Cell. Mol. Life Sci. 64 (2007) 3248 – 3260
1420-682X/07/243248-13
DOI 10.1007/s00018-007-7391-5

Cellular and Molecular Life Sciences

Mammary stem cells and breast cancer

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Online First 22 October 2007

Abstract. The mammary gland undergoes major developmental changes during puberty and pregnancy. It is thought that stem cells drive mammary gland development during puberty and are responsible for tissue maintenance as well as the major growth and remodelling that occurs with every pregnancy. The use of sophisticated cell separation procedures has facilitated the prospective isolation of mammary epithelial stem and differentiated cell subpopulations from the mouse mammary gland, while studies of primary human breast cancers have described sub-populations of tumourigenic cells capable of initiating tumour growth in immuno-compromised mice. These potential tumour 'stem cells' constitute an important therapeutic target population with respect to cancer therapy, as these are likely to be the cells which maintain tumour growth. Understanding the origin of these cells, their relationship to breast cancer subtypes, and how and why they differ from normal breast stem cells will lead to a revolution in tumour understanding, treatment and prevention. (Part of a Multi-author Review)

Keywords. Breast, mammary, tumour, stem, estrogen receptor, CD24, CD44, CD133, Sca-1, field cancerisation.

Introduction

Tumours are heterogeneous entities. In particular, it is well established that they are proliferatively heterogeneous, with only a subfraction of tumour cells able to grow either *in vitro* or in *in vivo* xenograft models. Two theories have been proposed to explain this. The stochastic theory suggests that all cells within a tumour have an equal but low probability of initiating tumour growth in *in vitro* or *in vivo* models. In contrast, the stem cell theory of cancer predicts that tumours contain a small number of tumour-initiating cells, or cancer 'stem cells', which drive tumour growth, and populations of more differentiated non-tumourigenic daughter cells, analogous to the transit amplifying and differentiated cells of the normal tissue [1]. The tumour is, in effect, a caricature of normal tissue development. These theories have profound consequences for tumour therapy. If the stem cell theory of cancer is correct, assessing anti-cancer agents according to their ability to shrink tumours may be flawed, as this approach may only eliminate cells which make up the bulk of the tumour, the progeny of the cancer stem cell, sparing the tumour-initiating cell population. Failure to destroy cancer stem cells would inevitably result in disease relapse. Therefore, a more targeted approach to cancer therapy may be necessary which aims at destruction or differentiation of the tumour-initiating cancer stem cells.

It must be emphasised that cancer stem cells do not have to arise from normal tissue stem cells. However, the longevity of normal stem cells increases the risk of these cells, over a lifetime, acquiring the multiple genetic mutations necessary for tumourigenesis. The stem cell is therefore the perfect candidate for tumour initiation. There are situations, however, in which transit amplifying – or even differentiated – cells could generate cancer stem cells. For instance, a first genetic lesion may reactivate a self-renewal pathway within these cells, effectively allowing them to reacquire stem cell characteristics. However, although transformation of progenitor cells with an MLL fusion gene has been demonstrated in a haematological setting [2],

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there is as yet no direct evidence that differentiated cells can generate cancer stem cells, to our knowledge. If it occurs, transformation of more differentiated cells into cancer stem cells is likely to be a very rare event – unless it occurs in the context of 'field cancerisation' (discussed further below).

This review will examine the recent evidence for mammary stem cells, the relationship between normal and cancer stem cells, and the possibility that transformation of different cell types in the breast may generate different types of breast cancer. It will also suggest that field cancerisation may be a significant but underappreciated complication of breast cancer treatment. For further discussion of some of the issues raised as well as a more detailed description of older studies see Smalley and Ashworth, 2003 [3], and Smalley and Clarke, 2005 [4].

Mammary gland development

The epithelium of the mature, non-pregnant mouse mammary gland resembles a branching treelike structure consisting of an inner layer of luminal epithelial cells which line the ducts, surrounded by a continuous layer of basal myoepithelial cells. A basement membrane separates these epithelial layers from the surrounding fatty stromal matrix in which they are embedded [5]. Luminal epithelial and basal myoepithelial cells can be identified by location and by the expression of cell-type-specific cytoskeletal markers, namely cytokeratin 8, 18 and 19 for the luminal epithelial layer, and cytokeratin 14 and α-isoform smooth muscle actin (SMA) for the basal myoepithelial layer [6].

In the mouse, the mammary gland develops embryonically from ectodermal thickenings which extend from the neck to the inguinal region, appearing at embryonic day (ED) 10–11 [5]. Epithelial buds begin to develop along this structure, gradually increasing in size until ED 16 when they begin to extend into the surrounding mesenchyme to form a primitive branched ductal system [7, 8]. The rudiments of the mammary gland remain quiescent during the first 3 weeks of life, until puberty. At this time, the increase in steroid hormones, oestrogen and progesterone, stimulate significant ductal growth [9]. This growth is driven by specialised structures at the tips of the elongating ducts, the terminal end buds (TEBs) [10]. These consist of two morphologically distinct cell types, an inner layer of body cells and an outer layer of cap cells [11, 12], which give rise to luminal and basal cell layers, respectively, of the subtending duct [5, 10, 13–15]. It is also clear that somewhere within the TEBs there is a stem cell activity [16] most likely located within the cap cells, such that they also give rise the body cells as well as the basal cell layer [10,12,15]. In humans, structures analogous to the TEBs have been observed [17].

Between three and approximately 10–12 weeks of age, the ducts grow into the mammary fat pad, branching at regular intervals, to create a network of primary and secondary ducts which form continuous channels from the site of milk production during lactation, the secretory alveoli, to the nipple. Once the mammary fat pad has been filled with glandular tissue, the TEBs regress and are converted to terminal ducts. During every oestrous cycle, small side branches form and disappear on primary and secondary ducts [5]. Depending on mouse strain, precursors of the secretory alveolar structures called alveolar buds will also appear and regress during the oestrous cycle. Older virgin animals tend to have more elaborate mammary epithelial networks than younger ones, suggesting that at least some of the cyclic proliferative changes are permanent.

During pregnancy, a surge of hormones results in major structural changes in the mouse mammary gland. Proliferation and maturation of the side branches occurs to form alveoli which are lined by functionally differentiated secretory luminal epithelial cells responsible for milk production [7]. The side branches and alveoli are apparently uniformly distributed within the mouse mammary gland. In humans, however, the milk secretory alveoli are localised in clusters which resemble bunches of grapes and are called Terminal Ductal Alveolar Units (TDLUs). The TDLUs contain smaller ducts which link the alveoli and are themselves linked by a network of larger ducts. Interestingly, the vast majority of breast cancers arise in the TDLU system – not in the large ducts which link them [18]. In humans, at least some TDLUs are already present prior to pregnancy, although they are probably not as complex as during pregnancy. The epithelial expansion during human pregnancy probably consists of a combination of an elaboration of the existing TDLUs and *de novo* ductal and lobular growth [19, 20], although this is not entirely clear, and new studies are required. The extent of the pregnancy-dependent proliferation is variable between individuals.

During suckling, milk is expelled by contractile myoepithelial cells, which form a basketlike network around the alveoli, down the ductal network to the nipple. The termination of suckling and the subsequent accumulation of milk initiates significant structural remodelling known as involution (for a review of mammary involution see Watson, 2006 [21]). Apoptotic cell death within the mammary epithelium during involution results in collapse of the alveoli

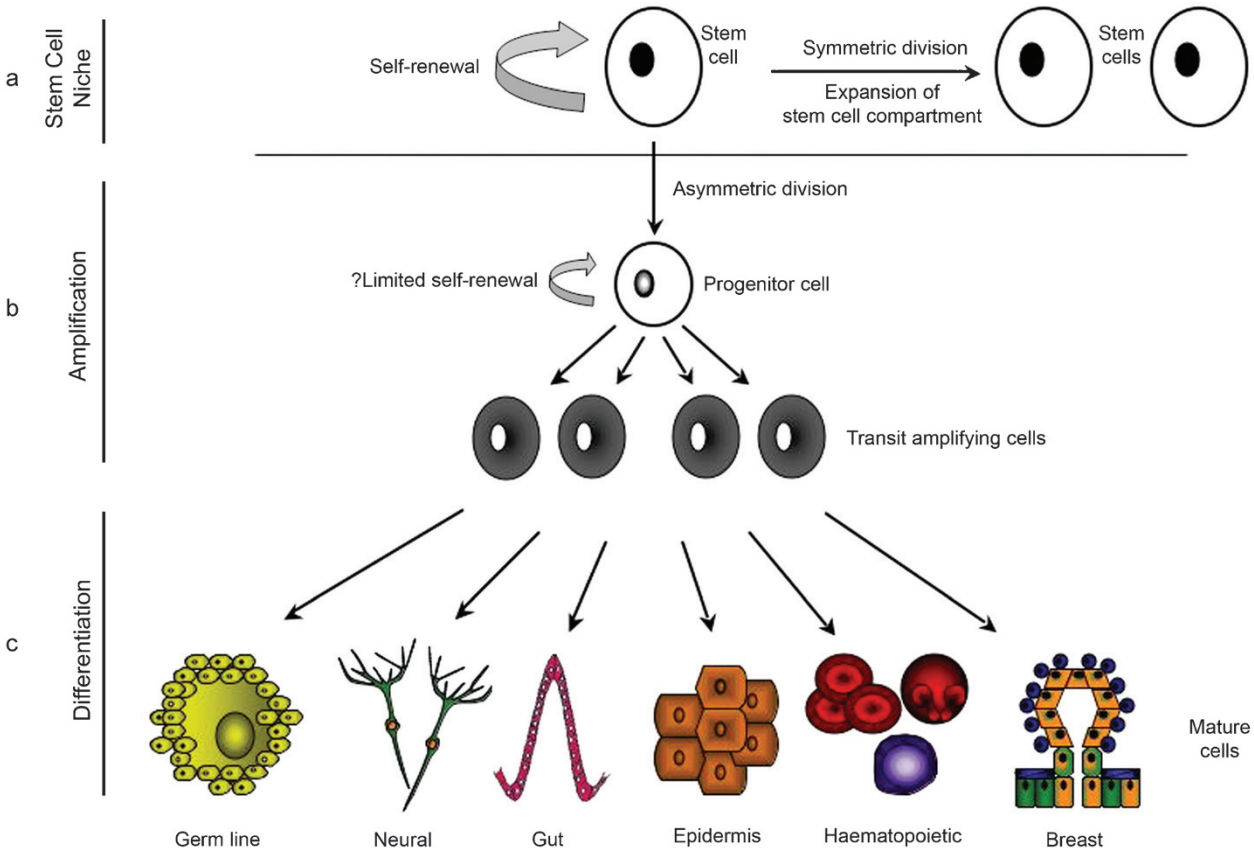

Figure 1. Adult stem cells self renew and undergo multilineage differentiation to maintain the adult tissues. (*a*) Stem cell self-renewal and expansion is regulated by signals from the stem cell 'niche' [97–100]. During symmetric division, each daughter cell is identical and retains the stem cell potential of the parent cell, resulting in an expansion of the stem cell compartment. (*b*) When cells divide asymmetrically, one of the daughter cells remains a stem cell while the other begins the process of cell number amplification and differentiation. This process may involve a number of intermediates, such as progenitor cells, which may have a limited self-renewal capacity and may retain multilineage differentiative capacity, and transit amplifying cells, which are the main proliferative compartment downstream of the stem cells and comprise the major source of cells for normal tissue renewal. (*c*) Ultimately, the transit amplifying cells differentiate into the functional cell types in each tissue. The self-renewal/differentiation of stem cells is regulated by signalling pathways such as Wnt, Notch and Hedgehog, all of which are known to play important roles in human carcinogenesis [101–104].

and a return to a predominantly ductal structure resembling the mature virgin gland. The mammary gland undergoes this cycle of proliferation, differentiation and involution with every pregnancy, and it has therefore been suggested that to sustain these developmental changes, a mammary epithelial stem cell must be present. However, until recently the identity of these cells has remained elusive.

In situ studies of stem cells in the normal mouse mammary gland

Studies of the haematopoietic system have been at the forefront of stem cell research for many years. Highly efficient strategies for cell isolation, using flow cytometry, in conjunction with *in vivo* analysis, have allowed the isolation and characterisation of small sub-populations of stem and progenitor cells within a larger more heterogeneous population. Such studies have contributed to the development of a well-characterised model (although one which is still being refined), describing the development of mature, terminally differentiated cells from a common stem cell via a series of cell divisions. This model may be applied to other organs of the body where constant production of terminally differentiated cells is necessary in tissue maintenance (e.g. the skin and gut) and repair and regeneration (e.g. the liver) (Fig. 1). The emerging common theme of the molecular pathways involved in the maintenance of this cellular hierarchy is the use of developmental signalling pathways such at Wnt, Hedgehog and Notch. Proving the applicability of the model to the mammary gland has taken many years, and it is still only just becoming generally accepted that this hierarchical stem / progenitor / differentiated cell model is of relevance to the mammary epithelium.

A number of approaches have been taken to identifying mammary stem cells [3]. *In situ* studies of the rat and mouse mammary gland [22] identified potential mammary stem and progenitor cells at the ultrastructural [23] and light microscopy levels [24] at all stages of mammary development. Cells described as small light cells (SLCs) were proposed to be stem/early progenitor cells on the basis of their small size, mitotic activity and lack of organelles. These SLCs made up approximately 3 % of the total cells in the rat mammary gland and were found to reside in a suprabasal position, in between luminal and myoepithelial cell layers, in putative stem cell niches [22, 24, 25]. A hierarchical arrangement of the cells within the mammary gland was suggested in which SLCs give rise to functionally differentiated large dark cells via transit-amplifying undifferentiated large light cells and differentiated large light cells [22]. Similar SLCs or basal clear cells have been identified in the human and bovine mammary glands [26–28].

The hypothesis that stem cells have a slow *in vivo* cycling time has led to the use of DNA label retention in pulse-chase type experiments to identify putative stem cells. In these experiments, a DNA label such as [^{3}H]-thymidine treatment or bromo-deoxyuridine (BrdU) is incorporated into the DNA of all cells during a pulse period. In the chase period following the label pulse, the label is lost as cells die or divide and dilute the label between their daughters. However, in the long-lived and slowly cycling stem cell populations, the label is retained. Both estrogen receptor α (ERα) and progesterone receptor (PR)-positive cells in the mouse mammary epithelium have been identified as cells that retain the DNA label [29–32]. Consistent with the slow cycling times is the observation that, in the normal human adult tissue, ER positive cells do not express markers of proliferation [33]. It has been suggested that ER downregulation occurs in these cells prior to the proliferative response, as stimulation with estrogen led to a decrease in ER expression within 4 h in mice [34]. These observations have led to the proposal that ER$^+$ cells may contain a stem/progenitor cell compartment.

These results need to be reinterpreted in the light of data on immortal strand retention – the 'Cairns hypothesis' [35, 36]. This suggests that stem cells will retain an 'immortal' DNA strand which is used as a template for all DNA replication cycles, whereas newly replicated DNA strands are lost to daughter cells destined to differentiate and die. This is proposed as a protective mechanism to prevent accumulation of DNA damage in stem cells. Direct evidence of immortal strand retention has been found in the mouse mammary gland using [^{3}H]-thymidine and BrdU double labelling. More than 80 % of [^{3}H]-thymidine-positive label-retaining cells (labelled during the pubertal ductal growth phase) were found to divide asymmetrically, retaining the [^{3}H]-thymidine-labelled DNA and passing the BrdU-labelled DNA onto the daughter cell [37] – in other words, rather than being quiescent, >80 % of label-retaining cells in the pubertal mouse mammary gland were actively cycling. However, it was also found that label-retaining cells were not a homogeneous population, with 27 % being ERα-positive (0.54 % of the total epithelial cells), although the majority of ERα positive cells (which formed 31 % of the total epithelial population) were not label retaining [32]. Heterogeneity of label-retaining cell populations has also been demonstrated by studies on human breast TDLUs explanted into mice, which showed that only approximately 15 % of label-retaining cells were positive for either one of two putative stem cell markers, p21^{Cip1} and Musashi-1 (Msi-1) [38]. It is therefore likely that label retention identifies more than just stem cell populations and casts doubt on the assumption that label retention and/or immortal strand retention *per se* are a defining feature of stem cells.

Identification of mouse mammary stem cells by cleared fat pad transplantation

The gold standard assay for stem/progenitor cell capacity in a cell population is the ability to regenerate the tissue in a transplantation model and produce all the terminally differentiated cells within that tissue. Ideally, it should be shown that a single transplanted candidate stem cell regenerates the tissue and its component cell lineages, while at the same time repopulating the stem cell compartment through self-renewal. In the mammary gland, stem cell assays are based around transplantation of mammary epithelial cells into syngeneic or immuno-compromised hosts using the cleared mammary fat pad transplantation assay. This technique, first described by DeOme et al. [39], depends on the incomplete development of the pre-pubertal mammary gland. Prior to pubertal ductal elongation, the mammary epithelium of the fourth (abdominal) fat pad is concentrated proximal to the nipple and has not yet passed the mammary lymph node. It is therefore possible to use the lymph node as a morphological marker to enable removal of endogenous epithelium, leaving the remainder of the fat pad clear for transplantation of candidate stem cell populations. If the candidate population does have stem cell activity, it will form ductal outgrowths with high frequency, even at limited dilutions, which resemble the normal endogenous epithelium (except

they are not connected to the nipple), contain basal and luminal cell layers and respond to pregnancy by generating alveoli. There is a caveat to this technique, however. It is possible that an endogenous epithelium-free mammary fat pad represents a stem cell niche with the ability to induce stem cell behaviour in a transit-amplifying cell. It has been recently demonstrated in a spermatogonial stem cell transplant system that a compartment of 'potential' stem cells exists in the transit-amplifying cell population in addition to the 'actual' stem cells within the tissue. These potential stem cells can replace the actual stem cells, should the latter compartment become depleted [40].

A number of different studies have used the cleared fat pad transplant technique to characterise the stem cell activity of the mammary epithelium. Fragments of mammary epithelia isolated from mature mouse mammary glands gave rise to complete glandular outgrowths when implanted into the cleared mammary fat pads of syngeneic hosts [41, 42]. Moreover, the location from which the fragments of mammary epithelia were removed did not affect outgrowth potential, suggesting stem cells are located throughout the mammary gland. Further studies demonstrated that at limiting dilution mammary epithelial cells were able to generate three different types of outgrowth which either showed only ductal, only alveolar or both ductal and alveolar development, suggesting the presence of three different stem/progenitor activities, possibly in a hierarchy [43]. Transplantation of retrovirally tagged mammary epithelial fragments provided evidence that transplanted outgrowths can be derived from a single stem cell [41]. Studies on parous (as opposed to virgin) but not currently pregnant mice identified a population of cells termed parity-induced mammary epithelial cells (PI-MECs), which had characteristics of differentiated alveolar luminal cells but which had not undergone apoptosis after weaning of pups and could contribute to epithelial outgrowths in the transplantation assay [44], suggesting that cells in the alveolar epithelial lineage were at least in part responsible for the stem/progenitor cell activity in the mammary epithelium.

Several approaches have been taken to prospectively isolate the stem cells whose presence was suggested by these mouse transplantation studies and characterise their relationship with the non-stem cell populations. Initial studies made use of the 'side population' phenomenon. This has been reviewed in detail [4]. In brief, flow cytometry for the simultaneous visualization of red and blue fluorescence emissions from Hoechst 33342-stained whole bone marrow reveals a unique cell-staining pattern – the side population (SP) and non-side population (non-SP) [45]. Isolation and characterization of these cell subsets has shown that the SP has cell surface markers characteristic (Sca-1^+/$lin^{neg/low}$) of haematopoietic stem cells (HSCs) and is enriched at least 1000-fold for HSC activity. It was suggested that the SP phenomenon may be a universal stem cell marker [46].

Staining of mouse mammary cell preparations revealed an SP that consisted of between $0.45 \pm 11\%$ ($n = 17$) [47] and 2–3% [29] of mouse mammary cells. RT-PCR demonstrated that mouse mammary SP cells had high levels of Bcrp1 expression, one of the molecular determinants of the SP phenotype [47], and when placed under mouse mammary epithelial clonal conditions they formed typical cytokeratin-expressing mammary epithelial cell clones [47, 48]. Thus, the mouse mammary SP cells did contain mammary epithelial cells with *in vitro* progenitor capacity. However, transplantation of pure, freshly isolated SP cells with no intervening culture period failed to demonstrate that they were enriched for transplantable stem cells, although they did generate some outgrowths of a mainly alveolar phenotype [47]. It was subsequently shown that Bcrp1 is expressed and functional in the mature secretory alveolar luminal epithelium of the lactating mammary gland [49]. Bcrp expression may therefore be an alveolar lineage marker, possibly expressed in some alveolar progenitors.

One problem with SP staining is that the Hoechst 33342 dye is toxic, so it is difficult to make comparisons of *in vivo* or *in vitro* outgrowth potential between SP cells (which by definition have less of the toxic dye) and non-SP cells (which have more) [4]. The most recent studies of mammary stem cells have therefore used cell surface markers to separate the different epithelial subpopulations. The first marker proposed was Sca-1, a marker of haematopoietic stem cells. The SP of mammary epithelial cells derived from mature virgin animals was found to be enriched for Sca-1^+ cells, and initial experiments found that Sca-1^+ mammary epithelial cells were enriched for transplantable stem/progenitor cells [29]. However, these cells were cultured for 72 h prior to cell sorting and transplantation to improve viability following Hoechst staining. Recent data have shown that culture of primary mouse mammary epithelial cells increases Sca-1 expression significantly [50, 51], and direct transplantation of uncultured Sca-1^+ mammary epithelial cells from either 8-week [52], 8–14-week [51] or 10–12-week [53] virgin animals has shown that they have very little transplantation capacity. It seems likely that the transplantation capacity of Sca-1^+ cells is a culture artefact, although differences in strains of mice used or in flow cytometry protocols and instrumentation cannot be ruled out.

The most significant breakthrough in the flow cytometric isolation of mammary epithelial stem cells was the report of a population of mammary epithelial cells highly enriched for stem cell activity which could be successfully single-cell transplanted and serially transplanted – proof of their stem cell nature. These cells were isolated, following removal of non-epithelial cells ('lineage-negative' or Lin^- sorting) according to the expression of CD24 and CD29 [51] or CD49f [52]. Lin^- $CD29^{hi}$ $CD24^+$ cells were found to be enriched for mammary repopulating units (MRUs), which when cultured in matrigel formed heterogeneous structures comprising of both luminal and myoepithelial/basal cells. Transplantation of this cell population, at limiting dilution, produced complete glandular outgrowths in syngeneic hosts. Indeed, a single Lin^- $CD29^{hi}$ $CD24^+$ cell was shown to produce a complete and functional mammary gland [52]. In contrast, Lin^- $CD29^{lo}$ $CD24^+$ mammary epithelial cells had low outgrowth potential in the cleared mammary fat pad transplantation assay, and when grown in matrigel, these cells formed mainly luminal alveoli-like structures. It was suggested that this population might represent differentiated progenitors restricted to a luminal cell fate [52].

In parallel, CD24 and CD49f were used to isolate analogous mammary epithelial cell subpopulations which were functionally analysed by *in vitro* culture and *in vivo* fat pad transplantation. Using these markers, MRUs were defined as Lin^- $CD49f^{high}$ $CD24^{med}$, myoepithelial cells were defined as Lin^- $CD49f^{low}$ $CD24^{med}$, while the luminal cells were Lin^- $CD49f^{low}$ $CD24^{high}$ [51].

CD24 is a small GPI-linked protein [54] which acts as a P-selectin ligand and may also modulate integrin function [55–57]. CD49f is α6-integrin and CD29 is β1-integrin, both of which are markers of basal epithelia, suggesting that it is the basal epithelial compartment which contains the mammary epithelial stem cells and that these two studies had essentially identified the same populations. This was further substantiated by data published at the same time which showed that mouse mammary epithelial cells can be divided into non-epithelial, basal and luminal epithelial cells (as defined by expression of lineage-specific cytokeratins and gene expression) on the basis of CD24 staining patterns alone (after $CD45^+$ haematopoietic cells had been excluded) [58]. The non-epithelial, basal and luminal epithelial cells were described as $CD24^-$, $CD24^{Low}$ and $CD24^{High}$, respectively. These populations corresponded to the $CD24^{Low}$ non-epithelial, $CD24^{Medium}$ basal/myoepithelial (MYO/MRU) and $CD24^{High}$ luminal epithelial (MaCFCs) populations described by Stingl et al. [51,53,58]. As expected, the majority of transplantation capacity in cells sorted on the basis of CD24 staining alone was found in the $CD24^{Low}$ basal cell compartment, although some activity was found in the luminal compartment [58]. Shackleton et al. did not distinguish between degrees of CD24 staining in their total $CD24^+$ population [52], most likely due to differences in staining protocols [53]. However, the group noted recently that the transplantable cells within the $CD24^+$ compartment tended to show lower levels of CD24 expression [59], consistent with a $CD24^{Low}$ [58] or $CD24^{Medium}$ [51] phenotype, depending on terminology. A consensus of opinion on CD24 staining and nomenclature in the mouse mammary gland is urgently required to facilitate stem cell isolation procedures.

Localisation of the mammary epithelial stem cells to the basal cell layer suggested they did not express ERα, as ERα is found in the luminal epithelial cells. This was confirmed both by demonstration that $CD24^+$ $CD29^{hi}$ stem cells were ERα-negative [59] but also by prospective isolation and transplantation of the ERα-positive luminal compartment. It was shown that Sca-1^+ cells within the $CD24^{High}$ luminal epithelial compartment were also positive for Prominin-1, the mouse homologue of CD133, and that these cells formed a specialised population which not only expressed ER and the PR but also the prolactin receptor. $CD24^{High}$ Sca-1^- cells, in contrast, did not express hormone receptors but did express genes associated with milk production, even in cells sorted from 10-week-old virgin mice [53]. As previously, cells in the $CD24^{Low}$ basal compartment were the most highly enriched for cleared fat pad transplantation potential, and it was directly demonstrated that $CD24^{Low}$ cells contained the MRUs previously defined by CD24 and CD49f staining [53]. Remarkably, it was also demonstrated that the small amount of fat pad repopulation capacity that had been observed in the $CD24^{High}$ luminal cells was located in the $CD24^{High}$ Sca-1^- ER^- compartment – the ER^+ compartment had almost no fat pad outgrowth potential at all [53]. The $CD24^{High}$ Sca-1^- ER^- compartment was also the most highly enriched for *in vitro* growth potential, whereas the $CD24^{Low}$ compartment had little or none, raising the possibility that the $CD24^{High}$ Sca-1^- ER^- cells contain a progenitor/transit amplifying compartment distinct from the basal stem cell compartment. Interestingly, the strategy previously used to identify PI-MECs used a milk gene promoter [44], which these new studies suggested was likely to be specifically active in the $CD24^{High}$ Sca-1^- ER^- luminal compartment [53]. It therefore seems likely that PI-MECs (or their cells of origin in the virgin) may be identical to the ER^- progenitor/transit amplifying compartment.

It is possible that the $CD24^{High}$ Sca-1^+ ER^+ population in the mouse mammary gland does contain a progen-

itor compartment for that population which is itself ER^+, although not competent for cleared fat pad transplantation. The existence of such a progenitor compartment is supported by the limited *in vitro* growth colony-forming potential of the $CD24^{High}$ $Sca\text{-}1^+$ ER^+ cells [53] and would be consistent with the labelling studies discussed above, which have clearly shown that some ER^+ cells are in cell cycle [32], although the majority of ER^+ cells in the mammary gland do not co-stain with cell cycle markers [33, 34]. It may be that previous studies which have suggested the existance of ER^+ stem cells have in fact been measuring such progenitors.

The demonstration that stem cells and ER^+ cells form different cell populations in the mammary gland means that circulating hormones must influence stem cell function through paracrine interactions. Indeed, data from tissue recombination and transplant experiments has demonstrated that different paracrine signals downstream of ERα/PR are required at different mammary developmental stages. In the pubertal mouse, the EGF (epidermal growth factor) family member amphiregulin is required for ductal elongation but is not required for pregnancy-mediated side branching and alveolar formation. This effect is likely to be mediated through stromal EGF receptors [60]. In contrast, Wnt-4 activity is required for ductal side branching during early to mid-pregnancy [61].

Stem cells in the normal human breast

As with the mouse, candidate normal human breast epithelial stem cells have been isolated using both SP staining and cell surface markers such as MUC-1 (a luminal epithelial marker), CALLA/CD10 (a basal/myoepithelial marker) and ESA (a general epithelial marker) [38,62–64]. *In vitro* studies using these markers identified $MUC\text{-}1^+$ $CALLA^-$ ESA^+ progenitor cells which generated colonies of a luminal cell phenotype (expressing CK8, 18, 19 and 9) as well as $MUC\text{-}1^{-/low}$ $CALLA^{low/+}$ ESA^+ progenitors which had bilineage potential *in vitro*, giving rise to cells with both luminal epithelial and basal/myoepithelial markers [63].

ESA^+ primary human breast epithelial cells stained with Hoechst 33342 gave rise to an SP and a non-SP comparable to that seen in the mouse. The SP was depleted of cells expressing markers of the mature luminal (MUC1) or myoepithelial (CALLA) phenotypes. *In vitro* analysis of the human breast epithelial SP cells showed them to be multipotent, with the ability to produce both luminal and myoepithelial cell types [38,64]. SP cells, but not non-SP cells, were able to form branched colonies in matrigel [38], although the caveat of the potential toxicity of Hoechst 33342 on the non-SP remains. Further analysis of the SP demonstrated that it was enriched for $ER\alpha^+$ cells, with approximately 60% expressing the marker, and also showed increased expression of the putative stem cell markers $p21^{Cip\text{-}1}$ and Msi-1 in comparison to non-SP cells [38]. Other studies, however, have found that the human breast SP is negative for both ERα and ERβ [64].

One disadvantage of human studies is lack of an *in vivo* stem cell transplantation model for normal cells that is equivalent of the gold standard mouse assay. It is hoped that the humanised mouse fat pad model of Kuperwasser et al. [65] will soon remedy this problem. In the meantime, researchers have turned to alternative *in vitro* systems for assays of stem cell behaviour. The 'neurosphere' culture system is a non-adherent assay in which neural stem cells are cultured as floating cell colonies, without inducing cell differentiation. This technique has been extended to mammary epithelial cells in so-called mammosphere culture. It was shown that human breast epithelial cells formed mammospheres after 7–10 days of culture which maintained a primitive phenotype and therefore did not express markers associated with terminal differentiation [66, 67]. In culture conditions which favoured cell differentiation, cells isolated from dissociated mammospheres were shown to have the capacity for multi-lineage differentiation in two-dimensional culture (as assessed by expression of cell-type specific markers) and in three-dimensional culture gave rise to lobular-alveolar structures [67]. Stimulation of Notch signalling resulted in a 10-fold increase in the number of secondary mammospheres obtained after dissociation of the primary spheres [68]. Stimulation of Hedgehog signalling had a similar effect [69]. Notch signalling also affected lineage commitment, proliferation and branching morphogenesis when mammospheres were put in two- or three-dimensional differentiating culture conditions [68]. This suggests that both Notch and Hedgehog signalling have a role to play in self-renewal and differentiation in mammary stem cells.

It remains to be definitively proven that the mammosphere culture system selects for stem cells. While *in vitro* cultured mammospheres can be transplanted [66, 69], it has yet to be shown that putative human mammary epithelial stem cells can be prospectively isolated from primary uncultured tissue, and then split between an *in vivo* functional transplant assay and an *in vitro* mammosphere assay. Only when it is shown that the cells that can be transplanted *in vivo* with no prior culture period are the same cells which generate mammospheres *in vitro* will the assay be truly

validated. Otherwise, the selective pressure of an artificial tissue culture system could be skewing the results.

Cancer stem cells

In 1997 it was demonstrated that less than 1 in 10000 human leukaemia cells could transfer the original tumour into non-obese diabetic/severe combined immunodeficient (NOD/SCID) mice [70]. This small population of cells had the normal stem cell surface phenotype of $CD34^+$ $CD38^-$ and were able to recapitulate the histologic phenotype of the initial tumour upon transplantation, consistent with a stem cell model of cancer and an origin from normal stem cells. Since then, the existence of cancer stem cells in leukemia has become well established [71, 72], and there is increasing evidence to suggest the presence of tumour-initiating cancer stem cells within solid tumours of various cellular origins, including colon [73, 74], brain [75, 76] and head and neck squamous cell carcinoma [77].

Studies on transplantation of breast cancer cells derived from eight pleural effusions and one primary tumour into etoposide-treated NOD/SCID mice showed that transplantable cancer-initiating cells were characterised by the expression of the cell surface markers $CD44^+$ $CD24^{low}$ lin^-. The introduction of as few as 200 of these cells, 1–10 % of the total cell population, formed tumours that recapitulated the phenotypic heterogeneity of the original tumour in NOD/SCID mice. However, the transplantation of up to 10^5 $CD44^+CD24^+$ lin^- cells failed to form tumours (except in one case). Only $CD44^+CD24^{low}$ lin^- cells derived from the transplanted tumours could go on to generate new tumours in secondary transplants [78]. These transplant studies are technically difficult and remain to be repeated. However, recent data have provided strong support for the $CD44^+$ $CD24^{-/low}$ phenotype being a marker of both normal breast and breast cancer stem cells. $CD44^+$ $CD24^{-/low}$ cells isolated from mammosphere cultures of human breast cancers had an enhanced transplantation capacity compared to a breast cancer cell line [66], although the caveats regarding the mammosphere assay remain. In a study on disseminated breast cancer tumour cells in bone marrow samples, 71 % of cytokeratin positive cells were found to have the $CD44^+$ $CD24^{-/low}$ phenotype [79]. Gene expression profiling of $CD44^+$ $CD24^{-/low}$ breast cancer cells compared to normal breast epithelium identified a 186-gene 'invasiveness' gene signature which had a significant association with poor outcome [80] and with a basal-like tumour phenotype [81], although it also has prognostic value in non-basal tumours [80]. Most significant, however, is the recent description of the isolation and gene expression profiling of putative stem cells from normal breast and breast tumours of different stages [82]. The putative stem cell compartment in both normal and tumour tissue was characterised as $CD44^+$ $CD24^-$ and was also positive for a basal breast epithelial cell marker, PROCR, but did not express CD10 (CALLA), a myoepithelial cell marker [83]. Analysis of gene expression profiles showed that $CD44^+$ $CD24^-$ $PROCR^+$ $CD10^-$ cells from normal breast and breast cancers were more similar to each other than to $CD24^+$ cells from the same tissue. Furthermore, the profile of $CD44^+$ $CD24^-$ $PROCR^+$ $CD10^-$ cells resembled that of stem cells [82]. Interestingly, $CD44^+$ $CD24^-$ $PROCR^+$ $CD10^-$ tumour cells and $CD24^+$ cells were clearly clonally related but were not genetically identical, suggesting that there is genetic progression within tumours.

The $CD44^+$ $CD24^-$ $PROCR^+$ $CD10^-$ phenotype of human breast stem cells is consistent with the identification of stem cells as basal, but not myoepithelial, in the mouse. The overall theme of basal stem cells and luminal progenitor/transit amplifying cells thus seems to be in common between mouse epithelium, human breast and human breast cancers.

The difficulties – and opportunities – that the existence of cancer stem cells presents have recently been demonstrated. It has been shown that $CD133^+$ glioma stem cells [75, 84] have increased radioresistence due to improved repair of DNA damage, as measured by a comet tail assay, compared to non-stem cells [84]. Furthermore, $CD44^+$ $CD24^{low/-}$ mammosphere-initiating cells derived from the MCF-7 and MDA-MB-231 cell lines have been shown to have increased radioresistence [85]. However, it has also been demonstrated that BMP4 'differentiation therapy' can be used to eliminate glioma stem cells [86] and that leukaemia stem cells can show increased sensitivity to molecularly targeted therapies compared to normal haematopoietic stem cells [87]. Thus, although cancer stem cells may be resistant to conventional therapies, a better understanding of the molecular mechanisms that control their self-renewal and differentiation may actually enable them to be eliminated with relatively minor toxicity to the rest of the body.

Field cancerisation

One potential aspect of the relationship between stem cell biology in the breast – and indeed other tissues – and cancer development which is not often fully appreciated is that of field cancerisation. The field cancerisation theory was first proposed by Slaughter

et al. [88] following the study of oral cancers. He showed that oral cancers developed from areas of pre-neoplastic tissue and often tumours were surrounded by abnormal tissue. They also often comprised multiple tumours occurring independently which had combined to form a single mass [88]. As has been discussed above, multiple genetic changes are usually required within a cell to result in tumour formation, and the long life of stem cells makes them ideal for accumulating such mutations. However, a stem cell that has accumulated two or three genetic lesions – but is not yet frankly transformed – is still carrying out its normal function within the tissue. In other words, it is generating short-lived transit-amplifying and differentiated daughter cells, which must themselves be carrying the same genetic lesions. Thus, mutations in a stem cell generate a field of tissue that is phenotypically normal but genetically abnormal. The stem cell – and the field – continues to accumulate mutations until only one more genetic change is required to generate a clinically recognisable lesion. Of course, all the time the stem cell has been accumulating lesions, the field of mutant daughter cells has been subjected to the same genetic insults, but these have not contributed to the accumulation of mutations in the field as daughter cells have only a limited lifespan. However, once the field has accumulated enough mutations through genetic insults to the stem cell that only one more mutation is required to generate a clinical lesion, then that lesion could come from any cell within the field. In such a case, the tumour has not been derived directly from a stem cell but has only been able to form because the stem has allowed mutations to accumulate. This begs the question, In a field cancerisation model, which is the cancer stem cell? Is it the mutant daughter cell that actually generated the tumour, or is it the mutant stem cell that generated the mutant field? Treatment of a tumour which does not also address the possibility of a field cancerisation effect inevitably runs the risk of the development of a new tumour from the same mutant field, as happens in oral cancers.

There is some evidence for such a field effect in breast cancer. It is known that there are clonally related areas ('patches') in the breast consisting of many TDLUs, as demonstrated by X-inactivation patterns [89]. Loss-of-heterozygosity (LOH) analysis of breast tumours and apparently normal luminal and myoepithelial cells surrounding them showed identical genetic lesions in each [90], whilst ductal carcinoma *in situ* (DCIS) recurrences have been shown by comparative genomic hybridisation (CGH) to be clonally related [91].

Breast cell biology and the origins of breast cancer subtypes

It is now known that breast cancers can be clustered on the basis of gene expression patterns into at least five, and probably more, molecular subtypes [92]. Luminal A and B tumours have a gene expression pattern resembling normal luminal breast cells. Luminal A tumours tend to have higher levels of ERα expression and better prognosis, whereas Luminal B tumours have lower levels, or are ERα-negative, and have poorer prognosis. The HER2 tumour group over-express HER2, and the normal breast-like tumour group, as the name suggests, have gene expression patterns resembling that of the normal breast. This group includes non-epithelial breast lesions. Finally, the basal group is $ER\alpha^{-}$ PR^{-} $HER2^{-}$ ('triple negative'), expresses basal cytoskeletal markers and is associated with younger women and with BRCA1 disease [93, 94]. Using what we are learning about mouse and human breast stem cell biology, and taking into account field cancerisation, we can model the possible origins of these tumour types.

Our model is shown in Figure 2. We suggest that basal tumours (Fig. 2b) may be genuine stem/early progenitor cell tumours of the mammary gland. Not only do they express basal cytokeratins but some also have a luminal component, and these two compartments can share common genetic lesions. In other words, they are bilineage, but with a common origin [95]. Furthermore, as discussed above, all the evidence points to breast stem cells being most highly enriched in the basal cell compartment.

If we accept that accumulation of genetic lesions in stem cells and the subsequent formation of cancer fields as a prime mechanism for tumour formation, then the other tumour types may be explained as resulting from this field effect. A basal stem cell accumulating mutations generates a field of cells in which only one more molecular pathway needs to be disrupted in order to generate a tumour (Fig. 2c, d). If that mutation occurs in an ERα-positive luminal cell, it generates an ERα-positive tumour (Fig. 2c). If it occurs in an ERα-negative luminal cell, it generates an ERα-negative tumour (Fig. 2d). If it occurs in a progenitor that has the potential to differentiate into either ERα-positive or -negative luminal cells, it could generate a tumour with a mix of ERα-positive and -negative cells – which is, of course, closer to reality. Similar scenarios have been previously suggested by Dontu and colleagues [96]. Alternatively, if the first genetic lesion in a non-stem cell promotes self-renewal, mutations could accumulate in a differentiated cell to generate a tumour without creating a field of abnormal cells (Fig. 2e). Of course, any model such as

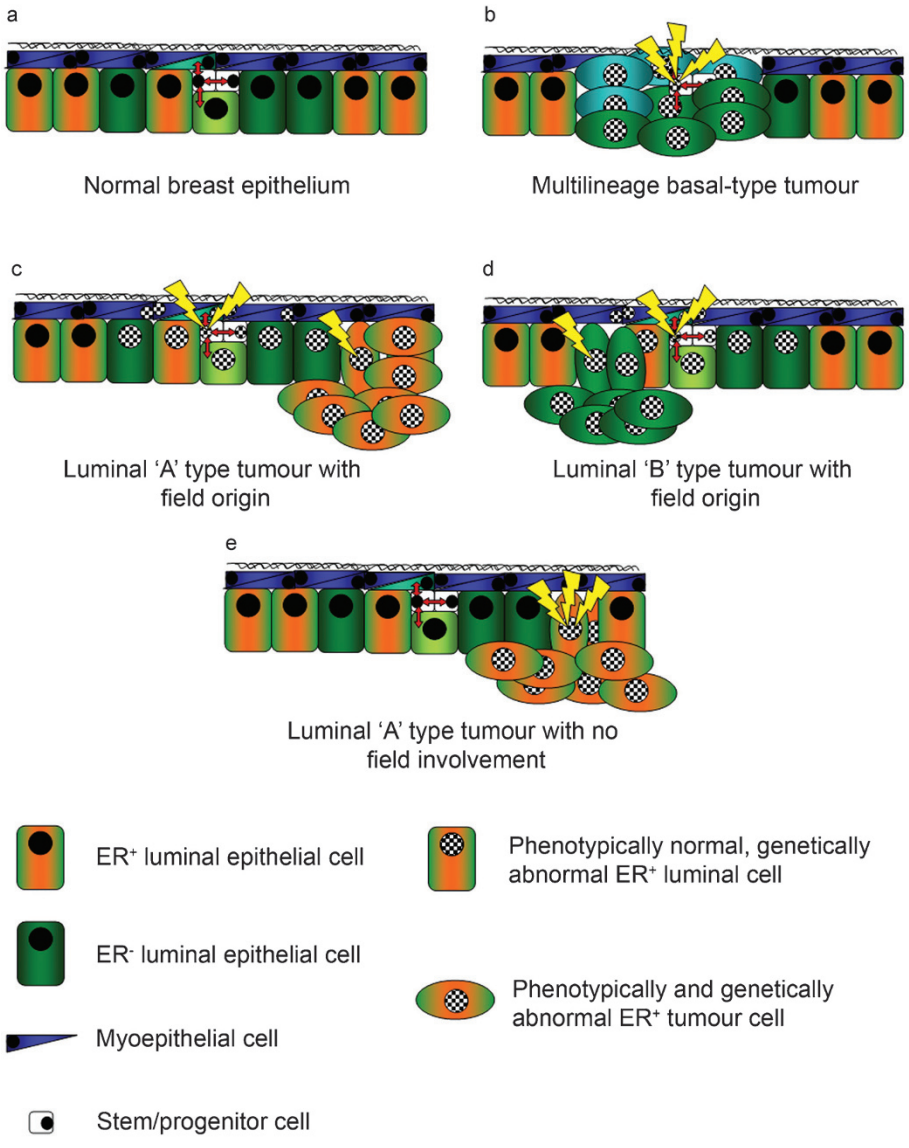

Figure 2. A model of the origins of breast cancer subtypes. The normal breast epithelium (*a*) is composed of myoepithelial (blue), ER^+ luminal (orange/green) and ER^- luminal (green) cells as well as a stem/progenitor compartment (white). Basal type tumours (*b*) may arise following transformation of a stem/progenitor cell as a result of accumulation of genetic damage (hatched nuclei). Luminal tumours may arise from a field of abnormal cells generated when stem cells acquire genetic damage without actually transforming. One final genetic lesion in an ER^+ luminal cell (*c*) or ER^- luminal cell (*d*) within the field generates a tumour. Alternatively, if the first genetic lesion in a non-stem cell promotes its self-renewal, or if it is an intrinsically long-lived non-stem cell, mutations could accumulate in a transit amplifying or differentiated cell to generate a tumour without creating a field of abnormal cells (*e*).

this is only one of many possible variants that could be proposed and does not include the possibility of transformation of lineage-specifc progenitors [96] or that a mutation in a gene which controls cell fate choices might generate a tumour of an apparently different cell lineage than that of the cell type in which the mutation occurred. In other words, mutations in cell-fate master regulators could generate basal-type tumours from luminal cells and vice versa. The core message of this model, and of similar models which have been previously suggested [96], is that breast tumour phenotype may not depend solely on genetic lesion, but also on the epithelial cell subtype in which that lesion occurs. In some cases the genetic lesion may be the prime determinant of tumour phenotype; in others it may be the cell of origin. In some cases, it may be the specific combination of the cell type and genetic lesion which is important.

Future prospects

Although stem cells have been identified in the mammary gland, there is still a long way to go in characterising these cells and understanding their role in mammary gland growth and development during puberty and pregnancy. Studies using primary breast tumours and tumours generated from conditional knock out mouse models are needed to increase our understanding of the cancer stem cell populations and, in the mouse models, to provide a platform in which to examine new therapies.

Comparison of normal mammary stem and differentiated cells and breast tumour cells, aided by more sophisticated mouse models, will provide information on the cells from which tumours are derived, while gene expression analysis will identify signalling pathways which can be targeted therapeutically. Finally, if definitive evidence of field cancerisation in breast cancer can be found, it will revolutionise our understanding of recurrent disease and lead to treatments aimed at eliminating the mutant field, as well as the primary tumour, and thus eliminating local recurrence.

Acknowledgements. Work in the laboratory is supported by Breakthrough Breast Cancer.

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