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14. ABSTRACT

This past year was the second year of the DOD award. Over this year we have made substantial progress in achieving the goals outlined in the proposal. In addition, we have found that the enzyme aldehyde dehydrogenase 1 is an excellent marker for cancer stem cells which can be readily detected in fixed histological sections. Development of this new tool will greatly facilitate future studies. Preliminary results both in xenograft models as well as in neoadjuvant trial are providing strong support for our hypothesis for resistance of cancer cells to chemotherapy. We have also made excellent progress at elucidating the pathways which regulate the cells including Hedgehog signaling and Bmi-1. Together these studies provide a rationale for the combination compounds which inhibit stem cell renewal pathways such as Hedgehog with chemotherapeutic agents.

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Chemo Resistance of Breast Cancer Stem Cells

Introduction

The proposal BC030314, Chemoresistance of Breast Cancer Stem Cells, was based on our previous description of the isolation of tumor cells from human breast cancers that have stem cell properties. These properties include the ability to self-renew as well as to differentiate into the non-tumorigenic cells which form the bulk of the tumor. The objectives of this study were to test the hypothesis that breast cancer stem cells are relatively resistant to chemotherapy compared to the differentiated cells which form the bulk of the tumor and thus may contribute to relapse following therapy. This was to be accomplished by utilizing mouse xenograft models as well as markers for stem cells in a clinical neoadjuvant chemotherapy study. The neoadjuvant chemotherapy studies are being performed in collaboration with Dr. Jenny Chang at Baylor University. Over the past year we have made significant progress in both aspects of this study. In addition, we have developed new technology which for the first time allows us to assess and quantitate tumor stem cells in sections of fixed tissue. This assay involves the expression of the enzyme aldehyde dehydrogenase 1 (see below). The development of and validation of this new marker should greatly facilitate present and future clinical studies.

Our preliminary results both in xenograft models as well as in the neoadjuvant trial support our hypothesis for relative resistance of breast cancer stem cells to chemotherapy. In light of this, it will be most important to develop new approaches to target this cell population. Although not originally included within the statement of work for this grant, we have made substantial progress in elucidating pathways which regulate stem cell behavior. These pathways include both Notch and Hedgehog signaling. The role of Hedgehog signaling as well as the polycomb gene Bmi-1 in regulating the self-renewal of both normal and malignant human mammary stem cells was published in Cancer Research. Together the results of our studies regarding the resistance of breast cancer stem cells to chemotherapy and the reliance of these cells on Hedgehog signaling, suggests that the use of the combination of chemotherapy to target the differentiated cells and Hedgehog inhibitors to target the cancer stem cells represents a rationale therapy strategy.

Body

1. Creation of New Xenografts

   As initially proposed, we have created several new breast cancer xenografts derived from patient samples. In particular, one of the new xenografts is estrogen receptor positive which will allow us to broaden our interpretation of our studies to this subset of breast cancer. We currently have over twenty tumor specimens

implanted in NOD-SCID mice which should be more than adequate to complete these studies.

2. Development of ALDH-1 As a Stem Cell Marker

We had proposed utilizing the cell surface markers CD44 and CD24 as markers of cancer stem cells since these had been the markers that we had initially described for these cells. One limitation of utilizing these markers is that they are not suitable for immunochemical detection of tumor stem cells since they require flow cytometric analysis with lineage positive cells being gated out. The need to utilize flow cytometry to assay stem cell phenotypes was a major limitation for neoadjuvant clinical studies since only small amounts of tumor tissue is obtained in biopsies. We thus sought to develop an alternative approach for identification of tumor stem cells which could also be used for immunochemical detection. Previously, aldehyde dehydrogenase had been described to be expressed in both normal and transformed hematopoietic stem cells. Based on this, we have developed evidence that both the activity and immunochemical localization of ALDH-1 is a powerful marker of tumor stem cells. Aldehyde dehydrogenase in tumor stem cells is an important enzyme which regulates the conversion of retinol to retinol acid which is involved in stem cell differentiation. A fluorescent probe Alduflor, which is commercially available, can be used to detect this enzyme activity. Fluorescently labeled Alduflor is freely permeable into cells, but becomes ionized and trapped in cells that contain ALDH activity. As show in Figure 1, ALDH positive tumor cells can be isolated and when tested in

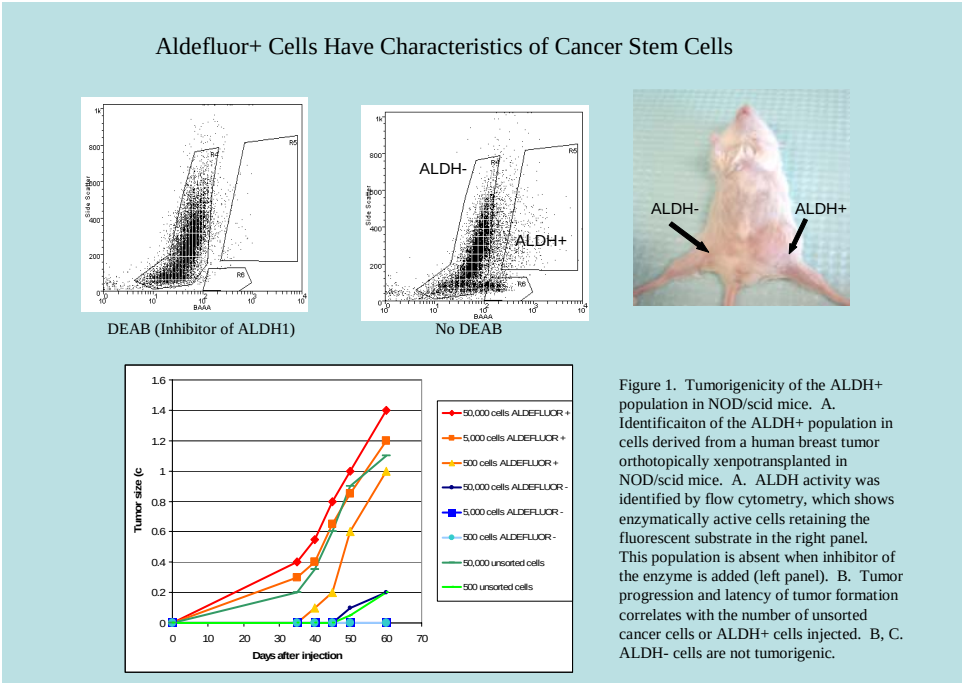

Figure 1. Tumorigenicity of the ALDH+ population in NOD/scid mice. A. Identificaiton of the ALDH+ population in cells derived from a human breast tumor orthotopically xenpotransplanted in NOD/scid mice. A. ALDH activity was identified by flow cytometry, which shows enzymatically active cells retaining the fluorescent substrate in the right panel. This population is absent when inhibitor of the enzyme is added (left panel). B. Tumor progression and latency of tumor formation correlates with the number of unsorted cancer cells or ALDH+ cells injected. B, C. ALDH- cells are not tumorigenic.

Figure 1

xenografts as little as 500 ALDH positive cells form tumors, whereas 50,000 ALDH negative cells do not. Consistent with ALDH identifying tumor stem cells, ALDH positive cells generate tumors composed of both ALDH positive and negative cells thus recapitulating the phenotypic heterogeneity found in the initial tumor. In tumor xenografts between 1% and 5% of cells are ALDH positive.

Using an antibody to ALDH-1 we have been able to detect ALDH positive cells in both normal mammy gland terminal end buds as well as in breast tumor specimens. We have validated the importance of expression of ALDH in tissue microarrays of over 300 human breast tumors. As shown in Figure 2, expression

ALDH1 Expression in Human Breast Cancer is Associated With Poor Prognosis

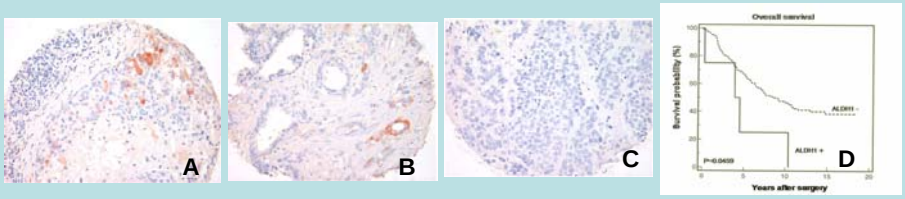

Figure 2. Examples of TMA breat carcinoma cores positive (A,B) and negative © for ALDH1 expression. C. Kapplan-Meyer survival curve shows ALDH positive tumors associated with Poor Prognosis.

Figure 2

of ALDH-1 was present in approximately 29% of tumors. Patients whose tumors contained ALDH detectable cells had a considerably worse prognosis than patients whose tumors failed to express ALDH-1. These studies (manuscript in preparation) demonstrate that ALDH is a valid marker of tumor stem cells. Furthermore, since we can detect this in situ it will allow us to further the proposed studies in the chemoresistance of breast cancer stem cells, since we can determine the percent of ALDH positive cells pre and post treatment facilitating their quantitation.

3. Chemoresistance of Breast Cancer Stem Cells

We have continued our studies of chemoresistance of breast cancer stem cells both in xenograft models as well as in collaboration with Dr. Jenny Chang at

Baylor University in patients undergoing neoadjuvant chemotherapy. As shown below, we have treated xenograft bearing mice with three weekly courses of Taxotere and assessed stem cell number before and after therapy. Preliminary results support our hypothesis that the percent of stem cells is increased after chemotherapy since these cells are more resistant to chemotherapy than are the differentiated cells comprising the bulk of the tumor.

Dr. Jenny Chang at Baylor University has been collaborating with us to test this hypothesis in patients undergoing neoadjuvant chemotherapy. Patient tumors are biopsied before chemotherapy and assayed for stem cell markers, including CD44+ CD24- lin- by flow cytometry following chemotherapy residual tumor is assayed. As shown in Figure 3 below in illustrating two patients, the percent of

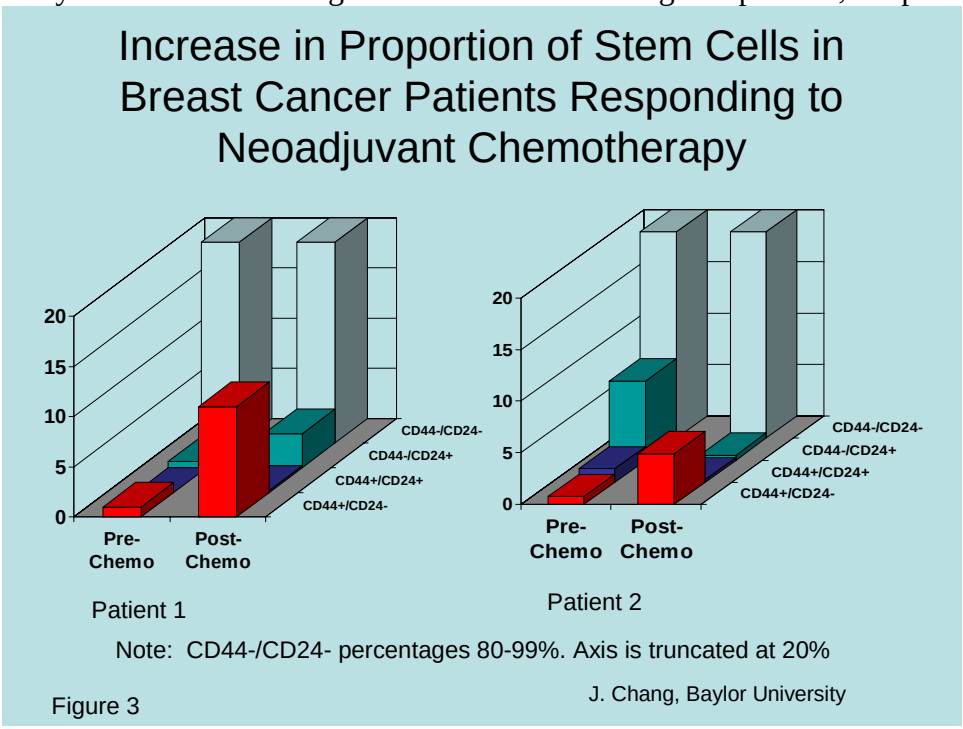

Figure 3

tumor cells bearing stem cell markers significantly increases following chemotherapy consistent with our hypothesis. To date, 12 patients have been accrued to this study and in all patients in which there is a shrinkage of tumors Dr. Chang has found an increase in the cancer stem cell percentage following chemotherapy. These studies will be continued and we will add ALDH as an additional stem cell marker.

The above work supports our hypothesis that breast cancer stem cells are inherently more resistant to chemotherapy than are the differentiated cells derived from these tumors. This suggests that new strategies will be necessary to target this cancer stem cell population. Over the past year we have made considerable progress elucidating other pathways which regulate stem cell behavior including the Hedgehog pathway and Bmi-1. We find that both normal and cancer stem cells utilize Hedgehog signaling pathway and Bmi-1 for self-renewal (Cancer

Research, 66:(12), June 15, 2006). These studies suggest that combinations of Hedgehog inhibitors with chemotherapy may be a rationale therapeutic strategy. We will pursue such strategies in future studies.

Key Research Accomplishments

- Generation of new xenografts from breast cancer patients, including estrogen receptor positive xenograft.
- Development and validation of ALDH as a marker for breast cancer stem cells.
- Use of ALDH expression to detect breast cancer stem cells in situ in fixed tissues.
- Tested affects of Taxotere on stem cells in xenograft models.
- Continued collaboration with Dr. Jenny Chang at Baylor to demonstrate in a new neoadjuvant study that chemotherapy treatment results in an increase in the proportion of surviving cancer stem cells supporting our original hypothesis.
- Demonstrated importance of Hedgehog signaling and Bmi-1 in regulation of self-renewal of both normal and breast cancer stem cells (Reference Breast Cancer Research).
- Based on above results, development of strategy combining chemotherapy and Hedgehog inhibitors for future studies.

Appendices

Liu S, Dontu G, Wicha M, Mammary stem cells, self-renewal pathways, and carcinogenesis. Breast Cancer Research, Vol. 7, No. 3, May 2005.

Dontu G, Wicha M, Survival of mammary stem cells in suspension culture: implications for stem cell biology and neoplasia. Journal of Mammary Gland Biology and Neoplasia, March 2005.

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Liu S, Dontu G, Mantle I, Patel S, Ahn N, Jackson K, Suri P, Wicha W, Hedgehog Signaling and Bmi-1 regulates self-renewal of normal and malignant human mammary stem cells, Cancer Research, 66:12, June 2006.

Review

Mammary stem cells, self-renewal pathways, and carcinogenesis

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Abstract

The mammary gland epithelial components are thought to arise from stem cells that undergo both self-renewal and differentiation. Self-renewal has been shown to be regulated by the Hedgehog, Notch, and Wnt pathways and the transcription factor B lymphoma Mo-MLV insertion region 1 (Bmi-1). We review data about the existence of stem cells in the mammary gland and the pathways regulating the self-renewal of these cells. We present evidence that deregulation of the self-renewal in stem cells or their progenitors **[AU: edit OK?]** might be a key event in mammary carcinogenesis. If 'tumor stem cells' are inherently resistant to current therapies, targeting stem cell self-renewal pathways might provide a novel approach for breast cancer treatment.

Introduction

The mammary gland in humans and in other mammals is a dynamic organ that undergoes significant developmental changes during pregnancy, lactation, and involution. It is likely that the cellular repertoire of the human mammary gland is generated by a stem cell component. These stem cells have a unique capacity for self-renewal as well as for generating the three lineages that comprise the lobulo-alveolar structure of the adult gland: myoepithelial cells forming the basal layer of ducts and alveoli, ductal epithelial cells lining the lumen of ducts, and alveolar epithelial cells synthesizing milk proteins [1,2]. Under the regulation of systemic hormones, as well as local stromal epithelial interactions, these cells proliferate extensively, differentiate during each pregnancy and lactation, and undergo apoptosis during mammary involution [2]. It has been shown previously that a subset of the luminal epithelial cells could convert to myoepithelial cells in culture, signifying the possible existence of a progenitor cell [3]. Recently, Stingl and colleagues characterized the multipotent epithelial cells in the normal adult breast [4]. In their experimental system, two distinct types of human breast epithelial cell (HBEC) progenitor population could be distinguished on the basis of their differential expression of the MUC-1 glycoprotein CALLA/CD10 and epithelial-specific antigen (ESA). MUC-1$^+$/CALLA$^-$/ESA$^+$ progenitors (luminal restricted progenitor, or alveolar progenitor) expressed typical luminal epitopes (keratin 8/18, keratin 19, MUC-1, and ESA) and showed low levels of expression of myoepithelial epitopes (keratin 14 and CD44v6). The second type of progenitor, MUC-1$^{-\text{ to }\pm}$/CALLA$^{\pm\text{ to }+}$/ESA$^+$ (bipotent progenitor, or ductal progenitor), generated mixed colonies of both luminal and myoepithelial cells when seeded in two-dimensional and three-dimensional cultures. Furthermore, they suggested that the MUC-1$^+$/CALLA$^-$/ESA$^+$ and the MUC-1$^{-\text{ to }\pm}$/CALLA$^{\pm\text{ to }+}$/ESA$^+$ progenitors are candidate *in vivo* alveolar and ductal progenitors, respectively [4]. HBEC clonal heterogeneity has also been reported by others [5]. Such clonal heterogeneity might be indicative of an underlying stochastic mechanism regulating HBEC differentiation independently of the presence of factors (such as epidermal growth factor and insulin **[AU: definitions OK?]**) that might be required to support the viability and/or stimulate the proliferation of these cells [4].

There is also increasing evidence that stem cells might be the targets of transformation during carcinogenesis. Carcinomas are believed to arise through a series of mutations that occur over many years. Adult stem cells are slowly dividing, long-lived cells, which by their very nature are exposed to damaging agents for long periods. They may therefore accumulate mutations that result in transformation [6]. In favor of the role of stem cells in carcinogenesis comes the observation that normal stem cells and cancer stem cells share several important properties such as the capacity for self-renewal, the ability to differentiate, active telomerase and anti-apoptotic pathways, increased membrane transporter activity, anchorage independence and ability to migrate and

Bmi-1 = B lymphoma Mo-MLV insertion region 1; Dsh = Dishevelled; **[AU: please confirm capitalization/lower-case initials on all protein names; there is inconsistency between the abbreviations list and throughout the paper]** ESA = epithelial-specific antigen; Fu = Fused; GSK = glycogen synthase kinase; HBEC = human breast epithelial cell; Ihh = indian hedgehog; Ptch = patched; Shh = sonic hedgehog; Smo = smoothened; SuFu = suppressor of fused.

form metastasis. The transformation of mammary stem and progenitor cells also contributes to the generation of tumor heterogeneity. There is now evidence for the existence of 'tumor stem cells' in human leukemias, myeloma, and brain tumors, as well as in breast carcinomas [7–12].

A unique property of stem cells is their ability to undergo self-renewal divisions. In normal organogenesis this process is tightly regulated. The deregulation of self-renewal might be one of the key events involved in carcinogenesis. Indeed, pathways involving cell signaling pathways and transcription factors involved in the self-renewal of normal stem cells have all been implicated in carcinogenesis. These pathways include hedgehog, **[AU: please confirm the capitalization of these and all other protein names, and their abbreviations]** Notch and Wnt, as well as the transcription factor B lymphoma Mo-MLV insertion region 1 (Bmi-1). In this article we review evidence that these pathways are involved in both stem cell self-renewal and carcinogenesis, which provides support for the concept that breast carcinogenesis results from the deregulation of self-renewal pathways of normal mammary stem cells. We then discuss the implications of these studies for the development of novel therapies that target these self-renewal pathways.

Mammary stem cells

Stem cells are defined by their ability to undergo self-renewal, as well as multi-lineage differentiation. This self-renewal can be either asymmetric or symmetric. Self-renewal is distinguished from other proliferative processes in that at least one of the progeny of self-renewal is identical to the initial stem cell. In all other replicative processes, the progeny of division undergo a series of differentiation events [13]. In asymmetric stem cell self-renewal, one of the two progeny is identical to the initial stem cell, whereas the other cell is a committed progenitor cell, which undergoes cellular differentiation. Because the product of an asymmetric self-renewal division is one stem cell and one differentiated cell, this process maintains stem cell number. In contrast, symmetric self-renewal results in the production of two stem cells; by its very nature this results in stem cell expansion. The processes that regulate the balance between asymmetric and symmetric divisions of stem cells are poorly defined, but recent evidence indicates a role for p53 and inosine monophosphte dehydrogenase [14]. Although stem cells themselves are slowly dividing, progenitor cells derived from them are highly proliferative [15]. This expanding progenitor cell also has the ability to differentiate into the lineages comprising the adult tissue.

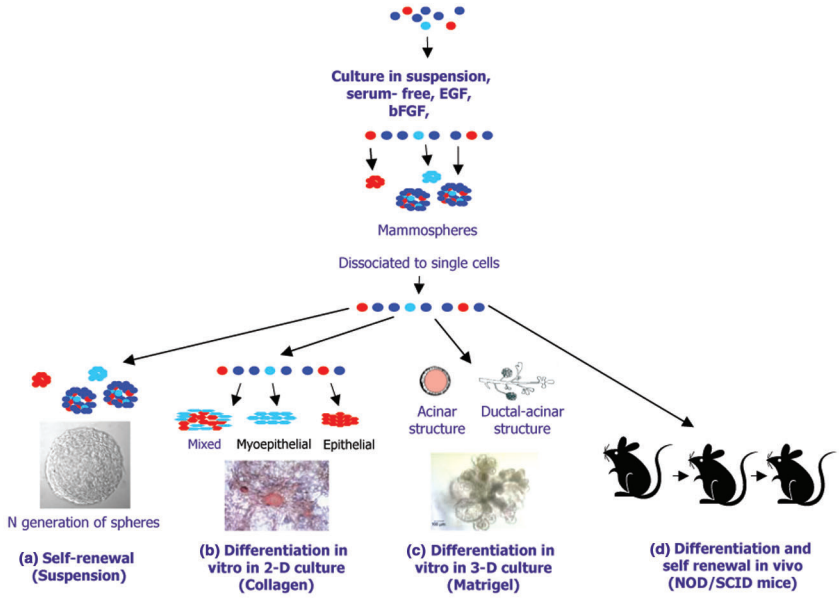
The existence of self-renewing multipotent mammary stem cells has been clearly demonstrated by transplantation studies in mice and rats [16–18]. Fragments of mammary epithelium marked with mouse mammary tumor virus were able to regenerate a new gland after transplantation into a mammary fat pad cleared of its epithelial components [19]. Serial transplantation of the clonally derived outgrowth recapitulated the entire functional repertoire of the gland, demonstrating the existence of self-renewing and multipotent mammary stem cells. A recent study in mice combining long-term labeling *in vivo* using bromodeoxyuridine with immunosorting and transplantation showed that mammary stem cell antigen-1 (SCA-1)-positive population is enriched in progenitor cells able to regenerate the gland *in vivo* [20].

The cultivation of normal mammary stem and progenitor cells has been limited by the lack of suitable systems that permit the propagation of these cells in an undifferentiated state. When primary cultures of mammary epithelium from rodents or humans are cultured on solid substrata, they undergo limited replication and differentiate in a process that is regulated by hormonal factors, extracellular matrix, and cell–cell interactions [21–23]. A major advance in neural stem cell research was achieved when it was found that an undifferentiated multipotent population of neural cells can be grown in suspension as neurospheres [24]. On the basis of the hypothesis that stem cells might be able to grow in anchorage-independent conditions, we developed a novel culture system for human mammary epithelial stem and progenitor cells. We demonstrated that human mammary epithelial cells, isolated from reduction mammoplasties, when grown on non-adherent substrata in the presence of growth factors, generate spherical colonies that we have termed 'mammospheres' [25], which are different from the three-dimensional structured mammospheres cultured from mammary organoids plated on extracellular matrix [26]. In our culture system *in vitro*, mammospheres are grown in suspension and are enriched in mammary stem/progenitor cells capable of self-renewal and multi-lineage differentiation (Fig. 1). We have also shown that mammospheres contain cells capable of clonally generating complex functional ductal alveolar structures in reconstituted three-dimensional culture systems in Matrigel (Fig. 1), and when combined with human mammary fibroblasts they are able to reconstitute the mammary tree in the cleared mammary fat pad of NOD/SCID mice (Fig. 1; **[AU: please give the names (with initials) of all those whose unpublished work is being cited]**, manuscript in preparation). The use of this culture system has enabled us to begin to elucidate the pathways that regulate the self-renewal and differentiation of normal mammary stem and progenitor cells (see below).

Tumor stem cells

There is increasing evidence that both stem and progenitor cells may be the targets of transformation during carcinogenesis. As described above, normal stem cells and cancer cells share several important properties, including the ability to self-renew and undergo differentiation. However, the mutations and/or epigenetic events involved in carcinogenesis may disregulate these pathways. Ensuing aberrant differentiation might in turn contribute to the phenotypic cellular heterogeneity found in tumors. Using different

Figure 1



Experimental design for assessing self-renewal and differentiation potential of cells grown as mammospheres. **(a)** Self-renewal is assessed by evaluating the ability of mammosphere-derived cells to form new spheres, containing multipotent cells. **(b)** Differentiation into all the three mammary lineage types on collagen in the presence of serum (immunostained with lineage-specific markers: brown, ductal epithelial (ESA); purple, myoepithelial (CD10); red, alveolar (β-casein)). **[AU: punctuation OK within parentheses now?]** **(c)** Generate complex ductal-alveolar structures in three-dimensional Matrigel culture. **(d)** Differentiation and self-renewal *in vivo* are tested by implanting human mammary epithelial cells into the cleared mammary fat pads of immunodeficient mice (NOD/SCID mice). EGF, epidermal growth factor.

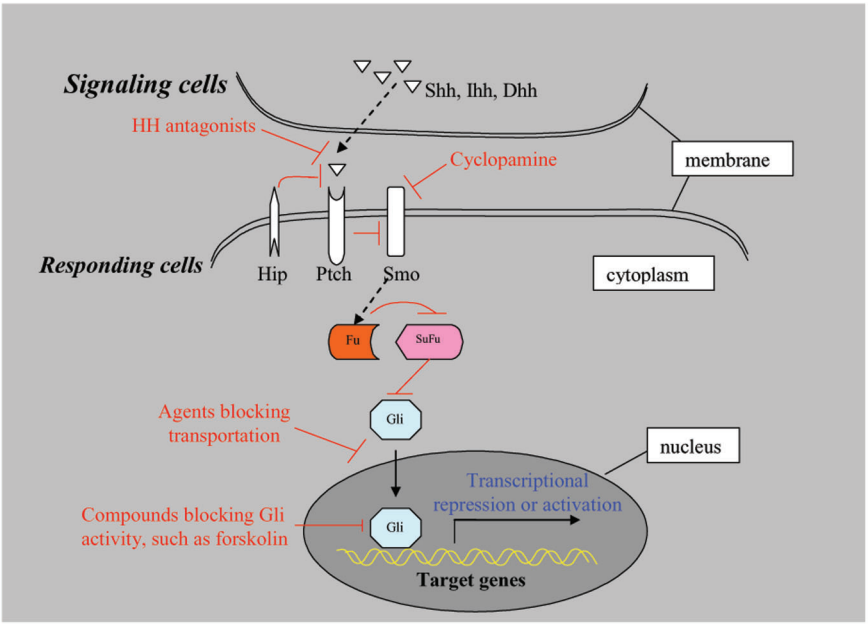
systems, several investigators have demonstrated that only a minority of cells in human cancers are capable of self-renewal. This has been most convincingly demonstrated by examining the ability of subpopulations of tumor cells identified by cell surface markers to form tumors when transplanted into immunosuppressed NOD/SCID mice. This approach was first successfully used to demonstrate the existence of leukemic stem cells [27].

We have used a similar approach to identify a subpopulation of human mammary cancer cells bearing the phenotype $ESA^+CD44^+CD25^{-/low}Lineage^-$ that have the properties of breast cancer 'stem cells'. As few as 100 of these cells, isolated from primary human breast carcinomas or metastatic lesions, are able to form tumors reproducibly in NOD/SCID mice. In contrast, tens of thousands of cells that do not bear this phenotype are unable to generate tumors in this model. Furthermore, consistent with a stem cell model is the observation that tumor stem cells are able to be serially passaged in NOD/SCID mice, each time generating a stem cell population, as well as the more differentiated non-tumorigenic cells forming the bulk of the tumor [27]. These 'tumor stem cells' thus share the properties of self-renewal and differentiation with their normal stem cell counterparts, although in tumors these processes are dysregulated. **[AU: 'dysregulated' OK, or do you mean 'deregulated'?]**

Recent studies have provided evidence for the existence of 'tumor stem cells' in human multiple myeloma and brain tumors in addition to acute leukemias and breast cancer [28,29]. An alternative model to the 'tumor stem cells' model is that cancers arise and evolve through stochastic mutations that are then expanded through clonal selection. Genetic instability and clonal selection undoubtedly do contribute to tumor heterogeneity and progression. However, the tumor stem cell model does not exclude the importance of these stochastic or selective events in tumor evolution. Both may in fact be operative in both tumorigenesis and tumor progression, and contribute to the heterogeneity found in cancer.

There has been some controversy about the nature of the cells that serve as targets of transformation. In a variety of malignancies, evidence for the clonal generation of tumors that display markers of multiple lineages has provided evidence for the stem cell as the cell of origin. However, in other cases, such as acute promyelocitic leukemia and chronic myelogenous leukemia, there is evidence for the transformation of progenitor cells. The transformation of progenitor cells might require mutations that allow them to undergo self-renewal, normally a process limited to stem cells. Indeed, we have recently proposed that the transformation of mammary stem and/or progenitor cells

Figure 2



A schematic diagram for the hedgehog (HH) signaling pathway. Ligands, such as Sonic Hedgehog (Shh), Indian Hedgehog (Ihh), and Desert Hedgehog (Dhh), are secreted by signaling cells and bind the transmembrane receptor patched (Ptch) in Hh **[AU: but abbreviation above is 'HH' – please confirm]** responding cells. In the absence of ligands, Ptch binds to Smoothened (Smo) and blocks Smo's function, whereas this inhibition is relieved in the presence of ligands, and Smo initiates a signaling cascade that results in the release of transcription factors Glis from cytoplasmic proteins Su **[AU: do you mean 'fused (Fu)'?[** and suppressor of fused (SuFu). In the inactive situation, SuFu prevents Glis from translocating to the nucleus; in the active situation, Fu inhibits SuFu and Glis are released. Gli proteins translocate into the nucleus and control target gene transcription. The red lines and the agents in red show the inhibitors of this pathway with potential therapeutic value.

might result in the heterogeneity of breast cancer types between different patients, reflected in molecular profiling data [6]. The molecular profile of tumors might be determined by both the cell of origin as well as the particular mutation profile, in turn determining the differentiation pattern of these cells, which comprise the bulk of the tumor. These categories defined by molecular profiling might have important diagnostic and prognostic implications. Regardless of the cells of origin, the common feature that might be required for transformation is the ability of the target cell to undergo self-renewal and subsequent expansion.

Thus, an understanding of the pathways that govern the self-renewal of normal stem cells, and the ways in which these pathways are dysregulated **[AU: 'dysregulated' OK, or do you mean 'deregulated'?]** during carcinogenesis, is of utmost importance. Several pathways found to have important roles in development and a transcription factor Bmi-1 have been shown to be involved in the regulation of stem cell self-renewal and differentiation. These pathways include Hedgehog, Notch, **[AU: please check capitalization – see below]** and Wnt. We review the role of these signaling pathways in stem cell self-renewal as well as evidence that these same pathways are important in the normal development of the mammary gland. We then discuss evidence that deregulation of these pathways is important in mammary carcinogenesis.

Hedgehog signaling

The hedgehog signaling pathway was first identified in *Drosophila*, where it is required for early embryo patterning. In recent years, great progress has been made in understanding the hedgehog signaling network [30,31]. This pathway is depicted graphically in Fig. 2. Three hedgehog ligands have been identified in mammals: Sonic Hedgehog (Shh), Desert Hedgehog (Dhh), and Indian Hedgehog (Ihh), all of which are secreted glycoproteins. After secretion, these ligands bind to the hedgehog-interacting protein 1 (Hip1) and Patched (Ptch), which are transmembrane receptors for these ligands. Two transmembrane proteins, Ptch and Smoothened (Smo), form the receptor complex in the absence of ligands. Ptch binds to Smo and blocks its function. This inhibition is relieved in the presence of ligands, and Smo interacts in a signaling cascade that results in activation of the transcription factors Gli1, Gli2, and Gli3. Gli proteins in turn translocate into the nucleus and control target gene transcription. In the absence of ligands, Gli proteins are tethered to the cytoskeleton by interacting with a multiprotein complex that includes Fused (Fu) and Suppressor of Fused (SUFU **[AU: this is SuFu in abbreviations list and Fig. 2 legend – please check which is correct]**) [32]. Gli regulates the

transcription of several genes, including those controlling cell proliferation such as cyclin D, cyclin E, Myc, components of the epidermal growth factor pathway, and angiogenesis components including platelet derived-growth factor and vascular endothelial growth factor.

Recent studies have indicated that hedgehog signaling is important in embryonic mammary gland induction, ductal morphogenesis, and alveolar development. A critical role for hedgehog signaling in mediating epithelial stromal interactions during ductal development has been shown by the genetic analysis of two hedgehog signal transduction network genes, Ptch1 and Gli-2. Disruption of either gene leads to similar, yet distinct, defects in ductal morphogenesis that are mainly ductal dysplasias **[AU: 'dysplasias' OK?]** similar to the hyperplasias of the human breast. We have used the mammosphere-based culture system to examine the role of hedgehog signaling in mammary cell fate determination. Our data show that the addition of recombinant Shh can stimulate the formation of primary and secondary mammospheres and can increase mammosphere size, a process that can be blocked by the Smo inhibitor cyclopamine (**[AU: please give the names (with initials) of all those whose unpublished work is being cited]**, manuscript in preparation). These studies suggest that hedgehog signaling is involved in mammary stem cell self-renewal.

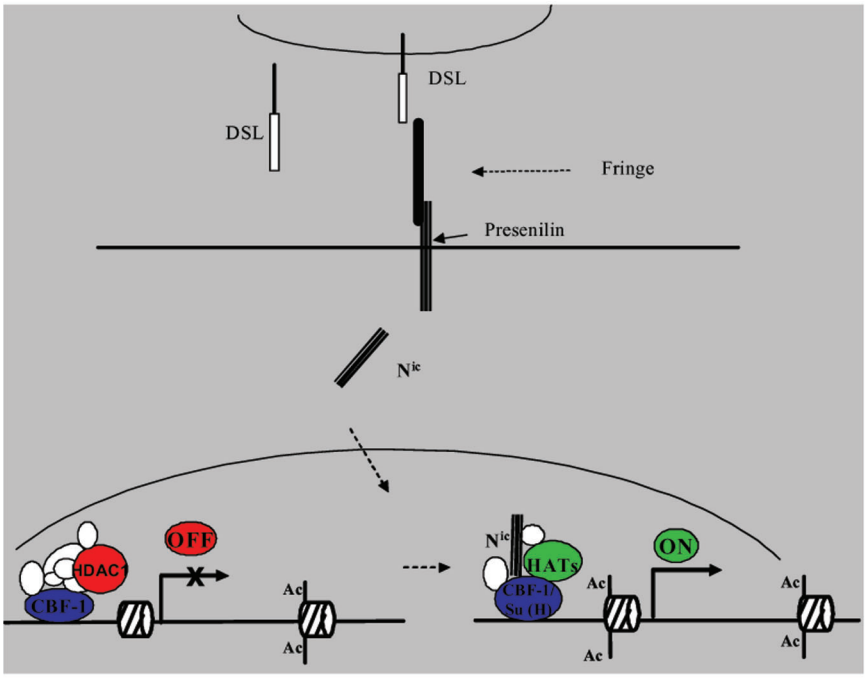
The importance of hedgehog signaling in carcinogenesis has been demonstrated by the fact that many of the genes involving hedgehog signaling are known oncogenes, including Smo, Shh, Gli-1, and Gli-2, or that Ptch1 can function as a tumor suppressor. Mutations in these genes have been linked to the development of many common cancers, which were shown to be dependent on activated hedgehog **[AU: lower-case OK?]** signaling [31]. Mutations in hedgehog signaling were first described in Gorlin syndrome and basal carcinomas of the skin. More recently, an important role for hedgehog signaling has been shown in medulloblastoma, prostate, and pancreatic carcinomas [33,34]. Similarities between hedgehog mutation-induced ductal dysplasias **[AU: 'dysplasias' OK?]** and human breast pathologies suggest a role for altered hedgehog signaling in the development of mammary cancer. There is also evidence that altered hedgehog signaling has a direct role in the neoplastic progression of the mammary gland. One study showed Ptch1 mutation **[AU: edit OK?]** in two of seven human breast cancers [35]. Recently, a natural polymorphism in the 3′ end of the Ptch1 coding region (C3944T; Pro1315→Leu) has been linked to increased breast cancer risk associated with oral contraceptive use [36]. Evidence for a role in breast cancer also comes from published genetic studies in mice showing hyperplastic defects in the mammary gland of ΔPtch1 plus and ΔGli1 **[AU: Greek letters OK? Should 'plus' be '+'?]** mutants [37]. Recently, Kubo and colleagues showed that a specific inhibitor of hedgehog signaling, cyclopamine, is able to inhibit the growth of mammary carcinoma cells *in vitro* [38].

Notch signaling

Notch transmembrane receptors are part of signaling pathways that are crucial in the regulation of the fate of cells in a variety of tissues [39]. The Notch **[AU: please check whether this should be capital or lower-case, throughout]** proteins, represented by four homologues in mammals, Notch 1 to Notch 4, are expressed in a variety of stem or early progenitor cells. They interact with several surface-bound ligands (DSL ligands: Delta, Delta like, Jagged1 and Jagged2 in vertebrates) [39]. These interactions are in turn regulated by a number of modifiers that form the fringe family [40]. Upon ligand binding, Notch receptors are activated by serial cleavage events involving members of the ADAM (for 'a disintegrin and metalloproteinase') protease family, as well as an intramembrane cleavage regulated by γ-secretase (presenilin **[AU: 'presenilin' OK?]**). This intramembrane cleavage is followed by translocation of the intracellular domain of Notch to the nucleus, where it acts on downstream targets (Fig. 3). Activation of the Notch pathway results in changes in cell fate, including self-renewal of stem cells or differentiation along a particular lineage [41]. The Notch pathway was shown to be involved in the normal development of the mammary gland. *In vitro*, overexpression of the constitutively active form of Notch4 inhibits the differentiation of normal breast epithelial cells. Smith and colleagues also demonstrated that, *in vivo*, Notch4 has an important role both in normal mammary development and in carcinogenesis. Transgenic mice harboring a constitutively active Notch4 under the regulation of mouse mammary tumor virus promoter exhibited arrested mammary gland development, and eventually developed poorly differentiated adenocarcinomas. Notch1 is also a downstream effecter of oncogenic Ras and its signaling activation maintains the neoplastic phenotype in human Ras-transformed cells [42].

We have recently used the mammosphere system described above to study the role of Notch signaling in mammary cell fate determination. Our findings suggested that Notch signaling is active in several distinct developmental stages of the mammary gland and that Notch acts as a regulator of asymmetric cell fate decisions. Notch activation promoted the self-renewal of stem cells, whereas in later stages of development it biased cell fate decisions in mammary progenitor cells toward the adoption of a myoepithelial cell fate versus an epithelial cell fate [6]. Musashi is a positive regulator of Notch signaling through an interaction with Numb mRNA and repression of its translation [43]. More recently, Musashi-1 and Notch1 were shown to be the two key regulators of asymmetric cell division in human breast epithelial stem cells [44,45]. These findings about the role of Notch in promoting the self-renewal of mammary stem cells, in addition to previous observations that it can function as a proto-oncogene [46,47], suggest that abnormal Notch

Figure 3



A schematic diagram for the Notch signaling pathway. Upon binding of the DSL ligand, **[AU: edit OK?]** Notch signaling is modulated by Fringe, **[AU: lower-case 'fringe' in the text]** and Notch receptors are activated by serial cleavage events involving members of the ADAM (for 'a disintegrin and metalloproteinase') protease family, as well as an intramembrane cleavage regulated by γ-secretase (presenilin **[AU: 'presenilin' OK?]**). This intramembrane cleavage is followed by translocation of the intracellular domain on Notch to the nucleus, where it acts on downstream targets. **[AU: please define CBF, HDAC and HAT]**

signaling might be involved in carcinogenesis, through the deregulation of normal mammary stem cell self-renewal.

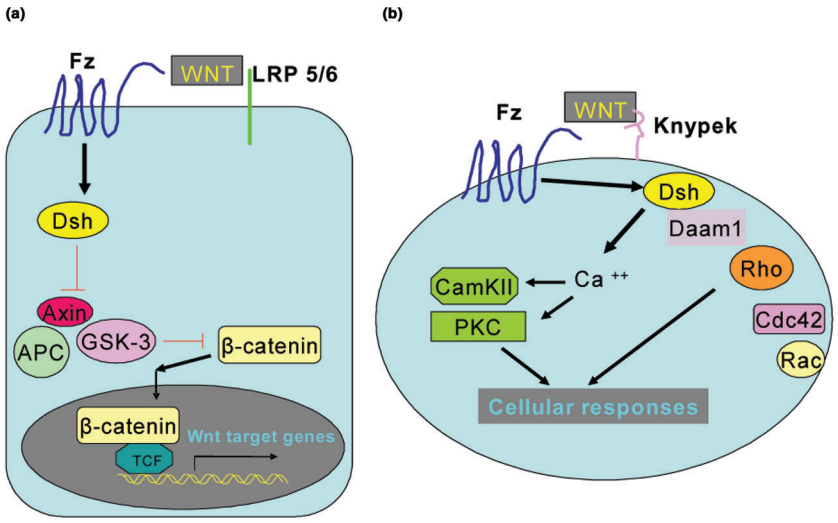
Wnt signaling

The Wnt pathway regulates cell fate determination in a number of tissues, including the mammary gland. The Wnts are a family of secreted proteins. So far, the most well-characterized Wnt signaling pathway is called the canonical Wnt pathway, in which Wnt ligands signal through the stabilization of β-catenin. More recently, several β-catenin-independent Wnt signaling pathways, known as non-canonical, have been shown to be crucial for different aspects of vertebrate embryo development [48]. In the canonical Wnt pathway, Wnt proteins bind to a family of Frizzled receptors in a complex with the low-density lipoprotein receptor-related proteins 5 and 6 (LRP5/6) [49]. Activation of these receptors results in the accumulation of intracellular β-catenin. In the absence of Wnt signaling, β-catenin remains in the cytoplasm, where it forms a complex with other proteins, including the tumor suppressor adenomatous polyposis coli and axin, and well as glycogen synthase kinase (GSK)-3β. GSK-3β is able to phosphorylate β-catenin, which targets the protein for ubiquitin-mediated degradation. When the Wnt pathway is activated, GSK-3β is inhibited, blocking β-catenin phosphorylation. Unphosphorylated β-catenin is stable and translocates to the nucleus, where it binds to and activates the transcription factors T cell factor/lymphoid enhancer factor (TCF/LEF), which then activate a variety of downstream target genes (Fig. 4a).

The noncanonical Wnt signaling pathway [48] involves Frizzled receptors and the proteoglycan co-receptor Knypek. A cytoplasmic signal transduction protein Dishevelled (Dsh) localizes to the cell membrane through its DEP domain. Dsh activates Rho through the bridging molecule Daam1. The precise roles of Rho versus other Rho-family small GTPases such as Rac and Cdc42 remain unclear, as is the potential role of the JNK pathway. Dsh can also stimulate calcium flux and sequentially activates the calcium-sensitive kinases protein kinase C and calmodulin-dependent protein kinase II (Fig. 4b).

Recently, several studies have provided evidence for a direct role of Wnt signaling in the self-renewal of hematopoietic, epidermal, and gut stem cells [50,51]. Retroviral transduction of activated β-catenin results in increased epidermal stem cell self-renewal and decreased differentiation. A direct role for dysfunction of this pathway in cancer was established by experiments in transgenic mice that showed that activation of the Wnt signaling pathway in epidermal stem cells leads to epithelial cancers [52]. Furthermore, in breast cancers, it has been demonstrated that there is upregulation of the

Figure 4



A schematic diagram for the Wnt signaling pathway. **(a)** The canonical Wnt/β-catenin pathway. Canonical Wnt signaling requires the Frizzled (Fz) and low-density lipoprotein receptor-related proteins 5 and 6 (LRP5/6) co-receptors to activate Dishevelled (Dsh). Then Dsh inhibits the activity of the β-catenin destruction complex (adenomatous polyposis coli (APC), axin, and glycogen synthase kinase-3 (GSK-3)), which phosphorylates β-catenin in the absence of the ligands. β-Catenin is stabilized and translocated to the nucleus, where it recruits transactivators to HMG-box **[AU: please define 'HMG']** DNA-binding proteins of the lymphoid enhancer factor/T cell factor (LEF/TCF) family. **(b)** The noncanonical Wnt signaling pathway. Noncanonical Wnt signaling requires Frizzled receptors and the proteoglycan co-receptor Knypek. In this pathway, Dsh localizes to the cell membrane through its DEP domain. A main branch downstream of Dsh involves the small GTPases of the Rho family. Dsh activation of Rho requires the bridging molecule Daam1. Dsh can also stimulate calcium flux and the activation of the calcium-sensitive kinases protein kinase C (PKC) and calmodulin-dependent protein kinase II (CanKII). At the end, the activation of this pathway induces the complex and dynamic cellular response.

uncomplexed transcriptionally active form of β-catenin without mutations afflicting downstream components [53]. A role for Wnt signaling in stem cell self-renewal of mammary stem cells was suggested by recent studies of Alexander and colleagues, who used transgenic mice to show that overexpression of Wnt ligands in mammary stroma or activated β-catenin in mammary epithelium leads to increased numbers of mammary stem cells [54]. Studies linking this process to mammary carcinogenesis include those showing that mammary stem cells and progenitors might be targets for oncogenesis by Wnt 1 signaling elements [55].

Bmi-1

Bmi-1 is a transcriptional repressor belonging to the polycomb (PCG) group of transcription factors. It was first identified in a B-cell lymphoma [56]. Recently, Bmi-1 has been shown to be a key regulator of the self-renewal of both normal and leukemic stem cells [57,58]. Bmi-1 has also been shown to be important in neuronal stem cell self-renewal [59]. Several recent studies have suggested a link between Bmi-1 and mammary carcinogenesis. Bmi-1 was shown to be overexpressed in several human breast cancer cell lines. Furthermore, it was found that Bmi-1 regulates telomerase expression in mammary epithelial cells. These studies suggest that Bmi-1 might have a role in mammary carcinogenesis [60]. Although the mechanisms by which Bmi-1 regulates stem cell self-renewal remain unclear, one important gene silenced by Bmi-1 might be P-16 [58]. However, P-16 only partly mediated the effects of Bmi-1 proteins in neural stem cells, thereby suggesting that other factors might participate in Bmi-1's effects on stem cell self-renewal. Recent studies by Tlsty and colleagues [61] have suggested that the epigenetic silencing of P-16 might be an important event in early mammary carcinogenesis. Together, these studies suggest that normal stem cell self-renewal might be regulated through Bmi-1, partly mediated through the repression of P-16. During carcinogenesis, this process might be dysregulated **[AU: 'dysregulated' OK, or do you mean 'deregulated'?]** by the epigenetic silencing of P-16 through methylation of the P-16 promoter [61].

Interaction between self-renewal pathways

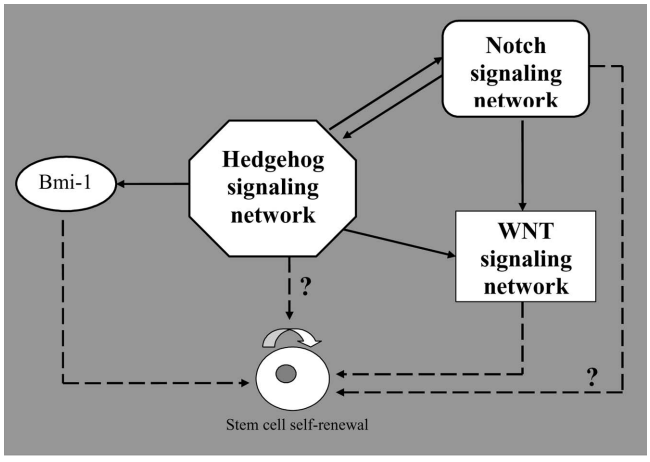
Although we have described signaling pathways that regulate stem cell self-renewal, individually it is clear that *in vivo* there are extensive interactions between the pathways. For instance, there is evidence for interaction between Hedgehog signaling and Notch signaling. One study provided evidence that secreted Shh might be involved in reinforcing the cell fate switch executed by Notch [62]. Moreover, a recent study presented intriguing evidence that Notch signaling regulates Gli-2 expression in mouse skin, and inactivation of the Notch-

1 gene in epidermis induces sustained expression of Gli-2 resulting in the formation of basal carcinoma-like tumors [63].

Recently we used our mammosphere-derived culture systems to examine the relationship between the hedgehog pathway and the Notch pathway, and we found that the activation of the Notch pathway resulted in the subsequent activation of the hedgehog pathway, including increased expression of Ptch and Gli. This activation could be blocked by γ-secretase inhibitor, which inhibits Notch signaling (**[AU: please give the names (with initials) of all those whose unpublished work is being cited]**, manuscript in preparation). These studies suggest that hedgehog acts downstream of Notch. In contrast, one study showed that Shh acts upstream of Notch to determine arterial cell fate during arterial endothelial differentiation [64]. Furthermore, we have evidence that activation of hedgehog pathway by the hedgehog ligands (Shh or Ihh) increased the expression of the Notch pathway target, HES1, in the mammospheres, and this effect could be blocked by the hedgehog inhibitor cyclopamine (**[AU: please give the names (with initials) of all those whose unpublished work is being cited]**, manuscript in preparation). Together, these studies indicate that Hedgehog and Notch might form a feedback loop regulating normal development.

Furthermore, deregulation of this loop might be involved in cancer formation. In the skin, the activation of two markers of active Wnt signaling, β-catenin and LEF-1, are associated with Notch-dependent transformation [65]. The activation of Smo might initiate processes during which transcription factors belonging to the Gli family are activated, and modify the transcription of Ptch and Wnt [65]. Wnt regulation has previously been observed in human basal carcinomas, indicating that tumor progression is mediated by interactions of distinct signaling pathways that regulate organ development during embryogenesis. All of these pathways are also intimately involved in the regulation of stem cell self-renewal. Interestingly, Bmi-1 expression was rapidly increased after the addition of Shh or after the overexpression of the Shh target Gli in cerebellar granular cells, which implies that Bmi-1 is a downstream target in the Shh pathway [66]. Overexpression of Bmi-1 correlated with overexpression of Ptch and SUFU, **[AU: SuFu elsewhere. Please also confirm that the comma is now correctly placed]** which suggests at least a partial activation of the Hedgehog pathway in Bmi-1 overexpression tumors [66]. In our preliminary data we showed that both the activation of Hedgehog pathway by Shh or Ihh and the activation of the Notch pathway by DSL resulted in the expression of Bmi-1 in the mammosphere culture system, and the induction of Bmi-1 expression could be blocked by the pathway-specific inhibitors cyclopamine and γ-secretase inhibitor, respectively **[AU: please clarify what respectively' is referring to]** (**[AU: please give the names (with initials) of all those whose unpublished work is being cited]**, manuscript in preparation).

Figure 5



A hypothetic interacting model in the regulation of stem cell self-renewal by the Hedgehog **[AU: but lower-case below]** signaling pathway, the Notch signaling pathway, the Wnt signaling pathway, and B lymphoma Mo-MLV insertion region 1 (Bmi-1). Interactions between the hedgehog, Notch, and Wnt pathways and Bmi-1 are shown by solid arrows; interactions between stem cell self-renewal regulation by the pathways and Bmi-1 **[AU: edit OK?]** are shown by dashed arrows; the question marks represent the postulated interactions.

Together, these studies demonstrate extensive interaction between the signaling pathways that regulate stem cell self-renewal. These interactions are depicted graphically in Fig. 5. In this model, Hedgehog and Notch signalings form a loop regulating normal development; both of these pathways might regulate the stem cell self-renewal by upregulating the expression of Bmi-1, which has been identified as a regulator of stem cell self-renewal. It has also been shown that the Wnt pathway can act downstream of both the Hedgehog pathway and the Notch pathway, and the Wnt pathway has been shown to be a regulator of stem cell self-renewal. However, it has not been determined whether the Hedgehog pathway and the Notch pathway can regulate stem cell self-renewal through downstream targets other than Bmi-1. Further elucidation of this model will be required for an understanding of the elements that regulate normal and malignant mammary stem cell self-renewal.

Conclusions and clinical implications

In this review we have presented evidence that carcinogenesis in the mammary gland, and in other organs, might result from transformation of stem and/or progenitor cells by the deregulation of self-renewal pathways. These pathways include Hedgehog, Notch, Wnt, and the transcription factor Bmi-1. The hypothesis that mammary carcinogenesis results from the deregulation of normal stem cell self-renewal pathways suggests that components of these pathways

might provide attractive targets for therapeutic development. This is of great importance because current therapies may be limited in their effectiveness by virtue of the fact that they might selectively target the more differentiated cells in a tumor. Tumor stem cells, by virtue of their slow cell cycle kinetics, transporter proteins, and anti-apoptotic mechanisms, might be resistant to these treatments (reviewed in [67]). The targeting of self-renewal pathways might provide a more specific approach to the elimination of cancer stem cells. A potential challenge in this regard is the development of therapies that selectively affect cancer stem cells while sparing normal stem cells that may rely on similar mechanisms for self-renewal. Recent studies have shown that inhibitors of hedgehog signaling, such as cyclopamine, can inhibit mammary tumor cells *in vitro* [38]. Furthermore, a small-molecule inhibitor of the Shh pathway – a Hedgehog antagonist (HhAntag) – has recently been reported to eliminate medulloblastoma in transgenic mice without apparent systemic toxicity [68]. These studies suggest that strategies aimed at targeting cancer stem cell self-renewal might provide a novel therapeutic approach for the treatment of breast and other cancers.

Competing interests

MW has financial holdings in OncoMed Pharmaceuticals, which has applied for a patent on cancer stem cell technologies.

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Research Article

Hedgehog Signaling and Bmi-1 Regulate Self-renewal of Normal and Malignant Human Mammary Stem Cells

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Abstract

The epithelial components of the mammary gland are thought to arise from stem cells with a capacity for self-renewal and multilineage differentiation. Furthermore, these cells and/or their immediate progeny may be targets for transformation. We have used both *in vitro* cultivation and a xenograft mouse model to examine the role of hedgehog signaling and Bmi-1 in regulating self-renewal of normal and malignant human mammary stem cells. We show that hedgehog signaling components *PTCH1, Gli1*, and *Gli2* are highly expressed in normal human mammary stem/progenitor cells cultured as mammospheres and that these genes are down-regulated when cells are induced to differentiate. Activation of hedgehog signaling increases mammosphere-initiating cell number and mammosphere size, whereas inhibition of the pathway results in a reduction of these effects. These effects are mediated by the polycomb gene *Bmi-1*. Overexpression of Gli2 in mammosphere-initiating cells results in the production of ductal hyperplasia, and modulation of Bmi-1 expression in mammosphere-initiating cells alters mammary development in a humanized nonobese diabetic-severe combined immunodeficient mouse model. Furthermore, we show that the hedgehog signaling pathway is activated in human breast "cancer stem cells" characterized as $CD44^+CD24^{-/low}Lin^-$. These studies support a cancer stem cell model in which the hedgehog pathway and Bmi-1 play important roles in regulating self-renewal of normal and tumorigenic human mammary stem cells. (Cancer Res 2006; 66(12): 1-9)

Introduction

Stem cells are characterized by their ability to self-renew as well as generate differentiated cells within each organ. There is increasing evidence that these cells or their immediate progeny may be targets for transformation. We have hypothesized that an early event in carcinogenesis may involve dysregulation of stem cell self-renewal leading to a clonal expansion of initiated stem cells (1, 2).

A number of developmental signaling pathways, such as Wnt, Notch, and hedgehog, have been found to play a role in regulating the self-renewal of normal stem cells in the hematopoietic system, the skin, the nervous system, and the breast (1, 3, 4). In normal breast development, the epithelial components of the mammary gland are generated by a stem cell able to give rise to the lineages found in the adult gland, including myoepithelial cells, ductal epithelial cells, and alveolar epithelial cells (5). In the past, characterization of the pathways that regulate self-renewal of mammary stem cells has been limited by the lack of systems that support propagation of these cells in an undifferentiated state *in vitro*. When primary cultures of mammary epithelium from rodents or humans are cultured on solid substrata, they undergo limited replication and terminally differentiate (6–8). Moreover, the *in vivo* study of human mammary stem cells has been precluded by the lack of xenotransplantation mouse models. We have recently described an *in vitro* system for the propagation of human mammary stem and progenitor cells in suspension culture. We showed that human mammary stem cells isolated from reduction mammoplasties generate spherical colonies in suspension culture. These colonies, which we have termed nonadherent mammospheres, are highly enriched in mammary stem and progenitor cells capable of both self-renewal and multilineage differentiation (9). We have previously used this culture system to show that the Notch pathway plays a role in cell fate determination of human mammary stem cells (10).

The characterization of mouse mammary stem cells and study of mammary development has been greatly facilitated by the use of transplantation models in which mammary cells can be transplanted into the cleared mammary fatpads of syngenic mice (5, 11). Recently, Kuperwasser et al. (12) described a system in which the fatpads of nonobese diabetic-severe combined immunodeficient mouse (NOD-SCID) mice, "humanized" by implantation of immortalized human mammary fibroblasts, were able to support the growth of human mammary cells. The use of *in vitro* human mammosphere cultures and their transplantation into humanized NOD-SCID mouse fatpads has allowed us to further elucidate the pathways that regulate self-renewal of normal human mammary stem cells.

In addition to addressing the cell involved in tumor initiation, the "cancer stem cell hypothesis" postulates that tumors are driven by a cellular subpopulation retaining stem cell properties (2, 3, 13). Consistent with this model, we recently identified a subpopulation of cells in human breast cancers with the phenotype $CD44^+CD24^-lineage^-$ that display stem cell properties. As few as 200 cells that display this phenotype were capable of generating tumors in NOD-SCID mice, whereas the bulk of the tumor population was not tumorigenic. Furthermore, consistent with a stem cell model, these tumor-initiating cells produce tumors that recapitulate the phenotype of the initial tumor. Thus, these tumor-initiating cells display the stem cell characteristics of self-renewal and differentiation. Over the past several years, tumorigenic stem

Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

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cells have been detected in myeloma, brain cancer, sarcoma, and prostate cancers (14–16), lending support to the cancer stem cell hypothesis. However, it remain unclear how pathways such as Hh regulate the self-renewal of normal stem cells and the role that deregulation of these pathways plays in carcinogenesis.

In the present studies, we have used both *in vitro* and mouse model systems to elucidate the role of hedgehog signaling and the polycomb gene *Bmi-1* in regulating the self-renewal of normal human mammary stem cells. Furthermore, we have examined the activation of these pathways in breast cancer stem cells. These studies provide support for the cancer stem cell hypothesis in which dysregulation of normal stem cell self-renewal pathways generates tumors driven by cells that maintain stem cell characteristics.

Materials and Methods

Dissociation of mammary tissue and mammosphere culture. One hundred to 200 g normal breast tissue from reduction mammoplasties were minced and dissociated, and single cells were cultured in suspension as described previously (9). Primary mammospheres were dissociated enzymatically and mechanically, and then cultured in suspension to produce mammospheres or on a collagen substratum, as described previously (9). After mammospheres were formed in suspension culture or cells reached 85% confluency on the collagen plate (~7 days), total RNA was isolated using RNeasy Mini kit (Qiagen) and used for real-time quantitative reverse transcription-PCR (qRT-PCR) assays in a ABI PRISM 7900HT sequence detection system with 384-well block module and automation accessory (Applied Biosystems) as described in Supplementary Data.

Treatments of mammospheres with hedgehog agonists and antagonist. Single cells from epithelial organoids were plated in six-well ultra-low attachment plates (Corning) as described previously (9). Biologically active, unmodified amino-terminal recombinant human Sonic hedgehog (Shh) and mouse Indian hedgehog (Ihh; R&D Systems), cyclopamine (TRC, Inc.) were used. We tested different concentrations of Shh and determined the optimum stimulation or inhibition was obtained with 3 μg/mL Shh (17) or 300 nmol/L cyclopamine (18) in our studies. Tomatidine was used as a negative control for cyclopamine. Mammospheres were then collected at days 1, 3, 5, or 7. All of these collected mammospheres were used for RNA extraction and qRT-PCR and the mammospheres treated for 7 days were also used for *in vitro* self-renewal assays as described in Supplementary Data.

Immunostaining. To assess lineage composition of the colonies, single-cell suspensions were plated on collagen-coated dishes and cultured as described previously (9) for 7 days. Cells were fixed on plates in −20°C methanol for 20 minutes and stained using Peroxidase Histostain-Plus and Alkaline-Phosphatase Histostain-Plus kits (Zymed), according to the protocol of the manufacturer. The primary antibodies, cytokeratin 18 for epithelial cells and cytokeratin 14 (Novocastra) for myoepithelial cells, were used at the dilutions indicated by the manufacturer. AEC and 3,3′-diaminobenzidine (Zymed) were used as substrates for peroxidase and nitroblue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate (Life Technologies) for alkaline phosphatase.

Virus production, infection, and cell culture. The retroviral plasmid DNAs for Vector only (SIN-IP-EGFP), Gli1 (SIN-GLI1-EGFP; ref. 19), and Gli2 (SIN-GLI2-EGFP; ref. 20) were generous gifts from Dr. Graham W. Neil. Retroviruses for SIN-IP-EGFP, SIN-GLI1-EGFP, and SIN-GLI2-EGFP were produced by stable transfection of 293 cells and were used to infect the single cells isolated from primary mammosphere (see Supplementary Data for details). A highly efficient lentiviral expression system (pLentiLox 3.7)[1] was used to generate Bmi-1-expressing (hBmi-1-GFP) and green fluorescent protein (GFP)–expressing (GFP alone) lentiviruses in University of Michigan Vector Core Facility.

Small interfering RNA constructions. Three human Bmi-1 (hBmi-1) siRNA oligos were purchased from Ambion, Inc. (Silencer Predesigned siRNAs) and were used to confirm the knockdown of Bmi-1 expression in primary human mammary epithelial cells. All the siRNA sequences were converted to small hairpins (shRNA) and inserted into lentivirus vector LentiLox 3.7. The GFP is expressed in lentivirus-infected cells as the marker to indicate that the cells express the shRNA for hBmi-1. In our experiments, >90% of cells were infected with the control (GFP alone) or siRNA lentiviruses (hBmi-1-siRNA1-GFP, hBmi-1-siRNA2-GFP, and hBmi-1-siRNA3-GFP).

Mammosphere implantation into the cleared fatpads of NOD-SCID mice. Three-week-old female NOD-SCID mice were anesthetized by an i.p. injection (21). The no. 4 inguinal mammary glands were cleared and humanized with 2.5×10^5 nonirradiated telomerase immortalized human mammary fibroblasts (a generous gift from John Stingl and Connie Eaves, Terry Fox Laboratory, Vancouver, British Columbia, Canada) and 2.5×10^5 irradiated (4 Gy) fibroblasts as previously described (12), following a previously established protocol (22). A 60-day release estrogen pellet (0.72 mg/pellet, Innovative Research of America) was placed s.c. on the back of the neck of the mouse by using a trocar, and 400 mammospheres were mixed with 2.5×10^5 normal human mammary fibroblasts and resuspended in 10 μL of 1:1 Matrigel: 5% serum Ham's F-12 and injected into each of the cleared fatpads. All of the implantation experiments were repeated five times using mammospheres from different patients with three mice implanted per patient sample.

Preparation of mammary fatpad sections. Approximately 8 weeks after the implantation, the fatpads were removed and fixed in Carnoy's solution for 1 hour and subsequently stained with carmine alum overnight. The tissue was then defatted through graded ethanol and cleared in 5 mL of xylene for 1 hour. The tissue was then embedded in the paraffin and sectioned for H&E staining.

Preparation of single-cell suspensions of tumor cells, xenografts, and flow cytometry. Human mammary tumors were passaged in NOD-SCID mice as previously described (21). Following tumor growth, which took 1 to 2 months, tumors were removed and single cells were obtained by collagenase digestion as described previously (21). One part of the single cells was used for flow cytometry to sort out the $H2K^{d-}CD44^{+}CD24^{-/low}lineage^{-}$ population and $H2K^{d-}CD44^{+}CD24^{+}lineage^{-}$ population as described previously (21). RNA was extracted from these two populations and real-time RT-PCR was used to quantitate gene expression.

Statistical analysis. Results are presented as the mean ± SD for at least three repeated individual experiments for each group. Analysis was done using Minitab statistical software for Windows (Minitab, Inc.). Statistical differences were determined by using one-way ANOVA for independent samples. $P < 0.05$ was considered statistically significant.

Results

Components of the hedgehog pathway are highly expressed in mammary stem/progenitor cells. We have previously described the development of an *in vitro* culture system and a xenograft mouse model for the propagation of mammary stem/progenitor cells. This system is outlined in Fig. 1*A*. When primary human mammary epithelium isolated from reduction mammoplasties are cultured in nonadhering conditions, the vast majority of cells undergo anoikis. However, a small number (mammosphere-initiating cells; ~4 per 1,000 cells) are able to form floating spherical colonies (mammospheres). Utilizing retroviral marking studies, we showed that these mammospheres could be dissociated and serially passaged at clonal density, with secondary and subsequent generation of mammospheres generated from single cells (9), maintaining a relatively constant number of mammospheres over a

[1] http://www.med.umich.edu/vcore/.

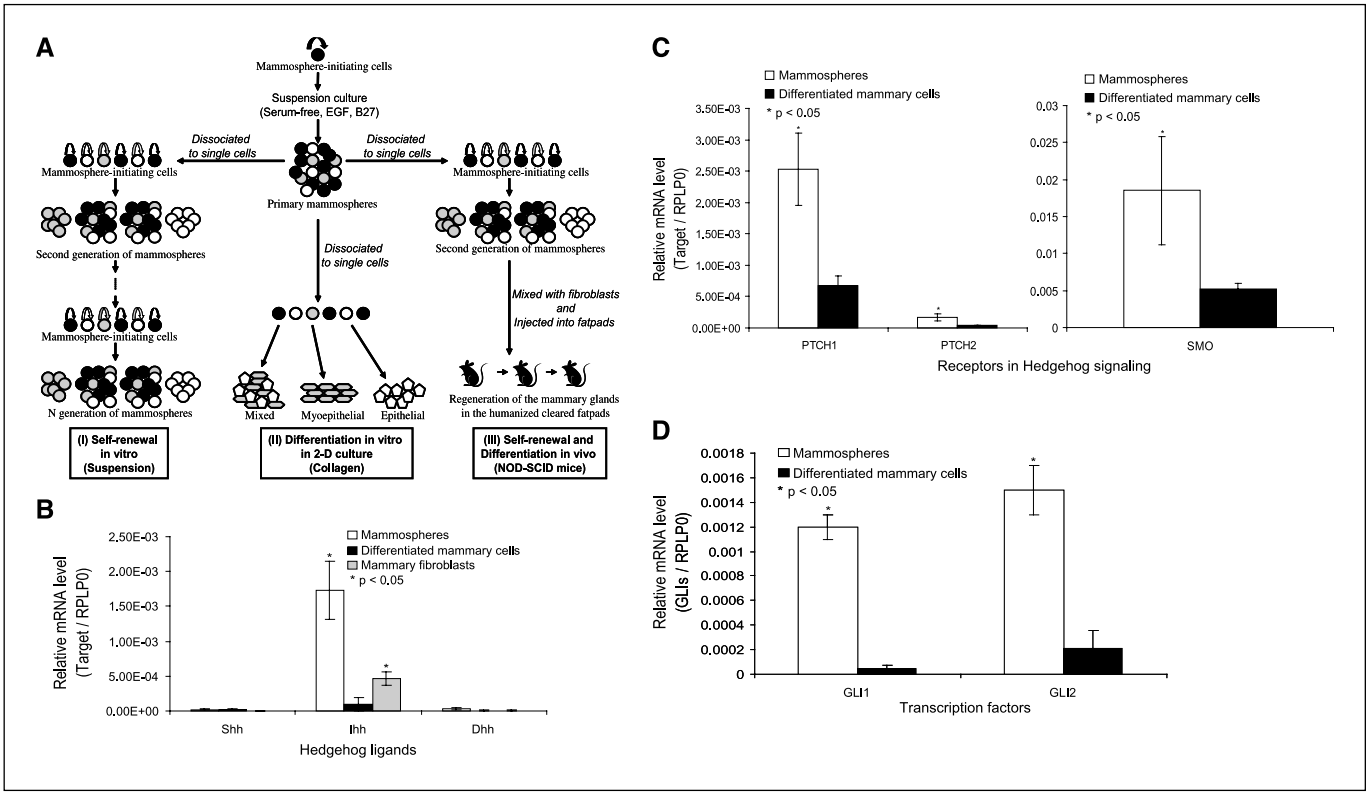

Figure 1. *A,* experimental strategies for testing self-renewal and differentiation of human mammary stem cells *in vitro* and *in vivo. I,* self-renewal is assessed by evaluating the ability of mammosphere-derived cells to form mammospheres containing multipotent cells. *II,* lineage-specific differentiation potential is assessed by culturing cells on a collagen-coated substratum in the presence of serum. *III,* differentiation and self-renewal *in vivo* are tested by implanting human mammary epithelial cells into the humanized cleared mammary fatpads of NOD/SCID mice. *EGF,* epidermal growth factor. *B* to *D,* components of the hedgehog pathway are highly expressed in mammary stem/progenitor cells. Mammary epithelial cells were cultured as mammospheres in suspension or as differentiated mammary cells on collagen substrata, and mammary fibroblasts from the same patient were cultured on collagen substrata. Total RNA was isolated and mRNA was quantitated by real-time RT-PCR. *Columns,* means; *bars,* SD. *, $P < 0.05$, statistically significant differences from the differentiated cells. *B,* mRNA expression of hedgehog ligands Shh, Ihh, and Desert hedgehog (*Dhh*). *C,* mRNA expression of hedgehog receptors PTCH1, PTCH2, and SMO. *D,* mRNA expression of transcription factors Gli1 and Gli2.

number of generations. The lineage-specific differentiation potential was assessed by plating these cells at clonal density on collagen substrata (9). These studies suggest that mammospheres are composed of a small number of stem cells capable of mammosphere formation and progenitors capable of multilineage differentiation, but not sphere formation. Similar findings have been reported for neural stem cells in neurospheres (1, 3, 4, 6). Attachment of cells to collagen substrata induces irreversible differentiation of these cells (9).

We compared expression of the genes in the hedgehog pathway in mammary stem/progenitor cells to that of differentiated mammary cells, using mammosphere-derived cells grown in suspension culture versus mammosphere-derived cells cultured on a collagen substratum. As shown in Fig. 1*B*, Ihh is the major Hh ligand expressed in mammary epithelial cells and its expression level is ~9-fold higher in stem/progenitor cells in mammospheres than in differentiated cells cultured on a collagen substratum. During normal mammary development, hedgehog signaling is present in the stroma as well as the epithelium (23). We found that mammary fibroblasts produce Hh ligands although at lower level than in mammospheres. Figure 1*C* shows that hedgehog receptors PTCHs and SMO are expressed in both cell populations. However, mammosphere-derived mammary stem/progenitor cells express ~4-fold higher levels of PTCH mRNA, and 3-fold higher levels of SMO mRNA than differentiated mammary cells. We measured the expression of transcription factors Gli1 and Gli2, which are downstream components of the hedgehog pathway, and found that mammary stem/progenitor cells have almost 25-fold higher levels of Gli1 mRNA and 6-fold higher levels of Gli2 mRNA than differentiated mammary cells (Fig. 1*D*). Taken together, these results indicate that hedgehog signaling pathway is activated in mammary stem/progenitor cells and is down-regulated during differentiation.

Hedgehog signaling agonists and antagonist regulate mammary stem cell self-renewal and multilineage differentiation. We have previously shown that mammosphere number upon multiple passages reflects stem cell self-renewal, whereas mammosphere size reflects progenitor cell proliferation (9, 10). We examined the effects of the hedgehog ligand Shh and hedgehog signaling inhibitor cyclopamine on primary and secondary mammosphere formation. We found that 3 μg/mL Shh increased primary mammosphere formation by 57% and the average cell number in these mammospheres by 62% (Fig. 2*A*). In F2 contrast, cyclopamine decreased primary mammosphere formation by 45% and the average cell number in the primary mammospheres by 51% (Fig. 2*A*). The specificity of Shh stimulation was shown by reduction of this effect by cyclopamine (Fig. 2*A*), but not by tomatidine, an inactive cyclopamine analogue (Supplementary Fig. S1). The degree of reversal was SF1 dependent on the concentrations of cyclopamine and Shh. This

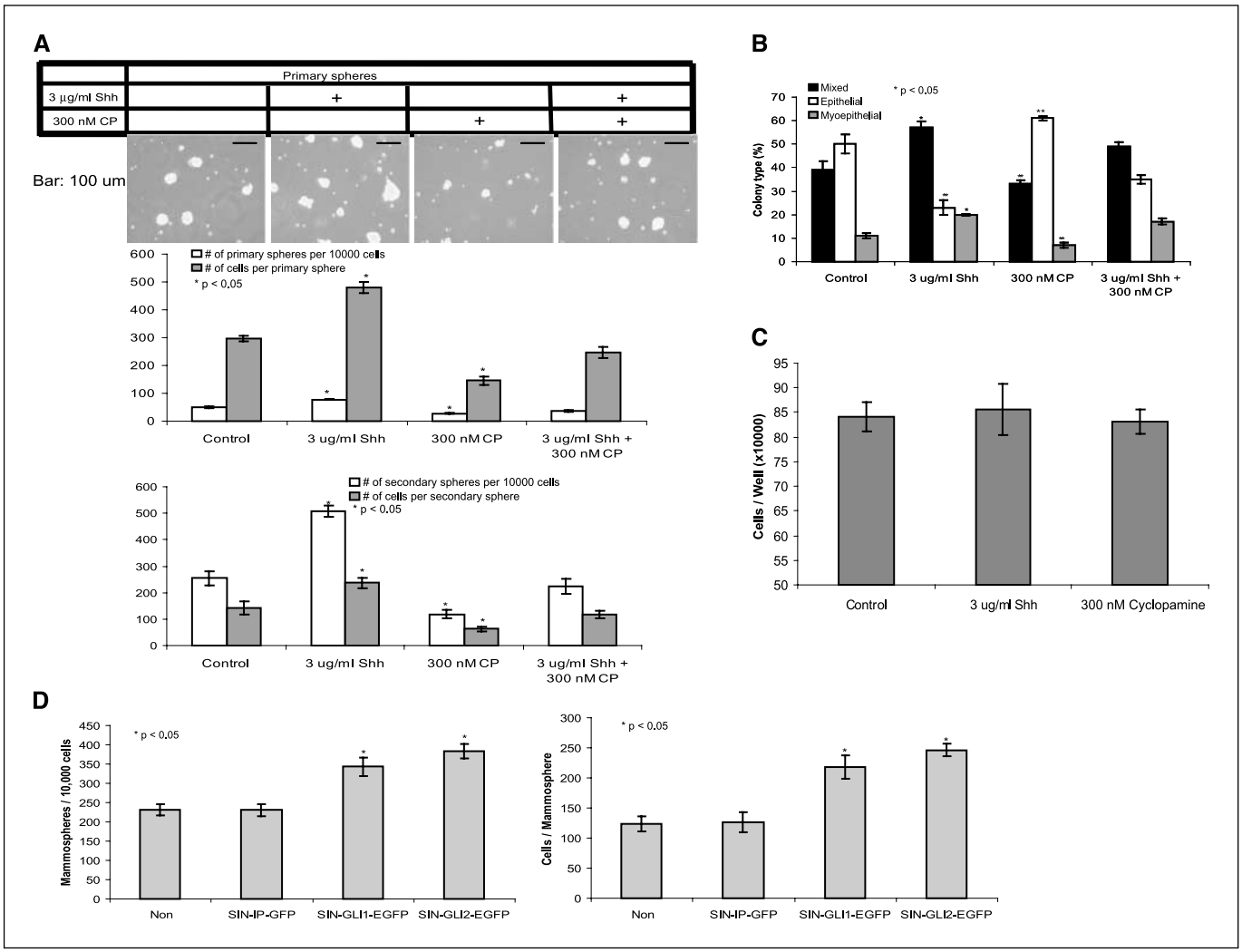

Figure 2. Hedgehog signaling regulates mammosphere number and size. *Columns,* mean; *bars,* SD. *, $P < 0.05$, statistically significant differences from the control group. Bar, 100 μm. *A,* effects of hedgehog agonist and antagonist on primary and secondary mammosphere formation. Primary mammospheres were grown in suspension for 7 to 10 days in the presence or absence of 3 μg/mL Shh, 300 nmol/L cyclopamine (*CP*), or both. Single cells dissociated from each group were grown as secondary mammospheres in suspension for 7 to 10 days without treatment. The number of mammospheres represents total mammospheres formed from 10,000 single cells; the number of cells represents the total cells per mammosphere. *B,* effect of hedgehog activation and inhibition on lineage specification of human mammary progenitor cells. *C,* effect of hedgehog agonist and antagonist treatment on differentiated mammary epithelial cells cultured on a collagen substratum. Primary mammary epithelial cells cultured on collagen-coated six-well plates were treated with 3 μg/mL Shh or 300 nmol/L of cyclopamine for 7 days, after which the cells were collected and counted. *D,* effects of Gli1 and Gli2 overexpression on mammosphere number and size. Secondary mammospheres were infected with SIN-IP-EGFP virus, SIN-GLI1-EGFP virus, SIN-GLI2-EGFP virus, or none as the control.

suggests that at low concentration of cyclopamine, inhibition of Smoothened was incomplete.

Modulation of hedgehog signaling had an even greater effect on secondary mammosphere formation. Shh-treated primary mammospheres formed 100% more secondary mammospheres and the average cell numbers per secondary mammosphere were increased 67% (Fig. 2*A*). This stimulation could be reversed by addition of 300 nmol/L cyclopamine (Fig. 2*A*). Single cells from primary mammospheres treated with cyclopamine generated 54% less secondary mammospheres and the average cell numbers per secondary mammosphere were decreased 56% (Fig. 2*A*) compared with controls.

To show that Hh stimulated self-renewal of undifferentiated cells, we examined the differentiation potential of cells treated with Hh ligand. If the hedgehog pathway acts on primitive cells, then stimulation of this pathway should increase the number of primitive mammary cells capable of multilineage differentiation. To assess this, we plated mammosphere-derived cells at clonal density on collagen plates in the presence of FCS, conditions that we have previously determined promote cell differentiation (9). We used cytokeratin 14 as a marker of myoepithelial cells and cytokeratin 18 as marker of epithelial cells. Similar results were obtained with the markers ESA and CD10, respectively (data not shown). Addition of Shh resulted in a 3.5-fold increase in the number of multipotent cells, whereas cyclopamine decreased the number of these cells by 1.8-fold (Fig. 2*B*), demonstrating that Hh activation increased the generation of undifferentiated cells.

Because Ihh was the main hedgehog ligand expressed in the mammospheres as assayed by real-time quantitative RT-PCR, we also determined the effects of recombinant Ihh on the system. Consistent with the previously reported interchangeability of hedgehog ligands, Ihh had same effects as Shh on mammosphere

formation and production of multilineage progenitors (data not shown).

In contrast to the effect on mammospheres, addition of 3 μg/mL Shh or 300 nmol/L cyclopamine to mammary epithelial cells cultured on a collagen substratum had no effect on the proliferation of these cells (Fig. 2*C*). This suggests that the Hh pathway primarily affects undifferentiated cell proliferation.

Mammary stem cell self-renewal is regulated by Gli transcription factors. To determine whether the effects of Hh signaling on self renewal of stem cells and proliferation of progenitor cells was mediated by the Gli transcription factors, we infected mammosphere-initiating cells with retroviral vectors containing Gli1 or Gli2 and determined the effect of constitutive expression of these transcription factors on mammosphere formation.

A highly efficient retroviral expression system (19, 24) was used to generate Gli1-, Gli2-, and EGFP-expressing human mammospheres. We found that compared with uninfected controls or the EGFP-expressing cells, overexpression of Gli1 and Gli2 in mammary epithelial cells in primary suspension culture stimulated mammosphere formation by 49% and 66%, respectively (Fig. 2*D*). Furthermore, overexpression of Gli1 and Gli2 increased the number of cells per mammosphere by 77% and 100%, respectively (Fig. 2*D*). Thus, Gli1 or Gli2 overexpression recapitulates the effects of hedgehog activation in this system.

Hedgehog effects on mammary stem cell self-renewal are mediated by the polycomb gene *Bmi-1*. *Bmi-1* is a polycomb gene, which has recently been shown to play a role in the regulation of hematopoietic (25) and neural stem cell self-renewal (26). Interestingly, we found that Bmi-1 mRNA levels are increased ~3.5-fold in mammospheres compared with differentiated mammary cells (Fig. 3*A*). We hypothesized that Bmi-1 might function as a downstream target of the hedgehog pathway. To test this hypothesis we investigated the effect of hedgehog activation on Bmi-1 expression. We found that activation of the hedgehog pathway by addition of Shh resulted in a 6-fold increase in expression of Bmi-1 in mammospheres, an effect that was blocked by the hedgehog pathway specific inhibitor cyclopamine (Fig. 3*B*). Furthermore, both Gli1-overexpressing and Gli2-overexpressing mammospheres displayed a 6-fold higher Bmi-1 expression compared with control cultures (Fig. 3*B*). Together, these results suggest that Bmi-1 expression can be up-regulated by Hh signaling in human mammary stem/progenitor cells.

To test whether Bmi-1 plays a role in regulating mammary stem cell self-renewal, we infected mammosphere-initiating cells with lentiviral vectors containing Bmi-1. We found that compared with uninfected controls or GFP-expressing cells, overexpression of Bmi-1 stimulated mammosphere formation by 80% and increased the number of cells per mammosphere by 67% (Fig. 3*C*).

To provide further evidence that Hh effects on stem cell self-renewal are mediated by Bmi-1, we used siRNA delivered in a lentiviral vector tagged with GFP to down regulate Bmi-1 expression in mammospheres. Two different siRNA lentiviruses significantly reduced the Bmi-1 expression at both the mRNA (over 80% reduction) and protein levels (over 70% reduction; Supplementary Fig. S2). We used these vectors to examine the effect of down-regulation of Bmi-1 on mammosphere formation in the presence or absence of Hh activation. Down-regulation of Bmi-1 expression reduced primary and secondary mammosphere formation by 80% (Fig. 3*D*) and 70% (Fig. 3*D*), respectively; and reduced the primary and secondary mammosphere size by 60% (Fig. 3*D*) and 70% (Fig. 3*D*), respectively. Furthermore, the effects of Hh activation on both primary and secondary mammosphere formation were significantly reduced by Bmi-1 down-regulation (Fig. 3*D*, Supplementary Fig. S3). Taken together, these studies suggest that Hh effects on mammary stem/progenitor cells are mediated by the polycomb gene *Bmi-1*.

Effects of Gli2 and Bmi-1 expression on mammary development in humanized NOD-SCID xenotransplants. Because Hh signaling modulates both Glis and Bmi-1, we determined the effects of Gli and Bmi-1 expression on mammary development. This was accomplished using a modification of the model described recently by Kuperwasser et al. (12) in which irradiated and nonirradiated human mammary fibroblasts are implanted into the cleared fatpads of NOD-SCID mice to support the growth of normal human mammary epithelial cells. The cleared fatpads of 3-week-old NOD-SCID mice were humanized with telomerase immortalized human mammary fibroblasts. Subsequently, they were implanted with control mammospheres, mammospheres overexpressing Gli2, or mammospheres with Bmi-1 overexpression or Bmi-1 down-regulation. After 8 weeks, the mammary glands were removed and examined by histologic analysis. Dense human mammary stroma was apparent in the humanized NOD-SCID mouse fatpad that expressed GFP (data not shown). Control mammospheres (SIN-IP-EGFP, GFP alone) produced limited ductal growth in these areas (Fig. 4*A* and *C*). In contrast, both Gli2-overxpressing mammospheres (SIN-GLI2-EGFP) and Bmi-1-overxpressing mammospheres (hBmi-1-GFP) developed substantially more outgrowths (Fig. 4*B* and *D*) than control mammospheres. Furthermore, down-regulation of Bmi-1 expression in mammospheres by siRNAs inhibited the mammary development (Fig. 4*E*). Microscopic examination indicated that Gli2-transfected mammospheres but not control mammospheres produced ductal hyperplasia (Fig. 4*B*). Gli2-transfected mammospheres produced ductal hyperplasia in ~90% of ductal structures, and these were not detected with the implantation of control mammospheres. The human origin of these cells was confirmed by immunostaining with human specific antibodies, such as ESA and cytokeratins (data not shown). These results show that mammospheres can generate human ductal/alveolar structures when implanted into the humanized cleared fatpad of NOD-SCID mice. Furthermore, generation of these mammary outgrowths is modulated by the expression of Gli2 and Bmi-1.

The hedgehog pathway and Bmi-1 are activated in breast tumor-initiating cells. We have recently reported that human breast cancers are driven by a small subset of cancer stem cells that are characterized by the cell surface phenotype $CD44^+CD24^{-/low}lin^-$. These cells functionally resemble normal stem cells in that they are able to self-renew generating tumors in NOD-SCID mice, as well as to differentiate into nontumorigenic cells that form the bulk of tumors (21). To determine whether the Hh pathway is activated in these cells, we used flow cytometry to isolate $CD44^+CD24^{-/low}lineage^-$ cells from a metastatic human breast carcinoma xenografted in NOD-SCID mice. CD44 is an adhesion receptor for extracellular matrix ligands, such as hyaluronic acid, whose expression has been linked to aggressive behavior and tumor metastasis (27). CD24 may regulate cell adhesion by down-regulation of CXCR4 an important receptor in stem cell homing and tumor metastasis (28). Mouse cells are eliminated in these studies by eliminating H2K-positive cells. Lin^- cells were depleted of cells displaying mammary differentiation antigens using a cocktail of monoclonal antibodies as previously described (21). The levels of

F5 mRNAs for Hh pathway components and Bmi-1 were measured by qPCR. As indicated in Fig. 5*A*, $CD44^{+}CD24^{-/low}lin^{-}$ cells displayed increased expression of Hh pathway components PTCH1, Gli1, and Gli2 by ~1.7-fold, 30-fold, and 6-fold, respectively, as well as 5-fold increase in Bmi-1 compared with the cells isolated from the same tumor, which lacked these cancer stem cell markers (Fig. 5).

Discussion

There is increasing evidence that stem cells or their immediate progeny may be the targets of transformation during carcinogenesis. Carcinomas are believed to arise through a series of mutations that occur over many years. Adult stem cells are slowly dividing long-lived cells, which by their very nature are exposed to

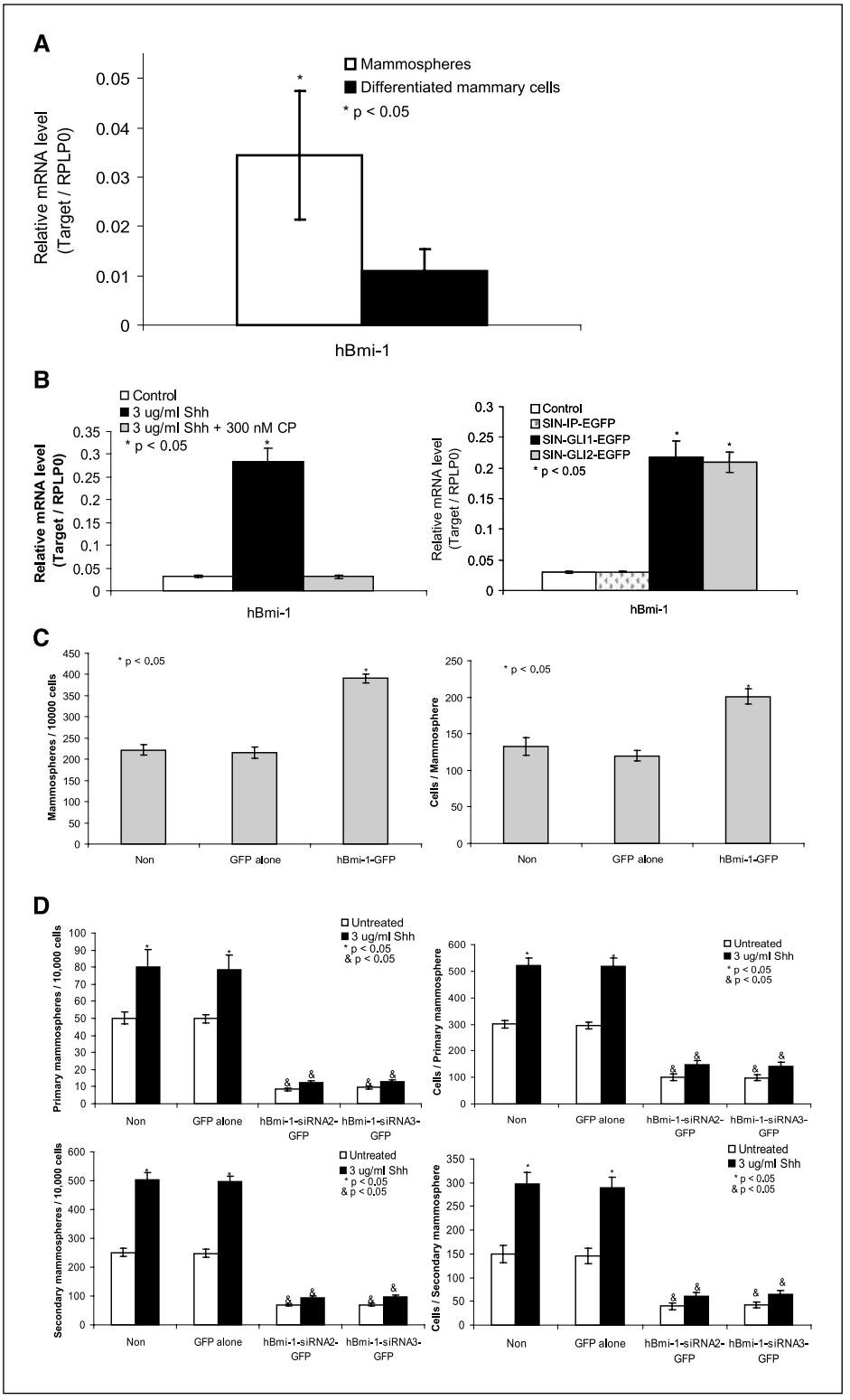

Figure 3. Hh effects on mammospheres are mediated by hBmi-1. *Columns,* mean; *bars,* SD. *, $P < 0.05$; &, $P < 0.05$, statistically significant differences from the control group or untreated group, respectively. *A,* hBmi-1 mRNA level in mammospheres compared with that in differentiated mammary cells. *B,* effects of hedgehog signaling on hBmi-1 mRNA level. *C,* effects of Bmi-1 overexpression on mammosphere number and size. *D,* effects of Bmi-1 knockdown on mammosphere number and size. Primary mammospheres were infected with the control virus (GFP alone) or siRNA lentiviruses (hBmi-1-siRNA1-GFP, hBmi-1-siRNA2-GFP, and hBmi-1-siRNA3-GFP), or uninfected (*Non*) as the control, and cultured in suspension in the absence (untreated) or presence of 3 μg/mL Shh for 7 days. The total number of mammospheres formed from 10,000 single cells and the total cells per mammosphere were counted. The single cells dissociated from each group of primary mammospheres were grown as secondary mammospheres in suspension for 7 to 10 days without treatment.

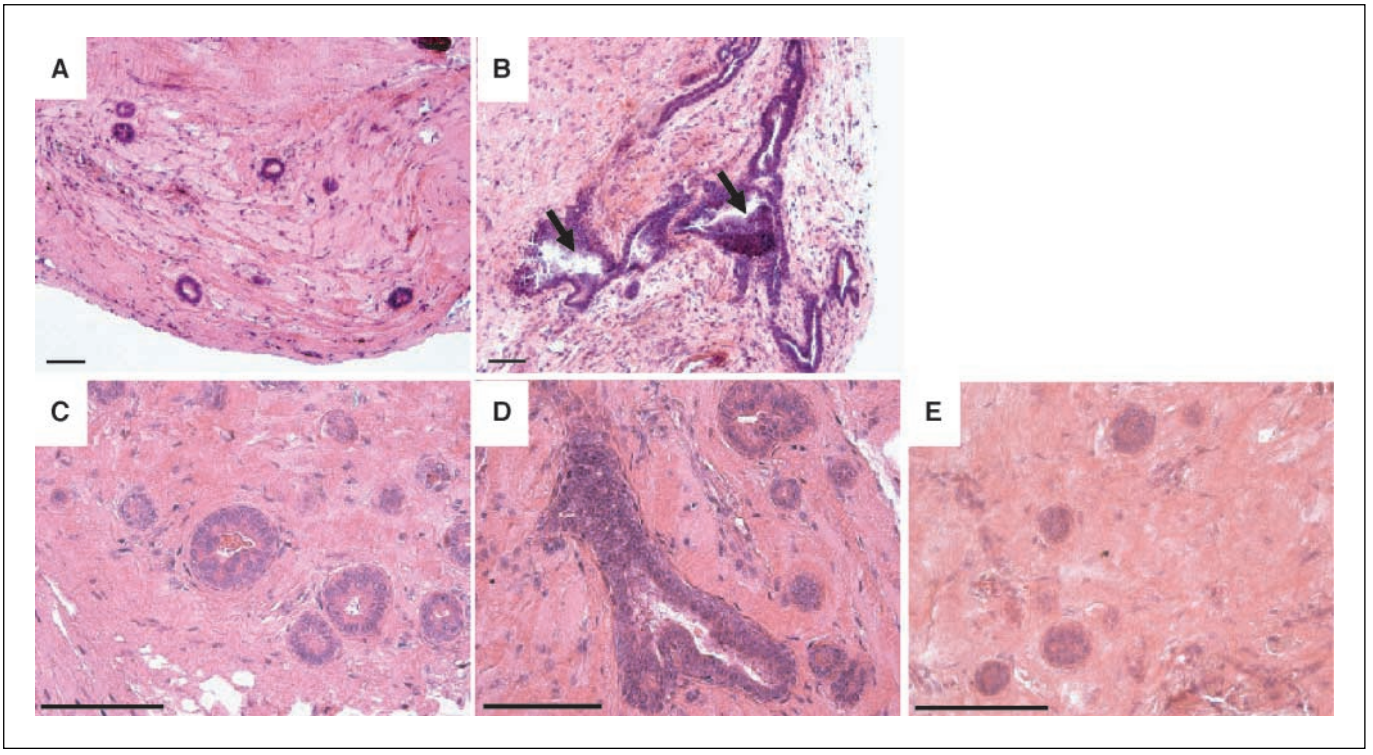

Figure 4. Gli2 and Bmi-1 moderates mammary gland development in humanized NOD-SCID mice. H&E staining of sections through the mouse fatpads for SIN-IP-EGFP control virus–infected mammosphere xenograft outgrowth (*A*), SIN-GLI2-EGFP virus–infected mammosphere xenograft outgrowth (*B*), GFP alone control virus–infected mammosphere xenograft outgrowth (*C*), hBmi-1-GFP virus–infected mammosphere xenograft outgrowth (*D*), and hBmi-1-siRNA-GFP virus–infected mammosphere xenograft outgrowth (*E*). *Arrow,* ductal hyperplasia. Bar, 100 μm.

damaging agents over long periods of time. Therefore, they may accumulate mutations that result in transformation (1). Stem cells are characterized by their ability to undergo self-renewal divisions, as well as to differentiate into cell lineages that form adult organs. The property of self-renewal in which a stem cell can produce one or two exact copies of itself is a property that is unique to stem cells. The development of *in vitro* culture system that maintain human mammary stem and progenitor cells in an undifferentiated state as well as NOD-SCID mouse models has permitted a more direct analysis of these pathways in normal and tumorigenic mammary stem and progenitor cells. We have shown that components of Hh signaling (PTCH1, Gli1, and Gli2) are highly expressed in normal mammary stem/progenitor cells compared with differentiated cells on a collagen substratum. Furthermore, we show that activation of this pathway with Hh ligands promotes the self-renewal of mammary stem cells, as evidenced by increased number of mammosphere-initiating cells. This effect was blocked by cyclopamine, a specific inhibitor of this pathway. Hh activation also increases the proliferation of mammary progenitor cells as reflected by increased mammosphere size.

We have used this system to investigate the downstream targets of Hh signaling responsible for mediating these effects. Addition of Hh ligands increases the expression of the transcription factors Gli1 and Gli2, which was inhibited by cyclopamine. Because Gli1 and Gli2 are positive mediators of Hh signaling, whereas Gli3 functions as a negative regulator of this pathway (29, 30), we focused on Gli1 and Gli2 in the current studies. Forced over-expression of Gli1 or Gli2 in mammosphere-initiating cells by

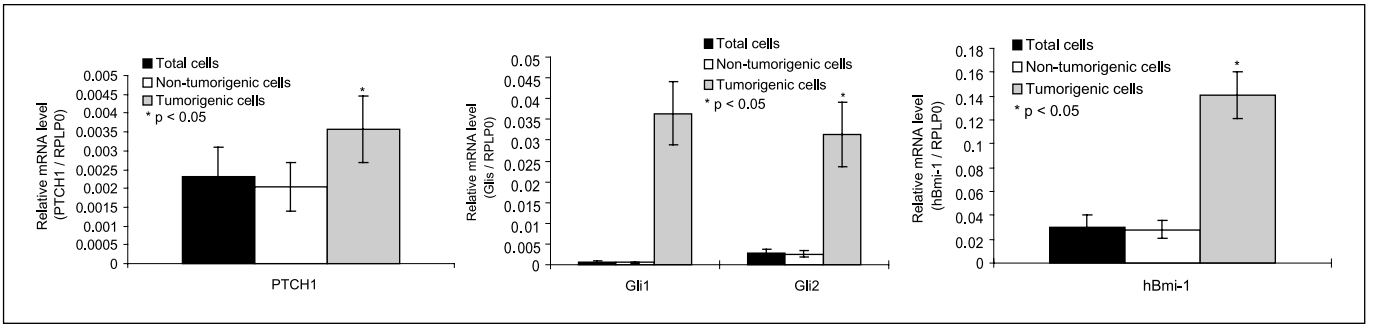

Figure 5. Activation of hedgehog signaling in breast tumor-initiating cells. Hh signaling components and Bmi-1 expression in breast tumor stem cells. Tumor cells were isolated from the mouse xenografts, both $CD44^+CD24^{-/low}lin^-$ population (tumorigenic stem cells) and $CD44^+CD24^+lin^-$ (nontumorigenic cells) were sorted by flow cytometry. Total RNA was isolated and mRNA for Hh components and Bmi-1 were quantitated by real-time RT-PCR. *Columns,* mean; *bars,* SD. *, $P < 0.05$, statistically significant differences from the control group.

retroviral transduction recapulated the effects of Hh ligands. These effects were unaffected by cyclopamine, an observation consistent with previous reports that Gli1 and Gli2 act downstream of smoothened, the target of cyclopamine (23).

It has recently been reported that the polycomb gene *Bmi-1* plays an important role in the regulation of self-renewal of hematopoitic (25) and neuronal stem cells (26). Bmi-1 is a transcriptional repressor that may regulate stem cell self-renewal through the repression of important cell cycle regulatory genes in the INK-4A/ADP ribosylation factor (ARF) complex, P16 INK-4A, and P19 ARF (25, 26). These studies have recently been confirmed and extended using mouse knockout models of INK-4A and P19 ARF (31). We have shown that Bmi-1 is expressed at increased levels in undifferentiated compared with differentiated mammary cells. Activation of Hh signaling increases Bmi-1 expression, and Bmi-1 overexpression promotes mammary stem cell self-renewal and proliferation as indicated by an increase in mammosphere number and size *in vitro* and increases ductal/alveolar development in humanized NOD-SCID mammary fatpads. In contrast, down-regulation of Bmi-1 using siRNA abrogates the effects of Hh signaling on mammosphere formation *in vitro* and inhibited ductal/alveolar development in NOD-SCID mice. These studies suggest that the effects of Hh signaling on mammary stem cell self-renewal may be mediated by Bmi-1.

We have previously proposed that deregulation of self-renewal may be one of the key events involved in the initial stages of carcinogenesis (1). Activation of the Hh signaling pathway as well as Bmi-1 has been shown to result in the generation of mammary carcinomas *in vitro* or in transgenic models (32, 33). PTCH mutations have been found in a subset of human breast cancers (33). A specific mutation in PTCH1 was linked to increased risk of breast cancers with oral contraceptives (34). Hh signaling was also shown to be activated in a subset of human cancers based on immunohistochemical staining of a set of 52 invasive breast cancers (35). We have found that overexpression of the Hh target Gli2 in mammospheres produces ductal hyperplasias when these cells are implanted into the humanized fatpads of NOD-SCID mice. These findings are consistent with a stem cell model of carcinogenesis in which early events involve deregulation of Hh signaling resulting in clonal expansion of stem or progenitor cells. These cells in turn may undergo further mutation to acquire a fully malignant phenotype.

We have recently described the existence of a cancer stem cell population in human breast cancers (21). In the present study, we show that these cancer stem cells display activation of Hh signaling components as well as increased expression of Bmi-1.

Taken together, these studies lend support to the cancer stem cell hypothesis in which carcinogenesis results from deregulation of self-renewal pathways in normal stem cells generating a cancer stem cell population that drives tumorigenesis. In normal mammary development, Hh and the downstream transcription factor Bmi-1 play an important role in regulating stem cell self-renewal. These processes are tightly regulated by factors in the stem cell niche. Deregulation of these processes during carcinogenesis may result in stem cell expansion, a key event in carcinogenesis. A hypothetical model depicting the role of Hh and Bmi-1 in the regulation of mammary stem cell self-renewal and deregulation of this pathway in cancer stem cells is shown in Fig. 6. F6
The clinical importance of this is highlighted by a recent report demonstrating a strong correlation between the expression of an 11-gene Bmi-1 stem cell signature and poor prognosis in patients with a wide variety of malignancies (36). Recently, inhibitors of Hh signaling, such as cyclopamine and related compounds, have been shown to have antitumor activity with minimal systemic toxicity in mouse tumor models (37, 38). Our studies highlight the importance of the Hh signaling pathway and Bmi-1 in the regulation of normal

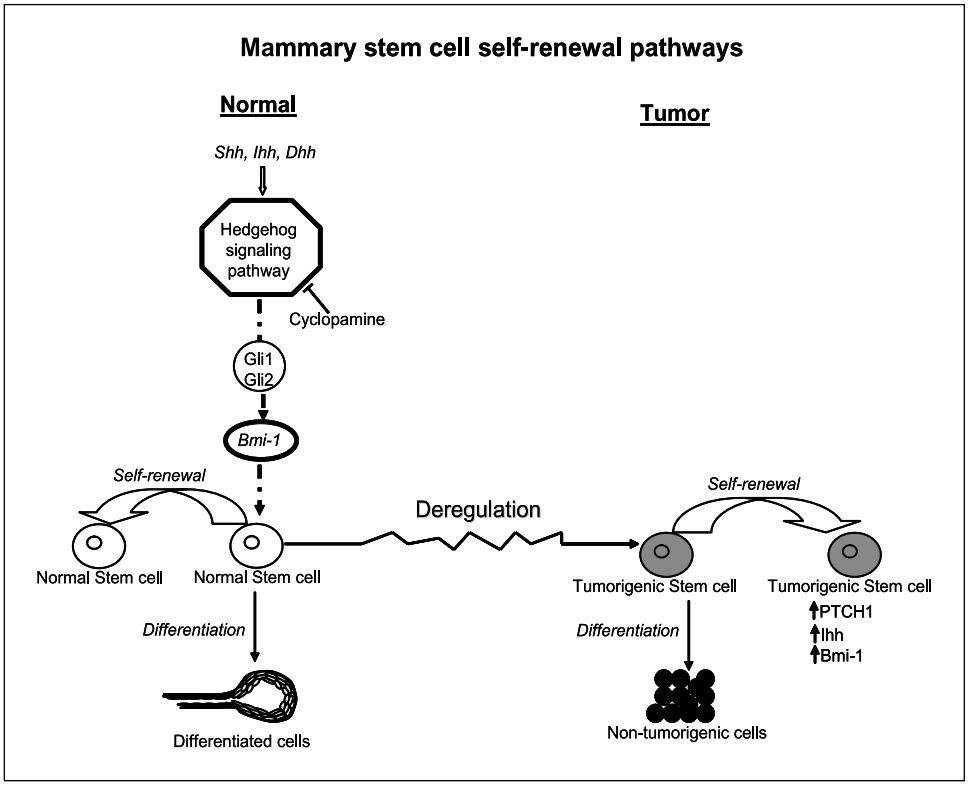

Figure 6. Hypothetical model depicting mammary stem cell self-renewal pathways in normal and cancer stem cells. *Arrows,* activation of the pathways by ligands; cyclopamine is an inhibitor of hedgehog signaling.

and malignant stem cell self-renewal and suggest that strategies aimed at inhibiting these pathways represent a rationale therapeutic approach to target cancer stem cells.

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Cancer Stem Cells: An Old Idea—A Paradigm Shift

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Abstract

Although the concept that cancers arise from "stem cells" or "germ cells" was first proposed ~150 years ago, it is only recently that advances in stem cell biology have given new impetus to the "cancer stem cell hypothesis." Two important related concepts of this hypothesis are that (*a*) tumors originate in either tissue stem cells or their immediate progeny through dysregulation of the normally tightly regulated process of self-renewal. As a result of this, (*b*) tumors contain a cellular subcomponent that retains key stem cell properties. These properties include self-renewal, which drives tumorigenesis, and differentiation albeit aberrant that contributes to cellular heterogeneity. Recent experimental evidence in a variety of tumors has lent strong support to the cancer stem cell hypothesis that represents a paradigm shift in our understanding of carcinogenesis and tumor cell biology. This hypothesis has fundamental implications for cancer risk assessment, early detection, prognostication, and prevention. Furthermore, the current development of cancer therapeutics based on tumor regression may have produced agents that kill differentiated tumor cells while sparing the rare cancer stem cell population. The development of more effective cancer therapies may thus require targeting this important cell population. (Cancer Res 2006; 66(4): 1-8)

Introduction

In a thought-provoking article published in *Fortune* in 2004, Leaf, a cancer survivor, posses the question, "Are we losing the war on cancer?" (1). In this article, he reviews data on the progress made since the "war on cancer" was declared in 1961. Over this time, there have clearly been dramatic advances in the treatment of such diseases as childhood leukemia, Hodgkin's disease, and testicular cancer. Furthermore, the overall mortality for some of the common epithelial malignances, such as breast cancer and prostate cancer, have been declining recently largely due to advances in early detection and prevention. However, as Leaf points out for the four most common epithelial malignancies (lung, breast, prostate, and colon cancers), the survival of patients with metastatic disease has not changed significantly over the past several decades. Despite these statistics, there is considerable optimism in the cancer research community that new targeted therapies will significantly improve on the results of empiric-based therapeutics. The ability to specifically target pathways deranged in cancer raises the hope of developing therapies with enhanced specificity and decreased toxicity. However, as our ability to attack specific targets increases, a fundamental question remains, "Are we targeting the right cells"? Evidence is accumulating that most, if not all, malignancies are driven by "a cancer stem cell compartment." Furthermore, these cancer stem cells may be inherently resistant to our current therapeutic approaches. The cancer stem cell hypothesis has fundamental implications for understanding the biology of carcinogenesis as well as for developing new strategies for cancer prevention as well as new therapies for advanced disease. In this commentary, we will discuss the cancer stem cell hypothesis, including recent evidence supporting its validity, and the implications of this model for cancer prevention and therapy.

The Cancer Stem Cell Hypothesis

All tissues in the body are derived from organ-specific stem cells that are defined by their capacity to undergo self-renewal as well as to differentiate into the cell types that comprise each organ. These tissue-specific stem cells are distinguished from embryonic stem cells in that their differentiation is largely restricted to cell types within a particular organ. The cancer stem cell hypothesis has two separate but related components. The first component concerns the cellular origin of tumors, including the question of whether tumors arise from tissue stem cells. A second related component of this hypothesis is that tumors are driven by cellular components that display "stem cell properties." The concept that cancer might arise from a rare population of cells with stem cell properties was proposed ~150 years ago (2–5). Over 40 years ago, it was postulated that tissue-specific stem cells may be the cell of origin of cancer (6). Over 30 years ago, Pierce (7) proposed that cancers represented a maturation arrest of stem cells. The concept that tumors contain cell populations with stem cell properties was also suggested by *in vitro* "clonogenic assays" that showed subpopulations of tumor cells with increased proliferative capacity as shown by colony formation in *in vitro* assays using cells isolated from tumor specimens (8). A major limitation of these studies, however, was that they measured *in vitro* proliferation rather than true self-renewal. In addition, it has been observed that the production of human tumor xenografts in animal models required a relatively large number of cells. However, it was unclear whether this was due to the inefficiency of these cells in promoting tumor growth or to the existence of rare subpopulations within a tumor that were uniquely tumorigenic in these systems.

Evidence supporting the cancer stem cell hypothesis has gained impetus due to recent advances in stem cell biology and the development of new animal models to measure self-renewal and more directly test the validity of this hypothesis. The concept that cancers arise from the transformation of stem cells is appealing for several reasons. Stem cells by their long-lived nature are subject to the accumulation of multiple mutations that are required for carcinogenesis. For example, women exposed to atomic bomb radiation in Hiroshima and Nagasaki developed breast cancer ~20 to 30 years after exposure (9). Mutations found in these women's breast cancers are consistent with those known to be induced by radiation (9). Furthermore, women exposed to radiation during

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late adolescents had the highest susceptibility to breast cancer development. This is thought to be the period when the mammary gland has the highest number of stem cells (10). Further evidence that stem cells may play a role in carcinogenesis is the observation that normal stem cells and cancer cells share several important properties. These include (*a*) the capacity for self-renewal, (*b*) the ability to differentiate, (*c*) active telomerase expression, (*d*) activation of antiapoptotic pathways, (*e*) increased membrane transporter activity, and (*f*) the ability to migrate and metastasize. Indeed, properties, such as anchorage independence, which have been thought to be a hallmark of transformed cells, have recently been described by us and others as a property of normal tissue stem cells (11–13). One of the key early events in transformation may be the dysregulation of the normally highly regulated process of self-renewal. Stem cells are the only cells capable of undergoing self-renewal divisions. In the steady state, these divisions are asymmetric in which a stem cell is able to produce an exact copy of itself as well as a daughter cell that undergoes differentiation into the lineages found in differentiated tissues. During stem cell expansion and tumorigenesis, stem cells may undergo symmetric divisions in which stem cells produce two identical stem cell progeny, thus allowing for stem cell expansion (ref. 14; Fig. 1). F1 During normal development, stem cell self-renewal is regulated by signals from the surrounding stem cell "niche." As has been elegantly shown in bone marrow transplantation models, a single hematopoietic stem cell introduced into a lethally irradiated mouse is able to repopulate the stem cell compartment resulting in reconstitution of the entire hematopoietic system. Extensive expansion in the stem cell population stops when this pool is replenished, illustrating the tight control of this process. We and others have hypothesized that deregulation of this self-renewal process leading to stem cell expansion may be a key early event in carcinogenesis. Recently, the pathways that regulate the self-renewal of normal stem cells, including Wnt, Notch, and Hedgehog, have begun to be elucidated. These signaling pathways have been implicated in regulating the self-renewal of hematopoietic, neuronal, and mammary stem cells (14, 15). The dysregulation of each of these pathways in rodent models leads to tumorigenesis. Furthermore, there is substantial evidence that dysregulation of these pathways also plays an important role in human carcinogenesis. Defects in the Wnt signaling pathway are seen early in colon cancer carcinogenesis. Alterations in Hedgehog signaling were first shown in human basal carcinomas of the skin (16). More recently, evidence for dysregulation of this pathway has been reported in human pancreatic, gastric, prostate, and breast carcinomas (17, 18). Alterations in Notch signaling have been observed in human T-cell acute lymphoblastic leukemia, cervical cancer, and breast cancer (19–23).

Recent studies have suggested that tumors may arise from progenitor cells and tissue stem cells. Transformation of these cells may require that they acquire the stem cell property of self-renewal. In support of this hypothesis, Jamieson et al. showed that chronic myelogenous leukemia (CML) blast crisis may originate in hematopoietic progenitor cells as a consequence of dysregulated Wnt signaling, allowing these cells to self-renew, a property normally restricted to hematopoietic stem cells (24). Similarly, by transfecting purified populations of hematopoietic progenitor cells, Kelly and Gilliland showed that AML-ETO may induce transformation of myeloid progenitor cells enabling them to acquire the property of self-renewal (25). We have recently proposed that human breast cancers may arise from the transformation of either mammary stem cells or early progenitor cells resulting in production of breast cancers with distinct molecular and clinical phenotypes (26). This concept is also consistent with recent descriptions in transgenic mouse models of mammary tumorigenesis, which suggest that distinct oncogenes may affect different stem and progenitor cells resulting in phenotypic differences in mammary tumors (27).

The second major component of the cancer stem cell hypothesis is that tumors contain and are "driven" by cellular components that display stem cell properties. This concept has gained substantial experimental support recently with the development of animal models that have permitted the direct assessment of stem cell properties of tumor cell subpopulations. These models have shown that prospectively identifiable subpopulations of tumor cells display the defining stem cell properties of self-renewal and differentiation. Self-renewal drives tumorigenesis, whereas differentiation (albeit aberrant in tumors) contributes to tumor phenotypic heterogeneity. In 1997, Dick et al. showed that the ability to transfer human leukemias into nonobese diabetic/severe combined immunodeficient (NOD/SCID) mice was retained in a small population of cancer stem cells that like their normal counterparts displayed the cell surface phenotype $CD34^{+}CD38^{-}$. These cells that comprised <1 in 10,000 leukemia cells could transfer the human leukemia into NOD/SCID mice, whereas the introduction of thousands of fold higher numbers of cells that did not bear this phenotype were nontumorigenic. Furthermore, the leukemias that were produced recapitulated the histologic phenotype found in the original tumor (28). More recently, this group used cellular marking studies to show that leukemic stem cells like their normal counterparts are heterogeneous with varying degrees of self-renewal potential. These findings suggest that leukemic stem cells, like their normal counterparts, exist in a hierarchy that is developmentally regulated. This supports the hypothesis that leukemic stem cells originate from the transformation of normal hematopoietic stem cells (29). Using a similar approach, in collaboration with Al-Hajj et al., we showed that human breast cancers contain a cell population characterized by the expression of the cell surface markers $CD44^{+}$ $CD24^{low}$ ^{lin} that have stem cell characteristics. As few as 200 of these cells, which comprise between 1% and 10% of the total cell population, are able to form tumors when implanted in NOD/SCID mice (30). In contrast, 20,000 cells isolated from the same tumor that do not display this cell surface phenotype are unable to form tumors. Furthermore, consistent with a stem cell model, cancer stem cells are able to generate tumors that recapitulate the phenotypic heterogeneity found in the initial tumor.

Confirming and extending our findings, Ponti et al. recently reported that, in addition to being tumorigenic, $CD44^{+}CD24^{-}$ human breast cancer cells form tumor mammospheres *in vitro*, a property that we described previously for normal mammary stem/progenitor cells (31, 32). Furthermore, the stem cell phenotype of these tumor cells was suggested by their expression of the stem cell markers Oct-4 as well as by the absence of Cx43 expression. Interestingly, these cells also produced vascular endothelial growth factor (VEGF) and were highly angiogenic (31). Lending further support to the cancer stem cell hypothesis and extending its generality, three groups have independently shown the existence of a cancer stem cell compartment in human brain tumors. These cancer stem cells, like their normal counterparts, are able to form neurospheres *in vitro* and express the neural stem cell markers CD133 and nestin. Furthermore, as few as 100 of these cells were

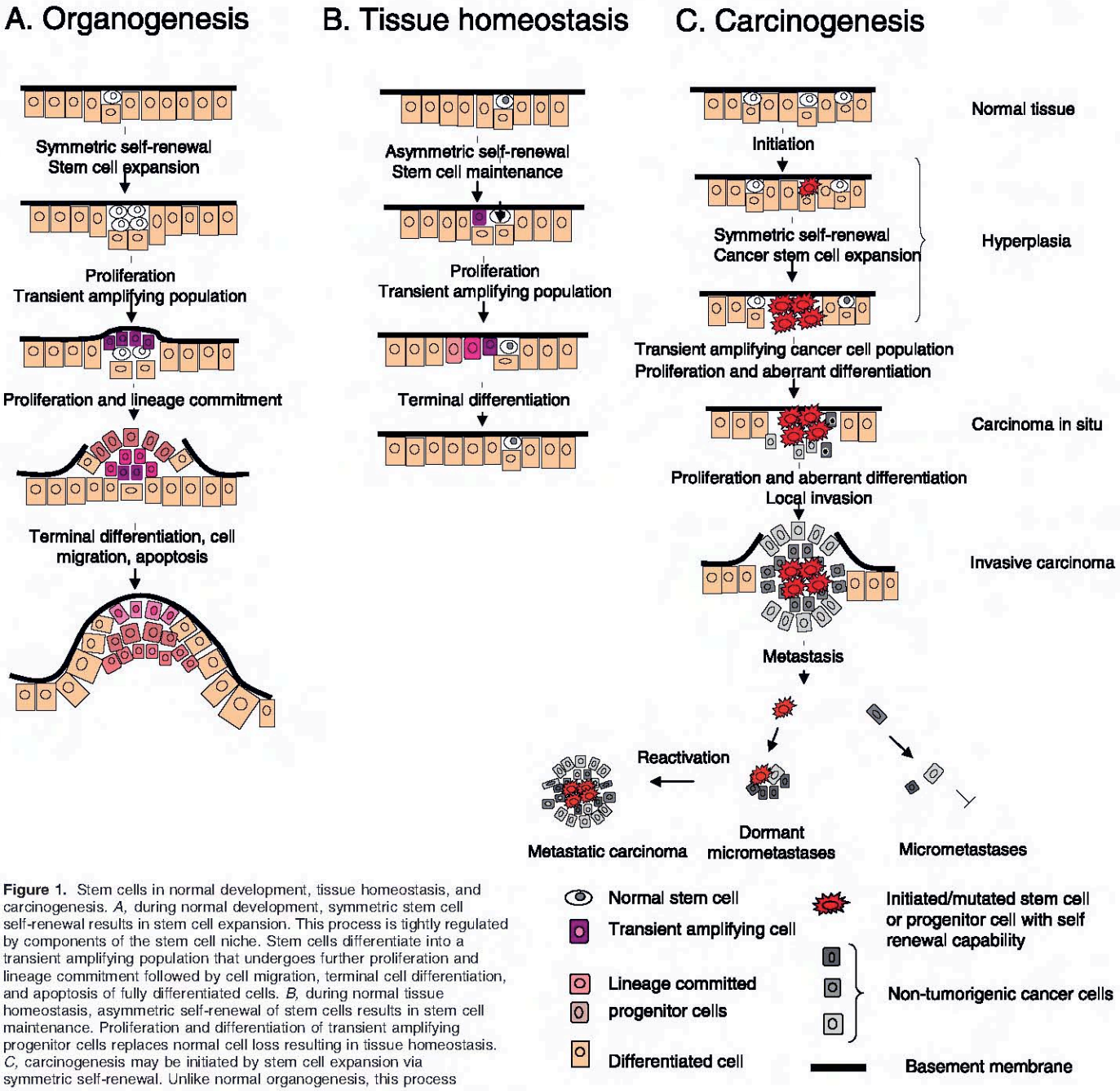

Figure 1. Stem cells in normal development, tissue homeostasis, and carcinogenesis. *A*, during normal development, symmetric stem cell self-renewal results in stem cell expansion. This process is tightly regulated by components of the stem cell niche. Stem cells differentiate into a transient amplifying population that undergoes further proliferation and lineage commitment followed by cell migration, terminal cell differentiation, and apoptosis of fully differentiated cells. *B*, during normal tissue homeostasis, asymmetric self-renewal of stem cells results in stem cell maintenance. Proliferation and differentiation of transient amplifying progenitor cells replaces normal cell loss resulting in tissue homeostasis. *C*, carcinogenesis may be initiated by stem cell expansion via symmetric self-renewal. Unlike normal organogenesis, this process is dysregulated resulting in cancer stem cell expansion. Aberrant differentiation of these cells generates tumor heterogeneity. Further mutations or epigenetic changes may accompany tumor invasion and metastasis. Metastases require the dissemination of cancer stem cells that may remain dormant and be reactivated resulting in tumor recurrence. In contrast, dissemination of differentiated tumor cells produces only micrometastasis that do not progress.

Legend

Q2

able to transfer the tumors when injected intracranially into NOD/SCID mice (33, 34). In contrast, 10^5 CD133 cells engrafted but did not produce a tumor. The tumors produced by the CD133$^+$ cells recapitulated the phenotypic heterogeneity found in the initial tumor (33). Evidence for existence of a clonogenic subpopulation of cells in human multiple myeloma was recently reported by Matsui et al. (35). Multiple myeloma cells express syndecan-1 (CD138). However, a small subpopulation resembling postgerminal center B cells were CD138 . Only the CD138 cells were clonogenic *in vitro* and in NOD/SCID mice (35). In the prostate, Xin et al. showed that stem cell antigen-1 (Sca-1) enriches for a prostate regenerating cell in mouse model and genetic perturbations of PTEN/AKT produced prostate cancer associated with a dramatic increase in Sca-1$^+$ cells (36). Further evidence for the existence of a cell population with stem cell properties in prostate cancer has been reported by Richardson et al. They found that

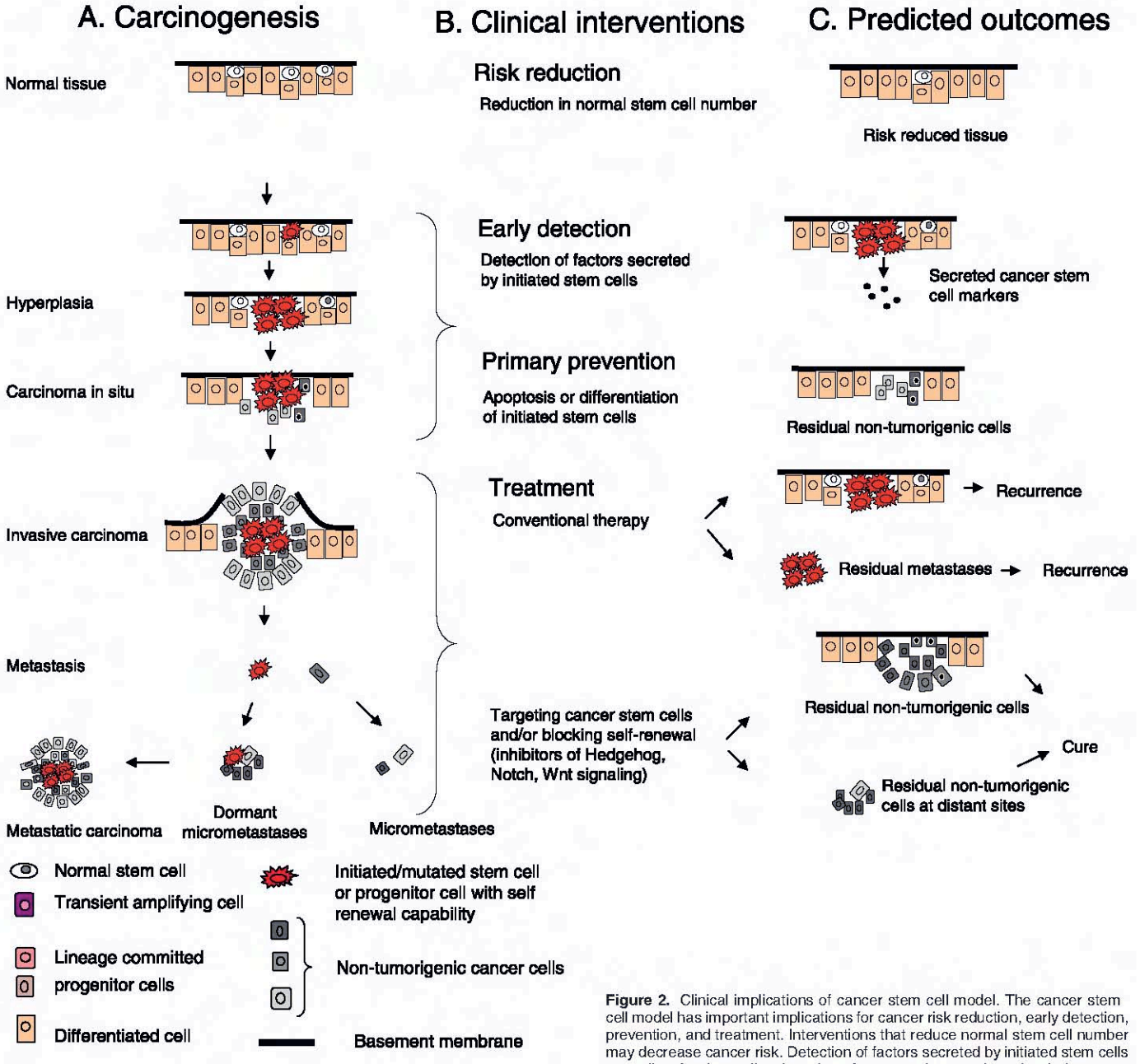

Figure 2. Clinical implications of cancer stem cell model. The cancer stem cell model has important implications for cancer risk reduction, early detection, prevention, and treatment. Interventions that reduce normal stem cell number may decrease cancer risk. Detection of factors secreted by initiated stem cells may allow for the earlier detection of cancers. Interventions that induce apoptosis or differentiation of initiated stem cells may be effective in cancer prevention. Conventional cancer therapies, including cytotoxic agents, selectively destroy differentiated cancer cells, sparing the cancer stem cell compartment resulting in cancer recurrence at primary or metastatic sites. Therapies that selectively eliminate cancer stem cells leave residual nontumorigenic cells resulting in potential cancer cures.

normal human prostate stem cells expressed CD133 (37). Furthermore, they identified a subpopulation of cells in human prostate cancer characterized as $CD44^+/\alpha_2\beta_1^{hi}/CD133^+$ with stem cell properties. As few as 500 cells with this phenotype that constituted 0.1% of total tumor cells formed tumors in NOD/SCID mice, whereas 5×10^5 CD44 cells failed to form tumors (38). Evidence for the existence of cancer stem cells in lung cancer has recently been presented by Kim et al. (39). They identified bronchial alveolar stem cells present at the bronchial alveolar duct junction. These cells exhibited the stem cell properties of self-renewal and multilineage differentiation. These stem cells could be transformed by K-ras *in vitro* and could form tumor in mice (39).

From these studies, it seems that several stem cell markers may be shared by cancer stem cells in multiple tumor types. These include CD44, α_6 integrin, β_1 integrin, and CD133 (Prominin). These cancer stem cells may not only share molecular markers but also display dysregulation of similar self-renewal pathways, such as Wnt, Hedgehog, and Notch. In support of this concept, we have recently found that Hedgehog signaling may regulate the self-renewal of normal mammary stem cells and that this pathway is

FN1 dysregulated in mammary cancer stem cells.[1] Taken together, these studies suggest that most, if not all, tumors contain a subpopulation of cells that display cancer stem cell characteristics. The relationship between normal and cancer stem cells is depicted graphically in Fig. 1.

Implications of the Cancer Stem Cell Hypothesis: A Paradigm Shift in Thinking about Carcinogenesis and Our Approach to Cancer Prevention and Therapy

Models of carcinogenesis. "Stochastic models" of carcinogenesis hold that transformation results from random mutation and subsequent clonal selection. In this model, any cell may be the target of carcinogenesis. The stem cell model of carcinogenesis, in contrast, suggests that cancers originate in tissue stem or progenitor cells probably through dysregulation of self-renewal pathways. This leads to expansion of this cell population that then may undergo further genetic or epigenetic changes to become fully transformed. In addition, epigenetic changes normally involved in cell differentiation contribute to the cellular phenotypic heterogeneity found in tumors. This model represents a paradigm shift in our thinking and has fundamental consequences for understanding the biology of carcinogenesis as well as important clinical implications for early detection, prevention, and therapy of human malignancies. These implications are summarized in Fig. 2. F2

Biological implications. The cancer stem cell hypothesis has important biological implications for the development of animal models of carcinogenesis as well as for understanding key biological processes, such as stromal-epithelial interactions and metastasis. Although there has been considerable progress in the development of mouse models of human cancer, in many cases, these models fail to recapitulate human disease. Many transgenic models use tissue-specific promoters to drive oncogene expression. However, these tissue-specific genes may be expressed only in differentiated cells. If stem cells or their immediate progeny are the true targets of transforming events, then the expression of oncogenes in more differentiated cells may fail to recapitulate actual carcinogenic processes. There is recent evidence that the expression of oncogenes in primitive cells using direct transfection technologies results in a fundamentally different phenotype than expression of the same genes driven by tissue-specific promoters. Welm et al. showed that expression of c-Met and c-Myc driven by the mammary-specific promoter mouse mammary tumor virus fails to produce carcinomas, whereas these genes transduced into primitive cells via a stem cell virus produced mammary carcinomas (40). Kim et al. have developed an animal model that targets normal lung stem cells to produce adenocarcinomas that resemble those found in human lung cancers (39).

The concept of the normal stem cell niche has direct relevance to understanding stromal epithelial interactions that occur during tumorigenesis in addition to understanding such complex processes as tumor metastases. For example, homing receptors found on normal hematopoietic stem cells, such as the cytokine receptor CXCR4, have been shown to play an important role in promoting metastases in a variety of tumors, including human breast and prostate carcinomas (41, 42). Recently, carcinoma-associated fibroblasts were shown to promote angiogenesis, in addition to tumor growth, by secreting SDF-1 that interacts with CXCR4 expressed by tumor cells and endothelial cells (43). In addition, properties, such as induction of angiogenesis, may be inherent in normal stem cells as well as in their transformed counterparts. Supporting this idea, we have recently found that Hedgehog signaling regulates the production VEGF by normal human mammary stem and progenitor cells as well as breast cancer stem cells.[2] The clinical course of micrometastases may also reflect stem cell characteristics of disseminated cells. Up to 30% of women with newly diagnosed breast cancer and men with prostate cancer exhibit micrometastases in their bone marrow as determined by immunochemical staining. However, after 10 years, up to 50% of these have patients have not developed clinically relevant macroscopic disease (44). A potential explanation for this is that although either stem cells or their more differentiated progeny may be capable of forming micrometastases, only stem cells have the self-renewal capacity to create a clinically relevant macroscopic metastases. In addition, the concept of "tumor dormancy" may directly relate to stem cell biology. Stem cells usually exist in a quiescent G_0 state and self-renew only when they receive appropriate signals from their niche environment. By analogy, cancer stem cells may remain dormant at metastatic sites until they are activated by the appropriate signals from the microenvironment. FN2

Implications for cancer risk assessment, early detection, molecular profiling, and prevention. The cancer stem cell model has important implications for many aspects of cancer risk assessment and prevention. If cancer stem cells or their immediate progeny are the targets for transformation, then cancer risk may be directly related to the number of stem cell targets. Pathways that influence target number may thus influence cancer risk. For example, it has been suggested that a previously unrecognized function of the hereditary breast cancer gene BRCA1 may be in the regulation of normal breast stem cell function (45). An important regulator of stem cell self-renewal of both normal and transformed stem cells is the polycomb gene Bmi-1 (46–48). It has recently been shown that Bmi-1 induced down-regulation of P-16 plays an important role in the regulation of hematopoietic and neuronal stem cell self-renewal (47, 49). Interestingly, recent studies by Holst et al. have suggested that one of the earliest events in carcinogenesis of the breast may be the silencing of P-16 expression by gene methylation (50). Together, these studies suggest that Bmi-1 may regulate normal stem cell self-renewal through down-regulation of P-16. During carcinogenesis, the silencing of this gene through methylation may result in the constitutive expansion of the stem cell population. In a similar manner, dysregulation of Wnt signaling may allow for the expansion of colonic stem cells during early colon cancer carcinogenesis.

The stem cell model also has important implications for the development of markers for the early detection of cancer. Most currently used tumor markers, such as prostate-specific antigen for prostate cancer or CA125 for ovarian cancer, are the products of differentiated cells within tumors. If tumors are to be detected during earlier stages of carcinogenesis, it may be necessary to characterize and detect markers made by the cancer stem cell populations. There has been considerable excitement generated by studies that show that important clinical prognostic and predictive

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[2] In preparation. Q3

information can be obtained from determining the molecular expression profile of tumors. This is consistent with the hypothesis that these molecular profiles represent the cell of origin as well as the differentiation pattern produced by subsequent oncogenic events. We have proposed previously that the molecular classifications of human breast cancers by gene expression analysis may reflect different cellular origins of these subtypes (51). If tumors are driven by a stem cell component, then elucidation of gene signatures characteristic of these stem cells may provide important prognostic information. In support of this, Glinsky et al. developed an 11-gene signature whose expression was regulated by the stem cell self-renewal gene Bmi-1. Remarkably, expression of this "stem cell gene" signature was associated with a poor prognosis for 10 different types of human malignancies (52). These studies summarized in an accompanying editorial, "Stem Cell-ness: A Magic Marker for Cancer" (53), provide strong evidence for the clinical relevance of the cancer stem cell hypothesis. Despite the important prognostic value of tumor profiling, the cancer stem cell hypothesis predicts that there will be considerably less value in using molecular profiling to identify new therapeutic targets. If cancer stem cells comprise only a minor fraction of total tumor cells and if these cells drive tumorigenesis, then the profiling of purified populations of cancer stem cells may identify more important therapeutic targets than profiling the entire tumor.

The cancer stem cell hypothesis suggests avenues for cancer prevention. If stem cells are the targets of transformation, then strategies that reduce stem cell number might reduce cancer risk. The use of tamoxifen in primary breast cancer prevention might occur through such a mechanism. Furthermore, if early events in carcinogenesis involve expansion of the stem cell pool, then interventions that induce either apoptosis or differentiation with a loss of self-renewal capacity in these cells represent a rational therapeutic approach to cancer prevention. Although the concept of differentiation therapy for cancer is not new (54), development of agents that can specifically target initiated stem cells may provide opportunities to intervene at the earliest stages of carcinogenesis before significant genetic instability occurs. This highlights the importance of elucidating the pathways that control differentiation and survival in these cells.

Implications for cancer therapeutics. The cancer stem cell model has fundamental implications for the development of new cancer therapeutic agents. Antineoplastic agents have largely been developed through testing in animal models as well as phase II human trials. In both of these, the measured outcome has been shrinkage of tumors. Tumor response is usually defined in the clinic as the shrinkage of a tumor by at least 50%. However, if cancer stem cells are inherently resistant to therapeutic agents and if these cells comprise only a minority of the tumor cell population, then shrinkage of tumors may reflect the effects of these agents on the differentiated cells in a tumor rather than the cancer stem cell component. This may explain why in clinical trials for advanced cancers, tumor regression often does not translate into clinically significant increases in patient survival. This has been shown in many tumor types, including solid tumors and multiple myeloma, where patient survival does not correlate with changes in the M-protein levels (55). If the cancer stem cell hypothesis is valid, then we may need to devise new experimental paradigms other than assessment of tumor regression for the evaluation of antineoplastic agents. To develop therapies that target the cancer stem cell population, it will be important to find and validate intermediate end points that predict ultimate patient survival. For instance, future clinical trial design may use such intermediate end points as time to tumor progression following delivery of an agent that can target cancer stem cells.

Therapeutic resistance of cancer stem cells. By virtue of their fundamental importance in organogenesis, normal stem cells have evolved mechanisms that promote their survival and resistance to apoptosis. For example, during normal mammary involution following lactation, there is massive apoptosis of differentiated cells, whereas stem cells are spared and regenerate the gland during subsequent pregnancies. Inherent resistance of normal stem cells to apoptosis is also observed in patients undergoing cytotoxic chemotherapy. When patients are given nonmyeloablative doses of cytotoxic chemotherapy, they experience a transient decrease in their WBC counts. This is caused by apoptosis of differentiated neutrophils and myeloid precursors. Stem cells in the bone marrow are not ablated by these doses of chemotherapy and are able to regenerate a normal hematopoietic system after several weeks. Similarly, many of the gastrointestinal side effects of chemotherapy are caused by induction of apoptosis in differentiating colonic epithelial cells. These dying cells are regenerated by gut stem cells that survive these chemotherapeutic insults. Just as normal stem cells may be resistant to the induction of apoptosis by cytotoxic agents and radiation therapy, cancer stem cells may display increased resistance to these agents compared with more differentiated cells that comprise the bulk of tumors. Supporting this concept, Guzman et al. have shown that leukemic stem cells are more resistant to chemotherapy than are the more differentiated myeloblastic cells that constitute the vast majority of cells in leukemia (56). Similarly, Matsui et al. have shown that myeloma stem cells are resistant to many therapies being used to treat myeloma including chemotherapy and the proteosome inhibitor Velcade (35, 57). There are several molecular mechanisms that may account for the resistance to apoptosis of cancer stem cells. These include (*a*) cell cycle kinetics. Many cancer stem cells are not cycling and are in G_0 and thus resistant to cell cycle–specific chemotherapy agents (58). (*b*) DNA replication and repair mechanisms. Stem cells may be resistant to DNA-damaging agents by virtue of being able to undergo asynchronous DNA synthesis in addition to displaying enhanced DNA repair (59–63). (*c*) During asynchronous DNA synthesis, the parental "immortal" DNA strand always segregates with the stem cell and not the differentiating progeny. This process may be regulated by P53 (64). This prevents the stem cell compartment from accumulating mutations associated with replication or from being affected by DNA-damaging agents. (*d*) Antiapoptotic proteins. Stem cells express higher levels of antiapoptotic proteins, such as members of the Bcl-2 family and inhibitors of apoptosis, than do differentiated cells (65). (*e*) Transporter proteins. Stem cells express high levels of transporter proteins, such as ABCG2 (BCRP), as well as P-glycoprotein. The development of effective immunologic approaches to cancer therapy may also be affected by the existence of cancer stem cells. Many of these therapies have involved targeting cells that express tumor-specific antigens. These antigens may be selectively expressed on differentiated tumor cells. Cancer stem cells that do not express these antigens may thus be spared by these immunologic interventions.

The concept of cancer stem cells also has implications for the development of targeted therapies. Arguably, the most successful targeted therapy has been the development of imatinib that targets BCR-Abl in patients with CML. The vast majority of patients with early stages of CML are put into a remission by administration of imatinib. However, recent studies have suggested that although

imatinib may target differentiated and progenitor CML cells, it does not eliminate CML stem cells that harbor this mutation. Following withdrawal of imatinib in animal models or the development of a resistant clone in patients, the disease reappears with kinetics predicted by a stem cell model (66). These studies suggest that the cure of CML will require the elimination of BCR-Abl containing CML stem cells.

If the ultimate cure of various cancers depends on the elimination of cancer stem cells, one can question why several malignancies, such as testicular carcinoma in men and choriocarcinoma in women, are curable with chemotherapy even in advanced disease, whereas the majority of common epithelial malignancies are not. One might speculate that the stem cell component of testicular carcinoma and choriocarcinoma are inherently different from those in other tissues, because these tumors arise in germ cells. Indeed, chemotherapy treatment of these tumors often produces residual masses that are benign teratomas composed of differentiated cells. An understanding of the inherent differences between stem cells of testicular cancer and choriocarcinoma compared with those from other tumors may provide new clues for the development of therapies for more common tumor types.

Opportunities for new therapeutics. The cancer stem cell model suggests that it may be necessary to alter the current paradigm in drug development. Eradication of cancers may require the targeting and elimination of cancer stem cells. Thus, one must devise strategies that can selectively kill these cancer stem cells while sparing normal stem cells, such as those in the gut and bone marrow. This represents a challenge because many pathways, such as those involved in self-renewal, are shared by cancer stem cells and their normal counterparts. However, a variety of recent studies using animal models that have targeted these pathways indicate the feasibility of this approach. For example, Notch signaling requires processing by the enzyme γ-secretase. An inhibitor of this enzyme has been recently shown to have activity against breast cancers that over express Notch1 (67, 68). Agents targeting Hedgehog signaling have recently been shown to have antineoplastic activity. The Hedgehog inhibitor cyclopamine that specifically inhibits Hedgehog signaling was used to treat animals bearing a variety of tumor xenografts. Cyclopamine caused dramatic regression of tumors that did not recur following cessation of treatment. Furthermore, at least over brief periods, the administration of these agents seemed to be nontoxic (17). A Hedgehog pathway inhibitor, HhAntag, with greater activity than cyclopamine has recently been shown to block medulloblastoma formation in a transgenic mouse model (69). These studies support the feasibility of selectively targeting the cancer stem cell population. The elimination of this key cell population may result in improved therapeutic outcomes for patients with even advanced cancers.

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Response

In the accompanying article, Hill presents his views that the current evidence is not conclusive regarding the existence of cancer stem cells in solid tumors. Although we agree that much remains to be learned about tumor stem cells, we feel that the substantial biological and clinical implications of this model justify intensive research in this area. Hill points to several theoretical and methodologic questions regarding the experimental evidence for the existence of cells with stem cell properties in solid tumors. He points out that the relative inefficiency of transferring human tumors to xenografts may be due to inherent inefficiencies in the systems rather than tumor subpopulations that differ in their tumorigenicity. We believe that the recent prospective identification of solid tumor stem cells in a variety of malignancies, including breast cancer, brain cancer, and prostate cancer, provide strong evidence that "not all cancer cells are equal." Only a relatively small percentage of cells with characteristic cell surface markers are able to be serially passaged in immunocompromised mice, a demonstration of their self-renewal capacity. We do, however, agree with Hill that tumor stem cells may themselves be heterogeneous with varying self-renewal capacity. Indeed, as stated in our article, this has recently been shown for human leukemias. We also agree with his statement that the microenvironment is important in determining the behavior of transplanted cells. In our review, we stress the importance of the stem cell niche in normal stem cell function and the tumor microenvironment in tumor growth and progression. For this reason, solid tumor xenograft models of breast and brain cancer have used orthotopic installation of tumors to the mammary fat pad or brain, respectively. Hill contends that genetic instability drives tumor development, so that the relationship between stem cell behavior and differentiation might change during tumor progression. Although genetic instability undoubtedly plays an important role in tumor progression, molecular profiling studies suggest that the biological behavior of tumors is inherent in the initial tumor. This is more consistent with a tumor stem cell model in which tumor behavior is largely determined by the cell of origin and its genetic profile. Hill also speculates that differentiated tumor cells that have "lost the ability to manifest as cancer stem cells might regain the ability next week or next month." However, we are unaware of any direct evidence of dedifferentiation of tumor cells. In xenograft models, differentiated tumor cells from tumors fail to form tumors when transplanted even after long periods of observation. Finally, it is important to distinguish between markers that may serve to identify tumor stem cells from molecules that play important roles in stem cell behavior. In many cases, the markers present on tumor stem cells mimic that of their normal stem cell counterparts. Thus, the argument that proteolytic digestion of solid tumors change cell surface markers has no direct bearing on the behavior of these cells when they are introduced into immunocompromised mice.

In summary, cancer stem cells were first described in human leukemias. Accumulating evidence in a variety of solid tumors suggest that these tumors may also be driven by a subset of cells that display stem cell properties. Further studies should lead to a greater understanding of the biology of these cells with significant implications for cancer treatment and prevention.

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Survival of Mammary Stem Cells in Suspension Culture: Implications for Stem Cell Biology and Neoplasia

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There is increasing evidence that a variety of neoplasms including breast cancer may result from transformation of normal stem and progenitor cells. In the past, isolation and characterization of mammary stem cells has been limited by the lack of suitable culture systems able to maintain these cells in an undifferentiated state *in vitro*. We have recently described a culture system in which human mammary stem and progenitor cells are able to survive in suspension and produce spherical colonies composed of both stem and progenitor cells. Recent observation that adult stem cells from other tissues may also retain the capacity for growth under anchorage independent conditions suggests a common underlying mechanism. We propose that this mechanism involves the interaction between the canonical Wnt signal pathway and E-cadherin. The Wnt pathway has been implicated in normal stem cell self-renewal *in vivo*. Furthermore, there is evidence that deregulation of this pathway in the mammary gland and other organs may play a key role in carcinogenesis. Thus, the development of *in vitro* suspension culture systems not only provides an important new tool for the study of mammary cell biology, but also may have important implications for understanding key molecular pathways in both normal and neoplastic stem cells.

KEY WORDS: stem cells; cancer stem cells; breast cancer; mammary gland development; Wnt signaling; self-renewal; differentiation.

INTRODUCTION

The isolation and characterization of tissue specific stem and progenitor cells has gained impetus due to the important insights that their study can provide into normal development and carcinogenesis. Adult stem cells are defined by their capacity for self-renewal and differentiation into cell lineages present in a specific tissue *(1,2)*. Self-renewal ensures the propagation of the stem cell compartment, which in turn sustains morphogenesis, tissue repair and maintenance. Differentiation generates the specialized cells that form each organ and ensure its normal functioning. In many organs, slowly dividing stem cells give rise to a transit, amplifying population of progenitor cells that proliferate actively in response to specific signals. These undifferentiated cells are multipotent and generate the lineage-restricted progenitors that subsequently undergo terminal differentiation *(3)*. Whereas mammary stem cells have not yet been isolated and characterized, their existence *in vivo* in mice was unequivocally demonstrated by

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Abbreviations used: ASMA, alpha smooth muscle actin; APC, adenomatosis polyposis coli; BCRP, breast cancer resistance protein; BMI, B-lymphoma MO-MLV insertion region 1; BMP4, bone morhogenetic protein 4; EGF, epidermal growth factor; ESA, epithelial specific antigen; ER, estrogen receptor; FACS, fluorescence-activated cell sorting; FITC, fluorescein isothyocyanate; bFGF, basic fibroblast growth factor; LIF, leukemia inhibitory factor; Muc 1, mucin 1; MMP7, matrix metalloproteinase 7; MTA3, metastasis associated gene 3; NuRD, Nuclear remodeling and deacetylation complex; PR, progesterone receptor; PTEN, phosphatase and tensin homolog; SP, side population.

serial transplantation studies *(4,5)*. Evidence for the existence of adult mammary stem cells and the strategies employed for their isolation and characterization were thoroughly reviewed in several recent papers *(6,7)*. The focus of this review will be on the development of methods for the cultivation of adult mammary stem/progenitor cells and the potential application of these systems in stem cell biology.

IN VITRO PROPAGATION OF NORMAL STEM CELLS IN SUSPENSION CULTURE

Ideally stem cells are purified by isolation directly from tissue to avoid any artifacts introduced by *in vitro* manipulation *(2)*. Purification of the stem cells is based on finding a number of morphological and molecular traits, including surface antigens, that distinguish them from all the other cells in the tissue of interest *(8)*. Most stem cell enrichment/purification protocols rely on fluorescence-activated cell sorting (FACS) or magnetic immunosorting and use sets of antibodies against cell surface proteins. When the molecular signature is not known, which is the case for mammary stem cells, the task of isolating the stem cells involves a tedious process of screening large numbers of putative markers to identify the ones that co-segregate with functional attributes of stemness. As a minimal working definition, these attributes include self-renewal and ability to generate differentiated progeny. *In vivo* generation of a functional tissue is the ultimate proof of "stemness" of any candidate marker. However, screening large numbers of candidate markers in *in vivo* transplantation assays in order to find the stem cell signature is impractical. On the other hand, until recently, the lack of a cultivation system that allows for the propagation of mammary progenitors in an undifferentiated state has precluded the testing of self-renewal *in vitro*.

We have developed a culture system, based on previous work in neural stem cell biology, which overcomes this inability of previous systems to maintain and propagate mammary stem and progenitor cells. In 1992 Reynolds and Weis published a study in which they showed that neural cells isolated from the subventricular zone of rat embryos can proliferate in suspension culture, clonally generating spherical colonies, which they termed neurospheres *(9)*. A clonal analysis of neurospheres demonstrated that 20% of these cells, proliferating *in vitro* in response to EGF and bFGF stimulation, were capable of both self-renewal and differentiation along multiple lineages, the defining characteristics of stem cells *(10)*. Two years later the same group showed that neural stem/progenitor cells from adult animals have the same ability to propagate *in vitro* as neurospheres *(11)*. Since then, numerous studies utilizing cells derived from either the central or peripheral nervous system, from embryonic and adult tissue showed that the ability to generate neurospheres *in vitro* correlates with the number of stem cells in the tissue of origin *(12–14)*. Implanted into the nervous system of an animal host, neurospheres generate cells that self-renew as well as differentiate and contribute to the various cellular compartments of the recipient animal's brain *(12,13)*. Therefore, these cells are capable of self-renewal and multilineage differentiation, both *in vitro* and *in vivo*. This experimental system has been an extremely useful tool in neural stem cell biology. It was used as an *in vitro* model for early development *(15,16)*, to identify factors involved in cell fate determination and to unravel signaling pathways active in stem and early progenitor cells *(17–19)*. A series of studies utilized this system for comparative transcriptional profiling in order to identify gene expression changes underlying self renewal or lineage specific differentiation *(20–23)*. Moreover, neurospheres were utilized in a number of *in vivo* studies to repair neuronal loss in mice with spinal cord and sciatic nerve injuries and in rat models with ischemic cerebral cortex *(24)*, partial Parkinson's disease *(25)* and Huntington's disease *(26)*. These studies demonstrated successful engraftment, migration to the site of the lesion and differentiation of sphere-derived cells into functional neurons with variable degrees of behavioral and anatomical recovery. These results make the case for the potential use of these progenitor cells, propagated *in vitro* in suspension culture, in repairing damaged tissue *in vivo*. The neurosphere culture is also routinely used now to assess the enrichment of stem cells in experiments using cell sorting for identifying markers of stem cells *(12,13)*. Neurosphere formation was also used to assess the size of the stem cell population in neural tissue of genetically manipulated mice in experiments aiming to investigate the role of genes such as BMI *(23)*, PTEN *(27)*, LIF and Notch *(28,29)* in self-renewal.

NONADHERENT MAMMOSPHERES

Recently we adopted the suspension culture as a strategy for the *in vitro* enrichment and propagation

of human mammary stem/progenitor cells *(30)*. Based on the model of neurospheres, we hypothesized that a small population of mammary cells with stem cell properties would be able to survive and proliferate in the absence of attachment to an exogenous substratum. We developed a culture system in which human mammary epithelial cells, isolated from reduction mammoplasties, are cultured on a non-adhesive substratum in serum-free medium in the presence of EGF and/or bFGF. Under these conditions the vast majority of cells undergo "anoikis." This term specifically applies to the apoptosis of non-transformed cells which occurs in the absence of anchorage to a substratum. We hypothesized that anoikis was a property of differentiated cells, but that stem cells could survive anchorage independent conditions. Consistent with this hypothesis, we found that approximately four in 1000 freshly isolated cells are able to survive and proliferate, and form multicellular spheroids. We termed these spheroids "mammospheres" by virtue of their resemblance to neurospheres cultured from primary neural cells. As is the case for neurospheres, we demonstrated that mammospheres are highly enriched in undifferentiated cells, as demonstrated by the ability of single cells isolated from mammospheres to generate multilineage colonies when cultured in the presence of serum on a collagen substratum which promotes their differentiation (Fig. 1(a)). Primary mammospheres contain eight times more bi-lineage progenitor cells than freshly cultured human mammary cells. Secondary and later passaged mammospheres consist of virtu-

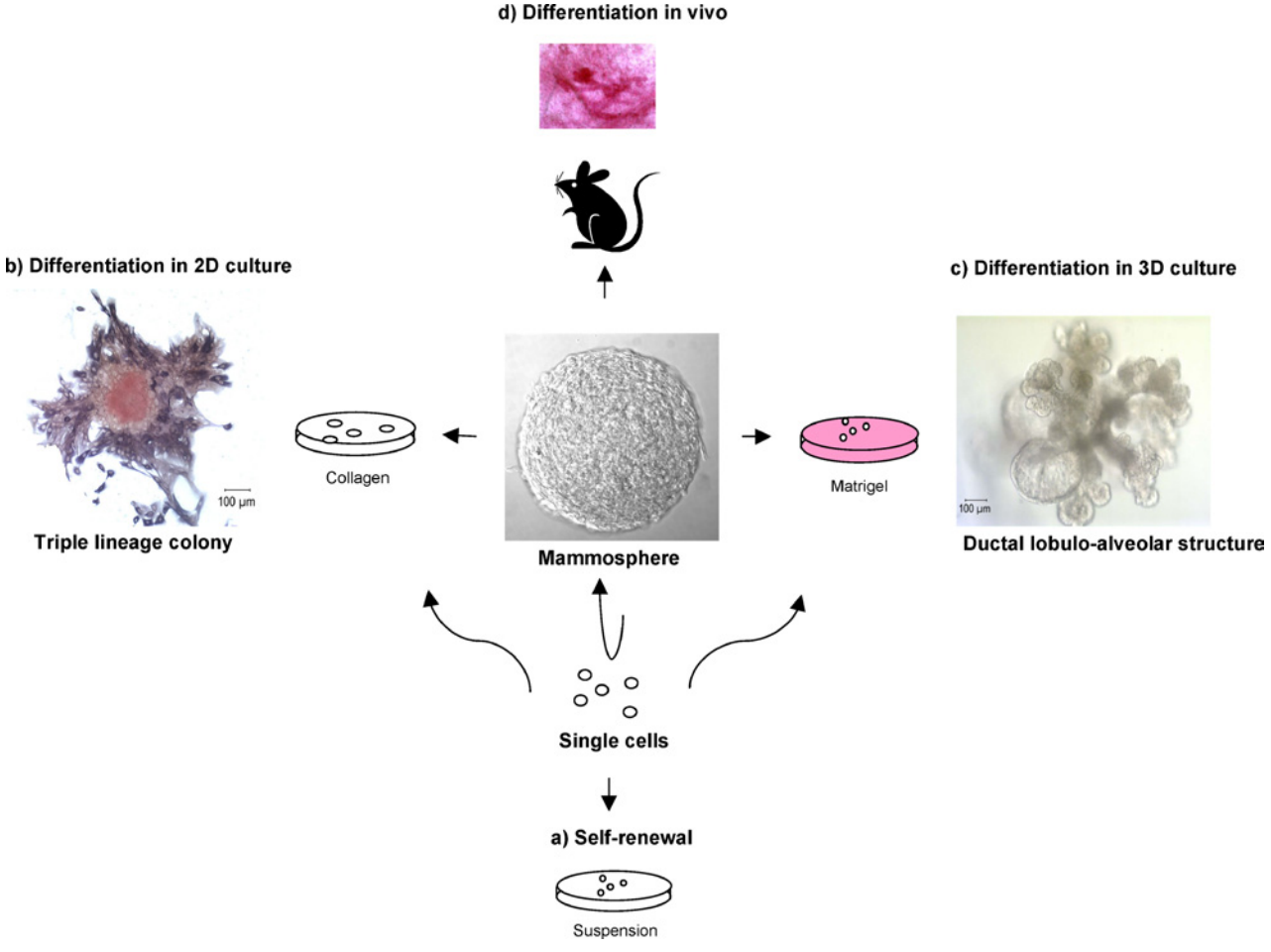

Fig. 1. Mammospheres are composed of stem/progenitor cells. Mammosphere-derived cells plated at clonogenic densities can: (a) self renew, (b) generate mixed colonies containing cells of all the three mammary lineage types (immunostained with lineage specific markers, ductal epithelial—ESA, brown; myoepithelial—CD10, purple; alveolar, beta-casein—red) (c) generate complex structures in 3D Matrigel culture, (d) generate ductal—alveolar outgrowths *in vivo*.

ally 100% bipotent progenitors. Furthermore, the majority of bipotent progenitors are able to generate colonies that contain all three lineages of the adult mammary gland, myoepithelial, ductal epithelial, and alveolar epithelial cells. We have also shown that mammospheres contain cells capable of clonally generating complex functional structures in reconstituted 3-D culture systems in Matrigel (Figs. 1(c) and 2).

Self-renewal of a cell population within mammospheres was demonstrated utilizing an assay in which single cells from mammospheres are able to generate second and later generation spheres (Fig. 1(b)). We demonstrated that mammospheres were clonally generated using retroviral marking. Furthermore, we demonstrated that mammospheres derived from these passaged cells also have multipotent differentiation potential. These results resemble those reported for neurospheres *(10,31)* and are consistent with a model in which the mammosphere-forming cell represents a mammary stem cell which undergoes limited self renewal and then gives rise to mammary progenitors still capable of multi-lineage differentiation *(32)*. Clonal experiments in which spheres were grown from single cells and single mammospheres were passaged, suggest that one or two self-renewal divisions are involved in the formation of a single mammosphere. This limited number of self-renewal divisions is in agreement with the majority of studies involving adult stem cells, which indicate that expansion of the adult stem population does not readily occur *ex vivo*, presumably due to asymmetric cell kinetic divisions that result in a large number of progenitors and differentiated cells and a small fixed number of stem cells. Our findings indicate that mammary stem cells are contained in the small population of cells that are anchorage independent and survive suspension culture to proliferate and differentiate into mammary progenitor cells *(30)*.

To determine if the ability of cells to form mammospheres correlates with enrichment in progenitor cells, we tested the sphere formation capability of the side population (SP) of mammary epithelial cells *(30)*. SP represents a subpopulation of cells capable of excluding dyes, such as Rhodamine and Hoechst, due to the expression of transporter proteins, such as BCRP (breast cancer resistance protein) and *P*-glycoproteins *(33)*. It has been shown that the SP fraction of hematopoietic and neural cells contains the long-term repopulating stem cells *(34)*. This phenomenon has also recently been demonstrated for mouse mammary cells with SP properties, which can regenerate the gland upon transplantation *(35)*. In our study SP and non-SP staining population from uncultured cells were separated by FACS and placed in suspension culture. Only cells contained in the SP fraction were capable of mammosphere formation in suspension culture as well as generation of multilineage colonies on collagen substrata *(30)*. In order to assess the cellular composition of mammospheres we used immunostaining with markers specific for the differentiated mammary cells of luminal epithelial (ESA, Muc1, cytokeratin 18) and myoepithelial lineages (CD10, ASMA, cytokeratin 14, alpha 6 integrin). Some of these markers were identified in previous studies as being associated with bi-potent progenitor cells (ESA, alpha 6 integrin) *(36,37)* or mammary stem/progenitor cells (cytokeratin 5). Mammospheres contained cells positive for alpha 6 integrin, cytokeratin 5 and CD10, ESA and cytokertain 14 *(30)*. ER and PR expression was also detectable by immunostaining in mammospheres and in colonies generated from mammospheres plated on a collagen substratum. A subpopulation of the ER positive cells was also positive for the Ki67 proliferation marker, indicating that these cells can divide *in vitro*. After 5–7 days of cultivation on a collagen substratum ER expression was no longer detected, suggesting that it is downregulated during differentiation *in vitro* (unpublished observations).

We have recently found that, when transplanted in the cleared mammary fat pad of NOD/SCID mice, mammospheres generated limited outgrowths with the morphological and cellular characteristics of a human mammary ductal alveolar structure (Figs. 1(d) and 2). As few as 500 transplanted mammospheres (10,000–25,000 cells) generated outgrowths, in the absence of human fibroblasts. Improved engraftment was obtained by using mammospheres combined with human mammary fibroblasts, as described by Kuperwasser *et al. (38)*. Titration experiments will be required to determine the absolute enrichment in gland reconstituting activity of mammosphere derived cells.

We have utilized the mammosphere cultivation system to delineate signaling pathways involved in cell fate specification of mammary stem/progenitor cells, such as Notch and Sonic Hedgehog *(32)*. Using assays for *in vitro* self-renewal and differentiation that we developed with this system, we showed that Notch signaling regulates cell fate decisions in the mammary gland at several distinct developmental stages. Notch activation increases self-renewal of mammary stem cells, as well as acting on

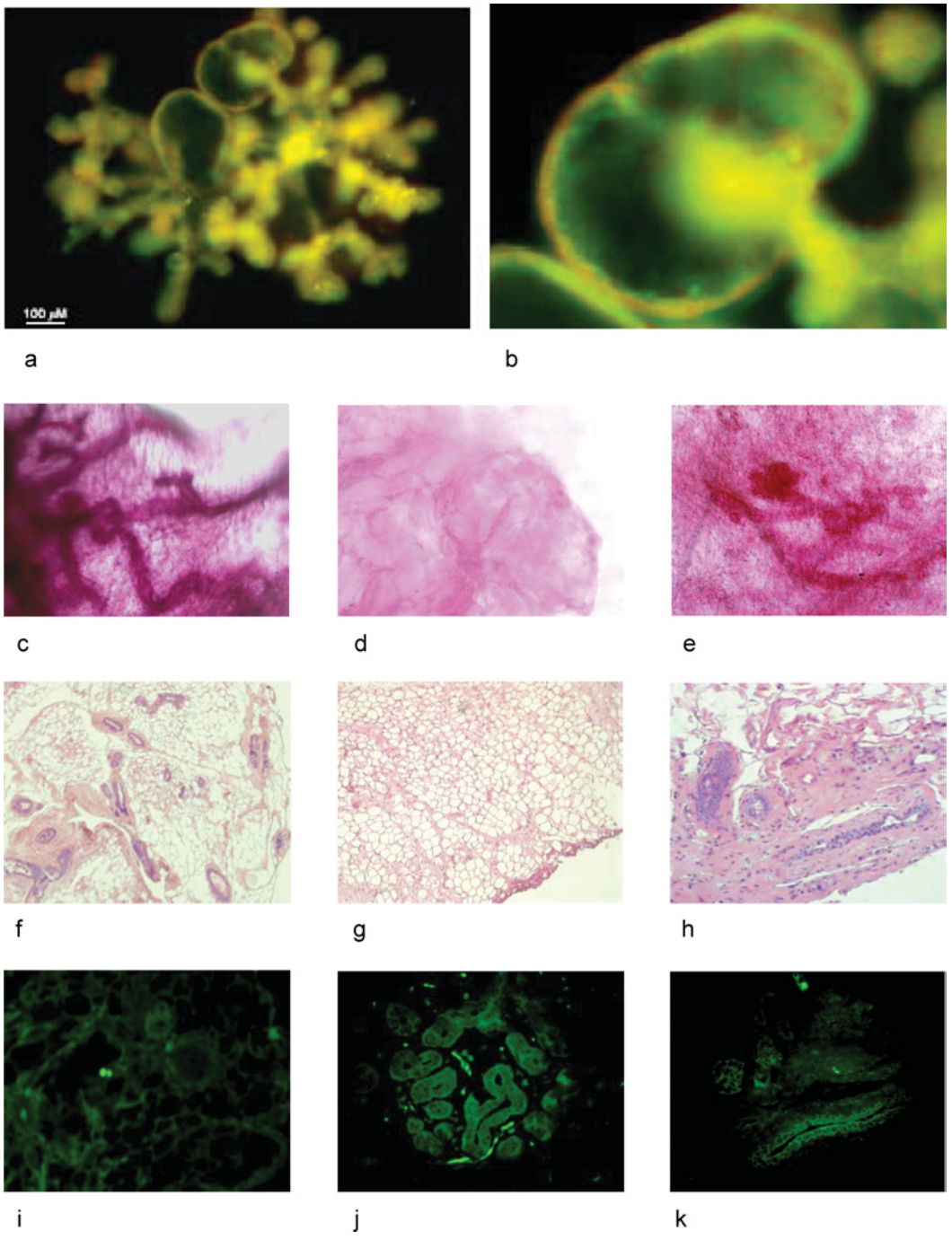

Fig. 2. (a) Fully developed ductal-alveolar structure grown in Matrigel from a secondary mammosphere, immunostained with lineage specific markers (myoepithelial cells are stained red with CD10-Texas red and ductal epithelial cells are stained green with ESA-FITC) (b) Same as a, higher magnification. (c-k)Mammospheres generate outgrowths in the cleared mammary fat pad. Whole mount staining (carmine-red) and H&E tissue sections through a mouse mammary fat pad: not cleared [(c) and (f)], cleared, non-implanted [(d), (g)], cleared and implanted with mammospheres derived from human mammary epithelial cells [(e), (h), (k)]. Immunostaining utilizing human specific ESA antibody - tissue section through a mouse mammary gland (i), human mammary gland (j), mouse cleared fat-pad implanted with human mammospheres (k).

progenitor cells to promote the adoption of the myoepithelial cell fate at a later step, during differentiation. Sonic hedgehog pathway also appears to have a role in regulating self-renewal of mammary stem cells, probably also interacting with the Notch pathway.

Based on the observations that embryonic stem cells, as well as at least two types of stem/progenitor cells from neural tissue and mammary tissue, have the ability to survive and proliferate in an anchorage independent manner, we speculated that suspension culture might be utilized as a method for isolating adult stem/progenitor cells from other tissues. Indeed this was recently confirmed in the case of skin *(39)*, cardiac *(40)*, and inner ear stem cells *(41)* that generated the same type of floating spherical colonies in suspension cultures, composed of cells with stem and progenitor functional properties.

CANCER STEM CELLS AND TUMOR SPHERES

We and others have recently proposed a model in which mammary carcinogenesis is driven by tumor stem cells derived from mutated adult stem or progenitor cells *(7,42)*. This model is based on the widely accepted concept that cancer arises through a series of mutations that may occur over many years. Since adult stem cells are slowly dividing, long-lived cells with a high proliferative capacity, they are able to accumulate the multiple mutations that occur during carcinogenesis *(1,42)*. Being exposed to damaging agents over long periods of time, they can accumulate and propagate the mutations induced by the genotoxic agents. These transformed stem or progenitor cells can in turn become "cancer stem cells," which maintain or acquire functional properties present in normal stem cells, including the capacity for self-renewal and differentiation. Mutations accumulated in the "cancer stem cell" disrupt the tight control of these stem cell functions, ultimately leading to deregulation of self-renewal, which drives the process of tumorigenesis, and to aberrant differentiation, which generates the cellular heterogeneity found in tumors. The existence of cancer stem cells was first demonstrated in hematologic malignancies and, more recently, in solid tumors *(43–46)*. In a previous review we analyzed in more detail the functional characteristics shared by normal stem cells and cancer cells, such as capacity for self-renewal, ability to differentiate, active telomerase, activation of antiapoptotic pathways, increased membrane transporter activity, anchorage independence and ability to migrate *(4)*. Comparing the transcriptional profile of stem/early progenitor cells with that of more differentiated cells of the mammary epithelium, we proposed that the former more closely resembles that of cancer cells than the latter. This observation suggests an underlying molecular circuitry that makes stem cells more readily transformed than differentiated cells. A key event in transformation may be the deregulation of pathways such as self-renewal, which are already active in stem cells.

Direct evidence for the existence of cancer stem cells was first provided by the work of John Dick's group in hematological malignancies *(43)*. They demonstrated the presence of a cancer stem cell population in human leukemias, representing a very small fraction of the total leukemic population, which was capable of transferring the disease to immunosuppressed mice. Furthermore, leukemic stem cells shared the expression of phenotypic markers with normal hematopoietic stem cells. Based on these studies, they proposed that different leukemic phenotypes resulted from particular mutations in hematopoietic stem cell populations, which resulted in aberrant differentiation of these cells *(43,47)*.

In collaboration with Clarke's group we recently presented evidence for the existence of human breast cancer stem cells *(45)*. Flow cytometry was utilized to separate subpopulations of cells based on their surface marker expression. A subpopulation of tumor cells, with the phenotype $CD44^{+}$ $CD24^{-}$ lineage-, which possesses highly tumorigenic characteristics, was thus identified. The tumorigenic subset, representing a minority of the total cellular population within a tumor, was defined by the same markers in the majority of tumors examined. As few as 200 of these cells consistently formed tumors in NOD/SCID mice. In contrast, the bulk of the tumor, which contained cells with different cell surface phenotypes, failed to form tumors even when tens of thousands of cells were injected. In order to determine whether this experimental system merely selected for a highly tumorigenic subset of cells, the phenotype of tumors produced in NOD/SCID mice by the prospectively isolated tumorigenic cells was analyzed. The results showed that the small population of tumorigenic cells was able to regenerate the entire phenotypic heterogeneity found in the initial tumor. These findings support a stem cell model of carcinogenesis, in which a small population of tumorigenic cells, with definable phenotype, is able to give rise to more

tumorigenic cells as well as the bulk tumor population, without tumorigenic properties. Hence, the tumorigenic stem cells, like their normal counterparts, are able to undergo both self-renewal and differentiation.

The existence of a cancer stem cell phenotype in brain tumors has also been suggested by two groups that utilized *in vitro* cultivation of brain tumor cells in suspension, as neurospheres *(46,48)*. Singh and coworkers used this experimental system to identify cancer stem cells from various human brain tumors *(46)*. Their study showed a correlation between the self-renewal capacity of cancer stem cells, demonstrated by sphere formation, and the clinical aggressiveness of the brain tumors from which these cells were derived. They also demonstrated that these cancer stem cells from brain tumors had the ability to generate differentiated progeny *in vitro*, similar to the tumor from which the cells were isolated. Most recently, they have shown that brain cancer stem cells, expressing the neural stem cell marker CD 133 are able to form tumors in NOD/scid mice, whereas CD 133 negative cells are not *(47)*. Moreover, when injected into animals, CD 133+ cells generated heterogeneous tumors composed of both CD 133+ and CD133− cells. Interestingly, only CD 133+ cells were capable of forming neurospheres.

Thus, as we have previously demonstrated for breast cancer, the stem cell model of carcinogenesis also applies to brain cancers.

HOW DO STEM CELLS AVOID "ANOIKIS"—A THEORETICAL MODEL

A fundamental question is whether the anchorage independence of normal stem cells is merely an *in vitro* phenomenon. Does the molecular mechanism responsible for this property also play a role in stem cell behavior *in vivo*? We will further speculate on this latter possibility and propose a mechanism that might coordinate proliferation and survival during stem/progenitor cell differentiation with cell–cell adhesion and integration into mature tissue architecture. Survival of single cells in suspension culture involves survival in the absence of cell-matrix interaction and cell–cell interactions.

Cell–Matrix Interactions

Our analysis of mammosphere showed the presence of matrix molecules including tenascin, decorin and laminin *(30)*. Interestingly, decorin and tenascin are present in the embryonic mammary gland, while laminin is also present in the basement membrane of the adult gland, suggesting that mammosphere formation may recapitulate some of the events that occur during embryonic and early mammary development *in vivo*. It appears that the cells that escape anoikis, represented mainly by stem cells and possibly early progenitor cells, synthesize and deposit extracellular matrix, creating an *in vitro* niche that supports their survival and proliferation in suspension. Undoubtedly, stromal-epithelial interactions are also involved in the generation of the stem cell developmental niche *in vivo*. Recent observations by Weinberg's group *(38)*, as well as our own, demonstrate that mammary stroma greatly potentiates the growth and differentiation of human mammary epithelium (from epithelial fragments and mammospheres) in NOD/scid mice. Interactions between the mammosphere-initaiting cells and their progeny and between the cellular and extracellular components of the spheres, dictate the types of divisions (self renewal vs. differentiation) and the cell fate adopted by the cells (lineage commitment). It remains to be determined to what extent this behavior recapitulates events occurring *in vivo*. One might speculate that signaling initiated by the matrix molecules engages specific sets of integrins expressed by mammary progenitor cells, promoting their survival. Signaling through growth factor receptors present in these cells probably also plays an important role in cell survival. The survival of cancer cells at sites of metastasis may employ similar mechanisms. One important caveat of extrapolating *in vitro* data to *in vivo* events has been recently illustrated in the neurosphere system. *In vitro* conditions altered the potential and fate specification of neural progenitor cells, generating tripotent cells, not seen *in vivo (49)*.

Cell–Cell Interactions

Cell–cell interactions are crucial for the survival of epithelial cells both *in vitro* and *in vivo*. An important component of this process is E-cadherin *(50)*. Loss of this mechanism of adhesion is probably involved in the massive apoptosis that occurs during mammary involution, following lactation *(51)*. Interestingly, the resistance of mammary stem/progenitor cells to apoptosis during involution preserves the populations which regenerate the gland

during subsequent pregnancies. E-cadherin is a member of a family of single-pass transmembrane glycoproteins that plays a role in establishing cell polarity and tissue morphology *(50)*. The extracellular domain of E-cadherin interacts homotypically with an E-cadherin molecule on an adjacent cell. The cytoplasmic domain of E-cadherin is linked to the cytoskeleton via interaction with catenins. An important interaction is with beta-catenin, a molecule in the canonical Wnt signaling pathway. The connection between E-cadherin and Wnt signaling and its role in normal breast development and carcinogenesis was the subject of an excellent recent review *(52)*. We will discuss this connection from the perspective of carcinogenesis which results from deregulated self-renewal of stem cells or acquired self-renewal by early progenitor cells. Beta-catenin is present in two cellular locations. In association with E-cadherin, it forms the adherens junctions. In the cytoplasm, beta-catenin is present in a complex with proteins such as adenomatous polyposis coli (APC) and axin. In the absence of Wnt signaling, beta-catenin is phosphorylated and targeted for degradation *(53)*. When Wnt is activated, unphosphorylated beta-atenin translocates to the nucleus, where it binds and activates the transcription factors TCF-LEF, which then activates a variety of downstream target genes, including c-Myc and cyclin D1 *(54,55)*. It has been recently proposed that the balance between the beta-catenin present in the adherens junctions and that involved in transcriptional activity is controlled by the pool of intracellular APC *(56)*. Furthermore, evidence from Drosophila studies indicates that APC regulates spindle orientation and asymmetric cell division *(57)*. An inverse correlation between E-cadherin and Wnt signalling has been noted in various tissues and organs, during development, as well as in cancers *(58)*. In some human epithelial cancers, E-cadherin gene mutations promote Wnt signalling, as judged by the detection of nuclear beta-catenin in the tumor tissue *(58)*. It has also been shown that E-cadherin can mediate growth suppression by inhibition of beta-catenin signaling, in an adhesion dependent manner *(59)*. In cultured mammary epithelial cells, inhibition of E-cadherin-dependent cell aggregation predisposes to cell death. *In vivo* studies, utilizing transgenic mice and the Cre/lox recombinase system, demonstrated that alveolar differentiation during lactation is dependent on the expression of E-cadherin *(60)*. Moreover, as indicated above, disruption of E-cadherin-dependent cell adhesion probably initiates the apoptotic program during mammary involution. The interaction of E-cadherin with beta-catenin appears to mediate these processes. Furthermore, recent studies provided evidence for a converse mechanism, namely nuclear beta-catenin/Lef1 mediated suppression of E-cadherin expression during normal development of hair follicles in mice *(61)*. The authors propose that the downregulation of E-cadherin may in turn perpetuate the Wnt signalling pathway, by increasing the pool of transcriptionally competent beta-catenin.

The shifting balance between the levels of E-cadherin and cytoplasmic beta-catenin, under the control of Wnt signaling, could remodel cellular junctions, coordinating cell proliferation with cell adhesion during differentiation. We speculate that stem cells and early progenitors, unlike their more differentiated progeny, are not dependent on cell–cell interactions for survival. In support for this hypothesis is the ability of a variety of stem/progenitor cells to survive in suspension culture, as discussed above. Moreover, the mammary small light cells (SLC), described by Smith and Chepko *(62)*, thought to be stem or very early progenitor cells, lack polarity and specialized membrane contacts with neighboring cells. The absence of gap junction proteins, including connexins, in mammary progenitor cells was described by Trosko *et al. (63)*. Utilizing, transcriptional profiling of mammospheres as well as differentiated cells derived from mammospheres, we found that E-cadherin expression level increases threefold during differentiation, while repressors of E-cadherin Snail and Slug are downregulated three- and twofold respectively *(30)*. In the absence of adherens junctions and E-cadherin, most of the beta-catenin will be localized in the cytoplasm. Signaling through the Wnt pathway results in beta-catenin-mediated transcriptional activity that activates different sets of genes, ultimately resulting in self-renewal or differentiation. The outcome probably depends on signals that modulate the Wnt pathway response and the interactions with other pathways involved in fate specification. While differentiation occurs, E-cadherin expression progressively increases, adherens junctions form and the antiapoptotic mechanisms are downregulated. Nuclear beta-catenin and its transcriptional activity decrease (Fig. 3). Consequently, differentiated cells are not able to migrate, depend on cell-cell adhesion for their survival, and proliferate less. In favor of this scenario gene expression changes induced by beta-catenin transactivation show upregulation of c-Myc, Cyclin D1 (proliferation), stromlysin-1, MMP7 and Twist (migration) and

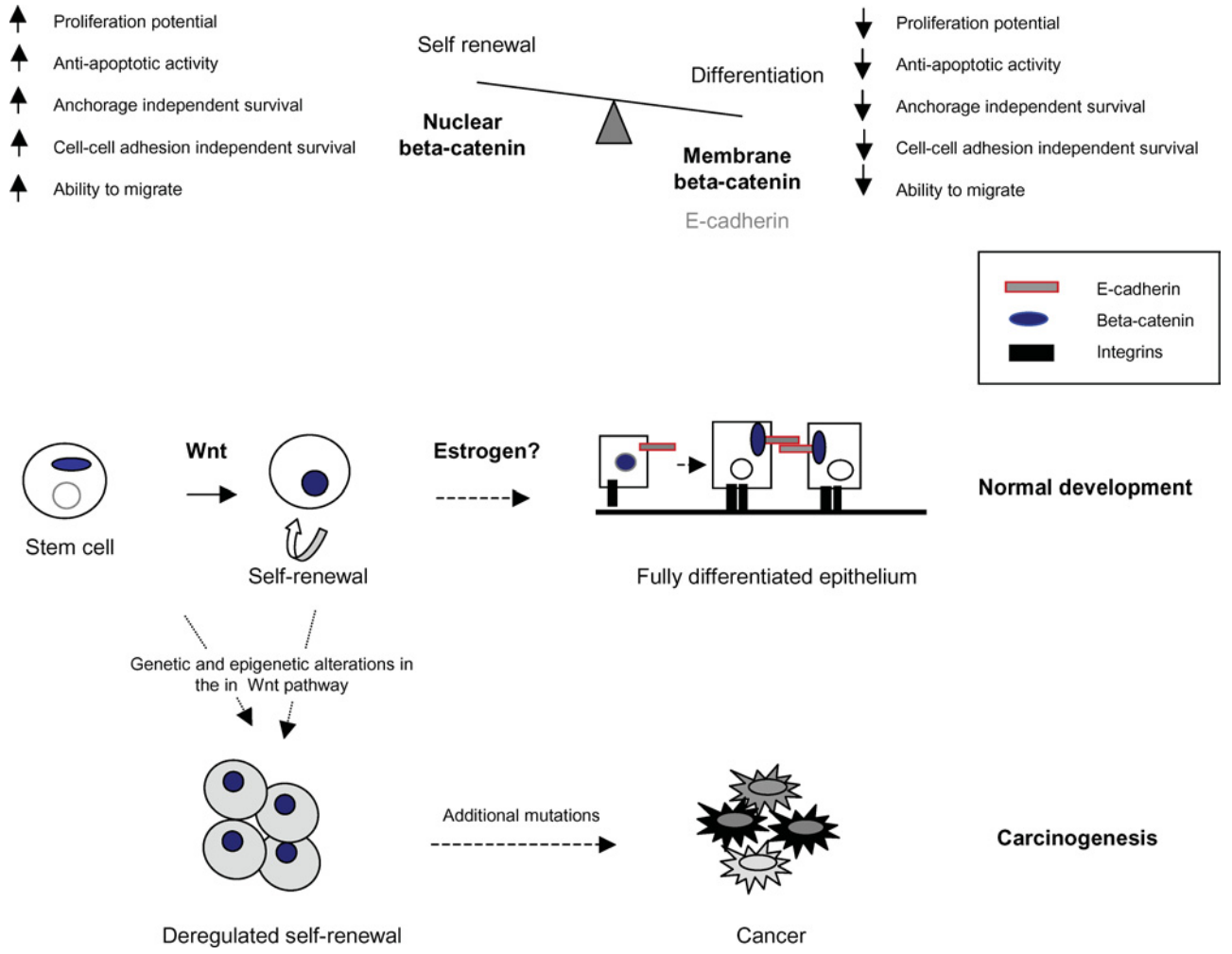

Fig. 3. Wnt–E-cadherin connection in stem cell self-renewal, differentiation and carcinogenesis. Levels of transcriptionally active beta-catenin and membrane E-cadherin are inversely correlated and change progressively during differentiation. In response to Wnt signaling, stem/progenitor cell E-cadherin is downregulated, the newly synthesized beta-catenin is directed towards the cytoplasmic pool and translocates to the nucleus, where it regulates self-renewal. During differentiation, expression of E-cadherin progressively increases and beta-catenin is recruited to the adherens junctions site, decreasing the pool of cytoplasmic beta-catenin available for transcriptional activity. Abnormal activation of Wnt signaling results in an increase in self-renewal of stem cells or early progenitor cells. Subsequent mutations may lead to cancer initiation and progression.

down regulation of E-cadherin, Ephrin, and BMP4. Furthermore, Fujita *et al.* reported that these events are under hormonal regulation, and involve the estrogen receptor *(64)*. They showed that in the mammary gland E-cadherin expression is regulated by the estrogen receptor through MTA3, a component of the Mi-2/NuRD transcriptional repressor complex which is an ER regulated inhibitor of Snail, a repressor of E-cadherin *(64)*.

During carcinogenesis a key event may be represented by an abnormal activation of Wnt signaling, resulting in an increase in self-renewal of stem cells or early progenitor cells. The Wnt pathway has been associated with both normal development of the mammary gland, from the very early stages of formation of anlage to terminal differentiation during lactation *(65–68)*. Also, increasing evidence points to a link between Wnt signaling and mammary neoplasia *(69–71)*. Moreover, it appears that the role of Wnt activation in self-renewal of stem cells might be the basis of its oncogenic potential *(72)*. A number of studies have recently provided evidence for a direct role of Wnt signaling in the self-renewal of normal hematopoietic, epidermal, and gut stem cells *(73–76)*. A role for Wnt signaling in self-renewal of mammary stem cells was suggested by recent studies of Alexander *et al.* who utilized transgenic mice to demonstrate that overexpression of Wnt ligands in mammary stroma or activated beta-catenin in mammary epithelium leads to increased numbers of mammary stem cells *(77)*. A direct link between self-renewal of stem/progenitor cells and carcinogenesis was suggested by the study of Li *et al.* who demonstrated in a mouse model that expression of the components of Wnt-signaling pathway preferentially induces mammary cancers derived from mammary progenitor cells *(70)*.

Subsequent mutations favored by the increased proliferative activity may occur during cancer initiation and progression. However, some of the traits that confer selective growth advantage to the cancer cells and contribute to local invasion and metastasis are normal attributes of stem cells. The observation that epigenetic mechanisms may be responsible for alterations such as the loss of E-cadherin, during carcinogenesis is consistent with this hypothesis.

CONCLUSIONS

In this article, we have reviewed the development of suspension-based culture systems for the propagation of stem and progenitor cells from the mammary gland, as well as from other organs. The ability of normal stem cells, as well as their malignant counterparts, to survive in suspension culture suggests a common molecular mechanism that may also have significance for the behavior of stem cells *in vivo*. We discuss evidence suggesting that the Wnt signal pathway and the E-Cadherin–beta-catenin interaction may play a key role in normal stem cell self-renewal and differentiation, as well as survival. Furthermore, there is accumulating evidence that deregulation of this pathway plays an important role in a variety of neoplasms, including breast cancer. These neoplasms, in turn, may be driven by a small stem cell component within tumors. This stem cell component, resistant to conventional treatments, may contribute to relapse following therapy. Inhibition of pathways, such as Wnt signaling, may thus prove to be a novel therapeutic strategy for selectively targeting this resistant cell population.

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