Cell Prolif. 2005, **38**, 375–386

REVIEW

Isolation and characterization of human mammary stem cells

R. B. Clarke

Breast Biology Group, Division of Cancer Studies, University of Manchester, Christie Hospital, Wilmslow Road, Withington, Manchester, M20 4BX, UK

Received 7 *July* 2005*; revision accepted* 22 *August* 2005

Abstract. Since stem cells are present throughout the lifetime of an organism, it is thought that they may accumulate mutations, eventually leading to cancer. In the breast, tumours are predominantly oestrogen and progesterone receptor-positive (ERα/PR+). We therefore studied the biology of ERα/PR-positive cells and their relationship to stem cells in normal human mammary epithelium. We demonstrated that ERα/PR-positive cells co-express the putative stem cell markers $p21^{CIP1/WAF1}$, cytokeratin (CK) 19 and Musashi-1 when examined using dual label immunofluorescence on tissue sections. Next, we isolated a Hoechst dye-effluxing 'side population' (SP) from the epithelium using flow cytometry and demonstrated them to be undifferentiated cells by lack of expression of myoepithelial and luminal cell-specific antigens such as CALLA and MUC1. Epithelial SP cells were shown to be enriched for the putative stem cell markers $p21^{CIP1/WAF1}$, Musashi-1 and ERα/PR-positive cells. Lastly, SP cells, compared to non-SP, were highly enriched for the capacity to produce colonies containing multiple lineages in 3D basement membrane (Matrigel) culture. We conclude that breast stem cells include two populations: a primitive ERα/PR-negative stem cell necessary for development and a shorter term ERα/PR-positive stem cell necessary for adult tissue homeostasis during menstrual cycling. We speculate these two basic stem cell types may therefore be the cells of origin for ERα-positive and -negative breast tumours.

INTRODUCTION

Tissue-specific stem cells are defined by their ability to self-renew and to produce the differentiated, functional cells within an organ. Differentiated cells are generally short-lived; in skin and blood for example, they are produced from a small pool of long-lived stem cells that last throughout life. (Dexter & Spooncer 1987; Jones 1997; Watt 1998; Orkin 2000). Thus, stem cells are necessary for tissue development, replacement and repair (Fuchs & Segre 2000). However, stem cell longevity makes them susceptible to accumulating genetic damage and they represent likely

Correspondence: R. B. Clarke, Breast Biology Group, Division of Cancer Studies, University of Manchester, Christie Hospital, Wilmslow Road, Withington, Manchester, M20 4BX, UK. Tel.: 44-161-446 3210; Fax: 44-161-446 3218; E-mail: robert.clarke@manchester.ac.uk

targets for carcinogenic transformation. The development, differentiation and function of the mammary gland would not be possible without tissue-specific stem cells. Full development of mammary epithelium occurs only during pregnancy and lactation to be followed at weaning by involution. The cycle of pregnancy-associated proliferation, differentiation, apoptosis and remodelling can occur many times during the reproductive lifespan of mammals and may be explained by the presence of a long-lived population of stem cells that have a near infinite propensity to produce functional cells. One implication of the 'multi-hit theory' of carcinogenesis is that cancer is a stem cell disease, suggesting that successful breast cancer prevention strategies must be targeted to mammary epithelial stem cells.

The resting adult human breast consists of a branching ductal system and terminal ductal lobulo-alveolar units (TDLUs) or lobules that are the functional glands of the pre-menopausal breast. Each lobule is lined by a layer of luminal epithelial cells surrounded by a basal layer of myoepithelial cells. TDLUs have been reported to be the site from which most breast tumours originate (Wellings *et al*. 1975). Furthermore, most breast tumours have the phenotype of luminal epithelial cells (Sorlie *et al*. 2001). The presence of stem cells in the mammary gland and susceptibility to carcinogens appear related. The greatest concentration of stem cells is in the terminal end bud and alveolar bud structures during pubertal development in rodents and it is during this period that the gland is most sensitive to carcinogens (Russo & Russo 1978a,b). Similar structures exist in the breasts of pre-pubertal and adolescent women, and it is this age group that suffered the highest rates of breast cancer after irradiation due to the atomic detonations in Japan in 1945 (Dawson 1934; McGregor *et al*. 1977).

Although breast cancer might be considered a stem cell disease, it is not clear whether the tumours seen clinically retain the characteristics of stem cells. Certainly, like putative stem cells, tumours express mainly markers of luminal cells, not myoepithelial cells (Taylor-Papadimitriou *et al*. 1989; Li *et al*. 1998; Perou *et al*. 1999, 2000; Sorlie *et al*. 2001; Korsching *et al*. 2002). On the other hand, it is feasible that the immortalizing or transforming step may occur during transit amplification or differentiation of stem cell progeny, which may explain why many breast tumours acquire features of differentiation. Indeed, the similarities between cancer cells and normal stem cells, such as increased proliferation potential and self-renewal ability, were noticed 150 years ago and provide a compelling potential reason why cancer chemotherapy may induce remission, yet rarely cures (Behbod & Rosen 2005). The therapies may affect descendent cells that are irrelevant for the persistence and propagation of the disease, leaving the rarer but more potent cancer stem cell unperturbed. Recently, the prospective isolation of breast tumour stem cells has been reported (Al-Hajj *et al*. 2003), followed closely by stem cell identification in other solid tumours such as brain (Singh *et al*. 2004). This increases the importance of investigating breast stem cell characteristics so that cancer stem cells can be targeted for therapy, preferably without inducing unwanted toxicities in normal stem cells.

Evidence for a breast epithelial stem cell

There is good evidence to suggest that the luminal and the myoepithelial cell types of the normal breast arise from a shared, pluripotent stem cell. Many years ago, it was demonstrated that small fragments of the rodent duct or TEBs transplanted in cleared mammary fat pads of syngeneic hosts could develop an entire and functional mammary tree (DeOme *et al*. 1959; Hoshino & Gardner 1967; Daniel *et al*. 1968; Ormerod & Rudland 1986). Serial transplantation, allowing full differentiation each time, revealed that the capacity for regeneration was not infinite, being lost by the seventh transplant generation (Daniel & Young 1971; Young *et al*. 1971). Surprisingly, the results indicated that the regenerative ability of mammary tissue from old mice was similar to that taken from young mice, and neither reproductive history nor reproductive state

affected regenerative capacity. The authors concluded from their experiments that the eventual growth senescence of serially transplanted mammary epithelium was essentially a function of the number of stem cell divisions that had taken place (Daniel & Young 1971). More recently, using viral integration to mark and follow individual clones, it has been reported that a fully differentiated mammary gland can be derived from a single cell clone (Kordon & Smith 1998).

Data demonstrating that the human breast is generated from stem cells have been provided by studies of the pattern of X-chromosome inactivation throughout the ductal and lobular epithelium. These showed that contiguous patches of epithelium with inactivation of the same X-chromosome were present, suggesting that the cells within the patch had been derived from the same stem cell (Tsai *et al*. 1996; Diallo *et al*. 2001). Further evidence for the existence of human breast stem cells comes from studies showing that the same genetic lesion can be detected throughout an individual lobule or duct within histologically normal mammary epithelium (Deng *et al*. 1996; Lakhani *et al*. 1996). Second, separated myoepithelial and luminal cells from the same breast region have similar patterns of loss of heterozygosity suggesting a common progenitor (Lakhani *et al*. 1999).

Steroid receptor expression patterns

Ovarian steroids play a critical role in mammary gland development and tumourigenesis acting through specific nuclear receptors expressed in target cells. Cells containing receptors for oestrogen and progesterone (ERα and PR, respectively) are located in the luminal epithelium of the ductal and lobular structures (Petersen *et al*. 1987), and it has been estimated that receptor-positive cells account for 10–20% of the epithelial cell population. ERα and PR are co-expressed in luminal epithelial cells but dividing cells are ERα/PR-negative and often adjacent to ERα/PR-positive cells (Clarke *et al*. 1997; Brisken *et al*. 1998; Russo *et al*. 1999; Ellis & Capuco 2002). This separation appears to be disrupted early in breast tumourigenesis because actively dividing steroid receptor-positive tumour cells can be found in premalignant lesions such as atypical ductal hyperplasia (Shoker *et al*. 1999). Since breast cancers are mainly ERα/PR-positive, the aim of our studies has been to examine the relationship of ERα/PR-positive cells to stem cells in normal human breast epithelium.

Intermediate human breast cells express steroid receptors

There is evidence from both rodents and humans that a population of division-competent mammary epithelial cells of a distinctive morphology can be found in a position intermediate between the basal and luminal cells (Smith *et al*. 1984; Ferguson 1985; Smith & Medina 1988; Chepko & Smith 1997). These intermediate cells are distinguished by their pale staining cytoplasm under both light and electron microscopy (Chepko & Smith 1997). They have few cellular organelles and pale nuclei and are found as small light cells (SLC) and as undifferentiated large light cells (ULLC). Their infrequent occurrence alone or in pairs and their undifferentiated character has made intermediate cells the focus of suggestions that they may be the mammary stem cell. There is some existing and some emerging evidence supporting this suggestion. First, when mammary epithelial cells from nulliparous mice are placed in culture, it is the pale staining cells that divide first irrespective of whether or not hormones are present in the medium (Smith & Medina 1988). In the presence of the lactogenic hormones insulin, hydrocortisone and prolactin, groups of pale cells gradually disappear and darker cells producing milk proteins appear. In the absence of lactogenic hormones, pale cells remain and no milk proteins are produced (Smith & Medina 1988; Chepko & Smith 1997). More recently, it has been shown that pale staining cells are depleted in growth senescent serial mammary epithelial transplants and their disappearance coincides with growth cessation (Smith *et al*. 2002). Thus, in rodents the results of *in vitro* and

in vivo experiments suggest that the pale staining or light cells situated between the luminal and the myoepithelial cell layers are the most likely candidates for a stem cell population.

These data seemingly contradict previous results in both human and rodent studies where a stem cell type that is multipotent in culture was demonstrated to be part of the luminal cell population (Stingl *et al*. 1998, 2001; Pechoux *et al*. 1999; Smalley *et al*. 1999). However, recent work on human breast cells used these contradictory observations to predict that luminal cells that did not contact the lumen would not express the apical membrane-specific sialomucin MUC1, but would express the luminal epithelial-specific antigen (ESA). Accordingly, an ESA+/MUC-1-population that can give rise to both luminal and myoepithelial cell types in culture has been isolated (Gudjonsson *et al*. 2002b). These cells were also shown to express cytokeratin (CK) 19 (Gudjonsson *et al*. 2002b). Therefore, we analysed the CK19 expression and its relationship to proliferating cells and those expressing steroid receptors. In many lobules CK19 expression was homogeneous in that all luminal cells were stained. In others, CK19 expression was scattered throughout the lobule and labelled the cytoplasm of infrequent cells that rarely contained the proliferation-associated nuclear antigen Ki67. However, these scattered CK19-positive cells were frequently steroid receptor positive. Second, when the position of ERα/PR-positive cells in relation to the luminal and myoepithelial cell layers was assessed, three-quarters of these ERα/PR-positive cells were in an intermediate position (Clarke *et al*. 2005).

Mammary epithelial side population (SP) analysis

In order to characterize putative epithelial stem cells further, we digested histologically normal breast tissue obtained from premenopausal women with collagenase and trypsin to obtain a single cell suspension and stained them with Hoechst 33342. Following flow cytometric analysis using a fluorescently labelled epithelial specific antibody (BER-EP4), an epithelial 'side population' (SP) was obtained, averaging 5% of cells that were able to efflux the fluorescent dye (Clarke *et al*. 2005). Haematopoietic cells that efflux Hoechst 33342 can reconstitute the bone marrow of lethally irradiated mice, suggesting that they are stem cells (Goodell *et al*. 1997; Jackson *et al*. 1999; McKinney-Freeman *et al*. 2002). The method has also been used to isolate an SP from mouse mammary glands. Mouse mammary SP cells are enriched for expression of three putative stem cell markers; Sca-1, α6-integrin and telomerase (Welm *et al*. 2002; Alvi *et al*. 2003; Liu *et al*. 2004). Mouse mammary SP cells were estimated to be 2–3% of epithelial cells in two studies (Liu *et al*. 2004; Welm *et al*. 2002) and 0.5% of total cells in the other (Alvi *et al*. 2003). Alvi *et al*. have further reported that nearly half of the SP cells were steroid receptor-positive (Alvi *et al*. 2003).

A similar SP to that observed in the mouse mammary gland has also been identified by several groups working on normal human breast tissue obtained from reduction mammoplasty and other non-cancer breast surgery (Alvi *et al*. 2003; Dontu *et al*. 2003; Clayton *et al*. 2004; Clarke *et al*. 2005). In the three groups who have performed human breast tissue SP analyses, the proportion of breast SP cells varied from ~0.2% (Alvi *et al*. 2003; Clayton *et al*. 2004) to ~1% (Dontu *et al*. 2003) to ~5% (Clarke *et al*. 2005).

The reason for this variation is partly that the SP cells are not a discrete population but form a continuum with the rest of the cell population, although methodological differences certainly account for some of it. For example, in our study where the highest percentage of SP cells was reported (Clarke *et al*. 2005), only epithelial cells were included in the flow cytometric analysis by using a fluorescence-labelled epithelial-specific antibody (BER-EP4). One caveat to this method may be that not all epithelial cells were counted since those with low levels of the marker may have been discounted. In each of the other studies (Alvi *et al*. 2003; Clayton *et al*. 2004; Clarke *et al*. 2005), epithelial cells were not separated from other breast cell types using

antibody recognition. However, they argue that because only epithelial cell colonies grew out in culture from the isolated SP cells, they must be epithelial. This finding does not exclude the likelihood that non-epithelial cells were present in the non-SP. Supporting this, in the study of mouse mammary cells, only 5% of the SP expressed CD45, a haematopoietic cell marker, but 40% of the non-SP cells were CD45-positive (Alvi *et al*. 2003). The actual percentage of SP in the epithelium may therefore be slightly higher than measurements given by Alvi *et al*. and Clayton *et al*. (Alvi *et al*. 2003; Clayton *et al*. 2004).

Cell culture studies using human breast SP cells

Although different proportions of isolated human breast SP cells were reported in the above studies, their stem cell nature has been analysed and compared to the non-SP cells using various *in vitro* cell culture methods. When using human tissue, it has obviously been difficult to test their 'stem-ness' by using transplantation into cleared mouse mammary fat pads because human breast cells do not form a mammary tree as xenografts. However, this approach may be feasible in the near future using a novel method for the humanization of the mouse mammary fat pad (Kuperwasser *et al*. 2004). Growing SP and non-SP at clonal densities in monolayer culture *in vitro* either on feeder layers or on collagen produced three types of colonies: those consisting of myoepithelial or luminal epithelial cells alone and mixed colonies of both cell types. However, depending on the substratum, SP cells produced two to seven times more colonies than non-SP cells. In support of their putative stem cell nature, only the SP cells possessed the ability to produce colonies with both myoepithelial and luminal epithelial cell types (Clayton *et al*. 2004; Clarke *et al*. 2005). Another published method for the culture of undifferentiated tissue-specific stem cells is the growth of colonies from single cells in non-adherent suspension culture, such as neurospheres grown from brain tissue, which are enriched in neural stem cells (Dontu *et al*. 2003). Where this has been applied to human breast cells grown as 'mammospheres', 27% of the total population of sphere cells were found to be fall into the SP region following Hoechst staining. Conversely, only SP, and not non-SP cells, from fresh breast cell digests were capable of forming mammospheres in non-adherent suspension culture (Dontu *et al*. 2003). Finally, in three-dimensional (3D) cultures in basement membrane preparations such as Matrigel (Fred Baker Scientific, Runcorn, UK), breast cells can differentiate to form acini (small hollowed out or solid colonies), or large branching structures reminiscent of lobular structures *in vivo*. In fact, it has been shown that isolated luminal epithelial cells produce acinus-like, polarized structures with a hollow lumen, and myoepithelial cells produce a solid acinus (Gudjonsson *et al*. 2002a). In contrast, single cells with stem/progenitor properties, for example, cells derived from mammospheres or other multipotent breast cells, produce branching lobule-like structures. We demonstrated in our study that human breast epithelial (BER-EP4+) SP cells produce branching type structures, while non-SP cells produced only acinus-like structures (Clarke *et al*. 2005). Furthermore, each individual branching structure contained separate populations of cells expressing cytokeratins (CK) of either myoepithelial (CK14) or luminal epithelial (CK18) type.

Stem cell marker expression in breast SP cells

Putative stem cell markers and differentiation markers have been analysed in the SP and compared to the non-SP cells by two research groups (Clayton *et al.* 2004; Clarke *et al.* 2005). In these studies, quantitative reverse transcriptase polymerase chain reaction (QRT-PCR) was used to assess gene expression, and antibody staining was used to analyse protein synthesis. Using antibodies to the cell surface markers of differentiated myoepithelial and luminal epithelial cells, CALLA and MUC1, respectively, it was demonstrated by both groups that ~70% of epithelial SP cells expressed neither protein, whereas most non-SP cells expressed one or the other of these

differentiated cell markers (Clayton *et al*. 2004; Clarke *et al*. 2005). This result strongly suggests that SP cells include an undifferentiated population of cells. In agreement with this, QRT-PCR analysis of SP cells by the Clayton group also demonstrated that most SP, but not non-SP, cells lacked these differentiation markers. Because the breast is a steroid hormone-responsive tissue, both groups analysed oestrogen receptor (ER) expression. Clarke *et al*. found a 6-fold increased ER-α expression in SP compared to non-SP cells using both QRT-PCR and antibody staining, whereas Clayton *et al*. (2004) found no SP cells expressing either the ER-α or the ER-β gene by QRT-PCR (Clarke). Again, the issue of measuring the proportion of human breast SP cells becomes relevant as these studies had a 25-fold difference in their analysis (~5% vs. ~0.2%), presumably because of the reasons outlined above and in the technical issues section below. This difference may help explain why these gene expression results are directly at odds with each other. On the other hand, Alvi *et al*. (Alvi *et al*. 2003) found that up to half of their mouse mammary SP cells expressed ER-α protein even though their SP fraction was similar in percentage to that of Clayton *et al*. (Clayton *et al*. 2004).

Finally, the expression of putative stem cell markers has been demonstrated to be increased in SP compared to non-SP cells. For example, $p21^{CIP1/WAF1}$ and Musashi-1 were reported to be 2-fold and 6-fold overexpressed as measured by QRT-PCR (Clarke *et al*. 2005). Interestingly, these proteins were co-expressed with ER-α in breast epithelial cells examined by dual label immunofluorescence, suggesting that SP cells may express all three proteins (Clarke *et al*. 2005). The proliferation marker Ki67 was absent in SP cells by QRT-PCR (Clayton *et al.* 2004), which would fit with the established fact that cells expressing ER-α do not proliferate in breast epithelium *in vivo* (Clarke *et al*. 1997) and the long-recognized quiescence of tissue-specific stem cells.

DNA label-retaining cells express ER-α, PR, $p21^{WAF1/CIP1}$ and Musashi-1

Two studies of the mouse mammary gland have used DNA label retention as a marker for the stem cell population. The technique has been used previously to label putative skin and intestinal stem cells (Potten & Morris 1988; Potten & Loeffler 1990). It relies on the dilution of DNA label when a cell divides such that it is undetectable after a small number of divisions. Since stem cells are thought to be quiescent compared to their progeny that undergo transit amplification and form the majority of proliferating cells within a tissue, they would be expected to retain label over long periods. In addition, stem cells may retain label through the selective segregation of old and new DNA strands at division as has been shown in the intestinal epithelium and more recently in breast (Cairns 1975; Potten *et al*. 2002; Smith 2005).

Two groups reported attempts to define mammary gland stem cells in the mouse using injection of DNA label and a pulse chase experiment to characterize label-retaining cells (LRCs). The studies differed in the DNA label that was used, the developmental stage at which labelling was conducted and the length for which LRCs were followed. Not surprisingly perhaps, the studies produced different results. In the first study, tritiated thymidine (^{3}H-dT) label was injected into 10–12 week old mice and the LRCs were followed for up to 3 weeks (Zeps *et al*. 1996, 1998). At 2 weeks, the number of LRCs was small, comprising less than 0.1–1% of epithelial cells depending on the stage of oestrous at injection (Zeps *et al*. 1996). Interestingly, of the heavily labelled LRCs, 95% expressed oestrogen receptor-α (ERα), whereas the remaining 5% of LRCs were ERα-negative basal cells (Zeps *et al*. 1998). In the second study, the DNA label was bromodeoxyuridine (BrdU) given continuously over a 2-week period to mice between the ages of 3 and 5 weeks and the LRCs followed until the mice were 13 weeks old (Welm *et al*. 2002). The period of labelling in this study covers the beginning of puberty in the mouse, and it seems probable that more stem cell division might occur in early puberty than after puberty (Zeps *et al*.

1996, 1998). Two weeks following the labelling period in the second study, less than 30% of LRCs expressed progesterone receptor (PR), and at 9 weeks, less than 2% of LRCs were PR-positive (Welm *et al*. 2002). No relationship between the strength of DNA label in LRCs and steroid receptor was reported. Some of the LRCs at 9 weeks following labelling were shown to be undifferentiated in character since they lacked cytokeratins 14 and 18 that are indicative of myoepithelial and luminal epithelial cells, respectively (Welm *et al*. 2002). The data from these two studies can be interpreted as indicating that there are at least two populations of stem cells: a long lived, mainly quiescent, steroid receptor-negative stem cell that survives throughout the pubertal period, and a shorter lived, less quiescent, steroid receptor-positive stem cell that is more active during post-pubertal oestrous cycles.

We studied LRCs in the human breast using an athymic nude mouse model in which the breast tissue is implanted subcutaneously (Clarke *et al*. 2005). The fate of lobular epithelial cells dividing in response to oestrogen was tracked by administering the DNA label 1 week after the start of oestrogen treatment and observing the LRCs for 2 weeks in the continued presence of the hormone (2 mg oestradiol pellet). We assessed the frequency of staining of LRCs with antibodies to two putative stem cell markers, the CDKI $p21^{WAF1/CIP1}$ (Topley *et al*. 1999; Cheng *et al*. 2000) and a novel stem cell marker, the RNA-binding protein Musashi-1 (Msi1) (Sakakibara *et al*. 1996).

The CDKI $p21^{CIP1}$ has been proposed to maintain skin and haemopoietic stem cell quiescence since its deletion in $p21^{CIP1}$ null mice leads to depletion of stem cells in these tissues (Topley *et al*. 1999; Cheng *et al*. 2000). Immunohistochemical detection of $p21^{WAF1/CIP1}$-positive cells in normal breast lobules indicated that they were infrequent in lobular epithelium (~0.5%), but 12-fold more frequent in LRCs (Clarke *et al*. 2005).

Msi1 is the human homologue of drosophila Musashi protein (Nakamura *et al*. 1994; Okabe *et al*. 2001). It is a 362-amino acid RNA-binding protein involved in the Delta/Notch signalling pathway that operates during asymmetric cell division (Imai *et al*. 2001; Okabe *et al*. 2001; Okano *et al*. 2002). Msi1 is strongly expressed in murine neural and intestinal stem cells (Sakakibara *et al*. 1996; Clarke *et al*. 2003). It is infrequently observed in lobular epithelium and has a distinctive pattern of staining that is punctate and perinuclear in appearance. Msi1 staining is also infrequent in lobular cells (~0.5%), but 14-fold more frequent in LRCs (Clarke *et al*. 2005). Msi1 was expressed at a similar frequency to $p21^{WAF1/CIP1}$ in lobular epithelial cells and each included cells both intermediate and luminal in position although no cells expressing both these markers were observed, suggesting that they mark separate stem cell populations.

Relationship of putative stem cell markers to steroid receptor expression

The majority of steroid receptor-positive cells lies in an intermediate position in lobular epithelium and the majority of epithelial SP cells is likely to be intermediate since they express neither the basal marker CALLA nor the apical membrane marker MUC1. Steroid receptor-positive cells express CK19, a marker previously associated with a stem cell population (Gudjonsson *et al*. 2002b). We demonstrated in our studies that $p21^{WAF1/CIP1}$ and Msi1 expression was tightly associated with ERα/PR-positive cells in immunofluorescent colocalization studies (Clarke *et al*. 2005). Since Msi1 has been shown to be a positive regulator of Notch signalling through its interaction with Numb mRNA and repression of translation, it has been proposed that Msi1 regulates asymmetric cell division at the stem cell/transit cell boundary through Delta/Notch signalling (Imai *et al*. 2001). Delta/Notch signalling is an evolutionarily conserved pathway that regulates the stem cell/transit cell boundary in both invertebrate and mammalian tissues (Lowell *et al*. 2000; Imai *et al*. 2001; Okabe *et al*. 2001; Kopan 2002). At this boundary, an asymmetric stem/progenitor cell division specifies one daughter cell to replace the stem/progenitor cell and

the other to enter the transit-amplifying population that multiplies to produce differentiated lineages. This suggests that in cells in which steroid receptors and Msi1 are co-expressed, numb would be down-regulated leading to cleavage of the Notch cytoplasmic domain and its translocation to the nucleus where it is known to positively regulate CSL transcription factors (Kopan 2002). We therefore analysed the location of the Notch1 cytoplasmic domain that has been reported to be associated with Msi1 expression in neural stem cells (Kanemura *et al*. 2001).

We found Notch1 expression was confined mainly to the membrane of epithelial cells where it is inactive. However, in Msi-positive cells membrane staining was absent suggesting that Notch1 had been cleaved from the membrane and translocated to the nucleus (Clarke *et al*. 2005). This explanation is in agreement with studies on murine neural cells where Notch1 translocates to the nucleus only in Msi1-positive stem cells (Imai *et al*. 2001). These patterns of Msi1 and Notch1 expression suggest that when a breast stem cell divides one daughter cell expresses Msi1 and replaces the stem cell while in the other Msi1-negative daughter cell, Notch1 remains inactive and the cell undergoes transit amplification before differentiating.

CONCLUSIONS

We have employed several complementary approaches to investigate human breast epithelial stem cells and their relationship to both the proliferative population and steroid receptor expressing cells. Our results are consistent with the hypothesis that normal lobular human breast epithelium is hierarchical in organization, with a small number of scattered quiescent stem cells (detected by $p21^{WAF1/CIP1}$ and Msi1 staining). These cells may overlap with the SP of potential stem cells which we found to be mainly 'intermediate' in character since they lack markers of either the basal myoepithelial cells (detected by CALLA staining) or the luminal cells that have an apical membrane (detected by MUC1 staining). ERα/PR-positive cells are also intermediate in position, and express CK19, $p21^{WAF1/CIP1}$ and Msi1. Overall, these data suggest that the pool of potential stem cells includes ERα/PR-positive cells.

In the normal breast, the production of transit-amplifying cells and differentiated cell lineages from stem cells is likely to occur through asymmetric cell division where one daughter cell remains a stem cell and the other undergoes transit amplification before differentiating. In both invertebrate and mammalian tissues, asymmetric cell division is regulated by a conserved pathway involving Delta/Notch signalling between daughter cells at the stem cell/transit cell boundary (Lowell *et al*. 2000; Imai *et al*. 2001; Okabe *et al*. 2001; Kopan 2002). Our data suggest Delta/Notch communication between Msi1/steroid receptor-positive cells and adjacent proliferating cells, perhaps following asymmetric division of a stem cell.

Overall, our data suggest a model where a hierarchy of stem cells produce and regulate the transit-amplifying population that are destined to differentiate following a small number of cell divisions (Fig. 1). Our model predicts that during breast development, the more primitive stem cells are necessary for the clonal derivation of large areas of epithelium similar to the patches seen in X-chromosome inactivation studies (Tsai *et al*. 1996; Diallo *et al*. 2001). The scattered 'intermediate' CK19/Msi1/$p21^{WAF1/CIP1}$/ERα/PR-positive stem cells may therefore be necessary for generating differentiated cells within a smaller patch of lobular epithelium in response to the release of hormones during menstrual cycles. A model where there is a hierarchical organization of generally quiescent epithelial stem cells surrounded by proliferating cells and differentiated progeny arranged in patches is similar to that seen in the basal epidermis of the skin (Potten & Morris 1988; Mackenzie 1997; Kolodka *et al*. 1998; Jensen *et al*. 1999). We speculate that the

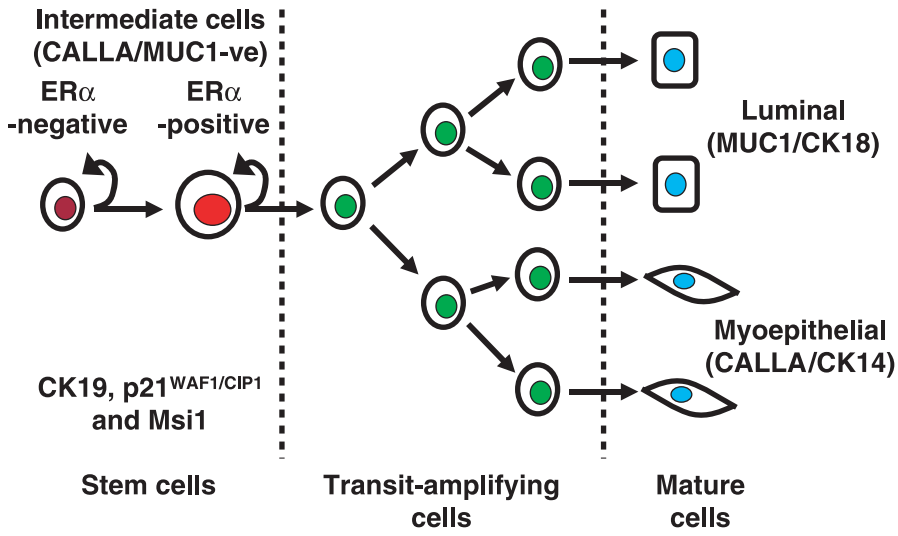

Figure 1. A model of the cellular hierarchies that may exist in the epithelium of the human breast lobule. Cells in an intermediate position include Hoechst dye-effluxing SP cells and some of these express steroid receptors, $p21^{CIP1}$, Msi1 and CK19. Msi1 and Delta/Notch signalling may regulate self-renewal of the stem cell and production of the transit-amplifying population through a process of asymmetric cell division. After a small number of cell divisions, transit-amplifying cells exit from the cell cycle and differentiate into myo- or luminal epithelial cell lineages characterized by the markers CALLA and CK14 (myoepithelial) or MUC1 and CK18 (luminal).

stem cells that persist in the breast epithelium during prolonged exposure to menstrual cycles uninterrupted by pregnancy would accumulate genetic changes leading to malignant transformation and this may explain the prevalence of CK19 and steroid receptor-positive breast tumours.

In summary, we have accumulated evidence to support a model where some steroid receptor-positive cells act as a stem cell type in the human mammary gland. Clonogenic assays and transplantation studies to confirm their function as long-lived stem cells remain more difficult than in the rodent. Application of SP isolation in combination with the markers that we have highlighted should provide the tools for future study of the stem cell type that resembles the phenotype of the majority of human breast tumours.

ACKNOWLEDGEMENTS

I am very grateful to colleagues for many useful discussions of this work, and apologise to any researchers whose relevant papers could not be cited due to lack of space. R.B.C. is funded by Cancer Research UK.

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