On mammary stem cells

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Journal of Cell Science 118, 3585-3594 Published by The Company of Biologists 2005
doi:10.1242/jcs.02532

Summary

Mammary gland stem cells are a quiescent and self-renewing population within the mammary gland that are capable of giving rise to the differentiated ductal, alveolar and myoepithelial cells. To identify mammary gland stem cells, several investigators have employed a variety of methods including: non-adherent mammosphere cultures; 5-bromo-2-deoxy-uridine (BrdU) label-retention studies; cell-surface markers, such as Sca1 and CD49f; and Hoechst dye efflux. These methods have helped identify and further characterize signal transduction pathways such as the Notch, Wnt and Hedgehog pathways that may be important for the self-renewal and fate determination of mammary gland stem cells. Stem cells within the mammary gland have been proposed to underpin many types of breast cancer. A better understanding of the signal transduction pathways and the molecules that are responsible for the self-renewal and survival of these cells will be essential in the design of more effective therapies aimed at the eradication of both cancer-initiating cells and breast cancer stem cells.

Key words: SP, Sca1, LRC, Stem cells, α6-integrin, ER

Introduction

Recent discoveries regarding the isolation and characterization of stem cells, the understanding of signaling pathways involved in their self-renewal and survival, and their potential role in diseases such as cancer have turned academic, political and public attention to the rapidly expanding field of stem cell biology. The most primitive stem cells – embryonic stem cells – have extraordinary differentiation potential and can mature into every cell type in a fully developed organism. Adult stem cells make up a small percentage of the cells found in mature organ systems, where they give rise to specific cell types, such as the skin, mammary gland, gut and central nervous system. Adult stem cells are long-lived, generally quiescent cells that generate new stem cells, and thereby maintain the stem cell pool, as well as more committed progeny, which populate the organ through proliferation (Molofsky et al., 2004; Reya et al., 2001). The most primitive adult stem cell population, which is able to give rise to all cell types within the organ, is thought to be maintained by signals found in the local environment – the stem cell niche (Ohlstein et al., 2004; Rizvi and Wong, 2005). When necessary, it can expand to generate a transiently amplified pool of progenitors to re-populate tissues.

Studies of model systems such as the hub cells in the *Drosophila* testis, the terminal filament and cap cells in the fly ovary (Yamashita et al., 2005), the bulge region of the hair follicle (Tumbar et al., 2004) and crypt cells in the gut have begun to provide insights into the stem cell niche (Radtke and Clevers, 2005). Stem cell quiescence in the niche, for example, is thought to be regulated by cell adhesion. This is mediated in part by homotypic interaction of cadherins from the surrounding niche and the stem cells, as well as interactions between integrins on stem cells and the extracellular matrix.

The mammary gland is organized into a tree-like structure composed of hollow branches. These have an inner layer of luminal epithelial cells that face the lumen and are surrounded by an outer layer of myoepithelial cells that secretes the basal lamina separating the mammary parenchyma from the stroma (Richert et al., 2000). Within the mammary arbor, the ductal cells are those that line the ducts of the mammary gland (Fig. 1c,d). Lobular cells form secretory acinar structures at the end of each branch and, upon pregnancy and lactation, become alveolar cells that produce milk proteins. The ability to replenish the mammary gland through cycles of pregnancy, lactation and involution throughout a woman's lifetime is attributed to stem cells that are proposed to reside in the mammary gland (Williams and Daniel, 1983; reviewed by Smith and Chepko, 2001). These cells are proposed to serve three functions: (1) to give rise to the tissues of the adult mammary gland during development; (2) to allow the enormous tissue expansion and remodeling that occurs in the mammary gland during multiple cycles of pregnancy, lactation and involution; and (3) rarely, to serve as a reserve for repair in the event of tissue damage. At the onset of puberty, the immature mammary gland undergoes rapid growth and differentiation at the tip of the terminal end buds (TEBs; Fig. 1b). The cap cell layer surrounding the TEB can take on a myoepithelial lineage or a luminal epithelial lineage, and therefore cap cells are thought to be multipotent stem cells. However, the TEBs are considered to be only a temporary niche since TEBs are transient structures that disappear once the duct reaches the end of the fat pad.

In the late 1950s, DeOme and colleagues elegantly demonstrated the existence of adult stem cells in mammary tissue by limiting-dilution transplantation experiments in which clonal progenitors can generate complete, functional, mammary outgrowths containing ductal, alveolar and

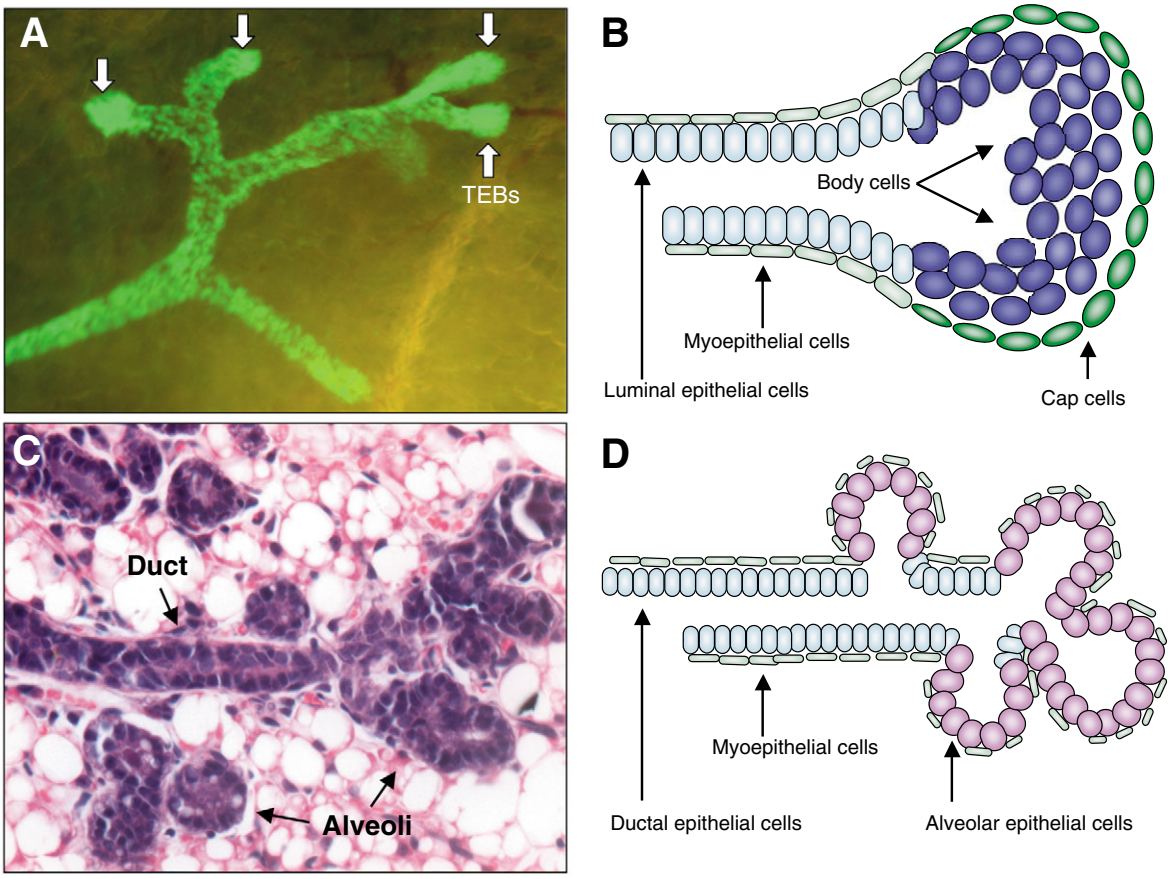

Fig. 1. The terminal end bud (TEB). The TEB appears at the onset of puberty, undergoing rapid growth and differentiation. (A) Expression of Sca1 is enriched in TEBs (arrows) and ducts of six-week-old mice. Micrograph showing live imaging of GFP expression in Sca1-GFP knock-in mice (Sca1-GFP mice kindly provided by T. A. Graubert, Washington University; glands prepared and image captured with help from M. T. Lewis, Baylor College of Medicine). (B) Schematic view of the TEB. A cap cell layer surrounds the body cells. The cap cells can take on either a myoepithelial lineage or a luminal epithelial lineage and therefore are thought to be multipotent stem cells. Differentiated myoepithelial and luminal epithelial cells line the neck of the TEB and the subtending duct. (C) Section, stained with hematoxylin and eosin, of a midpregnant mammary gland from C57BL/6 mice indicating the locations of the ductal and alveolar cells. (D) Schematic view of the ductal and alveolar cells during midpregnancy. The ducts are surrounded by a basal layer of overlapping myoepithelial cells, whereas the alveoli cells are surrounded by a basket-like layer of myoepithelial cells.

myoepithelial cells when transplanted into the cleared mammary fat pads of recipient mice (DeOme et al., 1959). Subsequently, other researchers extended the idea by demonstrating that samples taken from any portion of the mammary gland can give rise to mammary epithelial outgrowths that have complete developmental capacity regardless of their age and developmental stage (Smith and Medina, 1988). This impressive renewal capacity has been ascribed to a multipotent mammary gland stem cell population that resides and persists throughout the mammary parenchyma.

Stem cells are candidates for cells from which cancers originate (Sell, 2004). Bonnet and Dick provided direct evidence for the existence of cancer stem cells in leukemia by showing that only a minority of leukemic cells are pluripotent and can, therefore, reconstitute tumors in the bone marrow of NOD/SCID mice (Bonnet and Dick, 1997). More recently, several groups have prospectively identified cancer stem cells capable of recapitulating solid tumors from which they are derived, including glioma, pediatric meduloblastoma and breast cancer (Al-Hajj et al., 2003; Singh et al., 2003; Singh et al., 2004). Cancer therapy that targets this small population of cancer stem cells might thus be necessary to prevent cancer recurrence (Reya et al., 2001). In this Commentary, we examine current progress in identifying and characterizing adult stem cells in the mammary gland, the pathways responsible for maintaining stem cells in normal mammary tissue and, finally, the role of stem cells and stem cell self-renewal in breast cancer.

Identification of mammary stem/progenitor cell markers*

Several complementary approaches have been employed to isolate, identify and enrich mammary epithelial cells (MECs) that maintain stem/progenitor cell characteristics. Bone fide stem cell markers in general have remained elusive. Until

*Stem cells refer to the most primitive, pluripotent cells. We know that existing surrogate stem cells markers, such as Sca1 or CD49f, are not the only markers that characterize a stem cell. Here, we are designating the cells isolated by the existing surrogate stem cell markers as less primitive progenitor cells instead of stem cells.

recently, there were no known stem/progenitor cell markers in the mammary gland. Therefore, researchers have taken advantage of knowledge obtained in hematopoietic, neural, epidermal and other systems, and applied stem cell markers borrowed from these fields to search for potential stem/progenitor cells in the mammary gland. Below, we focus on recent progress in this area, and readers are referred to a review by Stingl and colleagues for discussion of earlier studies (Stingl et al., 2005).

Sca1

Stem cell antigen 1 (Sca1), a marker of hematopoietic stem cells, is one marker currently used to isolate and enrich for mammary gland progenitors. A population of Sca1$^+$ cells exists in the murine mammary gland (Welm et al., 2002). Label-retention experiments have demonstrated that this population is enriched in slowly dividing, largely quiescent cells (see below). A Sca1-green fluorescent protein (GFP) knock-in approach has shown that Sca1-GFP cells do not co-localize with the progesterone receptor (PR), a marker of differentiation, or peanut lectin, a differentiation marker that interacts with MUC4 on MECs. The highest level of Sca1-GFP expression is in the body cells of the rapidly proliferating TEBs at the tips of the growing ducts (Fig. 1a). In transplantation experiments, Sca1-GFP$^+$ cells isolated by fluorescence-activated cell sorting (FACS), or Sca1$^+$ cells isolated by magnetic bead sorting, have elevated outgrowth activity compared with Sca1-GFP$^-$ cells, which fail to give rise to outgrowths when transplanted into the cleared mammary fat pad. However, like most studies using single markers for FACS analysis, these experiments are highly dependent on the specific cell preparation and gating conditions used to isolate the Sca1$^+$ cells, and there appears to be a gradient of Sca1 expression. Thus, these experiments should not be over-interpreted to indicate that the presence of Sca1 represents an 'all or none' distinction with respect to stem/progenitor cell activity. Indeed, recent experiments have suggested that cells from the COMMA-D mammary epithelial cell line that have high Sca1 expression exhibit increased clonogenicity compared with COMMA-D cells that have intermediate or no Sca1 expression (M. Alfaro and J.M.R., unpublished observations).

Other markers, used to evaluate hematopoietic, epidermal and hepatic stem cells have also been assessed for their ability to allow the differential enrichment of MECs with outgrowth potential, and therefore putative mammary stem cell activity. Stingl and Eaves have reported preliminary evidence, documenting the success of this approach and the likelihood that a multiplicity of markers will be needed to discriminate stem and/or progenitor mammary cells from more differentiated mammary cells (J. Stingl and C. J. Eaves, BC Cancer Research Centre, Vancouver, Canada, personal communication). In addition, their findings suggest that mouse mammary stem cells retain the fluorescent dye Rhodamine-123, which is in contrast to results from adult mouse hematopoietic stem cells that actively efflux this dye. At present, very little is known about the regulation of mammary stem cell proliferation and the potential role regarding their interactions with the niche they occupy in vivo. Identification of surface markers expressed by mammary stem cells is therefore of additional interest because such information might provide clues to the molecular mechanisms involved in their regulation. Furthermore, to date, most of the published transplantation experiments have not looked at the long-term engraftment potential of any of these markers in serial transplantation experiments. Differences in the age and strain of the mice used for these analyses, as well as the methods used to isolate single MECs and the specific antibodies used for FACS analysis, might in part account for the lack of correspondence of Sca1 expression and outgrowth potential between these different studies.

Hoechst dye efflux

The DNA-binding dye Hoechst 33342 has been used as an unique method to identify potential stem cells in a host of tissues, including the bone marrow, heart, lung, muscle, eye and pancreas (Goodell, 2002; Goodell et al., 1996). The dual emission of the Hoechst dye generates a distinct 'side population' from the whole population of cells, which is enriched in Sca$^+$ and lineage$^-$ (B220$^-$, Gr-1$^-$, Mac-1$^-$, CD4$^-$, CD5$^-$ and CD8$^-$) cells. This unique segregation is conferred by the ATP-binding cassette family of multi-drug-resistant transporter proteins, such as the multi-drug-resistant protein (Mdr1 or p-glycoprotein), which actively pump out the Hoechst dye. In fact, when the whole cell population is treated with verapamil, an inhibitor of these transporters, the SP phenotype is lost (Goodell et al., 1996). In the bone marrow, these 'side population' or SP cells, are enriched approximately 1000-fold in hematopoietic stem cell activity in repopulation experiments, and provide an enrichment of 300-fold in radioprotection of lethally irradiated recipients (Goodell et al., 1996; Goodell et al., 1997). In addition, SP cells also contribute to both the myeloid and lymphoid lineages in the transplant recipients.

Rhodamine-123 is another fluorescent dye whose efflux can be used to enrich for potential stem cells. In bone marrow, the percentage of cells in the Rhodamine-123-effluxing subset is similar to that in the Hoechst-dye-effluxing SP cells (i.e. about 10%) (Spangrude and Johnson, 1990). However, the former does not segregate into a population as distinct as the Hoechst-dye-effluxing SP cells and, thus, must be used in combination with other surrogate stem cell markers for stem cell isolation.

Our laboratory has used a similar approach to identify mammary gland Hoechst-dye-effluxing SP (MG-SP) cells (Welm et al., 2002) and has shown that treatment with verapamil blocks their appearance. Interestingly, although the SP phenotype in hematopoietic cells depends on the presence of the ABCG2/BCRP1 (breast cancer resistance protein) transporter (Zhou et al., 2002), deletion of this gene does not lead to loss of the MG-SP population in BCRP1-null mice, which suggests compensation by other ABC transporters, such as Mdr1 (F.B. and J.M.R., unpublished observations). Interestingly, BCRP1 expression has been shown recently to increase in alveolar progenitors and during lactation, perhaps playing a role pumping xenotoxins into milk (Jonker et al., 2005). Mammary gland reconstitution experiments have demonstrated that MG-SP cells retain pluripotent outgrowth potential (Alvi et al., 2003; Welm et al., 2002). However, the Hoechst dye is toxic to MG-SP cells, which has restricted the functional characterization of these cells. Thus, one cannot yet

demonstrate, in limiting-dilution transplantation experiments, enrichment for stem/progenitor cell activity in the MG-SP cells compared with other cells. This MG-SP population is enriched in label-retaining cells (LRCs) compared with the $Sca1^+$ population alone, and is at least fourfold enriched compared with the non-SP population (Welm et al., 2002). LRCs are slowly cycling candidates for stem cells that retain the BrdU label after a prolonged chase. This suggests that the mammary gland contains stem/progenitor cells that have varying degrees of quiescence. However, the LRCs represent only a subpopulation of the MG-SP cells (<10%). Thus, if the MG-SP population represents 1 in 200 of the primary MECs, the MG-SP/LRCs, which may be a more quiescent, primitive population, appear to represent only 1/2000 cells. Interestingly, this is the number of stem cells present in the mammary gland predicted on the basis of limiting-dilution transplantation experiments (Smith and Medina, 1988). Thus, the majority of the MG-SP (and $Sca1^+$ cells) is probably more committed progenitors.

Smalley and colleagues have demonstrated that MG-SP cells exist in human as well as murine MECs (Alvi et al., 2003). By using known epithelial markers, these investigators showed that the MG-SP cells are relatively undifferentiated and express lower levels of cytokeratins K19 and K14 and higher levels of vimentin than non-SP cells. By characterizing in vitro cultures, they found both MG-SP and non-SP cells express K14 and K18, which are markers of myoepithelial and luminal epithelial cells, respectively. Transplantation into cleared fat pads demonstrated that MG-SP cells give rise both to lobuloalveolar and ductal outgrowths, which suggests that the MG-SP cells retain a full differentiative and developmental potential.

Using human cells obtained from mammoplasty reduction, Clayton et al. (Clayton et al., 2004) compared three candidates for stem cell populations: cells co-expressing the luminal and myoepithelial markers EMA and CALLA; EMA^- and $CALLA^-$ cells; and MG-SP cells. The EMA^+ $CALLA^+$ cells do not efflux Hoechst dye, and therefore these are not enriched in the MG-SP. By contrast, within the MG-SP, the majority of cells are EMA^- $CALLA^-$. Furthermore, the majority of the MG-SP cells are $K18^+$ or $K14^+$, and there is an increased proportion of $K18^+$ $K14^+$ cells. The MG-SP might thus be enriched for a population of bipotential cells, able to give rise to both the $K18^+$ luminal and the $K14^+$ myoepithelial lineages. This study suggested that these three populations represent three distinct cell lineages. The EMA^+ $CALLA^+$ population represents the more committed cell fate, ultimately becoming either luminal or myoepithelial cells. Whereas the EMA^- $CALLA^-$ population, lacking specific epithelial markers, represents a more primitive progenitor, capable of giving rise to both luminal and myoepithelial cell types.

Label-retention studies

Several investigators have used label-retention studies to identify mammary stem cells (Smith, 2005; Welm et al., 2002; Zeps et al., 1998). By labeling 4-week-old virgin mice with BrdU for 2 weeks (a time at which the TEBs are maximally active) and chasing the label for 9 weeks (during which ductal morphogenesis is completed), our laboratory was able to identify a small fraction of BrdU-LRCs in the total population of epithelial cells. Very few of these LRCs express differentiation markers, such as PR, which suggests that they represent a less differentiated state. The LRCs are twice as enriched in the MG-SPs compared with the $Sca1^+$ population, which supports the idea that the mammary gland contains stem/progenitor cells that have varying degrees of commitment. In general, the MG-SPs appear to represent a population of more-primitive stem/progenitor cells, whereas the majority of the $Sca1^+$ population might represent more-committed progenitors. However, since approximately 75% of the MG-SP cells are $Sca1^+$, a subset of these cells clearly overlap (Welm et al., 2002), and both the MG-SP and the $Sca1^+$ population appear to represent cells that have a range of activities.

More recently, Smith (Smith, 2005) asked whether mammary LRCs retain their template DNA strand and pass their newly synthesized chromatids to their daughter cells during asymmetric divisions, an idea originally proposed by Cairns (Cairns, 1975) and later by Potten et al. (Potten et al., 1978; Potten et al., 2002). Smith labeled mice receiving transplanted mammary tissue with [^{3}H]-thymidine (^{3}HTdR) for 5 days, chased for 3-4 weeks and, towards the end of the chase, gave a pulse of a second label, BrdU. LRCs retaining the ^{3}HTdR label (template strand) again were detectable in the mammary gland, and a large percentage of the ^{3}HTdR cells incorporated BrdU. Following the chase, the level of the BrdU label decreased in the daughter cells whereas the ^{3}HTdR was retained. This result suggests that the mammary LRCs selectively retain their ^{3}HTdR-containing template strand, while passing on the newly synthesized BrdU-labeled daughter strand to their progeny during asymmetric divisions. Furthermore, by transplanting LacZ-marked epithelial cells in a similar experiment, Smith demonstrated that the mammary gland stem cells can undergo self-renewal as well as asymmetric division in mammary gland outgrowths. The identification of a population of actively dividing LRCs demonstrated by the incorporation of BrdU indicates that these cells are not totally quiescent.

Hormone receptor status

One unique aspect of mammary gland development is its dependence on the circulating steroid hormones estrogen and progesterone. Furthermore, the majority of breast cancers are estrogen receptor (ER) positive and are responsive to hormonal therapy (Allred et al., 2004; Sorlie et al., 2003). Thus, whether mammary epithelial stem cells express steroid receptors such as ERα or PR is, therefore, a critically important question relevant to the etiology of ER-positive and ER-negative breast cancers. Clarke and colleagues (Clarke et al., 2005) have used several complementary approaches to characterize human breast epithelial stem cells with respect to ERα and PR. First, using long-term [^{3}H]-labeling of human breast epithelial xenografts implanted in athymic nude mice, they showed that the LRCs co-express putative stem cell markers such as $p21^{CIP/WAF1}$ and Msi1, an ortholog of the *Drosophila* Musashi protein involved in asymmetric stem cell division. A proportion of the cells also express steroid receptors. Next, by co-staining cells in the mammary gland for K19, a putative stem cell marker, and steroid receptors, they observed that $K19^+$ cells frequently express steroid receptors and, conversely, steroid-receptor-positive cells in the gland are likewise $K19^+$. Finally,

by analyzing Hoechst dye efflux, the authors observed that steroid-receptor-positive cells are highly enriched in the MG-SP cells compared with the non-SP population. Furthermore, the steroid-receptor-positive MG-SP cells can generate branching structures that include myoepithelial as well as luminal epithelial cell types, when grown on matrigel. Accordingly, Clarke et al. (Clarke et al., 2005) suggested that steroid-receptor-positive cells are enriched in breast epithelial stem cells with the capacities for self-renewal and differentiation.

Mammospheres

Neural stem cells cultured in suspension form clusters of apparently homogenous cells called 'neurospheres', which display an increased capacity for self-renewal. Using an analogous approach, Wicha and colleagues have developed a method to enrich for mammary stem cells in an undifferentiated state by culturing cells under anchorage-independent conditions (Dontu et al., 2003). The 'mammospheres' contain $CD49f^+$, $K5^+$ and $CD10^+$ cells. A few cells express luminal and myoepithelial markers, such as ESA and K14. When grown on a collagen substratum, mammosphere-derived single cells differentiate into colonies that express markers specific for only ductal or myoepithelial cells, or markers of both cell lineages. When grown on matrigel, the single cells can differentiate into functional complex branching structures similar to ductal and alveolar structures. In addition, when treated with prolactin, these mammospheres form functional alveolar cells that secrete β-casein into the lumen. By growing mammosphere-derived single cells, not only did these investigators show bipotential (giving rise to both luminal and myoepithelial cell types) and tripotential (giving rise to luminal, myoepithelial and alveolar cell types) differentiating capacity, they also demonstrated by retroviral tagging that mammospheres are clonally derived. In addition, they demonstrated that these cells can be propagated through multiple passages in an undifferentiated state and retain their multipotent capacity.

Cancer stem cells

Mutations that initiate breast cancer appear to accumulate slowly in cells that persist throughout a woman's lifetime, since there is an exponential increase in breast cancer incidence with age, and since girls exposed to excess radiation in adolescence have an increased risk of breast cancer 20-30 years after the exposure. It has been hypothesized that delayed cancers result from damage to a quiescent cell with unlimited potential for self-renewal that may persist for decades and ultimately give rise to a malignancy in response to an unknown proliferative signal. For this reason, stem cells make an attractive candidate for the cellular origin of cancer since they possess many features of the tumor phenotype, including self-renewal and essentially unlimited replicative potential (Reya et al., 2001).

Until recently, the prospective identification of tumor stem cells, which are a limited population of tumor cells responsible for giving rise to all components of a heterogeneous tumor, had remained elusive. However, Clarke and colleagues (Al-Hajj et al., 2003) have now used cell-surface markers to isolate a subpopulation of highly tumorigenic breast cancer cells from the bulk of human breast tumor cells. They observed that $CD44^+$ $CD24^-$ human breast tumor cells have an increased ability to form tumors when injected into the cleared mammary fat pad of etoposide-treated NOD/SCID mice, and that although as few as 100 $CD44^+$ $CD24^-$ human breast tumor cells can re-capitulate the human tumors from which they are derived, injection of 10,000 cells of other phenotypes fails to give rise to tumors. Tumors arising from $CD44^+$ $CD24^-$ cells are heterogeneous, giving rise to tumorigenic cells and a population of non-tumorigenic cells. In addition, the $CD44^+CD24^-$ cells can propagate indefinitely. Therefore, this subpopulation possesses stem cell characteristics, such as the ability to self-renew and to give rise to multipotent progenitors. Although these authors were unable to demonstrate tumor outgrowth from a single tumor stem cell, these data significantly advanced the hypothesis that tumor stem cells exist in human solid tumors and underscore the importance of better understanding of stem cell biology in the treatment of human tumors.

Signaling pathways implicated in stem cell self-renewal

Understanding the signaling pathways involved in the self-renewal of both normal and cancer stem cells is an important first step towards anti-cancer therapies targeting cancer stem cells. Studies of hematopoietic, intestinal, muscle and embryonic stem cell models have identified several key signaling pathways involved in self-renewal and maintenance of the stem cell pool (Yamashita et al., 2005). These include the Wnt/β-catenin, Notch, Hedgehog (Hh), transforming growth factor (TGF)-β, PTEN and Bmi signaling pathways (Andl et al., 2002; Boulanger et al., 2005; Brennan and Brown, 2004; Dontu et al., 2004; Hatsell et al., 2003; Ingham and McMahon, 2001; Korinek et al., 1998; Leung et al., 2004; Lewis et al., 1999; Machold et al., 2003; Molofsky et al., 2004; Reya et al., 2003; Stiles et al., 2004). Unsurprisingly, many of these pathways have been implicated in cancer, which is consistent with the hypothesis that dysregulation of normal stem cell self-renewal can lead to cancer initiation.

In the mammary gland, increasing evidence supports a role for Wnt/β-catenin, Notch and Hh signaling pathways in mammary stem/progenitor cell self-renewal. In addition, Deugnier et al. (Deugnier et al., 2002) have suggested that epidermal growth factor (EGF) signaling controls the developmental potential of transplanted murine mammary BC44 cells (a clonal derivative of HC11 mouse mammary epithelial cells, which express basal markers). This is interesting given the impressive pre-clinical and clinical data suggesting EGF receptor inhibitors potentiate the effects of radiation and improve overall survival in some cancers (Harari, 2004).

Wnt/β-catenin

Wnt is a secreted protein that binds to its receptor – frizzled (FZD) – and leads to the stabilization and translocation of β-catenin into the nucleus, where it binds the LEF/TCF transcription factors (Moon et al., 2002). Wnt signaling is involved in patterning during development and components of the pathway are mutated in several cancers, including

colorectal cancer, desmoid tumor and hepatoblastoma (Beachy et al., 2004). The *Wnt* gene was originally identified as a viral insertion in mouse mammary tumor virus- (MMTV) (Nusse and Varmus, 1992) induced mammary tumor. Stabilization of β-catenin has been demonstrated in >50% of human breast cancers, although overt mutations of pathway components in breast cancer have yet to be identified (Brennan and Brown, 2004). Loss of Wnt inhibitors such as SFRP1 and increased levels of β-catenin are associated with poor prognosis in breast cancer (Klopocki et al., 2004; Ugolini et al., 2001). In addition, Jain and colleagues have demonstrated that even transient loss of expression of the Wnt downstream target gene, *Myc*, can lead to irreversible loss of malignant cells (Jain et al., 2002). Recent studies also suggest that autocrine Wnt signaling plays a role in several human cancer cell lines, including breast and ovarian lines (Bafico et al., 2004).

β-catenin has been implicated as a stem cell survival factor in several systems, including neural crest cells, gastrointestinal crypts, epidermal follicles and hematopoetic stem cells (Reya et al., 2003). Inhibition of β-catenin signaling in mammary alveolar progenitors blocks mammary development and pregnancy-induced proliferation, implicating β-catenin as a stem cell survival factor in the mammary gland (Tepera et al., 2003). Alexander and colleagues provided the first direct evidence of Wnt signaling pathways in the maintenance of the stem/progenitor pool in the non-neoplastic mammary gland (Liu et al., 2004). They showed that the SP-enriched progenitor fraction is increased in the mammary gland of MMTV–Wnt-1 and MMTV–ΔNβ-catenin transgenic mice, and that ectopic Wnt-3a increases the SP fraction in MECs after 3 days in culture. The SP fraction in MECs expands in culture in response to radiation treatment, and this effect is significantly increased in MECs from MMTV–Wnt-1 mice. This suggests that Wnt signaling can mediate radiation resistance of the progenitor fraction in non-neoplastic MECs (W.A.W., M.S.C., F.B., J.M.R. and M. P. Alfaro, unpublished observations).

Recent studies have also demonstrated a role for Wnt signaling in neoplastic mammary stem-cell-like progenitors (Li et al., 2003). Li et al. have demonstrated an expansion of $Sca1^+$ progenitor cells in pre-neoplastic and neoplastic mammary gland lesions from MMTV–Wnt-1 mice and other transgenic mice in which the Wnt pathway is active, but did not observe this in other mammary tumor models in which the Ras pathway is activated (Li et al., 2003). In addition, these studies suggested that $K6^+Sca1^+$ cells present in neoplastic mammary lesions from Wnt-1-transgenic mice might represent bipotent cells, in this case capable of giving rise to both luminal and myoepithielial tumor cells, which represent target cells for stochastic mutations that result in mammary tumorigenesis.

Although the phosphatase PTEN, a tumor suppressor mutated in almost as many cancers as p53 (Stiles et al., 2004), has been implicated in stem cell renewal in embryonic stem cells, few studies have focused on its role in stem cell renewal in the mammary gland. Li et al. (Li et al., 2003) have demonstrated loss of PTEN heterozygosity in tumors derived from the progeny of MMTV–Wnt-1 mice crossed with $PTEN^{+/-}$ mice. Interestingly, the loss of PTEN occurs in both the luminal and myoepithelial lineages, which suggests that this occurs in a bipotent progenitor, perhaps analogous to the $K14^+K18^+$ cells observed in the SP population by Vivanca and colleagues (Clayton et al., 2004). The PTEN signaling pathway interacts with numerous signaling pathways important for development and can affect Wnt signaling indirectly through stabilization of the β-catenin cytosolic pool (Stiles et al., 2004). Therefore, the role of the interaction between Wnt and PTEN signaling in mammary stem/progenitor cell renewal remains to be elucidated.

In addition to this recent evidence for Wnt/β-catenin signaling in normal and neoplastic adult mammary stem/progenitor cells, there is substantial evidence for Wnt/β-catenin signaling in the developing mammary gland (Howe and Brown, 2004). Knockout mice lacking the Wnt/β-catenin pathway transcription factor LEF (van Genderen et al., 1994) and mice expressing the Wnt signal inhibitor Dickkopf driven by the K14 promoter fail to develop mammary glands (Andl et al., 2002). These data support the speculation that Wnt is necessary for maintenance of the stem cell pool in the mammary bud (Brennan and Brown, 2004; Korinek et al., 1998).

Notch

The interaction of Notch receptors with their ligands (Delta-like-1, -3 or -4 and/or Jagged-1 or -2) promotes cleavage of the intracellular domain. This involves the ADAM protease family and γ-secretase, and allows the intracellular domain of Notch to translocate to the nucleus and act on downstream target genes (Weng, 2004). Notch 4 is important both in normal mammary gland development (Smith et al., 1995) and was identified as an MMTV insertion site in mammary tumors in mice (Gallahan and Callahan, 1997). Accordingly, transgenic mice carrying constitutively active Notch 4 develop mammary tumors (Callahan and Egan, 2004). Dontu and colleagues have examined the role of Notch in the formation of mammospheres from human MECs derived from mammoplasty specimens (Dontu et al., 2004). A synthetic notch ligand shown to induce luciferase activity from the Hes promoter, a known downstream target of Notch signaling, increases secondary mammosphere formation tenfold. Conversely a Notch-4-blocking antibody completely abrogates secondary mammosphere formation (Dontu et al., 2004). These data from human specimens are the first to demonstrate directly a role for Notch signaling in stem cell renewal in the mammary gland.

Hedgehog

Regulation of Hh signaling occurs during normal development of virtually every organ system, including the mammary gland (Bailey et al., 2000; Cohen, 2003), and components of this pathway have been shown to be mutated or overexpressed in multiple cancers, including breast cancer, basal cell carcinoma, medulloblastoma, fibrosarcoma and rhabdosarcoma (Beachy et al., 2004). The core components of the Hh signaling network (Lewis et al., 2001) include ligands (Sonic hedgehog, Shh; Indian hedgehog, Ihh; and Desert hedgehog, Dhh), receptors (Patched-1 and -2, Ptc1 and 2), effector (Smoothened, Smo) and transcription factors (e.g. Gli1-3). In the central nervous system, Hh is required for neural stem cell proliferation in neurospheres, and inactivation of smoothened inhibits proliferation of neural stem cells in vivo and in vitro (Machold et al., 2003). Hh is also required for proliferation of somatic ovarian stem cells in *Drosophila* (Zhang and Kalderon, 2001).

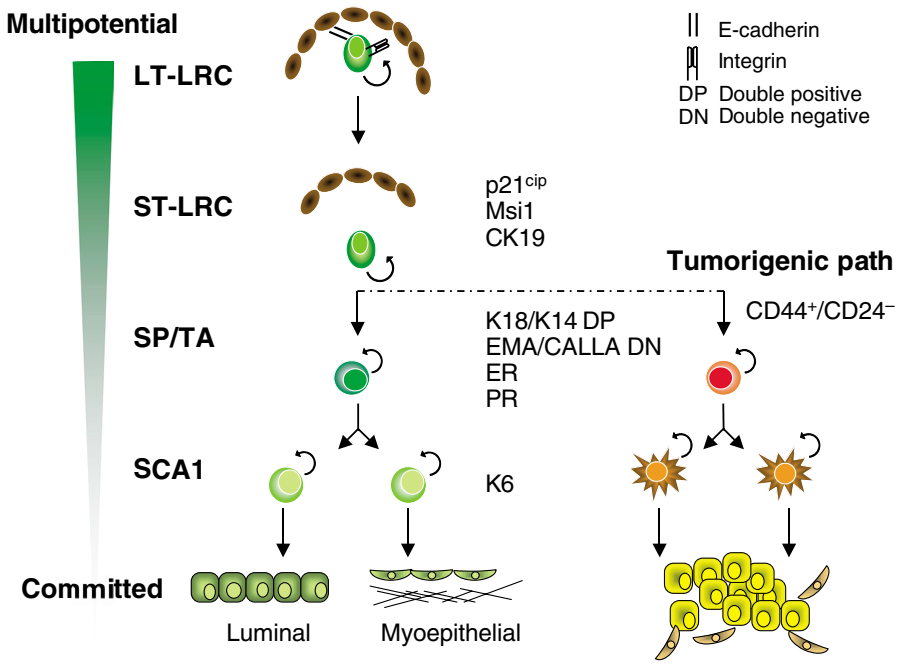

Fig. 2. Mammary gland stem/progenitor-cell fate. The degree of stemness potentially decreases from top to bottom: as the cell becomes more committed, the cell gradually loses its stemness. The stem cells are able to self-renew and proliferate within the niche, maintained in their un-differentiated state by cell-matrix and cell-cell interactions with the niche cells, involving integrins and cadherins, respectively. These cells can be distinguished by their long-term label-retaining cell (LT-LRC) properties, which are thought to reflect a state of quiescence. Responding to stimuli, stem cells exit the niche by becoming short-term (ST)-LRCs. These actively cycle and express stem cell markers such as $p21^{cip}$, Msi1 and CK19. As they become further committed, they become the transit-amplifying progenitors (TAs), comprising the side population (SP) that are able to efflux the Hoechst dye. The SP/TAs express bipotential markers, such as $K18^+$ and $K14^+$, or EMA^- $CALLA^-$, and may be steroid receptor positive. The SP/TA cells eventually give rise to more committed progenitors that are $Sca1^+$. The $Sca1^+$ population differentiates into luminal and myoepithelial cells. Stem cells are thought to possess many of the features that constitute the tumor phenotype, including self-renewal and unlimited replicative potential. Tumorigenic mutations are presumably sustained in the expanding SP/TA population. These cells give rise to tumorigenic progenitor cells. $CD44^+$ $CD24^-$ may be markers that distinguish tumorigenic progenitor cells from normal progenitor cells.

Studies of mammary gland ductal morphogenesis provide support for a role for Hh in interactions between the stroma and epithelial cells in the developing mammary gland (Gallego et al., 2002; Lewis et al., 1999; Lewis et al., 2001). In addition, recent studies have suggested that Hh signaling is activated in a majority of human breast cancers, based on immunohistochemical staining showing uniform overexpression of PTC1 and nuclear GLI1 (both markers for activated Hh signaling) in a set of 52 invasive breast cancers (Kubo et al., 2004). Furthermore, the Hh inhibitor cyclopamine can inhibit growth of a subset of breast cancer cell lines in vitro (Kubo et al., 2004). Preliminary studies in several laboratories have also suggested that the Hh pathway also plays a critical role in mammary stem cell self-renewal (G. Dontu and M. Wicha, personal communication; M. T. Lewis and J.M.R., unpublished observations).

A model for stem cell progression

The work of several laboratories has identified several distinct populations of stem/progenitor cells that display different degrees of commitment (Fig. 2). The [^{3}H]TdR-LRCs that do not retain BrdU, which we can term long-term LRCs (LT-LRCs), might represent the most primitive, quiescent, template-retaining stem cells present in the stem cell niche. Short-term LRCs (ST-LRCs) that actively cycle and are labeled by BrdU, but retain their original DNA template strand, would represent the next level. The heterogeneous MG-SP population appears to represent primarily a transient-amplifying population (SP/TA), but is also enriched in LRCs. It is more enriched in LRCs than the $Sca1^+$ population and, therefore, might be less differentiated than both the $Sca1^+$ and the EMA^+ $CALLA^+$ populations. To determine where cancer stem cells fit into this lineage, we have extrapolated from studies in the hematopoietic field to suggest that mammary gland stem cells are sequestered in a stem cell niche where their quiescence is maintained by adhesion. Increased activation of certain oncogene products, including Myc (Wilson et al., 2004), possibly as a result of the activation of the canonical Wnt/β-catenin signal transduction pathway, may decrease adhesion in asymmetrically dividing daughter cells. Once the stem cells exit the niche, they might become actively dividing early progenitor cells that retain their parental DNA template strand; subsequently, the more committed progenitors, TA cells, no longer retain the template DNA strand and continue to expand. These cells may then accumulate oncogenic mutations and be the primary targets for tumorigenesis.

Clinical implications

Data identifying cancer stem cells in leukemia and solid human tumors such as medulloblastoma, glioma and breast cancer highlight the need for a dramatic shift in the way we design cancer therapies. Since a small population of cancer stem cells can recapitulate the entire tumor, we must assess the efficacy of current cancer therapies at eradicating this small population, which probably drives cancer recurrence.

Clinically, radiation therapy is typically given in small daily doses to reduce normal tissue toxicity yet still achieving adequate tumor cell kill. Radiobiology studies from the 1980s demonstrated that tumors can undergo accelerated repopulation between daily fractions of radiation dose in both

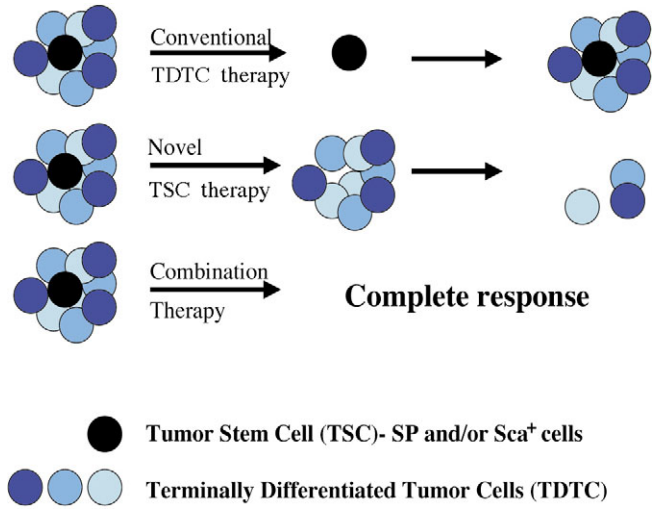

Fig. 3. Cancer therapy that does not kill tumor stem cells may provide gratifying initial results but ultimately result in recurrence. Conventional therapies target proliferating, terminally differentiated cells may leave tumor stem cells, which could lead to recurrence. Ideally, tumor stem cell therapies would specifically target tumor stem cells. Used alone, they might lead to tumor regression, but not dissolve tumor bulk, leading to questions regarding response rates and potentially untreated tumor-related symptoms. Combining conventional therapy with treatment targeting tumor stem cells may effectively eliminate both tumor bulk and tumor stem cells that might otherwise lead to recurrence.

in vivo and in vitro tumor models (Thames et al., 1996). This effect was demonstrated in clonogenic assays in which small single doses of radiation increased the number of tumor clonogens. An understanding of this biological phenomenon led to randomized trials of altered fractionation radiation therapy schedules, such as concomitant boost, whereby the last week of radiation therapy includes a second daily fraction during the fifth week to counteract the effect of accelerated repopulation. This scheme has been shown in a multi-center Phase III randomized clinical trial to improve overall survival in head and neck cancer (Fu et al., 2000). These data support the hypothesis that the clinical effect of accelerated repopulation derives from tumor stem cell clonogens responding to cellular stress that results from either radiation or potentially chemotherapy. Tumor stem cells might be more resistant to radiation than the differentiated cells that make up the bulk of the tumor, and it is possible that it is the resistant tumor stem cells remaining after definitive therapy that ultimately self-renew and amplify to give rise to tumor recurrence.

This phenomenon might apply in the mammary gland. Treatment of Sca1$^+$ immortalized mouse mammary cells with either taxol or radiation leads to an increase in the number of clonogens formed in matrigel, which is consistent with the hypothesis that progenitor cells are resistant to radiation and might expand in response to radiation (W.A.W. et al, unpublished observations). Interestingly, Ly-6E.1, a human ortholog of Sca1, is a marker of advanced tumorigenicity and upregulated in response to stress such as heat shock or serum starvation (Treister et al., 1998). Conventional cancer therapy that targets proliferating, terminally differentiated cells with limited replicative potential may initially lead to a favorable clinical response but fail to eliminate the small population of cancer stem cells that underpin recurrence. Thus, investigation of the mechanisms and signaling pathways that support stem cell renewal in normal and malignant tissue may provide new targets for therapies designed to complement existing approaches and reduce tumor recurrence (Fig. 3).

These studies were supported by grants CA16303 and CA84243 from the National Institutes of Health and a predoctoral fellowship DAMD-17-03-1-0699 from the Department of Defense. The authors would like to thank M. T. Lewis and G. Tucker for their help with the Sca1-GFP imaging studies, and S. L. Grimm for her help with graphics.

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