STEM CELLS AND BREAST CANCER: A FIELD IN TRANSIT

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Evidence that supports the existence of a tumour stem cell for breast cancer has given fresh impetus to the search for an adult mammary epithelial stem cell in the normal breast. Such a cell might be responsible for routine tissue renewal and the massive expansion in epithelial tissue that the breast undergoes during pregnancy, and might be the cell of origin of most, if not all, breast tumours. So, what evidence is there that an adult mammary epithelial stem cell exists, what is its possible identity and what opportunities might the identification of such a cell present for the development of novel therapeutic and prophylactic strategies for treating breast cancer?

TERMINAL DIFFERENTIATION
The process by which stem-cell or transit-amplifying-cell progeny commit to becoming the mature, fully functioning cell type of a tissue. Probably irreversible.

MICROENVIRONMENT
The local growth conditions of the cell, formed by interactions between the extracellular matrix, blood supply, local cell types, and circulating hormones and growth factors.

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Stem cells[1–6] (BOX 1) have a large replicative potential and a long life — enabling them to accumulate several mutations over time — which makes them excellent candidates for the cells of origin of cancer. This has resulted in them becoming an increasing focus of interest to cancer researchers as well as to developmental biologists.

Stem-cell biology has traditionally been dominated by haematologists. The accessible nature of blood and bone-marrow cells, the use of flow cytometry and the clinical drive for a better understanding of bone-marrow transplantation have led to the elucidation and definition of the details of the stem-cell hierarchy in the bone marrow and blood. The progression from the most primitive haematopoietic stem cell to the most differentiated cells is understood in great detail and much is known about the regulation of this process[7,8].

By contrast, our knowledge of stem-cell behaviour in solid tissues is patchy. In the skin, for instance, the genetic control of the progression from stem cell through to TERMINAL DIFFERENTIATION is becoming better understood and is providing insights into the ways in which the balance between stem cells and daughter-cell lineages is controlled[9,10]. In other tissues, such as muscle, potential stem cells have only been identified relatively recently, so their origin and relationship with their daughter cells is not entirely clear[11–13]. Furthermore, in all tissues, the analysis of stem-cell function can be complicated by the presence of transit/progenitor cells that have no self-renewal capacity — unlike the parental stem cell — but that undergo a population expansion to increase the number of fully differentiated cells that are ultimately produced by the original stem-cell-division event.

In the adult mammary gland (FIG. 1), no definitive identification has been made of an adult mammary epithelial stem cell (MESC), despite the proposal of several candidate cell populations. There is even an ongoing debate as to whether breast epithelial stem cells exist. Some have suggested that the idea of breast stem cells as a fixed cell population is an oversimplified view and that the crucial issue is whether a cell population has stem-cell-like ability, possibly as a result of its interactions with its MICROENVIRONMENT or 'stem-cell niche' (BOX 2). In this model, the stem-cell-like behaviour can be lost or gained — for instance, in the process of tumorigenesis. This issue could be a crucial factor in interpreting the results of experiments that test stem-cell-like activity. Here, we will use 'stem cell' as a shorthand for cells that show stem-cell-like behaviour *in situ* or *in vivo*. 'Stem-cell-like behaviour' will be used in situations in which experimental manipulation has occurred, to indicate the possibility of induced cell-type plasticity.

Role of adult mammary epithelial stem cells

The rodent mammary gland first appears embryonically along the mammary streak — a line of thickened ectoderm that extends from the anterior to the posterior limb bud — as an epithelial bud that penetrates the

LUMINAL EPITHELIAL CELLS
The cells that line the lumen — also used to refer to the non-myoepithelial component of the mammary epithelium in general.

MYOEPITHELIAL CELLS
Contractile cells that form a basket-like network around the secretory alveoli and a sheath around the ducts. These squeeze the milk down the ducts and out of the nipple in response to the hormone oxytocin.

APOPTOSIS
Programmed cell death that is characterized by regulated DNA digestion and phagocytosis of cell debris with minimal tissue inflammation.

LUMEN
The space in the centre of the mammary ducts and alveoli into which milk is secreted, and along which it passes to the nipple.

Summary

- Stem cells have a long life and a large replicative potential, making them good candidates for the cells of origin of cancer.
- The adult mammary gland requires stem cells, or a stem-cell-like activity, to fulfil the demands of pregnancy-dependent epithelial expansion and replace cells that are lost through routine cell turnover.
- Evidence for the existence of specialized adult mammary stem cells comes from transplantation, retroviral tagging and X-chromosome-linked gene-inactivation studies.
- Current evidence points to an undifferentiated, suprabasal cell, with a slow proliferative rate that has characteristics in common with stem cells from other tissues, as the best candidate for an adult mammary epithelial stem cell.
- Experimental evidence from the transplantation of tumour-cell subpopulations and from animal models supports the view that breast stem cells are the cells in which mammary cancers are initiated.
- Therapeutic or prophylactic targeting of breast stem cells provides a novel approach to breast cancer treatment that is aimed directly at the cells of origin of the tumour.

underlying mesenchyme to form a rudimentary branched ductal system. This rudiment remains inactive until approximately 3 weeks of age, when pubertal hormones stimulate the ducts to invade and branch through the fat pad. At this stage of development, it is generally accepted that stem-cell activity is found in terminal end buds (TEBs)[14] (FIG. 1b). These club-shaped structures form the growing tips of the extending ducts and consist of a mass of 'body cells' that are surrounded by a layer of 'cap cells'. It is thought that, as the ducts elongate and the TEBs move forwards, the body cells give rise to the inner LUMINAL EPITHELIAL CELL layer of the subtending duct and the cap cells give rise to the outer MYOEPITHELIAL CELL layer (FIG. 2). APOPTOSIS occurs in the middle of the mass of developing body/luminal cells to generate the ductal LUMEN. The cap cells can also be seen to migrate into the body cell mass[15], and this has led to the idea that the cap cells are stem cells. The TEBs and their subtending ducts grow, branch and ramify through the rodent mammary fat pad until they reach its edges. The TEBs then disappear and the gland settles into its normal adult cycle (FIG. 1).

The adult mammary gland must also have a role for stem cells. In the rodent, there is a massive burst of proliferation during pregnancy that results in the generation of side branches and SECRETORY ALVEOLI that resemble bunches of grapes (FIG. 1e). The inner, luminal cells of the alveoli produce the milk, whereas the outer, myoepithelial cells are contractile and act to squeeze the milk out of the alveoli and down the ducts. In some strains of mice, the alveoli develop from alveolar buds that are seen in the resting gland. By the time the mother is feeding her pups, her mammary glands are packed full of secretory epithelium with little fat (FIG. 1f), the complete opposite of the situation in the virgin or non-pregnant animal. Following weaning, these structures INVOLUTE by apoptosis until the gland once more resembles that of the virgin, with a fat pad that contains only well-spaced ductal structures in an ADIPOSE MATRIX. This cycle is repeated many times during the life of an animal. Only stem cells have the replicative potential that would be needed to maintain this process. If such stem cells do exist, they would fall into the class that are quiescent until responding to physiological cues (BOX 1). Even in non-pregnant animals, a similar process occurs as the oestrous cycle progresses, although it is much less elaborate and mainly involves expansion and regression of the alveolar buds. Similar processes to these occur during human pregnancy and the human menstrual cycle, although there are differences in the extent of *de novo* proliferation.

Box 1 | What are stem cells?

Stem cells, as classically defined, are cells with a capacity to self-renew and to generate daughter cells that can differentiate down several cell lineages to form all of the cell types that are found in the mature tissue. A stem cell might go through an asymmetric cell division to generate one cell that is identical to itself and one cell that is distinct. The identical cell provides for self-renewal of the stem-cell compartment; the distinct cell goes through a series of cell divisions and differentiative steps to generate the ultimate terminally differentiated cell populations. The cells that form the intermediates between stem cells and terminally differentiated cells are usually referred to as progenitor cells (especially if they give rise to a defined structure or cellular compartment), transit cells or transit amplifying cells. Stem cells could also generate distinct daughter cells by dividing symmetrically into two identical cells, followed by a random decision — based on, for example, variation in intensity of cell signalling — to establish one daughter cell as a new stem cell and the other as a transit cell. Furthermore, the stem-cell compartment can be expanded, or new stem cells established within a tissue, by symmetric cell divisions in which neither daughter cell progresses to produce transit amplifying cells. Stem cells could also divide symmetrically to give two transit cells, depleting the stem-cell compartment. It could be argued, however, that a cell that does this is not truly a stem cell, if self-renewal is accepted as one of the defining characteristics of stem cells. Whether this is a largely semantic point or a fundamental issue is debatable.

Stem cells can be divided into two functional classes. First, there are stem cells that are responsible for tissue renewal. Such cells are found, for example, in bone marrow, in the skin and in the intestine, and are responsible for replacing terminally differentiated cells as they mature and die or are shed from an epithelial surface. These cells are continually active, although at a slow rate. Second, there are stem cells that are inactive until required in response to environmental factors — for example, to repair tissue damage. Satellite cells of muscle might be an example of such a stem cell, as might putative liver stem cells that have been suggested to be responsible for liver regeneration. It is unclear at the moment whether such a functional division is reflected in phenotypic differences between stem cells in tissues or whether the same stem cells are responsible for both activities where they occur in a single tissue. If there are separate stem cells for these functions, there might be a hierarchical relationship between them.

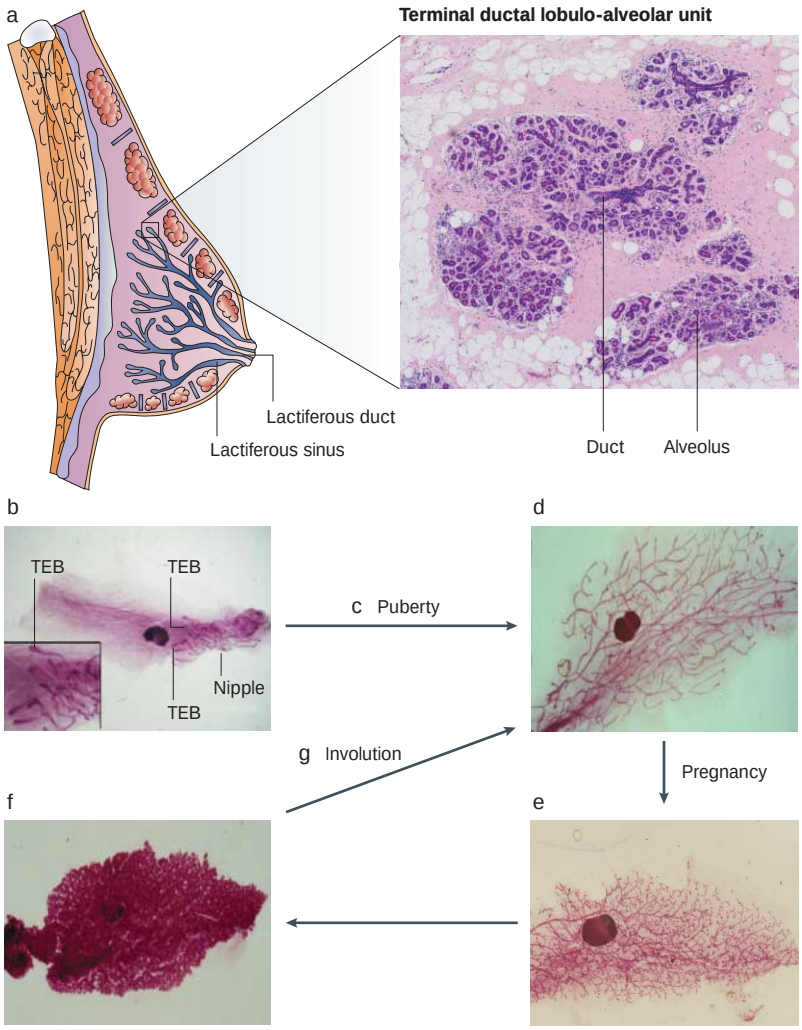


Figure 1| **The human breast and rodent mammary gland. a** | Schematic of the epithelial structure of the human breast with photomicrograph of a haematoxylin- and eosin-stained cross-section through a terminal ductal lobulo-alveolar unit. **b** | Ducts of a 3-week-old developing rodent mammary gland begin to grow out from the nipple towards the lymph node. The terminal end buds (TEBs) form the growing tips of the ducts. Inset: magnification of end-bud region. **c** | During puberty, the TEBs move through the fat pad to generate the ducts that ramify within the gland. **d** | The mature virgin mouse mammary gland consists of a branching ductal system within the adipose tissue and a few alveolar buds. **e** | In a mid-pregnant mouse mammary gland, secondary ductal branches and increasing numbers of alveoli develop. **f** | In lactating mice, the gland is full of secretory alveoli. **g** | After pregnancy, the epithelium is eliminated by apoptosis and the gland is remodelled until it once again resembles the mature virgin tissue. The authors are grateful to Jorge Reis-Filho (Breakthrough Breast Cancer Centre, Institute of Cancer Research, London) for the micrograph in **a**, and to Trevor Dale (Institute of Cancer Research, London) for the images in **d**–**f**, which are carmine-stained wholemounts. The image in **b** is a carmine-stained wholemount from the authors collection.

SECRETORY ALVEOLI
Structures that resemble bunches of grapes in the mammary epithelium that produce milk products. They are few in number in the virgin or non-parous animal, but appear in huge numbers during pregnancy and fill the gland during lactation.

Another area that might involve adult MESCs is in the replacement of cells that are shed from the epithelium into the lumen during routine cell turnover. This is certainly seen during lactation, as epithelial cells can be recovered from milk, as well as in experimental cell systems[16], and might be an important source of cell loss in the resting gland *in vivo*. Cells that are shed into the lumen of the alveolar and ductal systems must be replaced in some way, otherwise the epithelial tree could not maintain its integrity. Stem cells that have this role would fall into the class of continually active stem cells (BOX 1). So, there are clearly at least two potential roles for stem cells in the adult mammary epithelium — whether this means that there are two (or more) stem-cell types (possibly in a stem-cell hierarchy) or that one stem-cell type has the potential to perform several tasks depending on the cues it is given, remains to be seen. A model that illustrates the differences between stem cells that are involved in tissue renewal and those involved in the formation of alveoli is shown in FIG. 3.

Experimental evidence for breast stem cells

It is possible that there is no permanent specialized stem-cell type within the mammary epithelium. In this scenario, behaviour such as tissue renewal and the ability to generate alveoli in response to pregnancy could be a property of all cells, removing the requirement for specialist stem or progenitor cells, and whether or not any cell responds could be random. In this case, cells might undergo a transient stem-cell-like phase — for instance, under the influence of hormones during pregnancy — to generate new tissue structures (alveoli or ducts). Alternatively, there might be no stem-cell-like phase in these situations, and proliferation of myoepithelial and luminal epithelial cells in an independent, but coordinated, manner would give rise to new tissue structures. Although this remains a formal possibility, the balance of evidence detailed below indicates that this is not the case.

Opinion is divided between permanent stem-cell specialization and induced or transitory stem-cell-like behaviour in the mammary gland, but direct evidence for the existence of specialized mammary stem cells has come from several studies, including cleared fat-pad transplantation (BOX 3), RETROVIRAL TAGGING and studies of X-CHROMOSOME INACTIVATION. Transplantation of primary mammary epithelial cells at limiting dilution in the cleared fat-pad transplantation system resulted in three types of structure[17,18]. The most common structures were complete ductal systems that were able to respond to pregnancy by undergoing alveolar proliferation. Less common were structures that resembled groups of alveoli only, with no ductal element. Occasionally, ducts that could not respond to pregnancy and did not generate alveoli were formed. These results indicated that a stem-cell hierarchy exists, in which relatively common MAMMARY-TREE stem cells might give rise to less common alveolar bud-only stem cells and rare duct-only stem cells. However, in this model, all three progenitor cell types could produce the luminal and myoepithelial cell layers. Confirmation of this model awaits the identification of these three suggested classes of stem or progenitor cells.

Analysis of retroviral integration patterns in mammary epithelium that was transplanted into cleared fat pads and analysed over several transplant generations revealed that entire mammary epithelial outgrowths can be clonal in origin[18]. This would indicate the presence of stem or progenitor cells for the entire mammary epithelium within the adult gland. However, it is known that when marked mammary epithelial cells are mixed with

Box 2 | Stem-cell niches

Stem-cell niches are postulated to be specialized locations within a tissue that have the ability to support stem-cell function — namely, self-renewal, the generation of differentiated progeny and long life[73]. They are defined by the presence of supporting cells with specialized local signalling functions that act, possibly in association with extracellular-matrix signals, to maintain stem-cell function. They might have a specific location within a tissue — for instance, in the germaria of *Drosophila* ovaries, at the base of mammalian colonic and small-intestine crypts or in the basal epidermal layer of the skin.

There is evidence that such niches might have the inductive power to create stem cells from nearby daughter cells if the stem cells are depleted[5,74,75]. Stem-cell niches could, potentially, be created as a result of physiological conditions within a tissue, allowing transient stem-cell-like behaviour to be induced in cells that would not generally be considered to be stem cells. The epithelium-free mammary fat pad into which cells are transplanted in the cleared-fat-pad-transplantation assay (BOX 3) might also be an example of a tissue that could act as a stem-cell niche and induce stem-cell-like behaviour (that is, gland repopulation) in cells that would not normally show it (that is, differentiated daughter cells). This is one reason why such experiments must be treated with caution — they do not represent a normal physiological phenomenon.

The inductive power of stem-cell niches could also be important in cancer. The inappropriate expression of an inductive signal by a stem-cell niche or the inappropriate induction of stem-cell-like behaviour in a daughter cell, as a result of failure in the control of cell-signalling pathways, could lead to tumour formation[76]. Another important consequence of the potential inductive power of stem-cell niches is that any therapy that depletes stem cells might lead to repopulation of the empty stem-cell niche by daughter cells, which might, if the Cairns hypothesis (BOX 4) is correct, carry errors in their DNA. This would effectively 'fix' mutations in the stem-cell population and actually promote tumour formation. Use of anti-stem-cell therapy for prophylaxis must, therefore, be approached cautiously, and the benefits weighed against possible costs.

INVOLUTION
The name given to the process of apoptosis and tissue remodelling by which the mammary gland changes from the epithelial-cell-rich lactational state to the epithelial-cell-sparse non-parous state following weaning of offspring.

ADIPOSE MATRIX
The fatty connective tissue of the mammary gland that supports the epithelium.

RETROVIRAL TAGGING
Uses retroviral infection of cells as a lineage marker to enable the progeny of marked cells to be followed over many generations. Retroviral sequences incorporated into host DNA are detected by Southern blotting, and common insertion patterns are used to infer lineage relationships.

X-CHROMOSOME INACTIVATION
The process by which one X chromosome is stably transcriptionally inactivated. This occurs in almost all mammalian female (XX) cells to achieve comparable gene dosage to XY males.

non-marked cells and transplanted in the cleared fat-pad system at a non-limiting dilution, the resulting outgrowths are a mixture of marked and unmarked cells[15,17,19–21]. So, although clonality of the entire mammary epithelium is observed under certain experimental conditions, it does not necessarily occur in the normal gland. Rather, in the human breast at least, studies of X-chromosome-linked gene inactivation have indicated that the TERMINAL DUCTAL LOBULO-ALVEOLAR UNITS (TDLUs) are the clonal units[22]. Early in embryogenesis — day 16 in the human female — genes on either the maternally or paternally derived X chromosome are randomly inactivated in each cell and these events are stably inherited by the descendants of the cells. Analysis of genes that are polymorphic between the maternal and paternal X chromosomes allows tissue samples to be assessed for monoclonal or polyclonal derivation. The clonal origin of individual TDLUs supports a model of adult stem cells that are distributed throughout the gland.

Retroviral tagging and transplantation experiments have enabled an estimate to be made of the number of potential stem cells in the mouse mammary epithelium that could generate epithelial structures in a cleared fat-pad transplantation, and this is approximately 1 in 2,500 cells[17,18]. The number of epithelial cells in a mature virgin mouse gland (determined by measuring DNA content) was estimated at $2–2.5 \times 10^6$. So, each gland contains ~1,000 stem cells[17]. After limiting-dilution transplantation, in which the transfer of a single progenitor was assumed, some outgrowths were able to fill the fat pad with secretory alveoli at birth of the pups. The epithelial-cell numbers in fat pads at this time were estimated at $2–8 \times 10^7$ cells. In other words, a single cell would have to have undergone 25–27 population doublings to fill the fat pad with tissue at parturition[18]. Starting from a complete gland with 1,000 stem cells, only 15–16 population doublings would be needed, but this would occur at each pregnancy. Even stem cells might have difficulty supplying this proliferative capacity during the lifetime of a mouse. Much of this proliferation might, therefore, actually be occurring in transit amplifying cells, rather than the stem cells.

Candidate populations

The biology of the mammary gland indicates a role for stem cells, and data support their existence. So, in the light of the definitions of a stem cell that are discussed in BOX 1, is it possible to identify candidate stem-cell populations within the adult mammary epithelium? For clarity, we have discussed the evidence according to the types of study used, but this is a somewhat artificial division and there is, necessarily, some overlap.

In situ studies. For *in situ* studies, mammary epithelial tissue is examined with minimal disruption to the normal tissue architecture, using various histological and microscopic techniques, with the aim of understanding the association between the various cellular components. The difficulty with a pure *in situ* approach for stem-cell studies is that it is difficult to use them experimentally to show self-renewal and differentiative potential — they are, of necessity, purely correlative. Indications of potential candidate populations — based on degrees of differentiation, proliferative characteristics and cell-type-specific markers — are, however, provided by this approach.

HISTOLOGICAL and ULTRASTRUCTURAL studies in mouse and rat mammary epithelium have identified a candidate combined stem-cell and primary-transit-cell population on morphological grounds. The 'small light cell' (SLC) is an undifferentiated cell that is found in the luminal-cell layer, but in a basal or suprabasal location (that is, near the myoepithelium). These cells do not contact the lumen or, in general, the basment membrane, and are characterized by their small size and pale cytoplasm, which is sparse in organelles[23,24]. The researchers who identified these cells suggest a model, based on morphological intermediates, by which SLCs divide and differentiate progressively into undifferentiated large light cells (ULLCs), differentiated large light cells (DLLCs) and, finally, into the bulk population of the luminal-cell layer, large dark cells (LDCs). The ULLCs and DLLCs might form progressive progenitor/transit populations. Morphological intermediates might also exist in a separate pathway between ULLCs and myoepithelial cells[24]. The most up-to-date data provide evidence for the SLCs being organized into a stem-cell niche that is very similar to that seen in the *Drosophila* ovary[23]. Similar light cells have also been identified in cattle[25] and in the human mammary gland[26–28].

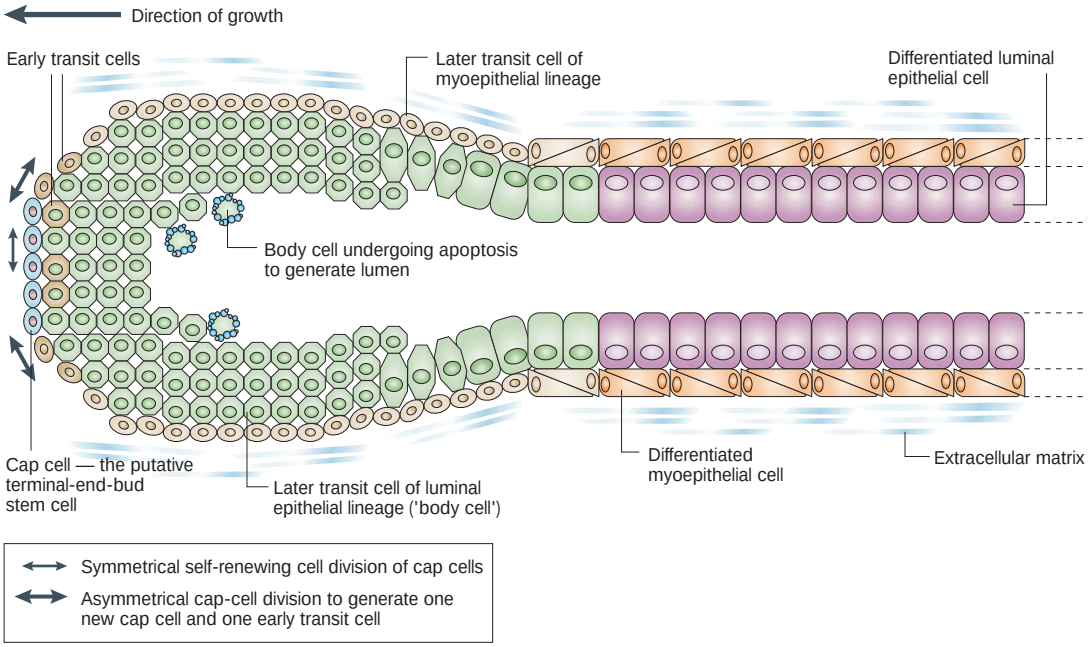


Figure 2 | **The terminal end bud.** The ducts of the developing mammary gland, with their inner luminal epithelial cell layers and outer myoepithelial cell layers, are established as the terminal end buds (TEBs) move through the fat pad. It is thought that the cap cells at the tip of the TEB generate transit cells of a myoepithelial lineage on the outer side of the TEB and generate transit cells — known as 'body cells' — of a luminal epithelial lineage to form the central TEB mass. The ductal lumen is formed as central body cells apoptose and outer cells differentiate into luminal epithelial cells. Extracellular-matrix enzymes degrade the stroma in front of the TEB to enable it to move through the fat pad, but it is unclear how the structures actually 'move' through the gland. It might simply be that progressive cell division building up cell bulk at the front of the mass of body cells, coupled with progressive apoptosis degrading cell bulk at the back of the body cell mass, creates the illusion of forward movement.

MAMMARY TREE
The complete epithelial structure of the mammary gland that, with its branching ducts and associated alveoli, resembles the branching of a tree.

TERMINAL DUCTAL LOBULO-ALVEOLAR UNITS
(TDLUs). The structures in the human breast that are equivalent to the secretory alveoli of the rodent. They have a higher order of organization than rodent alveoli, and consist of alveoli that are clustered around a distinct duct and ductal side branches. They also have a distinctive stromal component — the intralobular fibroblasts — as opposed to the interlobular fibroblasts of the breast connective tissue.

HISTOLOGY
The analysis of tissue samples by routine pathological methods and light microscopy.

ULTRASTRUCTURE
Cell and tissue morphology at the electron-microscope level.

CYTOKERATIN
A structural cellular protein that is typical of epithelia.

XENOGRAFTS
The growth of primary human tissue, cancer cells or cancer cell lines in animal hosts in an attempt to recapitulate aspects of normal, or cancerous, cell growth and morphology.

An *in vivo* labelling approach using bromodeoxyuridine (BrdU) in mammary epithelium identified cells that retained the label for an extended period of time. During the labelling period, BrdU is incorporated into DNA as the cells replicate, and is then diluted out of the cellular DNA by each cell cycle after the labelling period. Long-term maintenance of BrdU labelling indicates that cells have a very slow rate of proliferation — one of the potential characteristics of stem cells that enables them to maintain their proliferative potential throughout the life of the organism. Some of these label-retaining cells (LRCs) — which formed <5% of the luminal epithelial cell population — were negative for both luminal epithelial and myoepithelial CYTOKERATIN markers, whereas others were positive for the markers, indicating a transition from an undifferentiated LRC stem-cell population through a differentiated transit-cell population, which ultimately results in the mature differentiated-cell population[20]. Interestingly, this is similar to thymocyte differentiation — in which $CD4^-/CD8^-$ cells progress through a $CD4^+/CD8^+$ stage before maturing into either $CD4^+/CD8^-$ or $CD4^-/CD8^+$ cells — which indicates that this might be a common theme in stem-cell biology. LRCs might be related to SLCs, and recent studies of proliferation in the SLC–ULLC–DLLC–LDC axis indicate that most of the proliferation occurs in the ULLCs, DLLCs and LDCs — in other words, the transit cells and those cells that are becoming terminally differentiated. Interestingly, the results of this study also show that proliferation levels are lower in ducts than in lobules, indicating that either the microenvironments in those two structures differentially regulate the behaviour of their component cells, that the stem/progenitor cells in lobules differ from those in ducts, or that both situations exist (G. Chepko, personal communication).

In a model of *in situ* human breast epithelial proliferation, in which human breast lobules were cultured as XENOGRAFTS, cells that were positive for the oestrogen receptor (ER) were shown to be negative for a proliferation marker, Ki67, and had high levels of the cyclin-dependent-kinase inhibitor KIP1 (also known as p27)[29,30]. Again, it was suggested that these cells might be stem cells that had very slow rates of proliferation. By contrast, the number of ER-positive cells that are also Ki67-positive increases in tumours[31], indicating that these cells escape from growth control. Similar studies in mice, which used tritiated thymidine labelling of proliferating cells, found that most proliferating cells in the TEBs (including the cap cells) and ducts were ER-negative during pubertal development, although a significant minority of the proliferating cells (up to one-third) was ER positive. During the oestrous cycle of the adults, only 0.3% of cells were proliferating and 91% of these were ER-negative at pro-oestrous, whereas at oestrous, 5% of cells were proliferating, 58% of which were ER-negative. In the case of heavily labelled LRCs (0.6% of the cells examined), however, 37

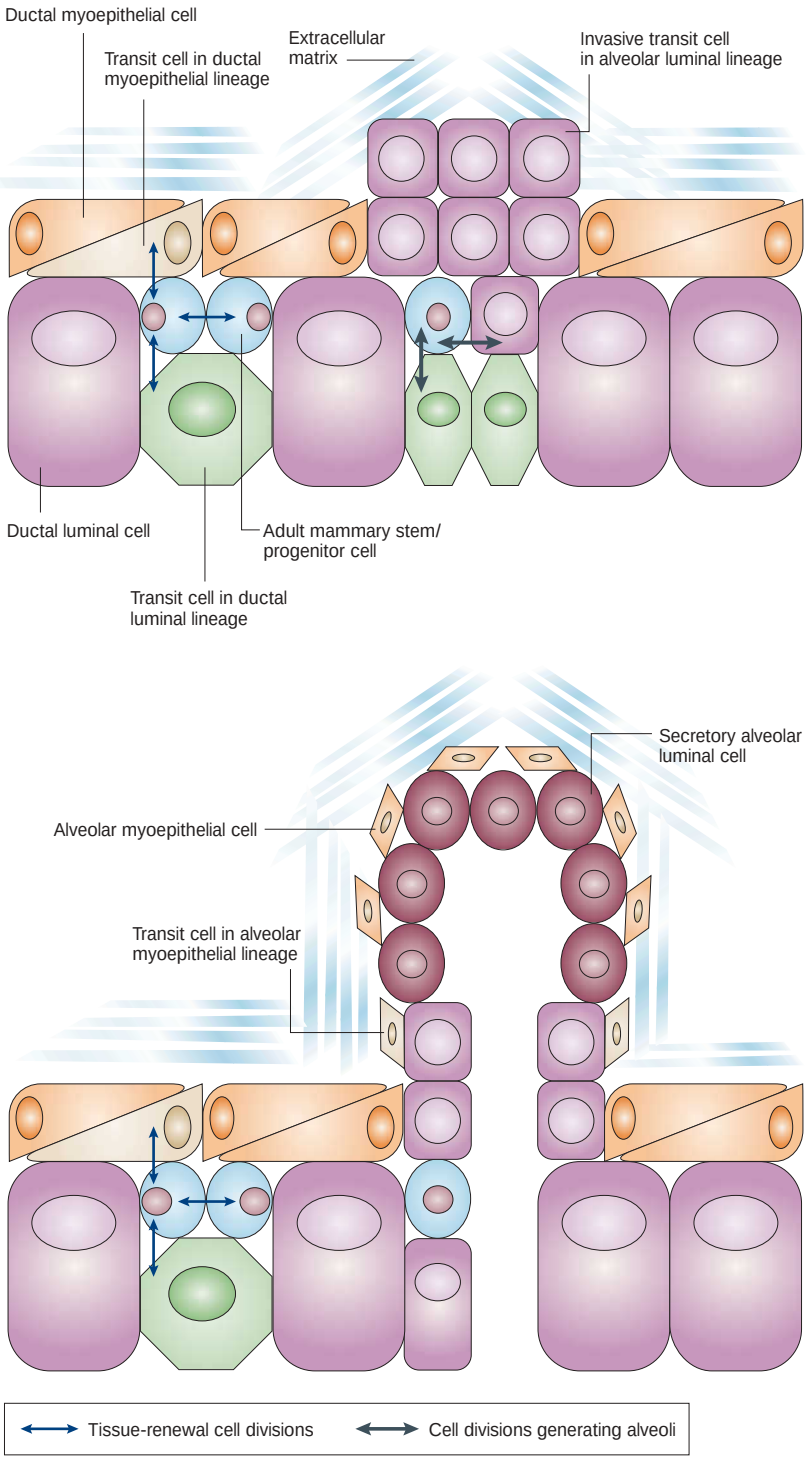


Figure 3 | **The role of stem cells in the adult mammary gland.** Stem cells that are responsible for tissue renewal undergo cell divisions that result in one daughter cell that remains in the stem-cell compartment or niche and one daughter cell that enters the transit-cell lineage of either the myoepithelial or luminal epithelial cells. By contrast, the immediate progeny of an alveolar-bud stem cell or progenitor cell must be an invasive transit cell type that breaks down extracellular matrix, invades stroma and then differentiates (bottom panel) into myoepithelial and luminal cells, or undergoes apoptosis to form the lumen. Such cells must also resist growth-inhibitory signals that might come from nearby ducts — normally, such signals are thought to regulate ductal spacing during growth of the epithelial tree in the virgin gland. Either these signals must be turned off during pregnancy or the nascent alveolar bud must no longer respond. It is easy to see how loss of control over these behaviours could lead to tumours.

of 39 were ER-positive luminal cells and only 2 were ER-negative basal cells[32,33]. So, although there does seem to be an association between ER expression and proliferatively quiescent putative stem cells in mice, the link between ER status and proliferation is not as strong as in humans.

In vitro studies. Attempts have been made to link *in situ* patterns of marker expression with *in vitro* differentiative capacity — that is, identifying the cell types that are present and then working out which, if any, have stem-cell properties. This usually means using cell-type-specific cytokeratins and cell-surface markers to divide isolates of primary mammary cells into luminal or myoepithelial/basal populations. The differentiative capacity of these cells in tissue culture is then tested in an attempt to define multipotential cells. The advantage of this approach over *in situ* studies is that self-renewal and differentiative capacity can be directly tested, but the disadvantage is that, no matter what culture system is used, there is always the possibility that *ex vivo* conditions might have no real bearing on what occurs in the whole organism.

Early studies of separated primary human luminal and myoepithelial cells that were cultured at clonal density showed a uniformity of marker expression that is consistent with their *in vivo* origin[34]. Freshly isolated, purified human luminal epithelial and myoepithelial cells could be grown as bulk cultures on completely defined, serum-free media for extended periods of time; however, when the luminal cells were switched to myoepithelial growth medium, a subpopulation of cells with myoepithelial markers slowly appeared. Cells that double-stained for both myoepithelial and luminal markers were also seen in these cultures. The same did not happen in the reverse experiment; that is, luminal-type cells did not appear when myoepithelial cells were switched to a luminal medium[35]. In an attempt to detect such intermediate cell types *in situ*, breast-tissue sections were stained for vimentin and α-isoform smooth-muscle actin (myoepithelial markers), and for cytokeratin 18 (a luminal marker). Occasional 'suprabasal' cells were observed that expressed vimentin and sometimes cytokeratin 18, but not smooth-muscle actin, indicating that an intermediate cell type does exist in the human breast[35]. However, self-renewal was not shown and the *in situ* intermediate cell types were not directly purified and analysed for their differentiative capacity.

Clonal culture of cells that were isolated on the basis of cell-surface-marker expression also provided evidence for a cell type with pluripotent capacity. Freshly isolated cells that expressed epithelial-specific antigen (ESA) and MUC1 — both of which are surface markers of luminal epithelial cells — gave rise to clones that expressed only luminal-specific cytokeratin markers. Cells expressing CD10 — a myoepithelial surface marker — generated clones that expressed only myoepithelial markers. However, cells that were positive for ESA, negative or weakly positive for MUC1 and weakly or strongly positive for CD10 ($ESA^{+}/MUC1^{-/\pm}/CD10^{\pm/+}$) generated clones that contained some cells that

Box 3 | Cleared fat-pad transplantation

Cleared fat-pad transplantation has been a standard technique in mammary-gland biology since the pioneering work of De Ome in 1959 (REF. 77). It is dependent on the peculiar nature of mammary development, in which most of the epithelial growth takes place after birth. In the fourth (abdominal) mammary fat pad (of which there are five pairs in the mouse) of the 3-week old mouse, the mammary epithelium is still concentrated in the nipple area and has not yet grown out beyond the mammary lymph node and penetrated the bulk of the fat pad. This provides an anatomical fixed point that enables the fat pad from the nipple to the lymph node to be cut away ('cleared'), leaving the bulk of the fat pad free of epithelium and ready to receive cells (see figure). The clearing of the endogenous epithelium is required, as otherwise the endogenous tree would rapidly overgrow the transplanted cells before they have had time to generate their own outgrowth. Transplantation of $>5 \times 10^5$ primary cells results in a >90% success rate and generates a mammary tree with luminal and myoepithelial cells that can respond to pregnancy by generating alveoli. This resembles the normal mammary tree in all ways except that it is not connected to the nipple. Note that immortalized mouse mammary epithelial cell lines do not, in general, generate a normal mammary tree when transplanted in this way — only freshly harvested primary cells, or those that have been cultured for a short time, generate a normal structure.

Transplantation of cells at limiting dilution ($<2 \times 10^4$) has become a key assay in mouse mammary stem-cell biology, as it is predicted that an epithelial-cell subpopulation that is enriched for candidate stem cells should produce more successful transplants at these limiting cell numbers than a subpopulation that has not been enriched. There are, however, technical issues relating to transplantation of very small numbers of cells that confound the issue. A refinement of the process is to transplant marked candidate stem cells mixed with unmarked cells that have been stem-cell depleted. The contribution of the marked cells to the outgrowths that are generated can then be examined.

One caveat to the use of this assay in testing stem-cell-like behaviour is that the epithelium-free fat pad might be a stem-cell niche (BOX 2), that promotes daughter-cell reversion to a more stem-cell-like phenotype. This could be a confounding factor in interpreting the results of this assay.

expressed myoepithelial lineage markers and some cells that expressed luminal lineage markers[36]. Later studies indicated that ESA$^+$/MUC1$^{-/\pm}$/CD10$^{\pm/+}$ cells were also positive for integrin-α6 (REF. 37), indicating that, *in vivo*, they might have a basal or suprabasal location within the luminal-cell layer.

Studies of suprabasal cells using cell-surface markers supported an ESA$^+$/MUC1$^-$ staining pattern and also indicated that they were positive for cytokeratin 19. Unlike the bulk of the luminal-cell layer, they did not contact the lumen. When ESA$^+$/MUC1$^+$ ('differentiated luminal') and ESA$^+$/MUC1$^-$ ('suprabasal luminal') cells were isolated and immortalized by transduction with HPV16 E6/7, the ESA$^+$/MUC1$^+$ line only generated ESA$^+$/MUC1$^+$ cells, whereas the line derived from ESA$^+$/MUC1$^-$/cytokeratin-19$^+$ cells also generated ESA$^+$/MUC1$^+$ and myoepithelial cells. In *in vitro* three-dimensional cultures and in xenografts, it also generated TDLU-like structures. Taken together, these results were interpreted as indicating that ESA$^+$/MUC1$^-$/cytokeratin-19$^+$ cells are TDLU precursors[50].

To show self-renewal and multipotent differentiative capacity — defining characteristics of stem cells — freshly isolated breast epithelial cells have been cultured in defined conditions under which the cells could not attach to the culture dishes, but instead formed non-adherent MAMMOSPHERES[38]. These mammospheres were disaggregated and the cells that were recovered were split between sphere-forming conditions and conditions that favour attachment and differentiation. This was repeated for each generation of spheres that arose. In early passages, clones that formed under attachment conditions expressed luminal markers, myoepithelial markers or a mixture of the two. After two passages as spheres, however, almost all colonies grown under attachment conditions expressed mixed markers, indicating that the number of multipotent cells in the spheres increased[38]. As the efficiency of sphere formation did not change with passage, however, each sphere-forming cell, as it divided to generate a new sphere, must have self-renewed only once, to give exactly the same number of sphere-forming cells. The bulk of these spheres might, therefore, have been made

MAMMOSPHERES
Balls of mammary epithelial cells that form under specialized culture conditions *in vitro* and that are capable of functional differentiation in the correct hormonal environment. Mammospheres that grow in suspension culture are analogous to neurospheres grown under similar conditions.

up of a progenitor-cell or transit-cell population. This study does indicate self-renewal of sphere-forming cells and the ability to generate restricted-lineage luminal-epithelial-type and myoepithelial-type colonies at early passage, which supports the existence of breast epithelial stem cells.

The localization of putative multilineage clonal progenitor cells within the luminal-cell layer in humans is consistent with studies that were carried out on primary rodent cells and on myoepithelial cell lines of rodent origin. Primary rat mammary luminal cells that were cultured at clonal density generated three clone types and the myoepithelial cells generated two clone types. The clones of myoepithelial origin and two of the clones of luminal origin expressed markers that are characteristic only of their cell type of origin. A third clone type, however, expressed both luminal and myoepithelial markers and generated all other clone types when subcloned[39,40].

By contrast, all clones that were cultured from isolated primary mouse luminal cells expressed both luminal and myoepithelial markers within a week of being placed in culture[41]. When cultured on a reconstituted basement-membrane gel (EHS matrix), a small proportion of clones of luminal origin developed a basal layer that expressed only myoepithelial cytokeratin markers (but not smooth-muscle actin). Myoepithelial clones on an EHS matrix only expressed myoepithelial markers and never developed a layer of luminal-type cells[42].

These *in vitro* studies provide evidence to support a luminal, possibly suprabasal, location for a breast epithelial stem cell, but a population with multipotent differentiative capacity has not yet been prospectively isolated, or been shown to be capable of self-renewal and subsequently localized *in situ*.

In vivo studies. Recently, attempts have been made to try and identify stem cells in the mammary epithelium by searching for cell populations that have characteristics shared by other stem cells. Two markers used for this approach have been side population (SP) and SCA1. SP was first identified as a marker for stem-cell activity in studies of haematopoietic stem cells in which the DNA-intercalating dye Hoechst 33342 was used as a method for determining the DNA content of cells, and therefore cell-cycle kinetics, by flow cytometry. During these studies, a distinct population of cells was found that lie to the side of the bulk population (which explains the name). It emerged that the SP occurs because the ABC TRANSPORTER PROTEIN ABCG2 pumps the dye out of the cells. Remarkably, most of the long-term POST-TRANSPLANT BONE-MARROW RECONSTITUTING ACTIVITY is found in the SP[43]. Since these first reports, an SP has been found in several tissues and putative stem cells and has been proposed as a universal stem-cell marker[44–46], although not all reports agree[47]. It should be noted, however, that *Abcg2*-knockout mice lose SP but still have bone-marrow stem cells — the SP phenotype is just a marker, it is not crucial to stem-cell function[48].

SP cells have been found in both human[38,49] and mouse[20,49] mammary epithelium, forming around 0.5–3% of the cells in the mammary tissue, depending on the study. The transplantation of purified mouse mammary SP cells into cleared fat pads at limiting dilution (2,000–5,000 cells per fat pad) resulted mainly in lobulo-alveolar-type structures, not complete mammary trees, although the rate of transplant success was low and enrichment of the SP population for stem-cell activity was not shown[49]. The transplantation of labelled SP cells mixed with unlabelled mammary epithelium resulted in CHIMERIC outgrowths of both labelled and unlabelled cells[20]. However, post-isolation viability of SP cells in these experiments was very low, making it difficult to say whether or not enrichment for stem-cell-like activity had occurred. The SP of the mouse mammary epithelium was found to be undifferentiated and lacked the cytoskeletal markers of the luminal- and myoepithelial-cell layers[49]. It was also enriched fourfold for BrdU-label-retaining cells[20]. Seventy-five percent of mouse mammary SP cells were positive for the haematopoietic stem-cell marker SCA1. In the tissue as a whole, 20% of epithelial cells were SCA1-positive. The transplantation of SCA1-positive-enriched cells at limiting cell dilution resulted in outgrowth formation at increased rates, which indicated that stem-cell-like activity was enhanced and, importantly, that SCA1-depleted cell populations had a reduced repopulation ability. All of the expected cell layers were found in the outgrowths[20]. However, self-renewal of transplanted SCA1-positive cells in a transplant assay has not yet been shown.

Interestingly, in the study in which human breast epithelial cells were isolated and grown as non-adherent mammospheres, the proportion of SP increased from 1% in freshly harvested cells to 27% in mammosphere cultures[38]. As the spheres did not show an increase in self-renewal capacity, but did show an increase in the number of cells that were capable of multilineage differentiation when placed in monolayer culture, this might indicate the expansion of a transit-cell population. The SP phenotype might, therefore, be a transit-cell — or combined stem/transit-cell — marker.

A synthesis of the evidence. We still do not know definitively that classically defined stem cells exist in the adult mammary epithelium, or, if they do, whether there is more than one type. Nevertheless, we can make a guess about the properties and identity of a generalized adult mammary epithelial stem cell. The location of such a cell is likely to be 'suprabasal' — that is, at the base of the luminal epithelial layer, next to the myoepithelium, and not contacting the lumen or the basement membrane[23,35–37,50]. This location might be a stem-cell niche[23]. It is likely to be a small, undifferentiated cell that does not express markers of fully differentiated myoepithelial and luminal epithelial cells[20,24,49,51], although combinations of certain markers (for example, cytokeratin 19, vimentin, ER, ESA and

ABC TRANSPORTER PROTEINS
Transmembrane protein pumps that can eliminate various small molecules from the cell.

POST-TRANSPLANT BONE-MARROW RECONSTITUTING ACTIVITY
The ability of bone-marrow cells, transplanted into an animal that has received lethal whole-body irradiation, to regenerate the haematopoietic stem-cell compartment and keep the animal alive.

CHIMERIC
Composed of cells that originate from more than one source.

integrin-α6) have been suggested as being characteristic of the stem cells. The cell probably has a slow proliferative rate, although this might change in response to pregnancy[20]. It might have characteristics that are common to other stem cells[20,38,49]. It might generate a transit-cell population that, at least in its early stages, is very difficult to distinguish from its parental stem cell, but that gradually acquires lineage markers[20,24]. Initially, such cells are likely to express markers of both the myoepithelial and luminal epithelial populations before becoming either luminal or myoepithelial.

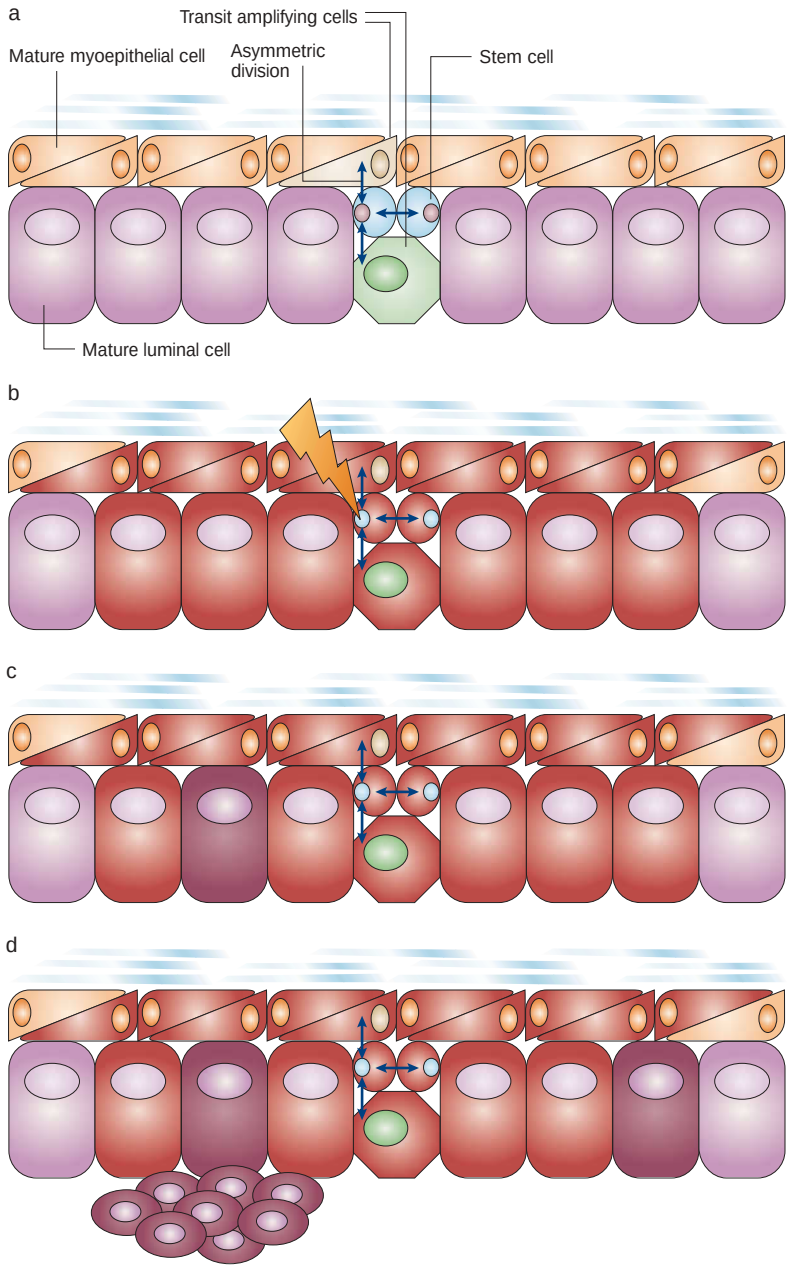


Figure 4 | **Field cancerization. a** | Stem cells divide (arrows) to self-renew and generate transit amplifying cells (green and light yellow) that terminally differentiate into mature luminal and myoepithelial cells. **b** | A mutation in a stem cell results in a field of transit and terminally differentiated cells that all carry the mutation (red cells). This is, however, still clinically silent. **c** | If the transit and terminally differentiated cells have a slow clearance time — or if the original mutation affects DNA stability or replication fidelity — then large numbers of cells are created that are targets for secondary mutations (maroon cells). **d** | The increasing number of cells that carry several mutations increases the chance that one cell will develop further mutations, resulting in a clinically apparent disease.

Breast cancer stem cells and tumour origins

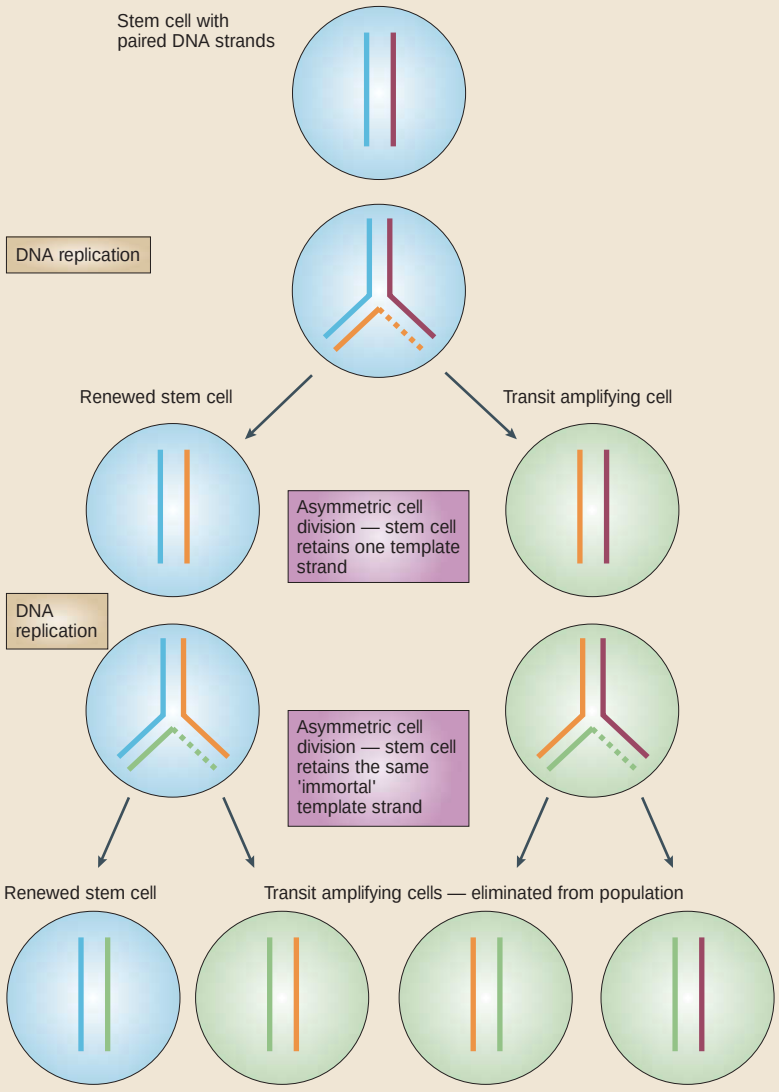
We shall now consider the association between mammary stem cells and breast cancer. The potential existence of a stem-cell-like cell in tumours is an old concept[52], but the recent paper by Al-Hajj and colleagues[53] has provided evidence for the existence of stem cells for breast cancer. This work identifies a putative breast tumour stem-cell-like population that is defined by the presence or absence of two cell-surface markers (CD44 and CD24, respectively)— although the functional significance of these markers in this context is unclear — and the lack of mammary epithelial lineage markers. This $CD44^{+}/CD24^{-/low}/lineage^{-}$ cell population lacks differentiated breast epithelial-cell-lineage markers and has a 10–50-fold increase in ability to form tumours in xenografts compared with the bulk of breast tumour cells. The presence of such a population in breast tumours has enormous implications for tumour therapy. Most traditional cancer treatments target proliferating cells and, although this might eliminate the mass of a tumour, relatively quiescent tumour stem cells could be bypassed. Recent therapeutic strategies, based on knowledge of specific molecular targets within bulk tumour cells, might be ineffective if these are not present in the stem-like cells. In both of these circumstances, the tumour will always regrow, no matter how often the tumour mass is reduced.

One of the most important questions is whether tumour stem cells originate from normal adult MESCs or from a transit-cell population in the normal breast. Tumour stem cells, and the tumours they generate, might have very different characteristics depending on which of these normal populations the tumour stem cells arise from — for instance, it could mean the difference between being poorly differentiated and highly aggressive or relatively well differentiated and non-invasive. Stem and transit cells, as the cells of origin of skin cancer, and the issues that arise from this concept, have been dealt with in depth in recent reviews in this journal[10,54]. Some experimental evidence supports the hypothesis that normal stem cells are indeed the primary targets for tumorigenesis in the adult mammary gland, and form the tumour stem-cell population. The reduced fat-pad repopulation ability of mammary epithelial cells that are derived from transgenic mice that carry a *Tgfβ* transgene under the control of the whey acidic protein (*Wap*) promoter was interpreted as being due to premature stem-cell senescence. These animals were more resistant to tumorigenesis that is induced by the mouse mammary tumour virus (MMTV), compared with wild-type animals[55]. In the tumours that did develop, the *Wap–Tgfβ* transgene was active, indicating that tumorigenesis had not been affected by presence of the Tgf-β protein, but rather by stem-cell senescence. In experimental mouse models, pregnancy is required for the establishment of terminal differentiation and, despite the massive apoptosis that

Box 4 | The Cairns hypothesis

In 1975, Cairns proposed that when stem cells undergo a series of DNA replications and asymmetric cell divisions (that is, to produce one new stem cell and one daughter transit cell in a non-random fashion), then at each division the same DNA template strands would always co-segregate to form the DNA compliment of the new stem cell[78] (see figure). This ensures that although the stem cell must, of course, have received a newly synthesized DNA strand following each cell division, this newly synthesized strand is eliminated in the subsequent division. The 'immortal' DNA strands are passed down through the stem-cell generations, ensuring that newly replicated DNA strands are never retained in the stem-cell compartment for more than one generation. This idea has profound implications for tumorigenesis, as it is during DNA-strand replication that 'unforced errors' — independent of radiation or carcinogen damage — might occur. By ensuring that daughter or granddaughter transit cells, which have a limited lifespan in comparison with the stem cells, receive the newly synthesized DNA strands, any errors in DNA replication will, sooner or later, be eliminated from the population, and mutations do not accumulate in the stem cells.

Recent evidence supports the Cairns hypothesis. Segregation of template DNA to parental stem cells and of newly synthesized DNA to daughter cells has been shown in the crypts of the small intestine[79] and in tissue culture in a mouse embryo fibroblast cell line[80].



occurs during postlactational involution of the gland, some of these terminally differentiated cells do survive. With successive pregnancies, the gland becomes increasingly composed of these terminally differentiated cells[19]. An analogous situation occurs in humans, in which there is an increase in the complexity of TDLUs following pregnancy and a decrease in proliferative activity of these lobules[56,57], supporting the view that there is unused proliferative capacity in the nulliparous breast. An early, first, full-term pregnancy is the single most important protective factor against breast cancer (although there is actually a slight increase in breast cancer risk during and immediately after pregnancy)[58,59], and it is tempting to speculate that the protective mechanism behind this is an increase in terminally differentiated cells at the expense of a stem-cell or progenitor-cell compartment. Other mechanisms have been proposed, however, such as a permanent change in the levels of circulating hormones in the body[60,61].

There are several mechanisms that might explain the link between breast stem-cell burden and risk of neoplasia. Stem cells are thought to be long-lived and have a large replicative potential. This means that not only will they persist in the body for long enough to accumulate the many mutations that are required to change a normal cell into one with neoplastic potential (a putative tumour stem cell), but they also have the proliferative capacity to actually generate a tumour mass. However, much of the proliferative capacity of normal stem cells might reside in a progenitor or transit-cell population — the initial daughter cells that are produced by the stem cell — as it is possible that replicative potential and long life might be maintained by means of a slow rate of division[20]. If these characteristics persist when normal stem cells progress to being putative tumour stem cells, they might therefore be resistant to traditional chemotherapy and so have the ability to survive an initial tumour kill and generate tumour transit cells, which might allow the tumour to regrow once the chemotherapy regimen has ended. Alternatively, such tumour stem cells could undergo selection for alternative mechanisms of resistance that can be passed on to the next generation of daughter tumour transit cells.

Stem cells might also explain 'field cancerization'[62,63] (FIG. 4). This concept suggests that preneoplastic fields of cells might develop because of their clonal origin from an original cell with a mutation — for instance, the loss of a tumour-suppressor gene. Such a mutation would be phenotypically silent, but would predispose all cells in the field to neoplastic development, even if they were relatively short-lived (compared with stem cells). So, even a single mutation in a stem cell could generate a cancer-prone field, leading to apparently independent tumours arising from nearby sites. The importance of this would relate to the rate of turnover of transit amplifying cells in the field — in other words, are they present for long enough for additional mutations to arise? Cells in the pre-neoplastic field have an advantage over cells outside the field, but additional hits still need to occur. LOSS OF HETEROZYGOSITY (LOH) analysis of

breast tissue has provided evidence for common genetic alterations in luminal epithelial and myoepithelial cells, indicating a mutation in a common stem cell that has given rise to a field of mutant progeny[64]. The most extreme case of field cancerization would be the inheritance of a germline mutation in a tumour-suppressor gene, such as *BRCA2* or *TP53*. In that case, the field comprises the whole body.

Breast stem cells as therapeutic targets

Whether stem cells themselves accumulate mutations to generate neoplasia, or whether they establish a clone of cancer-prone cells, they make attractive therapeutic targets. Targeting stem cells, or stem-cell-like cells, would target the cell of origin of the tumour and have the additional advantage of enabling treatment to be based on phenotype rather than genotype. In other words, instead of laboriously characterizing every genetic defect in a tumour, and tailoring treatment for each individual on the basis of that defect, tumours would be treated on the basis of a shared property that is characteristic of all mammary stem cells. Of course, to avoid side effects, this property would need to be absent from other stem cells.

If breast stem cells are the targets for malignant transformation, then the possibility arises of using anti-stem-cell therapy PROPHYLACTICALLY. Treatment of postmenopausal women, or younger women from an at-risk group, with an anti-breast-stem-cell therapy might severely deplete or even eliminate the cancer-prone cell population, with obvious benefits in reducing cancer incidence. Given that the breast is a non-vital tissue, this is an attractive approach. There are some caveats to such an approach in younger at-risk women, however, arising as a result of the Cairns hypothesis (BOX 4) and the possibility of repopulation of a depleted stem-cell niche by daughter cells in which mutations have occurred during DNA replication. This would fix a mutation in the stem-cell population, possibly increasing the breast cancer risk.

What if breast-tumour stem cells are not derived from adult mammary epithelial stem cells? This is not necessarily a problem — if tumour stem cells are derived from transit cells or differentiated cells[54], they might still have phenotypic characteristics of the normal breast epithelial stem cells. Perhaps some reversion to a more stem-cell-like behaviour is part of the neoplastic process. In this case, the phenotypic characteristics of normal breast stem cells could still be used to target tumour cells. Such are the possibilities that are offered by stem-cell research in the mammary gland.

The next steps

It is only a matter of time before the definitive identification of adult mammary epithelial stem cells is made. If we are to exploit this knowledge in the clinical setting, we need to decide what steps should be taken next. The route has already been mapped out by the studies of Stappenbeck, Ramalho-Santos, Ivanova and their colleagues[65–67]. Their studies and cross-comparisons of gene-expression patterns from various stem-cell types using microarray techniques have started to identify patterns of gene expression that define 'stem-ness'. Their studies will be a rich source of comparison for molecular characterization studies of mammary stem cells and will enable the definition of gene-expression sets that are limited to mammary epithelial stem cells, as well as genes that are potentially common to all stem cells.

Some studies are already beginning to give hints of the molecular pathways that might be important in mammary stem-cell biology. Mice null for syndecan 1 — a HEPARAN-SULPHATE PROTEOGLYCAN — are resistant to mammary tumour formation that is induced by WNT1 (REF. 68), indicating that syndecan is required to create a Wnt-responsive subpopulation of cells (it is thought that Wnt signals have an important role in the maintenance of stem-cell compartments)[69]. Wnt1-induced tumours also contain cells that have myoepithelial, as well as luminal, differentiation, indicating that they originate in a pluripotent stem or transit cell[70]. Other potentially important molecules that could be involved in regulation of stem-cell behaviour include components of the Delta–Notch pathway, MYC signalling molecules and p63, a recently discovered member of the p53 family[69,71,72].

Having defined mammary stem-cell genes, the next task will be to determine whether any of these are, in fact, important in breast cancer. Examination of expression patterns in tumours will indicate whether the stem-cell genes are potential therapeutic targets in tumour stem cells, although the potential paucity of such cells in bulk tumours might require enrichment strategies for these cells to make this a meaningful analysis. Mammary-gland-directed gene overexpression and ablation studies using transgenic and (conditional) knockout studies will address the potential for these genes to directly modulate the size and behaviour of the stem-cell compartment and to predispose, or directly cause, tumour formation. Isolation of stem-cell and transit-cell-specific gene promoters and their coupling to active, or inducible, oncogenes will enable the question of whether oncogene activity in stem cells is required for tumour formation. Methods of disrupting the target genes of interest within tumour models (possibly using RNA INTERFERENCE) will need to be developed so that the effect of anti-stem-cell therapy (which might cause stem-cell death or promote terminal differentiation) can be tested in various mammary tumours. Finally, the possibility that depletion of the stem-cell compartment might, in the long run, actually promote tumorigenesis as a result of niche repopulation will need to be rigorously examined.

Concluding remarks

We believe that multipotent differentiation and self-renewal of a mammary epithelial population will soon be definitively linked to an *in situ* cell type, and one of the mysteries that has both plagued and excited the field for many years will, at least partially, be solved. Several issues still remain to be addressed, however.

LOSS OF HETEROZYGOSITY
The elimination of the remaining normal copy of a gene from a cell that already carries one mutant copy of that gene. This results in the complete loss of function of the gene from that cell.

PROPHYLACTIC
Preventative treatment.

HEPARAN-SULPHATE PROTEOGLYCANS
Cell-surface molecules that might have a role in regulating cell–matrix or receptor–ligand interactions.

WNT1
A potent mammary oncoprotein that is activated by the mouse mammary tumour virus. Its normal role is as a developmental regulator.

RNA INTERFERENCE
A technique that triggers a natural defence mechanism against certain viruses and tricks the cells into suppressing expression of endogenous genes.

For instance, where do adult mammary epithelial stem cells come from? Are they deposited by symmetric cell divisions of the cap cells within the TEBs as they move through the fat pad? What is the lineage relationship between the three stem-cell types that are indicated by transplantation experiments? Is the stem- or transit-cell population depleted by a full-term pregnancy? Why, if stem cells are the targets of tumorigenesis, are tumours skewed to non-myoepithelial phenotypes? Finally, of course, can we actually develop methods of depleting the stem-cell compartment and will this have a tumour-protective effect?

These issues are going to keep the field of mammary stem-cell biology occupied for many years to come.

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