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Mammary stem and progenitor cells: Tumour precursors?

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ABSTRACT

Several groups have proposed that mammary epithelial cell (MEC) populations, in common with other epithelia, have stem and progenitor sub-populations that are long-lived and provide most of the growth potential during ductal (and perhaps lobuloalveolar) outgrowth. In this review, we describe what is known about normal development, particularly with respect to the growth potential and regenerative capacity of mouse MEC populations. We have developed a theoretical model in order to understand how the activity of the somatic stem/progenitor cell compartment during mammary gland development could affect the demographic of adult MEC populations. This demographic is likely to be key to understanding tumour risk, since long-lived cells provide great advantages in the process of cancer development.

1. Mammary stem and progenitor cells

1.1. *Definitions*

In contrast to the precursor cells that comprise early organogenic stages, the growth potential of cells in adult mammalian epithelia is unequally allocated across the population. Thus, in adult tissues, the majority of epithelial cells that comprise the internal organs are differentiated and have little growth potential. In several tissues, including the haematopoietic lineage, the gut and skin, different cellular compartments are recognised as part of a continuum, from those that are undifferentiated but have high growth potential, to those that are differentiated with low growth potential. These are typically labelled as stem/progenitor cells (undifferentiated cells with very high division potential, capable of the production of the entire lineage), transit amplifying (TA) cells (immature cells with relatively high division potential) and mature (MT) cells (fully differentiated cells with lower division potential). Since most mutational events rely on DNA replication and cell division for propagation, the ability to acquire multiple oncogenetic hits depends on the total division potential of the precursor cell and the number of cell divisions it encounters.[1,2] The majority of neoplastic risk is therefore likely to be focused in the minor component of adult epithelia that have high division potential and can be recruited into the cell cycle.

For the purposes of this article and for consistency with other literature, we will use the term 'stem cell activity' to denote cells with high growth potential as measured by assay in vivo. This is a property shared between stem and progenitor cells (and perhaps even TA cells, depending upon the assay conditions). The specific term 'stem cell' is reserved to describe an immortal cellular entity with molecular specialisations. For the purposes of our theoretical study, we specify that a stem cell can divide asymmetrically (to 'self-renew') or symmetrically (to increase the stem cell pool).

1.2. *Do mammary stem and progenitor cells exist?*

The stem cell activity of murine mammary glands has been functionally characterised by classic studies that used

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transplantation of limiting numbers of mammary epithelial cells (MECs) isolated from adult glands into new fat pads. Thus, small numbers of cells, challenged with de-epithelialised host tissue, will reconstitute a mammary ductal tree that is morphometrically indistinguishable from the normally formed gland and functionally competent.[3,4] Results of these assays suggest that there are cells with high growth potential in the mammary gland that are able to reconstitute the entire mammary tree, and there is some evidence that daughters of one (or just a few) progenitors predominate in the secondary ductal outgrowths.[5]

Explants can be serially transplanted, but the capacity for outgrowth declines after 4–6 transfers.[3,6] This decline suggests that 'immortal' stem cells are not responsible for the majority of growth potential observed in the mammary gland. Furthermore, the stem cell activity of mammary gland does not decline with age (of the mouse) or with parity,[3,6,7] implying that ductal stem cell activity is at equilibrium in adult mammary glands, and that these physiological processes create an insignificant challenge for the reserve of ductal growth potential in mammary gland.

Subpopulations have been purified from the majority of MECs based on their expression of functions or markers that are characteristic of stem and progenitor cells from other lineages. Welm and colleagues[8] were the first to use the precedent set by the study of haematopoietic stem cells to predict various characteristics of mammary progenitor cells. Thus, a Sca1-expressing undifferentiated population showed higher ductal reconstitution rates, and a side-population of cells (SP cells, characterised by their capacity to efflux Hoechst dye and their separation by flow cytometry; 2% total) were enriched in BrDu-retaining cells and did not express differentiated markers. These SP cells could reconstitute glands that contained both mammary lineages, and were enriched in telomerase expression.[9] Dontu and colleagues[10] found a subpopulation (0.4%) of cells that could, like neural stem cells, divide in suspension to form adhesion-independent cellular aggregates they called mammospheres. This clonogenic activity was long-lived, since primary mammospheres could be disaggregated and the resuspended cells would form new mammospheres. If single SP cells or mammosphere cells were seeded into three-dimensional collagen gels, they divided to form branching aggregates that contained from both mammary lineages, expressing either myoepithelial or luminal markers.[10–12] Our studies have shown that SP fractions can vary from 0.5% of total MECs in glands protected from Wnt-induced tumour development (*Sdc1*–/–), to over 10% in pre-neoplastic glands.[13]

More recently, functional studies using limiting dilution of whole cell populations into cleared fat pads have generated estimates for the frequency of stem cell activity in mouse MEC populations. For 2–3-month-old FVB mice, this frequency is 1/1400 cells (standard deviation (SD) 1/600–1/3000);[14] confirmed by our data, unpublished). Another study that tests the stem cell activity of MECs after flow cytometry (that excludes haematopoietic cells, Lin^-) suggests this frequency to be 1/4900.[15] These are likely to be conservative estimates, since the methods for cell preparation are relatively harsh, and any procedure that leaves cell–cell junctions intact enhances stem cell activity. For the purpose of this study, we will use an estimate of 1 stem cell/1000 total cells.

Cell fractions (double positive cells, DP) purified from the total population on the basis of their cell surface expression of high integrin levels (either α6 (CD49f) or β1 (CD29)), together with CD24, contain almost all the stem cell activity.[14,15] Some single cells from this fraction have enough stem cell activity to form a whole mammary gland. Only a small number of cells in these fractions have stem cell activity, and the activity of the purified fractions appears to be high (1/20 of 6% DP fractions, overall 1/333) when compared with the activity of the total population. However, we can use these results to drive estimates of frequencies of long-lived cell types in mammary gland. Notice that these DP cell subpopulations resemble myoepithelial cells, and are different from those isolated by dye exclusion, and different from fractions enriched in clonagen-forming units in culture. The relationship of these three fractions remains to be understood.

As yet, these data do not discriminate between stem and progenitor cells. At the simplest level, transplantation of a single undifferentiated cell to a fat pad assesses whether that cell can divide at least 20 times (to make a new ductal tree of 10^6 cells). Even without self-renewal, there will be sufficient division potential in that secondary tree to reconstitute another (using as few as 200 cells as an inoculum). To demonstrate conclusively that there are self-renewing, canonical mammary stem cells, single cells in secondary outgrowths of single cells should be capable of reconstitution.

A classic, but little understood, characteristic associated with stem cells of various lineages is their ability to retain DNA-associated molecules (measured for example, by incorporation of the substituted thymidine analogue BrdU into DNA or association with nucleosomal histone-2B) despite dilution by division. Thus in mammary gland, a population of label-retaining epithelial cells (LRECs) could be generated during juvenile ductal outgrowth, but not when adult ductal trees were labelled during oestrus.[16] One recent report suggests that there is a population of MECs able to divide asymmetrically to retain the 'immortal strand'.[1,17] Thus LRECs in mammary gland explants were shown to be transiently labelled with a second mitotic label (i.e. shown to be in cycle), suggesting that there are indeed canonical stem cells capable of conservative, asymmetric division.[18]

1.3. *Normal development of mammary gland*

The mammary gland develops as an extension of the skin (an ectodermal appendage) and develops from a thickened placode starting at embryonic day 11.5 (E11.5; reviewed by[19,20]). The invagination and extension of the mammary ductal tree is the result of a collaboration between the associated subdermal stroma, the fat pad precursors, and the epithelium.[19–22] There are extensive studies of the molecular and genetic regulation of this process that detail, for example, how male glands are induced to regress[23] and the importance of Wnt signalling to the expansion of the placode population.[24,25] At day 18, solid cords of epithelium approximately 15 cells thick undergo apoptosis of central cells to generate the lumen,[26] and the ducts extend as a bi- (and often thicker) layered tube into the fat pad. The outer layer comprises the myoepithelium (the basal layer), specialised to synthesise extracellular matrix, and to contract in response to oxytocin

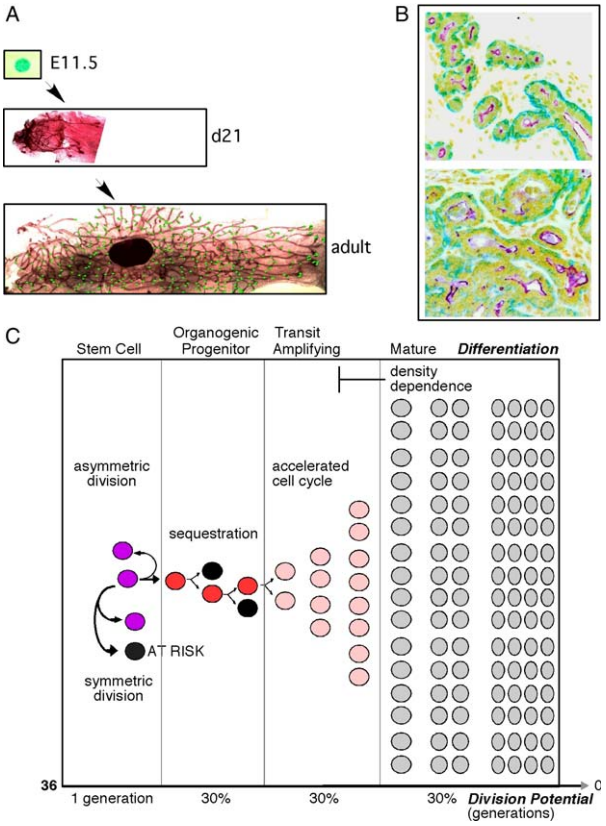

Fig. 1 – (A) Mammary development. Our theoretical model describes the expansion of the mammary rudiment, shown here as a cluster of cells invaginating from the ventral embryonic skin, and stained green-blue in this reporter strain to indicate active Wnt signalling (with thanks to John Wysolmerski[25]). The rudiment extends into a branching tree of bilayered ducts that colonises the fat pad from 21 d into adulthood (the tree is stained with carmine red and has expanded from left to right). Dilated end buds (far right-hand side) indicate ongoing growth, and the dark structure in the fat pad is a lymph node. Approximately 1000 artificial green dots have been applied to indicate the approximate frequency of cells with residual stem cell activity, distributed evenly throughout the ductal tree. We propose that these foci comprise clusters of stem/organogenic progenitors (OP)/ transit amplifying (TA) cells, surrounded by their mature daughters. (B) Mammary ducts comprise at least two cell types with a common ancestor, illustrated here by staining paraffin-embedded samples with antibodies to molecular markers. The mammary epithelium is (at least) bilayered, with a basal myoepithelial layer (specialised for contraction and stained here for expression of smooth muscle actin; green) and a luminal cell layer (specialised for secretion of milk; fuschia pink; counterstained in yellow for nuclei). (C) The main parameters that define the demographic equations. This illustrates the mammary lineage, characterised by cells that increase in age and differentiation as they divide. We assume that cells of the mammary lineage have a finite life-span (here 36 generations), that the lineage is divided into four parts, (i) canonical immortal stem cells, that divide asymmetrically to create a daughter progenitor cell (that enters the mortal lineage), together with a stem cell (classic self-renewal), (ii) organogenic progenitors (with high division potential; OP cells), (iii) TA cells (with mid-division potential; TA cells) and (iv) mature cells with low division potential (MT cells). The other parameters indicated are discussed in more depth in the text; briefly the OP compartment is regulated (by inactivation or sequestration) to promote organ differentiation, the TA compartment has a shorter cell cycle than OP and stem cells, and the ductal tree stops growing in response to secreted factors (density dependence).

during lactation (Fig. 1). Lumenal cells detach from their myoepithelial contacts during division, and multi-layered mammary epithelia are indicative of high division rates (CMA, unpublished). Morphogenesis and growth is focused to the terminal end buds of ducts; these are thick, multilayered structures with very high mitotic (and apoptotic) rates.[21,27] Developmentally, end buds are predicted to be the main site of stem/progenitor cell division and differentiation.[28]

Mammary rudiments form normally in mice in the absence of (either) oestrogen receptor (ERα or β), but ductal extension requires ERα (even though this occurs before puberty when oestrogen levels are low).[29–32] It is likely that the requirement for ERα during ductal development is mediated by stromal cells, since epithelial proliferation increases several-fold in response to ERα-positive stromal cells (measured by grafting of stroma-epithelial combinations under the kidney capsule.[33,34] However, the ductal lineage does not require oestrogen for survival, and is maintained after ovariectomy, despite expression of ERα by at least 25% of adult cells.[32]

In contrast, the lobuloalveolar epithelial lineage is stimulated to express progesterone receptor and to proliferate in response to the increased oestrogen levels associated with pregnancy. These cells are entirely dependent upon oestrogen for their growth and survival. Expression of ERα is heterogeneous, and cells are arranged into co-operative fields where the dividing cells are (almost) never Erα-positive.[35–37] It is likely that the effects of activated ER are mediated by progesterone and/or prolactin, since progesterone receptor knockout mice are entirely defective in lobuloalveolar development. These data suggest that the lobuloalveolar lineage is distinct and separate from the ductal lineage, and that the precursor cells have specific growth requirements. This leads some to propose that there are ER-positive and -negative (dependent and independent?) stem and progenitor cells.[38]

2. A model for stem-progenitor cell activity during mammary development

We devised a mathematical model to describe exponential expansion of MECs from progenitor and/or stem cells during development, to test the implications for tumour susceptibility and formation (Fig. 1). We used five criteria derived from classic observation of mammary gland and other epithelia, together with more modern molecular studies, to establish the basis for this model.

2.1. *There is a continuum of differentiated phenotypes for cells at different stages along the mammary lineage, and the lineage incorporates a finite life-span*

We assume that after the mammary organoid is specified from the ectoderm, the mammary precursor cells have equivalent, long life-spans (Fig. 1). Here we assume the lineage length is 36 generations (see below, 'Key factors that regulate stem/progenitor cell activity and number'). We also assume that the ordered acquisition of differentiated function is primarily dictated by generation number, and is largely irrespective of environment (this rationale is derived from the appearance of both mammary lineages in tumours arising from stem/progenitor precursor cells.[13]). We have not included any specific accommodation for the bifurcation of the mammary lineage tree, though independent regulation of each lineage could alter the final outcome of organogenesis.

2.2. *The size of the progenitor compartment was guided by experimental observation*

One of the goals of this model was to be able to monitor the size of the cell compartments (stem/progenitor, TA and MT cells) individually in order to have predictive value. Thus the first third of the lineage generations was designated as the stem/progenitor fraction (high division potential, 24–35 divisions; generations 1–12), the second third as the TA compartment (TA; generations 13–24; 12–23 divisions) and the last third as the MT compartment (MT; generations 25–36; 0–11 divisions).

In order to establish approximate boundaries for the progenitor fractions in adult MEC populations, we were guided by the estimates of stem/progenitor fractions established by studies in vivo and in vitro. Thus, if the lineage is divided into approximate thirds, the final progenitor cell (OP) fractions varies around 1%. Interestingly, this allocation made the demographics of the population mostly independent of the absolute length of the lineage.[39]

2.3. *Biological estimates of starting population (size of the mammary rudiment) and the population in the final adult ductal tree*

We assume that there is a developmental 'time zero' when precursor cells with equivalent division rates are specified and assume different roles in growth and maintenance. After time zero, the mode of division is regulated so that cells enter the ageing lineage.

This has precedent in several somatic lineages, including the gut and skin. For example, the epithelium in foetal mouse duodenum is stratified and equivalently proliferating at 14 d, but by 18 d is clearly patterned, thinning to a monolayer and becoming organised into fields of dividing cells in the crypts and differentiated cells in the villi.[40] These neonatal crypts mature into their adult clonal form as the lineage comes to equilibrium.[41] Gut stem cells depend upon active Wnt signalling for survival (as do haematopoietic stem cells) and deficiencies of Wnt signalling are manifest as loss of cellularity and cell division in the small intestine by embryonic day 16, leading to perinatal death soon after birth.[42–44]

In order to establish the adult branching ductal tree, the embryonic mammary placode grows to colonise the adult mammary fat pads (4–6 months old) with approximately 10^6 cells (depending upon strain and genetic background). The size of the rudiment at time zero was set to 1000 cells, based on a biological estimate,[25] since this timing coincides with an abrupt developmental switch in Wnt signalling.

2.4. *Ductal outgrowth is limited by density-dependent inhibition*

Unlike other exponentially growing lineages, most MECs are not terminally differentiated. That is, they are capable of dividing both in vivo and in vitro. We conclude that colonisation of the fat pad is actively limited by density dependent inhibition rather than by the accumulation of terminally differentiated cells. The final ductal density and the branching morphogenesis of the mammary tree is known to be regulated, so that the ducts of the normal gland never approach each other closer than 0.25 mm.[45,46] Diffusible factors are secreted by stromal cells during epithelial fat pad interaction,[46,47] and controlled also by epithelial cell properties in genetically modified glands.[48] To capture this density dependence in a mathematical model we altered the growth rate by a factor that is related to the total population.

2.5. *Cell cycle time accelerates through the lineage*

To design this model, we have extrapolated from the properties of other characterised lineages,[49] to propose that the cell cycle time of mammary progenitor cells is slower than TA and MT populations).

2.6. *Stem and progenitor cells*

The model was designed first to include only progenitor cells, and modified (in response to data[18]) to include stem cells.[50] Overall, the properties of these two models with respect to sensitive factors and growth potential are very similar.

3. The model fits the facts: predictable outcomes

3.1. *Outgrowth potential: a residue of organogenesis*

The difference between this model and one developed for continuously regenerating lineages, such as intestine, skin and haematopoietic lineages,[49–52] is that the mammary gland stem/progenitor population is not necessarily at equilibrium in the fully mature organ. Thus for continuously renewing tissues, there is exponential growth of TA cells and death of terminally differentiated cells. In comparison, the demands for regeneration in mammary gland are insignificant, and pregnancy- (and oestrus-) associated cell proliferation are easily accommodated (mathematically) by the expansion of cells from the TA compartment (that occur with a frequency approximately 1/100; potential lobuloalveolar progenitors), without impact upon the ductal progenitor or stem compartments.

When the colonisation of the fat pad is complete, we propose there is a large residual growth potential represented by

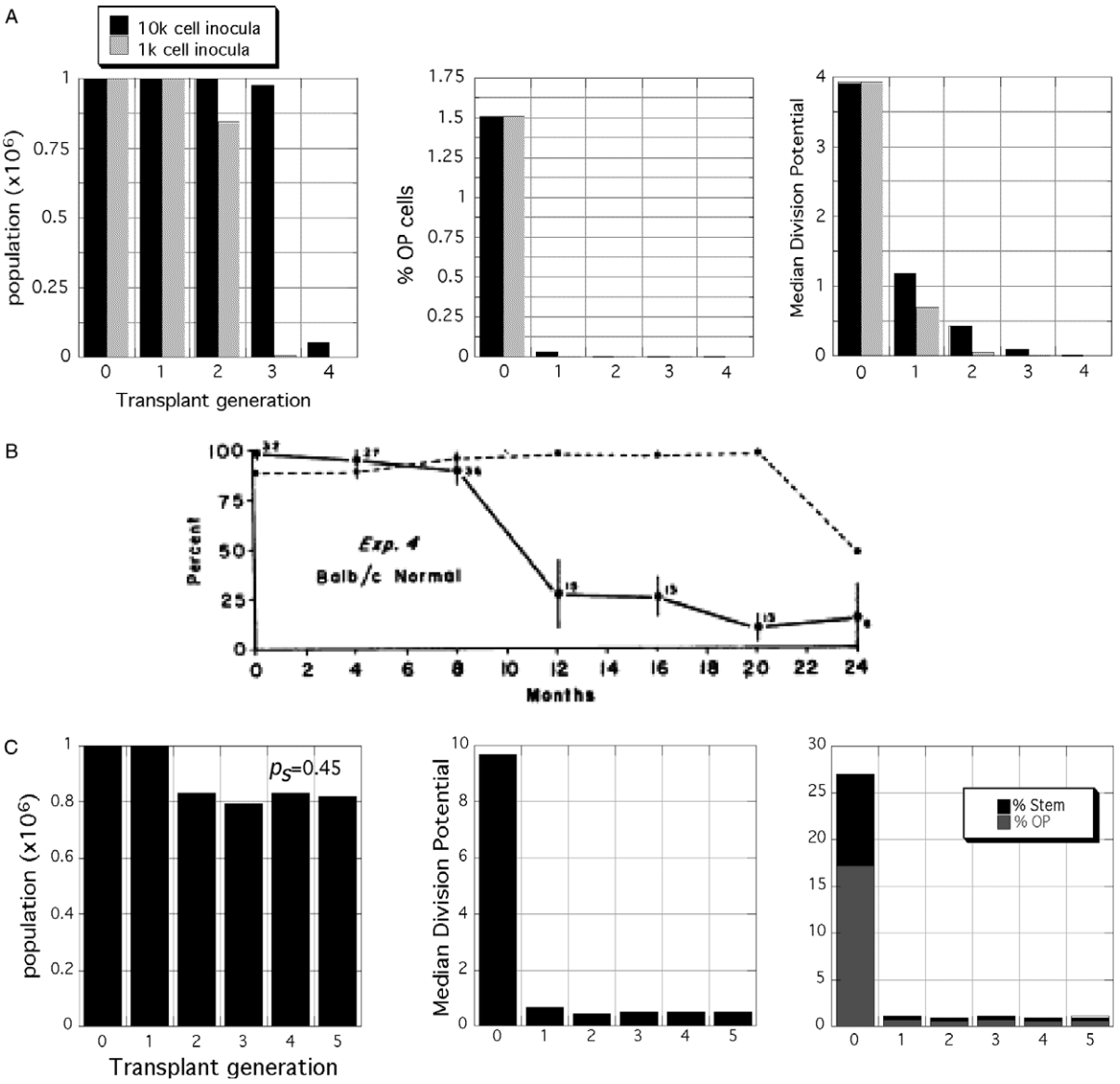

Fig. 2 – Simulated serial transplantation of mammary populations. (A) We have simulated ductal stem cell assays using fat pad transplantation of mammary tissue using the mathematical model, and (B) compared these data to those derived by experimentation. (A; left-hand side) The total population is plotted versus transplant generation, using two different sizes of cell aliquots, 1000 and 10,000 cells. This graph predicts the generation number at which there is insufficient outgrowth potential to form a new gland. (Middle panel) Average cellular age (median division potential, MDP) is plotted versus transplant generation, showing that the MDP drops from nearly 10 divisions (on average) in the first generation, to less than one in all subsequent generations (right-hand side). The stem/progenitor fraction is gone after the first transplantation generation. (B) Data reprinted from Daniel and colleagues[3] shows that for serial tissue transplants (estimated approximately 7000 cell equivalent), there was a steep decline in the percentage of glands colonised (defined as ⩾5-fold growth of the implant) after three generations. (C) When the probability of symmetric/total stem cell division was increased above 0.45, there was a dramatic effect on the serial transplantation behaviour. The population could be infinitely transplanted, the average age of the population was stable and resisted ageing, and the fraction of stem/progenitor cells was constant from generation to generation. Thus the population is immortal.

quiescent progenitors spread throughout the ductal tree. Thus if all the 1000 rudiment stem cells divide simultaneously, maturation of the population is seriously inhibited and most of the cells arrest in the undifferentiated TA compartment. To promote glandular differentiation, the number of stem cells active during development was lowered by consistently withdrawing them from cycle. More specifically, from the mathematical standpoint, we applied a probability of sequestration to each stem/progenitor division, a probability presumably governed by their molecular genetics (Fig. 1). This makes intuitive sense, allowing the developmental process to titrate the number of organogenic stem cells that are active during differentiation, and to reserve growth potential for regenerative purposes. This excess growth potential is

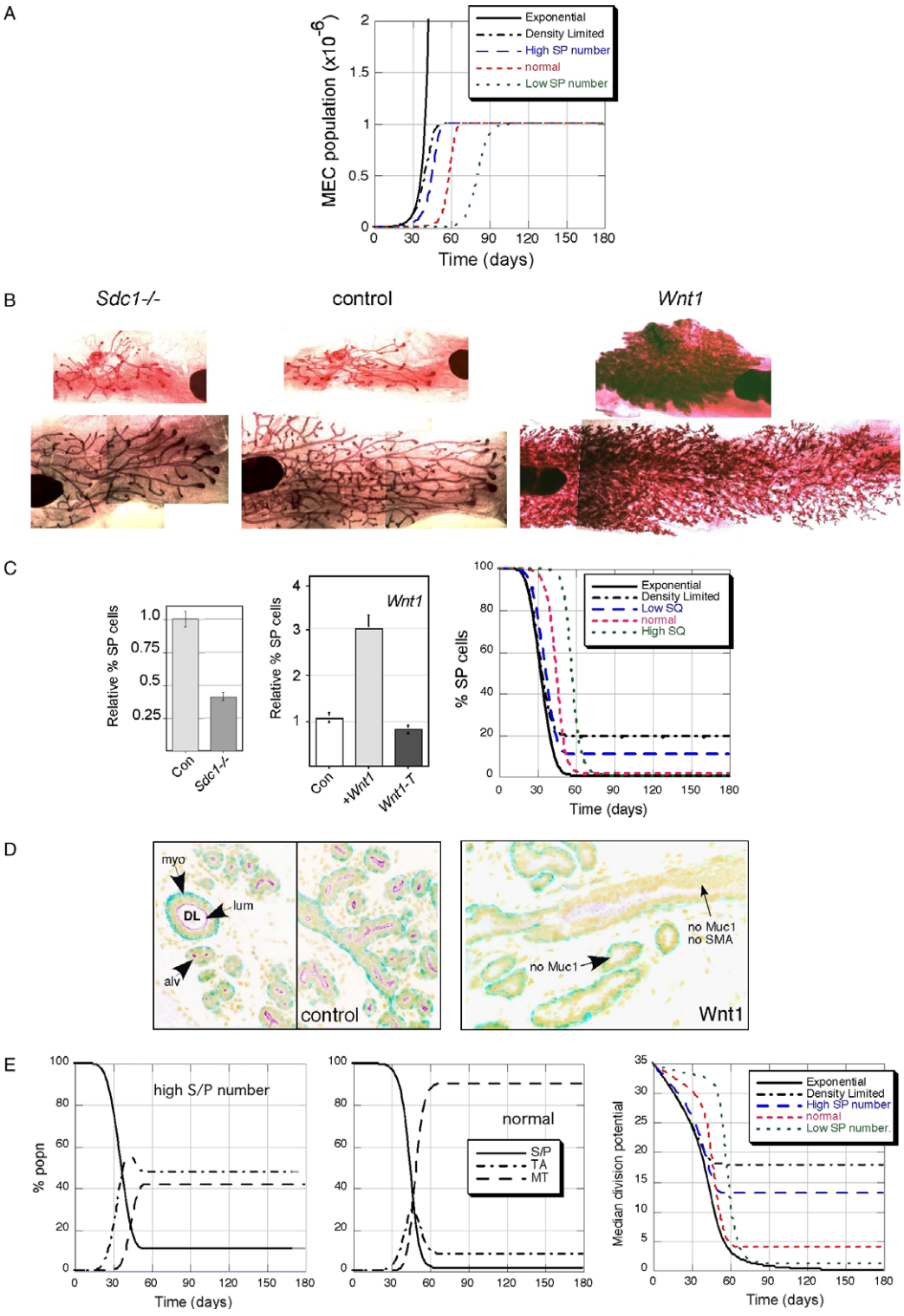

Fig. 3 – Biological data supporting our model. We propose that the stem/progenitor compartment of mammary glands from Sdc1–/– mice is hypomorphic, so that fewer stem/progenitor cells are dividing to make a ductal tree. In contrast, we propose that development of Wnt1-expressing glands is directed by more stem/progenitors than usual. If this is true, there are three behaviours we predict should be co-ordinately modified (as follows). (A,B) *Rate of population expansion*. Theoretically, the time required to achieve adult equilibrium colonisation depends upon the number of cells active per gland (modified here by sequestration/induced quiescence). (B) When whole mount preparations of mammary ductal trees were stained with carmine red, the Sdc1–/– glands were smaller at all time points (here 24 d), and the Wnt1-induced glands showed much more rapid population expansion. (C) *Adult stem/progenitor fractions*. Progenitor-enriched side populations (SP) were purified from adult mammary epithelial cell (MEC) populations using a flow cytometric assay for *Bcrp1* transporter activity.[13] These

typical of adult organs, tailored to meet the demands of morphogenesis and the response to injury. It is known that all parts of the mammary tree have equal outgrowth potential,[53] leading us to propose that sequestration distributes progenitors throughout the mammary tree during development (Fig. 1(A)).

This hypothesis has experimental support in other lineages where quiescent stem/progenitor cells have been demonstrated, notably in the continuously cycling hair follicles of skin (a continuously regenerating lineage). Thus, label-retaining cells have been described in the stem-cell-enriched bulge region of the hair follicle that are a substrate for tumour-initiating stimuli, such as myc and the tumour promoter TPA, and are mobilised in response to scratch-wounding.[54–56]

3.2. *A finite supply of growth potential*

We tested the theoretical outgrowth potential of aliquots of ductal populations (either 1000 or 10,000 cells) by inputting the cells from the primary outgrowth into a new developmental equation, in order to mimic classic ductal mammary stem cell assays. We assumed that when a new explant is started from a small cell inoculum, that the sequestered OP population from the mature adult is re-activated. In Fig. 2(A), we show the generation number when transplantation is expected to fail due to inadequate residual growth potential. Thus, for inocula of 1000 cells, explants senesce theoretically at outgrowth generation three, and for 10,000 cells (the approximate cell number in chopped organoid preparations) they senesce at generation five.

This theoretical outcome is a startling fit with the biological data from serial transfer experiments (an excerpt is reprinted in Fig. 2(B)).[3,7]

Since this outcome is correct, we predict that other reporters of the ageing process during serial transplantation will also be correct. The first is that the stem progenitor fraction should be almost ablated after just one explant generation. Thus, only the primary ductal tree will have a significant stem/progenitor fraction in normal glands (Fig. 2(A); middle). If there are 1000 or less canonical stem cells per gland, growth in secondary (and subsequent) ductal trees will be primarily directed by cells in the TA and MT compartments. As long as there is one (or a few) long-lived cells, outgrowth is likely be clonal (or pseudo-clonal), as has been reported previously, using lineage marking by retroviral tagging.[5] We predict that growth in subsequent generations is likely to be polyclonal.

The second is that the median division potential (MDP), an index of cellular ageing, should decline with transplantation (Fig. 2(A), right-hand side). Furthermore, altered stem/progenitor fractions should be associated with significant changes in the median division potential of the majority. Thus, for the comparisons detailed in,[39] a 10-fold increase in the stem/progenitor fraction (to approximately 12%) leads to an increase in the division potential of the whole cell population, predicting that 50% of the population will have 18 potential divisions instead of only three (Fig. 3(E), right-hand side).

3.3. *If the proportion of stem cell symmetric division is sufficient, mammary populations are immortal*

For our general model, we assume that stem cells almost always divide asymmetrically (undergo self-renewal), and enter the normal differentiation lineage as progenitors (see also the discussion of stem cells as tumour precursors). However, if the probability of symmetric division is increased, the fraction of stem cells increases. This has little effect on the serial transplantation behaviour of populations until the probability of symmetric division approaches 0.5 (i.e. the proportion of asymmetric and symmetric divisions is almost equal). At this threshold, the population becomes immortal, resisting ageing and maintains a constant stem/progenitor fraction, irrespective of transplant generation. Factors that regulate this probability would have this clear and predictable outcome for serial stem cell assays.

3.4. *Linked behaviours: predictors of molecular mechanism*

The data shown in Fig. 3 proposes that a hypoactive stem/progenitor compartment will be revealed in three linked phenotypes during mammary gland development: (i) a slow rate of population expansion during ductal outgrowth, (ii) hypermaturation of differentiated cells, and (iii) a lower fraction of stem/progenitor cells at equilibrium. On the other hand, glands with a hyperactive stem/progenitor compartment would show accelerated ductal outgrowth and a high proportion of stem/progenitor cells and TA cells in adult glands (TA = 50%; median division potential = 16.6 generations, compared with normal gland TA = 9%; MDP = 3.2 in 'normal'

fractions were depleted in Sdc1–/– glands (far left-hand side) and enriched in Wnt1 induced glands (middle), as predicted from the theory (far right-hand side). With a high probability of induced quiescence (sequestration; SQ) the percentage of stem/progenitor cells at equilibrium is low in adult glands. Vice versa, if most of the cells are kept in cycle, they remain at high fraction in the adult. (D,E) *Accumulation of undifferentiated cells in glands with high stem/progenitor activity*. Samples of Wnt1-induced (right-hand side) and control (mid and left-hand side) glands were analysed by immunocytochemical staining, as described in Fig. 1 (Muc1 antiserum stains luminal cells (pink) and smooth muscle actin (SMA) stains myoepithelial cells (green); counterstained with Hoechst (yellow). There are zones of the hyperplastic ducts that express no Muc1, and some that express neither Muc1 nor SMA, suggesting that they are not fully differentiated. (E) Accumulation of undifferentiated cells in glands where there are too many active stem cells is predicted from the theory (left-hand side, total percentage of the population occupied by each developmental compartment when the stem/progenitor number is high (more TA than MT cells); middle panel, same graph for the 'normal' activity; right-hand side. The average age of cells in glands that are formed with high stem/progenitor activity (blue) is compared with those with low activity (green). When the number of stem/progenitors is low, the demand on them is much greater, and the division potential of each daughter is almost exhausted.

glands). This would be expected to lead to lower maturation/differentiation of the population, evident in the proportion of cells expressing markers typical of the most differentiated luminal and myoepithelial cells.

We tested these predictions using two strains of genetically modified mice that we previously showed had different progenitor fractions in adult ductal trees. When a surrogate marker of stem/progenitor fractions was used to assay the first strain, with a mutation in the heparan sulphate proteoglycan, syndecan-1 (Sdc1), the adult ductal population showed a reduction of approximately 50% (measured by dye efflux, Fig. 3(C), left-hand side). Juvenile ductal trees from *Sdc1*–/– mice show fewer terminal endbuds, and population expansion was inhibited at day 24 (a reduction of 45% in terminal endbuds; Fig. 3(B)). The adult *Sdc1*–/– ductal trees colonise the full extent of the fat pad (though they are under-branched compared with controls). These behaviours are consistent with an increased rate of sequestration (due to, for example, slower stem/progenitor cell cycle time, increased apoptosis or inactivation of these cells or reduced mammary rudiment size; see below).

Another mouse strain tested here over-expresses Wnt1 under the control of the mouse mammary tumour virus (MMTV) promoter, and mammary glands from these mice have increased, pre-neoplasatic stem cell activity. In adult mammary trees, the stem/progenitor fractions were increased by (at least) three-fold (Fig. 3(C), middle). Fig. 3(B) (right-hand side) shows that population expansion during juvenile development is much more rapid than normal, and that in hyperplastic glands, the proportion of cells that express differentiated markers is lower than normal or tumour-derived populations (Fig. 3(D)). Thus, some ductal domains do not express the luminal marker, Muc-1, and some express neither Muc-1 nor the myoepithelial marker, smooth muscle actin (SMA). These domains show abnormal multilayered epithelia, and decreased cellular interactions (the myoepithelial cells resemble beads on a string). The theoretical outcome of developmental hyperactivity of ductal stem cells is shown in Fig. 3(E) (together with an estimate of average cellular ageing, Fig. 3(E), right-hand side). We conclude that these mice do indeed show the linked series of phenotypes we would predict if stem/progenitor cell activity was reduced or increased during development.

Note that we would also predict that the very high fractions of asymmetrically dividing label-retaining epithelial cells (LRECs) induced during explanation of cells in the presence of high ectopic oestradiol (2.1%) that were reported by Smith[18] should be associated with abnormal differentiation and glandular morphogenesis (or that there should be a high rate of apoptosis in the stem cell compartment to offset the accumulation).

3.5. *There should be two distinct populations of cells with growth potential in mammary populations*

At the molecular level, progenitors and stem cells are likely to be distinguishable, especially for their expression of molecular pathways that regulate asymmetric division. Interestingly, there do appear to be two different label-retaining cell types in human breast cells grown in rodent xenografts, one that is marked by elevated p21 expression (a cell cycle checkpoint mediator), and the other by the expression of Musashi (Msi-1), a developmentally important molecule that mediates asymmetric cell division and neural stem cell fates.[12] We would tentatively suggest that these markers are compatible with arrested progenitors and canonical stem cells respectively. Notice that these two populations will not necessarily be discriminated by stem cell assay in vivo.

3.6. *What are the key factors that regulate stem-progenitor cell activity and number?*

We can predict which factors are key regulators of stem and progenitor populations from our mathematical model. We find that many factors can be changed without dramatic effect on the output data.[39] On the other hand, other factors are exquisitely sensitive effectors.

Thus, the size of the rudiment at time zero is directly related to the stem/progenitor fraction in the final gland, and to the median division potential of the cell majority. Factors that regulate expansion of the rudiment and the timing of the time zero switch are therefore key. Factors that control sequestration of progenitors also regulate final progenitor fraction and ageing. If the efficiency of feedback inhibition of growth from the total cell majority is increased, the number of cells per gland decreases and the relative proportion of progenitor cells is increased. If the cell cycle time of the various compartments is changed with respect to one another, the demographic is considerably altered (and this is a factor with no supporting data). Other factors that would be powerful effectors of stem/progenitor scores are cell death (and renewal) in the stem/progenitor compartment and cell–cell interactions within regenerative compartments.

In contrast, the demographic of the mammary population is relatively insensitive to apoptosis of the MT cell compartment, to changes in the total cell number per gland (variable between strains) and to the number of generations in rudiment cells at time zero (i.e. the length of the lineage, an unknown factor).

Note that stem cell number and activity has been shown to be genetically determined for several lineages. Thus for various strains of inbred mice, the frequency of primitive haematopoietic stem cells was found to vary widely, and to correlate with mouse life-span. Genetic mapping has linked this phenotype to chromosomes 1 and 18 (the quantitative trait locus (QTL) on 18 is syntenic with human chromosome 5q).[57–59] The overall activity and frequency of stem and progenitor compartments does not impact overall blood counts, and even very low stem cell numbers are sufficient to provide blood cells for the life-span of the animal. This suggests that large variations in overall stem cell activity are entirely compatible with normal growth and development.

Notice also that when *BCL-2* (a factor that suppresses apoptosis) was over-expressed in mice, the haematopoietic stem cell count increased, together with their ability to engraft in vivo.[60] This shows that apoptosis is important at least to stem cell regulation in the haematopoietic lineage (a lineage whose differentiation and tumour development is highly regulated in general by programmed cell death).

Similarly, it was found that the number of clonogenic keratinocyte stem cells varied consistently with mouse strain, and was linked to a number of modifier loci that regulate the size of colony formation in vitro (chromosomes 1,4 6, 7, 8 and 9).[61] During tumour promotion of initiated skin, the frequency of colonies increased.[62] Furthermore, there was considerable overlap between the QTLs identified by these studies and those identified as regulators of the development of skin tumours in response to the DMBA-TPA two-step carcinogenesis protocol.[63]

Thus, though a direct link between stem cell frequency (or behaviour) and tumour development has not yet been drawn (see next section), it is very likely that the risk of neoplasia will be proportional to the number of cell divisions in the stem cell compartment, together with their mutational rate (proportional to exposure to mutagens, inherent rate of mutation, and perhaps also the number of mutations/pathways able to dysregulate growth in the tissue lineage). Factors influencing recruitment to cell cycle, apoptosis, stem cell-specific surveillance of genomic damage and the rate of self-renewal would all be expected to alter this risk, and to constitute a highly polymorphic genetic component.

3.7. *What is the significance of stem-progenitor cell kinetics for tumour development?*

There is increasing evidence that many human tumours contain subpopulations of cells with stem cell-like properties that drive and maintain tumour growth. Thus, stem cells (containing most of the tumourigenic growth potential) have been demonstrated in tumours from breast,[64] neural[65] and haematopoietic[66,67] lineages. However, it has not yet been shown that normal stem or progenitor cells are the antecedents of tumour stem cells.

If we assume that somatic stem and progenitor cells are potential tumour precursors, they are likely to be very different substrates for tumour development. Progenitors are mortal, but can create large numbers of daughter cells (under the correct growth conditions), and are sufficiently long-lived to accumulate more than one oncogenic mutation. Stem cells are immortal; this activity can complement growth-promoting mutations to create tumours with a single hit (or very few), vastly increasing their probability of being tumour precursors.

We propose that stem/progenitor cell dysregulation could promote tumour development by various means; (i) by changing the number of divisions of stem/progenitor cells (assuming that these fix and propagate mutagenic changes), or (ii) by altering the mode and regulation of stem/progenitor cell division to one that is more mutable (Fig. 4).

3.8. *Counting susceptible cell divisions*

If we assume that each stem/progenitor cell division is associated with an inherent risk of propagating potentially oncogenic mutations, the number of divisions in this cell fraction is key to assessing tumour susceptibility. Thus, signalling pathways that increase the number of cell divisions that occur in the stem or progenitor compartments[68,69] could increase tumour risk in parallel. Thus, using our model as a guide, if the rate of sequestration of progenitors is varied to achieve a 10-fold difference in stem/progenitor fraction at equilibrium, the total number of cell divisions in this compartment increases by a striking 100-fold. We propose that the range of stem/progenitor cell divisions we have observed in vivo could account for a 100-fold difference in risk of tumour development.

3.9. *What type of mutagenic changes would be predicted to induce tumour development?*

From our theory, there are at least two distinct classes of mutation that could induce a mammary tumour that derives from the stem/progenitor cell compartment.

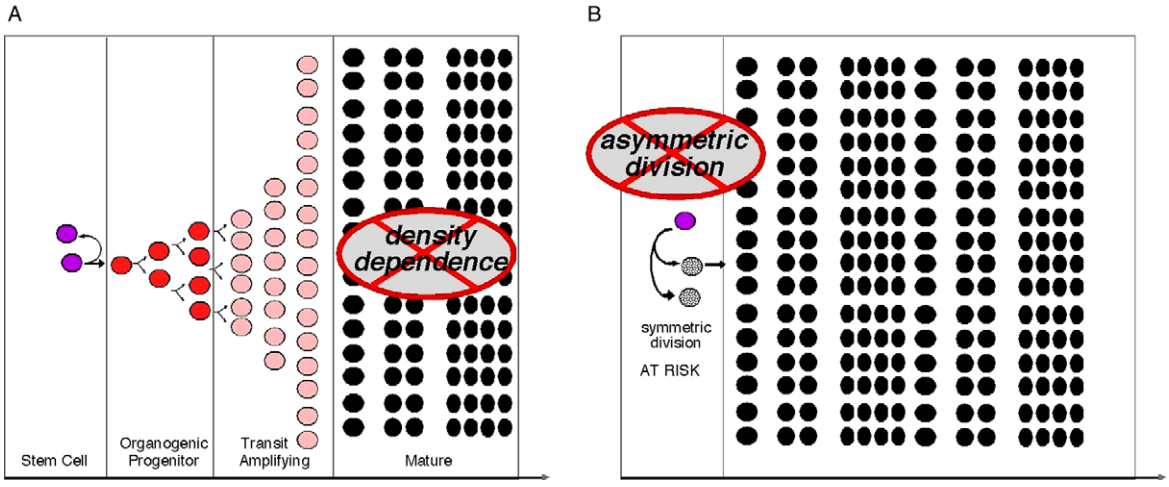

Fig. 4 – Models of tumour development. There are at least two types of mutations that could transform stem/progenitor cells into tumour precursor cells. (A) If density dependence is removed from the cell majority, the growth potential of the long-lived cells will be expressed. This growth potential is substantial and will allow the establishment of polyclonal adenomas that resemble the normal differentiated lineage (and benign until further mutated). (B) If the mechanism for maintaining asymmetric division is mutated, forcing stem cells to divide symmetrically, they will exit from the highly regulated mortal lineage in ways that are so far unknown.

The first type of mutation is one that disables density dependent inhibition of growth (Fig. 4(A)). Any cell drawn from generation 24 or above has enough division potential to grow into a benign mass that comprises more than 10^9 cells. This mass would likely obey most of the rules of this lineage, and comprise differentiated cells (as far as the dysregulated signalling pathway allows). During normal development, the division potential of these cells is actively suppressed. Our model predicts that cells with this property exist throughout the mammary tree at frequencies that depend upon the growth regulation prevailing during development. Adenomas induced by this route would be expected to comprise senescent cells and to express markers characteristic of ageing tissues.[70,71] If the mutation occurs in a stem cell this would create a truly potent tumour precursor cell. In this case, the stem cell is an immortal source of dysregulated daughter cells. However, given that stem cells are generally considered to have strict regulatory mechanisms that prevent mutational events, this mechanism may be unlikely.

The second type of mutation is one that targets the molecular components that regulate asymmetric division (Fig. 4(B)). Asymmetric division is considered to be a slow, self-renewing cell division process that is associated with the segregation of determinants of cell specification into two distinct daughter cells, including the specific retention of the immortal strand by the stem cell.[72,73] We have assumed that under normal growth conditions, the proportion of asymmetric divisions/total divisions for the stem cell compartment is high (greater than 90%). If the proportion of symmetric divisions increases to a threshold (50%), the frequency of stem cells increases concomitantly, so that the cell lineage becomes immortal and resists ageing during transplantation (Fig. 2(C)). Furthermore, nothing is known about stem cells that are created by symmetric division. If the argument above is extended to stem cells dividing symmetrically, both daughters cannot be genetically well-guarded, and the expanding populations may grow beyond the limits of their physical niche. The absence of appropriate daughter cell specification (epigenetic changes) is likely to inhibit the ageing and differentiation typical of the normal lineage. We predict then that tumours deriving from this type of mutation will (i) not show typical lineage-associated cellular compartmentation, (ii) will contain a large proportion of cells with stem-like properties, and (iii) that the tumour phenotype will be governed by the specifics of the collaborating growth-inducing mutation. Collaborating mutations are predicted to activate a growth-promoting pathway that is normally used during growth and differentiation of cells in this lineage (for mammary gland this is most likely epidermal growth factor (EGF), fibroblast growth factor (FGF) or insulin-like growth factor (IGF)).

Notice that these tumours are much harder to recognise for their stem cell origin. During the process of tumour development, the properties and phenotypes typical of stem cells are likely to be subverted. However, most human neural tumours that have been analysed comply with these predictions.[74] In this case, sub-populations of stem cell-like tumour cells (based on their cell surface expression of markers) were purified and shown to be able to recreate the tumour bulk (again by cell surface expression characteristics). In other words, these cells contained the tumour stem cell activity and could recreate a mini-lineage. These studies also confirm two other predictions from consideration of the regulation of a stem cell-based lineage; (i) tumours that originate in stem cells will contain cells with karyotypes that are not catastrophically rearranged (since the cells have not been selected to evade crisis), and (ii) tumours will have stem cell fractions higher than their normal tissue counterparts.

In conclusion, developmental modelling of the mammary lineage suggests various factors that could affect whether stem and progenitor cells are recruited to become tumour precursors. Thus, the total number of cell divisions that occur in this compartment (as opposed to more differentiated TA cells) will be related to risk. Mutational events that could keep these cells in cycle include those that enable daughters to evade growth inhibition. Tumours that arise by this mechanism are likely to be oestrogen-independent (ductal in origin) and of mixed lineage (for example the basal type of breast cancer described by transcriptional profiling.[75–78]

Conflict of interest statement

None declared.

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