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14. ABSTRACT
The main focus of this work is to study the effects of population demographics in the mammary gland on cancer risk using mathematical means and employ microtechnology for in vitro studies of primary cell characteristics. Specific attention has been paid to developing more quantitative methods for analyzing microfluidic cell cultures using In Channel Westerns. Also, understanding how the microfluidic culture platform differs from traditional macro-scale techniques is critical. By thoroughly understanding how this culture platform affects the cellular baseline first, better and more efficient data collection can be performed, thus requiring fewer primary cells. Preliminary results showing the effectiveness and ease of using In Channel Western protocols for studying protein expression in microfluidic cultures is promising.

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Introduction:

The main focus of this work is to study the effects of population demographics in the mammary gland on cancer risk using mathematical means and employ microtechnology for in vitro studies of primary cell characteristics. Specific attention has been paid to developing more quantitative methods for analyzing microfluidic cell cultures using In Channel Westerns. Also, understanding how the microfluidic culture platform differs from traditional macro-scale techniques is critical. By thoroughly understanding how this culture platform affects the cellular baseline first, better and more efficient data collection can be performed, thus requiring fewer primary cells. Preliminary results showing the effectiveness and ease of using In Channel Western protocols for studying protein expression in microfluidic cultures is promising.

Body:

Training Program:

All coursework for the PhD degree has been finished. Training for using microfluidics for culturing cell lines was performed during the first three months of the award due to it's start date being moved to June from September (instead of prior as stated in the Statement of Work). The use of immunocytochemical techniques was integrated into the microfluidic platform, including cell fixation, blocking and various staining protocols. The techniques were tested using a very well defined cellular reponse to a growth factor, discussed below. A poster was presented at the Biomedical Engineering Society Meeting in October 2006 on the topic (abstract is attached). Further analysis of the culture platform has since been performed, as described below.

I attended both the Biomedical Engineering Society annual conference in Chicago in October of 2006, and the Stem Cells and Cancer, Keystone Symposium in 2007. Both meetings were very useful for understanding how my work fits into both the engineering and biological communities. Insight into how best to approach the project was invaluable and led to the decision to focus more on the culture platform initially, and developing a robust method of quantitation.

I have continued to attend Cancer Biology Literature Groups, biweekly, along with relevant seminars on campus. In November 2007 I presented a paper for discussion at the Cancer Biology Literature Group. Also, I have attended both Dr. Alexander and Dr. Beebe's laboratory meetings, from which I have benefited greatly by working very cooperatively with the members of each lab.

Task 1:

The mice and reagents needed to perform the in vivo labeling of stem and progenitor cells that have become quiescent have not proved to be accurate and well defined enough to perform the experiments we proposed. The mice that had been described in the proposal would ideally expressed a GFP labeled Histone 2b conditionally while exposed to doxycycline. This would allow us to expose mice during early development to doxycycline as a pulse, then to later analyze the population for percentages of GFP-labeled cells after a chase period. These label retaining cells would have been quiescent during the chase period and that number could be compared with the mathematical model we've produced. While some cells of the glands in these mice do express GFP-Histone 2b, only ~30% of the cells are positive in virgins treated with a continuous pulse of doxycycline rather than 100% as would be expected. Due to the uneven expression of MMTV (mouse mammary tumor virus) in the virgin glands, the Tet transactivator that is under it suffers from heterogeneity within the glands as well. Thus, these mice are not ready to assay currently in an efficient and considerate manner, so work was focus on the other aims in the proposal.

However, a more thorough investigation of the math model was performed during the funding period. Another publication detailing the work was published in Cell Proliferation and is attached, in which all the details can be found. Briefly, this work provided a better understanding of how the different control factors in the model affect the final outcome of the population demographic was obtained. This will allow us to better be prepared for results when useful and defined mice strains become available. We identified that density dependence and cell cycle times will be critical parameters to thoroughly understand *in vivo* because the overall demographic is particularly sensitive to differences in them. Additional details on the variety of aspects of the model we tested can be found in the publication.

Tasks 2 and 3:

It was determined that further understanding of the use of microfluidic culture devices would be better performed first rather than last, as if this is not a suitable culture method, then another must be determined before undergoing the expensive pulse-chase experiments in Task 1. Cell lines have shown in our lab that there are differences in cell seeding and survival between the microfluidic cultures and traditional macro scale cultures[1]. I have begun to work to develop more robust microfluidic cultures, and to understand how dynamics of cell seeding, attachment and proliferation are different between the culture platforms.

In order to provide quantifiable readouts, I have begun work on characterizing a Western analysis protocol in microfluidic cultures. With this technique, I will then be able to quantify differences in cellular behavior in microfluidic culture in a variety of readouts such as activation of various stress pathways (phosphorylation of MAPK's, DNA damage, reactive oxygen species generation, etc), along with basic cell adhesion processes and proliferation. By understanding the status of cells in these cultures, I will then be able to improve culture conditions for primary cells in order to minimize any artifacts that may come from the culture conditions themselves. This will facilitate the generation of more accurate data from expensive and rare primary stem cells, thus requiring fewer cells and reduced reagents.

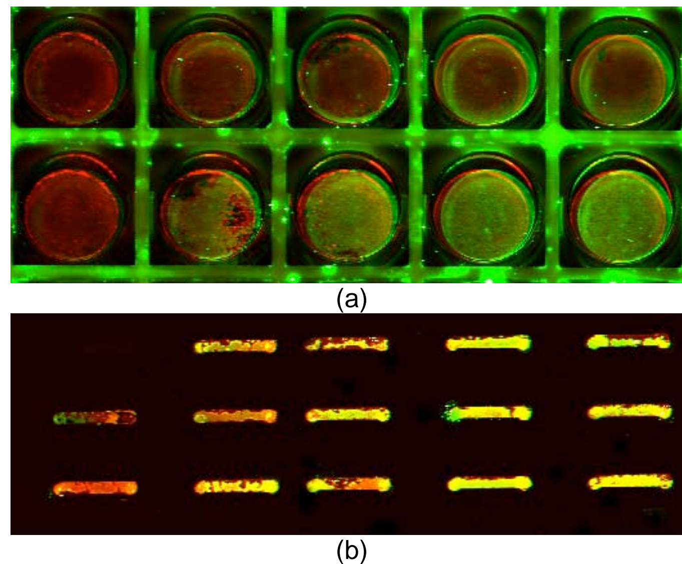
In Channel Westerns

The Western blot is a typical readout for macro scale cultures, but due to the requirement of very large total amounts of protein in order for the technique to provide accurate results, it cannot be used with current microfluidic techniques. Western blots currently require approximately 500 to 2,000 times the cell lysate that is typically present in microfluidic cultures. To date, very few methods have been used to provide quantitative data from microfluidic cultures. While immunocytochemistry techniques have been used, the readouts are qualitative (such as to prove that cells have differentiated, at best providing a percentage of cells positive for a marker), and are generally unable to analyze the entire population found in the cultures. Because microfluidic cultures require so few cells, a very useful characteristic of the technique is that all the cells of a specific culture population could be analyzed if the appropriate analysis techniques were available.

To improve our ability to derive quantitative readouts from the primary cell readouts we will be performing in the future, I have worked to adapt a new technique currently used for macro scale cultures into the microfluidic culture platform. The technique is the In Cell Western, in which immunofluorescence rated antibodies can be used *in situ* to stain for proteins of interest, and the entire culture area is then scanned using an infrared scanner (Licor). By integrating the intensity over the entire culture area, the total integrated intensity can be determined. By staining for nuclei, the fluorescence from the secondary antibodies can be normalized to the number of cells by comparing it to the fluorescence from the nuclear dye. This is similar to the loading control that would be typically found in a Western blot to control for total protein. The ratio of these fluorescence intensities can provide a relative measurement of the amount of a protein per-cell in various experimental conditions.

By performing this type of analysis *in situ*, we can measure the responses of cells to various treatments in micro scale cultures using Western techniques. To test the sensitivity of the In Channel Western (an In Cell Western only in a microfluidic channel), as compared to current macro scale results, I performed a side by side comparison of a macro and micro scale assay.

For the initial readouts, I tested the response of a normal murine epithelial cell line (NMuMG) to Transforming Growth Factor-b (TGF-b). It has been shown previously that this cell line undergoes epithelial to mesenchymal transition (EMT) when exposed to TGF-b *in vitro*[2]. The protocols for this transformation were repeated in the same way for both macro and micro scale cultures. Cells were seeded into macro and micro scale cultures at the same surface density, allowed to plate for 24 hours, then the media was changed to the experimental media with varying doses of TGF-b and 24 hours later the cells were fixed and stained. When these cells undergo EMT, they lose E-cadherin expression, in what is typically a dose-dependent manner.



(a)

(b)

Figure 1: Images obtained from scanning the macro (a) and micro (b) scale cultures for E-cadherin intensity (green) and nuclear stain (red). From left to right, columns in both macro and micro scale images correspond to: No Primary, Control, 1pM, 10pM, and 100pM TGF-b. Both cultures were seeded with the same surface density and exposed to TGF-b for 24 hours prior to fixation and staining.

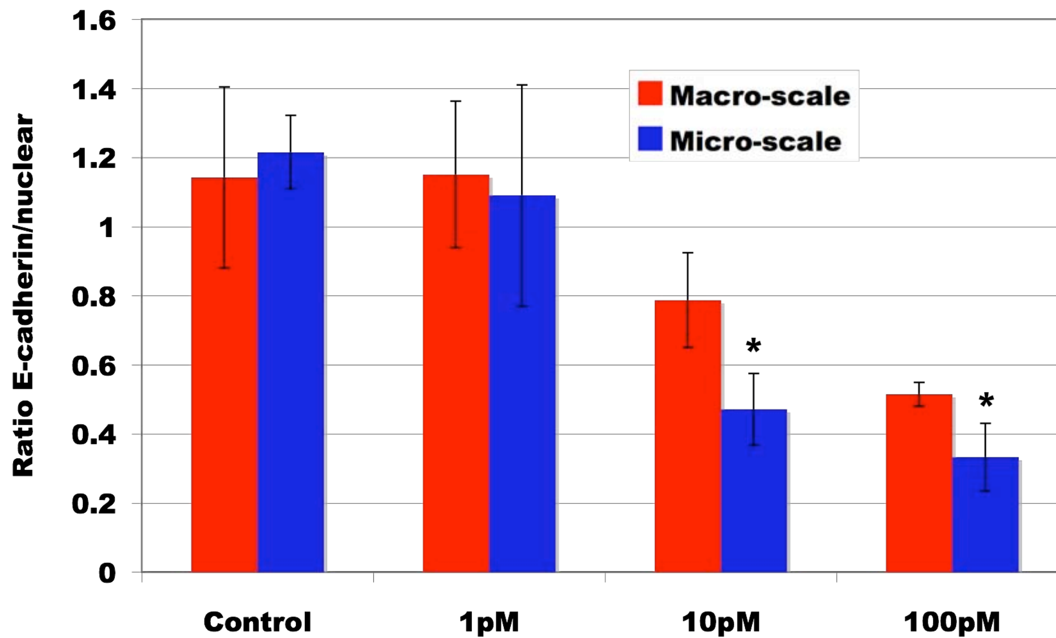


Figure 2: Plot of E-cadherin expression in NMuMGs after 24 hour exposure to TGF-b at various doses and in both macro and micro scale cultures. By taking the total integrated intensity of the E-cadherin images in Figure 1, normalizing them to the fluorescence of the nuclear stain in each channel they can be plotted for each condition. Error bars represent the standard error. Stars indicate statistically significant differences between the values and that of the control for the micro scale cultures ($p<0.002$).

The data indicate that the micro scale In Cell Western may prove to be more sensitive to changes in expression. No statistically significant ($p<0.10$) differences in fluorescence between the cultures dosed with TGF-b and controls were obtained in the macro scale cultures, due to large variations. The micro scale cultures, however showed very repeatable measurements and statistically significant differences in fluorescence between the 10 and 100pM doses and control ($p<0.001$). Further analysis of the sensitivity of the technique in the micro scale cultures will be performed for this and other assays of interest.

Key Research Accomplishments:

- Completed a detailed analysis of a mathematical model of stem cell regulation in the mouse mammary gland
- Completed training in microfluidic cell culture and other relevant lab techniques including image acquisition, image analysis and time lapse imaging system instruction
- Adapted In Cell Western protocol to microfluidic cell culture platform to provide a quantitative readout
- Begun using In Channel Western protocol for quantification of microfluidic cell culture conditions

Reportable Outcomes:

- A Publication in Cell Proliferation
- An Abstract and poster presented at Biomedical Engineering Society Meeting, 2006
- Significant training in Western blotting protocols and microfluidic assays
- Coursework for degree finished, and preliminary examination proposal written

Conclusion:

The mathematical model that was developed has now been thoroughly analyzed and its implications for assays of stem cells in vivo understood. As future analyses of primary cells from mouse breast cancer models will depend on a robust microfluidic culture platform and a method for quantitative analysis, work was focused on platform development. Without a thorough understanding of how this culture platform effects cell behavior, the results of assays done in the future may be affected by any artifacts from the culture technique. To eliminate this possibility, further examination of the cellular baseline using adapted Western techniques will be important. By using the In Cell Western technique and adapting it to use in microfluidic cultures, we have developed a very simple, accessible method for analyzing microfluidic assays. This technique provides the first, straightforward technique to produce quantitative data from an array of microfluidic culture channels.

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Appendices:
Cell Proliferation Paper
BMES Abstract

Cell Prolif. 2007, **40**, 106–124

Simulating mouse mammary gland development: cell ageing and its relation to stem and progenitor activity

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Abstract. *Background*: Somatic stem and progenitor cell division is likely to be an important determinant of tumor development. Each division is accompanied by a risk of fixing genetic mutations, and/or generating innately immortal cells that escape normal physiological controls. *Aim*: Using biological information, we aimed to devise a theoretical model for mammary gland development that described the effect of various stem/progenitor cells activities on the demographics of adult mammary epithelial cell populations. *Results*: We found that mammary ductal trees should develop in juvenile mice despite widely variant levels of activity in the progenitor compartment. Sequestration (inactivation) of progenitor cells dramatically affected the aging-maturation of the population without affecting the total regenerative capacity of the gland. Our results showed that if stem and progenitor cells can be demonstrated in glands regenerated by serial transplantation, they originated in a canonical primary stem cell (providing a functional definition of mammary stem cells). Finally, when the probability of symmetric division of stem cells increased above a threshold, the mammary epithelial population overall was immortal during serial transplantation. *Conclusions*: This model provides, (1) a theoretical framework for testing whether the phenotypes of genetically modified mice (many of which are breast cancer models) derive from changes of stem and progenitor activity, and (2) a means to evaluate the resolving power of functional assays of regenerative capacity in mammary epithelial cell populations.

INTRODUCTION

The ability to forecast how altered stem and progenitor cell function affects the development and maintenance of healthy tissue is now inportant to a variety of fields, from tissue engineering to oncology. Control mechanisms must exist that regulate somatic cell division, but they are still poorly understood. Mathematical models based on experimental data have been used to describe the controls of differentiation pathways that could exist for a variety of these systems (Deasy *et al.* 2002; Whitaker *et al.* 2003). For example, the haematopoietic lineage has been extensively modelled using various control mechanisms and differentiation pathways (Peng *et al.* 1996; Mahaffy *et al.* 1998; Zhang *et al.* 2001; Ostby *et al.* 2003). These models have

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incorporated examples of cell behaviour such as asymmetric cell division, stem cell self-renewal, programmed apoptosis and quiescence to demonstrate how the entire cell population is controlled both in number and in status of differentiation (Deasy *et al.* 2003).

Dysfunctional stem and progenitor cell control have been implicated in neoplasia in a variety of tissues including the colon and epidermis (Bjerknes 1996; Cairns 2002; Frank *et al.* 2003; Pelicci 2004). Yet, it has been difficult to determine how this dysregulation actually causes neoplasia. A number of theoretical models of these systems have been based on control mechanisms that are fundamentally different, despite the fact that they model the same lineages. In the colon, the epithelial cell lineage is characterized by their rapid cell turnover, and alterations of the stem cell compartment have been modelled either by increasing stem cell proliferation or by reducing stem cell senescence or apoptosis, both of which have been shown to have similar theoretical implications for neoplasia (Tomlinson & Bodmer 1995; Boman *et al.* 2001).

Mammary epithelial cell populations have been shown to be heterogeneous with respect to their division potential, and recently, evidence was presented to support the presence of true, self-renewing canonical mammary stem cells in mammary ductal trees (Smith 2005; Shackleton *et al.* 2006; Stingl *et al.* 2006). It has also been shown that tumour initiation in response to Wnt-controlled oncogenic stimuli is associated with dysregulated stem and progenitor cell numbers, and that the mammary tumour precursor cells are drawn from this stem/progenitor pool (Liu *et al.* 2004). It is therefore important to understand the consequences of stem and progenitor cell misregulation during development and pathogenesis. Stem cell activity is linked to growth and differentiation of progeny cells, and this complicated relationship is difficult to understand without a mathematical description of the outcomes of changes or variations in stem/progenitor activity.

Here we use a mathematical model to describe normal growth and differentiation of mammary glands, using variables that are known (as much as possible) from biological assay. We have analysed how mature population characteristics are altered by different developmental stem/progenitor activities. We find that although the stem cell activity can be varied over a wide range without affecting the success of organogenesis, different types of behaviour have profound consequences for the average age and differentiation of the cell majority. Biologically, this would be predicted to result in glands with very different growth properties (for example, senescent cells would show little response to growth factors such as EGF and IGF). Furthermore, if stem/progenitor/transit amplifying (TA) cells are indeed high-risk tumour precursor cells, the susceptibility of mammary gland cells to transformation will be determined, in part, by the number of these long-lived cells present. We propose that the adult stem cell fraction depends upon developmental processes during organogenesis.

BIOLOGICAL BASIS OF THE MATH MODELLING VARIABLES

Using what is known biologically as a basis, we can apply a mathematical perspective to identify the variables that most significantly affect the outcome of cell development. The model can then be used to narrow down the possible mechanisms of cell growth and regulation, by determining which mechanisms can produce glands with the experimentally established characteristics (size, rate of outgrowth or reconstitution ability, for example) and also produce predictions for trends in experimental results that could be used to distinguish one mechanism of regulation over another in a biological assay.

Here we have used some aspects of modelling equations that have been applied to studies of haematopoietic and colonic lineages. However, many characteristics helpful for applying

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quantitative mathematic variables used in models of other systems do not apply to the mammary gland. Principal amongst these is that, unlike the gut and haematopoietic lineages that renew themselves daily, the demand for cellular growth is low in solid epithelial organs such as the mammary gland. In fact, many of the regenerative requirements described in succeeding discussions for the mammary gland would be easily satisfied without the need for a self-renewing stem cell (for example, population expansion associated with pregnancy and the continuous cycling associated with oestrus).

Similarly, the colonic lineage has a stem cell niche that allows the geometry of the crypt to be part of the modelling scheme. This geometry has also allowed information about cell cycle times along the crypt to be directly measured and used to model population dynamics and the effects of dysregulation of the colonic stem cell (Boman *et al.* 2001). A similar niche has not been visualized for the mammary gland, and the observation of mammary stem cells lags far behind these other tissue lineages. Mammary stem cells are thought to exist amongst the long-lived, label-retaining epithelial cells (LRECs), observed throughout the mammary ductal tree (Zeps *et al.* 1996; Welm *et al.* 2002), alongside quiescent cells (both progenitors set aside and differentiated cells).

To assay the regenerative potential of single cells or populations of murine mammary epithelial cells (MECs), they can be transferred into 'cleared' fat pads, and tested for their ability to grow out into a ductal tree. There are some data that suggest that the outgrowths derived by transplantation are monoclonal (Kordon & Smith 1998), suggesting that they have a stem/progenitor cell precursor. When this assay had been applied to total populations of MECs, it was shown (albeit quite old studies) that stem cell activity was equivalent, regardless of where the gland sample was taken from, how old the mouse was, or whether it had been pregnant (approximately 4–6 transplants) (DeOme *et al.* 1959; Daniel *et al.* 1968; Daniel 1973). This implies that the long-lived cells are likely to be distributed evenly throughout the ductal tree, and to be at equilibrium in the adult gland (either because they are quiescent or are self-renewing). The frequency of stem/progenitor cells in total MEC populations (defined by their ability to reconstitute new ductal trees on fat pad assay) is estimated to be 1/1400 (Stingl *et al.* 2006).

Because the mammary gland does not regenerate on the scale of the haematopoietic and colonic lineages, the stem/progenitor compartment is not necessarily at equilibrium with the rest of the cells in the lineage in the adult gland. In other words, primary ductal outgrowth ceases when large enough to fill the available space, which is not dependent on achieving equilibrium between stem cell division and cell death. Unlike the terminally differentiated cells of skin or gut, most differentiated MECs retain the ability to divide; they are not terminally arrested. Mammary ductal growth is actively inhibited after 'full' colonization of the fat pad (so that ducts do not approach each other too closely). The ductal tree grows to fill the entire fat pad, but maintains a duct–duct spacing of no less than 0.25 mm (Faulkin & DeOme 1960; Neville *et al.* 1998). This even spacing implies that some local regulation mechanism is acting that prevents ducts from growing too close to each other (potentially a soluble or stroma-related factor). We have modelled the locally produced growth inhibitory factor with a factor that describes this density dependence. This limits the growth of individual ducts and also of the whole gland to the equilibrium size typically found *in vivo*.

The rudiment at time zero for this model was estimated to be approximately 500 cells (Chu *et al.* 2004), and these were assumed to be equivalent primitive mammary stem cells. Time zero, although not yet clearly defined in mechanistic terms, is marked by an abrupt change in Wnt signalling during development. The adult murine mammary gland size is assumed to be on the order of 1 million cells (the effect of varying this parameter is addressed in the 'Modelling Results and Analysis' section). Using a starting point of 500 primitive stem cells, we found that

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the growth potential of the stem/progenitor compartment had to be limited in some way to enable differentiation and maturation of the cells. In other words, if the initial 500 cells were allowed to divide simultaneously, the full size of the gland would be attained in approximately 10 divisions and the daughter cells would be relatively undifferentiated and difficult to organize into ducts. Thus, a factor was applied to the progenitor cell compartment that described the probability of entry into the cell cycle, and this phenomenon of stochastic quiescence was called 'sequestration'. Cells that were sequestered were permanently quiescent throughout development, but could be reactivated if transferred to a new fat pad.

We have incorporated these important biological considerations into the design of the complete lineage that we have modelled mathematically, as shown in Fig. 1. Finite lineage models have previously been used as a general model both for organ development and specifically for the haematopoietic lineage (Peng *et al.* 1996; Edelstein-Keshet *et al.* 2001; Whitaker & Portier 2002). Other relevant work includes using this type of compartment-based model to analyse the potential effects of therapy on cancer stem cells (for example, treatment of chronic myelogeneous leukemia with Imatinib (Huntly & Gilliland 2005; Michor *et al.* 2005)). We have constructed a lineage to include subpopulations relevant to the mammary gland. The two differentiated mammary cell types, luminal and myoepithelial, are probably derived from sublineages, but are combined in this model for the purpose of simplicity.

Design of equations

Cell maturation is assumed to progress from a stem cell compartment (stem), to an organogenic progenitor cell compartment (OP) through a TA compartment and finally a mature compartment (MT) (Fig. 1). These cell compartments each have specific characteristics, such as cell cycle time and mechanisms of regulation. Stem cells have the ability to divide asymmetrically (to generate one stem cell and a progenitor daughter) or to divide symmetrically (producing two stem cells, resulting in the expansion of the stem cell compartment). OP cells are long lived, have a relatively slow cell cycle progression time, and are subject to sequestration at a specific probability. The TA cells are more rapidly dividing cells that drive population growth and are not subject to regulation mechanisms present in the OP compartment. Finally, the mature stage consists of differentiated cells near the end of the lineage that have limited potential to divide, whose cell cycle is the fastest, and is thus most affected by density dependence. To describe the dynamics of each of the cell stages in the lineage mathematically, the stages were mapped to specific ranges of division potential (which is inversely related to generation number); the stem cell compartment corresponds to the highest division potential (generation number 1), then the OP stage corresponds to the first third of the lineage, the TA to the middle third and the MT to the last third. A justification of the choice of this particular mapping scheme is discussed in succeeding discussions.

To track cells through the proposed lineage of mouse mammary epithelial cells (estimated to be approximately 35 divisions long, effects of changing this parameter are addressed in succeeding discussions), the mathematical model was based on generational populations (rather than the total population). By tracking the cells through the entire lineage, the effects of changing time or other parameters upon the population demographic can be determined and matched to experimental data. For each cellular generation, g, specified probabilities exist for the cells to be sequestered ($p_s(g)$), to die ($p_d(g)$) or to divide, with the resulting daughter cells ageing to the next generation ($p_a(g) = 1 - p_s(g) - p_d(g)$). Sequestered cells are indefinitely quiescent during the process of ductal outgrowth. These cells are no longer actively dividing, although they are incorporated into the total population of the gland, affecting the rest of the lineage by contributing to density-dependent growth limitations. Although sequestered during ductal outgrowth, we assume

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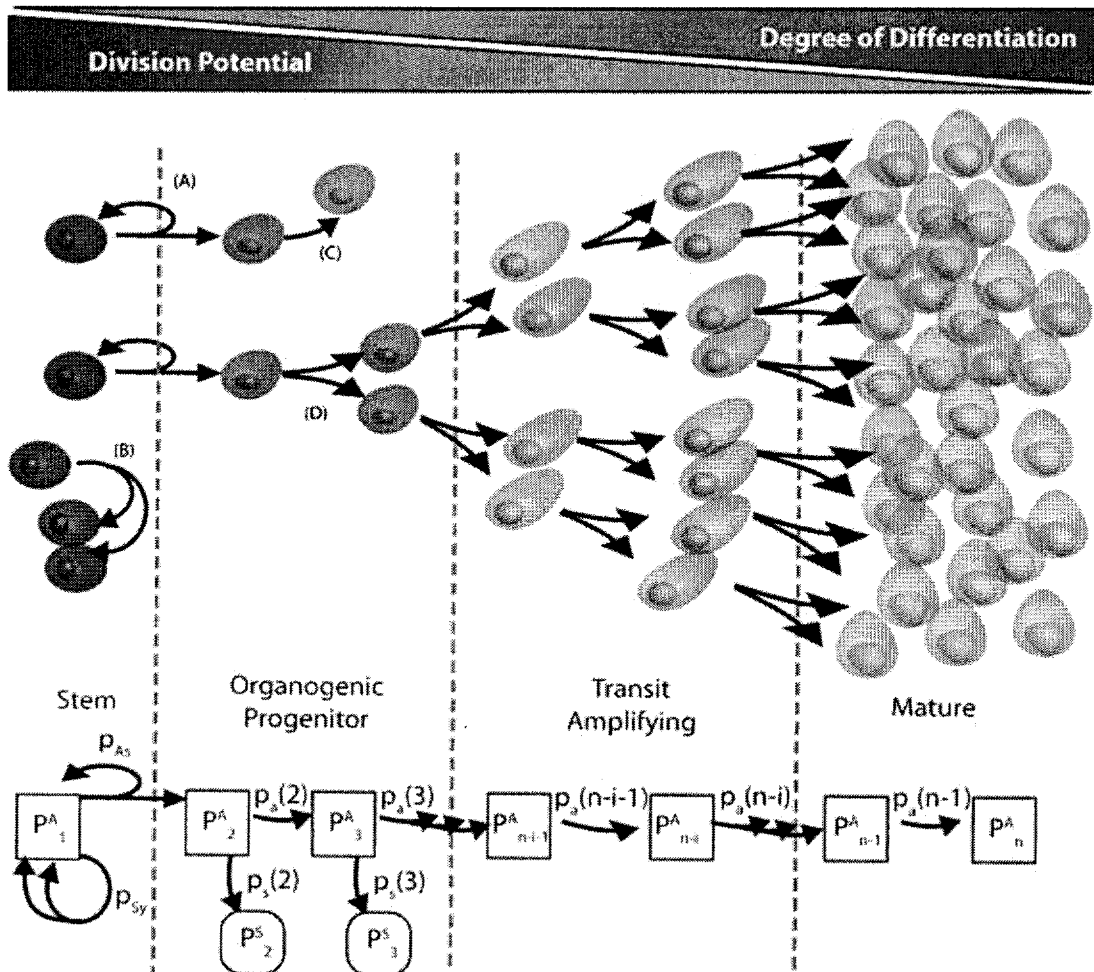

Figure 1. Summary of this theoretical model of mammary ductal development. In this model, the lineage is based upon four cell compartments, ranging from the least differentiated, but highest division potential stem cell compartment, to the most differentiated, lowest division potential mature cell compartment. Division potential is progressively lost as cells divide and differentiate along the lineage (defined by generations $1 - n$). Stem cells either asymmetrically renew themselves (division type A), producing one OP, and one stem cell or symmetrically renew (division type B), producing two stem cells. The frequency of these division types is governed by p_{As} (the probability of asymmetric division), and p_{Sy} (the probability of symmetric division), respectively, as shown in the lower schematic (that represents the mathematical translation of the cartoon). The OPs produced by asymmetric divisions of stem cells are subject to either sequestration (division type C), leaving one inactive progenitor, or division with ageing (division type D), which produces two daughter cells of lower division potential and higher level of differentiation. The balance between sequestration and ageing is governed by the probability of sequestration, p_s, whereas the probability of ageing is simply p_a, or $1 - p_s$. The sequestration rate ascribed to the progenitor compartment is not applied in subsequent cell compartments, namely the TA cells, then finally the mature cells.

that these progenitors can be reactivated to express their full growth potential when transplanted to a cleared fat pad. Stem cells have unique properties as well, described for this model as a probability of asymmetric division ($p_{As}(g)$) and of symmetric division ($p_{Sy}(g) = 1 - p_{As}(g)$). In general, we assume that the rate of asymmetric division is high compared to symmetric division

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(the effect of increasing symmetric division rate is discussed in succeeding discussions) and unless stated otherwise, was left at a probability of 100% asymmetric divisions.

These model parameters lead to the following differential equations. The population of active cells in each generation ($P_g(t)$) is given by Equation 1 for the first generation (stem cells) and by Equation 2, the more general case, for all successive generations where n is the total number of generations in the lineage. In addition, the number of sequestered cells in each generation's sequestered population, $P_g^S(t)$, is given by Equation 3 (although for this analysis, only the OP generations are assigned nonzero sequestering probabilities, and thus are the only generations with a nonzero sequestered population). Here, $\mu(g,t)$ is the growth rate (inversely related to the cell cycle time of cells in that generation) for generation g at time t, which is defined in Equation 4.

$$\frac{dP_1(t)}{dt} = -(p_a(1) + p_s(1) - p_{Sy}(1))\mu(1,t)P_1(t) \tag{1}$$

$$\frac{dP_g(t)}{dt} = 2(p_a(g-1))\mu(g-1,t)P_{g-1}(t) - (p_a(g) + p_s(g))\mu(g,t)P_g(t), \quad \text{For } g = 2, 3 \ldots n-1, n. \tag{2}$$

$$\frac{dP_g^S(t)}{dt} = p_s(g)\mu(g,t)P_g(t), \qquad \text{For } g = 1, 2 \ldots n-1, n. \tag{3}$$

$$\mu(g,t) = \left(1 - \frac{\sum_{g=1}^{n}(P_g(t) + P_g^S(t))}{P_{cap}}\right)\mu_{\max}(g) \tag{4}$$

It is anticipated that OP cells in the MEC system have a significantly slower growth rate than the mature cells, as differences in proliferation rates along lineages are known to occur and have even been modelled mathematically (Boman *et al.* 2001) (variations in this factor are analysed in succeeding discussions). The maximum cell cycle time ($\mu_{max}(g)$), was approximated for each compartment, to be 4.5 days, 3 days and 1.5 days, for the OP, TA and MT compartments, respectively. The actual values were tested in the model in order to obtain a fully developed gland in biologically relevant time periods (gland carrying capacity is obtained before 6 months *in vivo*), and also were of the same magnitude as cell cycle times for murine mammary gland cell lines *in vitro*, or approximately 2–3 days. The maximum growth rate is then modulated by the population density using the ratio of the total current population (the sum of each generation's active and sequestered populations, at each time point) to the carrying capacity of the gland (P_{cap}), as shown in Equation 4 (and is equal to 1 million cells in this model, except where noted). Thus, growth rate depends not only on generation but also density (assuming that the space available for the gland is fixed, density is proportional to total population). In order to estimate the cell cycle times of the stem cell compartment, we have related cell cycle time to the type of division that the cells are undergoing, therefore assuming that symmetric divisions could result in a cell cycle time of approximately 5 days, whereas asymmetric divisions could result in slower cell cycle times, or approximately 7.5 days.

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Table 1. Summary of the parameters used for the simulations

Parameter	High	Mid	Low	Zero
Probability of sequestration (p_s)	70%	40%	15%	0%
Final OP fraction	1.0%	2.3%	7.8%	13.3%
T50 (days)	119	70	53	45

Each level of sequestration results in an altered final fraction of OP cells in the glands as shown. The stem cell fraction remains the same (0.05%) for each model. Also, as sequestering levels increase, the rate of population expansion, as measured by the time required to reach 50% of the final equilibrium gland population, T50 tends to increase.

MODELLING RESULTS AND ANALYSIS

There are three principal ways in which stem/progenitor cell activity can be varied during organogenesis in this model: (1) the inactivation or sequestration of progenitors during organogenesis (2) altering the number of stem cells in the rudiment or (3) changing the proportion of symmetric divisions of the stem cells (increasing effective stem cell number during organogenesis). First, the effects of changing the probability of sequestration are considered with respect to population growth and adult gland demographics. We have used this model to analyse whether the effects of important variables that we have inferred or assumed, as a result of lack of existing data, are significant in order to understand the limitations of this type of model. The effects of changing the rudiment size on the overall level of differentiation of the resulting glands are then analysed and compared to those found for alterations in progenitor regulation. Finally, verification of the validity of this model *via* comparisons of what is predicted for serial transplantation assays is presented, and the effects of symmetric stem cell divisions are analysed in this context.

Stem and progenitor activity alterations *via* sequestration

Four rates of sequestration have been considered for all these analyses, none (0%; maximal activity of progenitor cells), low (15%), mid (40%) and high (70%; very few active progenitor cells). These values of p_s inversely correlate with the adult equilibrium OP fraction (Table 1). The day at which 50% of the limiting population is reached (*T50*) was analysed for each gland in order to compare the effects of sequestration on rate of outgrowth between the four models (Table 1). The normalized population growth, for each gland model, over a 6-month time period is shown in Fig. 2(a). As sequestration is reduced, more progenitors are actively cycling, resulting in faster population expansion of the glands, as would be expected.

By evaluating the stem, OP, TA and mature cell compartments, in each model over developmental time, it is clear that sequestration levels alter the relative contributions of each of the cell compartments to the overall growth of the gland. In Fig. 2(c)–(f), the proportion of cells in each compartment is shown for different levels of OP sequestration over time. All the populations come to equilibrium, but the distribution of differentiated and undifferentiated cells depends upon how many progenitor cells were dividing during development. Thus, with many cells in cycle (lower sequestration), the final proportion of mature cells is low, whereas that of transit cells are high (Fig. 2c,d), whereas when fewer progenitors are being used during development, the population is almost entirely mature with little to no residual TA population (Fig. 2e,f).

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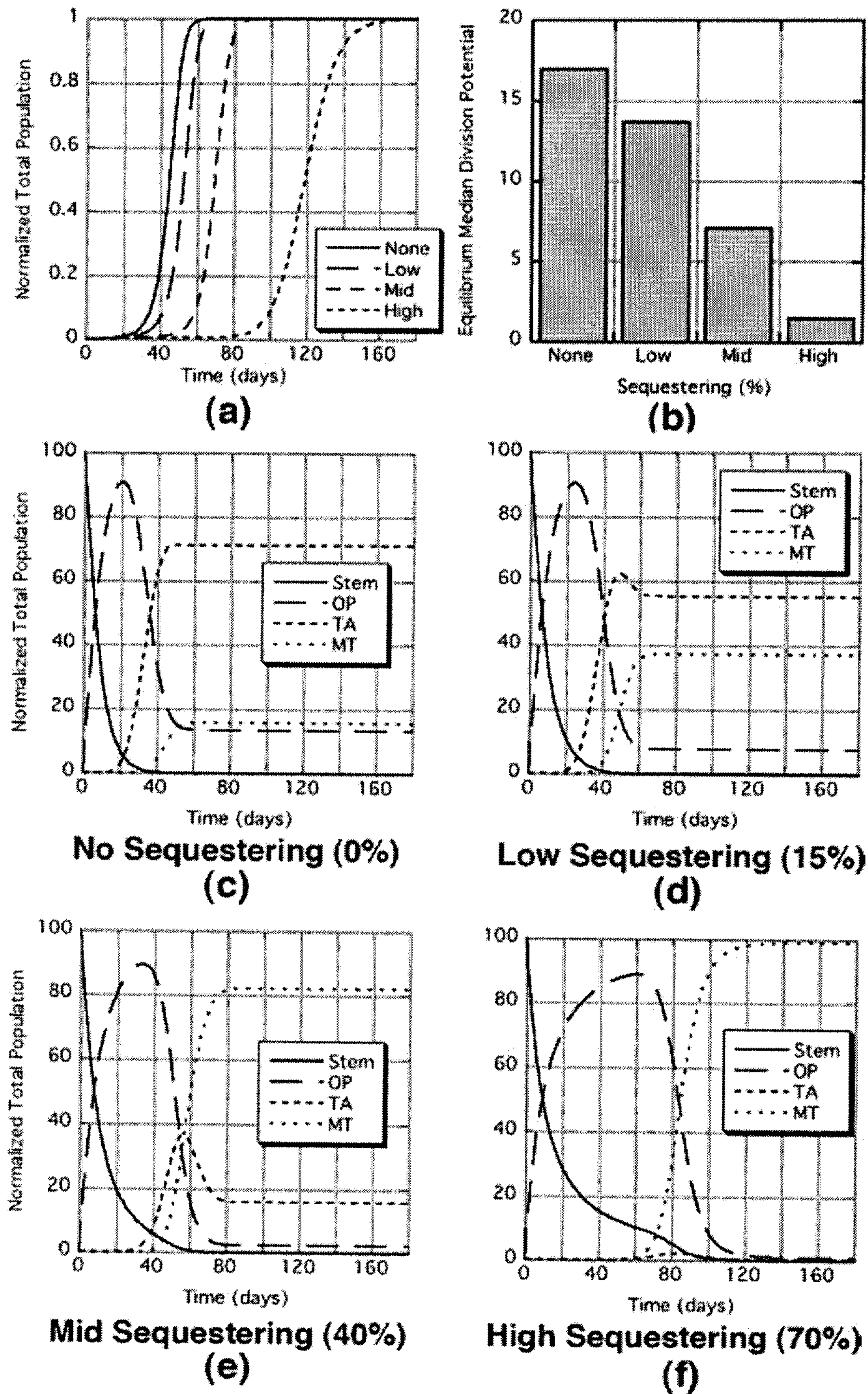

Figure 2. Regulation of progenitor activity leads to widely different rates of ductal development and average cellular age. The model was analysed with four different levels of sequestration of progenitor cells during development. For all levels of sequestration considered, we have shown the population growth over time (a), the final median division potential (b) and the fraction of the total population comprised by each cellular compartment over time (c–f). Cell compartments are labelled stem cell (stem), OP, TA or MT.

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In order to simplify the presentation of these changes in population demographics, we have devised a method for describing the degree of differentiation of a population using the median division potential (MDP), defined in Equation 5. Here, division potential, *DP(g)*, is the division potential of the cells in generation, *g*, and is given by Equation 6. The MDP is a weighted average of the population with respect to each cell's division potential.

$$\mathrm{MDP}(t) = \frac{\left(\sum_{g=1}^{n} DP(g)(P_g(t) + P_g^S(t))\right)}{\sum_{g=1}^{n} (P_g(t) + P_g^S(t))} \tag{5}$$

$$DP(g) = n - g \tag{6}$$

The final MDP values of each level of sequestration are shown in Fig. 2(b). As expected, there is an increase in the median division potential of the total population as sequestration is reduced, as a result of a decrease in the proportion of differentiated cells. Thus, the total growth potential of the embryonic rudiment is expressed differently when the number of progenitor cells in cycle is changed (all of these models began with the same number of stem cells with the same division potential). When many progenitor cells are in cycle, there are more cells of mid growth potential and low differentiation status; but when there are few, the population is highly polarized into a few very long-lived progenitors and a majority consisting of almost terminally differentiated mature cells.

Analysis of unknown model variables

We have used the mid-level sequestration model to determine the effects of changing the unknown parameters (that we have had to estimate) because the population demographic predicted by this model matches what characteristics are known biologically for the normal gland. The glands would be expected to be mostly differentiated, and contain relatively low levels of stem and progenitor cells as shown by the mid-level sequestration gland model). In succeeding discussions, we discuss the effects of lineage length, density dependence, cell cycle lengths along the lineage and apoptosis of mature cells upon the output data.

Lineage length

The length of the lineage, determined by the maximum division potential of the stem cells, is currently unknown for the MEC population. We have estimated that this maximum division potential is approximately 35 divisions using estimates from Hayflick (Hayflick 1965; Dimri 2005). We have found that if the relative fraction of progenitor cell generations is approximately a third of the total number of generations in the lineage, the resulting population demographic is not very sensitive to the absolute lineage length selected (shown in Table 2). Longer lineages than those considered do not produce MEC populations with properties that fit the biological data, whereas shorter lineages tend to not allow enough divisions to form glands that can differentiate or that can produce sufficient numbers of cells. Note that we assume that the mammary lineage is like other epithelial lineages, and that there is a succession of stem-OP-TA-MT cells, although there are little direct data to confirm this. We have presented the results of this one condition in order to focus our analysis on the implications of sequestration as a potential regulation mechanism, although a wide variety of lineage types could be imagined for this system.

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Table 2. Effects of the lineage length on the fractions of each cell compartment

Lineage length	Stem cell fraction (%)	Progenitor fraction (%)	Transit amplifying fraction (%)	Mature fraction (%)
24	0.05	1.2	5.6	93.2
30	0.05	1.6	10.0	88.4
36	0.05	2.3	15.7	82.0
42	0.05	3.1	22.5	74.4
48	0.05	4.1	30.1	65.8
54	0.05	5.3	37.8	56.8

Each version (with differing lineages lengths) has the same level of sequestration, and each cell compartment comprises one-third of the total lineage length. As the lineage grows longer, the average cellular age goes down, and all these simulated cell population demographics appear to be within the range of biological possibility.

Density dependence

The effects of changing the carrying capacity (or total cell number) on gland demographics is also important because the actual numbers of mammary epithelial cells in each type of gland is not known precisely and varies widely between mouse strains. To understand how density dependence can affect the final population, various carrying capacities (from 10 000 to 100×10^6) were applied (to a gland with a mid-level sequestration rate of 40%). Figure 3(a) shows that low carrying capacities inhibit differentiation of the gland (glands retain high MDPs), but when the carrying capacities reach 1 million or more, the MDP is not affected dramatically by changes in carrying capacity. The gland *in vivo* is likely to have at least 1 million cells (Kordon & Smith 1998), and potentially up to 100 million in some strains of mouse, and we predict that any of these values produce very similar population demographics. Thus, we consider that the choice of realistic gland carrying capacity is an insensitive parameter for the output data.

Cell Cycle

The model incorporates differences in cell cycle time over the entire MEC lineage as a decreasing function of cell compartment, so that mature cells have a lower maximum cell cycle time (higher growth rate) than progenitors. Although it is likely that growth rate changes over the lineage, exactly what the values are for each compartment are unknown. We have modelled three potential trends of cell cycle over the lineage to determine the effects of changing cell cycle time distributions along the lineage. Figure 3(b) shows the MDP over time under three conditions: (1) with an increasing growth rate as modelled previously (OP cell cycle time is longer than MT cell cycle time), (2) a constant, but average growth rate throughout the lineage (all compartments divide at the same rate) and (3) a decreasing growth rate (OP cell cycle time is shorter than MT cell cycle time). As the growth rate of the differentiated cells increases in relation to that of the progenitors, the populations tend to become more differentiated. We propose that cell cycle time distribution is a sensitive factor for this model and affects the predicted population demographics. For further analyses, we have used an increasing growth rate as this tends to produce a differentiated population, which would be expected biologically.

Apoptosis

Cell death through apoptosis was not specifically included in this model because it is not well characterized *in vivo* in such models. However, during gland development, apoptosis of differentiated

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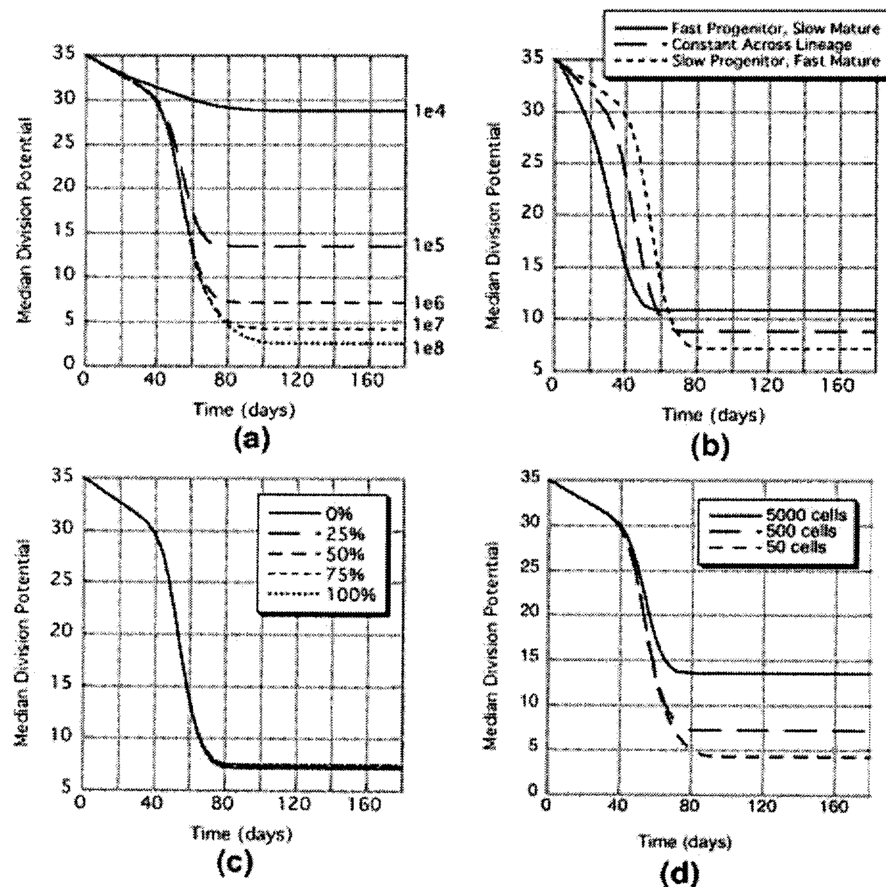

Figure 3. Effects of various parameters on the median division potential (MDP/age) of the mid-level sequestration gland model. (a) When the total cell number in the adult gland is increased (from 10^4 to 10^8, by reducing density dependence), the MDP decreases (cells are older). (b) Effects of changing cell cycle times with respect to development. Fast progenitor, slow mature, shows the result of a fast progenitor cell cycle and slow mature cell cycle, providing a decreasing growth rate along the lineage. Slow progenitor, fast mature is the opposite, with an increasing growth rate along the lineage. The slower the progenitor cell cycle time, the slower overall development is, and maturation of the majority is increased. (c) Apoptosis of mature cells (at varying probabilities from 0% to 100%) has almost no effect on cell MDP, regardless of the probability that the cells are undergoing apoptosis. (d) Rudiment size (from 50 to 5000 cells) determines adult cell MDP.

cells has been demonstrated, for example the cell death responsible for forming duct lumina. We have analysed the population demographics for a variety of rates of cell death (from 0% to 100%) from the last, most differentiated generation that would likely be responsible for the apoptosis seen *in vivo*. As shown in Fig. 3(c), MDP is insensitive to even high rates (100%) of apoptosis of these differentiated cells. The reserve of division potential in the MT and TA compartments easily accommodates loss of differentiated cells, without significantly affecting the overall population demographics during the time required for outgrowth to occur. Reserve of division potential in the MT and TA compartments may also be important to allow population expansion during growth phases (such as in oestrus and pregnancy), without drawing significantly upon the growth potential of the ductal stem/progenitor compartment. However, for the

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purposes of the analysis performed here, this factor is expected to have little to no effect on any predicted outcomes.

Rudiment size

The second major mechanism for altering activity levels of stem and progenitor cells in the developing glands is changing the rudiment size. Figure 3(d) shows the effects of altered rudiment size on the MDP of the population over time (using the mid-level sequestration model as a basis for this analysis). The total division potential (number of cells and their individual division potentials) of the rudiment plays an important role in determining the demographics of the outgrowth. Specifically, rudiment size reflects stem cell activity, and has a similar effect on the level of differentiation of the outgrowth, as modulating progenitor cell activity via sequestration. Both of these factors are potent regulators of final population differentiation, showing that both stem and progenitor cell activity regulations are important for the development of final gland cell demographics.

Serial transplantation simulations

We simulated biological serial transplantation experiments by taking 10 000 or 1000 cells (1% or 0.1% of the total population) from the equilibrium adult population in the primary ductal tree. We then used that as the input data for a new developmental model, and so on serially, until the outgrowth was unsuccessful (mature population size was not achieved). This paradigm is described in Fig. 4. The parameters that were applied to the serial transplants were the same as those for the primary outgrowth (in other words, levels of sequestration, cell cycle distributions, lineage characteristics, density dependence and rate of stem cell symmetric division, if applicable). Progenitor cells sequestered during each of the outgrowths were assumed to be reactivated and to enter the cell cycle again when transplanted.

The results for models with low-, mid- and high-level sequestration rates, together with results of modelling a gland with increased rates of symmetric stem cell renewal (equal to a probability of 40% per cell division) and a model lacking stem cells, are shown in Fig. 5. The level of symmetric stem cell renewal of 40% represents the minimum threshold at which glands will continue to reconstitute new fat pads upon transplantation of 1000 cells indefinitely (without significant deviations from the original population size in subsequent outgrowths). The example that shows the effects of having no stem cell compartment assumes that the 500 cells in the rudiment are not capable of asymmetric or symmetric self-renewal, and therefore behave as progenitors. The progenitor cell behaviour is kept constant (sequestration rate of 40%) for both the zero stem cell and for the symmetrically increasing stem cell examples (to illustrate the effects of varying the stem cell compartment behaviour alone). The output data shown includes the number of serial transplantations possible for each demographic, together with the average age of the cell populations after each transplantation, and the fraction of stem and progenitor cells found in the outgrowths. Table 3 shows the detailed quantitative trends for the stem cell fractions expected in each transplant generation for the same models analysed in Fig. 5 (except for the 'no stem cell' model).

These data illustrate the mortality of the cell populations during serial transplantation (that is, the populations cannot grow to the typical gland size of 1 million cells after only a few generations, depending on the level of sequestration and inoculum size, Fig. 5a,d,g,m). Panels 5b, e, h and n illustrate the trend to increased cellular age with each transplantation, and panels 5c, f, i and o show that the primitive stem/progenitor fraction is depleted during outgrowth of secondary transplants. Figure 6 shows the effects of developmental stem cell activity on the adult stem and progenitor fractions during serial transplantation (using a mid-level sequestration

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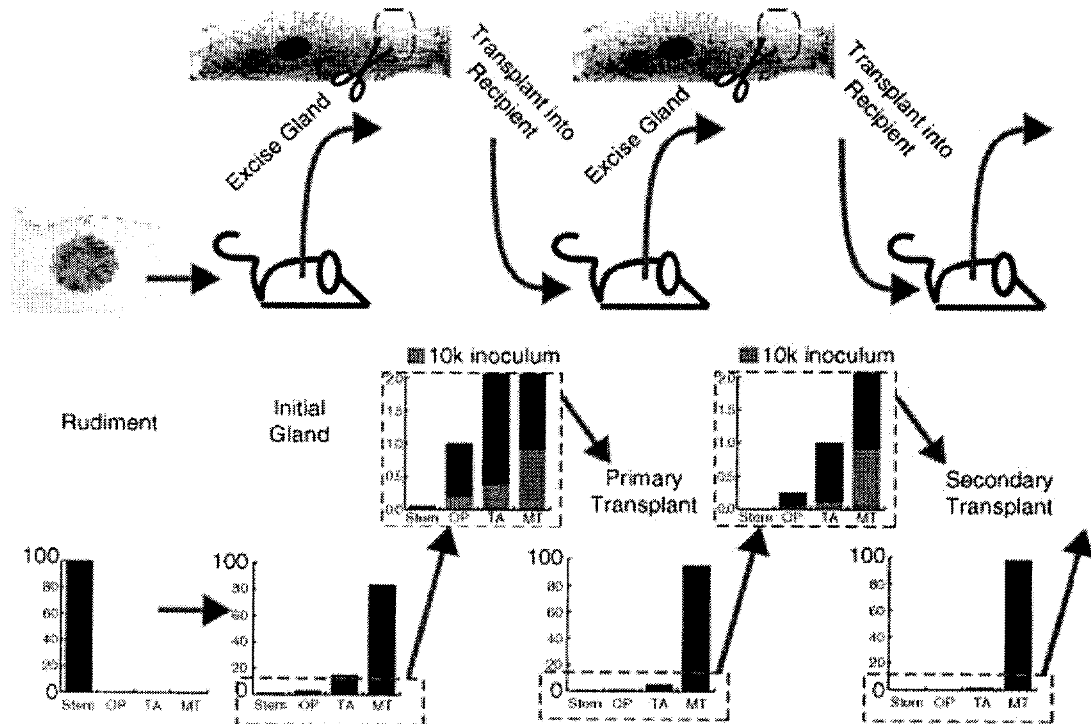

Figure 4. Scheme for simulating serial reconstitution. In order to simulate serial reconstitution assays using this mathematical model, we used the following protocol. Initially, the rudiment (100% stem cells, as shown in the demographic graph at the bottom left of the figure) is allowed to expand and develop the normal cell demographic found in the initial gland. For serial reconstitution, primary ducts are typically excised, and a random group of cells is removed, of either 1000 or 10 000 cells, which is then transplanted into the cleared fat pad of a host. The cells chosen for the transplantation are assumed to be randomly distributed along the lineage, so the demographic of the transplant inoculum is simply a fraction of that found in the source gland (as shown by the red bars for a 10 000 cell inoculum). The primary transplant was allowed to develop using the same parameters as for the rudiment, and the transplantation process was simulated repeatedly, using the same protocol each time. In the graphs shown, labels refer to the stem cells (stem), OP, TA and mature cells (MT).

rate). Specifically, regardless of the presence of an asymmetrically renewing stem cell, the behaviour of the stem and progenitor fractions during transplantation follows a similar decreasing trend. In order to maintain these populations during transplantation, symmetric renewal is required to expand the population faster than it is being diluted out during the selection for the transplant inoculum. When the probability of symmetric division is increased to 40%, the population of MECs in serial transplants comes to equilibrium. It can be endlessly transplanted (j), comes to equilibrium with respect to age (k), maintains a low but steady stem cell population (l) and (illustrated in Table 3), the progenitor population plateaus as well (Fig. 6).

DISCUSSION

We have constructed a theoretical description of ageing and differentiation of cells in the mammary gland during organogenesis. The model was designed to describe changes in the overall

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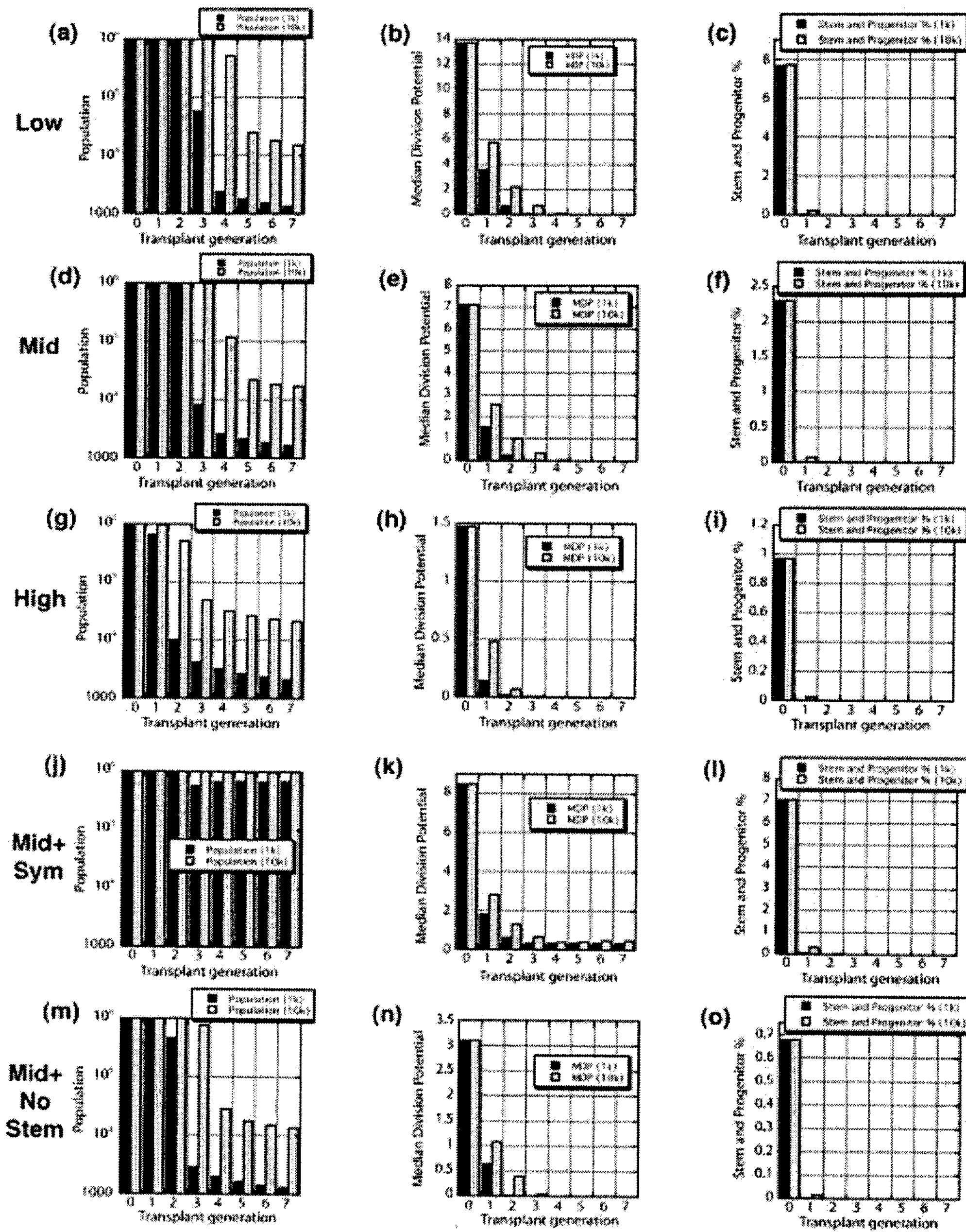

Figure 5. Serial reconstitution and cell ageing. Serially reconstituted gland populations, median division potentials and stem and progenitor fractions are presented for several models. Low- (a–c), mid- (d–f) and high- (g–i) level sequestering models show exhaustion of the regenerative capacity of the glands after a limited number of serial transplantations, along with large reductions in MDP and stem and progenitor fractions during transplantation. A mid-level sequestration model with a stem cell compartment capable of symmetric self-renewal (and therefore capable of the expansion of the stem cell compartment) at a probability of 40% is then shown (mid + sym, j–l). This model shows no depletion of the regenerative capacity of the gland, although the stem/progenitor cell fractions are low (but steady) in successive transplants, and the MDP comes to equilibrium (close to terminal differentiation). Finally, a mid-level sequestration model in which no stem cell compartment exists is shown for comparison (mid + no symm, m–o). This model behaves similarly to those containing only asymmetrically renewing stem cell compartments but exhibits slightly faster kinetics. Notice that a stem cell compartment is not required to generate an adequate ductal tree.

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Table 3. Stem cell fractions in each gland model during serial transplantation assays

Transplant generation	Stem cell fraction % (low sequestration)	Stem cell fraction % (mid sequestration)	Stem cell fraction % (high sequestration)	Stem cell fraction % (mid sequestration with symmetrical renewal)
0	5×10^{-2}	5×10^{-2}	5×10^{-2}	1×10^{1}
1	5×10^{-4}	5×10^{-4}	5×10^{-4}	3×10^{-2}
2	5×10^{-6}	5×10^{-6}	1×10^{-5}	2×10^{-3}
3	5×10^{-8}	5×10^{-8}	2×10^{-6}	2×10^{-4}
4	1×10^{-9}	4×10^{-9}	6×10^{-7}	2×10^{-4}
5	4×10^{-10}	2×10^{-9}	2×10^{-7}	3×10^{-4}
6	2×10^{-10}	1×10^{-9}	1×10^{-7}	3×10^{-4}
7	1×10^{-10}	6×10^{-10}	5×10^{-8}	3×10^{-4}

Stem cell fractions decrease by orders of magnitude with transplantation in all models, but as sequestration is increased, the population resists this depletion better. With 40% symmetrical renewal of the stem cell compartment, this compartment comes into equilibrium at 1/5000, regardless of transplant generation.

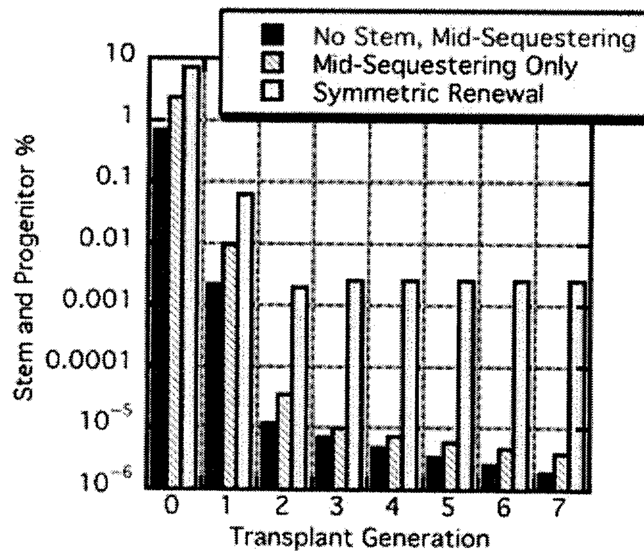

Figure 6. Detailed comparison of stem cell activity during serial transplantation for three contrasting models. The stem and progenitor fractions for each transplant generation (0, primary duct, 1–7 transplant generations generated by surgery) are shown for each of the three models, one without a stem cell compartment, one described by Fig . 1 (mid-level sequestering rate), and the last this same model with a high rate of symmetrically dividing stem cells (probability of symmetric self-renewal 40%). Symmetric renewal produces a low but constant stem/progenitor fraction ($1/10^5$ cells).

demographic of the final cell population in response to stem and progenitor cell regulation. We conclude that the cell population growth potential inherent in the embryonic mammary placode can be expressed in many different ways, with profound effect on the properties of the majority of epithelial cells. Thus, if more stem and progenitor cells are kept in cycle, the final population is 'younger' (has more division potential) and less differentiated. If only a few progenitor cells are used, and the rest are 'sequestered' or horded in the gland, the population demographic becomes highly polarized, comprising a few stem/progenitor cells with high growth potential

together with a majority of peri-senescent, highly differentiated cells (with very low division potential). This would be predicted to associate with major changes in the growth properties of the cells, and in their susceptibility to tumour development.

Does the model work?

Using few estimated factors, we find that we can describe mathematically the regenerative properties of adult ductal MEC populations. Thus, by taking fixed population aliquots from the primary demographic described by our simulations, and using them as the starting point for growing another gland, we can estimate the total outgrowth population possible, and the average cellular age of that outgrowth. Each generation can be used as the inoculum for the next fat pad, mimicking the actual serial transplantation studies that have been performed for MEC populations. Our model closely copies the pattern of senescence of the population that is observed by biological assay. This supports the validity of the population stratification and demographic predicted by the mathematical model, and that this model is likely to be useful as an aid to biological interpretation.

Developmental stem cell regulation – what effect does it have on the adult mammary gland?

We have considered three mechanisms for changing the number of stem and progenitor cells in cycle. The first is the sequestration of progenitors so that only some are in cycle, and the rest are reserved. This has the effect of balancing the demand for cellular differentiation with the excess growth potential required to accommodate organogenesis and regeneration. It has biological precedent in other epithelial systems (Owens & Watt 2003; Tumbar *et al.* 2004). The second is the number of cells allocated to the mammary rudiment. This is presumably controlled by signalling pathways that regulate the expansion of the rudiment (Chu *et al.* 2004) and prevent cellular differentiation associated with entry into the mortal lineage. Mutations that affect these pathways would be expected to have profound effects on the regenerative capacity of primary population outgrowth (for example, the depletion of stem cells in mice with a null mutation of the Wnt signalling receptor LRP5, Lindvall *et al.* 2006). The third is the probability of asymmetric stem cell division relative to symmetric division. Only when the rate of symmetric division is increased can there be a change in the overall growth potential of the cell population (via expansion of the high division potential stem cell compartment, rather than simply maintaining existing levels).

When the equations are applied, the outcome for each of these three conditions is different. Of particular interest is that when the sequestration rate of primitive stem cells is changed, the adult stem and progenitor fractions vary somewhat, but the regenerative capacity is approximately the same. The signature of this condition is that the outgrowth of the primary tree is slower as sequestration is increased, and the demographic of the final population is changed. For example, if this were measured for a duct that develops despite high rates of enforced quiescence/sequestration in the progenitor fraction, the stem and progenitor fraction in the mature gland would be lower, but the individual progenitor cells would be more potent and primitive (as a result of being sequestered earlier, when the cells still had high division potential), and the cell majority would be more differentiated and perhaps peri-senescent (pushed right to the end of the lineage). On the other hand, with low sequestration, the final gland population would be dominated by TA cells, and the majority would be much younger (higher MDP). We propose that sequestration of progenitor cells takes place during population expansion, and that successive sequestration during development results in long-lived, label-retaining progenitors scattered evenly throughout the mammary tree, in the wake of the advancing terminal end bud.

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When is a stem cell proven?

This model estimates the relative numbers of stem and progenitor cells. Thus, in the 'normal' (mid-level sequestration) model, the progenitor fraction is 45 fold more than the stem cell fraction, whereas this value decreased to 18× with higher sequestration, and increases to 153× with lower sequestration. However, many of the properties of progenitors and stem cells are shared. They are undifferentiated cells with high division potential. They may both be represented in the label-retaining population (for different reasons). We do not know how each of these cell types would be expected to score with respect to their clonogenic capacity (mammosphere formation or other culture clone), their expression of stem cell-associated molecular phenotype, or indeed their relative activity by stem cell assay in fat pads *in vivo*. This model can provide limits that can isolate expected trends in the presence of canonical stem cell activity.

If a population containing progenitor cells but no stem cells is used to generate a secondary ductal outgrowth, a substantial fraction of the growth potential contained in those cells is used, and a 310-fold reduction in progenitor fraction occurs, with an additional 190-fold reduction during secondary transplantation (in the tertiary outgrowth), giving stem cell fractions undetectable by current assays. Similarly, with only asymmetrically renewing stem cells, a 230-fold reduction in stem and progenitor fraction occurs initially, with another 285-fold reduction upon secondary transplantation. Thus, without the presence of a symmetrically renewing stem cell, the overall reduction in stem and progenitor fraction after two transplantations is between 57 000- and 67 000-fold. In contrast, when the stem cell compartment shows significant self-renewal, this reduction (during two transplantations) is 4000-fold, and the stem cell activity comes to equilibrium, able to create new MEC populations indefinitely. Thus, primary and secondary transplantations have the effect of essentially eliminating the stem/progenitor fraction (subsequent generations of transplants must then rely upon the growth potential of the transit-amplifying fraction) unless symmetric self-renewal in the stem cell compartment is occurring. If stem/progenitor fractions exist (by molecular criteria) in tertiary outgrowths, the cell/population that was used to generate this population contained a stem cell capable of symmetric self-renewal.

The model presented here provides a mathematical framework for the mammary gland lineage that will be a useful format for discussion and evaluation of stem cell activity. There are distinct outcomes that describe the expression of different stem cell activities during normal development that will be useful in the evaluation of phenotypes that affect stem cell regulation and function. For models that demonstrate these phenotypes, there are clear predictions with respect to their neoplastic behaviour.

It will be critical to incorporate data as they emerge, and to test it against our model. By modelling other modes of cell growth regulation and comparing model predictions with types of behaviour observed, mechanisms that are unlikely can be eliminated, helping to focus experimentation on more likely candidates. Using several characterized mouse strains with altered stem cell function, we are testing several of the key outcomes predicted by this model, including the development of experimental methods to track subpopulations in a lineage.

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Epithelial to mesenchymal transition (EMT) is an important set of cellular changes that occur during development allowing the formation of tissues in the embryo. These changes include increased motility, loss of epithelial characteristics such as E-cadherin localization and changes in the actin cytoskeleton, among others. These alterations have been linked to carcinoma formation and the ability of the carcinoma cells to metastasize. The pathways involved in this transition and how they function are topics of active research. Specifically, the induction of EMT by transforming growth factor - β (TGF-β), a growth factor which normally inhibits cellular proliferation, is of interest because tumor cells can acquire a resistance to this effect, allowing them to persist and metastasize.

Microfluidic analysis of cellular responses to growth factors allows the importance of the local environment to the cellular response to be studied. We have used normal mouse mammary epithelial cells at low and high surface densities in macro- and micro-scale cultures to analyze the dose response of this cell type to TGF-b at different timepoints. Differences in thresholds for EMT between cultures of differing scales or densities illustrate the importance of the culture conditions on the results of experiments involving cellular exposure to growth factors. By using microfluidic cultures for this type of experiment, more detailed studies of the activation of cellular responses from short-term cell surface alterations to longer-term transcriptional responses can be performed.