscopy. PI exclusion denoted viable cells. For all of the above assays, samples were run in six replicates for each condition tested.

1.4 Conventional and Real-Time PCR

Total RNA was isolated from GBM cells and from primary cells using TRIzol reagent (Life Technologies, Rockville, MD), and reverse-transcribed using SuperScript RNAse H⁻ Reverse Transcriptase (Life Technologies). The amounts of cDNA used as templates in the real-time PCR (RT-PCR) reaction were normalised with reference to glyceraldehyde-3-phosphate dehydrogenase (GAPDH). MCF-7 cell lines were used as positive controls for BMPRs. PCR products were visualised by electrophoresis in agarose (1%) gels stained with ethidium bromide.

Quantitative RT-PCR reactions were run in triplicate with the Brilliant® SYBR® Green QPCR Core Reagent Kit (Stratagene, La Jolla, CA). SYBR Green dye binds to any PCR product, and therefore does not require the use of sequence-specific probes. Fluorescent emission was recorded in real time (Chromo 4 Four-Color Real-Time PCR Detector**,** MJ Research, Bio-Rad). Gene expression profiling was completed with the comparative C_t method of relative quantification. Relative RNA quantities were normalised to two endogenous controls, GAPDH and 18S ribosomal RNA (18S rRNA). Each replicate was normalised and the average relative quantity (RQ) is reported for each gene. The mean fold changes were calculated together with the standard deviation and 95% confidence intervals of the three replicates.

1.5 Flow Cytometry Analysis

For cell cycle analysis, 1 million cells/sample were treated with BMP4 (100 ng/ml) for the indicated time. GBM cells were then resuspended in equal volumes of ice-cold PBS and 100% ethanol and incubated on ice for 30 min. After centrifugation, the cell pellet was washed three times with PBS and centrifuged for 5 min. Cells were then incubated overnight in the dark in 1 ml of PBS containing RNAse (12.5 μg /ml;

Sigma) and propidium iodide (3 μg/ml; Sigma) and analysed by flow cytometry.

1.6 Evaluation of Tumorigenicity by Orthotopic Injection and Immunohistochemistry

Tumorigenicity was determined by orthotopic transplantation of GBM cells either grown under control conditions or exposed for 48 h to the same conditions but with the addition of 100 ng/ml of BMP4. Prior to transplantation, cells were rinsed with PBS and resuspended in PBS to yield a final concentration of 10^8 cells/ml. Three microliters of this suspension were injected stereotactically into the right striatum of immunosuppressed mice, using the same coordinates and procedures described previously (Galli et al. 2004). Haematoxylin and eosin staining, as well as immunohistochemistry, were performed on paraffin-embedded, 4μm-thick microtome sections. Sections were processed as described previously (Vescovi et al. 1999; Galli et al. 2004).

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Strategies to Induce Nuclear Reprogramming

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Abstract. The cloning of mammals from adult donor cells has demonstrated that the oocyte can reprogram a differentiated nucleus into a pluripotent embryonic state. Reprogramming of committed cells into pluripotent cells can also be achieved by the explantation of germ line cells and by the fusion of differentiated cells with embryonic cells. The future challenge will be to stably convert a differentiated cell into embryonic stem (ES) cells by the transient expression of defined genes. Recent findings suggest that the exposure of adult cells to a few defined factors can indeed induce a pluripotent-like state resembling that of ES cells. This approach may allow for the generation of patient-specific stem cells in order to study and treat degenerative diseases without recourse to nuclear transfer.

1 Introduction

Cells of a multicellular organism are genetically homogeneous but functionally heterogeneous because of the differential expression of genes, which is the result of reversible epigenetic changes that are gradually imposed on the genome during development (Wakayama et al. 1998; Wilmut et al. 1997). The reversal of the differentiation state of a mature cell to one that is characteristic of the undifferentiated, embryonic state is defined here as nuclear "reprogramming". Reprogramming by nuclear transfer (NT) has been a unique tool for functionally testing nuclear potency and for distinguishing between genetic and epigenetic alterations of various donor cells (Eggan et al. 2004; Hochedlinger et al. 2004; Hochedlinger and Jaenisch 2002; Li et al. 2003, 2004). The successful treatment of animal models of disease by nuclear transfer-derived embryonic stem (NT ES) cells (Rideout et al. 2002) and the prospect of deriving patient-specific human embryonic stem (ES) cells by nuclear cloning have underscored the potential use of this technology for custom-tailored cell therapy. However, while nuclear transfer remains the tool of choice for studying reprogramming at a functional level, alternative, more amenable approaches are needed for dissecting reprogramming at the cellular, molecular, and biochemical levels.

It is the aim of this manuscript to discuss the different strategies that have been employed to induce the conversion of a differentiated cell into an embryonic, pluripotent state, including nuclear transfer, cell-cell fusion, culture-induced reprogramming, and the exposure of somatic cells to defined factors (Fig. 1). We critically discuss the criteria for assessing reprogramming at the functional and molecular levels with the different approaches and outline future challenges in the field.

2 Reprogramming by Nuclear Transfer

Most adult tissues contain a heterogeneous population of cells with a hierarchy of multipotent stem cells, progenitor cells, and terminally differentiated cells. When Dolly and other mammals were initially cloned from adult cells, the question of whether terminally differentiated cells are genetically totipotent remained. This was mainly due to the lack of genetic markers that could unambiguously prove the differ-

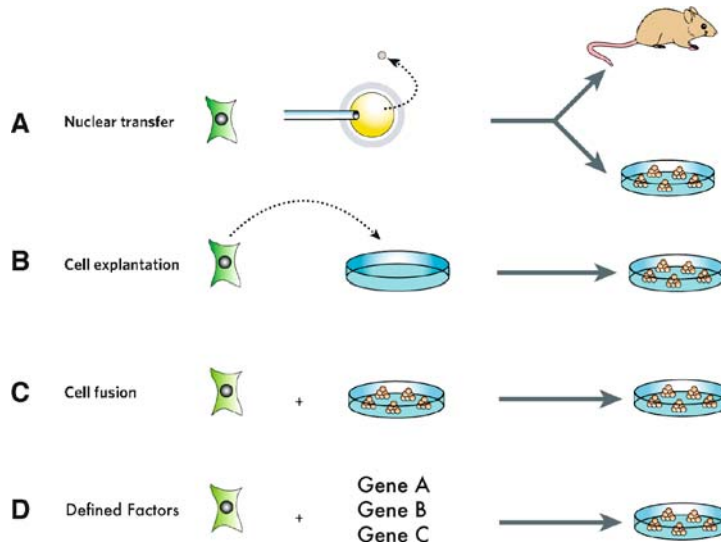

Fig. 1A–D. Illustration of four major strategies used for studying nuclear reprogramming. **A** Nuclear transfer involves the injection of a somatic nucleus into an enucleated oocyte, which upon transfer into a female recipient can give rise to a clone (reproductive cloning) or upon explantation into culture can give rise to ES cells (therapeutic cloning). **B** Explantation of germ line cells into culture selects for immortal cell lines that have regained pluripotency. **C** Cell fusion of differentiated cells with pluripotent ES cells results in the generation of hybrids, which show all features of pluripotent ES cells. **D** Introduction of defined genes into differentiated cells can give rise to ES-like cells. (Modified from Hochedlinger and Jaenisch 2006)

entiation state of the donor cell. The cloning of mice from mature lymphocytes that carried differentiation-associated immune receptor rearrangements (Hochedlinger and Jaenisch 2002) and from genetically labeled, postmitotic olfactory neurons (Eggan et al. 2004; Li et al. 2004) demonstrated that terminal differentiation does not restrict the potential of a nucleus to support the development of an animal. In other words, the epigenetic changes that direct terminal differentiation and permanent exit from the cell cycle are reversible.

The generation of animals by nuclear transplantation is extremely inefficient, with most clones dying soon after implantation and the few clones surviving beyond birth often being afflicted with severe abnormalities such as obesity (Tamashiro et al. 2002) and premature death (Ogonuki et al. 2002). Early experiments with amphibians demonstrated an inverse relationship between the age of the donor cell and clone survival (Briggs and King 1957). An important question has been, therefore, whether the state of donor cell differentiation affects the efficiency of reprogramming in mammals. Previous cloning experiments with blastomeres of the cleavage-stage embryo suggested that pluripotent nuclei support clone development at high efficiency (Cheong et al. 1993; Hiiragi and Solter 2005). Similarly, the cloning of mice from pluripotent ES cells (Eggan et al. 2001; Rideout et al. 2000) has been shown to be more efficient compared with adult cells such as fibroblast (Wakayama and Yanagimachi 1999), cumulus (Wakayama et al. 1998), or Sertoli cells (Ogura et al., 2000). Moreover, the derivation of ES cells from cloned blastocysts is significantly more efficient than the generation of mice from cloned blastocysts transferred into the uterus. Explantation of cloned blastocysts in culture may be, unlike fetal development, under fewer time constraints and may select for the outgrowth of rare, reprogrammed cells into stable ES cell lines, thus resulting in an apparently higher reprogramming efficiency. Alternatively, reprogramming may not be restricted to the oocyte stage but may continue in the inner cell mass of the blastocyst and hence give rise to NT ES cells at high efficiency. This observation may also explain why cloned mice from terminally differentiated neurons could only be generated with a "2-step" procedure involving first the derivation of NT ES cells, followed by the subsequent generation of cloned mice from NT ES cell nuclei by a second round of nuclear transplantation (Eggan et al. 2004). The finding that mature natural killer T cells can give rise to cloned mice by a single round of nuclear transfer (Inoue et al. 2005) argues, however, that some terminally differentiated cells can generate clones after direct implantation of clones into the uterus. Together, these results suggest (a) that mammalian nuclei, as has been observed in amphibians (Briggs and King 1957), become more refractory to reprogramming with differentiation, (b) that blastocyst formation and ES cell derivation, in contrast to fetal development, are less restrained by genetic and epigenetic

abnormalities, and (c) that ES cell derivation from cloned blastocysts is significantly more efficient than the potential of cloned blastocysts to grow into live pups.

The notion that reproductive cloning fails to fully reprogram a somatic nucleus raises the biologically and therapeutically relevant question of whether reprogramming is ever complete in ES cells derived from cloned blastocysts. Three lines of evidence suggest that this is indeed the case. First, NT ES cell lines, once established, grow as immortal cell lines and produce pluripotent tumors when injected into immunocompromised mice (Munsie et al. 2000). Second, analyses of the developmental potential of ES cells by tetraploid embryo complementation indicate that NT ES cells can give rise to entirely ES cell-derived, normal-appearing mice at an expected frequency (Eggan et al. 2004; Hochedlinger and Jaenisch 2002; Li et al. 2004; Rideout et al. 2002). Third, global gene expression profiling of NT ES cell lines derived from different donor cell types reveals transcriptomes that are indistinguishable from those of fertilization-derived ES cell lines (Brambrink et al. 2006). The process of ES cell derivation seems to rigorously select for immortal cells that have undergone or continue to undergo complete reprogramming to pluripotency, and therefore, ES cell lines derived by NT are expected to have the same therapeutic potential as ES cell lines derived from fertilized embryos.

3 Reprogramming by Cell Fusion

Cell fusion between different cell types has been used to study the plasticity of the differentiated state (Blau and Blakely 1999) (Fig. 1). In most hybrids the phenotype of the less differentiated is dominant over the phenotype of the more differentiated fusion partner. Consistent with this, Miller and Ruddle showed in 1976 that the fusion of pluripotent teratocarcinoma cells with primary thymocytes resulted in the formation of pluripotent hybrids that share all features with the parental EC cells, including their potential to induce tumors (Miller and Ruddle 1976). The dominance of pluripotent cells over differentiated cells has also been shown in cell hybrids made between somatic cells and murine EG (Tada et al. 1997, 2003) and ES (Tada et al. 2001, 2003) cells, and

this reprogramming potential seems to be conserved in human ES cells (Cowan et al. 2005; Yu et al. 2005).

A crucial question raised by these experiments was whether the chromosomes of the somatic cell had indeed been reprogrammed to pluripotency or whether they were simply retained as silent cargo. At the molecular level, the reactivation of the silent X chromosome in female lymphocyte-ES cell hybrids (Tada et al. 2001), the demethylation and reactivation of genes essential for pluripotency (Cowan et al. 2005; Tada et al. 2001), and the expression of genes representative for all three germ layers in teratomas produced from hybrids (Tada et al. 2003) supported the interpretation that the somatic chromosomes had undergone epigenetic reprogramming. To test reprogramming at a functional level, F9 EC cells that can normally only produce undifferentiated tumors were fused with thymocytes to score for an increase in the differentiation potency of tumors. The majority of hybrid cells gave rise to well-differentiated tumors, consistent with the notion that the thymocyte genome had been functionally reprogrammed to pluripotency by the EC cell (Rousset et al. 1983). However, independent fusion experiments between EC cells and differentiated cells came to the opposite conclusion (Oshima et al. 1981) and suggested that the differentiated phenotype of tumors may have been due to the loss or dilution of an amplified gene that blocked differentiation in EC cells, rather than the reprogramming of the somatic genome. To address the question of whether the ES cell genome is continuously required for maintaining pluripotency of hybrids, Matsumura and colleagues (Matsumura et al. 2006) attempted to selectively eliminate both ES cell copies of chromosome 6, which harbors the essential pluripotency gene Nanog, from hybrid cells. Surprisingly, while hybrid cells remained pluripotent, they became consistently trisomic for the somatic chromosome 6, suggesting either a dosage effect or incomplete reprogramming of one or more somatic loci.

A key question arising from fusion experiments is whether the ES cell nucleus or cytoplasm is required. Fusion experiments between neuronal cells isolated from neurospheres and the nuclear compartment (karyoblast) or the cytoplasmic compartment (cytoblast) from ES cells indicated that nuclear factors are essential for molecular reprogramming (Byrne et al. 2003; Do and Scholer 2004). In agreement, the fusion of

somatic cells with ES cells that are in the G_2/M phase of the cell cycle, where nuclear factors are present in the cytoplasm, results in an increased fusion efficiency (Sullivan et al. 2006). This observation is consistent with cloning experiments in amphibians and mice, which indicate that successful reprogramming depends on direct injection of nuclei into the germinal vesicle or into a metaphase oocyte.

Fusion experiments indicated that certain factors that are expressed in ES cells must contain reprogramming activity. Possible candidates for this activity may include pluripotency genes that are crucial for maintaining ES cells in an undifferentiated state. Indeed, overexpression of Nanog in ES cells results in an up to 200-fold increase in the number of hybrid colonies after fusion with neural stem cells (Silva et al. 2006). Interestingly, the effect was not so strong when fibroblasts were used as fusion partners, or when Nanog was ectopically expressed in the somatic cell partner before fusion, suggesting that the differentiation state and cellular context are critical for Nanog's effect on reprogramming. Alternatively, ectopic Nanog expression in ES cells may select for a rare subpopulation of cells that is more competent for reprogramming. Evidence in human ES cells suggests that cell lines are indeed heterogeneous (Stewart et al. 2006).

4 Culture-Induced Reprogramming

The approaches discussed so far require the exposure of somatic nuclei to nuclear/cytoplasmic factors of an oocyte or ES cell to elicit nuclear reprogramming. However, under certain physiological conditions, entire cells can dedifferentiate or transdifferentiate into another cell fate (Fig. 1).

Teratocarcinoma cells were the first pluripotent cells discovered in adult mammals (Andrews 2002). Teratocarcinomas represent a class of germ cell tumors that are composed of a rare population of undifferentiated embryonic cells called embryonal carcinoma (EC) cells as well as a variety of differentiated cell types. These tumors have been experimentally shown to originate from primordial germ cells (PGCs) (Andrews 2002), which normally differentiate into oocytes or sperm. The discovery of EC cells within teratocarcinomas prompted scientists

to find the equivalent cells in normal embryos, leading to the isolation of pluripotent ES cells (Evans and Kaufman 1981; Martin 1981) from preimplantation-stage embryos and pluripotent EG cells (Matsui et al. 1992; Resnick et al. 1992) from isolated primordial germ cells. Despite the phenotypic similarities of ES, EG, and EC cells, functional and molecular differences exist that likely reflect their different cellular origins. For example, EG and EC cells show a more restricted developmental potential than ES cells, which presumably reflects their origin from PGCs that have lost genomic imprints (Labosky et al. 1994; Tada et al. 1998). In addition, EC cells can form tumors when reintroduced into blastocysts (Rossant and McBurney 1982), and this behavior correlates with chromosomal changes that have accrued during tumor growth or in vitro culturing (Blelloch et al. 2004). Nonetheless, germ line contribution has been demonstrated for at least some EG (Labosky et al. 1994; Stewart et al. 1994) and EC (Stewart and Mintz 1982) cell lines, thus demonstrating their pluripotency.

The reprogramming of PGCs into EG and EC cells can be detected when comparing the developmental potencies of the cells of origin with their in vitro products. For example, inner cell mass cells of the blastocyst and derivative ES cells are both pluripotent and can give rise to all cell types of a mouse, including germ cells. While the derivation of ES cell lines (Evans and Kaufman 1981; Martin 1981) from inner cell mass cells likely induces epigenetic changes that facilitate immortal growth, no differences in the developmental potentials have been observed before and after culturing of the cells. In contrast to inner cell mass cells and ES cells, primordial germ cells do not contribute to tissues upon transfer into blastocysts (Durcova-Hills et al. 2006). However, EG cells derived from explanted PGCs and EC cells isolated from teratocarcinomas form tumors in SCID mice and contribute to chimeric animals after injection into host embryos (Durcova-Hills et al. 2006; Labosky et al. 1994; Stewart et al. 1994; Stewart and Mintz 1982). Convincing and reproducible evidence for the derivation of pluripotent cells has been confined to cells of the preimplantation-stage embryo (Chung et al. 2005; Evans and Kaufman 1981; Martin, 1981) and the germ line (Guan et al. 2006; Kanatsu-Shinohara et al. 2004; Matsui et al. 1992; Resnick et al. 1992). Germ line cells, in contrast to somatic cells, undergo major epigenetic changes during their differentiation, which may render them

more amenable for epigenetic reprogramming to pluripotency than somatic cells. It has been suggested, in fact, that all pluripotent cell lines characterized so far, including ES cells, are the product of germ cell precursors (Zwaka and Thomson 2005). Therefore, an important issue has been whether pluripotent cells can be derived not only from the embryo but also from adults without prior manipulation of their nuclei.

Recently, neonatal (Kanatsu-Shinohara et al. 2004) and adult (Guan et al. 2006) testis cells were shown to give rise at an extremely low frequency (1 in 15,000,000 testis cells) to ES-like cells when exposed to a specific combination of growth factors. ES-like cells expressed all the markers of pluripotent cells, formed teratomas after transplantation, and gave rise to chimeric animals that transmitted to the germ line. Thus these cells represent the only clear example for the derivation of pluripotent cells from a normal neonatal or adult mammal and may provide an alternative source of pluripotent human cells for the treatment and study of human diseases without recourse to nuclear transfer. However, a potentially serious concern for any therapeutic application of these cells is the unbalanced genomic imprinting. Parental imprints are erased in PGCs and sequentially re-established in a male- or female-specific pattern during subsequent gametogenesis (Hajkova et al. 2002; Lee et al. 2002; Yamazaki et al. 2005). Spermatogonial stem cells, which originate from PGCs, have undergone complete erasure of their imprints and seem to have re-established some male-specific imprints. Thus ES-like cells derived from the spermatogonial stem cells and EG cells derived from PGCs have an unbalanced imprinting status. Loss of imprinting by genetic manipulation of DNA methylation results in tumorigenesis in mice (Holm et al., 2005). Consequently, the therapeutic application of testis-derived ES-like cells may be problematic because the unbalanced, male-specific pattern of imprinted gene expression may inevitably result in tumorigenesis.

5 Reprogramming by Defined Factors

Based on the notion that ES cells contain factors that can elicit reprogramming, Takahashi and Yamanaka exposed fibroblasts to a pool of 24 retrovirally expressed genes (Takahashi and Yamanaka 2006) that

have been previously identified as ES cell specific (Mitsui et al. 2003), combined with a select set of other candidate genes, to screen for the emergence of ES cell-like colonies. In order to distinguish purely transformed fibroblast colonies from truly "reprogrammed" ES cell-like colonies, the authors selected for cells that had reactivated an ES cell-specific gene, Fbx15, and obtained ES cell-like colonies in 0.02% of infected fibroblasts. After selectively omitting individual retroviruses from the pool before infection, the authors could narrow down the minimal set of genes required for the induction of pluripotency to the four transcription factors Oct4, Sox2, c-myc, and Klf4. The reprogrammed cells formed differentiated teratomas, contributed moderately to mid-gestation chimeras, and showed a gene expression signature that resembled that of ES cells.

Several questions were raised by this experiment. For instance, how important is the cellular context for reprogramming? Nuclear transfer and fusion experiments suggest that less differentiated cells are generally more easily reprogrammed than fully differentiated cells. Klf4, c-myc, and Sox2 are expressed in multiple cell types in the adult, yet there is no spontaneous dedifferentiation, even when Oct4 is expressed ectopically (Hochedlinger et al. 2005), suggesting that the cellular context and/or the expression levels of the individual factors may be critical. Another question is whether the epigenome needs to be fully reprogrammed in order to reacquire a pluripotent state. It should be informative to assess whether the inactive X chromosome in female cells, DNA and histone methylation patterns are reprogrammed to the same extent in reprogrammed fibroblasts as they are in nuclear transfer, fusion, or explantation-derived pluripotent cells. A related question is whether the pluripotent state of cells is stable or requires the continuous expression of the four genes off retroviruses. Most importantly, are induced pluripotent cells functional? So far, their differentiation potential has only been characterized by morphological criteria and marker analysis. but no functional tests, such the ability to contribute to the germ line, have been performed.

6 Outlook

Despite the exciting progress that has been made in the field of reprogramming over the past few years, many questions remain. For example, it is unclear whether the same molecular mechanism underlies the different forms of reprogramming. While Nanog appears to be the rate-limiting molecule for the reprogramming of somatic cells by cell fusion (Silva et al. 2006), it seems to be dispensable for the direct reprogramming of fibroblasts upon forced expression of Oct4, Sox2, Klf4, and c-myc (Takahashi and Yamanaka 2006), and its role in germ cell reprogramming awaits to be determined. Most progress has been made in the mouse model, but it remains to be shown whether reprogramming by nuclear transfer, testis cell explantation, and defined factors is feasible in humans. In analogy to classic transformation experiments in human and murine fibroblasts, there may be different genetic requirements for reprogramming. Reprogramming by any of the discussed approaches is generally very low (<1%), and hence, finding strategies to improve efficiency will be critical for a potential therapeutic application of reprogramming research.

If four genes are sufficient to endow fibroblasts cells with pluripotency, it should be feasible to reprogram somatic cells directly into therapeutically relevant cell types such as blood, pancreatic beta-cells or neurons. Indeed, the forced expression of just two transcription factors in B cells induces their reprogramming into macrophages (Xie et al. 2004). This approach may allow for the generation of cell types that have so far been difficult to obtain through the directed differentiation of ES cells such as blood, pancreatic beta cells, or neurons.

Three independent studies have recently shown that embryonic and adult fibroblasts can be reprogrammed by retroviral expression of Oct4, Sox2, c-myc and Klf4 into pluripotent cells, which were epigenetically and functionally indistinguishable from ES cells including the ability to contribute to the germ line (Okita et al. 2007; Wernig et al. 2007; Maherali et al. 2007).

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The Intestinal Stem Cell Niche Studied Through Conditional Transgenesis

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Abstract. Despite a wealth of experimental data, the precise mechanisms governing the maintenance and regeneration of the intestine remain relatively poorly elucidated. After physical or genetic injury, stem cells from the intestinal crypt are killed and the subsequent repopulation process recruits new stem cells from sources currently unknown. Understanding the genetic elements that determine stem cell fate and the basis by which repopulation occurs will greatly aid our understanding of both stem cell plasticity and the contribution made by the stem cell compartment to malignant disease. It would also provide a better platform to develop therapies to regenerate damaged intestinal epithelia as seen after radiation injuries or inflammatory bowel disease such as Crohn's disease. In this review we discuss some of the basic mechanisms that regulate intestinal stem cell viability and maintenance, and also summarise recent data from our laboratory on the requirement for the Wnt pathway in these processes.

1 Introduction

Self-renewal is a necessary response to lethal cellular insult—from simple cell shedding to the likes of xenobiotic and ischaemic injury. Lifelong maintenance of self-renewal requires the presence of a long-term population of stem cells, the most primitive of which is the embryonic stem cell. However, a multitude of studies have indicated that most other adult tissues also harbour stem cells. These adult stem cells are normally involved in homeostatic self-renewal processes but can also be rapidly recruited to repair tissues upon injury. A better understanding of the nature and potential of these populations has clear implications for tissue engineering in the intestine. It is now also widely accepted that many cancers contain a minority population of self-renewing stem cells, the cancer stem cells, which are entirely responsible for sustaining the tumour, as well as giving rise to proliferating but progressively differentiating cells. Although many tumours probably have their origins in normal stem cells, evidence from the haematopoietic system suggests that genetic alterations in more committed progenitor cells can reactivate the self-renewal machinery, resulting in a further source of cancer stem cells. Thus, the identification of cancer stem cells and the factors that regulate their behaviour is likely to have an enormous bearing on the way we treat neoplastic disease in the future.

2 Stem Cells Within the Small Intestine

Stem cells are integrated into a microenvironment, called the stem cell niche, which enables them to maintain tissue homeostasis. The niche protects the stem cell from stimuli like differentiation, apoptosis and other processes which would endanger stem cell reserves, and also from excessive stem cell production which could lead to cancer. Stem cells must periodically divide by asymmetric cell division to give rise to one replacement stem cell and one transit amplifying cell (TA) that gives rise to various differentiated cell lineages. Stem cells, though highly clonogenic, are proliferatively quiescent in comparison to TAs. After labelling with BrdU or [^{3}H]thymidine, stem cells retain the label longer than the TA cell. This leads to a model termed the ‘immortal strand’ (Cairns 1975) whereby the stem cell retains the template DNA during

each division and its copy is allocated to the daughter cell (Potten et al. 2002). This process is hypothesised to protect the stem cells from accumulating errors during replication.

Our current understanding of intestinal self-renewal is grounded in classical studies using the adult mouse small intestine. The intestinal epithelium has a unique attraction as a biological model for stem cell experiments since the topographical position along the crypt-villus axis can be directly related to the position of cells in a lineage (Potten and Loeffler 1990; Potten 1998, 2004). Small intestinal and colonic stem cells reside at or near the base of crypts of Lieberkühn. The epithelial villus-crypt structure and its surrounding pericryptal fibroblasts and mesenchyme in the small intestine represent a physical unit that generates four cell lineages: absorptive enterocytes, enteroendocrine and goblet cells (that occupy the villi) and a fourth differentiated type, the Paneth cell, which resides at the bottom of crypts and secretes antimicrobial agents. Goblet, enteroendocrine and absorptive cells migrate towards the lumen of the gut, but Paneth cells migrate towards the bottom of the crypt. Crypts are monoclonal, but each villus receives cells from multiple crypts and is therefore polyclonal. The stem cells in the small intestinal epithelium have not been well defined, in part because of the lack of good molecular markers for stem cells. Recently, Musashi-1 has been suggested as a candidate marker for stem and early progenitor cells in the crypt base of the mouse small intestine (see e.g. Kayahara et al. 2003).

3 Analysis of Crypt Survival After Exposure to Ionising Radiation

A series of experiments using increasing doses of irradiation to reduce crypt stem cell numbers to different extents have been used to measure the ability of the crypt to survive and regenerate (see e.g. Bach et al. 2000). Such assays have led to the proposal of a hierarchical stem cell organisation, containing three categories of stem cells, with low, medium and high radiotolerance. A number of elegant studies by Potten and colleagues have suggested that there are normally 4–6 stem cells per crypt, but that another 30–40 clonogenic cells exist that can repop-

ulate the intestinal crypt if the original stem cells are deleted (Booth and Potten 2000). Potten has also shown that there is a hierarchy to this process, with increasing numbers of new cells being recruitable after 1, 8 and 15 Gy of irradiation (Roberts and Potten 1994). This process of recruitment and 'clonogenic' repopulation of the crypt is characterised by a marked burst of proliferation within the crypt, with an associated transient crypt enlargement (Ijiri and Potten 1986). The four to six stem cells present at the crypt base appear to be very sensitive to DNA damage, cannot repair such damage and are killed by 1 Gy of γ-radiation (Potten 1977), apparently as a result of p53-mediated apoptosis (Merrit et al. 1994). This extreme sensitivity may normally stop the crypts from maintaining a mutation in this long-lived cell, which could otherwise undergo carcinogenic transformation (Booth and Potten 2000). Approximately six additional clonogenic cells per crypt that survive 1 Gy can apparently maintain the crypt when the functioning stem cells are killed, but these cells are themselves killed by higher doses of radiation. These therefore represent a second tier of stem cells that under normal steady-state conditions would not exhibit their stem cell potential, but become dividing transit cells and ultimately differentiate. However, at this early stage, they retain some stem cell function (i.e. are uncommitted) and can be called upon to express their stem cell potential if required. Additionally, these cells also appear to have acquired the ability to repair their DNA, a process that involves a p53-mediated cell cycle arrest.

4 Signalling Pathways Regulating Intestinal Homeostasis: The Wnt Signalling Pathway

Differentiation and homeostasis of the intestine are controlled by signal transduction pathways, including Wnt, Notch, TGF-β and hedgehog, and their deregulation contributes to intestinal neoplasia (Radkte et al. 2006). The Wnt pathway is perhaps the most intensively studied of these, probably as a consequence of its very close association with intestinal cancer. For example, overexpression of the Wnt inhibitor Dickkopf1 (Dkk-1) results in loss of proliferative cells in both foetal and adult intestine coinciding with the loss of the crypt compartment (Pinto

et al. 2003; Kuhnert et al. 2004). Similarly, Korinek et al. (1998) have shown that mice lacking Tcf-4 have a depleted epithelial stem cell compartment. Tcf-4 complexes with β-catenin in response to Wnt signalling and activates transcription of Wnt target genes. β-Catenin activity is itself regulated along the length of the crypt, as reflected by the pattern of nuclear accumulation of β-catenin, which decreases in a gradient from the base of the crypt to the interface with the villus (Batlle et al. 2002; van deWetering et al. 2002). Further crypt roles have been demonstrated for some Wnt target genes, such as those belonging to the EphB/Ephrin-B family which have been implicated in restricting the intermingling and proliferation of differentiated cells in the crypt (see e.g. Batlle et al. 2002).

These studies have suggested that Wnt signalling is critical in regulating intestinal homeostasis, and the most consistent hypothesis is that Wnt signalling directly influences stem cell fate. To study the role of the Wnt pathway in intestinal homeostasis, we have used a Cre-Lox based strategy, which relies upon the cytochrome p450-inducible Cyp1A promoter to drive Cre-mediated gene deletion within the small intestine. Using mice bearing this Cre transgene, together with the Rosa26R LacZ reporter locus (Soriano 1999) we have shown that Cre induction results in near 100% intestinal recombination when scored through LacZ staining. If the Cre-Lox-induced mutation is not deleterious to the recombined cells, they remain stable for at least 1 year after induction. This pattern of retention is consistent with the observation that Cre induction occurs within the stem cells of the intestinal crypt (see e.g. Hay et al. 2005; Ireland et al. 2004).

Using this Cre-Lox strategy, we have investigated the phenotype of conditional loss of Apc (Sansom et al. 2004), which we find parallels some aspects of the phenotype reported for overexpression of OCT4 (Hochedlinger et al. 2005). Thus, both mutations lead to a ‘crypt progenitor-like’ phenotype characterised by hyperproliferation and perturbed migration and differentiation. The observed similarities between OCT4 overexpression and Wnt activation suggest some parallels in the control of the stem cell population. Critically, activation of the Wnt pathway following loss of Apc immediately confers many of the phenotypes associated with the early stages of neoplasia in the intestine. As might be predicted, conditional deletion of β-catenin with an in-

ducible Cre-Lox strategy leads to a directly opposing phenotype. Loss of β-catenin is not tolerated, with deficiency leading to rapid crypt ablation, increased apoptosis and the depletion of goblet cells (Ireland et al. 2004). β-Catenin-deficient cells are deleted from the intestine within 5 days after recombination. Subsequent to loss of the β-catenin-deficient cells, there is a rapid repopulation of the small intestine by wild-type cells (Ireland et al. 2004). These data demonstrate a requirement for functional Wnt signalling in crypt maintenance and also show that conditional deletion of genes which are critical for crypt survival, such as β-catenin, leads to a phenomenon of repopulation, whereby non-recombined cells are recruited to repopulate the crypt-villus axis.

We have also investigated the functional requirement for the Wnt target gene c-Myc within the intestine. Using the same inducible Cre-Lox strategy, we have conditionally deleted c-Myc from the adult intestine, and again find that c-Myc deficiency is not compatible with long-term crypt survival (Muncan et al. 2006). In this case, c-Myc-deficient cells persist within the intestine for up to 14 days after the initiation of gene deletion. c-Myc-deficient cells do undergo division, but this occurs at a reduced rate compared to controls. Furthermore, c-Myc-deficient cells show reduced metabolic activity. As with the scenario following β-catenin deletion, c-Myc-deficient cells are replaced by 'wild-type' (non-recombined) cells (Muncan et al. 2006). These observations therefore again necessitate a process of repopulation of the crypts from cells where gene deletion did not occur.

Remarkably, Apc-deficient crypts resemble crypts undergoing either repopulation following γ-irradiation or undergoing repopulation following loss of β-catenin or c-Myc. These similarities suggested that there could be a common mechanism shared between repopulating crypts and Apc-deficient crypts; with the most probable being an activation of Wnt signalling. Consistent with this, Paneth cells are mislocalised throughout the crypt in both Apc-deficient crypts and repopulating crypts, suggesting perturbations in the Ephrin/EphB gradients, as previously observed after loss of Apc (Sansom et al. 2004). We also observe nuclear localisation of β-catenin in repopulating crypts and the upregulation of two key Wnt targets, c-Myc and CD44. These data argue that activation of Wnt signalling mediates the repopulation process, and suggest that c-Myc may be central to this process.

We have been able to confirm a critical requirement for c-Myc in this repopulation process. In our laboratory, c-Myc deficiency leads to the relatively slow loss of crypt cells by day 14. Prior to this time point c-Myc-deficient cells are viable, and we have used this 'window' to show that c-Myc is absolutely required for the repopulation process following DNA damage. Thus, wild-type mice 3 days after irradiation show nearly complete repopulation, whereas c-Myc-deficient cells completely fail to repopulate. Notably, this c-Myc deficiency is revealed in an environment where there is potential competition in the repopulation process between wild-type and c-Myc-null cells (Sansom et al. submitted).

A key point here is that our studies of c-Myc deficiency differ markedly from those published by Andreas Trumpp's laboratory (e.g. Betteess et al. 2005). In that study, there was no detrimental effect of adult loss of c-Myc. By contrast, our own study showed that loss of c-Myc compromises crypt survival, leading to 100% loss of c-Myc-deficient cells by day 14. Both c-Myc alleles used in these studies are null after Cre-mediated recombination, and we therefore infer that the different phenotypes arise from the use of two different Cre transgenes. In the published studies, Villin-Cre was used and in our studies the Ah-Cre transgene was used. We now tentatively hypothesise that Villin-Cre drives recombination within those cells required for repopulation, and that therefore either no repopulation occurs or repopulation occurs by c-Myc-deficient cells (in the absence of competition from wild-type cells).

The precise origin of the repopulating cells remains undefined. It seems most likely that there is local recruitment of a 'quiescent' stem cell population from within the crypt-villus axis. However, several recent studies have suggested a somewhat more dramatic alternative, namely that particular lineages within the intestine may be replenished from the bone marrow (Brittan et al. 2002; Rivzi et al. 2006; Kraus et al. 2001).

5 Summary

Our studies indicate that repopulation occurs after a lethal insult (either physical or genetic), that this repopulation is driven by the Wnt path-

way and, furthermore, that it relies upon the recruitment of 'novel' stem cells from an unrecombined population. For example, deficiency of either β-catenin or c-Myc leads to the repopulation of the crypt-villus structure by wild-type cells. This process is driven either by death of the mutant stem cells or by selective overgrowth of the wild-type cells. The stem cells that subsequently repopulate the crypt-villus axis either lie quiescent in the crypt or are being recruited from outside the crypt. Critically, we observe repopulation of crypts, which are apparently wholly recombined for the target allele, as assessed by the Rosa26 reporter allele. Therefore, by definition, the 'novel' stem cells cannot all be recruited from the daughters of the functional stem cells within these crypts. Furthermore, the unrecombined cells destined to repopulate the crypt must represent the population of 'recruitable' novel stem cells. Taken together, our data show that the normal activity of the Wnt pathway is crucial for the survival of the intestinal crypt stem cell population. Thus, loss of key components of this pathway leads to loss of the stem cell population. Furthermore, we show that the Wnt pathway is required during the repopulation process that occurs either after exogenous damage or after genetic mutation, so establishing critical roles for this pathway in normal physiology.

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The Migrating Cancer Stem Cells Model–A Conceptual Explanation of Malignant Tumour Progression

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Abstract. Human colorectal cancer is one of the best, if not the best, understood tumour diseases. These tumours develop stepwise via an adenoma-carcinoma sequence. The steps in this process can easily be discriminated with light microscopy. The breakthrough in understanding carcinogenesis was the finding that mutations in tumour suppressor genes and oncogenes accumulate in parallel with these steps. This accumulation is the cause for the malignant progression of colorectal cancers, leading to highly invasive and migrating tumour cells. This concept is known as the multistep carcinogenesis model and has become the paradigm of tumour progression in general. But this model does not explain the complex, heterogeneous histology of colorectal tumours or the good differentiation of metastases, which are expected to have lost their differentiation because of the accumulation of mutations. Here, we present the model of migrating tumour stem cells, which explains these contradictions in the context of the histology of colorectal tumours. Thus colorectal tumours consist of tumour stem cells, which have recently been defined as a small CD133-positive population of tumour cells. These cells trans-differentiate into epithelial cells, which

represent the main mass of the colorectal tumours. Moreover, the tumour stem cells are the active component of migration and invasion, thus conferring the malignant phenotype. Taken together, mutations confer to the tumour cells the capability to live outside of their stem cell niche and intestinal compartment. In addition, the trans-differentiation potential of the tumour cell confers plasticity to the tumour and thus contributes to the heterogeneity of colorectal cancers.

1 The Multistep Carcinogenesis Model and Tumour Heterogeneity

The process of colorectal carcinogenesis is one of the best-understood mechanisms leading to neoplasia. Most colorectal cancers (CRC) develop via an adenoma-carcinoma sequence. The different steps in this linear progression can be easily discriminated histologically by light microscopy (Fodde et al. 2001). The breakthrough for understanding colorectal carcinogenesis was the finding that the adenoma-carcinoma progression is paralleled by the accumulation of mutations in oncogenes and tumour suppressor genes. In the majority of CRC (up to 80%; Bienz and Clevers 2000; Kinzler and Vogelstein 1996) the first detectable mutation is found in the tumour suppressor gene APC (adenomatous polyposis coli), which is therefore known as the gatekeeper of colorectal carcinogenesis (Kinzler and Vogelstein 1996). Other mutations occur frequently in the K-ras oncogene and the p53 tumour suppressor gene, Moreover, alterations in chromosome 18 are found in about 60% of CRCs, but only a list of possibly affected candidate genes has been defined to be responsible for the underlying genetic defect to date. These genes are members of the TGF-β (transforming growth factor β) pathway, like SMAD4/DPC4 (mothers against decapentaplegic/deleted in pancreatic cancer), MADRH2 or TGF-β RI (TGF-β receptor I) or DCC (deleted in colorectal cancer), which encodes the netrin receptor. The mutations and the subsequent alteration in the function of these proteins are thought to be responsible for driving the adenoma-carcinoma progression and thus leading to malignant progression. Consequently, colorectal tumour cells lose their epithelial differentiation (de-differentiation) and gain in parallel characteristics of migration and invasion, which are prerequisites for dissemination and metastasis. This complex

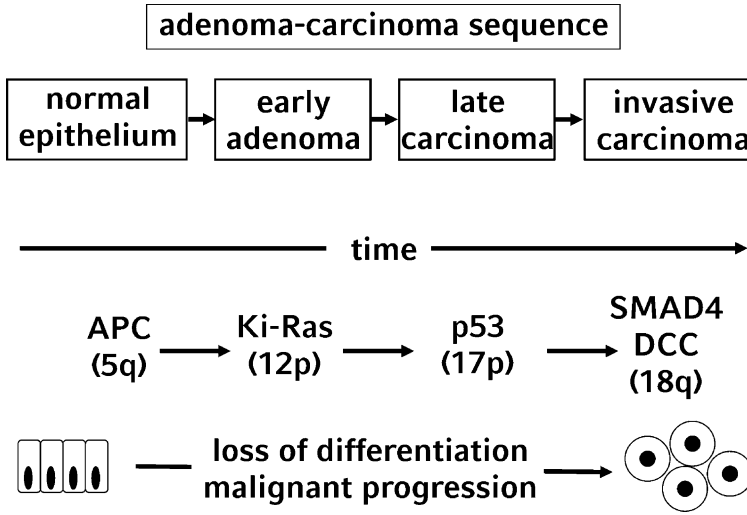


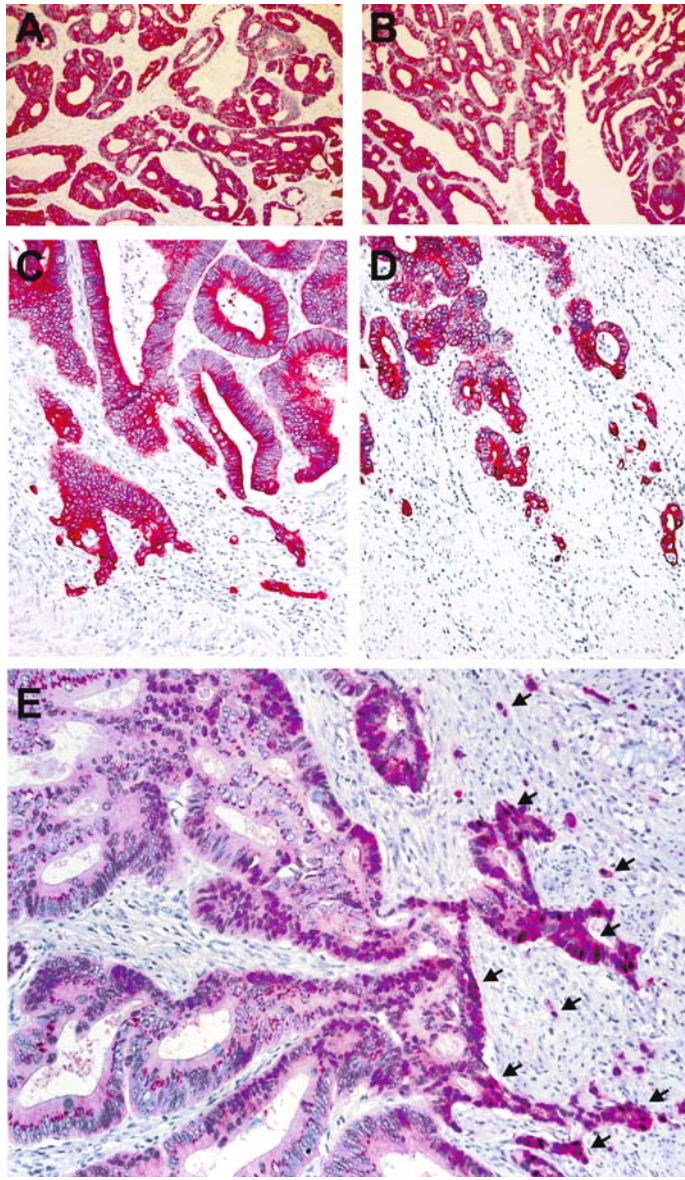
Fig. 1. The multistep carcinogenesis model. The adenoma-carcinoma sequence of CRC resembles a progression program. The driving force is the accumulation of mutations in time, mostly starting with mutations of the tumour suppressor gene APC (adenomatous polyposis coli), the gatekeeper of colorectal carcinogenesis. Other mutations mostly affect the oncogene K-ras and the tumour suppressor gene p53 as well as genes lying on chromosome 18, like SMAD4 or DCC. As a consequence of the mutations, the epithelial cells lose their differentiation, which is a prerequisite, and expression of malignant progression at the same time

model is known as the multistep carcinogenesis model and is a milestone in cancer research (Fig. 1) (Fearon and Vogelstein 1990).

According to this model metastases should be aggregates of de-differentiated tumour cells, as mutations are irreversible. However, taking a closer look at CRC and their corresponding metastases, it turns out that in most cases they are indistinguishable from each other. In some cases metastases are even better differentiated than their corresponding primary tumours (Fig. 2A, B) (Brabletz et al. 2001). But if de-differentiation is necessary for migration and invasion, the question arises as to how metastases develop at all, when CRC are epithelially

organised structures. Again a closer look may give a clue. Most if not all CRCs are growing heterogeneously with respect to their differentiation (Fig. 2C). Thus well-differentiated as well as de-differentiated cells are found in parallel in CRCs. However, these differentially differentiated tumour cells are not homogeneously distributed but accumulate in many cases in distinct areas of the tumours, thus giving rise to a histologically discernable growth pattern. Thus two zones can be defined on the basis of the state of differentiation of the tumour cells. The major part of the tumours is represented by cells with an epithelial organisation, and thus well differentiated. A tiny number of cells display a mesenchymal organisation, thus being de-differentiated. They are single lying cells without adherens junctions or small clusters of cells, called buds. They express marker proteins of mesenchymal cells like fibronectin and vimentin, and are located at the invasion front lining the tumour as a rim (Fig. 2C) (Brabletz et al. 2001). This zonal organisation into central tumour area with differentiated cells and invasion front with de-differentiated cells is found not only in primary tumours but again in their corresponding metastases (Fig. 2D). Thus it is tempting to speculate that this organisation is regulated and therefore not dependent on mutations. Interestingly, the number of de-differentiated cells or buds correlates highly significantly with low survival (Hase et al. 1993; Ueno et al. 2002). Therefore, the mechanisms governing the regulation of differentiation/de-differentiation seem to have biological relevance.

Fig. 2A-E. Human primary CRC and their corresponding metastases display indistinguishable growth patterns and are heterogeneously organised. The primary tumours (**A**) and metastases (**B**) of human colorectal tumours are histologically indistinguishable. Moreover, primary tumours (**C**) as well as the corresponding metastases (**D**) display a heterogeneous organisation. Whereas most parts of the tumours are organised as well-differentiated epithelial structures, the invasion front of CRCs with an infiltrative invasion front harbours single lying cells (**C**, **D** *arrow*) which are characterised by loss of E-cadherin and the parallel gain of mesenchymal markers like vimentin or fibronectin. Moreover, these cells express nuclear β-catenin (**E**). The nuclear expression of β-catenin is coupled with de-differentiation, whereas a membranous expression is characteristic for the epithelial organised zones in the tumours



2 The Wnt/β-Catenin Signalling Pathway

The simultaneous analysis of CRCs for mutations in the genes encoding APC, K-ras and p53 revealed that mutations in the three genes very seldom occurred at the same time (< 7%). But APC mutations were found in about 60% of all cases (Smith et al. 2002), thus emphasising the role of the APC gene for the process of colorectal carcinogenesis (Kinzler and Vogelstein 1996). APC is a large protein of about 312 kDa (Dikovskaya et al. 2001) harbouring many protein-protein interaction domains. Consequently, APC is involved in many cellular functions (Bienz 2002; Bienz and Clevers 2000; Dikovskaya et al. 2001). One of APC's functions is the regulation of the degradation of β-catenin. In this context APC is an integral component of a β-catenin degradation complex consisting of GSK-3β (glycogen synthase kinase 3β) and axin/conductin. The control of the stability of β-catenin and thus the activity of the β-catenin degradation complex is the central element of the Wnt/β-catenin signalling pathway (Bienz and Clevers 2000; Clevers 2006; Schneikert and Behrens 2007). This role of APC is thought to be the most important one for colorectal carcinogenesis for two reasons. On one hand, almost all mutations in the APC gene destroy its capacity to participate in the destruction complex. On the other hand, β-catenin is mutated in most of the remaining CRCs characterised by an intact APC gene in such a way that β-catenin becomes degradation resistant (Polakis 2000). As a consequence β-catenin accumulates in colorectal tumour cells. What are the consequences of the accumulation of β-catenin? β-Catenin is a protein with ambivalent functions. It is an integral component of adherens junctions, where it participates in and stabilises the epithelial phenotype of cells. In this context β-catenin works as a tumour suppressor gene. In the nucleus β-catenin complexes with DNA binding proteins of the TCF/LEF-1 (T-cell factor/lymphocyte enhancing factor) family of HMG (high mobility group) proteins. These complexes function as transcription factors. Of the four family members TCF-1, TCF-3, TCF-4 and LEF-1, TCF-4 seems to be essential for the development and maintenance of the gut (Korinek et al. 1998). In this context β-catenin works as an oncogene (Harris and Peifer 2005). Thus overexpression of β-catenin does not automatically lead to the nuclear accumulation of β-catenin and induction of

transcription but might induce even a higher grade of epithelial differentiation (Wong et al. 1998). The subcellular localisation of β-catenin and the state of differentiation differ with respect to the localisation of the tumour cells in the tumour and run in parallel. De-differentiated tumour cells located at the invasion front display a strong nuclear expression of β-catenin, whereas differentiated tumour cells located in central areas of tumours show membranous expression. The tumour tissue between the central areas and the invasion front is characterised by the gradual loss of differentiation connected with the parallel gain of the nuclear expression of nuclear β-catenin (Fig. 2D) (Brabletz et al. 1998, 2001, 2005b; Kirchner and Brabletz 2000). Additionally, the de-differentiated cells have lost several characteristics of epithelial differentiation. Among these is the loss of the membranous expression of E-cadherin. At the same time these cells gain characteristics of mesenchymal cells, such as the expression of the mesenchymal marker proteins fibronectin or vimentin (Brabletz et al. 2001). These observations are supported by the fact that nuclear β-catenin induces a mesenchymal state of differentiation in the epithelially organised cell line MDCK (Brembeck et al. 2004). Wnt/β-catenin induces the transcription of a variety of target genes which confer a progenitor phenotype to the cells (Brabletz et al. 2005b; van de Wetering et al. 2002). At the same time migration and invasion are induced (Brabletz et al. 2005b). The transcriptional portfolio of Wnt/β-catenin contains genes representing many of the cancer hallmarks (Hanahan and Weinberg, 2000). *Proliferation* might be induced by the target genes c-myc (He et al. 1998) or cyclin D_1 (Shtutman et al. 1999; Tetsu and McCormick 1999). *Migration and invasion* are mediated by genes like uPA (urokinase plasminogen activator) (Hiendlmeyer et al. 2004), uPAR (uPA receptor) (Mann et al., 1999), MMP-7 (matrix metalloproteinase 7) (Brabletz et al. 1999; Crawford et al. 1999), MT1-MMP (membrane type 1 MMP) (Hlubek et al. 2004), Laminin-5γ2 (Hlubek et al. 2001) or tenascin-C (Beiter et al. 2005). *Dissemination* is conferred by the expression of CD44 (Wielenga et al. 1999). *De-Differentiation* is granted by the down-regulation of E-cadherin, which might be mediated by factors like slug (Hajra et al. 2002), which is transcriptionally regulated by Wnt/β-catenin (Conacci-Sorrell et al. 2003). In parallel, the mesenchymal marker genes fibronectin (Gradl et al., 1999) and vimentin (Gilles et al. 2003) are trans-

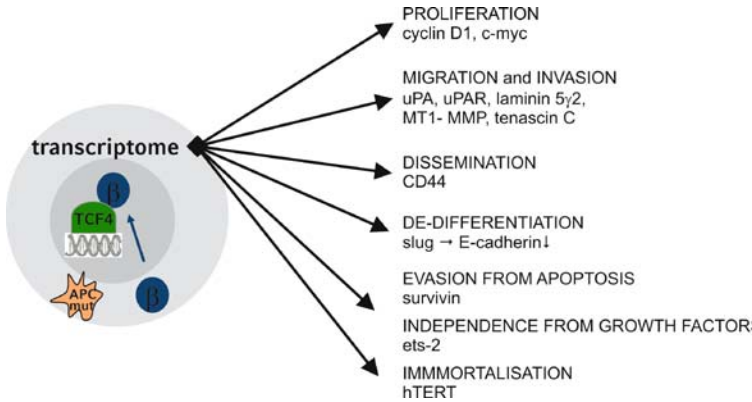


Fig. 3. Wnt/β-catenin target genes confer many characteristics necessary for the progression of colorectal tumours and associated with stemness of tumour cells. Wnt/β-catenin induces the transcriptional activation of genes driving proliferation, migration and invasion, dissemination, evasion from apoptosis, independence from growth factors and immortalisation as well as de-differentiation. Moreover, genes like *survivin* and *hTERT* (human telomerase RT component) are markers of adult stem cells, thus indicating the stem cell character of tumour cells characterised by the expression of nuclear β-catenin

activated by the function of Wnt/β-catenin. Suppression of *apoptosis* is mediated by the expression of survivin (Kim et al. 2003; Zhang et al. 2001). Moreover, the expression of transcription factors like ets is induced, which might confer *independence from growth factors* (van de Wetering et al. 2002). Finally, *immortalisation* is gained by the expression of hTERT (human telomerase RT component) (E. Hiendlmeyer, T. Brabletz, A. Haynl, H. Herbst, T. Kirchner, A. Jung, submitted). As expected, many of the above-mentioned genes are found to be overexpressed in the de-differentiated tumour cells expressing nuclear β-catenin at the invasion front (Brabletz et al. 2002, 2005a,b). In summary, quite a variety of genes are activated by Wnt/β-catenin transcriptional activity (Fig. 3), which alone might already be sufficient to drive colorectal carcinogenesis. This might be an explanation for why unleashing Wnt/β-catenin-signalling is an early and frequent event in the process of colorectal carcinogenesis (Fig. 3).

3 De-differentiated Tumour Cells at the Invasion Front Show Characteristics of Stemness

As c-myc and cyclin D_1 are targets of the Wnt/β-catenin transcriptional activity (He et al. 1998; Shtutman et al. 1999; Tetsu and McCormick 1999), it might be expected that colorectal tumour cells at the invasion front expressing nuclear β-catenin might display high proliferation. Unexpectedly, the opposite behaviour is found when employing immunohistochemistry using Ki67 as a marker (Brabletz et al. 2001; Jung et al. 2001; Palmqvist et al. 2000). Low proliferation is paralleled by the expression of the cell cycle inhibitor $p16^{INK4a}$ (Jung et al. 2001; Palmqvist et al. 2000), which correlates especially with the expression of nuclear β-catenin (Jung et al., 2001). Interestingly, $p16^{INK4a}$ is another target gene of Wnt/β-catenin (S. Wassermann, S. Scheel, T. Brabletz, E. Hiendlmeyer, R. Palmqvist, F. Hlubek, A. Haynl, S. Merkel, A. Jung, submitted). This raises the question of what the benefit might be for tumour cells to up-regulate proteins that stimulate or inhibit the cell division cycle at the time. Additionally, it might be that $p16^{INK4a}$ has another role in the regulation of invasion. Thus it is known that $p16^{INK4a}$ and the Wnt/β-catenin target gene laminin-5γ2 stimulate invasion (Natarajan et al. 2003). Low proliferation is also an indicator of adult stem cells and tumour stem cells (Melton and Cowan 2006; O'Brien et al., 2007; Pardal et al., 2003; Ricci-Vitiani et al., 2007). This view is further supported by the fact that Wnt/β-catenin induces the expression of the adult stem cells markers survivin (Kim et al., 2003; Zhang et al. 2001) and hTERT (Kolquist et al. 1998; Melton and Cowan 2006; S. Wassermann, S. Scheel, T. Brabletz, E. Hiendlmeyer, R. Palmqvist, F. Hlubek, A. Haynl, S. Merkel, A. Jung, submitted). Therefore, it is tempting to postulate that de-differentiated tumour cells at the invasion front displaying nuclear β-catenin resemble tumour-initiating cells or tumour stem cells. Tumour-initiating cells with characteristics of tumour stem cells have been defined in human colorectal tumours recently (O'Brien et al. 2007; Ricci-Vitiani et al. 2007). These cells are also characterised by lower proliferation and high expression of CD133. Thus a fundamental proof for the proposed stem cell function and thus model of the tumour cells at the invasion front will be the in situ detection of CD133.

In the tumour stem cell concept the biological activity is inherent to the small number of tumour stem cells (Pardal et al. 2003; Reya et al. 2001). Again this characteristic is reflected in colorectal tumours displaying an infiltrative invasion front. The number of de-differentiated cells or buds at the invasion front correlates very well with low survival (Hase et al. 1993; Ueno et al. 2002). Moreover, the nuclear localisation of β-catenin correlates with the appearance of de-differentiated cells (Brabletz et al. 1998; Kirchner and Brabletz 2000).

Taken together, the small number of tumour cells at the invasion front expressing nuclear β-catenin display many characteristics of cells which have been defined as tumour-initiating cells or tumour stem cells.

4 The Model of Migrating Tumour Stem Cells

On the basis of the histological, functional and cellular data we proposed the model of migrating tumour stem cells (Brabletz et al. 2005b). In this model (Fig. 4) tumour stem cells are characterised by nuclear β-catenin. They are found already in small numbers in adenomas and small carcinomas, as well as in infiltrative carcinomas. In adenomas and carcinomas they are found homogeneously scattered throughout the tumours. But in carcinomas displaying an infiltrative invasion front these tumour cells accumulate at the invasion front. Though both the cells at the invasion front as well as the cells scattered in the neoplastic tissue express nuclear β-catenin, the expression of factors inducing migration and invasion is found exclusively in the tumour cells at the invasion front. Thus tumour cells found in the central areas of CRCs might be defined as stationary tumour stem cells, whereas the cells at the invasion front would be migrating tumour stem cells. When assuming that metastases arise from a single cell descendent from primary tumours, it is very likely that the difference in stationary versus migrating tumour stem cells is induced by the microenvironment (Barker and Clevers 2001; Brabletz et al. 2001) rather than being caused by an cell-intrinsic mechanism like a mutation.

If de-differentiated tumour stem cells can give rise to differentiated tumour cells (O'Brien et al. 2007; Ricci-Vitiani et al. 2007) but not vice versa, then the tumour stem cells at the invasion front might give rise

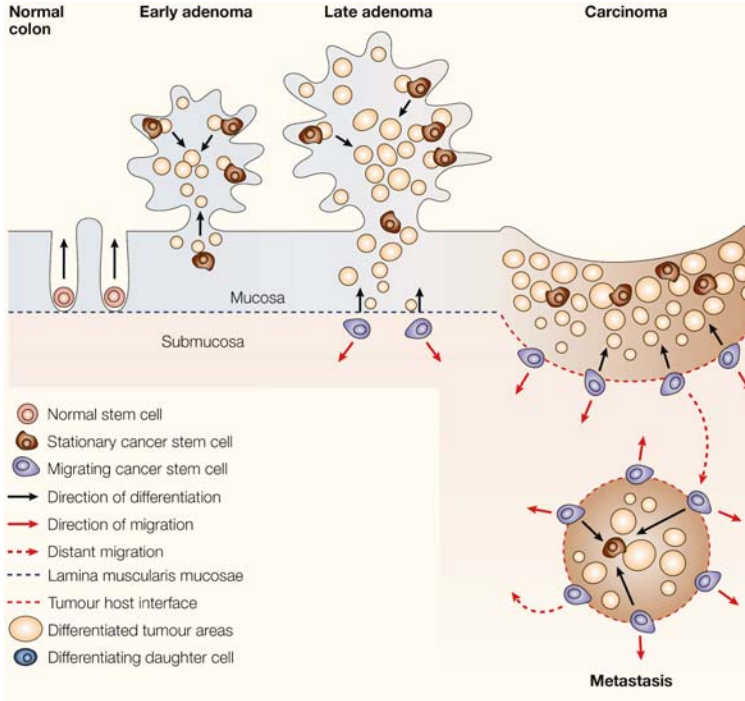


Fig. 4. The model of migrating tumour stem cells (Brabletz et al., 2005b). Adult stem cells are found at the base of the crypts building up normal colon mucosa. These stem cells are the origin of the later tumour stem cells which appear either as stationary stem cells (SCS) or migrating cancer stem cells (MCS). SCS are found scattered equally in adenomas and carcinomas as well as metastases in the differentiated areas of the tumours. When coming into contract with the stroma beyond the lamina muscularis mucosae tumour stem cells gain invasiveness and become MCS. In this model SCS are necessary for the maintenance of the main parts of the tumours, whereas the MCS might have a twofold role. They expand the border of tumour growth and establish metastases as well as giving rise to differentiated parts of the tumour by a process of MET (mesenchymo-epithelial transition)

to tumour buds and later epithelially differentiated tumour cells in the central areas of the tumour as the tumour grows. This would resemble

an MET (mesenchymo-epithelial transition) as is found during development. By this means migrating tumour stem cells show signs of plasticity in that they harbour the capacity to differentiate. Understanding the mechanisms of MET might lead to a therapeutic weapon against CRCs with an infiltrative invasion front and tumour stem cells defined by nuclear β-catenin. Moreover, the concept of EMT (epithelio-mesenchymal transition) as a tumour-propelling mechanism for CRCs (Brabletz et al. 2001, 2005b; Thiery 2002) and maybe other tumour entities as well (Thiery 2002) might have to be reconsidered in the light of the tumour stem cell concept (Brabletz et al. 2005b; Pardal et al. 2003; Reya et al. 2001).

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Bone Marrow Niche and Leukemia

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Abstract. Mounting evidence indicates that human cancers may originate from malignant transformation of stem cells. The most convincing proof is found in acute myeloid leukemia, where only a small subset of slowly dividing cells was able to induce *transplantable* acute myeloid leukemia. Normal hematopoietic stem cells (HSC) are characterized by their unlimited ability to self-renew, give rise to a multitude of cells that exhibit more differentiated features, and show slow division kinetics. Using human HSC and mesenchymal stromal cells (MSC) as models, we and others have demonstrated the vital role of the cellular niche in maintaining the self-renewing capacity, that is, "stemness" of HSC. Without direct contact with the cellular niche, HSC tend to differentiate and lose their stemness. Similar to their normal counterparts, leukemia stem cells divide slowly and maintain their self-renewal capacity through interaction with the niche. As a consequence, they are resistant to conventional chemotherapy strategies that target rapidly dividing cells. Thus it is of utmost importance to understand the interaction between cellular niche and normal HSC as well as

between leukemia stem cells and the niche to provide a basis for more efficient treatment strategies.

1 Stem Cells in Health and in Cancer

The hallmark of stem cells is their dual abilities to self-renew and to generate multiple cell lineages with more differentiated characteristics. Stem cells must therefore, at some time point during development, divide asymmetrically to give rise to one daughter cell that will self-renew and another that will give rise to more mature progeny cells (Ho 2005).

In analogy to physiological differentiation, a stem cell paradigm has been suggested for neoplastic development (Pardal et al. 2003). There is increasing evidence that human cancers may originate from malignant transformation of stem cells. The first hints were found in hematological malignancies, where only a small subset of slowly dividing cells was able to induce transplantable acute myeloid leukemia (Huntly and Gilliland 2005; Bonnet and Dick 1997; Lapidot et al. 1994). Normal stem cells are characterized by their ability to self-renew, their unlimited replicative potential, the production of a multitude of progeny cells with more differentiated characteristics, and their slow division kinetics (Table 1). Leukemia stem cells (LSC) have been reported to express stem cell markers, to self-renew and to live indefinitely upon serial transplantations in animal models, to give rise to leukemia cells that exhibit more differentiated features and to proliferate slowly (Bonnet and Dick 1997; Lapidot et al. 1994). The latter characteristic is associated with their resistance to conventional chemotherapy strategies that target rapidly dividing cells. Similar to their normal counterparts, the primitive HSC, the LSC are also extremely rare, estimated to be of the order of 1 per 10^5 or 10^6 mononuclear cells in the marrow.

Meanwhile, studies of other malignancies such as breast and brain tumors have provided further evidence for the cancer stem cell concept (Stingl et al. 2006; Ponti et al. 2005; Singh et al. 2004; Galli et al. 2004). Many groups have demonstrated that some cancer types harbor a subpopulation that maintains stem cell identity and might determine critical biological and clinical features of the cancer. These include high tumorigenicity upon transplantation, migration and invasion, chemo- and radio-resistance, and an affinity for specific tissue niches. Cancer stem

cells have also been reported to express stem cell markers and produce tumor cells that exhibit more differentiated features, proliferate slowly, and are resistant to conventional tumor therapies that target rapidly dividing cells (Stingl et al. 2006; Ponti et al. 2005; Singh et al. 2004; Galli et al. 2004).

2 Significant Role of the Stem Cell Niche

For normal hematopoietic stem cells (HSC), the fundamental decision process to self-renew or to differentiate is governed by interactions between HSC and their niche in the marrow (Fig. 1). In a series of experiments, we have demonstrated that direct contact between adult stem cells and cellular determinants of the microenvironment is essential in regulating asymmetric divisions and promoting stem cell renewal (Huang et al. 1999; Punzel et al. 2003; Wagner et al. 2004; Giebel et al. 2006). Using HSC and mesenchymal stem cells (MSC), both derived from human marrow as a surrogate model for the interaction between adult stem cells and their niche, we have defined the role of direct cell–cell contact between HSC and MSC in self-renewal and in the expression of specific genes that have been shown to regulate long-term hematopoiesis (Wagner et al. 2004). Without direct contact with the cellular niche, HSC tend to differentiate and lose their stem cell function. The marrow niche therefore protects the HSC from differentiating and maintains “stemness”.

It is also appreciated that not only the tumor cells per se but also the interaction with their microenvironment determine tumor growth (Mueller and Fusenig 2004). For leukemia stem cells (LSC), current

Table 1 Normal and leukemic stem cells share common features

Unlimited life span
Ability to self-renew
Quiescent or slow division kinetics
Heterogeneity and hierarchy of progeny cells
Well-protected by the stem cell niche
Extremely rare, difficult to define

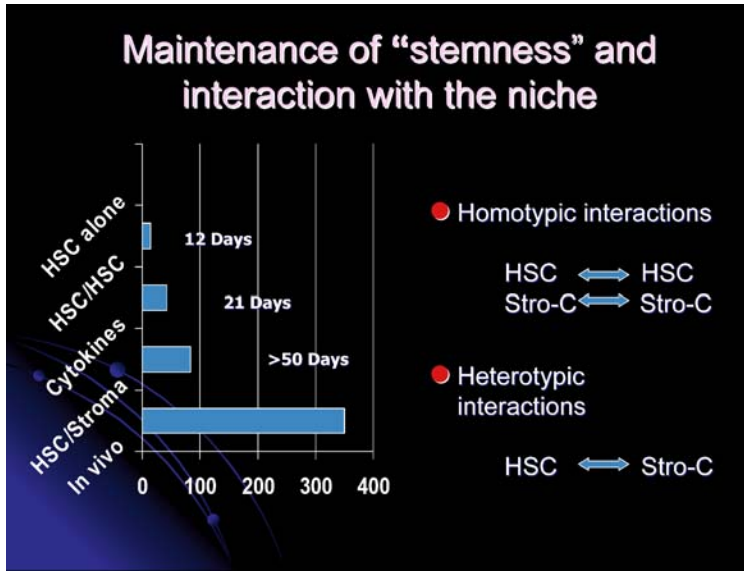

Fig. 1. Cell–cell contact between hematopoietic stem cells (*HSC*) and stroma cells is essential in maintenance of self-renewal. In vitro cell survival of HSC as single cells, maximum 12–24 h; of HSC in groups of more than 20 in a 96-well plate, approximately 12 days; with addition of cytokines, 21 days; of HSC in co-cultures with stroma cells (*STO-C*), more than 50 days; in animal models as long as the animal survives. Furthermore, our group has analyzed homeotypic interactions between mesenchymal stromal cells (MSC) as well as between HSC and heterotypic junctions between HSC and MSC

evidence indicates that the cellular niche of the marrow also maintains the "stemness" of the malignant stem cells. Thus not only do normal stem cells require support from the corresponding cellular niche (Wagner et al. 2005), but cancer stem cells are also dependent on interactions with the cellular determinants in the niche (Owens and Watt 2003). These determinants probably change with age, as suggested by age-dependent modulation of tumorigenicity (Campisi 2005).

Thus it is of utmost importance to understand the mechanisms of interaction between the cellular niche and HSC in comparison to that be-

tween the niche and LSC to provide a basis for more efficient treatment strategies. The application of such principles might induce long-term cure, as they could eradicate the ultimate source of leukemia.

3 Hematopoietic Stem Cells, Archetype of Adult Stem Cells

Modern-day stem cell research began with the discovery of assays to detect HSC some 40 years ago (Siminovitch et al. 1963). In a murine model, James Till, Ernest McCullough, and Lou Siminovitch demonstrated the existence of HSC in the bone marrow (BM) in the early 1960s (Siminovitch et al. 1963; Becker et al. 1963). Their series of experiments demonstrated that, first of all, cells from the bone marrow could reconstitute hematopoiesis and hence rescue lethally irradiated recipient animals. Secondly, by serial transplantations, they established the self-renewal ability of the original bone marrow cells. When cells from the splenic colonies in the recipients were transplanted into other animals that received a lethal dose of irradiation, colonies of white and red blood corpuscles were again found in the secondary recipients. Based on these experiments HSC were defined as cells with *the abilities of unrestricted self-renewal as well as multilineage differentiation.*

More than 40 years of HSC research has also shown that surface antigen markers are completely inadequate for the characterization of stem cells. Divisional kinetics and genotype markers, supplemented by xenogenic transplantation models, might be required to fully appreciate and define the genuine HSC (Weissman 2000; Ho and Punzel 2003).

4 Divisional Kinetics and Genotyping for Identification of Stem Cells

Using a combination of technologies for monitoring of cell division history and of long-term culture, Punzel et al. have correlated the division behavior of primitive $CD34^+/CD38^-$ cells with their long-term function at a single-cell level. Cells giving rise to primitive myeloid-lymphoid-initiating cells (ML-IC) were associated with asymmetric divisions and demonstrated a significantly lower division kinetics than

those giving rise to committed colony forming cells (CFCs) (Punzel et al. 2002, 2003). That most of the HSC with primitive phenotype, that is, $CD34^+/CD38^-/Lin^-$ cells, when analyzed directly after their isolation from the bone marrow, remained quiescent or had low cycling rates has been demonstrated by many other authors (e.g., Verfaillie 1993). In the nonobese diabetic/severe combined immune-deficient (NOD/SCID) mouse model, quiescent cells residing in G_0 had a significantly higher repopulating capacity than the proliferating fraction (Young et al. 1996; Metcalf 1998). By exploiting the difference in division history, $CD34^+/CD38^-$ cells could then be separated into self-renewing slow-dividing fraction (SDF) and committed fast-dividing fraction (FDF) (Fig. 2). Analyzing the gene expression profiles of SDF versus FDF derived from the same population of human $CD34^+/CD38^-$ cells, Wagner et al. found several markers that are associated with HSC to be highly expressed in the SDF (Wagner et al. 2004): *Prominin* (*CD133*), a marker associated with mobility and primitive function, the *multiple drug resistance gene 1 (MDR1)* and the *complement component 1 receptor 1* (*clqr1*) (Danet et al. 2002), the homeodomain proteins *Hoxa9,* and *cdx1*. Several adhesion proteins including *pecam-1*, *protocadherin beta 4, icam3, lfa-1,* and *integrin beta 2* were also more highly expressed in the SDF and might indicate the significance of adhesion proteins in protecting the stem cells from commitment to differentiate (Wagner et al. 2004).

Several authors have reported on the characteristics of the gene expression profiles of primitive HSC (Berardi et al. 1995; Ivanova et al. 2002; Ramalho-Santos et al. 2002). Despite the differences in the starting material used, for example, mouse versus human, in the methods of HSC preparation, and in the microarray platforms, comparative analyses have revealed several candidate genes that were highly expressed in all primitive subsets of HSC. Among the genes that were upregulated were: *frizzled 6 (fzd6)*, which functions as a receptor for the Wnt pathway, *RNA-binding protein with multiple splicing (rbpms), MDR1, Jak3*, and the homeodomain protein *Hoxa9*. These genes have been shown to play an important role in maintenance of primitive stem cell function and in asymmetric division.

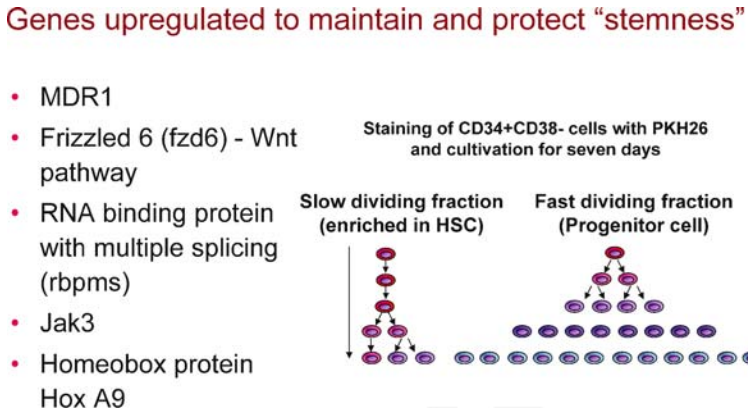

Fig. 2. Gene expression profiles of hematopoietic progenitor cells (HPC) were analyzed by microarray analysis to determine molecular mechanisms that maintain and regulate stemness. To enrich for HSC, we exploited the difference in division history: $CD34^+/CD38^-$ cells were separated into a self-renewing slow-dividing fraction (SDF)(A) and a committed fast-dividing fraction (FDF). Gene expression profiles of SDF versus FDF revealed that various molecules that have been associated with HSC and self-renewing divisions were more highly expressed in SDF(B)

5 Stem Cell Niche: The Importance of Being Connected

In a series of experiments with human HSC, our group has demonstrated that only direct cell–cell contact between HSC and specific feeder layer cells was able to maintain and promote asymmetric divisions of the former by recruiting significant numbers of primitive cells into the cell cycle (Huang et al. 1999; Punzel et al. 2002, 2003). This phenomenon of recruitment as well as the shift in asymmetric division could not be induced by cytokines (Huang et al. 1999). Thus dormant cells that are usually in G_0 can be recruited to cycle without loss of primitive function after cocultivation with stromal cells of murine and human origin. The stem cell niche thus provides the cue to regulate self-renewing divisions and subsequently to control cell numbers. In this process, intracellular as well as extrinsic mechanisms as a consequence of communication of

the daughter cells with each other or with surrounding cells might play a role (Ho 2005).

Thus local signals from the surrounding microenvironment might ensure the correct balance between stem cell self-renewal and differentiation. During spermatogenesis in *Drosophila* (Fig. 3), germ line stem cells were anchored to the hub through localized adherens junctions. Interactions between DE-cadherin on the surface of hub cells and germ line stem cells were shown to stabilize a localized binding site for β-catenin and Apc2 (Yamashita et al. 2003). The cadherin-catenin interaction and the associated cytoskeletal system therefore play a key role in this context.

6 Affinity of HSC Toward Stromal Cells

Migration of HSC toward and their subsequent communication with the niche have been shown to be mediated by podia formation (Holloway et al. 1999; Francis et al. 1998; Frimberger et al. 2001). We have demonstrated that HSC were able to migrate toward a gradient presented by murine AFT024 as well as by human MSC. Frühauf et al. and Wagner et al. have reported various types of podia formation in $CD34^+$ and $CD34^+/CD38^-$ cells and in the slow-dividing fraction of $CD34^+/CD38^-$ cells (Frühauf et al. 2002; Wagner et al. 2004). Different morphologic types of pseudopodia might be associated with specific functions (Frimberger et al. 2001; Francis et al. 2002).

HSC are characterized by rapid migratory activity and constantly changing morphology (Wagner et al. 2005). Brief interactions of podia with cells of stromal feeder layer and consistent extension of predominant pseudopodia in the direction of a stromal cell have also been reported by Frimberger et al. (2001). They suggested that podia formation was only observed among mobile cells but not among cells that adhered to a stromal cell. Wagner et al. have, however, demonstrated that most HSC remain attached to AFT024 cells through a uropod. At the site of contact CD44 and CD34 could be colocalized (Wagner et al. 2004). CD44 is known to bind fibronectin and hyaluronic acid and is essential for homing and proliferation of HSC as well as LSC (Holloway et al. 1999; Avigdor et al. 2004; Jin et al. 2006).

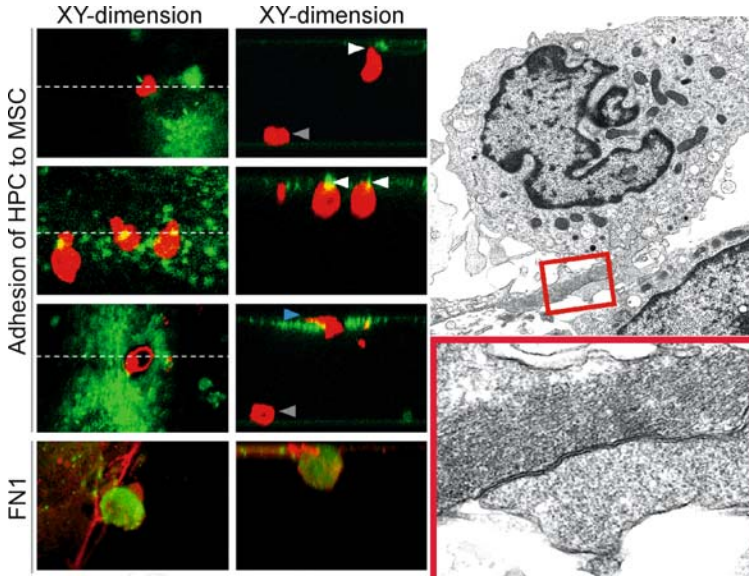

Fig. 3. Heterotypic cell–cell contact between HPC and stroma cells was analyzed with a novel adhesion assay based on gravitational force. Confocal microscopic analysis revealed that adherent HPC (*red*) remain attached to MSC (*green*) with their uropod, whereas nonadherent cells were separated by means of gravitational force and dropped to the lower glass slide (Wagner et al. 2007). HPC are interwoven in a network of fibronectin fibers. Heterotypic cell–cell junctions between HPC and stroma cells were further analyzed by electron microscopy

7 Homotypic and Heterotypic Interactions

To characterize the interactions between human HSC and stromal cells, Wuchter et al. have systematically analyzed the homotypic cell–cell contact among HSC and MSC. Whereas among HSC, defined as $CD34^+/CD38^-$ cells, no prominent junctions of cell–cell contacts were evident, remarkable junctions and junction complexes were found among mesenchymal stromal cells (MSC) (Wuchter et al. 2003). The molecular composition of these junctions was identified with a panel of antibodies specific for various components of tight junctions, adherens

junctions, and desmosomes by immunofluorescence microscopy, protein biochemical methods, as well as RT-PCR. The mesenchymal cells were interconnected by occasional gap junctions and two morphotypes of adhering junctions, namely, typical *puncta adhaerentia* and an abundant and elaborate form of variously sized, invaginating villi-to-vermiform junction complex (*complexus phalloides*) (Wuchter et al. 2007).

To explore the molecular sequelae of coculture of HSC with stromal cells, our group has studied the global gene expression profiles of HSC with or without coculture with stromal feeder layer (Wagner et al. 2005). We have shown that the regulation of gene expression in response to the cellular determinant is complex. Some genes showed upregulation within the first day of cocultivation, and these included the checkpoint (CHK1) homolog (CHEK1), early growth response protein 1 (EGR-1), and macrophage inflammatory protein-2-α precursor. Other genes showed an increasing differential expression in the subsequent 72 h of coculture. Among these were a number of genes that play a role in cell adhesion, proliferation, and DNA repair, which included CD69 (early T-cell activation antigen p60), tubulin genes, ezrin, and complement component 1q subcomponent 1 (c1qr1) (Wagner et al. 2005). Our results indicate that contact with stromal cells increased the expression of genes regulating adhesion and cytoskeleton rearrangement as well as genes controlling the genetic stability on DNA replication.

8 Significance of Adhesion Molecules for Regulation of Self-Renewal

By monitoring the division history and subsequent functional fate of HSC at a single-cell level, we could confirm that human MSC maintain stemness of HSC. Mounting evidence derived from the murine model has shown that osteoblasts at the endosteal areas of the bone might represent the HSC niche (Calvi et al. 2003; Zhang et al. 2003). However, in early phases of fetal development as well as in diseases such as myelofibrosis, human HSC survive and proliferate in extramedullary sites. Since MSC are ubiquitous in the human BM, spleen, and liver and generate osteoblasts, fibroblasts, and osteoclasts, MSC might represent an alternative niche in the human system.

By applying an anti-β_1-integrin function blocking antibody and a novel *in vitro* assay allowing the immediate identification and follow-up of HSC we have demonstrated that β_1-integrins play a significant role not only in the interaction between HSC and MSC, but also in the regulation of the long-term fate of HSC by favoring initial self-renewing divisions and the survival of primitive HSC (Gottschling et al. 2007) (Fig. 4). Treatment of human HSC with the mbAb against β_1-integrins inhibited the adhesion of HSC to MSC and induced a significant reduction in the proportion of self-renewing divisions and yield of long-term culture initiating cells (LTC-IC). Moreover, β_1-integrin-mediated contact in the first hours was crucial for the maintenance of stemness. β_1-integrin ligands alone (e.g., fibronectin, VCAM-1), however, showed no effect on the cell division symmetry and self-renewal of hematopoietic progenitor cells (HPC), indicating an indirect effect of these receptors. The relative significance of the various adhesion pathways on regulation of self-renewal are, however, not yet well defined and might be of utmost importance for understanding their relative roles in the maintenance and survival of LSC.

9 Conclusion

Cross talk with the cellular determinants in the niche LSC determines the balance between self-renewal versus differentiation of normal HSC. This probably holds true for LSC. Identification of stromal microenvironment will lead to an understanding of the intrinsic and extrinsic factors regulating normal self-renewing divisions. Molecular signals triggered by adhesion and junction complexes are responsible for the adoption of specific differentiation pathways, and the disturbances of this interaction might represent an essential mechanism for leukemic development. Through knowledge of the molecular mechanisms controlling self-renewing asymmetric divisions we might acquire the power to eradicate the ultimate source of leukemia–the LSC.

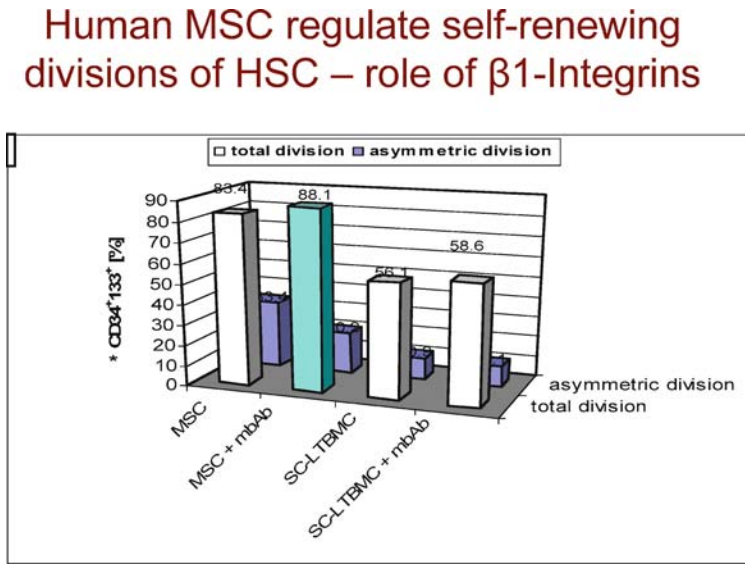

Fig. 4. β_1-Integrin plays a significant role in the adhesion between HSC and MSC. Subsequently this adhesion plays also a role in the regulation of long-term fate of HSC by favoring initial self-renewing divisions and the survival of HSC. Treatment of HSC with a blocking monoclonal antibody against β_1-integrins induced a significant reduction in the proportion of self-renewing divisions and yield of LTC-IC

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Breast Stem Cells and Cancer

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Abstract. Recent results have increased our understanding of normal stem cells and the signalling pathways which regulate them during the development of the mammary gland. Tumours in many tissues are now thought to develop from dysregulated stem cells and depend on activated stem cell self-renewal pathways such as Notch for their tumourigenic capacity. These cancer stem cells are recognised by specific cell surface proteins that they express and their capacity to grow tumours *in vivo* or spheres *in vitro*. We have described human breast DCIS mammospheres grown from cancer stem cells and demonstrated their dependence on the EGF and Notch receptor pathways. Stem cell self-renewal pathways such as these may represent novel therapeutic targets to prevent recurrence of pre-invasive and invasive breast cancer.

1 Introduction

Adult stem cells are defined by their capacity for self-renewal and differentiation into cell lineages present in a specific tissue (Morrison et al. 1997; Weissman 2000). The adult mammary gland has a lobulo-alveolar structure, composed of three cell lineages: myoepithelial cells which form the basal layer of ducts and alveoli; ductal epithelial cells which line the lumen of ducts and alveolar epithelial cells which synthesise

milk proteins (Richert et al. 2000; Rudland et al. 1998; Daniel and Smith 1999). The existence of a mammary stem cell is suggested by the cyclic development, involution and subsequent redevelopment of the mammary gland after pregnancy and lactation. Evidence to suggest that a pluripotent stem cell gives rise to both the luminal and myoepithelial cells was first demonstrated over 40 years ago when small fragments of the rodent duct or terminal end buds (TEBs) transplanted in cleared mammary fat pads of a syngeneic host could develop an entire and functional mammary tree (Deome et al. 1959; Hoshino and Gardner 1967; Daniel et al. 1968; Ormerod and Rudland 1986). Further studies observing the pattern of X chromosome inactivation throughout the ductal and lobular epithelium show that contiguous patches of epithelium with inactivation of the same X chromosome were present throughout the human breast, suggesting that the cells within each patch had been derived from the same stem cell (Novelli et al. 2003; Tsai et al. 1996).

Techniques to enrich for breast stem cells have subsequently been developed, including isolating a sub-population of mouse mammary epithelial cells defined by its ability to efflux the dye Hoecht 33342. The cells were termed the 'side population' (SP) and were found to include cells capable of regenerating a functional mammary gland system in a cleared fat pad (Welm et al. 2002). A similar side population has also been identified from human breast tissue (Clarke et al. 2005; Clayton et al. 2004; Alvi et al. 2003; Dontu et al. 2003a); this provided a functional parallel to the SP-containing haematopoietic stem cells in bone marrow (Goodell et al. 1997). More recently purification of mouse mammary epithelial stem cells with cell surface markers Lin^{-}, CD29hi and $CD24^{+}$ demonstrated these cells are highly enriched for mammary stem cells by transplantation and showed that a single cell, marked with a LacZ transgene, can reconstitute a complete mammary gland *in vivo* (Shackleton et al. 2006; Stingl et al. 2006).

There are many similarities between stem cells and cancer cells: Both self renew, although cancer cells are poorly controlled, unlike somatic stem cells, which are highly regulated. Differentiation also occurs where somatic stem cells generate normal, mature cells of the specific tissue; however, this is usually abnormal in cancer cells (reviewed by Pardal et al. 2003). Therefore, stem cells are an attractive candidate as the origin of cancer, as they would already have active self-renewal pathways

and over their long life span mutations and epigenic changes in the dysregulated pathways mentioned can occur, allowing for increasing evolution towards malignancy. Reviews have proposed a model describing cancer stem cells derived from mutated adult stem cells (Dontu et al. 2003b; Reya et al. 2001); the fact that leukaemic stem cells have a surface marker phenotype that is similar to normal haematopoietic stem cells supports the idea that they arise from haematopoietic stem cells. The model also suggests that in addition to stem cells, early or late progenitors could also serve as targets for these transforming events; however, if this was the case these cells would need to acquire mutations not only to promote malignancy but also to enable them to undergo self-renewal.

Signalling pathways involved in normal mammary stem cell regulation, including WNT (Liu et al. 2004; Li et al. 2003), Hedgehog (Dontu and Wicha 2005), Notch (Dontu et al. 2004; Stylianou et al. 2006), LIF (Kritikou et al. 2003), TGF-β (Ewan et al. 2005; Boulanger et al. 2005) and EGF families (Dontu et al. 2003a), prolactin (Dontu et al. 2003a), estrogen and progesterone (Clarke et al. 2005) are known to be dysregulated in many cancers (Fig. 1). In particular our group have reported that Notch receptor signalling is aberrantly activated in breast cancers compared to normal breast (Stylianou et al. 2006). Levels of Jagged 1 and Notch 1 have previously been correlated with poor prognosis (Reedijk et al. 2005). In a very recent study discussed below, we have demonstrated the importance of the Notch receptor signalling pathway in breast ductal carcinoma in situ stem cells (DCIS).

2 Cancer Stem Cells

There is now a large body of evidence showing that leukaemia originates from a cancer stem cell (Reya et al. 2001). The first evidence for cancer stem cells described a small but variable proportion of human acute myeloid leukaemia (AML) cells, which could be identified and purified with cell surface markers $CD34^{+}CD38^{-}$, and were found to be the only cells capable of transferring AML from human patients to NOD/SCID mice (Bonnet and Dick 1997), providing evidence that not all AML cells have clonogenic capacity and only a small subset of

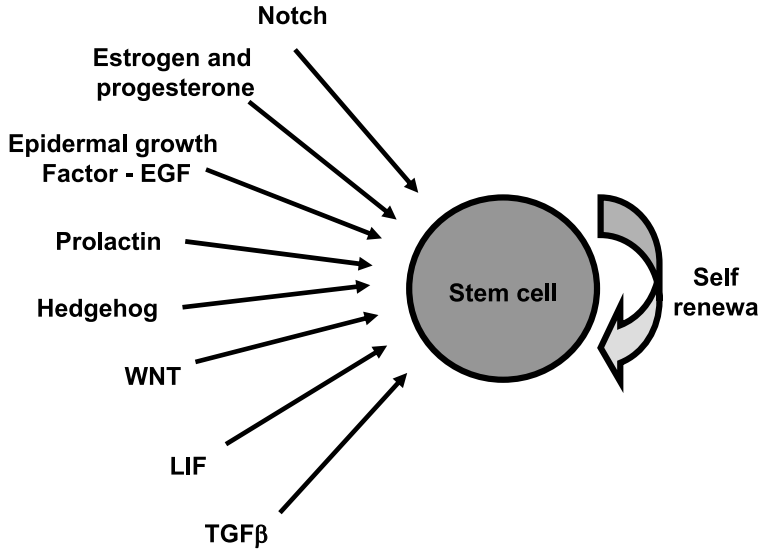


Fig. 1. Self-renewal pathways involved in regulating the normal breast stem cell

cells (the cancer stem cells, CSCs) are capable of regenerating the cancer. Solid cancers are known to be phenotypically heterogeneous and clonogenic in culture; therefore, many groups have extrapolated the cancer stem cell hypothesis from the haematopoietic system to solid cancers. Cells with stem cell characteristics from brain tumours were first isolated with clonogenic neurosphere culture technique from human glioblastoma (Ignatova et al. 2002), and now other groups have independently confirmed that brain tumours contain neurosphere-forming cells (Singh et al. 2003, 2004; Hemmati et al. 2003). These cells are highly enriched for cell surface marker CD133 and nestin, have a marked capacity for proliferation and self-renewal and are capable of differentiating *in vitro* into phenotypes identical to the tumour in situ. Cancer stem cell populations have also been found in prostate (Collins et al. 2005; Lawson et al. 2007), pancreas (Li et al. 2007)[39], colon (O'Brien et al. 2007; Ricci-Vitiani et al. 2007) and breast cancer (Al-Hajj et al. 2003).

For example O'Brien et al. (2007) reported a xenograft model using subrenal implantation of human colon cancer cell suspensions into pre-irradiated NOD/SCID mice where 17 out of 17 primary or metastatic colon cancer samples formed tumours which resembled the original tumour from which they were derived; these tumours could be passaged and re-form tumours in secondary and tertiary recipients. Fractionation of colon cancer cells based on expression of CD133, a potential cancer stem cell marker, revealed that the proportion of $CD133^+$ cells ranged from 1.8% to 24.5% within the colon cancer samples and that after implantation into NOD/SCID mice only 1 out of 47 mice injected with a $CD133^-$ population formed a tumour compared to 45 out of 49 when $CD133^+$ cells were implanted. Limiting dilution experiments determined that 1 in every 262 $CD133^+$ colon cancer cells was capable of re-initiating a tumour (O'Brien et al. 2007). Ricci-Vitiani and colleagues took a similar approach in sorting for $CD133^+$ primary colon cancer cells, where again they found that the $CD133^+$ population was enriched for cells which give rise to subcutaneous tumours in SCID mice, which could re-form tumours after re-implantation into secondary and tertiary mice. An *in vitro* culture system was also used to grow colon cancer cells as colon spheres similar to neurospheres, which allows the cells to grow in an undifferentiated state. Spheres were enriched for $CD133^+$ cells and were capable of growing tumours in mice, in contrast differentiated colon cancer cells were not tumourigenic. The study also demonstrated that primary colon cancer cells grown as spheres for over 1 year were still capable of initiating tumours with morphology similar to tumours formed before long-term culture (Ricci-Vitiani et al. 2007). Both studies are in line with the cancer stem cell hypothesis which suggests that tumours are generated and maintained by a small subset of undifferentiated cells able to self-renew and differentiate into the bulk tumour population (Wang and Dick 2005).

In the breast, the study by Al Hajj et al. (2003) was the first to identify a subpopulation of human breast cancer cells which initiated tumours in NOD/SCID mice, using a set of cell surface markers to sort cells with an increased tumourogenic capacity. Cells which were $CD44^+$, $CD24^{low}$, ESA^+ and $lineage^-$ (cells lacking markers CD2, DC3, CD10, CD16, CD18, CD31, CD64 and CD140b) isolated from one primary breast cancer and nine metastasis were able to form heterogeneous tumours

eight out of nine times. The tumours contained not only the $CD44^+$, $CD24^{low}$, ESA^+ and $lineage^-$ tumour initiating cells but also the phenotypically diverse non-tumourigenic cells which comprise the bulk of tumours. As few as 200 $CD44^+/CD24^{low}/ESA^+/lineage^-$ cells implanted into NOD/SCID mice could form tumours four out of four times, while no tumours formed when 200 cells from the $CD44^-/CD24^+/ESA^-$ cell population were used (Al-Hajj et al. 2003). A subsequent study carried out on 16 breast lesions with the sphere culture technique which has been used to enrich for normal breast stem cells (Dontu et al. 2003a) resulted in the production of three long-term primary cultures which had self-renewing capacity and could differentiate into the different breast lineages. The sphere forming cells were found to be 96%–98% $CD44^+/CD24^-$; however, cells with self-renewal only accounted for 10%–20% of the total cell number, showing that only a sub-group within the $CD44^+/CD24^-$ sorted cells had self-renewal capacity (Ponti et al. 2005), which is consistent with only 1 in 200 cells being capable of initiating a tumour in the previous study (Al-Hajj et al. 2003). This indicates that sorting for a $CD44^+/CD24$ population enriches for tumour-initiating cells; however, it highlights the need for additional markers to isolate the true CSC. Tumour-initiating capacity was measured with a long-term sphere culture of the breast cancer cell line MCF7, termed MCF-S. $CD44^+/CD24^-$ cells from the MCF-S or MCF7s (used as a control) were implanted into the mammary fat pad of SCID mice. The MCF7 cells gave rise to tumours when at least 1 million cells were implanted; however, the MCF-S cells gave rise to tumours with smaller numbers of cells (10^5, 10^4 and 10^3) with at least a 60% success rate, whereas the MCF7s showed no growth when a comparable cell number was implanted (Ponti et al. 2005), thus indicating that both the mammosphere culture system and the cell surface marker selection enriched for tumour-initiating cells.

Further studies in breast cancer cell lines and most recently in WNT-induced mouse mammary tumours have added to the growing evidence for CSC in the breast. Hoechst dye exclusion was used to isolate a side population (SP) in MCF7 cells (0.2%); this population had a greater tumourigenic capacity than the non-SP fraction when determined by tumour production subcutaneously in NOD/SCID mice. The MCF7 SP

also expressed higher levels of Notch1 and β-catenin mRNA compared to the non-SP population, suggesting that the SP cancer cells have some intrinsic properties of stem cells (Patrawala et al. 2005). The SP population within hyperplasic tissue from mouse mammary tumour virus (MMTV)-driven Wnt-1 transgenic mice was >2-fold increased compared to matched background controls (Woodward et al. 2007); radiation was shown to selectively enriched progenitors in the activated WNT cells compared to background-matched control mice. A recent paper suggests that breast cancer-initiating cells are radioresistant, firstly showing that MCF7 and MDA-MB-231 breast cancer cells grown as mammospheres have elevated numbers of $CD24^{-/low}/CD44^{+}$ cells; they were also found to be more radioresistant than the corresponding cells grown in monolayer when compared by clonogenic assay (Phillips et al. 2006). A comparable but more extensive study in glioblastomas showed not only that $CD133^{+}$ tumour cells were more radioresistant than the $CD133^{-}$ but that ionising radiation also increased the proportion of $CD133^{+}$ cells from glioblastoma specimens (Bao et al. 2006). The $CD133^{+}$ population preferentially activate the DNA damage checkpoint response to DNA damage and repair radiation-induced DNA damage more effectively than $CD133^{-}$ cells, suggesting that $CD133^{+}$ cells could be the source of tumour recurrence in patients after radiation. A specific Chk1 and Chk2 inhibitor used in this study was found to reverse this radioresistance *in vitro* and *in vivo*, indicating that targeting DNA damage check points may disrupt this resistance mechanism and improve tumour control with radiation treatment.

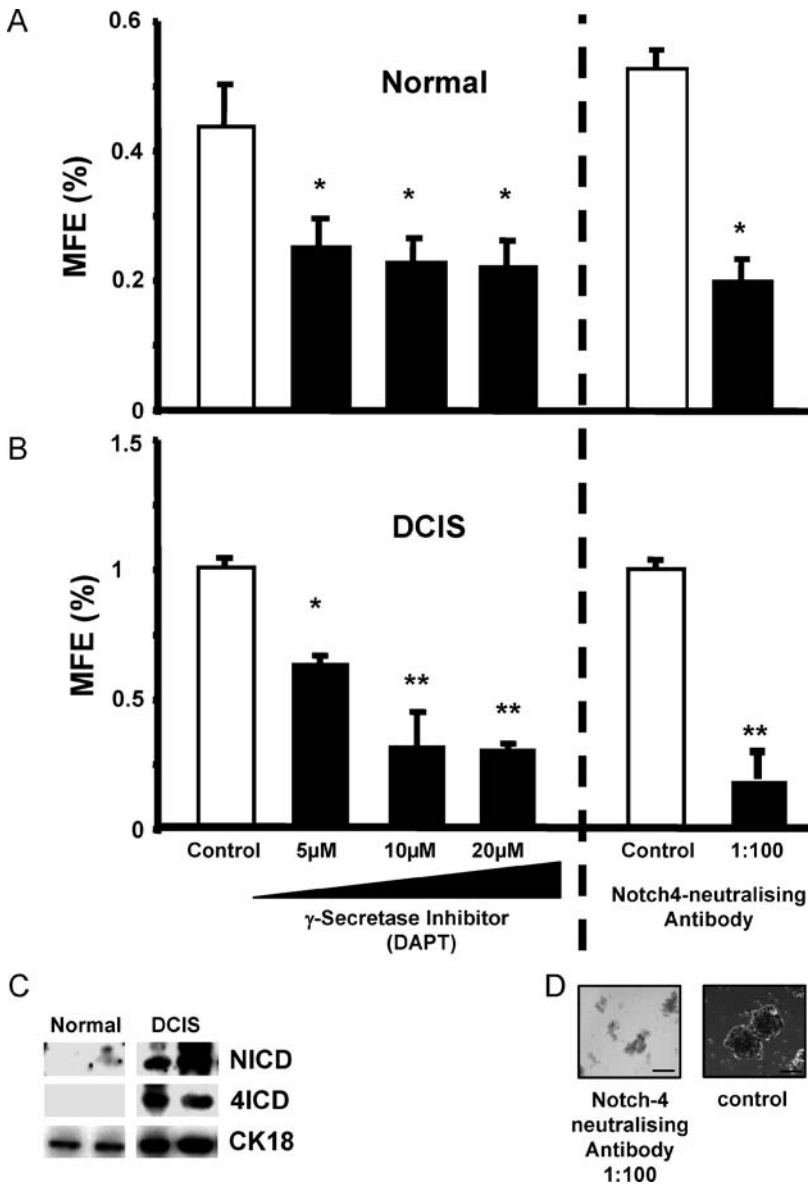
3 DCIS Mammospheres–Importance of EGF and Notch Signalling

We have adapted the mammosphere culture system which has been used to enrich for normal stem cells and populations of cells from cancers with a greater tumourigenic capacity to grow primary DCIS mammospheres (Dontu et al. 2003a; Singh et al. 2004; Ricci-Vitiani et al. 2007; Ponti et al. 2005; Farnie et al. 2007). Compared to normal breast cells grown under the same non-adherent culture conditions, primary DCIS tissue contained a greater number of cells with the ability to form mam-

Fig. 2. Mammosphere-forming efficiency (*MFE*) in normal breast (**A**) and DCIS (**B**) after treatment with DAPT and a Notch 4 neutralising antibody compared to equivalent DMSO or IgG control, respectively. Mean %MEF±SE, two-tailed test: $*P<0.05$, $**P<0.01$. (**C**) Western blotting showing expression levels of Notch 1 and 4 intracellular domain (NICD, 4ICD) in normal breast and DCIS tissue. Cytokeratin (CK) 18 is shown as a control for epithelial content of tissue. (**D**) Brightfield image of DCIS mammospheres after treatment with a Notch 4 neutralising antibody or an IgG control

mospheres (Fig. 2 A and B), DCIS mammosphere-forming cells also had a greater self-renewal capacity than normal breast mammospheres as they could produce a greater number of new mammosphere generations after passage. High-grade DCIS also had an increased MFE compared to low-grade DCIS; this is consistent with findings from both brain and colon cancers, where the most aggressive clinical samples have tumour stem cells with the highest sphere formation and self-renewal capacity (Singh et al. 2003; Ricci-Vitiani et al. 2007).

The non-adherent DCIS culture system has allowed the investigation of pathways which are involved in the survival and self-renewal of DCIS. Our previous xenograft work has shown that EGFR signalling is required for the growth of DCIS tumours (Chan et al. 2002). We found that high-grade DCIS mammospheres have a greater sensitivity to an EGFR inhibitor, gefitinib, compared to low-grade DCIS in the absence of exogenous EGF, suggesting secretion of an EGF-like ligand from the high-grade DCIS which is regulating mammosphere initiation and/or growth via the EGFR signalling pathways. Notch signalling has been shown not only to play a role in normal stem cell regulation but also to be frequently dysregulated in a number of cancers, including invasive breast cancer (Dontu et al. 2004; Stylianou et al. 2006). Western blotting confirmed that Notch signalling was highly activated in DCIS compared to normal breast tissue, showing that both Notch 1 and 4 intracellular domain (NICD and 4ICD, respectively) were elevated (Fig. 2C). We then used two Notch inhibitors in DCIS and normal breast mammosphere culture, mammosphere formation was reduced with both a γ-secretase inhibitor (DAPT) and a Notch 4 neutralising antibody. How-



ever ,the DCIS mammospheres were more sensitive to both inhibitors; in particular the Notch 4 blocking peptide where in two out of six cases caused 100% inhibition of mammosphere growth (Fig. 2D). Our results suggest that targeting both of these pathways may have therapeutic value as adjuvant therapy for DCIS.

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Prostate Cancer Stem Cells: A Target for New Therapies

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Abstract. Prostate cancer is now a common disease in men over 50 years of age. Medical therapies for prostate cancer are based on discoveries from the mid-twentieth century, and in the long term are rarely curative. Most treatments are directed towards an androgen receptor-expressing, highly proliferative target cell, which does indeed form the vast majority of cells in a prostate tumour. However, by invoking the existence of a cancer stem cell which, like normal epithelial stem cells in the prostate, does not express androgen receptor and is relatively quiescent, the observed resistance to most medical therapies can be

explained. The phenotype of the prostate cancer stem cells is that of a basal cell and cultures derived from cancers, but not benign tissues, express a range of prostate cancer-associated RNAs. Furthermore, stem cells purified on the basis of $\alpha_2\beta_1$ high integrin and CD133 cell surface antigen expression, from an established culture of Gleason 4 (2+2) prostate cancer (P4E6), were able to form multiple intraprostatic tumours in nude mice when grafted orthotopically in a matrigel plug containing human prostatic stroma. The final tumours re-expressed androgen receptor and displayed a histology similar to that of a Gleason 4 cancer.

1 Introduction

1.1 Prostate Cancer Therapy: A Historical View

Despite intensive study, we still know little about the aetiology of prostate cancer. The patient risk group is well defined, namely elderly men, and as a result most therapies and aetiological studies have centred on male sex hormones. Initially, as a result of the pioneering work of Charles Huggins almost 70 years ago (1941), removal of androgen supply by either orchiectomy or the chemical blockade of adrenal androgen and subsequent direct inhibition of androgen receptor binding to the activated (dihydrotestosterone) form of androgen (since the 1980s) provided almost instant relief from symptoms and longer-term tumour regression (Furr 1996). While these treatments remain palliative, prostate cancer eventually derives resistance to androgen-based therapies and returns in a frequently more aggressive, androgen independent form, which is fatal within 2 years of recurrence on average.

Even the more sophisticated medical therapies, such as optimised taxotere regimes (Tannock et al. 2004; Mackler and Pienta 2006), can do little to stem the inevitability of disease outcome, and the more common anti-mitotic drugs are equally ineffective. This poses a considerable challenge, not only to clinicians, but also to basic scientists. Recently, a first stage in describing the androgen-insensitive phenotype was described by Chen et al. and Holzbeierlein et al. (Chen et al. 2004; Holzbeierlein et al. 2004). Most strikingly, rather than an inactivating androgen receptor (AR) gene mutation a majority of recurrent prostate cancers show amplification of the AR gene, at its locus on the X chromosome (Xq11.2–12), which is present in only one copy in men. In-

deed, when AR mutations are observed, they frequently result in either enhanced androgen binding or a relaxation of the steroid specificity of the receptor, enabling it to activated gene expression after binding to other steroids, estrogens and even the commoner antagonists used for therapy (Culig et al. 1993; Linja and Visakorpi. 2004). Zero function mutations, such as those found in androgen insensitivity patients, are frequently toxic when re-expressed in prostate epithelial cells (Birnie and Maitland, unpublished data). However, the mechanism whereby the AR gene amplification arises remains unknown.

2 Transgenic Mouse Models of Prostate Cancer

A further role for androgens was provided by transgenic mouse experiments, such as the TRAMP system (Greenberg et al. 1995), where a potent viral oncogene (T antigen from SV40) was linked to a strong promoter (probasin) active in prostate luminal cells in rodents and introduced into the germ line of mice. The resulting TRAMP mice developed premalignant disease which eventually gave rise to carcinomas, which were androgen sensitive. A full description of this system and more recent developments using conditional and total knockouts of known prostate tumour suppressor genes such as PTEN can be found in Winter et al. (2003) and Kasper (2005).

3 Prostate Cancer: A Disease of Epithelial Differentiation

Our starting point was to treat prostate cancers not as an endocrinological disorder but as an epithelial disease, which had the added complexity of androgen responsiveness, in a proportion of cells. Inherent in most epithelial systems is a pre-programmed differentiation series in which the terminally differentiated cell, which produces the secretory proteins characteristic of the tissue, has resulted from a maturation through one or more epithelial cell intermediates. A major challenge in epithelial biology has been to define the phenotype of these intermediate cells, as they are frequently described by their expression of antigens and proteins, which have little to do with the whole differentiation process. As

such, the diversity of cytokeratin expression can provide insights into cellular diversity, and they have been exploited in prostate to establish rudimentary cell lineages (Hudson et al. 2001). The proposed maturation of epithelial cells in the normal human prostate is shown in Fig. 1. From the quoted references it is clear that the two major epithelial compartments in prostatic epithelium, namely the basal and luminal cells, can be subdivided into for example a basal-like transit amplifying cell and perhaps an immature luminal cell. Both of these cell types can be considered to be lineage committed with the resultant luminal cells as the terminal form, lacking both proliferative and long-term survival capacity. Within the true basal compartment, it is now accepted that there exist stem cells with a capacity for unlimited or prolonged self-renewal and the ability to produce at least one type of highly differentiated descendant. It is also accepted that between the stem cell and its terminally differentiated progeny there is an intermediate population of committed progenitors with limited proliferative capacity and restricted differentiation potential, sometimes termed transit amplifying (TA) cells. Furthermore, these normal tissue stem cells (nSC) have special protective mechanisms to resist phenotypic and genetic change such as drug efflux proteins and retention of the parental DNA strand during DNA replication and mitosis. The stability of the nSC may also partly be due to the immediate environment, sometimes called the ‘stem cell niche’, where the stem cell can sense and react to its contact with basement membrane as well as its spatial contact laterally with other basal cells and apically with more differentiated progeny. Definition of the essential signals for maintenance of ‘stemness’ has proved an elusive goal, but as methods for separation purification and tissue reconstruction from tissue stem cells have improved, it is becoming clear that these signals are shared with signalling pathways previously recognised to play a role in oncogenesis and indeed normal embryonic development (Pardal et al. 2003; Beachy et al. 2004).

4 Stem cell Expansion In Vitro

The challenge in all studies of this nature has been to achieve *purity* in the putative stem cell population and the paradox that a stem cell

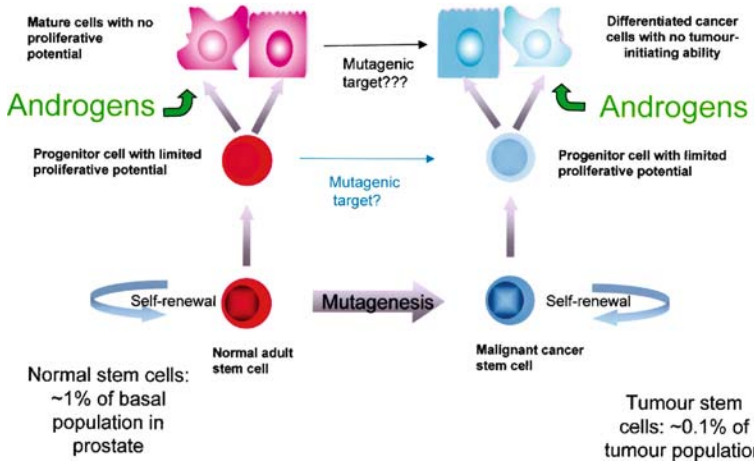


Fig. 1. Hypothetical epithelial cell differentiation in normal and malignant human prostate. The scheme shows major intermediates in epithelial cell differentiation and indicates possible points at which mutagenesis and hence carcinogenesis can occur. The influence of androgens on the epithelial cells is solely on the most differentiated (luminal) cells, although some prostatic stromal cells also express AR and can influence epithelial cell growth and differentiation

may sacrifice its 'stemness' when induced to proliferate. Development of stem cell media, and the discovery of small molecules (such as LIF, EGF and FGF2) which can promote expansion of the stem compartment relative to more differentiated forms, has also been a major step forward. However, the complex nature of epithelial tissues has made them more resistant to this type of analysis, relative for example to the haematopoietic system, where markers and lineage determination are more advanced, although as shown by some recent studies in T cell subsets (reviewed in Lefrancois and Marzo 2006) are not terminally defined.

5 Definition of the Stem Cell Phenotype in Prostate

In prostate epithelium, the existence of stem cells was originally proposed by Isaacs and Coffey (1989) based on the available tools at that

time. Further refinement of the putative stem cell compartment was possible with the use of defined cytokeratin profiles (De Marzo et al. 1998) However, these markers are of little use for tissue fractionation. What was needed were robust plasma membrane expressed proteins with the ability to define the stem cell compartments. Whilst it was clear that the multiply spliced CD44 antigen had discriminatory power for basal cells over the more abundant and protein content-rich luminal cells, a major breakthrough came from the study of cell adhesion molecules. As first reported by Collins et al. (2001), a heterogeneity can be observed in the basal epithelial layer of normal prostate with respect to the collagen binding (and hence basement membrane associating) $\alpha_2\beta_1$-integrin complex. It was proposed that these integrin 'bright' cells would adhere more rapidly to collagen matrix after tissue dissociation and would result in a cell preparation with enhanced clonogenicity and self-renewal capacity *in vivo* and *in vitro*. This was indeed the case, although the resultant preparation was by no means homogeneous. Further enrichment of this small fraction (1%–5%) of the basal cells was achieved by employing the CD133 (originally AC133) antigen (Yin et al. 1997; Corbeil et al. 2000) expression, which was found in a subset of the integrin-bright basal cells. The resultant population exhibited enhanced clonogenicity *in vitro* and, upon engraftment as a 'micro-prostate' in a matrigel plug containing prostate stromal cells into a subcutaneous site in nude male mice in the presence of androgen, a capacity to produce vestigial prostate glands. These luminal structures now expressed markers of differentiated prostate such as androgen receptor and prostatic acid phosphatase (Richardson et al. 2004).

The requirement for prostatic stromal cells, which also contain an androgen-responsive fraction was absolute, and recapitulated experiments carried out 30 years earlier, when Cunha (1975) showed that coengraftment of both ovarian and prostatic murine epithelium with male urogenital sinus mesenchyme resulted in development of prostatic epithelium independently of the source of the epithelium (male *or* female). Indeed, our own *in vitro* prostate reconstructions confirmed the requirement for prostatic stroma in order to recapitulate the entire differentiation program, including polarisation and secretion of 'prostate-specific' products (Lang et al. 2001).

The $\alpha_2\beta_1^{Hi}$/CD133$^+$/CD44$^+$ population therefore enabled a profound enrichment of the epithelial stem cell population from normal human prostate. However, it constituted a very small fraction of cells from any non-malignant human prostate tissue, and had a limited life span *in vitro*, despite growth medium optimisation. The stemness inherent in this cell type has now been independently verified in a number of other tumour types (recently reviewed by Clarke et al. 2006)

6 The Origins of Prostate Cancer

By treating prostate cancer as a disease of epithelium, rather than a consequence of androgen action, it is possible to reconsider its origins. The presence of an initially androgen receptor-expressing (largely luminal) phenotype in tumours has directed research towards the luminal cells as the target for oncogenic change (reinforced by the transgenic mouse studies). Whilst this hypothesis implies that the androgen-independent phenotype is the result of a forced de-differentiation from luminal to more basal characteristics, it is more logical to hypothesise that the basal cell, and indeed the stem cell within that compartment, is the target for the original oncogenic hit(s) and that the resultant phenotype is the consequence of an aberrant but close to normal differentiation pattern. There is now precedent for this in a number of other tumour systems, for example in human breast cancer where the most primitive cell appears not to express the estrogen receptor (Dontu et al. 2004), although this remains controversial (Clarke et al. 2005) and the proposed longevity of the normal stem cell would provide the life span and number of cycles of self-renewal necessary for the establishment of a founder clone within the prostate epithelium. Under a de-differentiation model, the heterogeneity of gene expression (Liu et al. 2004) and indeed genotypic changes seen in any one region of the prostate (Macintosh et al. 1998) can only be explained by a large number of independent and complex genetic changes. This could only be the result of extreme genetic instability, as a result of repeated mutagenic exposure–for example in the oral cavity where the term *field cancerisation* has been applied (see review by Perez Ordonez et al, 2006) – and the necessary components for various types of recombination and DNA repair-based instabilities are

rather rare in prostate cancers in comparison with most other common types (Rybiki et al. 2004).

If we consider that oncogenic change in a prostate gland starts as a relatively rare event, which becomes established in a particular gland because of enhanced survival of the founder clone of stem cells (with or without the influence of stromal factors), given the presumed rarity of tissue stem cell division (sometimes considered to be quiescence), a stem cell with a proliferation/survival advantage could become established as the major population in a particular gland. A mathematical model for this has recently been published, using the better fundamental knowledge available for stem cell kinetics in human colon (Calabrese et al. 2004).

What then causes this change? There are no known mutagens in prostate, although the gland will undergo proliferative changes in response to steroids and perhaps sexual activity. For example, low androgen and high estrogen levels can result in involution and prostatic remodelling. The prostate is also profoundly sensitive to infection, as shown by the frequency of prostatitis in the human population (MacLennan et al. 2006). The commonest result of this is an inflammatory response, with resultant cytolysis and requirement for repair (Karin 2006). Under these conditions the 'activated' stem cell is likely to accrue an advantage, and were an element of genetic instability to be present, then further changes in response to the new environment could occur. It has been suggested that the earliest such changes are observed as prostatic inflammatory atrophy (De Marzo et al. 1998; Nelson et al. 2004). The changes could be reversible, such as chromatin remodelling and methylation (Yegnasubramanian et al. 2004), resulting in a flexible stem cell compartment, which responds to environmental fluctuation (Feinberg et al. 2006). Such epigenetic changes can also become more permanent. A more committed change, such as gene translocation to 'fix' an advantageous gene in an active conformation, would result in a short-term advantage, but might be deleterious or at best neutral when the status quo is restored. Such translocations are common in leukaemias, but have only recently been observed in prostate cancers, where activation of the erg oncogene is one common result in more than 50% of advanced prostate cancers (Tomlins et al. 2005). Even with a simple theoretical treatment of oncogenic initiation, the potential for hetero-

geneity and the 'dead-end tumours' which are frequently found in the prostates of elderly men (Franks 1954) is apparent.

7 Isolation of Prostate Cancer Stem Cells

The difficulty with this hypothesis remains the final phenotype of prostate cancers. If the mutagenesis of a normal stem cell resulting in a tumour stem cell hypothesis is correct, then one possibility is that the cancer stem cell (CSC) will retain at least some of the phenotypic properties of its nSC origin. We decided to test this hypothesis by fractionating cells from malignant human prostates, using the cell surface markers which had been so successful in sub-fractionation of normal tissues. Using originally macrodissected radical prostatectomy specimens and lymph node excisions, we obtained rare epithelial cells , which proliferated *in vitro* after a distinct and reproducible 'lag' phase of 10–14 days. Thereafter, on a collagen matrix containing irradiated murine stroma, under conditions which did not favour epithelial differentiation, a rapid proliferation was observed.

With this technique (Collins et al. 2005) similar cultures were derived from multiple primary prostate cancer tissue samples and several primary lymph node metastases. The properties of these cells are summarised in Table 1. Despite the invasive nature of the prostate cancer stem cells *in vitro*, confirmation of oncogenic origin was dependent on both *in vivo* xenografting and gene expression profiling of the purified cell populations. Firstly, orthotropic xenografting of fewer than 1,000 P4E6 cells with the $\alpha_2\beta_1^{Hi}$/CD133$^+$/CD44$^+$ phenotype was tumour initiating when xenografted into the prostates of nude mice in a matrigel plug containing viable prostatic stroma (from a different patient) (Table 2). Significantly, the luminal (AR expressing) fraction of the same tissues (selected by expression of CD57-expressing cells) had much lower tumour-initiating capacity. This result is difficult to reconcile with the transgenic mouse models of prostate cancer, in which the TRAMP mouse was generated by expression of a powerful oncogene under the control of a luminal cell gene promoter (probasin). Other more recent models (Kasper 2005), particularly conditional knockouts of genes relevant to human disease and with no obvious reliance on AR expression,

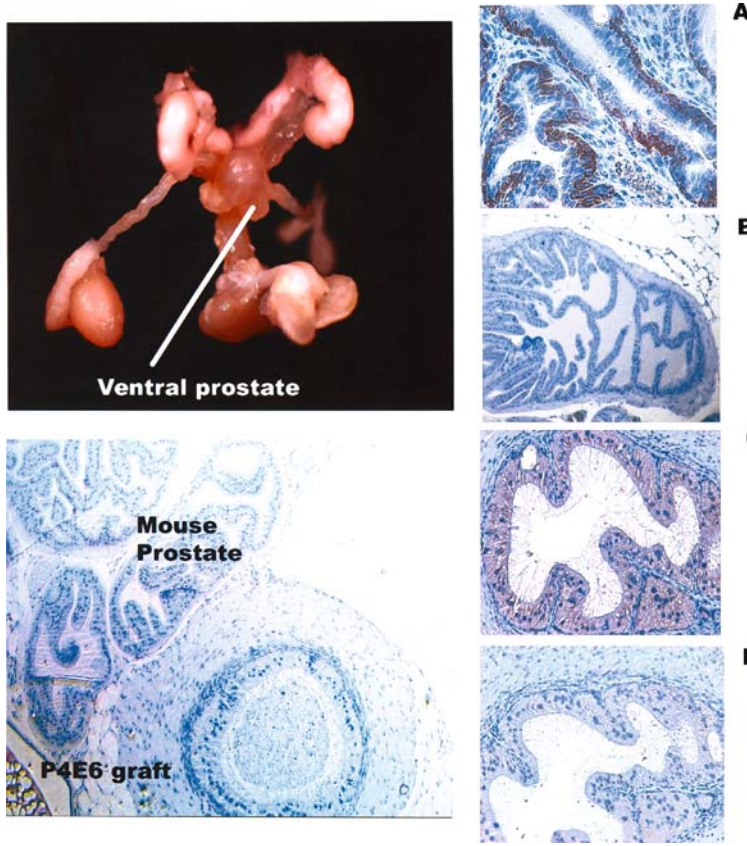
Fig. 2. Orthotopic xenografts of P4E6 stem cells. *Left panels* show the mouse prostate and indicate the target for the xenografted human cell plug. *Lower left panel* is a haematoxylin and eosin -stained section of a P4E6 tumour-bearing prostate, indicating the morphological differences between human and mouse prostate. *Four panels on the right* are immunohistochemically stained (brown peroxidase stain) sections of grafted tissues as follows: **A** P4E6 graft showing expression of basal cytokeratin; **B** Section of murine prostate stained with the same anti-human basal cytokeratin antibody (negative); **C** P4E6 graft stained for androgen receptor (nuclear stain in luminal cells); **D** P4E6 graft stained for prostatic acid phosphatase (cytoplasmic stain in luminal cells)

are more consistent with our hypothesis. The resultant tumours derived from the primitive basal phenotype of the CSCs did express differentiated (luminal) epithelial cell proteins such as luminal cytokeratin, prostatic acid phosphatase (PAP) and AR (Fig. 2).

Secondly, an Affymetrix gene expression array analysis of RNA from two cultures derived from different cancer patients (cells amplified from an original $CD133^+$ population) compared to three pooled benign prostatic hyperplasia (BPH) cultures revealed elevated expression of genes

Table 1 Properties of prostate cancer stem cell cultures

PCSCs have a greatly expanded life span (> 30 pd compared to a maximum of 10–12 for non-malignant tissue)
PCSCs are highly invasive (> 3-fold higher than most invasive prostate cell line PC3M)
PCSCs are highly clonogenic in vitro (2D and 3D)
PCSCs have a basal phenotype but can differentiate to an AR^+ luminal phenotype
PCSC-derived cell colonies express cancer-associated genes
PCSCs show some evidence of microsatellite instability
In a PCSC culture the content of $CD133^+$ cells does not vary significantly
The $CD133^+$ content of prostate tumours is not related to the stage or grade of the original tumour



previously associated with prostate cancers only in the cancer samples (Fig. 3). Furthermore, high expression of matrix metalloproteinases (not shown) provided a molecular explanation for the 2–3 fold increased invasive capacity of the stem cell-derived cultures, relative to the most invasive established prostate cancer cell line PC3M (Collins et al. 2005).

One striking feature of the long-term cultures was the constant proportion of $CD133^+$ cells, after up to 30 population doublings and independent of the original grade or stage of the tumour. While the former proportions have been observed in other *in vitro* culture systems (such as neuropheres and mammospheres) for cancer stem cells, there have been reports of higher proportions of CSCs in higher-grade neural tumours (Singh et al. 2003, 2004) . Thus the *in vitro* cultures are not optimised for cancer *stem cell* amplification alone. The constant $CD133^+$ proportion is suggestive of a self-renewal mechanism whilst the bulk of the culture is an amplifying (non-stem) population as illustrated in Fig. 4. The challenge of optimising *in vitro* culture conditions to amplify only the stem cell population therefore remains, and a longer-term risk of cross talk between the more committed amplifying cells and the primitive basal cells is frequently observed in prostate as in keratinocyte stem cells. Indeed, induction of the terminal differentiation program can be activated by inducing culture stress and the addition of androgens (Collins et al. 2005).

Table 2 P4E6 stem cell xenografts

P4E6 cell fraction		5×10^5 Cells	$<1{,}000$ Cells
Unselected cells	Total Culture	5/5 mice Multiple tumours/section	
$\alpha_2\beta_1{}^{hi}/CD133^+$	Stem		5/5 mice Multiple tumours/section
$\alpha_2\beta_1{}^{low}/CD133^-$	Transit amplifying	2/5 mice Two or less tumours/section	
$\alpha_2\beta_1{}^{low}/CD57^+$	Luminal	0/5 mice	

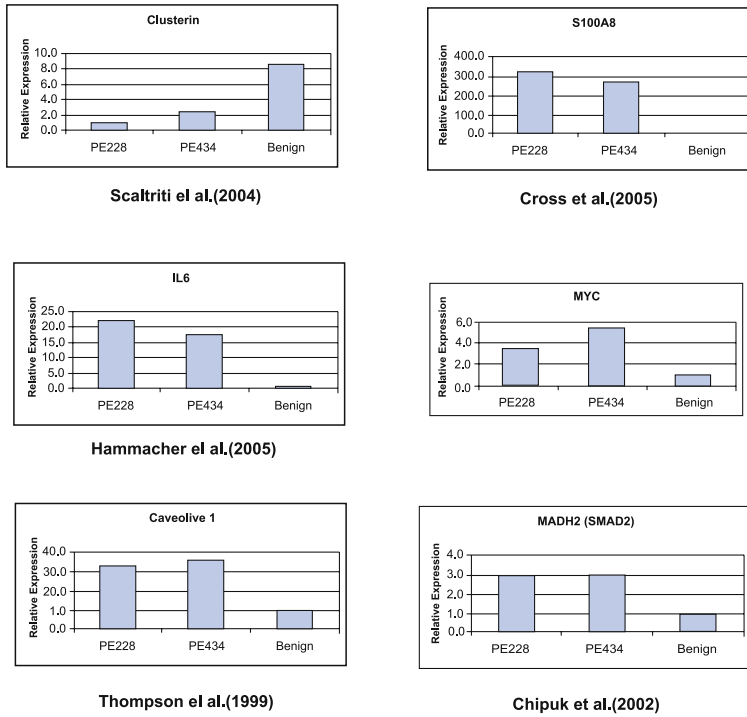


Fig. 3. Expression array analysis of cell cultures derived from malignant and non-malignant stem cells. Below each panel is a literature reference to the cancer-specific expression pattern of each gene. c-myc is generally upregulated in all tumour types. Note that the cells used to produce the RNA for the expression analysis are mainly TA cells originating from CSCs (PE228 and PE434)

8 Gene Expression in Prostate Cancer Stem Cells

The ability to amplify different cell populations *in vitro* has allowed us to identify sets of genes which form a 'fingerprint' for the cancer stem cells relative to the amplifying and terminally differentiated cells. The same comparison can be made between these cell classes from non-malignant and cancer tissues (Collins et al., manuscript in preparation). Novel sets of genes from therapeutically important classes

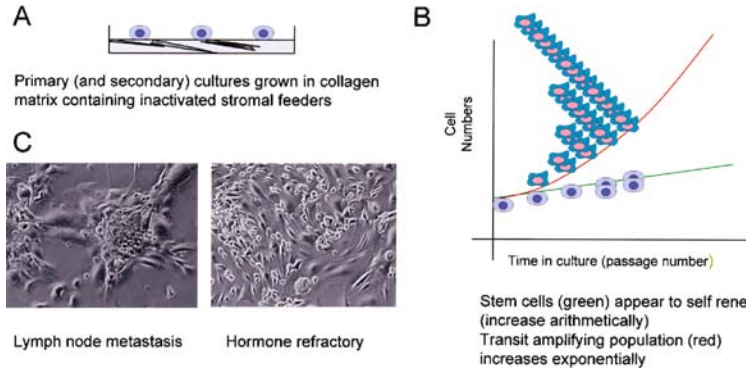


Fig. 4A–C. Culturing cancer stem cell from human tissues. **A** Feeder requirements for CSC culture (shown as larger cells in **C**). **B** Hypothetical growth kinetic scheme for CSCs. **C** Photomicrographs of CSC cultures from a lymph node metastasis and from a hormone refractory tumour. Note the difference in appearance of the colonies and the small size of the CSC-derived culture relative to the inactivated feeder cells (slightly out of focus)

such as cell surface proteins, transcription factors, kinases and other signalling molecules show variable levels of expression in the cancer stem cells, which display a consistent phenotype with a lower level of patient:patient expression change than is seen in gross expression studies with microdissected prostate tissues.

Expression analysis has highlighted technical restraints in the detection and isolation of prostate cancer stem cells for diagnostic purposes. Firstly, even laser capture microdissection is incapable of enriching for more than an epithelial fraction, whereas tissue disaggregation by enzymatic means and the specificity of antibody-mediated sorting (by either FACS or magnetic bead techniques) is capable of distinguishing epithelial subsets (Fig. 5). However, unlike haematopoietic tumours, in which disaggregation is efficient, resulting in single cells, epithelial tissues form aggregates of two or more cells which compromise gene expression analysis. In our original experiments the Affymetrix arrays confirmed the antigen staining reported by Collins et al. (2005), namely that the CSC phenotype was basal, after a brief amplification of the cells *in vitro*. When prostate tumour tissues were disaggregated, fractionated

and analysed without culture amplification, expression of luminal features was clearly detected. There were two potential explanations for this result. Firstly, *in vitro* culture was changing the phenotype, or producing a genotypic change as seen in glioblastomas when cultured in the presence of serum (Lee et al. 2006). Since similar short-term *in vitro* amplification has been successful in studies of the haematopoietic tumour stem cell population, we thought this to be the least likely explanation for the discrepancy (although some phenotypic changes may well result from culture and disaggregation stress). The likely explanation was provided by FACS analysis of the disaggregated tumours. This revealed a 1%–2% contamination of the fractionated stem cell population to be responsible for the observed 30 to 50 fold over-expression of luminal features (e.g. luminal cytokeratins and AR).

Comparison of overall gene expression, and the magnitude of changes between the various separated cell populations, also revealed the importance of obtaining pure cell populations. As shown in Fig. 5, little comparison can be made between results of microarray assays of gene expression using RNA extracted from whole tumours, even after microdissection to obtain pure epithelium. The transition from normal stem cell to malignant stem cell resulted in relatively modest changes in the expression of a number of genes (3-fold), whereas the stem to TA transition change produced much higher changes (30-fold), probably due in part to a combination of differentiation and proliferation in the TA population. The comparison between stem and luminal cells in terms of expression was even more polarised, with several thousandfold upregulation of the expression of genes such as AR, PSA luminal cytokeratin and PAP in the luminal cells. Thus even a minor contamination between populations results in an apparent gene expression change. For example in Fig. 5B mis-diagnosis of the pathology of tissue (i.e. tumour cell content in needle biopsies) also results in a ‘false’ stem cell signature. The sample indicated by an arrow represents a cancer biopsy with extremely low (5%) tumour cell content, which has been misallocated. The expression array analysis can clearly distinguish the differences between normal and tumour stem cells.

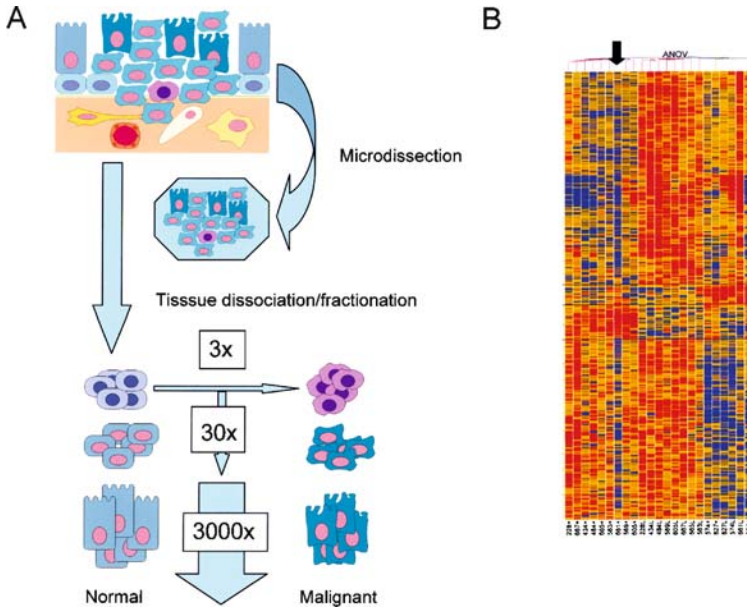


Fig. 5A, B. Cell purity requirements for expression analysis. **A** Tissue fractionation techniques, contrasting the mixed population of epithelial cells after laser capture dissection and the more homogeneous populations after dissociation and separation using cell surface antigens. The panel also indicates the relative gene expression levels and changes between the various epithelial cell populations. **B** Microarray analysis of purified cell fractions cultured from cancer stem cells. The *arrow* indicates a largely benign sample which was wrongly classified as such (only 5% tumour content), which results in a mixed expression type

9 Implications for Prostate Cancer Therapy

Cancer stem cells have been proposed as the residual therapy-resistant fraction for many years (Wodinsky et al. 1968; Hamburger and Salmon 1977; Heppner 1984). Thus the concept is not new. In prostate cancer a stochastic treatment regime, where all tumour cells are identical and of equivalent tumour-intiating capacity, has been standard. Therefore the withdrawal of androgens results in regression of human prostate tissue – both normal and malignant. However, such hormone therapy is either

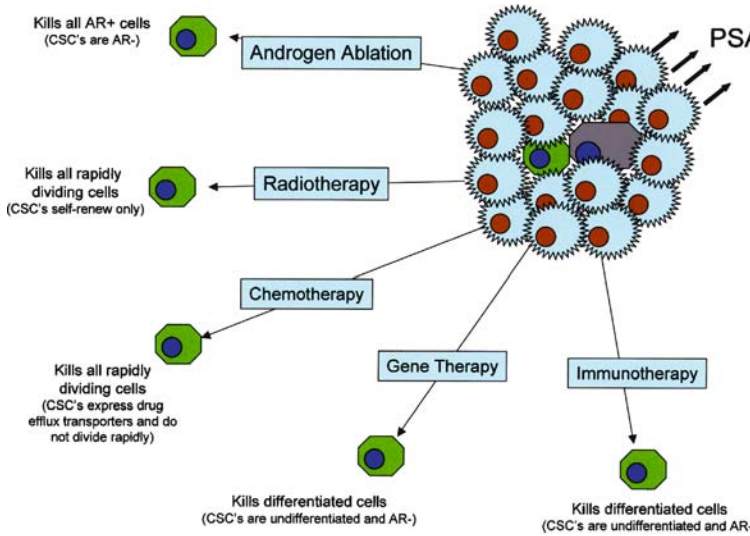


Fig. 6. Consequences of prostate cancer medical therapy according to a cancer stem cell model

transient in effect (for cancers) or reversible in the case of castration effects on normal prostate. But the presence of androgen-independent tissue stem cells within the human prostate has been known for just as long. The pioneering studies of L. M. Franks in the 1950s indicated the presence of multiple tumours within the prostates of elderly men. It should be no surprise then that therapies designed to 'fit all' patients and most tumours eventually fail in prostate cancer. While the introduction of medical castration in the 1980s made the therapy more acceptable (Furr 1996), the strategic thinking behind prostate cancer therapy has consisted of refinements and modifications of the standard therapeutic approaches. These are summarised in Fig. 6. If one considers the putative phenotype of the prostate cancer stem cells, the existence of a minor CSC population within each tumour can provide a logical explanation for the observed development of therapy resistance, and testable hypotheses for the development of curative therapies.

9.1 Surgical Therapy

It is now accepted that surgery to remove disease that is organ confined has a high chance of complete cure. However, if small numbers of viable stem cells have escaped the prostatic capsule, then their ability to support clonogenic growth as micro- and then macro-metastases would result in tumor recurrence. Indeed, the recurrence need not have the same phenotype or genotype as the original tumour, as the selective pressure to survive and grow in the new tumour location (presence of hormone, local matrix adhesion, stromal environment and growth factor secretion) could result in a novel phenotype. There is also the spectre of over-treatment, when patients with frankly non-malignant disease which has the morphological appearance of cancer are subjected to unnecessarily radical surgery (Miller et al. 2006).

9.2 Radiotherapy

Much the same can be said for radical radiotherapy. While some benefit can accrue from pre-operative treatment, in order to eliminate cancer stem cells either within a tumour mass, or at a distant site within the prostate a focussing of highest-energy therapy would be required. While focussed external beam therapy can minimise off-target effects, it may not be capable of reaching remote sites, something also shared by ultrasound-guided brachytherapy. Indeed, the mutagenic effects of sublethal irradiation could act to stimulate latent or unsuccessful cancer stem cells.

9.3 Medical Therapy

Of more biochemical significance are the results of stem cell phenotype on resistance to chemo- and androgen therapies. It has been recognised for many years that stem cells from haematopoietic systems are found in the ‘side population’ of drug-effluxing cells. There is now evidence to support a similar phenotype for tissue stem cells, including those from prostate(Bhatt et al. 2003). If our initial hypothesis concerning the origins of prostate cancer and preservation of some patterns of gene expression between non-malignant and malignant stem

cells are correct, then the CSCs will also express the ABC transporter genes found in their normal equivalents. Equally, most cancer therapeutics require cycling cells (to differentiate normal from tumour) and it is likely that CSCs could also escape the toxic effects of genotoxic and indeed more modern non-genotoxic anti-mitotic drugs. One good example would be taxotere, which does show some efficacy in hormone-relapsed prostate cancers, but only rarely effects a cure, normally extending life expectancy by little more than 20% (reviewed by Mackler and Pienta 2005).

When we consider resistance to hormone ablation, the results of a CSC which does not express AR are readily apparent. Hormone ablation therapy should result in the destruction of AR-expressing cells within a tumour, leaving the CSCs (and normal SC) unaffected in the short term. However, both stem cell types are now in an altered androgen environment, namely low exogenous androgens (although there are some recent data to suggest that adrenal androgens are not completely ablated and that altered steroid metabolism may supply a secondary source) and a high anti-androgen concentration. Under the latter conditions there are good grounds to induce expression of the androgen receptor in the replicative progeny of the residual (stem) cells in a similar manner to over-expression of dihydrofolate reductase under methotrexate treatment (Alt et al. 1978) or HPRT (also X-encoded like AR) during HAT selection (Fuscoe et al. 1983). Ultimately in both of the latter cases, not only is the metabolising gene over-expressed but the genes are also amplified in a tandem manner. This provides one explanation for the observed AR gene amplification and over-expression recently observed after anti-androgen treatment of prostate tumour xenografts and in human hormone refractory tumours (Chen et al. 2004; Holzbeierlein et al. 2004).

9.4 Gene and Immunotherapy

Gene and immunotherapy for prostate cancer have also been developed, exploiting the existence of a range of ‘prostate-specific’ genes such as PSA, PAP and AR (Maitland 2000). However, on closer examination, most prostate-specific functions can be equated with prostate-luminal expression. In essence, these therapeutic strategies simply mimic the

androgen-based therapies (which destroy luminal AR-expressing cells) and the anti-mitotic therapies, both key properties of the majority of cells in a tumour.

However, as long as the 0.1% CSC population persists, even the most targeted and sophisticated therapies can be no more than palliative. A good analogy is the use of defoliant weedkillers versus systemic weedkillers. If cancer stem cell therapies can be developed and the tumours are indeed derived from a 'root' of such stem cells, then curative therapies are a possibility. CSC-specific genes whose normal functions are essential for the survival of the stem cells would be the best targets, but any inherent genetic instability and the inheritance of the capacity of normal stem cells to survive chemical and radiological stress could make the design of CSC-directed therapies challenging. Perhaps the Achilles' heel of the CSC lies in its primitive nature and its dependence on a protective niche for its longevity.

Another potentially useful target is clearly the chromosomal translocation products, commonly observed in chronic myeloid leukaemia (CML) and now found for the first time in prostate cancers. Although the TMPRSS2-erg fusion in prostate does not appear to result in a novel fusion protein, similar to the tyrosine kinase in CML, Imatinib has revolutionised the therapy of CML, and is directed against the fusion product. However, not all leukaemic stem cells (also defined by CD133 expression) are susceptible to Imatinib, perhaps because of quiescence, and for complete elimination some form of combinatorial therapy will ultimately be required (Pirson et al. 2006; Goldman and Gordon 2006). We know from the data presented here and elsewhere in other cellular systems that the induction of differentiation, perhaps by inappropriate culture conditions, is sufficient to reduce the life span of most epithelial stem cells *in vitro*. Differentiation therapy was proposed many years ago for many types of cancer, and may now be resurrected, as a result of modern genetic analysis, in much the same way that technological advances in cell separation and culture have restored the stem cell hypothesis of human cancer.

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The Hedgehog Signaling Network, Mammary Stem Cells, and Breast Cancer: Connections and Controversies

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Abstract. Several signal transduction networks have been implicated in the regulation of mammary epithelial stem cell self-renewal and maintenance (Kalirai and Clarke 2006; Liu et al. 2005). These signaling networks include those of the Wnt, Notch, TGFβ, EGF, FGF, IGF, and most recently, the Hedgehog (Hh) families of secreted ligands. However, we currently know very little about the cellular and molecular mechanisms by which these signaling pathways function to regulate normal epithelial stem/progenitor cells. What is clear is that the regulatory signaling networks thought to control normal stem/progenitor cell self-renewal and maintenance are, with the current sole exception of the hedgehog network, well-documented to have contributory roles in mammary cancer development and disease progression when misregulated. In this review, genetic regulation of mammary gland development by hedgehog network genes is outlined, highlighting a developing controversy as to whether activated hedgehog signaling regulates normal regenerative mammary epithelial stem cells or, indeed, whether activated hedgehog signaling functions at all in ductal development. In addition, the question of whether inappropriate hedgehog network activation influences breast cancer development is addressed, with emphasis on the prospects for using hedgehog signaling antagonists clinically for breast cancer treatment or prevention.

1 Introduction

Organ-specific tissue stem cells are considered to be a rare population of long-lived cells that are defined operationally by the ability to self-renew and to give rise to all other cell types in a given tissue. They not only function in organ formation in the embryo, but also participate in tissue repair, regeneration, and remodeling in the adult organism (Molofsky et al. 2004; Wicha et al. 2006). In the mammary gland, regenerative stem cells, and division-competent progenitor cells derived from them, are the driving force behind gland development and function. These cells are also thought to be the primary targets for breast cancer initiation (Behbod and Rosen 2005).

Elegant transplantation studies initiated nearly five decades ago (Daniel et al. 1968; DeOme 1958; Faulkin and Deome 1960) demonstrated that the adult mouse mammary gland contains relatively growth-quiescent epithelial stem cells that are distributed throughout the entire gland. These stem cells could be activated to self-renew upon transplantation of small duct fragments or dissociated cells, and were capa-

ble of regenerating morphologically normal, functional ductal trees for multiple transplant generations. Estimates of the frequency of regenerative stem cells made by limiting-dilution transplantation analyses have climbed from about 1 per 2,500 epithelial cells in the adult mouse mammary gland a decade ago to about 1 per 1,100 more recently (Shackleton et al. 2006; Sleeman et al. 2006; Smith 1996; Stingl et al. 2006; Moraes et al. 2007) as techniques for their isolation, identification, and transplantation have improved.

Several properties of mammary epithelial stem and progenitor cells make them attractive candidates for the cells of origin for cancer. Unlike more differentiated cells that have a restricted life span, mammary stem cells are thought to be long-lived, and therefore have the opportunity to accumulate oncogenic mutations over time. Also, unlike differentiated cells, stem/progenitor cells already possess self-renewal capacity and extensive differentiation potential and thus would not need to reacquire these abilities as would their differentiated counterparts. Whether, in fact, normal mammary stem/progenitor cells are the cells of origin for breast cancer is one of the central unanswered questions in breast cancer biology (Beachy et al. 2004; Behbod and Rosen 2005; Smalley and Ashworth 2003).

Related to the question of whether normal stem/progenitor cells are the cells of origin for breast cancer is the question of the existence of "breast cancer stem cells", or more precisely, "breast cancer-initiating cells" (Reya et al. 2001; Sell 2004). Breast cancer-initiating cells are conceptualized as a distinct subpopulation of cells within a tumor that possess self-renewal and differentiation capacities similar to those of normal tissue stem cells. Thus these cells should have the ability to give rise to new tumors on transplantation, and resulting tumors should display the same level of cellular and molecular heterogeneity as the tumor of origin. Such cells may also be responsible for metastasis, disease relapse, and treatment resistance (Al-Hajj et al. 2004; Behbod and Rosen 2005; Dean et al. 2005).

Evidence for a distinct subpopulation of cancer-initiating cells is established firmly in leukemias of the hematopoietic system, where single transplanted cancer cells have been demonstrated to give rise to new tumors in host animals (reviewed by Sell 2004). Evidence for a distinct subpopulation of cancer-initiating cells in breast cancer has only re-

cently been obtained (Al-Hajj and Clarke 2004). With the use of cancer cells isolated primarily from pleural effusions of patients with metastatic breast disease, a subpopulation of epithelial cells was identified by flow cytometry that was enriched for tumorigenic cells relative to the bulk tumor using limiting-dilution transplantation into mammary fat pads of immunocompromised mice. This subpopulation was negative for a panel of nonepithelial cell lineage markers (Lin^-), showed low-to-undetectable expression of the CD24 cell surface antigen ($CD24^{-/low}$), but expressed high levels of the cell surface marker $CD44^+$, a protein involved in cell adhesion.

Recent work has implicated several signal transduction networks in regulation of normal mammary stem and progenitor cell self-renewal and maintenance (Kalirai and Clarke 2006; Liu et al. 2005). These signaling networks include those of the Wnt, Notch, TGFβ, EGF, FGF, IGF, and most recently, the Hedgehog (Hh) families of secreted ligands. Despite the implication of these signaling pathways in control of mammary epithelial stem cell behavior, we currently know very little about the cellular and molecular mechanisms by which they function to regulate such cells. Indeed, for some, it remains unclear whether the network functions within the stem cell itself, or whether the network can function indirectly to regulate stem cell behavior, perhaps via activity within a stem cell "niche." What is clear is that the regulatory signaling networks thought to control normal stem/progenitor cell self-renewal and maintenance are, with the sole exception of the hedgehog network, well-documented to have contributory roles in mammary cancer development and disease progression when misregulated (Liu et al. 2005).

The focus of this review is to discuss data linking the hedgehog signaling network to control of mammary gland ductal development. In particular, this review highlights a developing controversy as to whether activated hedgehog signaling regulates normal regenerative mammary epithelial stem and progenitor cells, or, indeed, whether activated hedgehog signaling functions at all in ductal development. In addition, this review addresses the question of whether inappropriate hedgehog network activation influences breast cancer biology, and, if so, what the prospects are for using hedgehog signaling inhibitors clinically for breast cancer treatment or prevention.

2 Stem and Progenitor Cells in Mammary Gland Ductal Development

Mammary gland development begins with positioning of the mammary gland along the anterior-posterior and dorsal-ventral axes and establishment of mammary epithelial cell identity from unspecified surface ectoderm. In the mouse, placement of the mammary glands is initiated with the formation of an elevated ectodermal ridge on each flank of the embryo, known as either the mammary line, the mammary streak, or the milk line (Sakakura 1987; Veltmaat et al. 2003). This mammary line is visible at about embryonic day E10.5 and spans the region of the prospective mammary placodes 2, 3, and 4. Molecularly, the mammary line is defined by expression of *Wnt10b* (Veltmaat et al. 2004). This initial mammary line is complemented by separate axillary and inguinal streaks, also defined by *Wnt10b* expression, that develop slightly later and eventually connect with the initial mammary line.

At around embryonic day 11.25 (E11.25), inductive signals from the somites (Veltmaat et al. 2006) and underlying dermal mesenchyme direct formation of five pairs of mammary placodes that emerge as disk-shaped surface ectodermal thickenings (reviewed by Sakakura 1987; Veltmaat et al. 2003). Shortly after induction (around E12.5), the placodes transform into spherical buds that are closely associated with a few cell layers of condensed mesenchyme called the mammary mesenchyme. At around E15.5, the buds elongate into a mammary sprout, the distal tip of which penetrates a second type of mesenchyme, the underlying fat pad precursor mesenchyme (Kimata et al. 1985), which will give rise eventually to the mammary fat pad proper. At around E16.5 the epithelial sprout initiates branching morphogenesis within the fat pad precursor mesenchyme to form a rudimentary ductal tree. Once formed, the rudimentary gland ceases growth, and will remain relatively growth-quiescent until hormone-dependent growth is initiated at puberty.

Mammary epithelial stem cell identity is established as early as day E13-14 of embryonic development, as shown by the ability to regenerate a mammary ductal tree by transplantation of embryonic tissue into the epithelium-free "cleared" fat pad of a host mouse. Mammary epithelial cells can revert to an epidermal fate until at least E15.5 (Foley et al. 2001; Satokata et al. 2000), suggesting that, once established, mammary

epithelial cell identity must be maintained. Commitment of the epithelium to a mammary fate occurs as early as E17, as demonstrated by tissue recombination experiments in which grafts of embryonic mammary epithelium with nonmammary mesenchyme retained the ability to produce milk proteins (Sakakura et al. 1976, 1979).

At puberty, ovarian steroids stimulate epithelial cell proliferation associated with rapid and invasive ductal elongation and branching morphogenesis. At the growing tips of elongating mammary ducts are bulb-like structures called terminal end buds (TEBs) (Daniel and Smith 1999; Daniel 1987). Histologically, TEBs consist of two highly proliferative cell compartments, an outer "cap cell" layer, and an inner "body cell" compartment consisting of four to six layers of relatively undifferentiated luminal epithelial cells. As the TEB invades the fat pad, cap cells serve as a self-renewing progenitor cell population and differentiate into myoepithelial cells near the neck of the TEB as the duct elongates (Williams and Daniel 1983). Body cells serve as a second stem and progenitor cell population that is thought to give rise to all luminal epithelial cell subtypes of the mature duct, including new multipotent mammary stem cells. Multipotent mammary epithelial stem/progenitor cells laid down during TEB growth persist long-term as demonstrated by label retention studies (Smith 2005; Welm et al. 2003; Zeps et al. 1996). At least some label-retaining stem/progenitor cells divide asymmetrically and traverse the cell cycle more frequently than thought previously (Smith 2005).

As ducts form, they are surrounded by a periductal stroma that actively condenses around the ducts at the neck of the TEB. This condensed periductal stroma consists mainly of fibroblasts, macrophages, eosinophils, and associated extracellular matrix material (Gouon-Evans et al. 2002; Williams and Daniel 1983). These structures are further surrounded by adipose, vascular, and immune system cells within the confines of the mammary fat pad. On reaching the limits of the fat pad at ductal maturity, ductal elongation ceases and TEBs regress to leave a branched system of differentiated ducts.

3 The Hedgehog Signaling Network in Mammals

The hedgehog signaling network functions in cell-cell communication and regulates pattern formation, proliferation, cell fate, and stem/progenitor cell maintenance and self-renewal in many organs. A greatly simplified version of "canonical" hedgehog signaling in mammals (Fig. 1) (Cohen 2003; Evangelista et al. 2006; Hooper and Scott 2005; Nusse 2003) typically involves two types of cells, a signaling cell expressing a member of the Hedgehog family of secreted ligands (*Sonic Hedgehog* (Shh), *Indian Hedgeho*g (Ihh), or *Desert Hedgehog* (Dhh)) and a responding cell expressing one or more *Patched* family hedgehog receptors (*Patched-1* (PTCH1) and *Patched-2* (PTCH2)). In the absence of ligand, PTCH1 and PTCH2 can function to inhibit downstream signaling by antagonizing the function of the *Smoothened* (SMO) transmembrane effector protein. Under these conditions, expression of hedgehog target genes is inhibited by repressor forms of one or more members of the *Gli* family of transcription factors (GLI2 or GLI3), whose formation is positively regulated by a cytoplasmic intermediate called Suppressor of Fused (SUFU). In the presence of ligand, PTCH1 releases inhibition of SMO, which leads to induction of target genes by transcriptional activator forms of *Gli* transcription factors (GLI1, GLI2, or GLI3). Formation of transcriptional activator forms of GLI proteins is under negative regulation by SUFU (see Cohen 2003; Evangelista et al. 2006; Hooper and Scott 2005; Nusse 2003 for detailed models).

Of course, the true picture is not that simple. Other network components such as those regulating ligand posttranslational modification, release, and availability, or those modulating ligand response in the cytoplasm, are not included in the model described. Furthermore, some components are known to have both ligand-dependent and ligand-independent functions. For example, in addition to its signal transduction activities, PTCH1 can also function to sequester hedgehog ligand, thereby restricting the range over which free ligand can signal (reviewed by Hooper and Scott 2005). Finally, there is evidence to suggest that PTCH1 can function as a "dependence receptor" to induce apoptosis in cell types dependent on ligand-bound PTCH1 for survival (Chao 2003; Guerrero and Ruiz i Altaba 2003; Thibert et al. 2003).

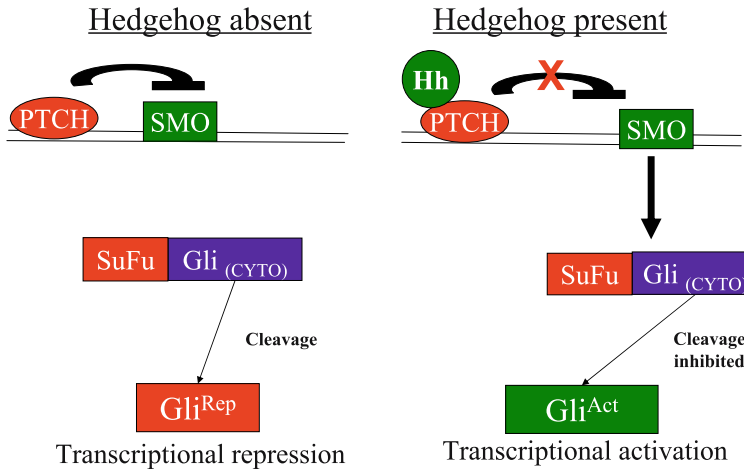

Fig. 1. A two-state model for hedgehog signaling in the absence and presence of hedgehog ligands. Positively acting components are shown in *green*; negatively acting components are shown in *red*. The cytoplasmic form of GLI proteins is represented in *purple*

Still more complexity is introduced at the level of the downstream transcription factors. In some organs, hedgehog ligands can regulate transcription of target genes through the COUP-TFII transcription factor, which may occur in a GLI-independent manner (Krishnan et al. 1997a,b; Lamont and Childs 2006; Lee et al. 2006a). Finally, hedgehog ligands can also regulate transcription via the ZIC family of zinc-finger transcription factors (ZIC1-5) (left-right asymmetry; neural tube formation) (Aruga 2004; Brewster et al. 1998; Wallis and Muenke 2000), although it is not clear whether transcriptional regulation by these factors is independent of GLI proteins.

Evidence for "non-canonical" hedgehog signaling via SMO-coupled heterotrimeric G-protein activation is also beginning to come to light. SMO is a seven-pass transmembrane protein that is evolutionarily related to G-protein-coupled receptors such as rhodopsin and β-adrenergic receptor. However, until recently, evidence to support a role for heterotrimeric G-proteins in activated hedgehog signaling has been mini-

mal (Chen et al. 2004; DeCamp et al. 2000; Fremion et al. 1999; Hammerschmidt and McMahon 1998; Kasai et al. 2004; Meloni et al. 2006; Riobo et al. 2006; Wilbanks et al. 2004). Two recent studies in cell lines have demonstrated functional interactions between SMO and selected Gα subunits primarily of the $G\alpha_i$ family (Kasai et al. 2004; Masdeu et al. 2006; Riobo et al. 2006). Together, these two studies indicate that SMO can couple with G_{i1}, G_{i2}, G_{i3}, G_0, G_z, and G_{15}. In contrast, SMO could not couple with G_s, G_q, G_{qzIc}, G_{qG66D}, $G_{qG66Dx5}$, $G_{12,}$ G_{13}, or G_{16} (but see also Kasai et al. 2004).

These studies are complemented by other recent work showing that signaling via SMO is enhanced by catalytically active G-protein-coupled receptor kinase 2 (GRK2), but not catalytically inactive GRK2. GRK2 activity promoted association of SMO with β-arrestin-2 (Chen et al. 2004; Meloni et al. 2006). These activities of β-arrestin-2 and GRK2 contrast sharply with their interactions with most, but not all, other G-protein-coupled receptors, where they typically function to desensitize cells to ligand stimulation (Reiter and Lefkowitz 2006). Intriguingly, G-protein coupling by SMO could be genetically separated from GLI activation with a truncated SMO protein (Riobo et al. 2006), offering the possibility that these two functions might be uncoupled in vivo, under certain conditions. Very little is yet known about the roles of these genes in mammary gland development, mammary stem cell self-renewal, stem cell maintenance, or breast cancer. Thus a possible role for G-protein-coupled hedgehog signaling in gland development or breast cancer is not discussed here, but should be kept in mind in the interpretation of experimental results.

4 Expression and Function of "Canonical" Hedgehog Network Genes During Mouse Mammary Ductal Development

Over the last few years, we and others have conducted expression and phenotypic analyses of many of the genes in the canonical hedgehog signaling network, including *Shh, Ihh, Dhh, Ptch1, Smo, Gli1, Gli2*, and *Gli3*, throughout mammary gland development. Of these, single-gene loss-of-function mutations in *Shh, Ihh*, and *Gli1* have yielded only

negative data with respect to a role in mammary ductal development. However, our laboratory has demonstrated critical functions for two network genes, *Ptch1* and *Gli2,* in postnatal mammary ductal development (Lewis et al. 1999, 2001; Lewis and Veltmaat 2004), as well as a unique ductal phenotype associated with overexpression of constitutively activated *Smo* in transgenic mice (Moraes et al. 2007). Recently, two other groups have demonstrated a key role for the *Gli3* gene in the induction of the embryonic mammary gland (Hatsell and Cowin 2006; Veltmaat et al. 2006), but a role for *Gli3* in postnatal gland development has not been demonstrated.

An important caveat for analysis of the hedgehog ligands, PTCH receptors, and GLI transcription factors by genetic approaches is that genes within these groups can have redundant functions and might therefore compensate for one another in vivo (Zhang et al. 2001). Thus two or more genes in a given family may need to be disrupted simultaneously to achieve a phenotypic effect. Simultaneous disruption of multiple ligands, multiple receptors, or multiple transcription factors has not been performed in the mammary gland. Therefore, potential roles for some hedgehog network genes in mammary gland development have not been ruled out formally.

5 The Ligands

As detected by in situ hybridization, hedgehog ligands are expressed in the epithelium of the mouse mammary gland at several stages of development. Both *Shh* and *Ihh* can be detected in the mammary epithelium as early as E12.0 (Michno et al. 2003; Mill et al. 2003). During ductal elongation, low levels of *Ihh* and *Dhh* mRNA have been detected in the TEB and in differentiated ducts (Kouros-Mehr and Werb 2006; Lewis et al. 1999). During pregnancy and lactation, levels of *Ihh* mRNA in the alveolar epithelium increase dramatically as seen by in situ hybridization analyses (Lewis et al. 1999). SHH protein has been detected by immunohistochemistry in alveolar cells in glands of pregnant mice, but expression in TEB or ductal epithelium was not reported (Gallego et al. 2002).

In normal human mammary epithelium, mRNA for all ligands has been detected, but *Ihh* appears to be the primary ligand expressed (Liu et al. 2006; Mukherjee et al. 2006). Unlike in the mouse, *Ihh* message was detected in both mammary epithelial cells and mammary fibroblasts. Expression of SHH protein in normal mammary epithelium is currently a matter of debate (Kubo et al. 2004; Mukherjee et al. 2006).

Despite their expression, functional analysis of hedgehog ligands in mice has yielded only negative results during ductal development or alveolar morphogenesis. Individual targeted disruption mutations for both *Ihh* and *Shh* have been examined in detail. However, since disruption of either *Shh* or *Ihh* is embryonic lethal (St-Jacques et al. 1998, 1999) transplantation of epithelial fragments from mutant embryos into the epithelium-free fat pad of host mice was required for their analysis. In both cases, transplantation of epithelial fragments showed no changes in the ability to fill the epithelium-free mammary fat pad of host mice or in the ability to form alveolar structures that express milk proteins (Gallego et al. 2002; Michno et al. 2003). The targeted disruption mutation for *Dhh* has not yet been analyzed systematically for a mammary phenotype (Bitgood et al. 1996; Endo et al. 2002). However, no mammary phenotypes have been reported, and since these mice can be maintained by crossing homozygous females with heterozygous males, homozygous dams maintain a milk supply at least adequate to support their litters (http://jaxmice.jax.org/strain/002784.html.)

6 The PTCH Receptors

As shown by in situ hybridization, *Ptch1* is expressed in both ectodermally derived epithelium and mesodermally derived mesenchyme after mammary placode formation (as early as day 12.5) (Michno et al. 2003) and shows slightly reduced levels in the epithelial bulb relative to the overlying ectoderm at E14 (Boras-Granic et al. 2006; Lewis et al. 1999). Similarly, during ductal development, *Ptch1* mRNA is expressed in the body cells of the TEB, in differentiated ducts, and in the periductal stroma, as demonstrated by both in situ hybridization (Lewis et al. 1999) and immunolocalization (Moraes et al. 2007). As shown by in situ hybridization, *Ptch1* mRNA levels are elevated in alveolar cells rel-

ative to associated ducts during pregnancy and lactation (Lewis et al. 1999). Curiously, evaluation of *Ptch1* promoter activity with the *Ptch1* targeted disruption allele in which bacterial β-galactosidase has been inserted in frame (allele $Ptch1^{tm1Mps}$) does not allow detection of LacZ activity in mammary epithelium at any time during either embryonic or postnatal gland development (Hatsell and Cowin 2006). The reason for this discrepancy is entirely unclear, but it may have to do with the level or stability of β-galactosidase produced, or with the stability of the mRNA generated by the mutant *Ptch1* allele in mammary cells.

In normal human breast, as in the mouse, *Ptch1* mRNA and protein are expressed in both the epithelial and stromal compartments (Liu et al. 2006; Mukherjee et al. 2006; Moraes et al. 2007; but see Kubo et al. 2004). *Ptch2* expression has not yet been investigated.

Targeted disruption of *Ptch1* (allele $Ptch1^{tm1Mps}$) in mice is early embryonic homozygous lethal (~embryonic day9.5). *Ptch1* heterozygous mice in a mixed 129:B6D2F1 genetic background developed medulloblastomas (brain), rhabdomyosarcomas (muscle), and basal cell carcinoma-like lesions (skin) at detectable frequencies (Aszterbaum et al. 1999; Goodrich et al. 1997), but mammary tumors were not observed in these long-term studies. Similar lesions were observed with a second targeted disruption allele of *Ptch1* (allele $Ptch1^{neo6/7}$) in outbred CD1 mice (Hahn et al. 1998); again, no mammary tumors were observed. Interestingly, expression of tumor phenotypes with the $Ptch1^{neo6/7}$ allele has been shown to be dependent on genetic background (Pazzaglia et al. 2004). Genetic crossing of $Ptch1^{neo6/7neo}$ heterozygotes to two independent inbred cancer-resistant and cancer-sensitive mouse lines dramatically alters the relative frequency of tumor formation. The effect of genetic background on tumor formation for the $Ptch1^{tm1Mps}$ allele has not been reported, but is likely to show a similar effect.

In the mammary gland, mice heterozygous for targeted disruption of *Ptch1* (allele $Ptch1^{tm1Mps}$) resulted in morphological changes in TEB structure in postpubescent virgin animals, as well as to atypias and ductal dysplasias characterized by multilayered ductal walls and dissociated cells that frequently filled the ductal lumen (Lewis et al. 1999). Our more recent work suggests that the ductal dysplasias observed in these mice are due primarily to increased proliferation within the ducts without changes in caspase-3-mediated apoptosis (Moraes et al. 2007).

However, apoptosis in the TEB has not yet been analyzed to the degree necessary to rule out a change in non-caspase-3-mediated apoptosis. Remarkably, defects observed in virgin mice are reverted during late pregnancy and lactation, but return on involution and gland remodeling (Lewis et al. 1999), suggesting that wild-type levels of *Ptch1* function are required to maintain ductal histoarchitecture but wild-type levels are not required during pregnancy or lactation.

Similar to tumor formation due to heterozygosity of the $Ptch^{6/7neo}$ allele, the mammary ductal phenotype readily observable in progeny from $Ptch1^{tm1Mps}$ heterozygotes serially backcrossed to B6D2F1 mice (Lewis et al. 1999) is reportedly not present in an outbred genetic background (Hatsell and Cowin, unpublished results cited by Hatsell and Cowin 2006). Whether this variability in penetrance as a function of genetic background is due to polymorphisms at the *Ptch1* locus or to the presence of genetic modifiers in the outbred background is an open question.

Whole mammary gland transplants demonstrated that the observed dysplasias reflect an intrinsic developmental defect within the gland in that the dysplastic phenotype could be partially recapitulated in the transplanted gland (Lewis et al. 1999). However, *Ptch1*-induced epithelial dysplasias were not stable on transplantation of epithelial fragments into a wild-type epithelium-free fat pad, suggesting that *Ptch1* functions in the stroma, or in both the epithelium and the stroma, to control epithelial cell behavior.

Ptch2 expression and function in mouse mammary gland development have not been investigated systematically. Homozygous *Ptch2* mutants display few phenotypic abnormalities, but they do show hair loss (alopecia) and skin hyperplasias (Lee et al. 2006b; Nieuwenhuis et al. 2006). However, homozygous dams are able to support litters, indicating that mammary function is not altered dramatically. As with the ligands, the possibility exists of functional redundancy among the two *Ptch* genes (Lee et al. 2006b). Mice homozygous for *Ptch2* disruption as a single-gene mutation do not show increased incidence of tumor formation, but loss of *Ptch2* shows functional synergy with *Ptch1* heterozygosity and led to an increased frequency of tumor types normally observed in *Ptch1* heterozygotes, as well as to a broader range of tumors observed (Lee et al. 2006b).

7 The GLI Transcription Factors

In the mouse, *Gli1* mRNA expression is not detectable in mammary epithelium or in epithelium-associated mesenchymally derived tissue during either embryonic or postnatal mammary gland development (Hatsell and Cowin 2006). Rather, by the use of a LacZ knockin allele, *Gli1* appears to be expressed exclusively in the lymphatic vasculature within the mammary fat pad. Efforts to detect phenotypic effects of *Gli1* disruption in the mammary gland have failed thus far (M.T. Lewis, unpublished results).

In contrast to *Gli1*, *Gli2* expression has been detected in both the embryonic and postnatal mammary gland. In the embryo, *Gli2* mRNA is detectable in the epithelial compartment by in situ hybridization as early as E14.5 (Boras-Granic et al. 2006). In the postnatal virgin mouse, expression of *Gli2* mRNA undergoes a tissue compartment switch and is detected solely in condensed periductal stroma (Lewis et al. 2001), with expression being lower in actively condensing stroma near the tip of the TEB but increased in stroma that has already condensed around the duct. Curiously, with pregnancy, *Gli2* mRNA expression undergoes a second tissue compartment switch and becomes both epithelial and stromal throughout pregnancy and lactation (Lewis et al. 2001).

Unlike the *Ptch1-LacZ* knockin allele, the expression pattern observed with a *Gli2-LacZ* knockin allele appears to be identical to those observed with in situ hybridization during both embryonic and postnatal virgin development. With the knockin allele, *Gli2-LacZ* expression can be observed in the dermal mesenchyme and underlying somites in E11.0 embryos in the region corresponding to the mammary line between the fore- and hindlimbs (Hatsell and Cowin 2006). Later, at E14.5, expression can be observed in both the epithelial bud and surrounding mammary mesenchyme. By E16.5, LacZ expression becomes restricted to the basal layer of cytokeratin-14+ and p63+ epithelial cells within the mammary sprout, and to the condensed mammary mesenchyme immediately surrounding the sprout.

During postnatal ductal development, Gli2-LacZ staining is restricted to the periductal stroma, consistent with our previous in situ hybridization results (Hatsell and Cowin 2006; Lewis et al. 2001). However, during pregnancy, expression patterns observed for the Gli2-LacZ knockin

allele at pregnancy day (P)14.5 do not correspond to those determined by in situ hybridization at P6/7 and P18 (Hatsell and Cowin 2006; Lewis et al. 2001). Whereas in situ hybridization at both P6/7 and P18 showed expression in both alveolar epithelium and stroma, the *Gli2-LacZ* allele shows expression in stroma and myoepithelium; alveolar epithelium did not stain positively for LacZ expression. The reason for the apparent discrepancy between these data and our in situ hybridization data is not known, but again may have to do with the level or stability of β-galactosidase produced, or with the stability of the mRNA generated by the mutant *Gli2-LacZ* allele in mammary cells.

Phenotypic analysis of a targeted disruption mutant for *Gli2* revealed that *Gli2* function is required for normal mammary ductal development in virgin mice (Lewis et al. 2001). Transplantation rescue of embryonic mammary glands into immunocompromised host mice demonstrated aberrant ductal morphogenesis in transplants harvested 8 weeks post-transplantation. Ductal dysplasias consisted mainly of swollen and distended ducts, but could also be abnormally branched and histologically altered in a manner similar to ductal hyperplasias of the human breast. As with the *Ptch1* heterozygotes, transplantation of epithelial fragments into epithelium-free fat pads of immunocompromised host mice failed to recapitulate the ductal phenotypes observed in intact glands. As with *Ptch1*, these results indicated that *Gli2* functions primarily in the mammary stroma to affect mammary epithelial cell behavior in virgin mice during ductal development.

Like *Gli2*, *Gli3* mRNA is also expressed in mammary structures during both embryonic and postnatal development. During embryonic development, *Gli3* expression was detected in the in the hypaxial buds of the somites underlying the developing mammary streak as early as E10.0 by in situ hybridization (Veltmaat et al. 2006), with lower expression in the dermal mesenchyme and no detectable expression in the overlying ectoderm at this stage. By E13.5 Gli3 protein is expressed in both the epithelial bud and the surrounding mesenchyme as shown by immunohistochemical staining (Hatsell and Cowin 2006). In postnatal mammary gland development, *Gli3* mRNA and protein were detected in both the TEB epithelium and surrounding stroma in the 4-week-old virgin. During pregnancy mRNA and protein were relatively strongly expressed in the epithelial compartment, as well as in associated stroma.

While no overt mammary phenotypes have thus far been detected by transplantation rescue of embryonic mammary glands derived from *Gli3*-null embryos (both epithelial fragments and whole mammary glands; M.T. Lewis, unpublished results), loss of *Gli3* leads to failure of mammary placodes 3 and 5 to form during embryonic development (Hatsell and Cowin 2006; Veltmaat et al. 2006). With respect to placode 3, elegant work by Veltmaat et al (Veltmaat et al. 2006) has demonstrated that *Gli3* function is required in the hypaxial buds of thoracic somites as early as E10.5 (36 somite stage) to induce somitic FGF10 expression. This FGF10 signal is received by the surface ectoderm via the FGFR2b receptor, where it then induces expression of Wnt10b in the mammary streak. Loss of *Gli3* leads to reduced FGF10 expression, and failure to induce Wnt10b in the region corresponding to mammary placode 3. Thus, at least for mammary gland 3, *Gli3* function is required in the somites to establish mammary epithelial cell identity, but *Gli3* function does not appear to be required in the epithelial cells themselves at these early phases of growth.

Genetic analysis indicates that *Gli3* functions as a transcriptional repressor during embryonic mammary gland development (Hatsell and Cowin 2006). Expression of two copies of the *Gli1* gene (encoding a transcriptional activator) knocked into the *Gli2* locus (and therefore under the control of the *Gli2* promoter) had no phenotype on its own, but could lead to loss of mammary placodes 3 and 5 at high frequency in the context of a heterozygous $Gli3^{xt}$ (extra-toes) allele. These data suggest that *Gli1,* which functions exclusively as a transcriptional activator, can antagonize remaining *Gli3* function in the heterozygous state, leading to placode loss. Thus *Gli3* likely functions as a transcriptional repressor during embryonic development. If so, induction of FGF10 by *Gli3* during normal gland development is likely to be mediated indirectly via an unknown intermediate.

In normal human mammary tissue, *Gli1* mRNA and protein have both been detected in mammary epithelium and stroma (Liu et al. 2006; Mukherjee et al. 2006; but see Kubo et al. 2004). In addition, *Gli2* mRNA has been detected in both tissue compartments by QPCR. *Gli3* expression has not been demonstrated. These data contrast sharply with data in the mouse, suggesting there may be species-specific differences in hedgehog network function.

8 A Working Model for Hedgehog Network Regulation of Mammary Ductal Development

In our original working model for hedgehog signaling in mammary ductal development (Lewis 2001), we proposed that the hedgehog signaling network may be active transiently in the body cells of the TEB to regulate its growth. This interpretation was based primarily on low, but detectable, expression of *Ihh* in the body cells of the TEB and elevated expression of *Ptch1* mRNA relative to the immediately subtending duct. The recent discoveries that *Dhh* and *Gli3* are also expressed in the TEB (Hatsell and Cowin 2006; Kouros-Mehr and Werb 2006) are consistent with this hypothesis, given the possibility that *Gli3* could function as a transcriptional activator during ductal elongation in the presence of ligand. Also, the possibility of "noncanonical" hedgehog signaling has not been ruled out.

We hypothesized that as TEB elongate, signaling must be inhibited in mature ducts as body cells differentiate into luminal epithelium in elongating ducts. This interpretation was based primarily on reduced expression of *Ihh* and *Ptch1* mRNA in mature ducts relative to body cells of the TEB, as well as on the *Ptch1* targeted disruption phenotype (Lewis et al. 1999).

In an updated version of this model (Lewis and Veltmaat 2004), we proposed further that hedgehog signaling must also be inhibited in periductal stroma surrounding mature ducts. This interpretation was based on the similarity between the *Ptch1* and *Gli2* ductal dysplasia phenotypes, the requirement for mutant stroma for the presence of the ductal dysplasia phenotypes in the *Ptch1* and *Gli2* mutants, and the lack of a demonstrable phenotype in *Ihh* or *Shh* knockout mutants during ductal development. Thus, if GLI2 protein is indeed expressed in the periductal stroma, it would be expected to function as a transcriptional repressor (Lewis and Veltmaat 2004). The observation that *Gli3* is widely expressed in mammary epithelium and periductal stroma in mature virgin mice further suggests that the hedgehog network is not normally active in the ducts or periductal stroma of the mature virgin mammary gland once ducts are formed (Hatsell and Cowin 2006).

Thus our working model for hedgehog network function during ductal development remains one in which the hedgehog signaling network

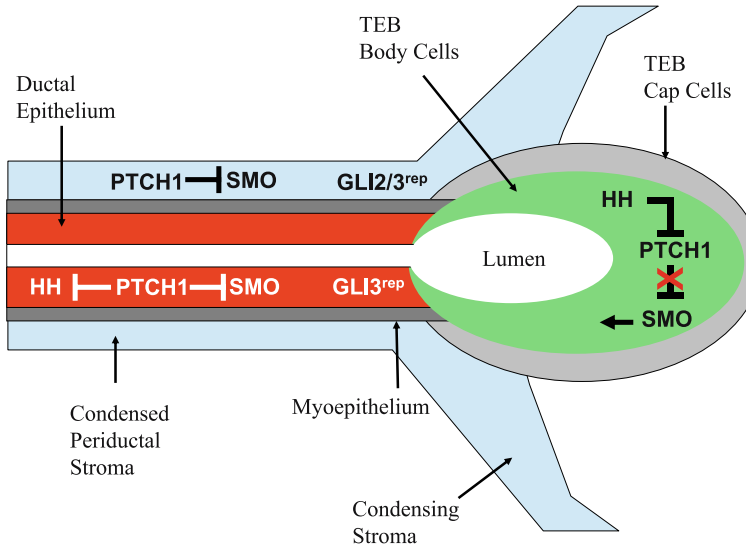

Fig. 2. Our current working model for hedgehog pathway function during ductal development. In this hypothetical model, hedgehog signaling (canonical? non-canonical?) is activated transiently in the body cells of the TEB. As luminal epithelial cells differentiate, we propose that signaling must be inactivated (by PTCH1, in part). Under these conditions, GLI3 is predicted to function as a transcriptional repressor. Similarly, in the condensed stroma, hedgehog signaling must also be inhibited (by PTCH1, in part). Under these conditions, GLI2 and GLI3 are predicted to function as transcriptional repressors. It should be noted that of the network genes shown, only *Ptch1* and *Gli2* show a phenotypic effect as single-gene loss-of-function mutations, and Smo loss-of-function has not been tested

is active transiently in the TEB, perhaps to regulate stem/progenitor cell behavior, but that it must be inhibited normally in both the epithelium and periductal stroma in the adult virgin mammary gland (Fig. 2) (Lewis and Veltmaat 2004). This model has allowed specific and testable hypotheses to be formulated regarding the functional consequences of hedgehog network mutations in vivo.

9 Does Activated Hedgehog Signaling Regulate Mammary Epithelial Stem Cell Self-Renewal or Maintenance?

Consistent with the hypothesis that activated hedgehog signaling could contribute to regulation of mammary epithelial stem and progenitor cells, Liu and colleagues (Liu et al. 2006) demonstrated that treatment of reduction mammoplasty-derived, normal human breast epithelium with recombinant hedgehog ligand increased both primary and secondary mammosphere formation (in vitro assays of anchorage-independent survival and growth, and of self-renewal of stem and progenitor cell types (Dontu et al. 2003; Dontu and Wicha 2005). Treatment with ligand also increased the relative proportion of mammospheres capable of multilineage differentiation in vitro. Conversely, treatment with the hedgehog signaling antagonist cyclopamine decreased mammosphere formation and also decreased the relative proportion of mammospheres capable of multilineage differentiation. Expression of either *Gli1* or *Gli2* with viral vectors also increased primary and secondary mammosphere formation. Growth stimulation by *Gli2* resulted in dysplastic outgrowth on transplantation of transduced mammospheres into the fat pad of immunocompromised mice.

Mechanistically, hedgehog network activation increased the expression of the polycomb gene, *Bmi1*, which has been shown to be required for self-renewal in hematopoietic and neural stem cells (Molofsky et al. 2005; Park et al. 2003), suggesting that *Bmi1* might be a downstream target of the hedgehog signaling network (Liu et al. 2006). Transduction of primary mammary epithelial cells with a lentivirus expressing shRNA against *Bmi1* decreased mammosphere formation significantly. Importantly, *Bmi1* overexpression immortalizes mammary epithelial cells (Dimri et al. 2002). Taken together, these data suggested that activated hedgehog signaling could regulate stem/progenitor cell self-renewal via regulation of *Bmi1*.

Recent work from our laboratory in mice is also consistent with the hypothesis that activated hedgehog signaling can function to regulate mammary epithelial stem or progenitor cells in vivo (Moraes et al. 2007). We have conducted a phenotypic analysis of a new transgenic mouse model in which we expressed activated human SMO (SmoM2)

under the mouse mammary tumor virus (MMTV) promoter selectively in mammary epithelium. Expression of this transgene leads to increased proliferation, altered differentiation, and ductal dysplasias distinct from those caused by *Ptch1* heterozygosity, despite similar increases in proliferation in both models. As in the study by Liu et al. (Liu et al. 2006), SMO-mediated hedgehog signaling activation increased the mammosphere-forming efficiency of primary mammary epithelial cells approximately two-fold relative to wild-type cells. However, limiting-dilution transplantation of isolated mammary epithelial cells showed a decrease in the frequency of regenerative stem cells in *MMTV-SmoM2* epithelium relative to wild type, suggesting that enhanced mammosphere-forming efficiency by *MMTV-SmoM2* cells was due to increased survival, or activity, of downstream division-competent progenitor cell types under anchorage-independent growth conditions, rather than an increase in the proportion of regenerative stem cells per se.

On the other side of this debate, Hatsell and Cowin (Hatsell and Cowin 2006) have interpreted their gene expression and genetic analysis of *Gli3* in mice to indicate that activated hedgehog signaling plays no role whatsoever in embryonic or postnatal virgin mammary gland development, and thus could not possibly regulate stem or progenitor cells. Indeed, we previously posed the question of whether mouse mammary gland development might be entirely hedgehog ligand independent, since data available at the time could be interpreted as such (Lewis and Veltmaat 2004). In addition to the lack of phenotype in either the *Ihh* or *Shh* targeted disruption mutants, the assertion by these authors that the hedgehog network is entirely inactive during mammary gland development is based on the observations that *Gli3* (primarily a transcriptional repressor), is the only *Gli* gene expressed in the luminal epithelium, as well as on the lack of detectable Gli1-LacZ or Ptch1-LacZ expression in any tissue compartment in the mammary gland aside from the lymphatic vessels.

Obviously, somethin's gotta give.

The problem with *all* of these studies is that none of them constitutes a definitive test for the requirement of activated hedgehog signaling in stem cell self-renewal or maintenance specifically, or in mammary gland development generally.

The analysis of human mammary epithelial stem cells is hampered by our current inability to demonstrate regenerative stem cell function formally by xenograft transplantation and regeneration of a functional mammary ductal tree. Thus the increased proportion of mammospheres possessing multilineage differentiation capacity in response to ligand treatment or *Gli* overexpression could reflect an increase in a multipotent progenitor population (possibly lacking regenerative capacity), rather than an increase in the regenerative stem cell population itself. Until xenograft transplantation techniques improve, this hypothesis cannot be tested. However, if true, this alternative interpretation of the human data would then be entirely consistent with our observations with the *MMTV-SmoM2* model that mammosphere formation efficiency can be increased dramatically as a consequence of hedgehog network activation, yet still be associated with a decrease in the frequency of regenerative stem cells (Moraes et al. 2007).

Despite the possible biological consistency with the study by Liu et al, our analysis of the *MMTV-SmoM2* transgenic mouse also has significant limitations. As with all genetic analyses using transgenes, our analysis of mouse mammary gland development and epithelial stem cell behavior using the constitutively activated *SmoM2* allele only indicates what hedgehog network activation *can* do with respect to gland development, not what it *does* do in vivo. In addition, the *MMTV-SmoM2* transgene only showed detectable expression in a relatively small fraction of epithelial cells (~5%) in virgin mice. These cells were almost certainly not all stem cells, and there are some data to suggest that the MMTV promoter is expressed preferentially in more differentiated cell types (Welm et al. 2005). As such, we may not have driven expression in the correct cell type to observe an effect due to SMO function within the regenerative stem cells themselves. Thus it remains possible that SMO expression in regenerative stem cells might have different functional consequences than SMO expression in a downstream progenitor or differentiated cell type.

Finally, the analysis of *Gli* gene expression and phenotypes by Hatsell and Cowin also suffers from limitations. For example, transplantation rescue of embryonic mammary glands from *Gli3*-null embryos was not performed by these authors (Lewis et al. 2001). Thus phenotypic consequences of *Gli3* loss during ductal (or alveolar) development

were not examined. As a result, nothing can be said about how *Gli3* might, or might not, function in postnatal gland development. Even if these transplants had been performed, functional redundancy is known to exist at the *Gli* transcription factor level, particularly between *Gli2* and *Gli3*. As pointed out previously, this fact poses a significant challenge if one wishes to study these components functionally in vivo. For example, if endogenous *Gli2* expression is derepressed in the epithelium as a consequence of *Gli3* loss-of-function, simultaneous disruption of both *Gli2* and *Gli3* may be necessary to achieve a phenotypic effect. With respect to the *Gli* and *Ptch1-LacZ* knockin reporter systems mentioned above, the lack of detectable expression of Ptch1-LacZ or Gli-LacZ in the mammary gland could be explained by the mechanisms already mentioned related to protein levels or mRNA stability from the LacZ mutant alleles. Furthermore, it is known that elevated expression of *Ptch1* and *Gli1* occurs only at intermediate and high levels of hedgehog signaling (e.g., the floorplate of the neural tube) and not at low levels of signaling (discussed in Hooper and Scott 2005). Thus, if only low-level hedgehog signaling is required for mammary gland development, induction of *Ptch1* and *Gli1* would not necessarily be expected. In addition to these issues, the possibilities of non-*Gli*-mediated, or "non-canonical", hedgehog signaling were not acknowledged.

A definitive test for the requirement of activated hedgehog signaling in these processes would require complete ablation of *all* hedgehog signaling in a given tissue compartment in vivo. Elimination of all hedgehog signaling could be accomplished by tissue-specific or cell type-specific disruption of the *Smo* gene, which, based on current data, is essential for all ligand-activated hedgehog signaling and is among the few genes in the network to be nonredundant. This critical test is in progress in our laboratory thanks to the availability of an appropriate genetic model for cre-recombinase-mediated *Smo* disruption (Long et al. 2001). This model, and a similar model for conditional disruption of *Ptch1* (Ellis et al. 2003), now offer us the opportunity to perform the key in vivo genetic experiments necessary to demonstrate conclusively whether activated hedgehog signaling is required in normal mammary epithelial stem cells, and to determine whether hedgehog pathway activation is required in the epithelium at any time for normal mammary gland development generally.

10 Evidence for a Role of the Hedgehog Network in Mammary Cancer

Altered hedgehog signaling is now implicated in the development of approximately 20%–25% of all cancers (Briscoe and Therond 2005), especially soft tissue cancers. In most cases, it appears that hedgehog activation plays a causal role (Fig. 3). Network activation can be achieved by a number of gain-of-function mutations in positively acting components (e.g., ligands, *Smo*, *Gli1*, *Gli2*), as well as by loss-of-function mutation in *Ptch1*, a key inhibitor of hedgehog network function, as well as in *Su(Fu).* With respect to human breast disease, data are accumulating gradually that suggest a role for altered hedgehog signaling in mammary cancer development or disease progression. However, efforts to determine whether activated hedgehog signaling leads to altered tumor development or metastatic behavior have thus far been inconclusive.

In mutational analysis, an early study found *Ptch1* mutations in two of seven human breast cancers (Xie et al. 1997). Additionally, a *Ptch1* polymorphism was linked to increased breast cancer risk associated with oral contraceptive use (Chang-Claude et al. 2003). More recently, array comparative genomic hybridization (CGH) analyses indicate that genomic loss at the *Ptch1* locus was the fourth most commonly detected change among the tumor suppressor genes identified in the study, occurring in 19% of human breast cancers and 33% of breast cancer cell lines (Naylor et al. 2005). However, until very recently (Sjoblom et al. 2006), no mutations in other network components had been identified in breast cancer (Vorechovsky et al. 1999). With exhaustive sequencing efforts, three missense mutations in the *Gli1* gene were identified in 11 breast cancer samples examined, but these mutations were carried in the heterozygous state and have not yet been analyzed functionally.

By expression analysis, a recent immunohistochemical staining study suggested that hedgehog signaling is activated in a majority of human invasive breast cancers based on ectopic expression of PTCH1 and GLI1 (Kubo et al. 2004) in 50 out of 52 clinical samples investigated. However, as mentioned previously, neither SHH, PTCH1, nor GLI1 expression was detected in normal tissue in this same study, whereas these genes have been detected in normal tissue by others. For example, a second study examining differential expression of hedgehog network genes

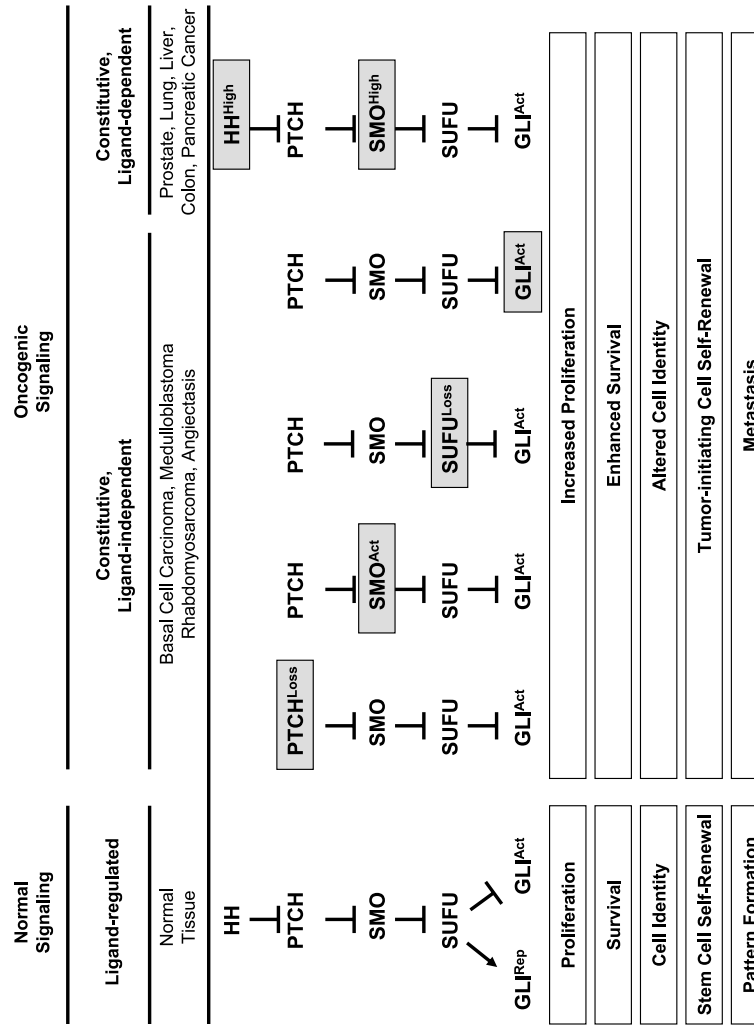

Fig. 3. Models of tumorigenesis resulting from constitutive hedgehog/GLI signaling. Ligand-independent tumor growth arises from loss of *Ptch1* ($PTCH^{Loss}$), or loss of *SuFu* ($SUFU^{Loss}$), as well as from activating mutations in *Smo* (SMO^{Act}) or Gli genes (GLI^{Act}) (*gray boxes*). In contrast, other cancers are ligand dependent. In these tumors constitutively elevated pathway activity can be due to increased ligand (HH^{High}) and *Smo* ($SMOH^{High}$) expression (*gray boxes*). Both ligand-dependent and ligand-independent tumors are characterized by an increase in GLI^{Act} (transcriptional activator) activity, and organ-specific GLI target gene expression. General cancer-related processes activated downstream of HH/GLI (e.g., proliferation, survival, metastasis, and stem cell activation) are shown. (Modified from Kasper et al. 2006)

in at least eight patient-matched samples total showed SHH, PTCH1, and GLI1 expression (Mukherjee et al. 2006) in both normal and cancer tissue. In this study, expression of SHH was increased more than twofold in the epithelium of five of eight samples, with a more than twofold increase in GLI1 expression in cancerous epithelium in four of eight samples examined. There was also a greater than two-fold increase in PTCH1 expression in three of nine samples examined. Immunostaining of these three proteins in normal or cancer-associated stroma was reduced relative to the epithelium. With microdissection, Gli1 protein and mRNA expression were positively correlated with one another. However, Ptch1 protein and mRNA expression were not positively correlated in these samples, as might be expected based on current models for hedgehog network function. With respect to mRNA expression, one striking observation was that *Smo* mRNA levels were significantly higher than normal in four of 10 samples examined.

We have also conducted an immunohistochemical study for expression of PTCH1 and SMO in a large panel of normal ductal carcinomas in situ (DCIS) and invasive breast cancer (IBC) samples (Moraes et al. 2007). In our study, like that of Mukherjee et al. (Mukherjee et al. 2006), PTCH1 was detectable at moderate levels throughout the epithelium and in isolated stromal cells of the normal breast. While some samples in our study showed strong PTCH1 expression, PTCH1 expression was decreased or absent in ~50% of DCIS and IBC. With respect to SMO, expression was undetectable in normal breast, but SMO was ectopically

expressed in ~70% of DCIS and ~30% of IBC. PTCH1 expression was not correlated significantly with SMO expression in either DCIS or IBC. Altered SMO protein expression in human breast cancer was generally consistent with the Q-PCR results of Mukherjee et al. (Mukherjee et al. 2006) that showed increased mRNA expression in ~40% of their samples. Together, these data suggest that altered hedgehog signaling occurs early, and often, in human breast disease progression.

With respect to a possible predictive or prognostic utility of altered hedgehog network gene expression, expression of PTCH1 or SMO did not correlate with histological grade (DCIS only) or with expression of any clinically relevant marker tested (estrogen receptor, ErbB2, p53). It will be of considerable interest to determine whether SMO or PTCH1 expression correlates with any other clinically relevant parameter such as metastatic or osteolytic behavior (Sterling et al. 2006), given the recent finding that expression of *Gli2* in MDA-MB-231 cells mediates osteolytic behavior of these cells via induction of parathyroid hormone-related protein.

11 Could Activated Hedgehog Signaling Regulate "Breast Cancer Stem Cells"?

If, in fact, activated hedgehog signaling regulates behavior of normal mammary epithelial stem cells, it is entirely possible that it might also influence the behavior of breast cancer-initiating cells or "breast cancer stem cells." In their study of hedgehog regulation of normal mammary epithelial stem/progenitor cell self-renewal, Liu and colleagues explored this possibility (Liu et al. 2006). With $CD44^{+}$;$CD24^{-/low}$;Lin^{-} cells isolated from a xenograft of a metastatic human breast cancer, the subpopulation shown previously to be tumorigenic in xenografts in NOD/Scid mice, showed increased expression of *Ptch1*, *Gli1*, *Gli2*, and *Bmi1* mRNAs relative to all other cells isolated from the same tumor, as well as from the bulk tumor. These data suggested that hedgehog signaling might be activated in the tumorigenic subpopulation of cancer cells. In addition to these data in cancer xenografts, overexpression of either *Gli1* or *Gli2* in normal mammosphere-initiating cells led not only to increased mammosphere formation but also to dysplastic growth

as xenografts. While tumor formation was not tested, these provocative results suggested that hedgehog network activation might also regulate the behavior of breast cancer-initiating cells.

Further support for a potential influence of activated hedgehog signaling on malignant stem/progenitor cells comes from our immunohistochemical analysis of PTCH1 and SMO expression in human breast cancer. As part of our study (Moraes et al. 2007) we explored the relationship between PTCH1 or SMO expression and proliferation, using dual immunofluorescence analysis of PTCH1-Ki67, and of SMO-Ki67, in a subset of DCIS and IBC. In tumors expressing PTCH1 there was extensive co-localization with the Ki67 proliferation marker. However, in tumors expressing SMO, SMO expression rarely colocalized with the Ki67 proliferation marker. This observation in human breast cancer was remarkably consistent with our observation that SMO protein expression does not colocalize frequently with proliferation markers in the *MMTV-SmoM2* mouse model. These results were entirely unexpected given reports that hedgehog signaling activation appears to increase proliferation directly in other cell types (e.g., Detmer et al. 2005; Hutchin et al. 2005; MacLean and Kronenberg 2005; Palma et al. 2005).

12 Can Hedgehog Signaling Antagonists Be Used for the Treatment of Breast Cancer?

Hedgehog signaling inhibitors have been used successfully in vitro to inhibit growth of a variety of cancer cell lines and in vivo for the treatment of medulloblastoma (Berman et al. 2002; Romer et al. 2004). Amazingly, mice treated with these agents show little evidence of adverse side effects. If, indeed, activated hedgehog signaling plays a role in breast cancer development or progression, perhaps directly within the stem cells themselves or indirectly via a paracrine mechanism, small-molecule hedgehog signaling antagonists would appear to be attractive candidates for breast cancer treatment (Lewis and Veltmaat 2004).

Two groups have recently shown that the hedgehog signaling inhibitor cyclopamine can inhibit growth of a subset of breast cell lines in vitro (Kubo et al. 2004; Mukherjee et al. 2006) at doses of ~10 μM and above. Cyclopamine was shown to inhibit proliferation and to induce

apoptosis, as well as to inhibit expression of a *Gli*-dependent luciferase reporter in sensitive cell lines (Mukherjee et al. 2006). These data have suggested that hedgehog signaling may be active in a subset of human breast cancer cell lines, and that hedgehog signaling antagonists might inhibit breast cancer growth in vivo.

However, as aptly pointed out by Mukherjee et al. (Mukherjee et al. 2006), the specificity of cyclopamine at doses required for growth inhibition remains an open question. For example, in most cell lines used to examine the molecular mechanism of hedgehog signaling, cyclopamine and other, more potent, antagonists have IC_{50} values in the 300 nM range or below (Chen et al. 2002a,b; Masdeu et al. 2006; Mimeault et al. 2006; Williams et al. 2003) using a number of accepted readouts for network activity, including reduction of *Ptch1* and *Gli1* expression (nearly "universal" targets induced by activated hedgehog signaling). Thus several questions must be addressed before hedgehog signaling antagonists can be considered as viable target-based therapeutic agents for the treatment of breast cancer. For example, if breast cancer cell line growth is, in fact, being promoted by activated hedgehog signaling (which has never been demonstrated formally), and signaling antagonists truly function in the nanomolar concentration range, why then does it require micromolar doses of cyclopamine to inhibit proliferation in breast cancer cell lines? Similarly, if hedgehog signaling is truly active in a subset of breast cancer cells in vitro, why does treatment with high doses of cyclopamine have only minimal (*Gli1*) or no (*Ptch1*) effect on expression of these two "universal" molecular readouts of activated signaling (Mukherjee et al. 2006)? Thus given current data, it remains possible that the doses of cyclopamine required to inhibit growth can inhibit not only hedgehog signaling but also a secondary target (or targets) that may, in reality, mediate the proliferative and apoptotic responses to antagonist treatment.

13 Concluding Remarks

While conclusive data regarding the controversies outlined above are forthcoming, we do not, as yet, have definitive evidence that *activated* hedgehog signaling has a role in regulating normal mammary epithelial

stem cell behavior specifically, or mammary gland development generally. Similarly, we do not have definitive data demonstrating a role for activated hedgehog signaling in breast cancer development or disease progression. However, current genetic data do indicate that *inhibition* of hedgehog signaling is important for control of proliferation and histoarchitecture, and that activated hedgehog signaling *can* regulate important aspects of the behavior of both normal and malignant mammary epithelial stem and progenitor cells.

An important missing piece to this puzzle is whether activated hedgehog signaling can function directly within the stem/progenitor cells themselves, or whether it can also function indirectly via a paracrine influence of neighboring epithelial cells in which signaling is not activated. A second missing piece of the puzzle is whether there is also a role for activated signaling in the mammary stroma in the aberrant behavior of mammary epithelial cells in cancer development.

Should activated hedgehog signaling ultimately be shown to play a contributory role in breast disease development or progression, in either the epithelium or the stroma, the good news is that small-molecule antagonists of hedgehog signaling already exist, and appear to be well tolerated in vivo at effective doses (at least in animal models). Thus, in time, these and similar compounds could indeed emerge as a new class of therapeutic agents–either alone, or in combination with conventional therapies–for the treatment or prevention of breast cancer.

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Strategies to Eliminate Cancer Stem Cells

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Abstract. Therapeutic advances over the past three decades now allow most cancer patients to achieve major clinical responses. Although clinical responses can clearly decrease side effects and improve quality of life, most cancer patients still eventually relapse and die of their disease. Emerging data suggest that initial responses in cancer represent therapeutic effectiveness against the differentiated cancer cells making up the bulk of the tumor, while rare biologically distinct cancer stem cells resistant to the therapies are responsible for relapse. Better understanding the biology of cancer stem cells, and reexamining both our preclinical and clinical drug development paradigms to include the cancer stem cell concept, have the potential to revolutionize the treatment of many cancers.

1 The Paradox of Response and Survival

A cardinal principle of cancer therapeutics has been that evidence of a clinical response will translate into improved survival. The major advantage of using clinical response as the primary end point of thera-

peutic trials is that it is measurable over weeks to months, allowing the stepwise process of drug development to occur more rapidly and efficiently. In contrast, demonstrating a survival benefit adds significant complexity to clinical trial design, usually requiring the accrual of large patient numbers and long follow-up to provide statistical significance.

Although clinical responses can clearly decrease side effects and improve quality of life, there is surprisingly little evidence that disease response is an appropriate surrogate for survival (Huff et al. 2006). In fact, there are numerous examples in which even dramatic responses are not predictive for improved survivals. Indolent lymphoma patients who achieved a complete remission with conventional-dose therapies in the pre-rituximab era did not experience a survival advantage over similar patients treated with a "watch and wait" approach (Horning 1993). In multiple myeloma (MM), neither the magnitude nor the kinetics of clinical response had an impact on survival (Durie et al. 2004). Even the most intensive therapy for MM, blood or marrow transplantation (BMT) (Attal et al. 1996; Child et al. 2003), provided no overall survival advantage in the recently published national intergroup trial (Barlogie et al. 2006) or a recent meta-analysis (Levy et al. 2005). Furthermore, despite new treatments that now produce complete remissions in the majority of women with ovarian carcinoma, cures are rare (Armstrong et al. 2006).

This apparent paradox that treatment response and survival are not necessarily linked may be explained by the presence within the malignant clone of small populations of biologically distinct cells (i.e., cancer stem cells) that are responsible for initiating and sustaining the growth of the cancer (Jones et al. 2004; Huff et al. 2006). It had long been accepted that chronic myeloid leukemia (CML), although characterized by an excess of differentiated myeloid cells, arose from hematopoietic stem cells (HSC) (Fialkow et al. 1977); this was confirmed nearly 15 years ago when several groups identified and isolated CML stem cells with characteristics known to define normal HSC (Bedi et al. 1993). Since normal plasma cells are terminally differentiated, we hypothesized that MM might parallel CML: That is, there are MM stem cells that are biologically distinct from the malignant plasma cells forming the bulk of the tumor. Accordingly, we found that MM plasma cells are progeny of less-differentiated, self-renewing cancer stem cells with a memory B cell phenotype (Matsui et al. 2004b). Although not stem

cells in the classic sense, memory B cells can be considered "honorary" stem cells, in that they are long-lived and self-renew in order to maintain long-term immune memory as well as differentiate into plasma cells. Cancer stem cells that are biologically distinct from the differentiated cells that make up the bulk of the tumor have also been found in myelodysplastic syndromes (Nilsson et al. 2002), breast cancer (Al Hajj et al. 2003), brain cancer (Singh et al. 2003; Hemmati et al. 2003), and lung cancer (Kim et al. 2005).

2 The Dandelion Phenomenon

Emerging data from some of the most successful new anticancer agents have helped shed light on this paradox that response and survival are not always linked. Imatinib has replaced interferon-α as the standard of care for newly diagnosed CML patients, based on an interim analysis of a multicenter, randomized trial showing higher response rates for imatinib (O'Brien et al. 2003). With up to 5 years of follow-up, most of the complete cytogenetic remissions to imatinib remain durable (Roy et al. 2006). However, it now appears that imatinib may not be able to completely eradicate CML. CML patients who achieve the best responses to imatinib (reverse transcriptase polymerase chain reaction negativity for BCR-ABL transcripts) can relapse quickly when the drug is discontinued (Cortes et al. 2004; Merante et al. 2005; Mauro et al. 2004), or even progress while remaining on the drug (Mauro et al. 2003).

BCR-ABL gene amplification or mutations prevent productive imatinib binding (Gorre et al. 2001), and secondary genetic mutations capable of driving BCR-ABL-independent leukemic growth may also be present, even at initial diagnosis (Hochhaus et al. 2002). However, these genetic mechanisms of resistance are probably not responsible for the persistent CML in most patients treated with imatinib. Several investigators have now provided evidence that imatinib has differential effects on CML cells depending on their state of differentiation: While imatinib is toxic to differentiated CML progenitors, CML stem cells may be relatively or even completely resistant to the drug (Graham et al. 2002; Holtz et al. 2002; Angstreich et al. 2005). The basis for the differential activity of imatinib toward CML stem cells and their differ-

entiated progeny is likely multifactorial (Angstreich et al. 2005). CML stem cells share many biological properties with their normal counterparts (Bedi et al. 1993) that likely limit the effectiveness of therapeutic strategies targeting BCR-ABL signaling. Hematopoietic stem cells are largely quiescent and normally express high levels of ATP-binding cassette (ABC) transporters, such as the multidrug resistance-1 gene (Raaijmakers et al. 2002) and ABCG2 (Bunting 2002). Both of these factors may limit the cellular uptake of imatinib, which is a substrate for the ABC transporters (Burger et al. 2004; Mahon et al. 2003). Maybe most importantly, BCR-ABL expression appears to be required for the survival of CML progenitors, but the same does not appear to be true for CML stem cells, where the BCR-ABL gene can be silent (Bedi et al. 1993; Jiang et al. 2003). These data suggest that BCR-ABL may produce only subtle effects in CML stem cells, and thus its inhibition may similarly have only minor consequences for these cells (Angstreich et al. 2005). Therefore, based on the longevity of their normal counterparts, CML stem cells likely survive for years even if BCR-ABL activity is completely inhibited (Bedi et al. 1993); eventually, because of intrinsic genomic instability, CML stem cells and their progeny may develop true genetic resistance to imatinib.

We recently found that the novel anti-MM agents bortezomib and lenalidomide have little activity against MM stem cells *in vitro* despite being quite active against the MM plasma cells (Matsui et al. 2004a). Conversely, rituximab and alemtuzumab (Campath) eliminated MM stem cells *in vitro*, but had no activity against MM plasma cells that lack the relevant target antigens (CD20 and CD52, respectively). Although rituximab's activity in MM has been disappointing (Treon et al. 2002), parameters typically used to follow clinical response in MM (i.e., monoclonal immunoglobulin level and percentage of plasma cells in the marrow) primarily measure the effect of therapies on the terminally differentiated plasma cells. The long survival of the MM plasma cells could have obscured activity against the MM stem cells responsible for maintaining the disease. Perhaps a longer duration of rituximab treatment could ultimately have demonstrated clinical responses according to traditional criteria, by inhibiting new MM cell production for a sufficient period of time to allow terminally differentiated MM plasma cells to undergo spontaneous apoptosis.

The rapid responses induced by imatinib in CML (O'Brien et al. 2003) and bortezomib in MM (Richardson et al. 2003) are likely a consequence of their impressive activity against differentiated progenitors that make up the bulk of the tumor. Emerging data suggesting that these early responses may not be durable (Richardson et al. 2003; Cortes et al. 2004; Mauro et al. 2003, 2004; Merante et al. 2005) are consistent with findings that cancer stem cells from these diseases are resistant to these agents (Angstreich et al. 2005; Matsui et al. 2004a). This pattern of activity is analogous to mowing a dandelion; although this will eliminate the visible portion of the weed, the unseen root also must be eliminated to prevent regrowth of the weed (Jones et al. 2004; Angstreich et al. 2005; Huff et al. 2006). Conversely, responses to interferon in CML are slow and gradual, often taking years to develop, but can be durable (Bonifazi et al. 2001). This pattern of response fits our data showing that interferon's activity is directed principally at the rare CML stem cells (Angstreich et al. 2005). Our data with rituximab and alemtuzumab in MM (Matsui et al. 2004a) are other examples of drugs that selectively target cancer stem cells. Such a treatment effect mimics attacking just the root of the dandelion; although this has no immediately discernible effect on the weed, over time the weed will eventually wither and die if its root has been eliminated (Jones et al. 2004; Angstreich et al. 2005; Huff et al. 2006).

3 Eliminating Cancer Stem Cells

Currently, the search for novel anticancer therapies is primarily focused on oncogenes that are specific for (e.g., BCR-ABL in CML), or overexpressed in (e.g., c-myc or bcl-2), selected cancers. However, attacking cancer-specific targets has met with variable success, and many of the most effective anticancer therapies, such as rituximab in lymphomas, high-dose cytotoxic therapy, or the allogeneic graft-versus-tumor effect, show limited or even no tumor selectivity. Targeting a cancer-specific pathway could fail for several reasons. It is likely that many cancers have already acquired multiple oncogenic mutations, even at initial diagnosis, capable of driving tumor growth; in such cases, targeting only one oncogene might be expected to generate limited activity. Further, as

already discussed, even when the initiating oncogenic event is targeted as with imatinib in CML, inherent properties of stem cells may make the target inaccessible or no longer relevant (Angstreich et al. 2005; Jones et al. 2004).

Properties shared with normal stem cells not only appear to be responsible for cancer stem cell resistance to many anticancer agents, they may also lead to the development of novel therapies active across many malignancies. In fact, prospective targets shared with normal stem cells may have particularly strong anticancer potential since their conserved expression suggests a critical function retained by the cancer stem cells. Several signaling pathways that are important for the generation and maintenance of normal stem cells during embryonic development [e.g., Notch, Wnt, and Hedgehog (Hh)] (Reya et al. 2001) and/or postnatally [e.g., telomerase (Harrington, 2004) and growth factors] also appear to be important for the growth of many cancers. Preliminary data suggest that inhibition of these pathways, even when they are not mutated or overexpressed, may produce potent antitumor activity across a range of malignancies, possibly because of the key roles these pathways play in stem cell maintenance and growth.

While toxicity from lack of tumor specificity is an obvious concern for shared stem cell targets, there are several potential differences between normal and cancer stem cells that may provide a therapeutic ratio for shared targets. Normal stem cells have normal cell cycle checkpoints that are likely to protect them from cellular damage or crisis. The stage of differentiation at which cancers arise may also provide a therapeutic ratio for approaches targeting cancer. Although many cancers may arise from tissue stem cells, they may not be the most primitive tissue stem cells as exemplified by CML (Bedi et al. 1993). Accordingly, if a therapy equally eliminated both CML stem cells and their normal counterparts, the existence of more primitive normal stem cells should replenish the normal progenitor pool (Angstreich et al. 2005).

Another example of a shared stem cell target potentially providing tumor selectivity is telomerase, where differences between cancer stem cells and their normal counterparts in the interplay of telomere length and telomerase should provide a therapeutic ratio. Normal stem cells require telomerase to prevent telomere shortening leading to replicative senescence. However, even in the absence of telomerase, normal stem

cells can maintain replicative capacity for some period of time because of their relatively long telomeres. Accordingly, telomerase-knockout mice show deficits only after four to six generations (Hao et al. 2005). In addition, the major cause of death in dyskeratosis congenita, a congenital disease that results from loss-of-function mutations in telomerase components, is bone marrow failure, but this usually does not manifest until the second or third decade of life (Dokal and Vulliamy 2003). In contrast, uninterrupted telomerase activity may be absolutely required for the maintenance and growth of most malignancies, in order to stabilize the short telomeres that characterize cancer cells. In fact, crossing telomerase-knockout mice with *INK4a*$^{-/-}$ (Greenberg et al. 1999) or *APC*^{min} (Rudolph et al. 2001) mice predisposed to cancer significantly lowered the development of cancers in these mice. Thus the differential in telomere length between normal (long) and cancer (short) stem cells should provide differential sensitivity to telomerase inhibition.

Because of the difficulty in assessing the effects of therapies on the rare cancer stem cells responsible for relapse, the development of such approaches requires new clinical paradigms and methodologies (Huff et al. 2006). We believe these new paradigms should rely heavily on preclinical modeling, employ nontraditional measures of clinical response as trial end points, and utilize novel preclinical assays to evaluate the fate of cancer stem cells. Preclinical studies should assess the effects of therapies on both cancer stem cell and differentiated cancer cell populations. With the correct preclinical models, it may be possible to develop a detailed understanding of the mechanisms of action of new treatments, as well as strategies for optimizing activity; this could potentially allow a fully developed new approach to be taken directly "from the bench to the bedside". However, effective preclinical models for cancer stem cells may ease the task of clinical trial development, but will not eliminate the need for new clinical paradigms. Evaluating the efficacy of treatments against cancer stem cells should be possible by utilizing these treatments after debulking the differentiated cells that constitute the majority of the tumor. In cancers where clinical debulking is successful (i.e., complete remissions are common but transient), studying therapies after induction of remission should permit the use of duration of remission as a measure of activity against the can-

cer stem cells. The fate of cancer stem cells could also be assessed as secondary laboratory end points with newly developed preclinical assays.

4 Conclusions

Initial responses in cancer represent therapeutic effectiveness against the differentiated cancer cells making up the bulk of the tumor, while resistant cancer stem cells often appear to be responsible for relapse. Since many currently active treatments have been developed to target the differentiated cancer cells, they may have little activity against the biologically distinct cancer stem cells. Traditional response criteria measure tumor bulk and may not reflect changes populations of rare cancer stem cells (Huff et al. 2006). Standard response parameters may not only potentially overestimate the effect of therapy on the minute population of stem cells, but may also underestimate it. As with interferon for CML and rituximab in MM, therapy selectively directed at cancer stem cells will not immediately eliminate the differentiated tumor cells; such therapy therefore might be prematurely abandoned if clinical activity is judged solely by standard response criteria that reflect the effects of treatment on the bulk of the cancer. Thus improving the results of cancer therapy requires identification and better understanding of the biology of cancer stem cells, as well as reexamination of both our preclinical and clinical drug development paradigms to include the cancer stem cell concept.

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DNA Repair in Stem Cell Maintenance and Conversion to Cancer Stem Cells

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Abstract. Genomic stability is essential for cell and organism longevity. Without genomic stability, replication errors and external stress as well as direct forms of DNA damage can induce mutations, which decrease cell survival, cause altered gene expression, and can lead to cellular transformation. All represent the antithesis of maintenance of normal stem cell function. We argue here that genomic stability is essential for stem cell maintenance and longevity. This concept is supported by human diseases associated with premature aging and animal models of DNA damage repair abnormalities all of which lead to abnormalities of stem cell survival. Furthermore, with competitive repopulation, hematopoietic stem cell survival can be assessed in the face of DNA repair defects, and results from these studies support the general conclusion that

chemotherapy and other forms of DNA damage lead to stem cell failure syndromes and malignant transformation most commonly along the myeloid and lymphoid pathways. Thus one origin of the cancer stem cell phenotype is the inability to maintain genomic stability among the stem cell population leading to mutational alterations and transformation. Capturing stem cells at this transition point represents an exciting field of discovery possibly leading to early detection and therapeutic interventions.

1 Human Diseases Linked to Inherited Defects in DNA Repair Genes

Numerous human diseases are associated with defects of DNA repair. Many of these are associated with stem cell failure syndromes, malignant transformation, and/or premature aging (Table 1). For instance, the Fanconi anemias complementation groups, FANC A, FANC C, FANC G, FANC D1, FANC D2, and FANC F, all have abnormalities in DNA double-strand break pair processing (Yamashita and Nakahata 2001). Mutations in these genes are associated with multiple physical abnormalities, mental retardation, renal failure, pancytopenia, and malignant transformation to acute leukemia, as well as myeloplastic syndrome (Kook 2005). Similarly, we found that Li-Fraumeni syndrome caused by a mutation in p53 is associated with multiple malignancies including leukemia. Various syndromes associated with abnormalities of nonhomologous end-joining including mutations and truncations in the DNA-PK, Ku70, and Ku80 genes are associated with abnormalities of T and B cell function (Gennery 2006). Clinically, individuals have severe combined immunodeficiency (Buck et al 2006; Tachibana 2004). Mice have a tendency to develop lymphoma (Prochazka et al 1992).

A number of abnormalities in nucleotide excision repair including the syndromes xeroderma pigmentosum, Cockayne syndrome, and trichothiodystrophy are associated with abnormalities of organ function and maintenance, skin disorders, sun and radiation hypersensitivity, malignant transformation, and, to a lesser extent, premature aging with the loss of hematopoietic function and bone structure. These syndromes are also associated with premature cancers, both skin and epithelial cancers such as prostate cancer and lung cancer, and leukemias (de Boer

Table 1 Human diseases caused by inherited defects in DNA repair genes

Li-Fraumeni syndrome; p53
SCID: DNA-PK, Ku70/80 [NHEJ]
HNPCC; Msh2, Mlh1 (MMR)
Xeroderma pigmentosum (XP); Xpa to Xpg (NER)
Cockayne syndrome; Csa, Csb (TC-NER)
Trichothiodystrophy; Xpd (NER)
Werner, Bloom, Rothmund-Thomson syndromes; RecQ DNA helicases
Fanconi anemia; FancA to FancG (DSB repair)
Nijmegen breakage syndrome; Nbs (DSB repair)
Ataxia telangiectasia; Atm (DSB repair)
Breast, ovarian cancer; BRCA-1, BRCA2 (DSB repair)

and Hoeijmakers 2000; Hansson 1992; Hoeijmakers 1994; Lockett et al. 2005; Prochazka et al. 1992).

Animal models have been used to analyze the general human phenotype for these disorders (Ishikawa et al. 2004; Meira et al. 2001; van Steeg et al. 2001). In addition, some studies have identified hematopoietic abnormalities associated with these syndromes (Bender et al. 2002; Carreau et al. 1998; Ishikawa et al. 2004; Meira et al. 2001; Prasher et al. 2005; Shultz 1991; van Steeg et al. 2001). However, it is clear that certain nucleotide excision repair abnormalities such as seen in ERCC-1 deficiency have defects in hematopoiesis due to DNA damage hypersensitivity (Prasher et al. 2005). Other syndromes involving DNA repair defects present with a mild phenotype of stem cell abnormality when introduced into mice, including Cockayne syndrome, XPA, and p53 mutation (Kuramoto et al. 2002). These mice are fertile, however, and do not have perceptible hematopoietic abnormalities, although often these strains have a slightly greater tendency to develop lymphoid malignancies, especially T and B cell lymphomas. While there has been much excitement in the study of these abnormalities because of the onset of premature aging, few studies have focused on hematopoietic stem cells.

2 Accelerated Stem Cell Aging After Significant DNA Damage

Mice and humans show evidence of premature aging and loss of stem cells after radiation and chemotherapy exposure. For instance, after chemotherapy or radiation exposure, patients may show evidence of chronic pancytopenia, a conversion to myeloplastic syndrome, and acute leukemia and have delayed onset of secondary lymphoid malignancies as well. Each of these disorders suggests an abnormality of stem cell maintenance, perhaps due to the chronic effects of the mutation caused by radiation and chemotherapy or due to other more subtle changes within the stem cell population. We have observed that C57BL/6J mice 6 months after chemotherapy or radiation exposure develop evidence of premature aging with a vertebral hump and premature graying hair; they also have a shorter life span of approximately 16–18 months compared to the normal life span of over 26 months (Fig. 1). In our studies, mice occasionally develop late evidence of stem cell failure after chemotherapy or radiation exposure, with pancytopenia and death due to hypoplastic marrow. Furthermore, humans who appear to be at greatest risk for leukemic transformation or bone marrow failure syndrome after chemotherapy or radiation exposures appear to have shortened telomeres, suggesting that the inability to maintain chromosomes during replication represents yet another form of DNA damage abnormality. Clonal hematopoiesis has been observed after chemotherapy and is associated with telomere shortening. In addition, stem cell transplantation itself can be associated with telomere shortening (Fern et al. 2004; Lahav et al. 2005).

3 Robust Stem Cell Transplantation as a Marker of Stem Cell Maintenance

Stem cell transplantation represents the most vigorous stem cell stress, explaining why telomere shortening is commonly observed in humans. It has been known for many years that young marrow cells are capable of repopulating recipient marrow spaces and blood more efficiently and at the expense of older marrow cells in competitive repopulation experi-

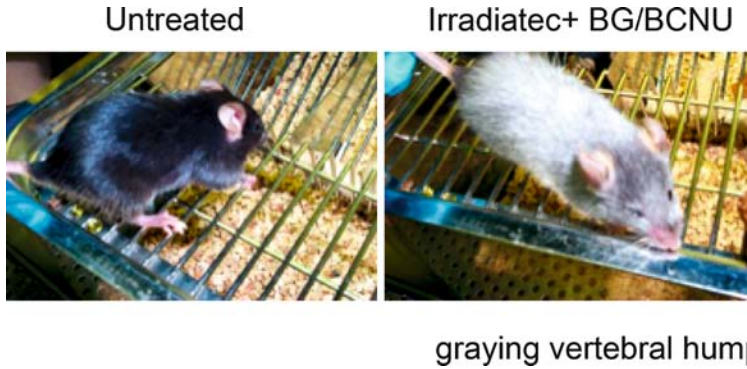

Fig. 1. DNA damage induces aging in normal mice. C57BL/6 mice that have been irradiated with 8.5 Gy of ^{137}Cs and treated with BCNU appear frail and gray at 6 months of age (*right*). The photo on the *left* represents an age- and strain-matched mouse that has not been exposed to DNA-damaging agents

ments in which equal numbers of bone marrow cells derived from young and older donor mice are transplanted into lethally irradiated recipient mice. Furthermore, human umbilical cord blood has a much greater proliferative repopulating potential in the allogenic stem cell setting than adult sibling marrow, again providing clinical evidence for more potent stem cell maintenance at birth compared to adulthood.

Few studies have focused on the role of DNA repair genes in the early stem cell phenotype. Perhaps the most important study by Melton and colleagues (Ramalho-Santos et al. 2002) examined commonly expressed DNA repair genes in hematopoietic stem cells, neural stem cells, and embryonic stem cells. Using comparative hybridization of expressed genes, they identified four key DNA repair genes expressed at high levels in all of the stem cell types. These include Ku 80, a member of the nonhomologous end-joining pathway; MSH 2, a member of the mismatch pair pathway; ERCC5, a protein important in both nucleotide incision repair and radiation double-strand break repair; and RAD23, a gene that is induced during double-strand break repair and after radiation damage. Thus many of the DNA repair pathways are expressed at high levels in early stem cells. Interestingly, some DNA repair genes are expressed throughout early stem cell lineages, whereas

others are expressed at restricted times during development and differentiation. For instance, at the onset of T-cell rearrangement and B-cell rearrangement, nonhomologous VDJ end-joining takes place in each developing T and B cell. RAG 1 and RAG 2 complement the expression of DNA Pk Ku70 and Ku80 proteins to facilitate VDJ rejoining. However RAG 1 and RAG 2 do not appear to be present in hematopoietic stem cells. Mice with knockout of RAG 1 and/or RAG 2 function show absence of lymphoid cells but normal hematopoietic reconstitution. Thus differential expression of DNA damage repair mechanisms as well as tissue-specific aging is seen in DNA repair-defective hereditary diseases, suggesting a lineage-specific function for many of these DNA repair pathways.

These studies lead to the further hypothesis that defects in DNA repair capacity cause mutations in stem cell populations and stem cell exhaustion, abnormalities in differentiation, and/or malignant transformation. We propose that abnormalities in DNA repair leading to stem cell exhaustion may be responsible for stem cell failure syndromes as well as for the onset of improper selective outgrowth of subclones with leukemic transformation potential. Promoting this process is the emergence of genomic instability in the stem cell pool.

4 The Role of Mismatch Repair in Stem Cell Maintenance

Mismatch repair identifies and corrects nucleotide misincorporations and insertions in the nascent DNA strand during replication (Modrich 2006). Common abnormalities identified by mismatch repair include single base mispairs, such as G:T and A:C, or insertions in the nascent DNA strand during DNA replication across large repeats consisting of three or more of the same base (Fig. 2). The critical role of the mismatch repair pathway in malignant transformation has been shown in hereditary nonpolyposis colon cancer, in which inherited defects in one or another of the mismatch repair genes due to mutation result in loss of mismatch repair function and premature onset of colon carcinogenesis as well as a high incidence of endometrial and gastric cancer (Curia et al. 1999). In all of these instances, loss of the normal allele or pro-

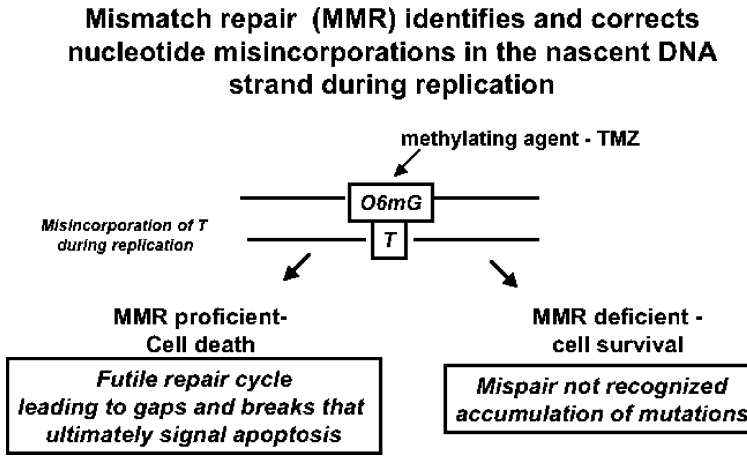

Fig. 2. Mismatch repair-mediated repair links to apoptosis and microsatellite instability

moter silencing due to methylation of the remaining allele is observed. Loss of mismatch repair results in both unrepaired point mutations and replication-related insertions. This gives rise to an accumulation of genomic instability at sites of multiple base repeats known as microsatellites. Polymorphisms of microsatellites are inherited constant regions that are maintained throughout life because of the function of mismatch repair. In the absence of mismatch repair, microsatellite instability occurs at a reasonable frequency in single cells, leading to subclonal populations of cells with the same microsatellite alteration. In precursor or stem cells, these subpopulations can be responsible for malignant transformation. Of interest, lymphocytes from such patients do not show evidence of chromosomal abnormalities (Lindor et al. 1998). Age appears to be an important factor in this process because of either a decline in mismatch repair function or the cumulative effects of acquisition of genomic instability over the decades of replication that occur in human stem/precursor cells. For instance, previous studies have shown that peripheral blood T cells from healthy individuals show increased microsatellite instability (MSI) as a function of age (Krichevsky et al. 2004). However, whether this occurs in all lineages or is representative

of stem cell populations, in particular hematopoietic stem cells, has not been determined.

Our understanding of mismatch repair is based on the bacterial systems identified by Modrich (Modrich 2006). The mouse msh-alpha complex in human cells consists of MSH 2 and MSH 6, which recognize base mispairs, or msh-beta, which consists of MSH 2 and MSH 3, which recognize insertions. Binding of the MSH complex leads to recruitment of MLH1 and PMS 2, which navigate the parental strand and form a lariat-loop structure, which leads to excision of upwards of 500 bases, with resynthesis of the nascent strand and ligation with the assistance of endonuclease 1, PCNA, and ligase I or III.

Mismatch repair can also be induced as a consequence of chemotherapy and can be responsible for apoptosis-mediated cell death. One such example of this is the methylation of the *O*-6 position of guanine by the chemotherapeutic agent Temozolomide and related methylating chemotherapeutic agents. During DNA replication, the persistent *O*-6 methyl guanine preferentially base pairs with thymine forming a O6mG:T mispair that can be recognized by mismatch repair. The accumulation of high levels of O6mG:T mis-pairings leads to recognition by the mismatch repair complexes and then to futile repair cycles, gaps, strand breaks, and ultimately apoptotic signaling and cell death (Liu et al. 1996) . Conversely in the absence of mismatch pairs, G to A point mutations accumulate that, however, are nonlethal as futile repair cycles are not initiated and thus the MMR-deficient cell is said to be resistant to the effects of methylating agents.

5 Stem Cell Defects Due to Loss of Mismatch Repair

We have studied the survival and impact of methylation damage on the bone marrow of mismatch repair-defective mice alone and after transplantation in competition with normal hematopoietic stem cells.

Our research confirms that marrow hematopoietic progenitor stem cells defective in mismatch repair are markedly resistant to Temozolomide chemotherapy. In bone marrow transplants mixed with wild-type cells, mismatch repair-defective cells were enriched 10-fold in vivo after treatment with Temozolomide (Reese et al. 2003). More than 90%

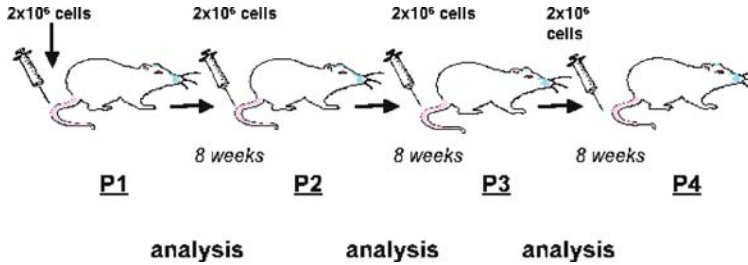

Fig. 3. Competitive serial transplantation model. Lethally irradiated mice were transplanted with a mixture of 10% mismatch repair-defective bone marrow and 90% wild-type bone marrow. At 8-week intervals, cells were harvested from the transplant recipient and either serially transplanted into another recipient mouse or analyzed for genotype, cell cycle, telomere length, and MSI

of the bone marrow progenitors from the recipient wild-type mice were derived from mismatch repair-defective donor stem cells 8 weeks after drug exposure. This conversion toward mismatch-defective cells was maintained during serial transplantion in the secondary recipient; however, mismatch repair-defective stem cells were lost in tertiary recipients, resulting in bone marrow failure (Fig. 3). In contrast, wild-type mice treated with Temozolomide survived quaternary transplants. In the absence of Temozolomide, wild-type cells predominated during competitive serial transplants, and tertiary recipients were repopulated entirely with wild-type hematopoietic stem cells. This suggested not only that mismatch repair-defective stem cells are resistant to methylating chemotherapy, but also that they were defective during replication and hematopoietic constitution, leading to a marrow failure syndrome.

To identify the source of these stem cell abnormalities, we investigated the difference in cell cycle between wild-type and MMR-deficient hematopoietic stem cells. The G_0 to G_1 ratio is an indication of the proportion of primitive stem cells present in bone marrow and would identify whether there was a loss of ability to maintain stem cells in a quiescent phenotype. We found similar values of resting G_0-G_1 cells in wild-type and mismatch repair-defective animals. We also found similar telomere lengths in the marrow of wild-type and mismatch repair-defective mice. Interestingly, we found that mismatch repair-defective

stem cells exhibited genomic instability (Reese et al. 2003). Neither wild-type colony forming units (CFU) nor young hematopoietic stem cells from mismatch repair-defective mice showed evidence of microsatellite instability . However, microsatellite instability at a frequency of 11% was observed in the mismatch repair-defective CFU derived from secondary recipients. From these data, we can conclude that microsatellite instability in hematopoietic stem cells accumulates and can be a cause of marrow failure. Furthermore, mismatch repair-deficient hematopoietic cells give rise to T-cell lymphoma. Whether this occurs in humans has not been identified, but it is the case that approximately 15% of leukemias and myelodysplastic syndromes and even greater percentages of secondary leukemias have evidence of microsatellite instability. This suggests that hematologic cancers can form from a high mutation frequency that leads to cellular transformation and mutations as observed in the absence of mismatch repair and that this first leads to marrow failure followed by clonal expansion of rapidly proliferating transformed cells.

6 Current Studies of Genomic Instability During Stem Cell Aging and Malignant Transformation

This evidence raises the important question of whether or not humans accumulate microsatellite instability with age and whether these mutational events are associated with disease. Few studies have examined the accumulation of microsatellites with age. In one study, lymphocytes subcloned from the peripheral blood showed evidence of microsatellite instability and its frequency with age. Our research group has performed preliminary studies with hematopoietic CFU derived from cord blood, young adults, and older individuals up to the age of 80 (Fig. 4). We are beginning to see evidence of microsatellite instability in a subset of hematopoietic stem cells (HSC) within individuals of all ages and are now assessing the relationship between age and frequency of microsatellite instability in various hematopoietic progenitor cell populations. Furthermore, we are attempting to determine the relevance of MSI accumulation to the development of cancer, prognosis, and treatment options.

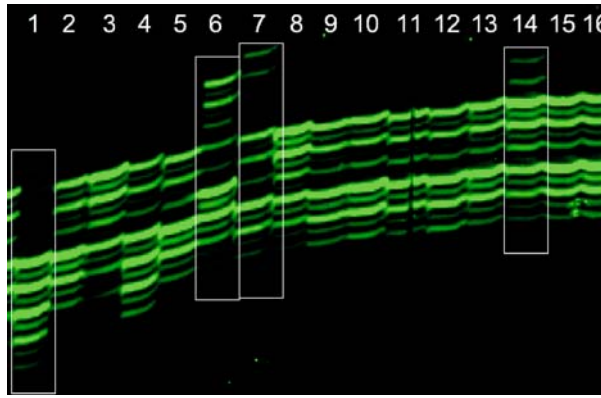

Fig. 4. Microsatellite instabiity in human hematopoietic stem cells. This gel image illustrates PCR products from 16 human myeloid lineage CFU taken from an individual over the age of 60. The CFU DNA was amplified with primers for the microsatellite marker D17S250 labeled on the forward primer at the 5′ end with the HEX fluorophore. *White boxes* indicate individual CFU positive for MSI, indicating genomic alterations in the somatic sequence

7 Summary

There is growing evidence that DNA repair is important in stem cell maintenance. A number of DNA repair genes are expressed at high levels in all lineages of stem cells. It is clear that the loss of DNA repair is capable of leading to a loss of regulated gene expression, replication, genomic stability, and/or coordinated cell division. These events in turn open the possibility of global stem cell loss in every organ system. In humans, a number of DNA defects are associated with a loss of the stem cell phenotype. Repair pathways in which such defects have been identified include mismatch repair, nonhomologous end-joining, V(D)J end-joining, double-break strand repair, and nucleotide excision repair. These fundamental processes suggest a clear requirement for maintenance of DNA repair during stem cell proliferation.

The extent to which microsatellite instability and other error-prone pair processes lead to transformation of the stem cell toward a cancer stem cell in humans remains to be determined. Furthermore, it is un-

clear whether or not there exists a cancer-prone stem cell with a defect in DNA repair that can be identified before the switch toward a malignant cell type occurs. Characteristics of this stem cell would be retention of the reconstitution capabilities coupled with irreversible replication defects that give rise to a proliferative advantage and eventually malignant transformation. Understanding this transition may be one of the most important areas of research into stem cells responsible for cancer growth and dissemination. Thus, just as the stem cell phenotype is so closely related between normal and malignant cells, the role of DNA repair appears to be equally tightly linked between normal and malignant stem cells. Understanding this transition remains an exciting area of research.

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Tumorigenic Epithelial Stem Cells and Their Normal Counterparts

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Abstract. ABC transporters are highly conserved and represent a major protective mechanism for barrier tissues as well as adult tissue stem cells. Emerging data support the existence of a cancer stem cell that shares features of tissue stem cells, including the ability to self-renew and undergo dysregulated differentiation. Here we show that a rare population of cells coexpressing MDR transporters and stem cell markers is a common feature across therapy-naive epithelial cancers as well as normal epithelial tissue. MDR^+ and MDR^- candidate

tumor stem and progenitor populations were all capable of generating highly anaplastic transplantable human tumors in NOD/SCID. The finding that rare cells bearing stem cell markers and having intrinsic MDR expression and activity are already present within the tumorigenic compartment before treatment with cytotoxic agents is of critical importance to cancer therapy. Just as damaged normal epithelial tissues regenerate after chemotherapy by virtue of highly protected resting tissue stem cells, the existence of malignant counterparts in therapy-naive epithelial cancers suggests a common mechanism by which normal and tumor stem cells protect themselves against toxic injury.

1 Background

Multiple drug resistance (MDR) was early recognized as a barrier to cancer therapy (Biedler et al. 1970). The common mechanism responsible for cross-resistance to multiple structurally unrelated agents was determined to be reduced cellular permeability (Ling and Thompson 1974), mediated by a family of highly conserved proteins known as ATP-binding cassette (ABC) transporters (Leslie et al. 2005). Although ABC transporter expression is recognized as a significant cause of chemotherapy resistance, the prevalent paradigm understands MDR in cancer to result from drug-mediated selection of cells with ABC transporter gene amplification (Chen et al. 2002) or regional gene activation (Wang et al. 2006). More recently, it has become apparent that normal adult tissue stem cells, including hematopoietic (Udomsakdi et al. 1991; Chaudhary and Roninson 1991; Goodell et al. 1996), airway (Giangreco et al. 2004), pituitary (Chen et al. 2005), small intestine (He et al. 2005), and testes (Riou et al. 2005), express high levels of MDR transporter activity. Persistence of tissue stem cells is essential to tissue maintenance and repair, and constitutive MDR activity is thought to be one of several mechanisms by which normal tissue stem cells protect themselves from toxic insults, including those resulting from damage by chemotherapeutic agents (Donnenberg and Donnenberg 2005). A dramatic example can be found in chemotherapy-induced alopecia, which results from damage to the rapidly cycling progenitor cells of the hair follicle (Paus and Cotsarelis 1999; Alonso and Fuchs 2003). However, alopecia is reversed on cessation of therapy because the common precursor of the four distinct cell types within the follicle, as well as skin epithelial cells

themselves, is a resting epithelial stem cell (Rendl et al. 2005), which is protected by constitutive MDR activity (Yano et al. 2005).

The cancer stem cell paradigm (Fiala 1968; Hamburger and Salmon 1977; Reya et al. 2001; Dick 2003; Al-Hajj et al. 2004; Donnenberg and Donnenberg 2005; Dick and Lapidot 2005; Wicha et al. 2006; Polyak and Hahn 2006) envisions the cancer-initiating cell as a genetically damaged tissue stem cell, or a more mature cell that has reacquired stem cell attributes through mutation. The unique insight that we derive from the study of adult tissue stem cells is that drug resistance is a normal self-protective mechanism that may be retained by the nascent neoplasm on transformation of the tissue stem cell. The notion that the cancer stem cell, or a subset of these cells, may have constitutive drug resistance agrees with the observation that cancers often recur after apparently successful therapy.

2 Results

2.1 ABGG2$^+$ Cells Are Present in Therapy-Naive Tumor and Normal Lung and Express Stem/Progenitor Markers

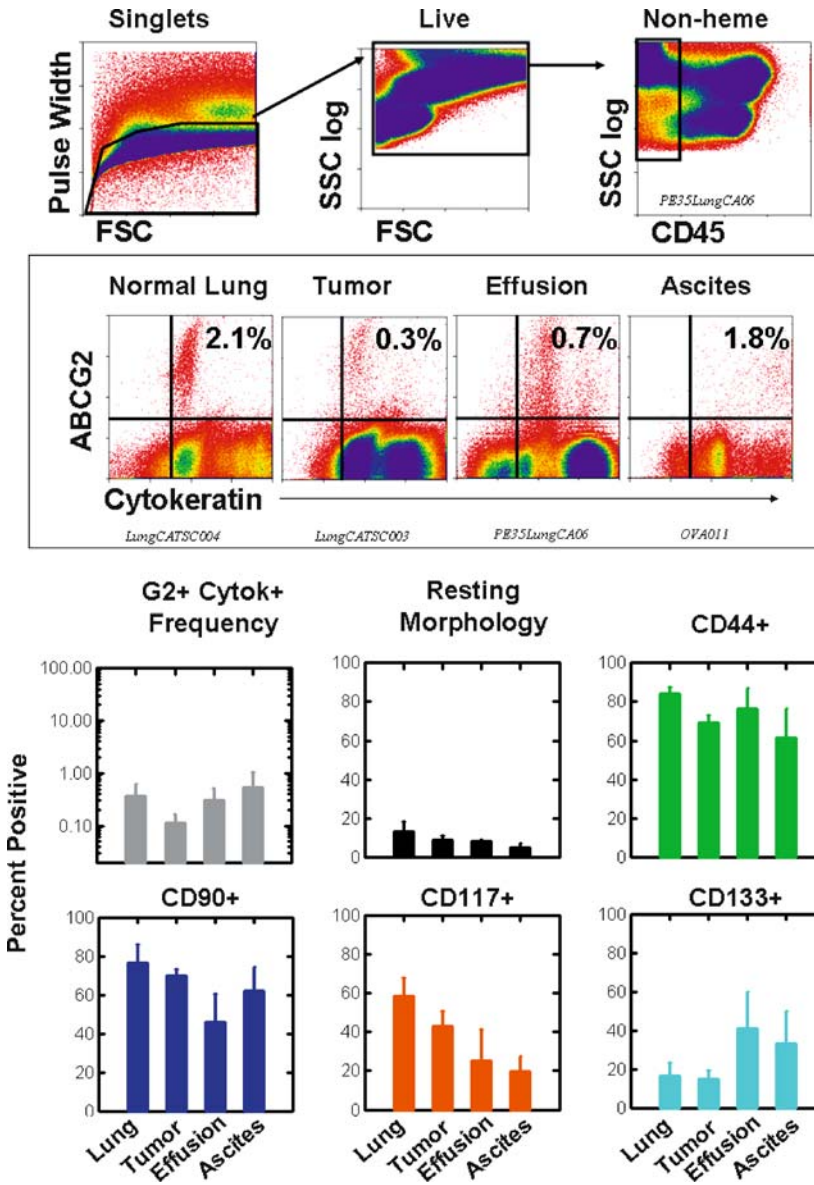
Stem cells from a variety of epithelial tissues have been enriched by sorting for cells with constitutive MDR transporter activity. To investigate expression of the MDR transporter ABCG2 in freshly isolated therapy-naive epithelial tumor cells, single-cell suspensions were prepared from solid tumors, malignant ascites, and effusions. Normal lung tissue was also investigated as a positive control. A population of non-hematopoietic, cytokeratin$^+$, ABCG2$^+$ cells was present at low frequency in both neoplastic and normal tissues (Fig. 1).

All newly diagnosed untreated epithelial tumors contained a rare subset of CD45$^-$ cytokeratin^{dim} ABCG2$^+$ cells (0.43±0.57% of CD45$^-$ cells, mean±SD). ABCG2$^+$ cytokeratin^{dim} cells also expressed CD44 (69±18%) and the stem/progenitor markers CD90 (62±20%), CD117 (34±23%), and CD133 (25±23%). Eight ± five percent of ABCG2$^+$ cells (0.03% of CD45$^-$ cytokeratin^{dim}) had low forward and side light scatter profiles compatible with small resting morphology.

None of these markers, alone or in combination, was able to distinguish normal lung from primary epithelial tumors. In contrast, ABCG2$^+$ cells from previously untreated metastatic cancers (effusions and as-

Fig. 1. Expression of ABCG2 and stem cell markers on freshly isolated normal lung tissue and therapy-naive malignant cells. To investigate expression of the MDR transporter ABCG2 in freshly isolated therapy-naive tumor cells, single-cell suspensions were prepared from solid tumors (lung cancer 7, ovarian cancer 3) and malignant ascites and effusions (lung cancer 2, ovarian cancer 6, gastric cancer 1) by mechanical dissection and collagenase digestion. Samples were stained by seven-color flow cytometry for expression of ABCG2, CD45, intracellular cytokeratin, CD44, CD90, CD117, and CD133. An average of 2.5 million events were acquired for each sample (min=200,000, max=6,000,000). The *first row* shows the gating strategy used in this and subsequent analyses. Forward scatter pulse height (*x-axis*) and pulse width (*y-axis*) are used to define singlet cells and eliminate cell clusters. Forward and side light scatter are then used to eliminate debris and dead cells. CD45 expression and side light scatter are used to eliminate hematopoietic cells. The *second row* shows ABCG2 and cytokeratin expression in the gated population of representative normal lung, lung tumor, metastatic lung pleural effusion, and ovarian ascites. The percentages of $ABCG2^+$ $cytokeratin^+$ cells are shown. The average frequency of these cells at each site is shown in the *first bar graph* (*error bars*=SEM). The *remaining bar graphs* show the frequencies of cells with resting morphology (lymphoid light scatter), CD44, CD90, CD117, and CD133 expression in the $CD45^-$, $ABCG2^+$, $cytokeratin^+$ population

cites) had significantly lower proportions of $CD90^+$ and $CD117^+$ cells ($p = 0.034$, and 0.011, respectively) and a higher proportion of $CD133^+$ cells ($p = 0.015$) than either normal lung or primary tumor. These data demonstrate that therapy-naive epithelial tumors contain a rare subpopulation of MDR-positive, stem cell marker-positive cells, a phenotype shared with an equally rare subset present in normal lung tissue. Further, this subpopulation was detectable in malignant effusions and ascites, sites unlikely to harbor normal tissue stem cells. Together these data suggest that stem cells within the tumor are not simply normal stem cells engaged in wound healing, and that these tumor cells share mechanisms with normal tissue stem cells that may equally confer resistance to cytotoxic therapy.



2.2 ABC Transporters Are Constitutively Active in a Small Subset of Therapy-Naive Tumor Cells

Functional measurement of ABC transporter activity is important, since expression and activity are not always well correlated (Webb et al. 1996). In Fig. 2 we show simultaneous transport of the MDR transporter substrates Hoechst 33342 and R123 in freshly isolated cells from a therapy-naive non-small cell lung tumor. The SP phenotype (ABCG2- and ABCB1-mediated transport) comprised 4% of nonhematopoietic cells, 29% of which had concomitant R123 efflux (ABCB1 transport; Fig. 2, color-evented red). None of the SP negative cells (Hoechst bright) transported rhodamine.

Virtually all of the *dual transporting* cells exhibited low light scatter, consistent with a resting morphology. The ABC transporter specificity of dye efflux was demonstrated with the ABCG2-specific inhibitor fumitremorgin, which abrogated 75% of the SP phenotype. $CD90^+$ cells were present in both SP^+ and SP^- fractions, indicating that not all cells bearing this stem cell marker have MDR activity. Furthermore, when we examined R123 efflux among the $CD45^-$ $CD117^+$ subset of untreated ovarian and lung tumor cells, MDR activity was restricted to the subset with low morphologic complexity and G_1/G_0 cell cycle phase ($n = 5$, data not shown). All epithelial tumors contained a small subpopulation of stem marker$^+$ cells having resting morphology. Figure 3 shows imaging flow cytometry performed on an untreated freshly isolated non small cell lung tumor. The $CD45^-$ $CD90^+$ stem fraction shown in panel A comprised 5% of $CD45^-$ singelt cells and were of uniform small morphology with high nucleus to cytoplasm ratio. In contrast, $CD90^-$ cells comprised the vast majority of $CD45^-$ tumor cells and were heterogenous with respect to morphology. Taken together, these data demonstrate that resting stem cell marker-positive tumor cells with low morphologic complexity express both ABCG2 (breast cancer resistance protein 1) and ABCB1 (P-glycoprotein) and exhibit the highest constitutive MDR activity.

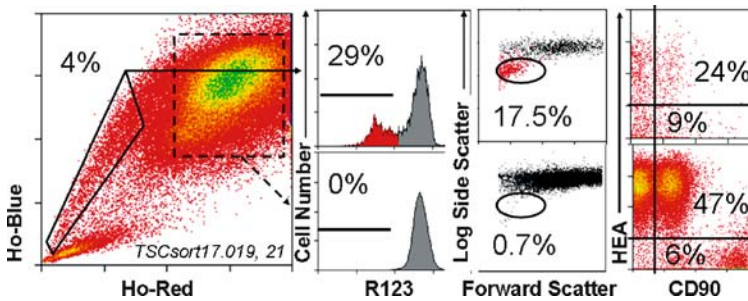


Fig. 2. ABCG2 and ABCB1 activity in freshly isolated therapy-naive non-small cell lung cancer. Antibody-stained suspended tumor cells were incubated simultaneously with the ABCG2/ABCB1 substrate Hoechst 33342 (8 μM) plus the ABCB1 substrate rhodamine 123 (R123, 0.13 μM) for 90 min at 37°C (Bertoncello and Williams 2004). Hoechst emission was separated with a 510-nm dichroic long-pass filter. Blue and red fluorescence were measured with 450-65 nm and 670-20 nm bandpass filters, respectively. Propidium iodide (PI, 10 μg/mL) was added immediately before sample acquisition. All events were gated on PI-excluding (live), nonhematopoietic singlets. Five million events were collected. The *leftmost panel* shows a small population (4%) of Hoechst 33342-excluding cells in the typical pattern of the side population (SP). SP (*top panels*) and non-SP cells (*bottom panels*) were further characterized: A proportion of SP cells also excluded the ABCB1 substrate dye R123. These accounted for 29% of the SP cells (color-evented red in the dot plots) and accounted for almost all of the cells with low forward and side light scatter, consistent with a resting morphology (Fig. 3). Non-SP cells did not transport R123 and were exclusively of high light scatter. A significant proportion of both SP and non-SP cells expressed CD90, often in combination with epithelium-specific antigen HEA. Coincubation of tumor cells with Hoechst 33342, R123, and the ABCG2-specific inhibitor fumitremorgin (10 μM) resulted in 75% inhibition of the SP phenotype

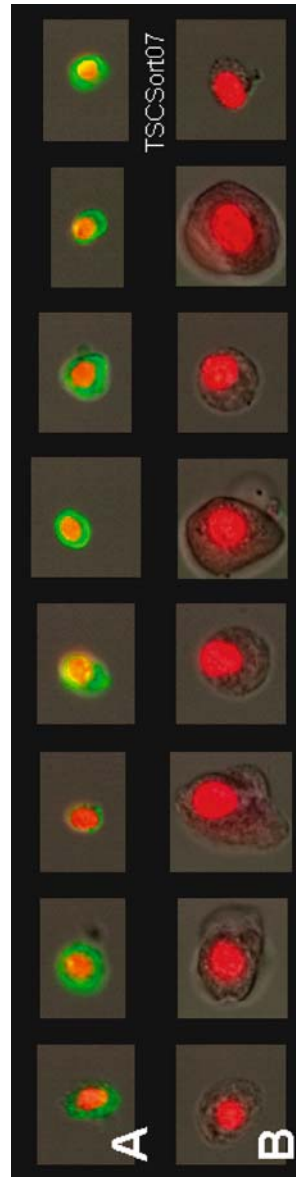
2.3 Transporter-Positive and -Negative Cells Are Tumorigenic

In order to determine tumorigenicity of ABCG2 protected fractions, particularly the resting fraction, ABCG2-positive and -negative $CD90^+$ cell populations were sorted from a recurrent breast cancer pleural effusion. These populations were defined within the $CD45^-$ $CD44^+$ frac-

Fig. 3. A,**B** $CD45^-$ $CD90^+$ cells isolated from primary tumors have small resting morphology. A freshly resected untreated non-small cell lung cancer was collagenase digested and stained with CD45, CD90, and the nuclear stain Draq5 (5 μM). Virtual sorting was performed with an Amnis ImageStream100 imaging flow cytometer (Amnis Corporation, Seattle WA). All analyzed cells were singlets, as determined by a histogram of brightfield area versus brightfield aspect ratio. Images are composites of brightfield, CD90 (*false-colored green*), and Draq5 (*false-colored red*). **A** Images of nonhematopoietic ($CD45^-$) $CD90^+$ cells. **B** Images from consecutive $CD45^-$ $CD90^-$ cells. $CD90^+$ tumor cells were small, with a relatively high nucleus-to-cytoplasm ratio

tion shown previously to contain tumorigenic breast cancer cells (Al-Hajj et al. 2003). The tumorigenicity of low light scatter (resting) and high light scatter (differentiated) tumor populations were examined separately. For each of the four fractions, the total cells recovered after sorting were divided into four equal aliquots and injected into the mammary fat pad admixed with 10,000 heavily irradiated (10,000 rads) sorted $CD45^-$ tumor cells suspended in Matrigel. Thus animals received 43–58 sorted cells from the resting cell fractions and 633–13,200 cells from the more prevalent high scatter fractions (Fig. 4). Additionally, two groups of two mice each were injected at four sites each with 10,000 sorted $CD45^-$ unirradiated or irradiated tumor cells, respectively. All sorted $CD90^+$ cell fractions generated tumors, even the rare resting fractions where 43 and 58 cells were injected. The proportion of mice developing tumors and the day of sacrifice are shown in Fig. 4, with characteristic immunohistochemical staining for human cytokeratins. Tumors grew slowly and were first palpable at 5–10 months. Irradiated cells were not tumorigenic. None of the mice injected with sorted $CD45^-$cells evidenced tumors at the time that mice injected with $CD90^+$ cells were sacrificed. However, small tumors were observed in two of eight sites injected with $CD45^-$ cells when the experiment was terminated at day 371. Tumors were poorly differentiated with atypical nuclei.

Despite the homogeneity of the injected human cell populations, flow cytometry of tumor xenografts revealed a heterogeneity strikingly similar to the clinical isolate, regardless of the sorted population of origin. Figure 5 shows a detailed flow cytometric analysis of the freshly



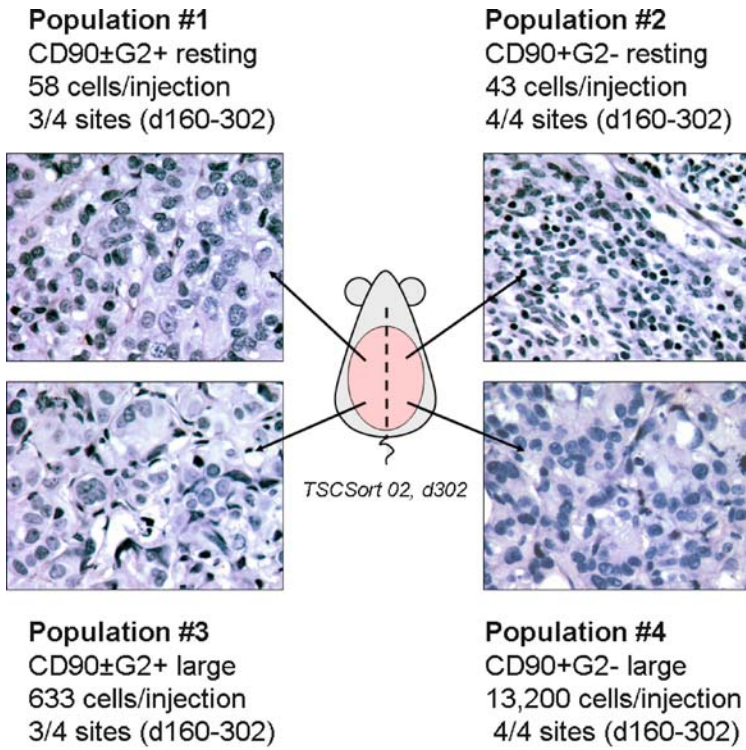


Fig. 4. In vivo tumorigenicity of ABCG2$^+$ and ABCG2$^-$ breast cancer cells. Twenty NOD/SCID mice were injected with FACS-sorted freshly isolated breast cancer pleural effusion cells as indicated. Sorted cells were admixed with 10,000 heavily irradiated CD45$^-$ tumor cells to minimize loss of small numbers of sorted tumor cells. The proportion of mice developing tumors and the days of sacrifice are indicated. Photomicrographs (×40 objective, H&E stain) illustrate histology on tumor xenografts harvested 302 days after injection of sorted cells. Tumors from all fractions were poorly differentiated, with abundant human cytokeratin$^+$ cells in most (not shown), but not all, tumors

isolated pleural effusion, sorted population 4 ($CD45^-$ $CD44^+$ $CD90^+$ $ABGG2^-$), and a tumor that was harvested 204 days after injection of this population. With the exception of human $CD45^+$ lymphohematopoietic cells, which were prevalent in the effusion and absent in the xenograft, all major effusion populations were observed in the xenograft. Interestingly, the injected population was present at a similar frequency in the original tumor and the effusion. This provides evidence for self-renewal and expansion of the $CD90^+$ $ABCG2^-$ fraction, since only 13,200 cells were injected and the resulting tumor measured 3 mm. Importantly, the injected $ABCG2^-$ cells gave rise to $ABCG2^+$ cells that were seen in both $CD90^+$ and $CD90^-$ fractions, as they were in the fresh clinical isolate.

To determine whether tumor xenografts could be passaged, tumors 1 and 4, harvested on day 302, were disaggregated and sorted into $CD45^-$ $CD44^+$ HEA^+ $CD90^+$ $ABCG2^-$ and $CD45^-$ $CD44^+$ HEA^+ $CD90^+$ $ABCG2^+$ fractions. Sorted cells were admixed with 10,000 irradiated unsorted tumor cells, suspended in Matrigel, and injected into the mammary fat pads of NOD/SCID mice (2 animals/fraction). The $CD90^+$ $ABCG2^-$ fraction from tumors 1 and 4 yielded 875 and 1,285 cells per injection site, respectively. The $CD90^+$ $ABCG2^+$ fraction from tumors 1 and 4 yielded 43,750 and 30,000 cells per injection site, respectively. Mice were sacrificed on days 129 and 231 with 3- to 8-mm subcutaneous tumors at the injection sites of all fractions.

3 Discussion

In this report we have demonstrated the existence of a rare population of $CD44^+$ $cytokeratin^+$ $ABCG2^+$ $CD90^+$ cells across a spectrum of previously untreated epithelial cancers, as well as in normal lung tissue. A proportion of these cells has resting morphology and coexpresses the stem/progenitor markers CD117 and CD133. The unexpected finding that the $ABCG2^+$ population and its subsets are detected at similar frequency in normal and neoplastic tissues, as well as across epithelial cancers from different organs, suggests that elements of normal epithelial stem cell function and differentiation are universally retained after neoplastic transformation. In contrast, great variability was seen in expres-

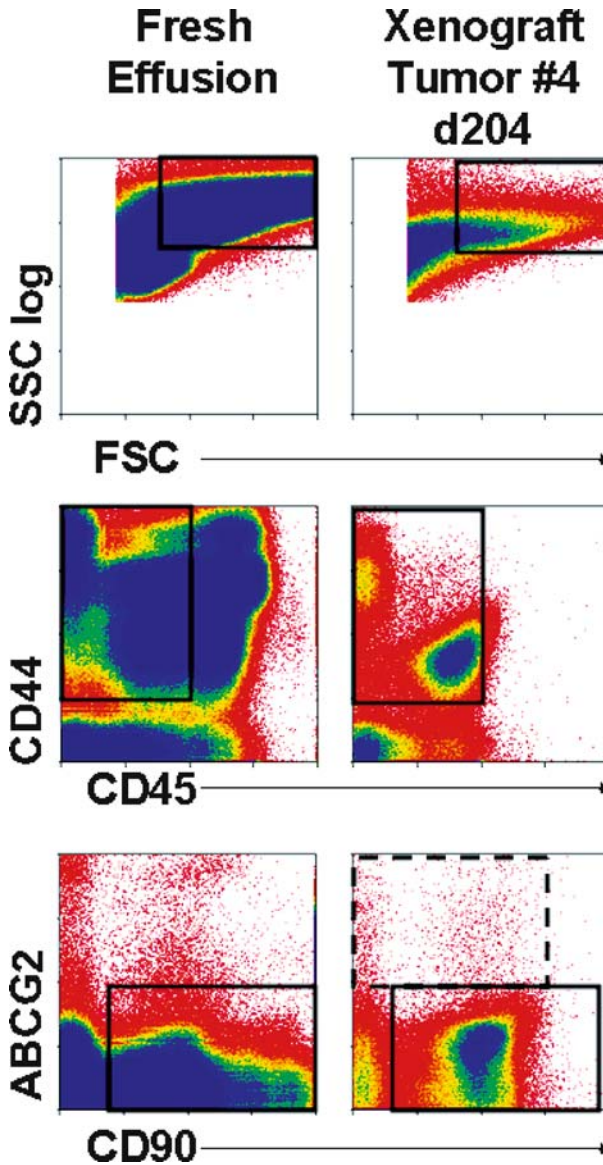


Fig. 5. Self-renewal and differentiation in a breast cancer tumor stem cell xenograft. The *first column* shows seven-color flow cytometry performed on the freshly isolated breast cancer pleural effusion, which was sorted and injected into NOD/SCID mice. Superimposed are gates identical to those used to sort population 4 (high light scatter, $CD45^-$, $CD44^+$, $CD90^+$, $ABCG2^-$; 13,200 cells injected/mouse). The s*econd column* shows the tumor xenograft, which has differentiated substantially, showing light scatter heterogeneity, and the emergence of CD44 and CD90 negative populations. Most importantly, a population of $ABCG2^+$ cells (*dashed box*, 0.9%) was observed, indicating that MDR expression can be induced in the progeny of $ABCG2^-$ cells. Self-renewal can also be seen in the *solid boxes* (*column 2*), which indicate the xenograft tumor population falling within the original sort logic used to isolate population 4. Similar to the original pleural effusion, these cells comprised 13.1% of the xenograft tumor. However, since they arose from only 13,200 injected cells, the original $CD45^-$ $CD44^+$ $CD90^+$ $ABCG2^-$ cells expanded substantially within the xenograft tumor. Note: All histograms were gated on singlets (not shown). A total of 6 million effusion cells and 1.1 million xenograft tumor cells were analyzed

sion of maturation/differentiation markers (cytokeratin, MUC-1, HEA) between tumors from different organs, reflecting the different tissues of origin (data not shown). Interestingly, the frequency of $CD90^+$ and $CD117^+$ cells (candidate stem fraction) was lower and the frequency of $CD133^+$ cells (candidate progenitor fraction) was higher in untreated metastatic sites (Fig. 1).

Sorted $CD44^+$ $CD24^-$ breast cancer cells (Al-Hajj et al. 2003), as well as sorted $CD133^+$ cells from brain tumors (Singh et al. 2004) and prostate cancer (Collins et al. 2005), have previously been shown to be tumorigenic in NOD/SCID mice. Although these studies have been widely quoted as supporting the cancer stem cell hypothesis, they did not attempt to distinguish between stem and progenitor compartments and did not determine whether the tumorigenic fraction was protected by mechanisms common to normal tissue stem cells. In this report we used the markers CD90, CD117 and CD133 to identify the stem/progenitor fraction within the $CD45^-$ $CD44^+$ compartment. Within this population, low morphologic complexity and the differentiation

marker HEA were used to provisionally distinguish between resting stem cells and more differentiated progenitor cells. We found that both stem and progenitor populations are tumorigenic, and both have a subset that expresses the ABC transporter ABCG2. However, only the resting stem cell fraction had a subpopulation with constitutive activity of both ABCG2 and ABCB1 transporters (Fig. 2). Further, the stem cell fraction was tumorigenic at very high frequency.

Biologically, the salient finding is that untreated epithelial tumors retain a vestige of the ordered growth and differentiation of the parent tissue, including the persistence of resting stemlike cells (some of which are protected by MDR transporters), a more differentiated tumorigenic progenitor fraction, and their postmitotic nonclonogenic progeny. Despite the phenotypic heterogeneity of tumorigenic cells, the most critical population from a therapeutic standpoint is the resting stem cell-like population. We hypothesize that this population is as resistant to cytotoxic therapy as its normal counterpart, by virtue of constitutive MDR activity and possibly other protective mechanisms afforded by the niche in which it persists (Arai et al. 2005). This population provides an attractive candidate for the cancer stem cell postulated by Weissman (Reya et al. 2001; Al-Hajj et al. 2004) Dick (Dick 2003; Dick and Lapidot 2005) and others: a resting, drug resistant tumor cell that can lay dormant after initially successful therapy, providing a seed for later recurrence and metastasis.

The finding of intrinsic MDR activity within a rare resting tumorigenic population is not explained by the conventional MDR paradigm, which views ABC transporter-mediated drug resistance as a trait that tumor cells acquire on drug exposure through substrate-driven induction, gene amplification, or regional gene activation. By concentrating on freshly isolated therapy-naive clinical isolates, we have demonstrated that ABC transporter expression and activity is present before exposure to cytotoxic agents. Given the central role of MDR transporters in protecting normal tissue stem cells, our data support a broadened interpretation of the cancer stem cell paradigm, and provide a unified explanation for the successes and failures of cytotoxic antineoplastic therapy. Namely, the ultimate target, the MDR-protected resting cancer stem cell, is spared along with its normal tissue stem cell counterparts. Since cytotoxic regimens must be designed to minimize irre-

versible toxicity to normal tissue, the therapeutic index has traditionally been thought of as the differential sensitivity of measurable tumor versus that of the highly protected adult tissue stem cell compartment, which is required for regeneration. Our findings recast this concept as the differential sensitivity of MDR-protected tumor stem cells and their normal tissue counterparts.

4 Methods

4.1 Patient Samples

Thirty-four patient samples (tumor, adjacent normal tissue, ascites, and pleural effusions) were acquired under protocols approved by the University of Pittsburgh Internal Review Board. With the exception of the sample described in Figs. 4 and 5, all were obtained from patients at the time of tumor resection and before cytotoxic or radiation therapy.

4.2 Tissue Digestion

Solid tissues were minced with paired scalpels, digested with type I collagenase (4% in RPMI 1640 medium, Sigma Chemicals, St. Louis, MO) (Elder and Whiteside 1992) and disaggregated through 100 mesh stainless steel screens. Ten to 500 million viable cells were recovered from 5 to 10 mm^3 specimens of tumor or normal lung parenchyma. Pleural effusions and ascites were concentrated, collagenase digested, and separated on a ficoll/hypaque gradient.

4.3 Staining and Flow Cytometry

Single cell suspensions were stained according to a protocol described in detail elsewhere (Donnenberg and Donnenberg 2003). Five minutes before staining with fluorochrome-conjugated monoclonal antibodies, neat mouse serum (5 μL) was added to each cell pellet to minimize nonspecific antibody binding. Before cytokeratin staining, cells were stained for surface markers and permeabilized with 0.1% saponin (Beckman Coulter, Fullerton, CA) in phosphate-buffered saline with 0.5% human serum albumin. Antibodies and dyes used in these studies included HEA-FITC (Miltenyi Biotech, Bergisch Gladbach, Ger-

many, Cat. No. 12000420), pan cytokeratin-FITC (Beckman Coulter, Cat.
No. IM2356), CD44-PE (Serotec, Oxford, UK, Cat. No. MCA 89PE); CD90-biotin (BD, Cat. No. 555594), streptavidin-ECD (Beckman Coulter, Cat. No. IM3326), ABCG2-PC5 (Chemicon, Temecula, CA, Cat. No. MAB4155PC), CD117-PC7 (Beckman Coulter, Cat. No. IM3698), CD133-APC (Miltenyi Biotech, Cat. No. 120001241), CD45-APCC7 (BD, Cat. No. 557833), propidium iodide (Calbiochem, La Jolla, CA, Cat. No. 537059), rhodamine 123 (Sigma Chemicals, St. Louis MO, Cat. No. R8004), Hoechst 33342 (Invitrogen, Carlsbad, CA, Cat. No. H3570), and Draq5 (Alexis Biochemicals, Lausen, Switzerland, Cat. No. BOS-889-001-R200). Fumitremorgin was purchased from Alexis (Cat. No. ALX-350-127). Seven-color analysis was performed with the three-laser, nine-color CyAn LX cytometer (DakoCytomation, Fort Collins, CO). Sorting and analysis requiring an ultraviolet laser was performed on a three-laser, eight-color DakoCytomation MoFlo. An effort was made to acquire a total of 5 million cells per sample at rates not exceeding 10,000 events/s. The cytometers were calibrated before each use with SpectrAlign beads (DakoCytomation, Cat. No. KO111) and eight-peak Rainbow Calibration Particles (Spherotech, Libertyville, IL, Cat. No. RCP-30-5A). Color compensation matrixes were calculated for each staining combination within each experiment, using single-stained mouse IgG capture beads (Becton Dickinson, Cat. No. 552843) for each antibody and single-stained cells for rhodamine 123. Off-line analysis was performed with Summit software (DakoCytomation). In all analyses, doublets and clusters were eliminated with forward scatter peak width versus height as a discriminator. Propidium iodide staining was used to eliminate nonviable cells.

4.4 Tumor Xenografts

Female NOD.CB17-Prkdcscid/J mice 6–8 weeks of age were purchased from The Jackson Laboratory (Bar Harbor, ME), and housed five to a cage in a specific pathogen-free environment. Before injection of tumor cells, mice were anesthetized by methoxyflurane inhalation. Sorted cells were admixed with sorted $CD45^-$ tumor (irradiated with 10,000 rads from a ^{137}Cs source) and suspended in 25 μL of ice-cold DMEM, 15%

FBS, plus 25 μL of Matrigel (Becton Dickinson). Fifty microliters of ice-cold cell suspension were injected subcutaneously into the mammary fatpads (4 injections/animal). Animals were examined twice weekly for behavioral changes and evidence of tumor.

4.5 Statistical Analysis

The frequencies of cells expressing stem cell markers were compared with Student's *t*-test for two groups (2-tailed test). Statistical tests, descriptive statistics, and graphic analysis (other than cytometry) were performed with Systat version 11 (Systat Software Inc, Richmond, CA).

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