Review

Mammary gland stem cells: More puzzles than explanations

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Mammary gland stem cells (MaSC) have not been identified in spite of extensive research spanning over several decades. This has been primarily due to the complexity of mammary gland structure and its development, cell heterogeneity in the mammary gland and the insufficient knowledge about MaSC markers. At present, $Lin^{-}CD29^{hi}CD49f^{hi}CD24^{+/mod}Sca\text{-}1^{-}$ cells of the mammary gland have been reported to be enriched with MaSCs. We suggest that the inclusion of stem cell markers like Oct4, Sox2, Nanog and the mammary gland differentiation marker BRCA-1 may further narrow down the search for MaSCs. In addition, we have discussed some of the other unresolved puzzles on the mammary gland stem cells, such as their similarities and/or differences with mammary cancer stem cells, use of milk as source of mammary stem cells and the possibility of in vitro differentiation of embryonic stem (ES) cells into functional mammary gland structures in this review. Nevertheless, it is the lack of identity for a MaSC that is curtailing the advances in some of the above and other related areas.

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1. Introduction

Neonates of mammals depend on their mother's milk for food. Milk is a nutritionally rich secretion synthesized in the mammary gland of the mother from parturition until weaning the offspring. A form of milk called colostrum is the first lacteal secretion produced by the mammary gland in late pregnancy, prior to the production of milk. In all mammals, the mammary gland expands prolifically during pregnancy and remodels to its virgin state after weaning. During each cycle of pregnancy, the mammary gland passes through three different phases, viz. mammary gland expansion, lactation and involution (Sakakura 1987). This re-appearance and remodeling of mammary gland tissue and its ability for expansion indicate the possible existence of stem and progenitor cells in the mammary gland. Today, it is well established that there are cells residing in the mammary gland that are capable of generating a whole mammary gland from a single cell when placed in a suitable mammary microenvironment (Shackleton *et al.* 2006; Stingl *et al.* 2006). Although extensive studies have been carried out on isolating mammary stem cells, none have succeeded till date in isolating an absolute mammary gland stem cell (MaSC) population, the main constraint being the heterogeneity of cell types in the mammary gland. This review discusses the major hurdles in the identification and isolation of mammary stem cells, such as the complexity of the structure of mammary gland and its development, heterogeneity of the MaSC populations and the insufficient knowledge about markers for identifying MaSCs. It also discusses the possible similarities of MaSCs with ES cells and the feasibility of differentiation of ES cells to mammary gland stem cells, in view of their prospective applications in the study of functional genomics of mammary cancers.

Keywords. Epithelial cells; mammary gland; markers; mouse; parity; stem

Abbreviations used: CK, cytokeratin; EBs, embryoid bodies; ER, estrogen receptor; ES cells, embryonic stem cells; LRC, label retaining cells; MaSCs, mammary gland stem cells; PI-MEC, parity-induced mammary epithelial stem cells; Sca-1, stem cell antigen-1; SLC, small light cells; TEBs, terminal end buds; ULLC, undifferentiated large light cells

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2. The mammary gland: A complex organ with a highly heterogeneous population of cells

Lactation is one of the signature characteristics of mammals, and the mammary gland is the functional complex responsible for this. During evolution, lactation has developed as the most efficient, effective and adaptable means of nutrient provision that has ever arisen among the vertebrates (Blackburn 1993). The mammary gland is thus accountable for much the evolutionary success of the Class Mammalia.

The mammary gland is unlike most vertebrate organs that are patterned during embryogenesis and maintain their basic structure throughout adult life. In males, it is present in a rudimentary and generally non-functional form. In females, it is a highly dynamic organ that undergoes dramatic morphogenetic changes during puberty, pregnancy, lactation and regression. Considering the mouse as a model system, the development of the mammary gland can be traced to proceed in distinct phases. During embryonic development, there occurs the formation of bilateral milk lines and mammary buds form at specific locations along the mammary line. Each bud penetrates the underlying mesenchyme and enters the cluster of pre-adipocytes that become the mammary fat pad. A limited number of branches sprout from the invading anlage and this forms the rudimentary ductal tree that is present at the time of birth (Robinson *et al.* 1999; Veltmaat *et al.* 2003). Each branch is composed of a single layer of epithelial cells that surround a central lumen: the cells bordering the lumen are called the luminal epithelial cells. The myoepithelial cells form a basal layer beneath the epithelial cells (Richert *et al.* 2000). The myoepithelial cells are contractile in nature and are responsible for the secretion of milk from the alveoli and its movement down the ducts during lactation (Asch and Asch 1985; Richardson 2009; Dulbecco *et al.* 1986). With the onset of puberty, the hormonal and local cues induce the anlage to respond rapidly and establish a ductal network. The ducts lengthen and branch to form secondary and tertiary ducts. This occurs through the formation of bulbous terminal end buds (TEBs) at the tips of the ducts and their bifurcations. This continues until the entire fat pad of the young adult is filled by an extensive system of branched ducts. The primary duct is large and consists of a layer of epithelial cells surrounded by a thick layer of dense stroma, whereas the secondary and tertiary ducts are composed of a single layer of cuboidal epithelial cells surrounding a central lumen (Sekhri *et al.* 1967). The TEBs are composed of multiple layers of epithelium with an outer layer of undifferentiated, pluripotent stem cells called cap cells that sit on the basal lamina (Richert *et al.* 2000; Williams and Daniel 1983).

With the onset of pregnancy, instigated by an increase in serum prolactin and progesterone, the ducts branch laterally and form side branches with concomitant epithelial proliferation (Brisken 2002; Oakes *et al.* 2006). Alveolar structures, composed of a single layer of epithelial cells enveloping a circular hollow centre, form on the expanded ductal tree and differentiate into lobular alveoli (Richert *et al.* 2000). At around the time of parturition, the lobular alveoli differentiate into secretory epithelium, ready to synthesize and secrete milk for the suckling pups upon parturition (Nguyen *et al.* 2001). At this stage, the mammary gland would be almost filled by the expanded epithelium and the large fat cells would have dedifferentiated into smaller pre-adipocytes. Upon involution, the secretory epithelium apoptoses, the fat cells, redifferentiate and the gland remodels back to a state to resemble that of an adult virgin mouse (Watson 2006; Lund *et al.* 1996).

Taken together, this well-orchestrated chain of events in the female mammary gland involves the participation of a heterogenous population of cells, namely, the mammary stem cells, luminal progenitor cells, alveolar progenitor cells, myoepithelial cells, luminal cells, alveolar epithelial cells, secretory epithelial cells, etc. (Dulbecco *et al.* 1982). These cells display different cell surface markers and/or their expression levels that distinguish them from each other (table 1). Mammary stem cells provide the dynamic and flexible attributes to the mammary gland in undergoing the events discussed above apart from the normal tissue homeostasis. These cells give

Table 1. Different types of cells in the mouse mammary gland and the typical cell surface markers distinguishing them from each other

Mammary gland cell type	Characteristic markers on cell surface	References
MaSCs	$Lin^{-}CD29^{hi}CD49f^{hi}CD24^{+/mod}Sca\text{-}1^{-}$	Shackleton *et al.* 2006; Stingl *et al.* 2006; Visvader and Smith 2011; Asselin-Labat *et al.* 2007
Luminal progenitor cell	$Lin^{-}CD29^{lo}CD49f^{+}CD24^{+}CD61^{+}KIT^{+}ER^{+}$or ER^{-}	Asselin-Labat *et al.* 2007; Sleeman *et al.* 2006
Alveolar progenitor cell	$Lin^{-}CD49f^{+}CD24^{+}Sca1^{-}$	Asselin-Labat *et al.* 2007
Ductal epithelial cell	$Lin^{-}CD29^{lo}CD49f^{+}CD24^{+}CD61^{-}Sca\text{-}1^{+}ER^{+}$or ER^{-}	Visvader 2009; Stingl *et al.* 2006; Shackleton *et al.* 2006
Alveolar epithelial cell	$Lin^{-}CD29^{lo}CD49f^{+}CD24^{+}CD61^{-}ER^{-}$	Visvader 2009; Stingl *et al.* 2006; Shackleton *et al.* 2006
Myoepithelial cell	$Lin^{-}CD29^{hi}CD49f^{hi}CD24^{+}CD61^{+}$	Visvader 2009; Stingl *et al.* 2006; Shackleton *et al.* 2006

rise to the mature epithelium of either the luminal or myoepithelial lineage via a series of lineage restricted intermediates such as the luminal and myoepithelial progenitors respectively (Visvader 2009). The myoepithelial cells vary in appearance from being a sheath around the epithelial cells during development and involution to discontinuous layer of cells that circle the alveoli during lactation (Richert *et al.* 2000). The luminal lineage can be subdivided into ductal and alveolar luminal cells which arise through their respective progenitors. The luminal progenitor cells can either be positive or negative for ER (estrogen receptor) and can give rise to ER-positive or ER-negative ductal luminal cells, respectively (Visvader 2009; Zeps *et al.* 1998). The ductal luminal cells line the ducts, whereas the alveolar luminal cells constitute the alveolar units that arise during pregnancy as mentioned before. Given the heterogeneity of the mammary cells, it has become difficult for the identification and isolation of a pure mammary stem cell population per se.

3. Mammary gland stem cell populations are heterogeneous

There are at least two different populations of stem cells in mammary gland, viz. mammary stem cells and parity-induced stem cells. These two subsets of mammary stem cell populations are highly similar to each other based on their cell surface markers. A brief account on their discoveries and current status of knowledge about them are given below.

3.1 *Mammary gland stem cells*

Mammary stem cells are the self-renewing cells in the mammary gland which can give rise to a functional mammary gland when placed on the mammary fat pad. Until Kordon and Smith conclusively showed the existence of MaSCs through mammary epithelial transplantation experiments (Kordon and Smith 1998), it was not certain if stem cells existed in the mammary gland. Before this discovery, there were only speculations and indirect evidences about their existence; for example, Rama 25 cells that were isolated from a dimethyl benzanthracene-induced adenocarcinoma of rat appeared to be a type of pluripotent mammary epithelial stem cell that could form two further cell types, an alveolar-like and a myoepithelial-like cell (Rudland *et al.* 1980), and in a different approach, Dulbecco and group had labelled female rats with radioactive thymidine at various phases of estrus cycle to study the different cell types and their involvement in the mammary gland, and their results suggested that stem cells for mammary development are present in the terminal end buds and they generate a lineage for luminal cells and possibly a distinct one for myoepithelial cells (Dulbecco *et al.* 1982). The most difficult task in studying MaSCs is the isolation of a pure mammary gland stem cell population. As described before, the mammary gland consists of various populations of cells, namely, the mammary stem cells, luminal progenitor cells, alveolar progenitor cells, myoepithelial cells, luminal cells, alveolar epithelial cells, secretory epithelial cells, etc. (Dulbecco *et al.* 1982). All these cells share many cell surface markers and they express these markers in variable amounts. Search for potential markers to separate the stem cell population out of the whole mammary gland cell pool had begun well before their discovery. Earlier, a group of cells from the rat mammary gland carrying the cell surface markers 1A10, 24B42 and 57B29 were considered as the mammary stem cells (Dulbecco *et al.* 1986). Now, CD24, CD29 (β1integrin), CD49f (α6 integrin), CD14, CD61 (β3 integrin) and Sca-1 (stem cell antigen-1) are the most widely used markers to separate the MaSCs from the total mammary cell population (Shackleton *et al.* 2006; Stingl *et al.* 2006; Asselin-Labat *et al.* 2007; Visvader and Smith 2011). Further details of mammary glands stem cell markers are discussed later in this review.

From the 1980s to date, extensive research has been carried out to isolate MaSCs from the pool of differentiated, partially differentiated and undifferentiated cells in the mammary gland. Smith and Medina tried to identify mammary stem cells based on their morphological appearance (Smith and Medina 1988). They observed that a group of pale coloured cells with large nuclei, clear cytoplasm, round smooth curved shape and tight cell junctions exist in mouse mammary gland from the 16th day of its embryonic growth. These cells generated cells capable of differentiating in the presence of lactogenic stimuli. In yet another morphological study, cells identified using microscopic techniques, which were described as small light cells (SLCs), were proposed as stem cells based on their small size, high mitotic activity and absence of organelles. These SLCs were found to give rise to darker cells, which were thought to be the differentiated population of cells. Other than SLCs, there were undifferentiated large light cells (ULLC), which were thought to be multipotent stem cells (Chepko and Smith 1997, 1999). Limited dilution of mammary gland cells followed by mammary fat pad transplantation experiments also provided evidence for the existence of three kinds of mammary epithelial progenitors, viz. lobular epithelial progenitors, ductal epithelial progenitors and both lobular and ductal epithelial progenitors (Smith 1996). The lobular and ductal epithelial progenitor cells could be the basal MaSCs capable of giving rise to ductal epithelial progenitors. Using transgenic mice with mammary tumour viral insertions in its genome, Kordon and Smith suggested that an entire functional

mammary gland may comprise progenies from a single stem cell. In their study, random fragments from the mammary glands of the transgenic mouse when transplanted into epithelium-free fat pads generated clonal mammary epithelial growths. The epithelial fragments from this growth again generated clonal epithelial growths upon denuded mammary fat pad transplantations, showing the self-renewal potential of the original stem cell. Kordon and Smith were also able to obtain three multipotent progenitor populations from the limiting dilution transplantation studies conducted with the cell cultures derived from third generation clonal outgrowths (Kordon and Smith 1998).

Kenney and coworkers (Kenney *et al.* 2001) focused their study to locate mammary stem cells by identifying them as long-lived, label-retaining mammary epithelial cells (LRCs) in growth-active (developing) or growth-static (aged) mammary ducts. They incorporated Brdu : Bromodeoxyuridine into primary epithelial cells and transplanted them into cleared juvenile syngeneic mammary fat pads and suggested that LRCs could be MaSCs. It was earlier reported that Sca-1 antigen is expressed on functional hematopoietic stem cells (Spangrude *et al.* 1988) and that the efficient efflux of the fluorescent dye Hoechst-33342 is a mechanistic characteristic of pluripotent hematopoietic stem cells (Goodell *et al.* 1996). Welm and group reported that these LRCs present in the mammary gland were found to express Sca-1 antigen on their surface and were effluxing Hoechst dye (Welm *et al.* 2002). Smalley and Clark also isolated a side population cells from mammary gland cells which could efflux Hoechst33342 dye. This population of cells was capable of differentiating into both luminal and myoepithelial cells (Smalley and Clarke 2005). However, they were not able to prove conclusively that the side population cells were enriched for mammary stem cells.

In 2006, Shackelton and coworkers identified a subpopulation of cells that are enriched for the mammary stem cells. They used FACS to sort the cells which were Lin^{-} $CD24^{+}$ $CD29^{hi}$ cells, and when these cells were transplanted onto denuded mammary fat pads of virgin mice, they were able to generate the whole functional mammary gland. Transplantations of single cells to denuded mammary fat pads following serial dilution of each of these subpopulations demonstrated that they contain mammary stem cells at a frequency of 1/48 (Shackleton *et al.* 2006). Meanwhile, Stingl and coworkers showed that yet another subpopulation of mammary gland cells (Lin^{-} $CD24^{+}$ $CD49^{hi}$) were also able to generate the complete functional mammary gland from a single cell transplanted on the mammary fat pad. These cells were able to generate progenitors in culture and successively, adherent colonies as well (Stingl *et al.* 2006). Both these cell populations identified using cell surface markers were self-renewing for multiple generations.

CD24 is a heat-stable antigen present on the cell surface, while CD29 and CD49f refer to β1 integrin and α6 integrin, respectively (Wang 2006; Smith 2006). It is still not known whether these cell surface markers are the cause or the effect of stemness in the mammary stem cells. If they are the cause of the stemness, then the interaction of the cells with the extracellular matrix through these cell surface receptors might determine whether the cell has the potential to be a stem cell or not. The role of extracellular matrix in mammary gland development is well known (Wicha 1984). The integrins act as adhesion receptors for the mammary epithelial cells, which in turn act to pass the developmental cues for these cells. They also assist the cells in sensing hormonal and growth factor signals (Katz and Streuli 2007). If the presence of these markers is the effect of the stemness, then there could be other factors that determine the stemness of these stem cells. Ibarra and coworkers studied the role of microRNAs in the maintenance of mammary progenitor cells and found that mir205 and mir22 are abundant in the mammary progenitor cells while let7 and mir 93 are depleted. When let7 microRNA was forcefully expressed, the cells were not able to self- renew any further (Ibarra *et al.* 2007). Together, these evidences illustrate that the differentiated and undifferentiated cells in the mammary gland are different at many levels. The differentiation of mammary stem cells to epithelial cells is not as straightforward as speculated. It could be the result of a network of factors at many levels starting from epigenetic to translational.

3.2 *Parity induced mammary stem cells*

The mammary gland undergoes a cycle of events, viz. epithelial cell expansion, alveologenesis, lactation and involution, during each cycle of pregnancy. Involution is characterized by extensive apoptosis. It was believed that all the differentiated epithelial cells undergo apoptosis during involution and the MaSCs expand during the next cycle of pregnancy to reconstitute the differentiated secretory epithelium until Wagner and coworkers (Wagner *et al.* 2002) provided genetic evidence to show that the new mammary gland epithelial cells can originate from a subpopulation of the mammary epithelial cells that skipped apoptosis during the preceding term of pregnancy. These cells located in the terminal ducts within alveolar units were showing the properties of progenitor cells as evidenced by the mammary fat pad transplantation experiments and were also closely similar to multipotent MaSCs. When transplanted to mammary fat pads, these cells gave rise to ductal and alveolar morphogenesis. Wagner and his coworkers proposed that one of the differences between the nulliparous and multiparous mice would be the absence of parity induced mammary stem cells in nulliparous mice. Boulanger supported these findings of Wagner and showed that the parity induced MaSCs can

differentiate into any cell type of the mammary gland and also discovered the self-renewing property of these cells (Boulanger *et al.* 2005). But the concept of the absence of the parity induced MaSCs in nulliparous females was disproved by Booth and coworkers in 2007 (Booth *et al.* 2007). They showed that the differentiating mammary epithelial cells from the mammary explants of a nulliparous female mouse can also develop into mammospheres in culture and into mammary out-growths in transplantation assays which supported the existence of these stem cells in virgin female mice. The proportion of these cells in the mammary gland of nulliparous female varies from 0.8–4 % depending on the estrus cycle of the female. It increases to 20–30 % in non-pregnant multiparous mouse (Wagner and Smith 2005).

The different cells in the mammary gland can be classified based on their cell surface markers. In 2006, Stingl and coworkers separated a population of mammary gland cells which were $CD24^{+}/CD49^{hi}$ and found that these cells contained multipotent MaSCs at high frequency (Stingl *et al.* 2006). Matulka and coworkers classified parity-induced MaSCs into multipotent MaSCs after observing that these cells are $CD24^{+}$ / $CD49^{hi}$ (Matulka *et al.* 2007). This probably indicates that the parity-induced mammary stem cells could be a subpopulation of the stem cells identified by Stingl and coworkers (Stingl *et al.* 2006). Booth and coworkers have shown that a single parity-induced mammary epithelial stem cells (PI-MEC) cannot give rise to mammospheres in culture and they need the association of other cells for this development (Booth *et al.* 2007). Besides, the fact that the PI-MEC is lobule limited disqualifies them from being classified into the basal mammary gland stem cell. Rather, they are progenitor cells capable of differentiating into lobule limited cells and self-renewal. Identification of more markers capable of isolating pure population of MaSCs would help in distinguishing the mammary repopulating units (MRU) from the PI-MECs among the $CD24^{+}$/ $CD49^{hi}$ population of cells.

4. Current knowledge about mammary stem cell markers is insufficient for their identification and isolation

The mammary gland contains various cell types that are different from each other by their positions in the mammary gland, functions and/or markers expressed on their cell surfaces. Although there are sets of cells with specific markers that classify them into myoepithelial cells, luminal epithelial cells or secretory epithelial cells, there are no markers to differentiate mammary stem cells from the rest of the population. However, researchers have found that $Lin^{-}CD29^{hi}CD49f^{hi}CD24^{+/mod}Sca\text{-}1^{-}$ cells in the mammary gland are highly enriched in mammary stem cells (Visvader and Smith. 2011). Sorting of cells in the mammary gland for Lin^{-} feature excludes hematopoietic and endothelial cell populations. Similarly, excluding the $CD24^{-}$ or $CD24^{high}$ cells excludes non-epithelial or luminal epithelial cells from the heterogeneous mammary gland cells, respectively (Sleeman *et al.* 2006). CD29 and CD49f are markers present on the surface of skin and colonal stem cells, respectively (Jones *et al.* 2004; Kawase *et al.* 2004). Expression of CD29 and CD49f is high in mammary stem cell populations. However, these markers alone are not sufficient for the isolation of mammary stem cells as myoepithelial cells in the mammary gland are also $CD29^{hi}CD49f^{hi}$ (Visvader 2009). Similarly, Sca-1, a phosphatidylinositol-anchored protein and a member of the Ly-6 antigen family (van de Rijn *et al.* 1989), was one of the initial markers identified as a stem cell marker. However, it was later shown that culturing mammary epithelial cells induces high levels of Sca-1 expression. Also, subsequent studies that identified mammary stem cells to a higher degree of purity show that these cells are $Sca\text{-}1^{low/-}$ (Shackleton *et al.* 2006; Stingl *et al.* 2006). Inclusion of new markers for sorting out mammary stem cells from the total mammary gland cell populations has increased the proportion of MaSCs among the sorted population (table 2). Maximum proportion of mammary repopulating units was obtained (1/20) when $CD45^{-}CD31^{-}TER119^{-}$ (Lin^{-}) $Sca\text{-}1^{low}CD49f^{hi}$ $CD24^{+}$ cells were used in transplantation experiments (Stingl *et al.* 2006).

Bai and Rohrschneider (2010) showed that s-SHIP (an active gene in ES cells and hematopoietic stem cells) promoter is active in the activated stem cells of the mammary gland and they constitute 9 % of the total population of $Lin^{-}CD24^{+}$ $CD49^{hi}$ cells and 22.4 % of total population of $Lin^{-}CD24^{+}$ $CD\ 29^{hi}$ cells during puberty. At pregnancy, they constitute 10.8 % and 4 %, respectively. The frequency of

Table 2. Frequency of cells among the heterogeneous population of mammary gland cells capable of repopulating the denuded mammary fat pad (MRUs) and the corresponding marker/s used for selecting the cells for the repopulation experiment

Marker for selecting MRUs	Frequency of repopulating MRUs	References
Lin^{-}	1/4900	Shackleton *et al.* 2006
$CD45^{-}$ $Ter119^{-}$ $CD49f^{hi}$	1/200	Stingl *et al.* 2006
$Lin^{-}CD49f^{hi}CD24^{+}Sca\text{-}1^{low}$	1/20	Stingl *et al.* 2006
$Lin^{-}CD29^{hi}CD24^{+}$	1/64	Shackleton *et al.* 2006
$Lin^{-}CD49^{hi}CD24^{+}s\text{-}SHIP^{+}$	1/14	Bai and Rohrschneider 2010

mammary repopulation units in $Lin^{-}CD24^{+}$ $CD49^{hi}$ population with active s-SHIP promotor was 1/14. Nevertheless, the inclusion of s-SHIP promoter activity as a marker along with the other known MaSC markers has increased the probability of identifying the MaSCs (Bai and Rohrschneider 2010).

Differentiating known cell surface markers from stem cell markers has proven to be useful for sorting MaSCs (Shackleton *et al.* 2006; Stingl *et al.* 2006; Asselin-Labat *et al.* 2007). It would be worth testing the expression of ES cell markers like Oct4, Sox2 and Nanog and their utility in further purification of mammary stem cells. Oct4 has already been reported to express in human mammary stem cells (Tai *et al.* 2005). Oct4 and Nanog are known to be expressed in mammary tumours (Liu *et al.* 2004).

BRCA1 can be another candidate as a marker for sorting mammary gland stem cell. Among the mammary gland cells, the least expression of BRCA1 is seen in human mammary stem cells. As the stem cell undergoes differentiation, the level of BRCA1 expression is seen to increase (Ginestier *et al.* 2009). A complete knock-down of BRCA1 increases the population of mammary stem cells (Liu *et al.* 2008), whereas BRCA1 heterozygocity leads to expansion of luminal progenitors (Lim *et al.* 2009).

5. Embryonic stem cells versus mammary stem cells

One of the unsolved questions in stem cell biology is a mechanism to convert ES cells into MaSCs. A differentiation system for converting ES cells to MaSCs would be of paramount utility as a model for breast cancer research. However, only a little is known about the ontology of the MaSCs. In mouse, during embryonic development, pluripotent stem cells appear in the form of inner cell mass of blastocyst (Evans and Kaufman 1981; Martin 1981). However, it is not known whether MaSCs are present in the embryo during any of its developmental stages. The first visible embryonic mammary gland structures are the milk lines, the multilayered ectodermal ridges stretching from anterior to the posterior limbs on the ventral side of the embryo (Robinson *et al.* 1999; Veltmaat *et al.* 2003). These ridges are formed by the migration of some of the cells in the embryonic ectoderm and their aggregation. These structures eventually develop into mammary glands. Examination of the cells in the milk line for stem cell potential might be crucial in explaining how the milk line develops into mammary gland.

Comparative analysis of gene expression in ES cells and MaSCs would be helpful in designing a strategy for differentiating ES cells into MaSCs. The major hurdle in conducting this study is the lack of knowledge about markers to isolate a pure population of MaSCs. Even though CD24, CD29 or CD49 markers are in use for separating the MaSCs from mammary gland cells, they are only capable of isolating a population of mammary gland cells enriched with stem cells. A set of markers for purifying mammary stem cells are yet to be identified.

Research indicates that a few pluripotency markers expressed in ES cells are common to mammary gland progenitor cells as well. Oct4 (Tai *et al.* 2005), Sox2 and Nanog are expressed in human mammary gland progenitor cells and their expression reduces as these cells differentiate (Tai *et al.* 2005; Simoes *et al.* 2011). In addition, ectopic expression of Nanog and Sox2 increases the potential of breast cancer cells to develop into mammospheres and their ability for invasion (Simoes *et al.* 2011). This suggests that these genes may account for the pluripotency of the MaSCs similar to ES cells. Rohrschneider and coworkers showed that promoter of a gene called s-SHIP is active in blastocyst and many other tissues including some of the epithelial cells in the developing mammary gland (Rohrschneider *et al.* 2005). Later Bai and Rohrschneider showed that these cells are activated mammary stem cells (Bai and Rohrschneider 2010). It suggests that s-SHIP could be a pluripotency- associated gene in mammary stem cells.

Recently, some of the miRNAs have also been implicated for the pluripotency and self-renewal of the MaSCs. miR 205 has been shown to have a role in the pluripotent population of Comma D β Geo cells (Greene *et al.* 2010). When overexpressed, miR 205 increases colony formation of stem cells in the Comma D β Geo cells and their proliferative potential. Further, it was also shown that miR 205 expresses at higher levels in different populations of stem cells in the mouse mammary gland. It would be interesting to study the differentially expressed miRNAs in ES cells and MaSCs and design a strategy for differentiation of one cell type into the other.

6. Future directions in mammary stem cell biology

Some of the questions still unanswered in mammary stem cell biology are: How similar are mammary gland stem cells and mammary cancer stem cells? Does milk contain mammary stem cells? Whether other cell types can be transdifferentiated into mammary gland cells, and are there any specific marker/s for mammary stem cells?

The claudin-low and normal-breast-like subtypes of breast cancers resemble mammary stem cells (Prat *et al.* 2010). If the relationship between breast cancer subtypes is traced back through the epithelial hierarchy of mammary gland cells, then these two subtypes may be traced back to the MaSCs (Visvader 2009). Research focusing on identification of mammary stem cells and markers associated with them would aid in the prognosis and treatment of these subtypes of breast cancers. Potential markers for isolating mammary stem cells are already in use for identifying

tumorogenic cells. Zhang and coworkers (Zhang *et al.* 2008) found that the Lin^{-} $CD29^{hi}$ $CD24^{hi}$ cells (described by Shackelton *et al.* in 2006 as markers for enriching mammary stem cells during fluorescence- activated cell sorting of mammary gland cells), isolated from p53-null mouse mammary gland tumours, are capable of generating mammary tumours upon transplantation into cleared mammary fat pads. However, this population of cells showed differential expression of genes involved in DNA damage response and repair as well as the previously reported genes involved in epigenetic regulation of stem cell self-renewal, compared to bipotent stem cells of the mammary gland. In MMTV-*Wnt1* breast tumour extracts of mouse, Cho and coworkers identified a subpopulation of cells which are $Thy1^{+}$ $CD24^{+}$ and are capable of regenerating tumours upon transplantation to cleared mammary fat pad, Thy1 being a hematopoietic stem cell marker (Cho *et al.* 2008). In WAP-T transgenic mouse, where the SV40 large and small T antigens drive the carcinogenesis in mammary glands, cells extracted from the mammary carcinoma (G2 cells), sorted on the basis of CD24a, CD49f, CD61, Epcam, Sca1, and Thy1 or metabolic markers, reconstituted the initial cell population during repopulation assay (Wegwitz *et al.* 2010).

The presence of stem cells in milk is a well-known fact since decades (Grieve and Kitchen 1985; Buehring 1972, 1990). In 2007, Cregan and coworkers identified a population of cells in human breast milk which were nestin-positive and were excluding Hoechst 33342 stain (Cregan *et al.* 2007). This discovery prompted scientists to look at milk as a non-invasive source of stem cells. In 2010, Fan and coworkers found that these stem cells in milk do not grow under established culture conditions (Fan *et al.* 2010). In addition to that, the so-called stem cells in the milk have to undergo the tests of pluripotency and mammary fat pad reconstitution to be proven as mammary stem cells. However, milk as a source of stem cells is an emerging theme in the mammary stem cell biology.

Earlier, attempts have been made to generate an *in vitro* model of the functional mammary gland. (Huang *et al.* 2011; Zhou *et al.* 2010; Jiang *et al.* 2010) (figure 1). One approach for achieving this end was through the direct transplantation of ES cells into mammary gland fat pads. Huang and coworkers tried this and found limited success in it. They were able to generate cells expressing K18 epithelial marker after 2 weeks of transplantation of ES cells into cleared mammary fat pads (Huang *et al.* 2011). Jiang and coworkers conducted 3D culture of murine ES cells and found that in culture, they can form acini which contain cells expressing CK5 and CK14. However, these cells were not able to differentiate into mammary gland cell types. But when they differentiated the mouse ES cells into 14-day-old hematopoietic embryoid bodies (EBs) followed by transplantation of the cells constituting them on denuded mouse mammary fat pads,

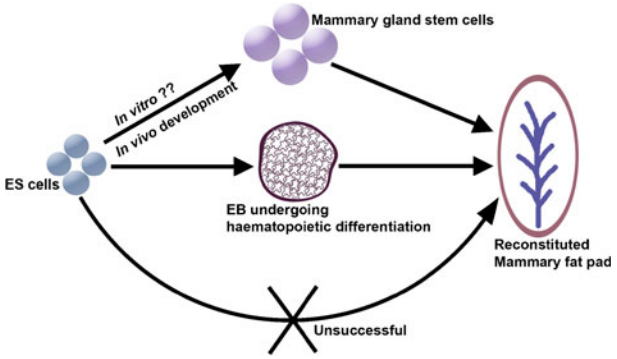

Figure 1. Repopulation of denuded mammary fat pad with ES cells failed to generate functional mammary gland. However, ES cells from embryoid bodies undergoing haematopoietic differentiation were able to generate functional mammary glands in low frequencies, indicating that a small population of cells among them resembles mammary stem cells. Direct differentiation of ES cells into mammary stem cells has not been successful so far.

mammary tissue reconstitution was found to occur in 25 % of the transplanted fat pads and the frequency of reconstitution was found to be increasing with number of hematopoietic EB cells injected (Jiang *et al.* 2010). However, the number of cells contributing to the mammary gland reconstitution seems to be very low, considering the high number of cells that were injected. It probably indicates that the cells in the hematopoietic EBs are heterogeneous and a small population of the cells in hematopoietic EBs is capable of reconstituting the mammary tissue. A lot of research is needed in deciphering the ability of hematopoietic cells to generate a functional mammary gland. Nevertheless, it would be of great utility if ES cells can be exploited to generate an *in vitro* mammary gland model.

7. Conclusion

Stem cells provide mammary glands with the ability to undergo cycles of expansion and involution. The isolation of pure population of MaSCs has not yet been successful because of the heterogeneity of mammary gland cells and also the insufficient knowledge about precise mammary stem cell markers. Researchers are exploring new sets of markers for this purpose and it is hoped that the isolation of MaSCs would become a reality in near future. This accomplishment would help to locate and target cancer stem cells for therapeutic purposes and also in isolating MaSCs from milk, which could be a non-invasive source of stem cells. In addition, there is a need of a protocol for differentiating ES cells in to MaSCs as it would help in functional studies of genes associated with mammary gland development and cancers.

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