Chapter 6
Self-Renewal Pathways in Mammary Stem Cells and Carcinogenesis

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Abstract Accumulating evidence shows the presence of a subpopulation of cancer stem cells (CSCs) in many cancers including breast cancer. The breast cancer stem cells (BCSC) are resistant to traditional treatments and able to initiate tumorigenesis, suggesting that they may contribute to therapy resistance and relapse. The expression of specific markers in BCSCs and development of mouse model has facilitated the study and several intrinsic and extrinsic pathways maintaining BCSC population have been exploited. Several signal transduction pathways such as Wnt, Notch, Hedgehog, Bmi-1, PI3K/AKT and IL6 are known to regulate self-renewal pathways in normal stem cells; while in CSCs these pathways are normally dysregulated due to accumulated mutations and epigenetic changes. Understanding the signaling pathways through which CSCs regulate their self-renewal and maintenance, and hence tumor growth and metastasis is important for developing targeted therapies to abrogate CSCs.

Keywords Mammary stem cells • Breast cancer stem cells • Carcinogenesis • Self-renewal pathways

1 Introduction

The mammary gland is a dynamic organ which undergoes massive morphological changes during puberty, pregnancy, lactation and involution. Under the influence of systemic sex hormones including estrogen and progesterone, the mammary epithelium proliferate and differentiate to accumulate fat and develop lobulo-alveolar structure and lactating ducts during puberty, pregnancy and lactation, and undergo regression with massive apoptosis during mammary involution. The dynamic cycle

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of mammary gland suggests the existence of stem-like cells within mammary epithelium (Hennighausen and Robinson 2005; Smalley and Ashworth 2003). Recent prospective isolation and characterization of mammary stem cells and progenitor populations in the mouse (Asselin-Labat et al. 2006; Stingl et al. 2006; Shackleton et al. 2006; Sleeman et al. 2005) and human (Shipitsin et al. 2007; Villadsen et al. 2007; Lim et al. 2009; Eirew et al. 2008; Keller et al. 2012) mammary gland provide further evidence that the mammary epithelium is organized in a hierarchical manner, and that a single mammary stem cell (MaSC), which resides in the basal/myoepithelial layer, can functionally reconstitute a mammary gland by giving rise to differentiated progenies through various lineage-restricted progenitor cells (Stingl et al. 2006; Shackleton et al. 2006; Visvader 2009). Therefore, abnormalities of normal development and proliferation are not surprisingly seen in breast tissue related to the frequent proliferation/involution cellular events.

There is also increasing evidence that stem cells might be the targets of transformation during carcinogenesis. Breast cancer is defined as a malignant tumor arising from uncontrolled proliferating breast epithelial cells, and results in lumps or thickening of the breast tissue. Breast cancer is highly heterogeneous; the heterogeneity of breast cancers is manifested by their classification into a number of distinct subtypes, each with a characteristic transcriptome and molecular expression signature (Visvader 2009). The categorization into different types helps clinicians to choose the most effective therapies. For example, estrogen and progesterone receptors (ER and PR) are important prognostic indicators in breast cancer; tumors with these two receptors tend to be well differentiated and responsive to hormone-therapies, and such patients tend to have a prolonged disease-free survival rate. Tamoxifen that inhibits cell proliferation by competing with endogenous estrogens on the ER site is the most frequent prescription medicine for ER positive breast cancer patients. Although early detection and development in adjuvant systemic chemotherapy and radio-therapy increase relapse-free and overall survival rates, development of therapy-resistance and disease recurrence remain problematic. These features are key characteristics of the cancer stem cell (CSC) hypothesis: the existence of a group of cancer cells able to survive conventional therapies and give rise to tumor growth later. Accumulating evidence suggests the existence of subpopulations within a tumor that are resistant to treatments and display “stem cell” properties (Wicha et al. 2006; Liu and Wicha 2010; Charafe-Jauffret et al. 2008).

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, the transcription factor B lymphoma Mo-MLV insertion region 1 (Bmi-1), PI3K/AKT, as well as IL6 pathway. 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 also highlight the potential therapeutic approaches and clinical implications of targeting these signaling pathways for breast cancer treatment.

2 Mammary Stem Cells Versus Breast Cancer Stem Cells

Stem cells are defined by their ability to self-renew and differentiate into multiple lineages. They are important for embryonic development and tissue regeneration in adults. In adult tissues, stem cells sit in specific niches, which are important in stem cell regulation and maintenance by responding to intrinsic and extrinsic signals. In human mammary gland, the presence of breast epithelial stem cells were initially described by their ability to form colonies, which morphology resemble myoepithelial or luminal phenotypes or express markers exclusive for these two populations (Stingl et al. 1998). Mammary epithelial stem cells exhibit the following properties:

1. Expression of distinct proteins has been used to identify stem cell populations for a long time, although the functions of these markers are not always well understood. In mammary epithelial cells, MUC-1 glycoprotein $(MUC\text{-}1)^{+}$/common acute lymphoblastic leukemia antigen $(CALLA)^{-}$/epithelial-specific antigen $(ESA)^{+}$ and the $MUC\text{-}1^{-\text{ to }+/-}/CALLA^{+/-\text{ to }+}/ESA^{+}$ were suggested as the progenitor markers for ductal and alveolar cells by the group that first identified human breast epithelial progenitor (Stingl et al. 1998). Recent studies show that the use of cell surface markers CD49f (α6 integrin) and CD29 (β1 intergrin) together with CD24 (heat stable antigen) or EpCAM (epithelial specific antigen), have been shown to enrich for mammary stem/progenitors in the mouse and human mammary gland (Stingl et al. 2006; Shackleton et al. 2006).
2. The ability to eject fluorescent dyes such as Hoechst due to high activity of several transmembrane transporters leads to formation of a side population (SP). Research on normal mammary epithelium found limited SP cells, but these cells were able to differentiate into ductal and lobular cells both in vitro and in vivo (Alvi et al. 2003), indicating their stem/progenitor property.
3. The ability to display aldehyde dehydrogenase activity, which can be assessed by the ALDEFLUOR assay via flow cytometry. In vivo study showed only $Aldefluor^{+}$ cells of normal mammary epithelium were able to repopulate fat pad in mouse but not the $Aldefluor^{+}$ population. And the structures formed by $Aldefluor^{+}$ mammary epithelial cells resembled the human mammary duct phenotype as well as expressed same pattern of cytokeratins (Ginestier et al. 2007).
4. The potential to survive and proliferate when grown in anchorage-independent environment with the presence of growth factors in the form of spheroids. These floating 3D structures, termed mammospheres were capable to differentiate into both epithelial and myoepithelial lineages. More importantly, this culture technique maintained the self-renewal and multilineage potential of the mammary epithelial stem/progenitor populations (Dontu et al. 2003).

Taken together, the establishment of biomarkers, in vitro and in vivo models from studies of normal mammary stem cells has facilitated the isolation and characterization of such cells in malignant breasts.

The concept of stem driven carcinogenesis was first proposed in 1855 by The German pathologist Rudolf Virchow. For years, direct evidence to prove cancer

stem cell existence is not found. In 1997, cancer stem cell (CSCs) was first identified in acute myeloid leukemia when specific cell surface protein markers became available for distinguishing a rare population of cells (Bonnet and Dick 1997). Since then, researchers have shown many human cancers, including breast cancer, might have a population of cells that display stem cell properties (Wicha et al. 2006; Liu and Wicha 2010; Charafe-Jauffret et al. 2008). These properties include self-renewal, which gives rise to tumorigenesis, and differentiation, which contributes to cancer cell heterogeneity. These cells may mediate metastasis and, by virtue of their relative resistance to chemotherapy and radiation, contribute to treatment relapse following therapy.

Flow cytometry utilizing cell surface markers is one of useful ways to identify putative breast cancer stem cells (BCSCs). Our laboratory first isolated breast cancer initiating cells based on the expression of three unique cell surface antigens, which are epithelial specific antigen (ESA) and CD44 but not CD24. $ESA^+CD44^+CD24^-$ cells were capable to generate tumors when as few as 200 cells were injected into mammary fat pad of NOD/SCID mice, whereas cells without these markers isolated from the same tumors did not, even 100-fold more cells were injected (Al-Hajj et al. 2003). Subsequent studies show $CD44^+CD24^-$ can define a population enriched in BCSCs. The $CD44^+CD24^-$ BCSCs possess the ability to self-renew and to differentiate which undergoes xenograft mammary tumor formation and progression. Other cell surface markers, like CD49f and CD133, can be used to identify BCSCs in different breast cancer subtypes combining with $CD44^+CD24^-$. In breast cancer, elevated CD49f expression is associated with reduced survival (Friedrichs et al. 1995) and knockdown of its partner CD104 decreases in vivo tumorigenicity (Lipscomb et al. 2005). The cells enriched in $CD44^+CD49f^{hi}CD133^{hi}$ subset displays heightened tumorigenicity and self-renewal in vivo, and the capacity to give rise to functional and molecular heterogeneity (Meyer et al. 2010).

BCSCs can also be isolated or studied using the Aldefluor assay based on aldehyde dehydrogenase (ALDH) activity (Ginestier et al. 2007). ALDH is an enzyme responsible for the oxidation of intracellular aldehydes, it plays an important role in stem cell differentiation via retinoic acid metabolism. The commercially available Aldefluor kit (StemCell Technologies, Inc., Vancouver, British Columbia, Canada) contains a BODIPY-aminoacetaldehyde (BAAA) substrate labeled with a fluorochrome that is converted into BODIPY-aminoacetate (BAA) by ALDH catabolism. Cells expressing high ALDH activity have brighter fluorescence and can be indentified using DEAB (an inhibitor of the enzymatic reaction) as the isotype control for FACS analysis. The combination of Aldefluor positivity with other unique stem cell surface markers such as $CD133^+$ and $CD24^-CD44^+$ has been shown to further label and locate BCSCs (Charafe-Jauffret et al. 2009).

Furthermore, in primary breast xenografts, $CD44^+CD24^-$ and ALDH identified overlapping, but non-identical cell populations, each capable of initiating tumors in NOD/SCID mice. Tumor cells that expressed both CSC markers (i.e. $CD44^+CD24^-$ and $ALDH^+$) displayed the greatest tumor-initiating capacity, generating tumors in

NOD/SCID mice from as few as 20 cells. The $EpCAM^{+}CD24^{-}CD44^{+}$ and $ALDH^{+}$ populations across different subtypes of breast cancers identify anatomically distinct BCSCs with respective EMT (epithelial-to-mesenchymal transition) and MET (mesenchymal-to-epithelial transition) gene expression profiles, and they dynamically transit between the mesenchymal and the epithelial states reflective of their normal counterparts in the mammary epithelial hierarchy (Liu et al. 2014).

BCSCs can subsequently be sorted and assayed for clonogenic potential in vitro and tumorigenicity in vivo by xenotransplantation using immune-compromised mice. The latter is the gold standard for assessing BCSC activity. Cells isolated from tumorspheres exhibit multi-lineage differentiation potential when given serum and extracellular matrix such as collagen (Dontu et al. 2003). Another use is based on the ability of stem cells to exclude DNA dye such as Hoechst 33,342 by membrane transporters, and the SP has been shown to contain the most tumorigenic population within breast cancer cell line when injected in vivo (Dontu et al. 2003; Hadnagy et al. 2006). A more recent method to characterize CSCs in vitro is the cell membrane label-retaining assay. This assay uses the PKH fluorescent dye series, which consist of a fluorophore attached to a peptide backbone that irreversibly binds to the lipid bilayer of cell membranes. The use of PKH dye label-retaining mammosphere assay has recently been used to identify both normal MaSCs and BCSCs (Pece et al. 2010; Cicalese et al. 2009).

3 Self-Renewal Signaling Pathways

Several pathways such as Hedgehog, Notch, and Wnt and a transcription factor Bmi-1 are known to regulate self-renewal pathways in normal stem cells; while in CSCs these pathways are normally dysregulated due to accumulated mutations and epigenetic changes. Conventional cancer therapies normally target aberrant pathways in the rapid proliferating bulk tumor cells, but often spare the CSCs leading to tumor recurrence and metastasis. Therefore, the design of new therapies must be based on targeting the signaling pathways that affect both CSCs as well as bulk tumor cells. We review the role of these signaling pathways in stem cell self-renewal as well as evidence that deregulation of these pathways is important in mammary carcinogenesis. And we review the main pathways that are involved in CSC self-renewal along with their potential therapeutic implications.

3.1 Hedgehog Signaling

The hedgehog signaling pathway was first identified in Drosophila, where it is required for early embryo patterning. Recent studies show that Hh signaling pathway regulates cell proliferation, cell fate determination and stem/progenitor cell

maintenance (Cohen 2003; Lewis and Veltmaat 2004). 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) to activate Gli transcription factors. In the absence of ligands, two transmembrane proteins, Ptch and Smoothened (Smo), form the receptor complex. Ptch binds to Smo and blocks its function. 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. Smo interacts in a signaling cascade that results in activation of the transcription factors. Gli proteins, include Gli1, Gli2, and Gli3, in turn translocate into the nucleus and control target gene transcription (di Magliano and Hebrok 2003). 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.

Ptch1, Gli1 and Gli2 genes are expressed in normal human mammary stem/progenitor cells cultured as mammospheres and are down-regulated during differentiation. Overexpression of Ptch1, Gli1 and Gli2 has been shown in $CD24^{-}CD44^{+}$ BCSCs compared to non-stem cells. Activation of Hh signaling using Hh ligand or Gli1/Gli2 overexpression increases mammosphere formation, mammosphere size and multi-lineage progenitors, whereas inhibition of the pathway via cyclopamine results in a reduction of tumorigenic potential. Moreover, overexpression of Gli2 in human mammary stem/progenitor cells enriched in mammosphere culture produces ductal hyperplasias when these cells are implanted into the humanized fatpads of NOD-SCID mice (Liu et al. 2006).

The Hh pathway was targeted using cyclopamine, a steroidal alkaloid that down-regulates Gli1 by binding to Smo and hence suppresses the growth of breast cancer cells (Kubo et al. 2004). Subsequently, new Hh inhibitors have been developed by chemically modifying cyclopamine (Tremblay et al. 2008). At present, GDC-0449 (Vismodegib, trade name: Erivedge), the first Hh pathway inhibitor approved by FDA (Robarge et al. 2009), is undergoing clinical trials in combination with the Notch signaling inhibitor RO4929097 (a gamma-secretase inhibitor, GSI) for metastatic breast cancers where tumors cannot be surgically removed, but this trial has been suspended owing to side effects associated with this therapy, and other combination therapies are currently undergoing (http://clinicaltrials.gov/) (Hui et al. 2013). It would be particularly interesting to see the effect of these Hh inhibitors on CSCs as the Hh pathway may be activated in CSCs in response to chemotherapy or during recurrence. Since Hh signaling also imparts chemoresistance (Olive et al. 2009), the most effective cancer therapy would likely include Hh inhibitors along with cytotoxic chemotherapy.

3.2 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. The Notch proteins, involves four homologous trans-membrane receptors, Notch 1 to Notch 4, are expressed in a variety of stem or early progenitor cells. Upon binding to their cognate ligands (DSL ligands: Delta, Delta like, Jagged1 and Jagged2), the intracellular domain (ICD) of Notch is cleaved and translocates into the nucleus to activate its target genes (Chiba 2006). This process is 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). Notch signaling has emerged as a key regulator involving stem cell maintenance, cell-fate specification, and differentiation (Chiba 2006) and dysregulated Notch signaling has been implicated in a number of human malignancies (Roy et al. 2007; Radtke and Raj 2003). In vitro, overexpression of the constitutively active form of Notch4 inhibits the differentiation of normal breast epithelial cells. 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. Knockdown of the canonical Notch effector Cbf-1 in MaSC-enriched population was found to increase stem cell activity whereas constitutive Notch signaling specifically targeted luminal progenitor cells for expansion, leading to hyperplasia and tumorigenesis (Bouras et al. 2008). 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 (Uyttendaele et al. 1998; Soriano et al. 2000), suggest that abnormal Notch signaling might be involved in carcinogenesis, through the deregulation of normal mammary stem cell self-renewal. In human breast cancers, co-expression of Jag1 and Notch1 is associated with poor overall survival (Reedijk et al. 2005). In $ESA^{+}CD24^{-}CD44^{+}$ BCSCs, Notch-4 and Notch-1 activity was found to be eightfold and fourfold higher respectively compared to the differentiated bulk tumor cells (Harrison et al. 2010). Pharmacologic or genetic inhibition of Notch1 or Notch4 reduced stem cell activity in vitro and reduced tumor formation in vivo (Harrison et al. 2010). Elevated Notch-1 signaling also contributes to drug resistance as down-regulation of Notch-1 signaling in human breast cancer cells increases chemo-sensitivity to doxorubicin and docetaxel (Zang et al. 2010).

Several important oncogenic pathways such as ErbB2, Jak/Stat, TGF-β, NF-κB, Wnt and Hedgehog interact with the Notch pathway (Olsauskas-Kuprys et al. 2013). For example, ErbB2 has been shown to induce Notch-1 activity through Cyclin D1 induction (Lindsay et al. 2008). Combined treatment of DAPT, a Notch inhibitor with ErbB2 inhibitor Lapatinib effectively targets stem/progenitor cells both in vitro and in vivo in breast ductal carcinoma in situ (DCIS) (Farnie et al. 2013). Another study showed that Notch-1 signaling is decreased in ErbB-2 overexpressing SKBR3,

BT474 and MCF7/HER2 cells and that HER2-targeted therapies using trastuzumb or lapatinib reactivated Notch-1 and rendered them sensitive to GSIs (Osipo et al. 2008). These studies suggest that combined treatment of GSI with HER2 targeted therapies may be more beneficial and could potentially reverse the resistance of HER2 targeted therapies especially in CSCs. It has also shown the association between Notch3 and EGFR receptors. Inhibition of EGFR kinase activity leads to activation of Notch transcriptional targets in a gamma secretase inhibitor sensitive manner and causes Notch activation, leading to an increase in ALDH+ cells (Arasada et al. 2014). Together these studies suggest that treatments aimed at molecules that affect multiple stem cell pathways could present a novel strategy for targeted therapies.

3.3 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 glycoproteins. The well-characterized Wnt signaling pathway is called the canonical Wnt pathway, in which Wnt ligands signal through the stabilization of β-catenin. Several β-catenin-independent Wnt signaling pathways, known as non-canonical, have been shown to be crucial for different aspects of vertebrate embryo development (Veeman et al. 2003). 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 or LRP6). 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 leading to its ubiquitin-mediated degradation. When the Wnt pathway is activated, GSK-3β is inhibited, blocking β-catenin phosphorylation. Activation of the Wnt pathway phosphorylates GSK3β and hence stabilizes β-catenin which then translocates to the nucleus, where it binds to and activates the transcription factors T-cell factor/lymphoid enhancer factor (TCF/LEF), which then activates several oncogenes such as ID2, MMP7, and c-Myc (Klaus and Birchmeier 2008). The noncanonical Wnt signaling pathway is known to act through Rho family small GTPase, calcium and protein kinase A signaling. It 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. Dsh can also stimulate calcium flux and sequentially activates the calcium-sensitive kinases protein kinase C and calmodulin-dependent protein kinase II (Veeman et al. 2003).

The Wnt pathway regulates cell fate determination in several tissues including the mammary gland (Logan and Nusse 2004). In LRP5 knockout mammary glands, very few stem or progenitor cells were present compared to wild type mammary

glands (Badders et al. 2009). Activation of Wnt signaling and its components have been implicated in variety of cancers including breast (Bafico et al. 2004; Klopocki et al. 2004; Nagahata et al. 2003; Nakopoulou et al. 2006). Transgenic mice overexpressing Wnt-1 in mammary glands were enriched for epithelial cells expressing progenitor cell markers keratin 6 and Sca1 and tumors that developed in these mice contained cells expressing keratin 6 (Li et al. 2003). This suggests that mammary stem cells and/or progenitors may be the targets for oncogenesis by Wnt pathway. Furthermore, the transforming activity of Wnt effectors was shown to be correlated with their ability to induce accumulation of mammary progenitor cells (Liu et al. 2004). The AKT/β-catenin pathway is also activated by anti-angiogenic agents such as sunitinib and bevacizumab which drives CSCs expansion through HIF1 alpha (Conley et al. 2012). Targeting of the Wnt pathway could be achieved by several approaches. For example, methylation-associated silencing of SFRP1 was shown to inhibit Wnt signaling in breast cancer (Yang et al. 2009). In breast cancer cell lines including MCF7, HuL100 and SKBR3, incubation with Wnt1 monoclonal antibody has been used to inhibit Wnt-1 signaling and induce apoptosis (He et al. 2004). The redundancy between different ligands may suggest that antibody directed Wnt inhibition would not be a successful approach, but some cancers have been shown to rely heavily on specific Wnt isoforms, it may be a viable approach in those cancers. For tumors which do not rely on specific Wnt, the use of pan-Wnt inhibitor may be more efficacious. A recent study demonstrated that a soluble ligand binding domain of Fzd8, Fzd8-CRD-Fc, inhibited autocrine Wnt signaling in vitro, as well as in multiple xenograft models (DeAlmeida et al. 2007).

3.4 Bmi-1 Signaling

Bmi-1 is a transcriptional repressor belonging to the polycomb group (PcG) of transcription factors. It was first identified in a B-cell lymphoma (Alkema et al. 1993). Bmi-1 has been shown to be a key regulator of the self-renewal of many normal and cancer stem cells. Recent studies have shown that Bmi-1 is a marker of CSCs. Bmi-1 is found to be overexpressed in several human breast cancer cell lines, playing a pivotal role in maintaining stem cells phenotype and carcinogenesis. Several recent studies have demonstrated a role of Bmi-1 in regulating EMT and migration of breast cancer cells. Bmi-1 is overexpressed in primary human breast cancer and metastatic breast cancer cells, regulating EMT and metastasis of cancer cells (Li et al. 2014). In Twist-induced EMT, Twist1 and Bmi-1 act cooperatively to repress expression of epithelial marker, E-cadherin, and promote tumor-initiating capability (Yang et al. 2010). Overexpressed of Bmi-1 due to positive feedback loop between Bmi-1 and Wnt is likely to increase the fraction of CSCs and endow tumors resistance to drug (Cho et al. 2013).

Since its essential role in cancer cell and contribute to drug resistance, Bmi-1 might be a new target in breast cancer therapy. Researchers have found that elevated Bmi-1 expression is correlated with advanced stage of breast cancer, especially

basal-like breast cancer (Guo et al. 2010; Wang et al. 2012). Drugs aimed at Bmi-1 or its regulation pathway may be potent in controlling or even completely removing cancer. Several attempts have been made. Joon-Ho et al. suggests that since Akt phosphorylating Bmi-1 inhibiting self-renewal of hemopoietic stem cells, overexpression of Akt may reduce the fraction of CSCs (Liu et al. 2012). Kreso and colleague found that, PTC-209, a Bmi-1 inhibitor, can effectively block self-renewal of cancer initiating cells in vitro and tumor growth in mouse xenograft in colon cancer (Kreso et al. 2014). Besides, there is report about Bmi-1 autoantibody, pointing out that Bmi-1 autoantibody acting as a new potential biomarker for cervical carcinoma, implying an antibody therapy (Tong et al. 2011). Yet whether this compound and antibody is also apply to breast cancer, more studies need to be explored.

3.5 PI3K/AKT Signaling

The PI3K/Akt signaling pathway plays a pivotal role in cell survival, proliferation, migration, metabolism, angiogenesis, and apoptosis. Akt kinase is activated after activation of PI3K in growth factor receptor-mediated signaling cascades. A simple model for activation by growth factor is that, upon growth factor combining to receptor tyrosine kinase, PI3K is recruited to the plasma membrane via its Src-homology (SH) domain. Catalytic subunit of PI3K then phosphorylates phosphatidylinositol-4,5-bisphosphate (PIP2) and thus generates phosphatidylinositol-3,4,5-trisphosphate (PIP3). The increased PIP3 acts as an anchor point and recruits PH-domain-containing protein, such as Akt. The recruited Akt is then phosphorylated at Thr-308 and Ser-473 by phosphoinositide-dependent kinase 1 (PDK1) and PDK2, respectively, and then phosphorylates downstream effectors in cytoplasm and nuclear (Franke 2008).

The PI3K/Akt signaling pathway participates in a wide spectrum of tumor types. The aberrant activation of the PI3K/Akt pathway and mutations in the phosphatase and tensin homolog (PTEN) gene, whose product inhibits downstream products of PI3K and activation of Akt, have been validated by epidemiolodical and experimental studies as a key step in the initiation and maintenance of human cancers. Study has found that PTEN/PI3K/Akt pathway plays an essential role in maintenance prostate cancer stem-like cells (CSLCs). A $CD133^{+}/CD44^{+}$ population of cells enriched in the prostate cancer progenitor cells have the potential to initiate tumor. These cells preferentially activate PI3K/Akt pathway in the sphere-forming condition. Inhibition of PI3K can repress the growth of these cells. What's more, shRNA-mediated knockdown of PTEN increases the sphere-forming, clonogenic and tumorigenic potential (Dubrovska et al. 2009). Study in colitis indicated that, PI3K/Akt signaling cooperates with the Wnt to increase activation of β-catenin via phosphorylating on Ser-552, thus promoting activation of progenitor cells in the process from chronic ulcerative colitis to colitis-associated cancer (Lee et al. 2010). Anne and colleagues suggested that PTEN/PI3K/Akt may have a role in regulating mouse and human gliomas, for the observation that Akt can regulate the activity of ABCG2,

which endows CSLCs with chemoresistance, and that knockdown of PTEN can increase the fraction of CSLCs (Bleau et al. 2009).

The alteration of PI3K/Akt signaling pathway, through either activation of oncogenes or inactivation of tumor suppressors, is also a commonly disrupted pathway in human breast cancer and has a major role in anti-cancer drug resistance. Zhou et al. observed that in MCF7 cell line, PI3K/PTEN signaling has a pivotal role in maintaining the survival and proliferation of side population cells that are rare cell populations known to enrich CSLCs within cancers and cell lines (Zhou et al. 2007). There is study suggests that, different PI3K/Akt pathway aberrations may play distinct role in the pathogenesis of different breast cancer subtypes. PI3K mutations are more common in hormone receptor-positive and HER2-positive than in basal-like tumors, while Akt and PTEN mutations are restricted to hormone receptor-positive tumors. Besides, PI3K and PTEN mutations are more common in cell lines than in tumors, while Akt mutations are absent in cell lines. Thus PI3K-targeted therapy in hormone receptor-positive breast cancer might be a potential therapy (Stemke-Hale et al. 2008). On the other hand, Serra et al. found that, in HER2-overexpressing breast cancer cells, inhibition of PI3K can abolish Akt activation but result in a compensatory activation of the ERK signaling pathway due to activation of HER family receptors, which can be prevented by either MEK inhibitor or anti-HER2 monoclonal antibodies and tyrosine kinase inhibitors. Therefore they proposed a combined therapy administrating PI3K inhibitors with either HER2 or MEK inhibitors (Serra et al. 2011). But whether these therapies is of use in clinical and is there any other interaction between PI3K/Akt and other pathway, much more need to be done.

3.6 IL6 Signaling

Cytokines generated by cells within the tumor microenvironment stimulate CSC self-renewal, which then may promote tumor growth and metastasis (Sansone et al. 2007; Ginestier et al. 2010). IL6, one of the Cytokines, plays a crucial role in the pathophysiology of cancer (Rose-John et al. 2006). In cancer patients, high levels of IL6 are associated with poor patient outcome and in pre-clinical models IL6 has been shown to promote tumorigenesis, angiogenesis and metastasis (Scheller and Rose-John 2006; Fisman and Tenenbaum 2010). IL6 triggers the gp130 and IL6R proteins to form a complex after IL6 interacting with its receptor, IL6R, and this complexes could activate Stat3 (Heinrich et al. 1998). Stat3 activation in turn leads to transcriptional activation of NF-κB in inflammatory cells which secrete additional IL6 and IL-8 acting on tumor cells. Thus, these cytokines generate a positive feedback loop between immune cells and tumor cells which further stimulates the tumor stem cell components accelerating metastasis and therapeutic resistance. Utilizing mouse xenografts, we have demonstrated that bone marrow mesenchymal stem cells are recruited to sites of growing breast cancers by gradients if IL6 (Liu et al. 2011). IL6 could interact with a lot of factors to affect the cancer. IL6 regulated

the transcriptional and epigenetic mechanisms of CYP2E1 and CYP1B1 in colorectal cancer to promote colorectal carcinogenesis (Patel et al. 2014). In pancreatic carcinoma, heparanase induced macrophages to produce more IL6 to induce STAT3 signaling and to augment pancreatic carcinoma cell proliferation (Hermano et al. 2014). Maria Ouzounova et al. has confirmed the relationship between IL6 and p53/PTEN (Ouzounova et al. 2014). They developed transformed MCF10A model by simultaneous knockdown of p53 and PTEN and in this model, they demonstrated that enhanced the expression of SOCS3 could reduce the tumor growth and inhibited metastasis. Importantly, SOCS3 negatively regulated the IL6/Stat3/NF-kB pathway and this is why it could have the effect on tumor. All of above suggested that IL6 plays critical role in cancers and IL6 has been used as a therapeutic target. Some data suggest that in ER+ breast cancer the patients have "high-producer" IL6 genotypes and poor prognosis. And those tumor cells had a high expression of IL6 gp130 receptor, JAK/STAT signaling and cyclin D, suggesting targets for intervention in these patients (Demichele et al. 2014). Clinical trials utilizing IL6 blocking antibodies have been initiated for the treatment of multiple myeloma with early encouraging results (Fulciniti et al. 2009). Furthermore, anti-IL6R antibody, tocilizumab, has been approved for the treatment of arthritis (Ohsugi and Kishimoto 2008) with little clinical toxicity.

4 Interaction Between Self-Renewal Pathways

The signaling pathways regulating stem cell self-renewal described above have interactions between each other in vivo. Accumulating evidences have showed the interactions between the Hedgehog signaling and the Notch signaling pathways. Notch, executing the cell fate, can be reinforced by the secretion of Shh (López et al. 2003). In order to examine the relationship between Hedge signaling and Notch signaling, we utilized mammosphere-derived culture systems, and it was found that when we activated the Notch pathway, the hedgehog pathway was subsequently activated and the expression of Ptch and Gli was also up-regulated. Additionally, if we blocked activation of Notch signaling by γ-secretase, the hedgehog pathway remained unactivated (Liu et al. 2006). However, studies have shown that during arterial endothelial differentiation, Shh, one of the hedgehog ligands, acts upstream of Notch to determine arterial cell fate (Lawson et al. 2002). As Shh activates hedgehog pathway, expression of HES1, the Notch pathway target in the mammospheres, is also up-regulated, which could be blocked by the hedgehog inhibitor cyclopamine (Liu et al. 2006). So, it might be a feedback loop that Hedgehog and Notch forms to regulate normal and cancer development.

Studies have shown that Wnt pathways are involved in the regulation of multiple pathways. β-catenin and LEF-1 are the two markers of active Wnt signaling.

Evidences have shown that in the skin, the activation of β-catenin and LEF-1 correlates with Notch-dependent transformation (Kopper and Hajdú 2004). In chronic myeloid leukemia, Activation of Stat3 inducing by hyperactive Shh signaling in CD34+ CML up-regulates expression of downstream target genes Wnt3a, Lef1, CyclinD1, Gli1 and p21, leading to overactive Wnt signaling. The hyperactive Wnt in turn together with Shh promotes the expression of Lef1 and CyclinD1, causing uncontrolling proliferation of cells (Sengupta et al. 2007). Recent studies represent that the Wnt pathway interacts with Notch through Wnt/TCF target Jagged-1, a Notch ligand, and Mel-18, a negative regulator of Bmi-1. Knockdown of Mel-18 has been shown to enhance the self-renewal of BCSCs whereas its overexpression inhibited the number and self-renewal activity of BCSCs. Mel-18 blockade up-regulated Jagged-1 expression and consequently activated the Notch pathway (Won et al. 2012). Activation of Wnt signaling and its components have been implicated in variety of cancers including breast cancer, indicating cancer arise and develop through interactions of different pathways.

Evidences have shown that Bmi-1 acts as a downstream target in Shh pathway, as its expression rapidly increased after addition of Shh or overexpression of the Shh target Gli in cerebellar granular cells. Due to the association between overexpression of Bmi-1 and overexpression of Ptch and Sufu, the Hedgehog pathway is at least partially activated in Bmi-1 overexpression tumors (Leung et al. 2004). Our studies indicate that activation of Hedgehog pathway and Notch pathway resulted in the expression of Bmi-1 in the mammosphere culture system, and this induced expression could be aborted using inhibitors targeting Hedgehog and Notch (Liu et al. 2006). Recent studies have explored the relationship between Bmi-1 and Wnt signaling pathway. Joon-Ho et al. demonstrates a positive feedback loop regulating the autoregulation of Bmi-1. Bmi-1 can transcriptionally repress DKK family protein, a Wnt inhibitor, and up-regulate the Wnt factors, hence up-regulating the canonical Wnt signaling pathway, resulting up-regulation of c-Myc, which in turn promotes up-regulation of Bmi-1 (Cho et al. 2013).

Furthermore, recent study demonstrated that IL6 treatment triggered Notch-3–dependent up-regulation of the Notch ligand Jagged-1 and promotion of MS and MCF-7–derived spheroid growth (Sansone et al. 2007). And some papers indicated that IL6 meditated Jagged1-Notch1 promotes breast cancer bone metastasis (Sethi et al. 2011). All of those suggests that IL6 may regulate stem cells through Notch pathways. On the contrary, it has been reported that suppression of IL6, GM-CSF, and MMP-3 production by DLL-1 blockade might be responsible for the amelioration of arthritis in a mouse model of RA (Sekine et al. 2014). What's more, ADAM10 mediates a canonical Notch-dependent regulation of IL6 through Dll4 in human endothelial cells (Pabois et al. 2014). Thus, IL6 and Notch could interact with each other to regulate CSC.

Together, all these studies demonstrate extensive interaction between the signaling pathways that regulate stem cell self-renewal as elucidated in Fig. 6.1.

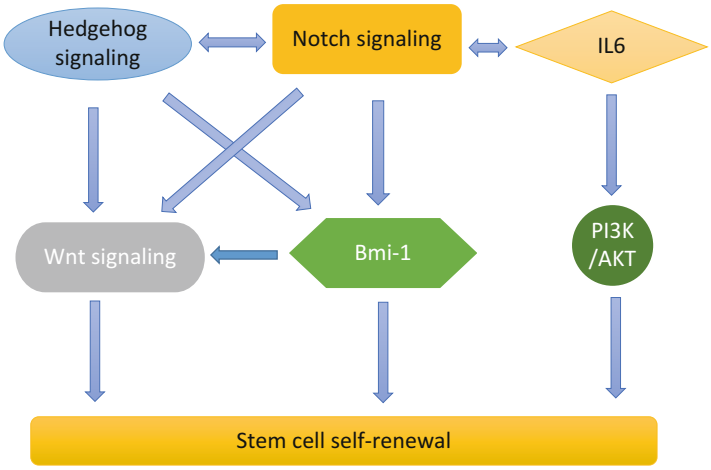

Fig. 6.1 Interaction between the signaling pathways that regulate stem cell self-renewal

5 Conclusions and Future Perspectives

In this chapter, we demonstrated that several transduction pathways, including Hedgehog, Notch, Wnt, the transcription factor Bmi-1, PI3K/Akt, and IL6 regulate self-renewal pathways in normal stem cells, while in CSCs these pathways are normally dysregulated due to accumulated mutations and epigenetic changes. Conventional cancer therapies normally target aberrant pathways in the rapid proliferating bulk tumor cells, but often spare the CSCs leading to tumor recurrence and metastasis. Therefore, the design of new therapies must be based on targeting the signaling pathways that affect both CSCs as well as bulk tumor cells. One challenge here is understanding the signaling pathways through which CSCs regulate their self-renewal and maintenance and hence tumor growth and metastasis, so we can use that as a target to abrogate CSCs. Another challenge is the combination of drugs targeting different pathways for better treatment outcome. The combination of conventional cancer therapies together with specific CSC targeted therapies brings the promise of eradicating a cancer with no possibility of recurrence.

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