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/progenitors 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) 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 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].

Bmi-1 = B lymphoma Mo-MLV insertion region 1; Dsh = Dishevelled; 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.

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, 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 monophosphate 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].

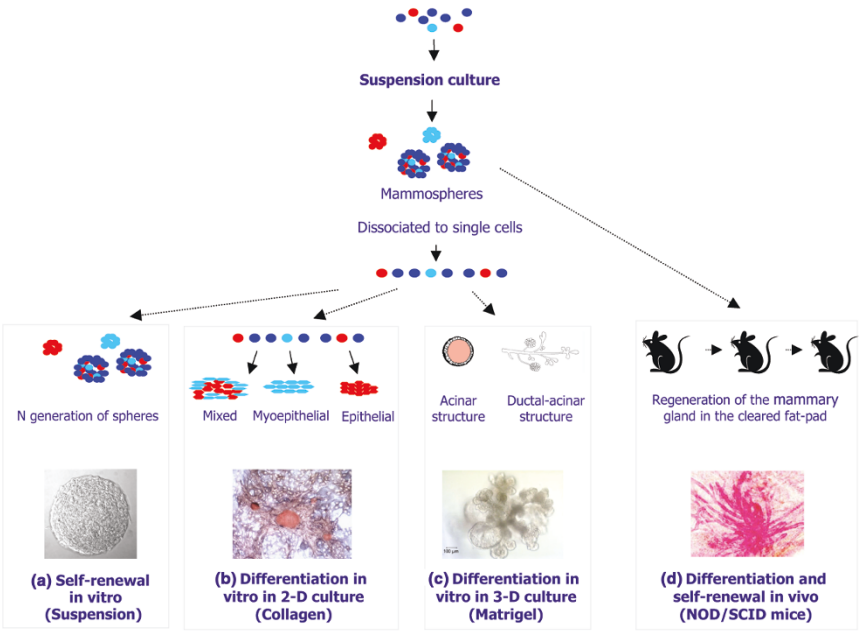
The cultivation of normal mammary stem and progenitor cells has been limited by the lack of suitable systems that permit the propagation of these cells in an undifferentiated state. When primary cultures of mammary epithelium from rodents or humans are cultured on solid substrata, they undergo limited 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; Liu S and Mantle I *et al*., 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 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

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)]. **(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.

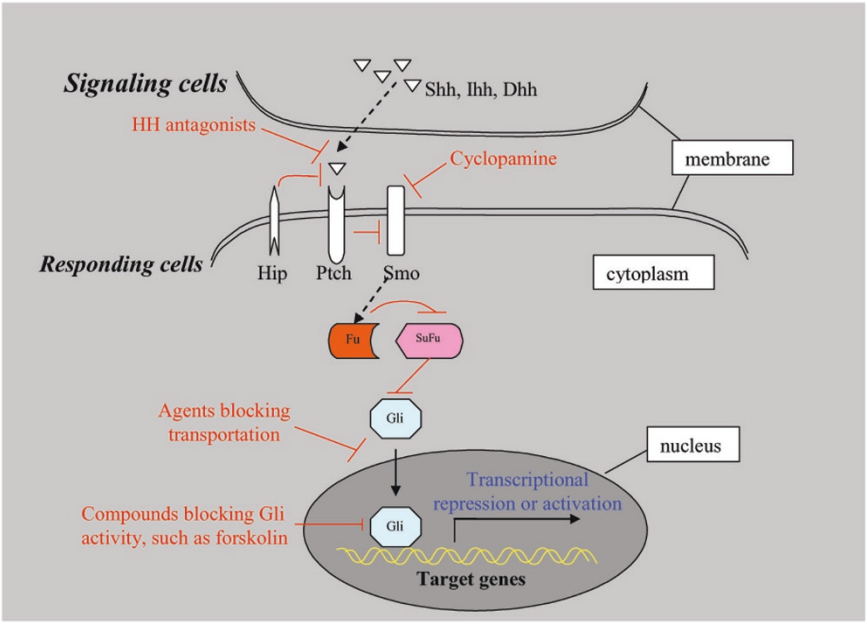
breast cancer 'stem cells'. As few as 100 of these cells, isolated from primary human breast carcinomas or metastatic lesions, are able to form tumors reproducibly in NOD/SCID mice. In contrast, tens of thousands of cells that do not bear this phenotype are unable to generate tumors in this model. Furthermore, 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 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 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

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 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 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.

pathways are 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, 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) [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 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 (Liu S *et al*., 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 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 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 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 + and ΔGli1 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

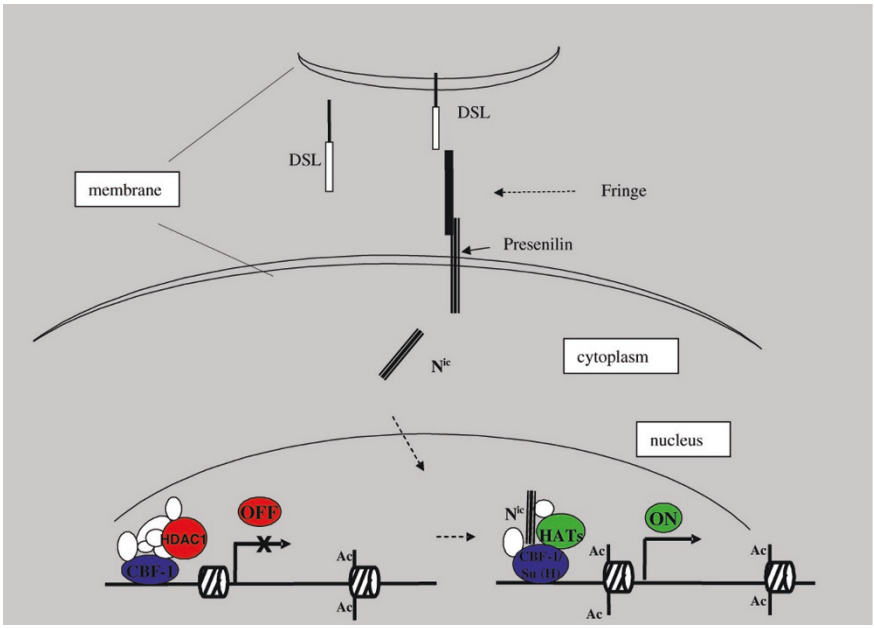
Notch transmembrane receptors are part of signaling pathways that are crucial in the regulation of the fate of cells in a variety of tissues [39]. The Notch proteins, represented by four homologues in mammals, Notch 1 to Notch 4, are expressed in a variety of stem or early progenitor cells. They interact 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). 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 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. Unphosphory-

Figure 3



A schematic diagram for the Notch signaling pathway. Upon binding of the DSL ligand, Notch signaling is modulated by fringe, 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). This intramembrane cleavage is followed by translocation of the intracellular domain on Notch to the nucleus, where it acts on downstream targets. CBF, C promoter binding factor; HDAC, histone deacetylase; HAT, histone acetyltransferase.

lated 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).

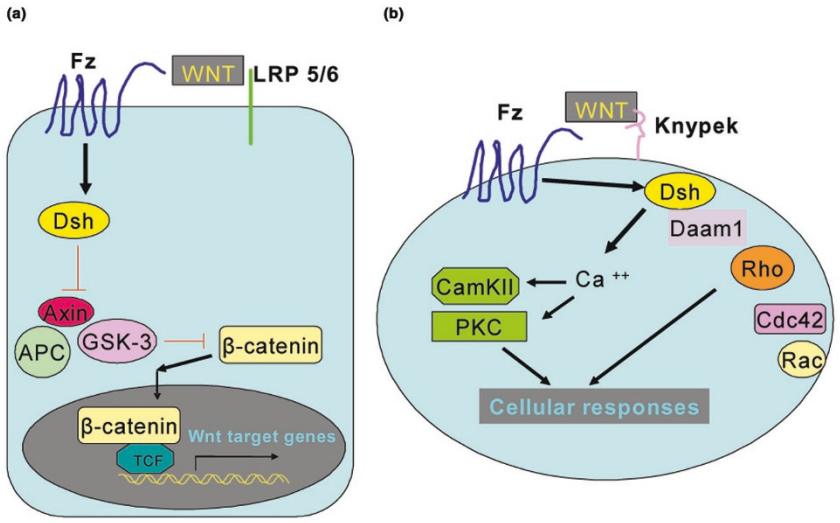
The noncanonical Wnt signaling pathway [48] involves Frizzled receptors and the proteoglycan co-receptor Knypek. A cytoplasmic signal transduction protein Dishevelled (Dsh) localizes to the cell membrane through its DEP domain. Dsh activates Rho through the bridging molecule Daam1. The precise roles 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 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

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 high mobility group (HMG)-box 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.

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 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].

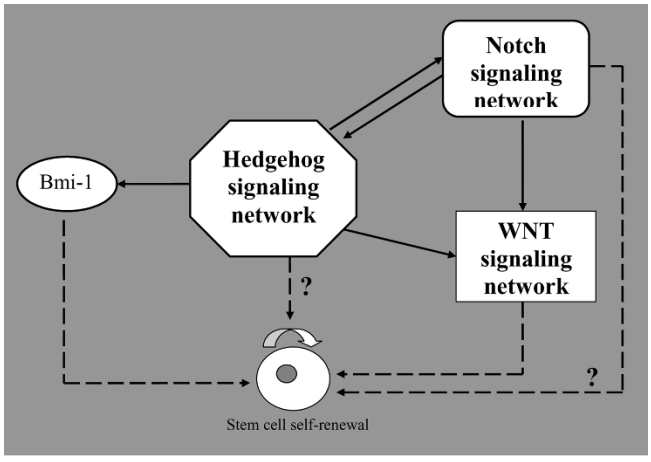
Recently we used our mammosphere-derived culture systems to examine the relationship between the hedgehog pathway and the Notch pathway, and we found that the activation of the Notch pathway resulted in the subsequent activation of the hedgehog pathway, including increased expression of Ptch and Gli. This activation could be blocked by γ-secretase inhibitor, which inhibits Notch signaling (Liu S *et al.*, 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 (Liu S *et al.*, 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, 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 in the Hedgehog pathway and γ-secretase inhibitor in the Notch pathway, respectively (Liu S *et al*., manuscript in preparation).

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.

Figure 5



A hypothetic interacting model in the regulation of stem cell self-renewal by the Hedgehog 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 are shown by dashed arrows; the question marks represent the postulated interactions.

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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References

1. Rudland PS, Barraclough R, Fernig DG, Smith JA: **Mammary stem cells in normal development and cancer.** In *Stem Cells.* Edited by Potten CS. San Diego: Academic Press; 1997:147-232.
2. Hennighausen L, Robinson GW: **Signaling pathways in mammary gland development.** *Dev Cell* 2001, **1**:467-475.
3. Gudjonsson T, Villadsen R, Nielsen HL, Rønnov-Jessen L, Bissell MJ, Petersen OW: **Isolation, immortalization, and characterization of a human breast epithelial cell line with stem cell properties.** *Genes Dev* 2002, **16**:693-706.

4. Stingl J, Eaves CJ, Kuusk U, Emerman JT: **Phenotypic and functional characterization in vitro of a multipotent epithelial cell present in the normal adult human breast.** *Differentiation* 1998, **63:**201-213.
5. Karsten U, Papsdorf G, Pauly A, Vojtesek B, Moll R, Lane EB, Clausen H, Stosiek P, Kasper M: **Subtypes of non-transformed human mammary epithelial cells cultured in vitro: histo-blood group antigen H type 2 defines basal cell-derived cells.** *Differentiation* 1993, **54:**55-66.
6. Dontu G, Al-Hajj M, Abdallah WM, Clarke MF, Wicha MS: **Stem cells in normal breast development and breast cancer.** *Cell Prolif* 2003, **36**(Suppl 1):59-72.
7. Bonnet D, Warren EH, Greenberg PD, Dick JE, Riddell SR: **CD8$^+$ minor histocompatibility antigen-specific cytotoxic T lymphocyte clones eliminate human acute myeloid leukemia stem cells.** *Proc Natl Acad Sci USA* 1999, **96:**8639-8644.
8. Dorrell C, Takenaka K, Minden MD, Hawley RG, Dick JE: **Hematopoietic cell fate and the initiation of leukemic properties in primitive primary human cells are influenced by Ras activity and farnesyltransferase inhibition.** *Mol Cell Biol* 2004, **25:**6993-7002.
9. Singh SK, Clarke ID, Terasaki M, Bonn VE, Hawkins C, Squire J, Dirks PB: **Identification of a cancer stem cell in human brain tumors.** *Cancer Res* 2003, **63:**5821-5828.
10. Singh SK, Clarke ID, Hide T, Dirks PB: **Cancer stem cells in nervous system tumors.** *Oncogene* 2004, **23:**7267-7273.
11. Warner JK, Wang JC, Hope KJ, Jin L, Dick JE: **Concepts of human leukemic development.** *Oncogene* 2004, **23:**7164-7177.
12. Al-Hajj M, Wicha MS, Benito-Hernandez A, Morrison SJ, Clarke MF: **Prospective identification of tumorigenic breast cancer cells.** *Proc Natl Acad Sci USA* 2003, **100:**3983-3988.
13. Smalley M, Ashworth A: **Stem cells and breast cancer: a field in transit.** *Nat Rev Cancer* 2003, **3:**832-844.
14. Sherley JL: **Asymmetric cell kinetics genes: the key to expansion of adult stem cells in culture.** *Stem Cells* 2002, **20:**561-572.
15. Dontu G, El-Ashry D, Wicha MS: **Breast cancer, stem/progenitor cells and the estrogen receptor.** *Trends Endocrinol Metab* 2004, **15:**193-197.
16. DeOme KB, Medina D: **A new approach to mammary tumorigenesis in rodents.** *Cancer* 1969, **25:**1255-1258.
17. Smith GH, Chepko G: **Mammary epithelial stem cells.** *Microsc Res Tech* 2001, **52:**190-203.
18. Kim ND, Oberley TD, Yasukawa-Barnes J, Clifton KH: **Stem cell characteristics of transplanted rat mammary clonogens.** *Exp Cell Res* 2000, **260:**146-159.
19. Kordon EC, Smith GH: **An entire functional mammary gland may comprise the progeny from a single cell.** *Development* 1998, **125:**1921-1930.
20. Welm BE, Tepera SB, Venezia T, Graubert TA, Rosen JM, Goodell MA: **Sca-1^{pos} cells in the mouse mammary gland represent an enriched progenitor cell population.** *Dev Biol* 2002, **255:**42-56.
21. Muschler J, Lochter A, Roskelley CD, Yurchenco P, Bissell MJ: **Division of labor among the α6β4 integrin, β1 integrins, and an E3 laminin receptor to signal morphogenesis and β-casein expression in mammary epithelial cells.** *Mol Biol Cell* 1999, **10:**2817-2828.
22. Romanov SR, Kozakiewicz BK, Holst CR, Stampfer MR, Haupt LM, Tlsty TD: **Normal human mammary epithelial cells spontaneously escape senescence and acquire genomic changes.** *Nature* 2001, **409:**633-637.
23. Reynolds BA, Weiss S: **Clonal and population analyses demonstrate that an EGF-responsive mammalian embryonic CNS precursor is a stem cell.** *Dev Biol* 1996, **175:**1-13.
24. Weiss S, Reynolds BA, Vescovi AL, Morshead C, Craig CG, van der Kooy D: **Is there a neural stem cell in the mammalian forebrain?** *Trends Neurosci* 1996, **19:**387-393.
25. Dontu G, Abdallah WM, Foley JM, Jackson KW, Clarke MF, Kawamura MJ, Wicha MS: **In vitro propagation and transcriptional profiling of human mammary stem/progenitor cells.** *Genes Dev* 2003, **17:**1253-1270.
26. Lochrie JD, Phillips K, Shand JH, Price NC, Allan GJ, Flint DJ, Beattie J: **The insulin-like growth factor binding protein-5 (IGFBP-5) profile in primary cultures of differentiated mouse mammary epithelial cells.** *Endocr Abstr* **8**-GO6.
27. Dick JE: **Normal and leukemic human stem cells assayed in SCID mice.** *Semin Immunol* 1996, **8:**197-206.
28. Pellat-Deceunynck C, Bataille R: **Normal and malignant human plasma cells: proliferation, differentiation, and expansions in relation to CD45 expression.** *Blood Cells Mol Dis* 2004, **32:**293-301.
29. Singh SK, Clarke ID, Terasaki M, Bonn VE, Hawkins C, Squire J, Dirks PB: **Identification of a cancer stem cell in human brain tumors.** *Cancer Res* 2003, **63:**5821-5828.
30. Cohen MM Jr: **The hedgehog signaling network.** *Am J Med Genet* 2003, **123A:**5-28.
31. Lewis MT, Veltmaat JM: **Next stop, the twilight zone: hedgehog network regulation of mammary gland development.** *J Mamm Gland Biol Neoplasia* 2004, **9:**165-181.
32. Pasca di Magliano M, Hebrok M: **Hedgehog signalling in cancer formation and maintenance.** *Nat Rev Cancer* 2003, **3:**903-911.
33. Olsen CL, Hsu PP, Glienke J, Rubanyi GM, Brooks AR: **Hedgehog-interacting protein is highly expressed in endothelial cells but down-regulated during angiogenesis and in several human tumors.** *BMC Cancer* 2004, **4:**43.
34. Karhadkar SS, Bova GS, Abdallah N, Dhara S, Gardner D, Maitra A, Isaacs JT, Berman DM, Beachy PA: **Hedgehog signalling in prostate regeneration, neoplasia and metastasis.** *Nature* 2004, **431:**707-712.
35. Xie J, Johnson RL, Zhang X, Bare JW, Waldman FM, Cogen PH, Menon AG, Warren RS, Chen LC, Scott MP, Epstein EH Jr: **Mutations of the PATCHED gene in several types of sporadic extracutaneous tumors.** *Cancer Res* 1997, **57:**2369-2372.
36. Chang-Claude J, Dunning A, Schnitzbauer U, Galmbacher P, Tee L, Wjst M, Chalmers J, Zemzoum I, Harbeck N, Pharoah P, Hahn H: **The patched polymorphism Pro1315Leu (C3944T) may modulate the association between use of oral contraceptives and breast cancer risk.** *Int J Cancer* 2003,**103:**779-783.
37. Lewis MT: **Hedgehog signaling in mouse mammary gland development and neoplasia.** *J Mammary Gland Biol Neoplasia* 2001, **6:**53-66.
38. Kubo M, Nakamura M, Tasaki A, Yamanaka N, Nakashima H, Nomura M, Kuroki S, Katano M: **Hedgehog signaling pathway is a new therapeutic target for patients with breast cancer.** *Cancer Res* 2004, **64:**6071-6074.
39. Mumm JS, Kopan R: **Notch signaling: from the outside in.** *Dev Biol* 2000, **228:**151-165.
40. Wu JY, Rao Y: **Fringe: defining borders by regulating the notch pathway.** *Curr Opin Neurobiol* 1999, **9:**537-543.
41. Krause DS: **Regulation of hematopoietic stem cell fate.** *Oncogene* 2002, **21:**3262-3269.
42. Weijzen S, Rizzo P, Braid M, Vaishnav R, Jonkheer SM, Zlobin A, Osborne BA, Gottipati S, Aster JC, Hahn WC, *et al.*: **Activation of Notch-1 signaling maintains the neoplastic phenotype in human Ras-transformed cells.** *Nat Med* 2002, **8:**979-986.
43. Okano H, Imai T, Okabe M: **Musashi: a translational regulator of cell fate.** *J Cell Sci* 2002, **115:**1355-1359.
44. Clarke RB, Anderson E, Howell A, Potten CS: **Regulation of human breast epithelial stem cells.** *Cell Prolif* 2003, **36(Suppl 1):**45-58.
45. Clarke RB, Spence K, Anderson E, Howell A, Okano H, Potten CS: **A putative human breast stem cell population is enriched for steroid receptor-positive cells.** *Dev Biol* 2005, **277:** 443-456.
46. Uyttendaele H, Soriano JV, Montesano R, Kitajewski J: **Notch4 and Wnt-1 proteins function to regulate branching morphogenesis of mammary epithelial cells in an opposing fashion.** *Dev Biol* 1998, **196:**204-217.
47. Soriano JV, Uyttendaele H, Kitajewski J, Montesano R: **Expression of an activated Notch4(int-3) oncoprotein disrupts morphogenesis and induces an invasive phenotype in mammary epithelial cells in vitro.** *Int J Cancer* 2000, **86:**652-659.
48. Veeman MT, Axelrod JD, Moon RT: **A second canon. Functions and mechanisms of beta-catenin-independent Wnt signaling.** *Dev Cell* 2003, **5:**367-377.
49. Schweizer L, Varmus H: **Wnt/Wingless signaling through beta-catenin requires the function of both LRP/Arrow and frizzled classes of receptors.** *BMC Cell Biol* 2003, **4:**4.
50. Brittan M, Wright N: **Gastrointestinal stem cells.** *J Pathol* 2002, **197:**492-509.
51. Reya T, Duncan AW, Ailles L, Domen J, Scherer DC, Willert K, Hintz L, Nusse R, Weissman IL: **A role for Wnt signalling in self-renewal of haematopoietic stem cells.** *Nature* 2003, **423:**409-414.

52. Honeycutt KA, Roop DR: **c-Myc and epidermal stem cell fate determination.** *J Dermatol* 2004, **31:**368-375.
53. Bafico A, Liu G, Goldin L, Harris V, Aaronson SA: **An autocrine mechanism for constitutive Wnt pathway activation in human cancer cells.** *Cancer Cell* 2004, **6:**497-506.
54. Liu BY, McDermott SP, Khwaja SS, Alexander CM: **The transforming activity of Wnt effectors correlates with their ability to induce the accumulation of mammary progenitor cells.** *Proc Natl Acad Sci USA* 2004, **101:**4158-4163.
55. Li Y, Welm B, Podsypanina K, Huang S, Chamorro M, Zhang X, Rowlands T, Egeblad M, Cowin P, Werb Z, *et al.*: **Evidence that transgenes encoding components of the Wnt signaling pathway preferentially induce mammary cancers from progenitor cells.** *Proc Natl Acad Sci USA* 2003, **100:**15853-15858.
56. Alkema MJ, Wiegant J, Raap AK, Berns A, van Lohuizen M: **Characterization and chromosomal localization of the human proto-oncogene BMI-1.** *Human Mol Genet* 1993, **2:**1597-1603.
57. Lessard J, Sauvageau G: **Bmi-1 determines the proliferative capacity of normal and leukaemic stem cells.** *Nature* 2003, **423:**255-260.
58. Park IK, Qian D, Kiel M, Becker MW, Pihalja M, Weissman IL, Morrison SJ, Clarke MF: **Bmi-1 is required for maintenance of adult self-renewing haematopoietic stem cells.** *Nature* 2003, **423:** 302-305.
59. Molofsky AV, Pardal R, Iwashita T, Park IK, Clarke MF, Morrison SJ: **Bmi-1 dependence distinguishes neural stem cell self-renewal from progenitor proliferation.** *Nature* 2003, **425:**962-967.
60. Dimri GP, Martinez JL, Jacobs JJ, Keblusek P, Itahana K, Van Lohuizen M, Campisi J, Wazer DE, Band V: **The Bmi-1 oncogene induces telomerase activity and immortalizes human mammary epithelial cells.** *Cancer Res* 2002, **62:**4736-4745.
61. Crawford YG, Gauthier ML, Joubel A, Mantei K, Kozakiewicz K, Afshari CA, Tlsty TD: **Histologically normal human mammary epithelia with silenced $p16^{INK4a}$ overexpress COX-2, promoting a premalignant program.** *Cancer Cell* 2004, **5:**263-273.
62. Lopez SL, Paganelli AR, Siri MV, Ocana OH, Franco PG, Carrasco AE: **Notch activates sonic hedgehog and both are involved in the specification of dorsal midline cell-fates in *Xenopus*.** *Development* 2003, **130:**2225-2238.
63. Nicolas M, Wolfer A, Raj K, Kummer JA, Mill P, van Noort M, Hui CC, Clevers H, Dotto GP, Radtke F: **Notch1 functions as a tumor suppressor in mouse skin.** *Nat Genet* 2003, **33:**416-421.
64. Lawson ND, Vogel AM, Weinstein BM: **sonic hedgehog and vascular endothelial growth factor act upstream of the Notch pathway during arterial endothelial differentiation.** *Dev Cell* 2002, **3:**127-136.
65. Kopper L, Hajdu M: **Tumor stem cells.** *Pathol Oncol Res* 2004, **10:**69-73.
66. Leung C, Lingbeek M, Shakhova O, Liu J, Tanger E, Saremaslani P, Van Lohuizen M, Marino S: **Bmi1 is essential for cerebellar development and is overexpressed in human medulloblastomas.** *Nature* 2004, **428:**337-341.
67. Al-Hajj M, Becker MW, Wicha M, Weissman I, Clarke MF: **Therapeutic implications of cancer stem cells.** *Curr Opin Genet Dev* 2004, **14:**43-47.
68. Romer JT, Kimura H, Magdaleno S, Sasai K, Fuller C, Baines H, Connelly M, Stewart CF, Gould S, Rubin LL, *et al.*: **Suppression of the Shh pathway using a small molecule inhibitor eliminates medulloblastoma in $Ptc1^{+/-}$ $p53^{-/-}$ mice.** *Cancer Cell* 2004, **6:**229-240.