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Mammary Development and Breast Cancer: The Role of Stem Cells

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Abstract

The mammary gland is a highly regenerative organ that can undergo multiple cycles of proliferation, lactation and involution, a process controlled by stem cells. The last decade much progress has been made in the identification of signaling pathways that function in these stem cells to control self-renewal, lineage commitment and epithelial differentiation in the normal mammary gland. The same signaling pathways that control physiological mammary development and homeostasis are also often found deregulated in breast cancer. Here we provide an overview on the functional and molecular identification of mammary stem cells in the context of both normal breast development and breast cancer. We discuss the contribution of some key signaling pathways with an emphasis on Notch receptor signaling, a cell fate determination pathway often deregulated in breast cancer. A further understanding of the biological roles of the Notch pathway in mammary stem cell behavior and carcinogenesis might be relevant for the development of future therapies.

Keywords

Breast cancer; mammary stem cells; notch signaling

INTRODUCTION

Stem cells in adult tissues are of key importance for physiological tissue renewal and regeneration after injury. Understanding the molecular pathways that govern normal stem cell function may aid in the development of tissue-specific cell replacement therapies whereby stem cells adopt specific cell fates and functions in any desired organ. Although still in its infancy, cell replacement therapy using autologous stem cells holds great promise for overcoming genetic disorders and tissue regeneration after damage. Under pathological

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conditions such as uncontrolled proliferation and metastases formation in cancer, populations of tumor cells have been identified that are collectively referred to as “tumor-initiating cells (TIC) or cancer stem cells (CSC)” controlling cancer cell maintenance and growth. These cells are thought to harbor many properties of normal stem cells in that they exhibit self-renewing capacity, multipotency and their ability to initiate and sustain neoplastic growth. Increasing evidence indicates that these CSC are responsible for cancer cell maintenance that underlies malignant progression and recurrence after treatment failure.

Breast cancer affects almost 1 in 8 women in the Western world with a total of about one million new cases per year worldwide of which 35% will eventually die. Breast cancer is a complex and heterogeneous disease with several histological and molecular manifestations within tumors and between patients. A comprehensive understanding of the etiology of breast cancer is paramount to the identification of novel therapies and improving existing strategies for treatment and prevention of the disease. The mammary gland is a dynamic organ that goes through significant changes during the menstrual cycle, development, pregnancy, lactation, and involution. Normal mammary gland development and homeostasis is a stem cell driven process and key signaling pathways have been identified that control these processes. Mounting evidence indicates that the same genes that control physiological organ development and function are often deregulated in cancer.

Here we provide an overview on the functional and molecular characterization and identification of mammary stem cells in the context of normal breast development and their role in breast cancer. We highlight the key molecular pathways controlling these processes with a special emphasis on the role of Notch receptor signaling, a highly conserved cell fate determination pathway frequently altered in human breast cancer.

STEM CELLS

Stem cells can be categorized into two types: pluripotent and multipotent. Pluripotent stem cells are cells that can give rise to the three germ layers, endoderm, mesoderm and ectoderm and propagate by symmetric cell division; i.e. the capacity to give rise to identical daughter cells with identical fates. Examples are embryonic stem cells and embryonic germ cells, which will not be discussed further here. Unlike embryonic stem cells, adult stem cells are multipotent and lineage-restricted and divide by asymmetric cell divisions producing a daughter and a mother at every division with distinct cell fates.

Multipotent stem cells are present in many if not all adult tissues and are rare immature cells characterized by the ability to undergo unlimited self-renewal and differentiate into all cells of a given lineage [1]. Until recently it was assumed that stem cells are quiescent or slow-dividing so-called label retaining cells (LRCs) [2], but that dogma has recently been challenged by the identification of rapidly dividing populations of stem cells in the epidermis and gut where tissue replenishment is a continuous process [3-5]. It has become clear that these organs have both slow and fast dividing stem cell populations that perform different and overlapping functions in regulating tissue homeostasis [6].

The enormous interest in stem cell research over the last decade has led to the identification of many different markers that allow the identification and purification of cell types with

long term repopulating activity in various tissues and organs. So far the most well characterized stem cells are those which form blood, namely Hematopoietic Stem Cells (HSCs) [1]. Currently, there is a good understanding of hierarchical organization of hematopoietic lineage commitment [1]. A similar organization has been observed in other self-renewing tissues such as the intestinal epithelium [7]. Approaches analogous to the ones applied for the hematopoietic system and intestinal stem cells have been employed for the identification of mammary stem cells. Not surprisingly the continuous cell renewal required during each menstrual cycle and for lactation as well as regression in the mammary gland is also controlled by mammary stem cells [8]. The existence of a slow (LRCS) and a fast proliferating stem/progenitor populations within the mammary gland has been postulated [9].

Cancer Stem Cells

Stem cells are obvious targets of accumulating oncogenic mutations due to their longevity and capacity for indefinite proliferation [10]. However, it was only in nineties until John Dick and colleagues established the existence of tumor initiating cells or CSCs from myeloid leukemia. By careful immunophenotyping and fractionation of subpopulations of tumor cells, they established that only a fraction of the bulk of the myeloid tumor had the capacity to self-renew *in vitro* and give rise to transplantable leukemias *in vivo* [11, 12]. Whereas all cancer cells harbored the initiating mutation, only a fraction was able to initiate and maintain neoplastic growth. From these studies the concept was put forward that leukemia's are composed of a hierarchy of undifferentiated immature cells to more differentiated cells with limited potential for self-renewal. Soon hereafter colon [13], brain [14], breast [15], pancreas [16], prostate [17], and melanoma [18] CSCs have been postulated. According to this hypothesis, normal stem cells that acquire mutations during tumor evolution continue to exist within tumors and are responsible for the initiation and maintenance of neoplastic growth. TICs retain key stem cell properties such as self-renewal and the capacity to generate progenitor cells, in contrast with the bulk of tumor cells. There is increasing evidence that TICs are enriched in breast cancer patients after conventional treatment, indicating their intrinsic therapeutic resistance [19]. Thus, the first step towards understanding breast carcinogenesis is to identify the pathways that regulate normal breast development and homeostasis. This understanding may lead to insight into the pathways that drive cancer formation, progression, maintenance and resistance to therapy.

MAMMARY GLAND DEVELOPMENT

In mammals the first step in mammary morphogenesis is a thickening of the ventral ectoderm also referred to as the milk or mammary line. This structure gives rise to placodes: the precursor to the mammary bud that will give rise to a ductal branching network rooted within the fat pad and attached to the nipple. Whereas humans only have one pair of placodes that develop into two breasts, mice have 5 pair symmetrically distributed along the rostral-caudal axis between the upper- and hind limbs developing in 10 functional mammary glands. Early mammary gland morphogenesis relies on coordinated signaling between the epithelium and the underlying mesenchyme similar to the development of other epithelial appendages (e.g. limbs, hair follicles). There are important differences however between

murine and human mammary gland development. A brief overview of the key steps in mouse mammary gland development is given below and indicated where human development differs significantly.

Mouse Mammary Gland Development

Mouse mammary gland development starts around embryonic day 10.5 (E10.5) and is complete just before birth at E19 day. Just around the time of milk line development Wnt10b expression marks the epithelial and mesenchymal cells destined to form the future mammary gland [20]. Canonical Wnt signaling promotes mammary placode development and is essential for initiation of mammary gland morphogenesis and maintained in the ducts until E15.5. Activation of Wnt signaling induces placode formation and size [21, 22]. Canonical Wnt signaling is mediated by the transcription factor Lef1 and epithelial Wnt10b expression is driven by Fgf10 produced by mesenchymal cells from the somites, which is essential for midline and placode formation [23]. Together with Lef1, Tbx3 is also expressed during early mammary gland development. The combination of signaling pathways Tbx3, Fgf and Wnt regulates epithelial-mesenchymal interactions during this time. Both Fgf and Wnt signaling seem to maintain Tbx3 expression while this leads to the expression of Lef1 [24]. It is important to note that due to the spatial distribution of placodes along the rostro-caudal axis, each pair is also exposed to unique signaling cues [25]. During the embryonic development, the mammary gland remains quiescent until E15.5-E16.5 when ductal growth is stimulated by steroid hormones [26]. A combination of steroid and locally acting growth hormones like Insulin-like growth factor [27], estrogen, progesterone, and somatotropin [28] mediate developmental signals and work synergistically in the transmission of these signals to the stromal and epithelial components of the mammary gland [29]. The lumen is generated by apoptosis of central cells in the multilayered epithelium while ducts expand into the fatpads by growth centers with high mitotic rates at the tip of ducts called terminal end buds (TEBs) [30] where stem cells are thought to reside [31].

After birth, ductal growth and branching from TEBs give rise to fully developed mammary outgrowths between 3-12 weeks of age. Mammary gland goes through changes during pregnancy such as lobulo-alveolar outgrowth (pregnancy), differentiation-secretion (lactation), and apoptotic regression (involution) as part of the normal reproductive cycle [32]. Peptide and steroid hormones together with the interactions of extracellular matrix are responsible of regulating these different phases. Estrogen, progesterone, and prolactin and their cognate receptors are well known regulators required for the successful completion of this developmental cycle [33].

The adult mammary gland is composed of a highly branched system of ducts that terminate in a lobulus. Lobules are composed of alveoli, secretory cells that produce milk. Ducts are composed of two major cell types: an inner layer of luminal epithelial cells and an outer layer of contractile myoepithelial cells which are responsible for contraction in response to oxytocin during lactation [34]. The rapid proliferation and differentiation of mammary gland stem cells at the onset of lactation is necessary to provide secretory activity for milk production [35]. After weaning milk production ceases and the gland involutes [36].

Morphological and Functional Differences Between Mouse and Human

Mammary development in humans starts as a primary ectodermal outgrowth in 4-6 months old embryo [37]. At this stage, the primary bud contains a central and a peripheral-basal cell population which will give rise to different cell layers [38]. The existence and organization of these distinct cell layers is important in both mice and humans for the correct functioning of the gland and a unique feature of the mammary gland. The epithelial buds start to form from the primary bud in the 21-25 weeks of embryonic age [38] (Fig. **1**). Breast development differs between individuals of mice at the level of birth. While some individuals have a few branched ducts at that point of development, highly structured ductal tree together with regular lobules as it is observed in adults are also observed [39-41]. After birth, the effect of maternal hormones lessens and the newborn's breast involutes. In females, the ductal tree development and the stromal enlargement continue further only during puberty [42].

Mouse and human mammary gland also differ in terms of morphology. In humans, branching ducts connect to groups of small terminal ductal and alveolar structures, which are collectively called terminal ductal lobular units (TDLUs). On the contrary, TDULs are not a feature of mouse mammary epithelial tree. Instead, in the mouse, once the mammary gland is stimulated by hormones, ducts branch and elongate from TEBs. Despite these differences, there is ample evidence supporting overall similar morphology and function including the existence of mammary epithelial stem cells [43].

Developmental Patterning and Breast Cancer

Pathways involved in patterning and morphogenesis during mammary gland development are also implicated in breast cancer formation. Among these are neuregulin3 (NRG3) (an epidermal growth factor receptor (EGFR) ligand [44] involved in placode induction), Wnt signaling (essential for midline specification [20]), fibroblast growth factor (FGF) (critical for inductive signaling to the placode [45]) and Notch signaling [46] important in luminal cell fate commitment which are all frequently activated/mutated in human breast cancer. Importantly, the same pathways have also been identified by insertion mutagenesis as common proviral insertions in mouse mammary tumor virus (MMTV) induced breast cancer [47] indicating also a causal role for these signaling cascades in breast cancer in mice. Similar insertions by a complete proviral sequence, 95% to 99% homologous to MMTV, were observed also in human breast cancer tissue. This virus was named as Human Mammary Tumor Virus (HMTV) [48]. The percentage of HMTV occurrence is shown to reach up to 42% in breast cancers occurring in Europe, North America [49] and Australia [50] compared to the healthy breast tissues, which do have 1 to 2 % HMTV prevalence. High percentage of insertions seems to play a role in some human breast cancers.

EVIDENCE FOR MAMMARY STEM CELLS

The postnatal development of the mammary gland makes it possible to surgically remove the rudimentary gland leading to an epithelium free (cleared) fat pad and to study mammary development by transplantation from donor mice. The first functional evidence for the presence of murine mammary epithelial stem cells comes from transplantation experiments

of DeOme [51]. This study showed that small tissue fragments isolated from a randomly selected portion of the mammary gland were capable of regenerating a functional ductal tree upon transplantation into a cleared mammary fat-pad. Moreover, explants taken from a regenerated gland could be serially transplanted to other fat-pads [52]. While these experiments strongly pointed to a long-lived pool of epithelial progenitors, whether these contained “true” stem cells was questioned since transplant ability and ductal outgrowth could only be maintained for up to 7 generations [53-55]. More conclusive evidence for a mammary stem cell was obtained by clonal analysis using insertion mutagenesis with MMTV. These elegant studies demonstrated for the first time that an entire mammary gland composed of luminal and myoepithelial cells was a clonal derivative of a single cell. This “stem cell” was also capable of forming restricted lineages that produced only lobules or only ductal outgrowths without lobules that cannot expand upon impregnation [56]. Accordingly, it appears that mammary epithelial cell populations generated by a single stem cell comprise distinct and multipotent epithelial cells which are able to proliferate. Moreover, MMTV tagged progenitors survived multiple rounds of pregnancy and involution demonstrating that these cell had the capacity for robust proliferation and differentiation and were protected from apoptosis. It is important to note that such a stem-cell population would be exquisitely sensitive to oncogenic transformation.

It took almost another decade for researchers to identify markers that could be used to enrich for this multi-potent mammary stem cell (MaSC) and corroborate these earlier findings [57, 58].

Molecular Characterization of Mammary Stem Cells (MaSC)

Researchers have been using several complementary methods to isolate and study mammary stem/progenitor cells. The isolation of stem cells based on the expression of cell surface markers has been the most commonly used technique. The knowledge obtained in many different systems such as neuronal, hematopoietic and epidermal lineage has been an advantage for the researchers working on the characterization of mammary epithelial stem cells. The following part will provide an overview of the techniques used to identify mammary stem cells.

1. The side population technique; The Side Population (SP) is a population of cells that are characterized for their ability to actively exclude vital dyes from being taken up by the cell such as Hoechst and Rhodamine 123 while maintaining other stem cell-like markers [59, 60]. Dye uptake is an active process mediated by ATP-binding cassette family of multi drug resistance proteins e.g. MDR1/P-glycoprotein) and SP staining can be inhibited by drugs such as verapamil. Drug efflux is restricted to normal stem cells and CSC and not observed in differentiated cells. Expression of ABC is closely correlated with stem cells from a wide variety of tissues sources ranging from embryonic stem cells to bone marrow [61]. Mammary gland SP isolated from reduction mammoplasty [62], normal human [63, 64] and mouse mammary gland [65, 66] respectively have been shown to be enriched by stem/progenitor cells based on their ability to generate lobuloalveolar and ductal outgrowths upon transplantation.

2. Label retention; Label retaining cell (LRC) is another approach to identify stem cells. This approach is based on differential retention of nucleotide analogs (^{3}H-Thymidine (^{3}HTdR) or 5-BromodeoxyUridine (BrdU)) that are incorporated during S-phase between slowly proliferating versus fast proliferating cells. The LRC hypothesis has been very popular for many years particularly for epidermal and intestinal stem cells [2, 67] however recent work has challenged this hypothesis by the isolation of fast-cycling stem cells [6]. LRCs have also been reported in the mammary gland [66, 68, 69]. The undifferentiated nature of LCRs in mammary gland has been identified by the lack of differentiation markers [9]. This population of cells has been shown to divide asymmetrically and is able to repopulate stem cell niches. Although asymmetric cell divisions of stem cells have been proposed before, in a more recent study, Smith *et al*. tried to answer the question by labeling mice first with ^{3}HTdR and then with BrdU. ^{3}HTdR label was retained by LRCs in the mammary gland. Following the pulse of BrdU as a second label, most of ^{3}HTdR labeled LRCs were shown to have the BrdU label as well. As a next step, it was shown that these cells were able to undergo asymmetric divisions and pass the newly synthesized BrdU labeled strand to their progeny while retaining the ^{3}HTdR label [69].

3. Hormone receptor status; steroid hormones have a huge impact on mammary development and the majority of breast cancers are estrogen receptor (ER) positive and are an important target for anti-hormonal therapy [70]. In view of this, it is not surprising that researchers have been trying to characterize MaSCs with respect to steroid receptor expression. Previously, studies of Clarke *et al*. demonstrated the existence of steroid receptor positive cells within LRC and SP with full self-renewal and differentiation capacity [71]. On the other hand, more recent studies revealed that both mouse and human MaSCs show a ER, PR and ErbB2 negative phenotype [72]; but yet they respond to ovarian hormone signaling [73].

4. Mammosphere culture; this approach has been used to functionally identify MaSC and TICs based on their ability to form mammospheres in culture that give rise to tumors containing all the differentiated cell types present in the original tumors. [64]. Using this technique, the differentiation capacity (based on the markers specific for different lineages of the mammary gland) and clonality (i.e. by retroviral tagging) of cells enriched in the mammospheres was demonstrated. Cells from mammospheres can be serially passaged and retain their multipotency for multiple passages.

5. ALDEFLUOR assay; this is another promising approach to identify stem cells such as hematopoietic and neural stem cells which have high aldehyde dehydrogenase 1 (ALDH1) activity [74]. ALDH1 is a detoxifying enzyme responsible for the oxidation of retinol to retinoic acid and it may have a role in the early differentiation of stem cells [75, 76]. ALDH1+ cells were shown to possess functional and phenotypic characteristics of mammary stem cells even though they seem to be restricted luminal epithelial layer [77]. It has been shown that ALDH1+ cells can survive and proliferate under anchorage independent conditions and they are capable of self-renewal. Furthermore clonogenic assays demonstrated that these

cells do have the ability to give rise to mixed colonies of myoepithelial and luminal cells. ALDH1 expressing cells have been demonstrated in the normal human mammary gland and human breast cancers [78].

6. Cell surface markers; to date, different combinations of cell surface antigens have been used for the isolation of human and mouse mammary epithelial stem/progenitor cells. Although several research groups reached to an agreement on the cell surface expression profiles of stem and certain progenitor cell groups, it is important to keep in mind that the interpretation of the FACS data obtained so far mostly depends on the use of antibodies conjugated to different fluorochromes, antibody titrations and good controls. In the following part, we will review markers used to identify mouse or human mammary stem cells that are summarized in Table **1**.

Mouse Mammary Stem Cells

Defined subsets of mouse mammary epithelial cells have been used to show stem cell capacity by being able to reconstitute mammary glands when transplanted into cleared fat pads [43]. So far, the most useful markers for mouse MaSCs have been CD49 (6 integrin), CD29 (1 integrin), CD61 (3 integrin), CD24, and Sca-1 [79]. Mouse MaSCs could be purified based on $CD24^{+}CD29^{hi}CD49f^{hi}Sca1^{-}$ profile following the removal of stromal and hematopoietic cells [57, 58, 80].

A single genetically tagged cell from this population could give rise to an entire epithelial tree upon transplantation [57]. The self renewal and multi-lineage capacity of these cells was demonstrated by serial transplantations into cleared fat pads leading to mature functional glands with the formation of milk producing alveolar units upon impregnation. Moreover, when these genetically tagged cells were cultured together with wild type cells, they were able to produce chimeric structures [57, 58]. By these experiments, researchers were able to show that the purified cells based on the markers mentioned above did indeed contain stem cells. It is important to note that only a small group of MaSCs is characterized by the $CD24^{+}CD29^{hi}CD49f^{hi}Sca1^{-}$ population, others have identified other combinations of markers that indicate stemness (e.g. $CD45^{-}Ter119^{-}CD31^{-}CD49f^{hi}CD24^{med}$. Such populations are not pure since committed progenitors and differentiated cells are also present that express these markers as well [58].

Some of the markers used to enrich MaSC populations are interesting due to their regulatory roles. For example high expression levels of integrin 6 (CD49f) and 1 (CD29) both important for adhesion and migration indicates an important role for stemness and microenvironment interactions [81]. It has been previously shown that both 6 and 1 integrin expression decreases during breast cancer progression [82, 83]. Reduced levels of integrins might give rise to reduced interaction of MaSCs with the microenvironment and a failure in the self-renewal and proliferation. Deletion of 1 integrins from Keratin-5 expressing basal cells led to defects in mammary morphogenesis, proliferation and survival of mammary epithelial cells. 1 integrin deficient transplants efficiently regenerated an entire new gland albeit slower than control transplants and were characterized by disorganized ductal outgrowth indicating a lack of regenerative potential in the absence of integrin 1 [84]. A

comprehensive review on the role of integrins and extracellular matrix in mammary gland development has recently appeared [85].

Human Mammary Stem Cells

Common patterns of X chromosome-inactivation and loss of heterozygosity in adjacent areas of breast epithelium (so-called “Field effect”) have provided proof for the clonality of lineages within the normal and neoplastic human mammary gland [86, 87]. Further evidence for a hierarchical model of human breast epithelial development has been obtained by *in vitro* clonogenic assays where the human mammary stem cells have been shown to reside within the ducts since it was possible to serially passage ductal fragments [64, 88, 89]. The attempts to characterize human mammary stem cells have resulted in identification of a combination of markers such as epithelial cell adhesion molecule (EpCAM), ALDH1 and CD49f [88-92].

Eirew *et al*. developed a xenotransplantation approach to study the *in vivo* characteristics of human mammary epithelial cells. A population of cells having $EpCAM^{low}CD49f^{hi}$ profile was sorted by FACS from reduction mammoplasty samples and these cells were subsequently transplanted into immunodeficient mice together with irradiated fibroblasts. The *in vivo* experiments of Eirew *et al*. and Lim *et al*. revealed that $EpCAM^{low}CD49f^{hi}$ population are positioned basally within the mammary gland ducts and posses stem cell characteristics [90, 93]. Even though these two markers do identify different cell types when used alone, together they define a subset of human mammary cells, which have regenerative capacity in a xenograft mouse model. The self-renewal and regenerative capacity of $EpCAM^{low}CD49f^{hi}$cells was further supported by transplantation into humanized mouse mammary fat pads -Human in Mouse model (HIM)-[93, 94]. In this model, the mouse mammary fat pads are humanized by pre-injection of immortalized human fibroblasts. Using the same method, Ginestier identified another subpopulation of human stem/progenitor cell population expressing high levels of ALDH1, with high engraftment capability into NOD/SCID mice [77]. It should be noted that not only MaSCs but also luminal lineage restricted cells express ALDH1 therefore it is necessary to use a combination of different markers together with ALDH1 such as EPCAM, CD49f, MUC1 and others to obtain the highest enriched fraction of cells with MaSC ability.

Evidence for Tumor Initiating Cells in Breast Cancer

A large body of evidence has demonstrated the similarities between stem cells and cancer cells. It is well established that stem and progenitor cells are likely targets of genetic mutations necessary for carcinogenesis. The stem cell thory of cancer proposes that there is a small group of cells–cancer stem cells (CSCs) or tumor initiating cells (TICs)- within tumors that foster tumor initiation and maintenance [95, 96]. The first evidence for TICs was based on studies which showed human acute myeloid leukemia originated from a small proportion of cancer cells [12]. Since then, many groups have investigated and demonstrated the existence of tumor cells capable of self-renewal from hematopoietic to solid cancers by using different techniques [26]. A frequently applied approach has been the transplantation of tumor cells classified by cell surface markers into NOD/SCID mice to investigate the ability of subpopulations of tumor cells to give rise to new tumors. Similar approaches have

been used to identify TICs in breast cancer, which will be discussed below. The risk of developing breast cancer is 1 in every 8 women in the western world and it increases with age. This is thought to be related to the accumulation of multiple mutations in cells of the mammary gland over lifetime of women. Damaged stem cells with unlimited potential of proliferation can give rise to delayed cancers such as breast cancer after having a normal phenotype for decades. This is important because once it becomes possible to identify such cells; approaches to eliminate them would decrease the risk of breast cancer incidence and recurrence. The first prospective identification of breast cancer stem cells from human breast cancer specimens reported that within lineage - (tumor cells depleted with expression of normal antigens CD2, CD3, CD10, CD16, CD18, CD31, CD64, and CD140b) population up to 35 % of cells displayed a $CD44^{+}CD24^{-/low}$ marker profile that was able to efficiently generate transplantable tumors in mice [15, 97]. Interestingly, $ALDH1^{+}$ phenotype within a number of human breast tumor samples defined a subpopulation of cancer stem cells not overlapping with the $EpCAM^{+}CD44^{+}CD24^{-}$ phenotype [62]. When the cells were isolated based on $ALDH1^{+}EpCAM^{+}CD44^{+}CD24^{-}$ phenotype, this population was highly enriched with TIC. Another study showed that ALDH1 expression did not differ between BRCA1 (breast cancer 1) mutation carriers (who are at increased risk of developing breast cancer) and non-carriers [78]. Others however have shown a close correlation between Brca1-deficiency and ALDH1 FLUOR positivity in clinical samples and experimental models of Brca1-related cancer [98]. Even though numerous studies [99-102] have proposed the existence of breast TICs, to date the exact nature of these cells remain undefined and far from clinical utility.

SIGNALING CUES REGULATING MAMMARY STEM CELL RENEWAL AND BREAST CANCER

Normal mammary gland development is tightly controlled by TGF-β , Wnt, FGF Hedgehog, EGF, Estrogen and Notch signaling pathways [103]. It has been demonstrated that deregulation of these pathways in the mammary gland can give rise to tumor development. In the following part, we will discuss the role of these pathways with a focus on Notch signaling.

Wnt/β-Catenin

Wnt proteins are highly conserved secreted signaling molecules that regulate cell fate throughout metazoan development and homeostasis. Canonical Wnt proteins transduce signals through frizzled (FZD) transmembrane receptors which lead to inactivation of the tumor suppressor protein APC and stabilizes β-catenin which is the intracellular effector of the Wnt pathway [104]. The importance of Wnt pathway activation in breast cancer was demonstrated by identification of Wnt a common insertion site of MMTV [105]. Besides promoting maintenance and proliferation of stem cells, Wnt signaling is also shown to be crucial in pluripotency. The expression of Nanog, a gene known to be associated with pluripotency and self-renewal of stem cells, is suppressed by the Wnt-responsive transcription factor T cell factor 3 (TCF3) [106]. Wnt involvement in pluripotency is further supported by the effect of upregulation of Oct-4, a transcription factor mostly known through its involvement in the inhibition self-renewal of undifferentiated embryonic stem

cells [107]. Aberrant activation of the canonical Wnt pathway is also associated with cancer development in early-onset familial adenomatous polyposis (FAP) and late-onset spontaneous forms of colon cancer [108-109]. Upregulated Wnt activity due to the loss of the Wnt inhibitor SFRP1 (Secreted Frizzled-related protein 1) and high levels of β-catenin is correlated with poor prognosis in breast cancer [110, 111]. Moreover, β-catenin expression has been shown to be associated with survival of mammary epithelial stem cells and more efficient self-renewal [112, 113]. Transplantation of mammary epithelial cells overexpressing constitutively active β-catenin into cleared mammary fat pads gave rise to hyperplasia's [112]. These results illustrate that upregulated canonical Wnt signaling increases the mammary stem cell activity *in vivo*. Conclusive evidence for a role of Wnts in mammary stem cell renewal was recently obtained by the Nusse lab that demonstrated that purified Wnts are sufficient to promote clonogenic growth of mammary stem cells in culture and support their long-term repopulation *in vivo* [114]. It is likely that Wnt induced mitogenic effects in the mammary stem cell niche are causative to tumor initiation.

Hedgehog

The Hedgehog (Hh) pathway is another highly conserved pathway essential for early embryonic patterning and cell fate determination and in the self-renewal and maintenance adult tissues including mammary gland [115-117]. In mammalian cells, signaling takes place between the three secreted ligands; Sonic Hedgehog (Shh), Desert Hegdehog (Dhh) and Indian Hedgehog (Ihh) and cells that express the transmembrane receptor for Hedgehog ligand Patched-1 (PTCH1) and Patched-2 (PTCH2). In the absence of ligands, Ptch forms a complex with another transmembrane protein called Smoothened (Smo) inhibiting the binding of Smo to Gli: a transcription factor. In the presence of Hh, Patched binds to Hh, enabling Smo to activate Gli proteins (Gli1, Gli2 and Gli3) that activate gene transcription. Deregulated Hh signaling has been shown to be associated with a range of malignancies. Mutations found in the Hh pathway genes leading to ligand independent activation have been shown to be associated with several malignancies such as medulloblastoma, sarcoma and basal cell carcinoma [118, 119]. Overexpression of Hh ligands is frequently observed in gastrointestinal tract and lung carcinomas [120].

The evidence supporting Hh involvement in breast carcinogenesis is increasing. In one of the first studies, Lewis and colleagues showed that heterozygous disruption of Ptch1 and Gli-2, impairs ductal morphogenesis resulting in ductal hyperplasias and dysplasias [121]. Constitutive activation of Smo also leads to aberrant proliferation and ductal dysplasia [122]. The Hh pathway was first implicated in breast cancer by Kubo and colleagues who showed a correlation between high levels of Shh, Ptch and Gli1 with growth inhibition by the Hh inhibitor cyclopamine [123]. Although activating mutations in Hh pathway are common in many cancers, they are not frequently associated with breast cancer [124]. Instead, epigenetic events may be more important in Hh pathway activity in breast cancer as demonstrated by the finding that demethylating and deactylating agents can upregulate Ptch expression in breast cancer cell lines [125]. Finally, Patched polymorphisms have been shown to be associated with the risk of oral contraceptive use on breast cancer risk [126]. This suggests that Hh signaling pathway might an important role in hormone induced development of breast cancer.

Furthermore, in an *in vitro* study, addition of Shh ligand or overexpression of Gli2 into mammosphere cultures increased primary and secondary mammosphere formation and size, which was reversed by cyclopamine, a specific inhibitor of the pathway. Finally, Hh signaling has been associated with the tumorigenic phenotype of $CD24^{+}/Cd44^{-/low}/Lin^{-}$ TICs [127]. Altogether, the Hh pathway plays pivotal roles during normal mammary gland development and likely also plays unrecognized roles in breast cancer as well.

Transforming Growth Factor–Beta TGFβ

The transforming growth factor beta (TGFβ) family of polypeptide growth factors comprises secreted proteins of which there are three isoforms: TGFβ1, TGFβ2, and TGFβ3. TGFβ proteins bind to Type II receptors that heterodimerize to Type I receptors which triggers serine/threonine phosphorylation and activation of receptor SMAD proteins that in turn heterodimerize with other SMAD proteins which enter the nucleus and activate gene-transcription. TGFβ signaling controls many cellular processes during development and in adult tissues including cell proliferation, differentiation, and apoptosis, in species from worms to mammals. Defects in TGFβ signaling occur in many inherited and acquired diseases including cancer [128]. The function of TGFβ in mammary morphogenesis appears to be cell and context dependent. Several groups demonstrated the importance of different local concentrations of TGFβ within ductal epithelium as an inhibitory factor in the ductal growth and lateral branching [129, 130] TGFβ within luminal epithelial cells controls alveolar development [131, 132]. The role of TGFβ in breast cancer is complex as TGFβ has both growth suppressive as well growth promoting activities. In general TGFβ promotes tumor progression in several ways including suppression of immune responses, stimulation of angiogenesis and promotion of epithelial-to-mesenchymal transition [133], [134].

Estrogens and Progestagens

The steroid hormones estrogen and progesterone have key roles during normal mammary gland physiology from puberty to menopause. Deregulation of this hormonal regulation also markedly influences breast cancer risk forming the basis for anti-hormonal therapies in breast cancer treatment. Although the role of hormones in cancer risk has already been known for a long time it was only recently that two groups reported the underlying mechanism [73], [135]. The thought that TICs would directly respond to hormones is attractive however these cells do not express the receptors for either hormone. It appears that proliferative effect of hormones on breast tissue occurs during the normal reproductive cycle and pregnancy when hormones rise causing significant increases in MaSC numbers. The proliferative effect is indirectly mediated by the response of the Niche (basal and luminal cells) surrounding the stem cells. These produce a Wnt4a signal that stimulates MaSC proliferation. These findings pave the way to understanding why hormones can modulate growth and proliferation of the normal mammary gland and may sustain malignant growth. They also suggest that during such hormonal surges stem cells may be particularly vulnerable for accumulating mutations that lay dormant until later in life when women develop breast cancer. Such breast cancers may ultimately be unresponsive to hormonal therapies if their origin is driven by TICs. In hindsight the relationship between estrogens, MaSC and breast cancer may not be totally unexpected given the interplay between transcriptional regulation of estrogen/progesterone and cyclinD1; a gene frequently

upregulated in breast cancer [136, 137]. Mice lacking the cyclinD1 gene have defects in hormone-induced proliferation during pregnancy [138] and are protected from breast cancer [139]. Paradoxically these findings do not explain why pregnancy and breast-feeding in the long term protects against breast cancer in humans [140].

Notch

Notch signaling is a short-range cell-cell communication pathway that controls virtually every aspect of metazoan development and cellular responses to maintain tissue homeostasis in adults. Notch signaling occurs between transmembrane bound ligands and receptors on adjacent cells and is conserved from flies to mammals. Flies have a single Notch receptor and two ligands (Delta and Serrate/Jagged) whereas mammalian cells have four receptors and at least five Delta/Jagged type ligands (Fig. **2**). Notch proteins are single pass type I transmembrane receptors, with an extracellular domain involved in ligand binding, and a cytoplasmic domain involved in signal transduction [141]. During maturation in the trans-Golgi network, Notch precursors are first cleaved at Site-1 (S1) by Furin-like convertase producing a heterodimeric type I receptor with the Notch extracellular domain (NECD) non-covalently bound to a transmembrane/intracellular fragment (TMIC). The extracellular domain of Notch proteins is composed of 29-36 epidermal growth factor (EGF) like repeats involved in ligand interactions, three cysteïne rich LIN12/Notch (LNR) repeats and a heterodimerization domain (HD). The intracellular domain of Notch (NICD) contains nuclear localization signals together with a transcriptional activation domain (TAD) and a PEST domain [142]. The canonical Notch signaling cascade is regulated by proteolysis. In the absence of ligand, mature Notch receptors are held into an inactive “proteolysis resistant” state because the Negative Regulatory Region (NRR) composed of the HD domain and the Lin12/Notch repeats (LNR) inhibits Notch activation. Ligand binding to Notch receptors unfolds the NRR permitting cleavage by the metalloproteases ADAM10/Kuzbanian at a site close to the membrane termed site-2 [142] leading to shedding of the Notch extracellular domain [142, 143]. Extracellular cleavage of Notch triggers the intramembranous cleavage by the multi-subunit protein complex termed γ-secretase containing the aspartyl protease Presenilin. This leads to the release and translocation of Notch intracellular domain (NICD) to the nucleus where it interacts with the transcription factor CSL (CBF1 in humans; RBP-J_k in mice) to activate Hes/Hey family genes, which are involved in growth and proliferation, differentiation, and survival [144]. In the absence of Notch signaling, CSL inhibits the transcription of target genes.

Most if not all Notch signaling requires metalloprotease and γ-secretase cleavage to release the NICD and induce transcriptional activation by binding to CSL [145]. Notch activity is frequently deregulated in human cancer by overexpression or mutation [146]. Mutations are found in the extracellular HD and intracellular PEST domain and induce ligand-independency and increased stability of Notch [147]. Mutations that affect the activity of Notch are also found in negative regulators such as the ubiquitin ligase Fbw-7 [148, 149]. Given the frequent involvement of Notch in human malignancies, targeting Notch cleavage appears an attractive drug target. At the same time this provides challenges for drug development since such drugs would also target physiological Notch activation. Several clinical trials are underway that evaluate the efficacy of γ-secretase inhibitors (GSIs) as anti-

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cancer drugs [150]. While targeting γ-secretase has shown encouraging results in targeting tumors with activated Notch it has many pitfalls as well. Among these is the lack of specificity and the mechanism based toxicity caused by attenuating physiological Notch function. For example, the gastrointestinal toxicity caused by precocious secretory differentiation of intestinal epithelial cells prevents long-term use because of intestinal stem cell depletion [151-153]. Real *et al*. showed that such side-effects may be overcome by combination treatment with glucocorticoid and GSIs which suppressed gut toxicity through inhibition of KLF4 [154]. Since γ-secretase has many different substrates, pleiotropic effects are unavoidable when targeting this enzyme, although modulators have been identified that may show selectivity for specific substrates [155]. Despite the fact that GSIs have been studied intensely for the past decade, a safe-drug has yet to enter clinical practice [150].

Notch in Mammary Development

Members of Notch family have been shown to play important roles in normal breast development by several studies (Fig. **3**). In one of the earliest reports, Uyttendaele *et al*. demonstrated that normal breast epithelial cells failed to differentiate upon overexpression of a constitutively active form of Notch4 *in vitro* [156]. This was supported by other reports where a constitutively active form of Notch4 was expressed in transgenic mice and lead to a failure in mammary development followed by the progression of mammary tumors [157-159]. The increase in mammosphere numbers in mammosphere culture systems upon activation of Notch signaling *via* an exogenous peptide showed that Notch signaling has a promoting role in self-renewal in human primary mammary epithelial cells [160]. In addition, Notch activation facilitated proliferation and branching morphogenesis which could be inhibited by using Notch antagonists. Altogether, these reports demonstrate the importance of Notch signaling in the regulation of ductal branching in normal development where aberrant Notch signaling seems to disrupt differentiation and causes excessive proliferation.

Notch3, another member of the family, was shown to be upregulated in mammospheres formed by normal breast tissue [160] suggesting its role in self-renewal. Notch3 has also been shown to be upregulated in breast cancer cells [161]. Causality between deregulated Notch signaling and mammary carcinogenesis was exemplified by the identification of Notch1 and Notch4 loci as common insertions in MMTV induced tumors [162, 163]. The integration of MMTV into the Notch4 locus results in a Notch protein lacking most of the extracellular domain creating a ligand-independent protein [164-167]. WAP (whey acidic protein) or MMTV (mouse mammary tumor virus)-driven expression of Notch4 in transgenic mice also leads to developmental defects and formation of mammary tumors [158]. The failure in development leads to differentiation defects and hyper-proliferation of immature ductal cells. A possible explanation can be that specified cells within the mammary epithelium govern a proliferating stem cell fate that makes the cells vulnerable to mutational events driving tumorigenesis [158].

Notch activation is likely to be important for alveolar development since Notch4 overexpression leads to aberrant alveolar growth and lactation in both WAP and MMTV-Int3 female mice [157-159]. This observation was supported by the observation that loss of

RBP-J_k and Pofut1, a fucoslytransferases necessary for the activity of Notch proteins, leads to a disturbed balance in basal cells at the expense of luminal cell fates [168].

Notch and Mammary Stem Cells

Dontu *et al*., were among the first who demonstrated the involvement of Notch signaling in mammary stem cell renewal [160]. By utilizing the mammosphere system, they were able to show that human mammary stem/progenitor cells formed an increasing number of mammospheres when Notch signaling was activated, and that sphere formation was inhibited when Notch signaling was blocked. In the same study, they demonstrated the role of Notch4 in myoepithelial lineage commitment and branching morphogenesis of human mammary stem/progenitor cells in three dimensional matrigel cultures. More genetic approaches have also been used to block the Notch pathway, such as the targeted disruption of RBP-jk in the mouse mammary gland [168]. Even though no aberrant changes were observed in virgin animals, alveolar cell maintenance and basal cell proliferation increased dramatically by the loss of RBP-jk upon pregnancy. Thus Notch appears to be regulating alveolar development during pregnancy by controlling the luminal cell fate since the luminal cells fail to differentiate upon loss of Notch signaling. The role of Notch signaling in luminal cell fate commitment was identified by Bouras who demonstrated high Notch1 activity in luminal progenitor cells *in vivo* [169]. Moreover, constitutive activation of Notch1 in $CD29^{hi}CD24^{+}$cells was shown to stimulate luminal cell fate commitment leading to excessive proliferation and eventually tumorigenesis. Conversely, knockdown of RBP-Jk led to an increase in number, size and clonogenic capacity of $CD29^{hi}CD24^{+}$ mammary epithelial cells. Notch pathway inhibition induced aberrant luminal cell differentiation and expansion of basal cells. In contrast, Notch4 was shown to be down-regulated in luminal restricted stem/progenitor cells whereas Notch3, was shown to be upregulated [169]. Functional studies supported this further where Notch3 signaling was blocked and this retarded the luminal cell fate commitment where it stimulated myoepithelial cell differentiation. This was further confirmed by Rouf who performed a transcriptome analysis and showed that upregulation of Notch3 and down-regulation of Notch4 characterized the luminal cell lineage [91].

Notch in Mammary Carcinogenesis

MMTV insertions in the Notch1 locus were first identified in MMTV-Neu mammary tumors [162] as a collaborating oncogene in ErbB2/Neu driven mammary tumorigenesis. Notch1 was shown to be rearranged in 2 out of 24 MMTV-Neu mammary tumors investigated. These insertions caused expression of constitutive active truncated Notch1 proteins in a similar manner to that observed in the case of Notch4. Wnt and Notch signaling also collaborate in the transformation of human primary mammary epithelial cells (HMECs) [170]. In this study, ectopic expression of Wnt1 was shown to induce overexpression of Notch ligands (Dll1, Dll3, and Dll4). Further, it induced Notch receptor cleavage, which leads to Notch activity resulting in mammary epithelial transformation. HMECs with constitutive activation of Notch1 detached from culture plates and began to form spherical structures. However these spheres failed to proliferate, indicating that Notch1 activation alone was not sufficient to induce transformation of these cells. Interestingly, Notch1 activation in 3 dimensional cultures of MCF-10A, an immortalized human mammary

epithelial cell line, yields heterogeneous phenotypes such as large and hyper-proliferative structures or small and growth arrested structures [171]. Based on these results, it is postulated that different phenotypes are related with dose and context dependence of Notch pathway [172].

There is increasing evidence that activation of Notch signaling is a frequent event in human breast cancers. In one of the earliest studies, immunohistochemical analysis of Notch1 was observed in high percentage (~57%) of breast cancers in concert with high Ha-RAS expression [173]. Further *in vitro* experiments revealed that oncogenic Ha-RAS acts at a post-transcriptional level to increase Notch1 activity and that Ha-RAS induced transformation requires Notch1 activation. High Notch1 levels have been correlated mainly with poorly differentiated breast tumors suggesting a possible defect in cell fate decisions, whereas Notch2 expression is inversely correlated to poor prognosis [174]. These data support the notion that different members of the Notch family might have different roles depending on cellular context. Analysis of several human breast cancer cases showed high JAG1 levels besides high Notch1 and Notch3 expression in association to poor overall survival [100, 161, 175]. These findings are in line with research demonstrating the tumor-promoting role of Notch1 and Notch3 in mice [100, 176]. Similarly, Pece *et al*. showed that loss of NUMB expression (a negative regulator of the Notch pathway) is commonly observed in primary human breast cancer [177].

Notch Signaling as a Prognostic Tool and a Therapeutic Drug Target

To date, it is clear that the Notch pathway plays a crucial role in breast cancer development. Therefore, targeting Notch activity in cancer may provide a bona fide approach for the development of novel therapeutic intervention strategies. Strategies include: (i) preventing ligand-receptor interaction using soluble ligand [178, 179], (ii) ectodomain blocking antibodies [180-183] (iii) targeting metalloprotease cleavage [143, 184] and (iv) targeting γ-secretase cleavage [173]. Crosstalk between Notch signaling and other pathways frequently altered in breast cancer are exploited to sensitize breast cancers to common cancer therapeutics drugs by combined Notch inhibition [185, 186]. Finally, approaches to block transcriptional activation have also been successful and may be implemented for future treatment regimes [187].

Inhibition of γ-secretase activity has been so far the most well developed approach to prevent the activation of Notch signaling. γ-secretase inhibitors (GSIs) have been heavily investigated since the gamma secretase complex also cleaves Aβ peptide which plays an important role in Alzheimer's disease [188]. These inhibitors can also block the Notch pathway, generating increasing interest for their potential usage as new cancer therapeutics. In case of breast cancer treatment, GSIs seem to have potential in combination with other therapies for individualized therapy. The optimal use of Notch inhibitors was demonstrated to be dependent on ER status of the tumor. It has been suggested that GSIs might be more efficient in combination with chemotherapy for the treatment of ERα/PR negative tumors [189] which do not overexpress Her2/Neu. Accordingly, another study showed that inhibition of Her2 overexpression by trastuzumab or tyrosine kinase inhibitors (TKI)

increases Notch1 activity, which then sensitized breast cancer cell lines [185] for GSI treatment.

Expression patterns of Notch pathway genes are also likely to have a prognostic relevance. Recent studies stressed the importance of the detection of Notch expression as a potential prognostic marker in breast cancer. Yao *et al*. demonstrated the correlation of the expression of Notch1, Notch4 and Jag1 with known prognostic factors such as ER status, tumor grade, Ki67, lymphovascular invasion, lymph node status and tumor size [190].

CONCLUSION

The existence of MaSCs was already demonstrated more than 50 years ago but we are only beginning to understand the identity of these cells, the signaling pathways that regulate their homeostasis and their role in diseases such as breast cancer. Currently, there are a number of important questions remaining to be answered; what are the functional differences between human and murine stem cells in the mammary gland? Where do these stem cells reside within their organs and in what micro-environment (niche)? This knowledge is needed to improve our understanding of breast stem cells and their role in cancer formation and progression. Notch signaling has emerged as an important cell fate determinant in mammary gland morphogenesis where it plays seemingly opposite roles in cell renewal and differentiation. Notch is also frequently deregulated in breast cancer and in some cases expression of Notch or its ligands has shown prognostic significance. The γ-secretase inhibitors are emerging as potent drugs that attenuate Notch signaling and are currently being evaluated in clinical trials including those with breast cancer. The coming years will reveal if and how breast cancer patients may benefit from treatments with Notch inhibition.

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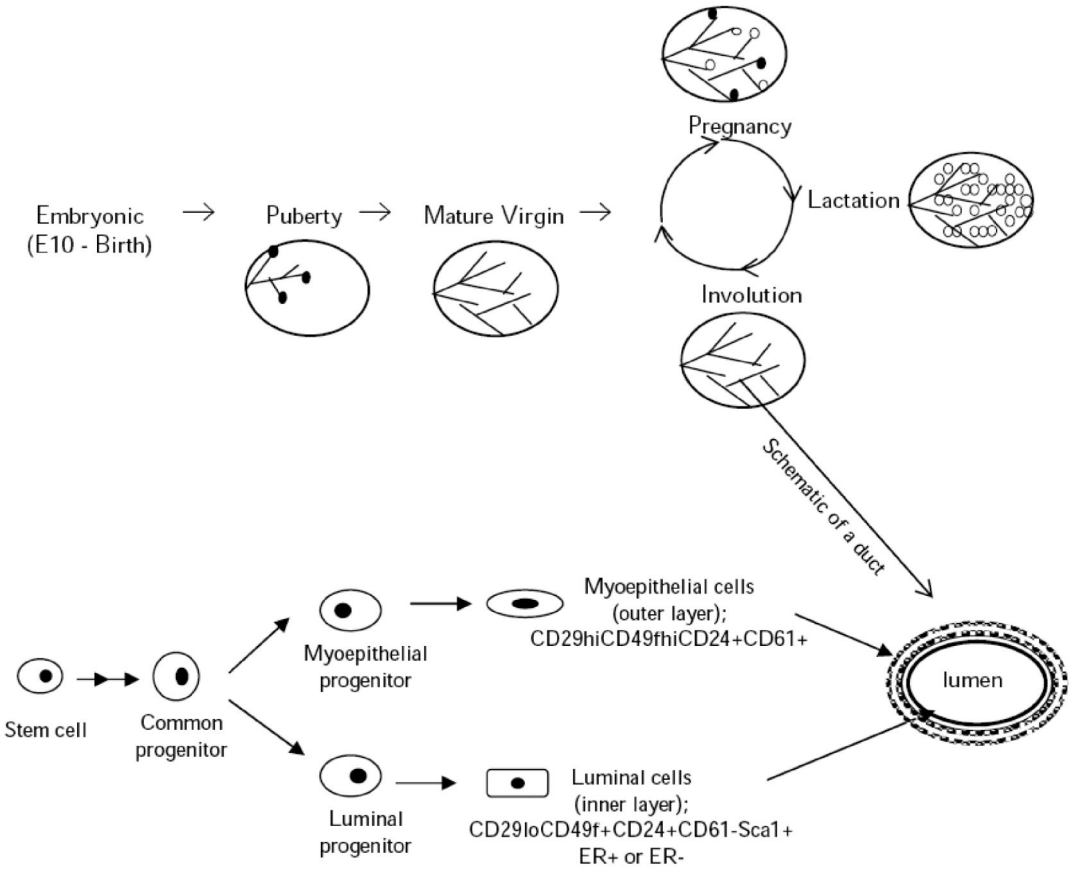

Fig. (1). A schematic representation of mouse mammary gland development and a duct

The closed circular structures appearing at the ductal tips during puberty represent terminal end buds and during pregnancy, also alveolar buds. Mature alveoli are symbolized by the open circles.

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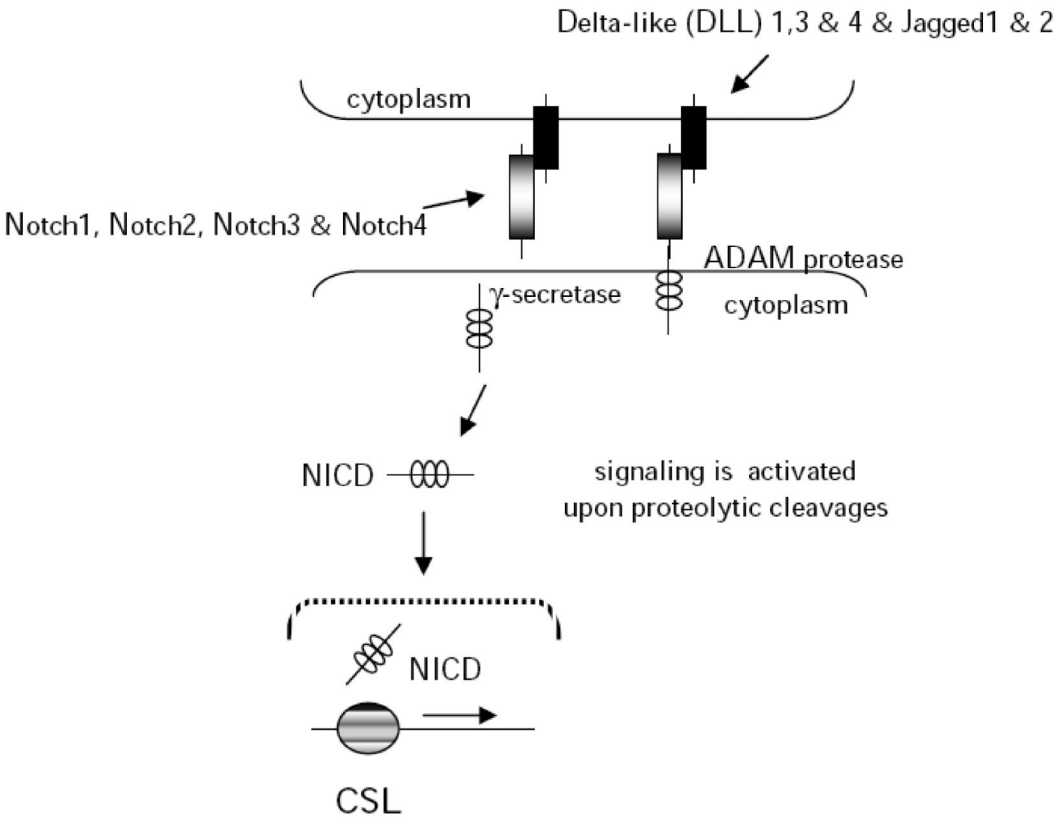

Fig. (2). A schematic of activation of the Notch pathway

Notch signaling is activated by proteolytic cleavages followed by the release and translocation of Notch intracellular domain to the nucleus where it acts on downstream targets by binding the transcription factor CSL to activate *Hes/Hey* family genes which are involved in cell growth, differentiation, and survival.

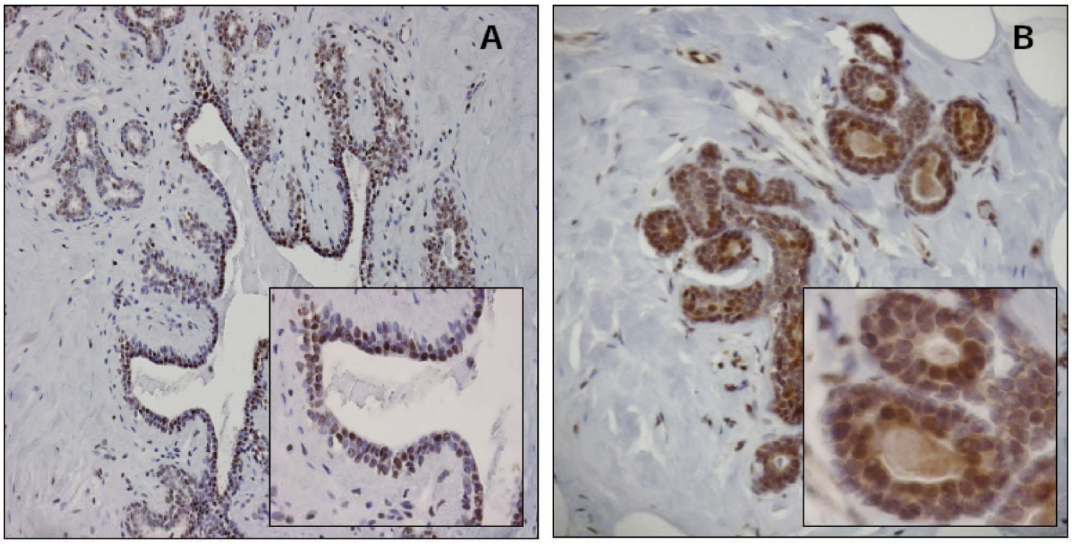

Fig. (3). Notch1 Signaling in Normal Human Breast

We have observed high Notch1 activity in normal human breast epithelium, indicating the activation of this pathway during normal breast development. Normal human breast tissue probed with an antibody against (**A**) cleaved Notch1, representing Notch1 activity (10× and 40× magnification), and (**B**) Hes1 (a downstream target of Notch pathway) (10× and 40× magnification). Similar patterns of expression are evident.

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Table 1

Commonly Used Surface Markers to Identify Mouse and Human Mammary Stem Cells

Mammary Gland Stem Cells	Marker
Mouse	CD24, CD29, CD49f, CD61, Sca-1.
Human	ALDH1, c-KIT, CD10, CD24, CD44, CD49f, CD90, CD133, EpCAM, MUC-1