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John W. Pepper
Kathleen Sprouffske
Carlo C. Maley

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John W. Pepper[a,d], Kathleen Sprouffske[b,c], and Carlo C. Maley[c]

[a] Department of Ecology and Evolutionary Biology, University of Arizona, Tucson, Arizona, U.S.A. (corresponding author, email: jpepper1@email.arizona.edu)

[b] Genomics and Computational Biology Program, University of Pennsylvania, Philadelphia, Pennsylvania, U.S.A.

[c] Systems Biology Division, The Wistar Institute, Philadelphia, Pennsylvania, U.S.A.

[d] The Santa Fe Institute, Santa Fe , New Mexico, U.S.A.

Abstract

Cell differentiation in multicellular organisms has the obvious function during development of creating new cell types. However, in long-lived animals with extensive cell turnover, cell differentiation often continues after new cell types are no longer needed or produced. Here we address the question of why this is true. It is believed that multicellular organisms could not have arisen or been evolutionarily stable without possessing mechanisms to suppress somatic selection among cells within organisms, which would otherwise disrupt organismal integrity. Here we propose that one such mechanism is a specific pattern of ongoing cell differentiation commonly found in metazoans with cell turnover, which we term “serial differentiation”. This pattern involves a sequence of differentiation stages; starting with self-renewing somatic stem cells and proceeding through several (non-self-renewing) transient amplifying stages before ending with terminally differentiated cells. We test this hypothesis using an agent-based computer simulation of cell population dynamics and evolution within tissues. The results indicate that, relative to other, simpler patterns, tissues organized into serial differentiation experience lower rates of detrimental cell-level evolution. Self-renewing cell populations are susceptible to somatic evolution, while those that are not self-renewing are not. We find that a mutation disrupting differentiation can create a new self-renewing cell population that is vulnerable to somatic evolution. These results are relevant not only to understanding the evolutionary origins of multicellularity, but also the causes of pathologies such as cancer and senescence in extant metazoans including humans.

Introduction

The puzzle of ongoing cell differentiation

Most mature tissues of long-lived metazoans exhibit ongoing cell differentiation, with tissue-specific somatic stem cells dividing to renew populations of more differentiated cells that are not self-renewing. Although ongoing cell replacement is clearly necessary for long-lived organisms, it is not obvious why tissue renewal should involve ongoing differentiation from somatic stem cells. In principle, tissues could be maintained by self-renewal of fully functional cell types, using the same kind of "cell memory", or direct epigenetic inheritance of cell state that is typical of unicellular organisms [1]. Indeed, some metazoan tissues do seem to replace lost cells through such self-duplication of functionally differentiated cells [2]. Such a simple system is both evolutionarily conserved and metabolically efficient. Instead, however most adult tissues contain sequences of semi-differentiated "transient amplifying", or "transit" cell stages, with each sequence starting with a self-renewing population of stem cells, and ending with fully differentiated cells that no longer divide [3-8]. Because this more elaborate system presumably requires greater genetic complexity, as well as a metabolic cost in supporting the additional cells, it would seem unlikely to have evolved unless it provided some important advantage to the organism. Here we propose that this advantage lies in the suppression of somatic selection and thus somatic evolution

The challenge of somatic evolution

A multicellular organism can be viewed as a population of cooperating cells. This population is subject to the same evolutionary processes as any other population undergoing reproduction, death, mutation, and competition for limiting resources. Selection within a metazoan will inevitably favor those cells that are better at reproductive competition and survival. Yet, the characteristics that help cells compete effectively within the organism are generally detrimental to organismal integrity and fitness. Thus there is a fundamental conflict between selection among cells within organisms (somatic selection) and selection among organisms within populations (organismal selection). Multicellular organisms could not emerge as functional entities before organismal selection had led to the evolution of mechanisms to suppress somatic selection [9-12]. Differentiation has been recognized as a mechanism to control somatic evolution and its potential for carcinogenesis during development [13]. However, even in mature tissues, the combination of cell turnover and somatic mutation creates the conditions for somatic evolution. The conflict between the cellular and organismal levels of selection is exacerbated in long lived organisms with extensive cell turnover. Humans are estimated to contain approximately 10^{14} cells with extensive cell turnover [14]. For example, each day the small intestine and the hematopoietic system shed 10^{10} and 10^{11} cells, respectively [15,16]. Furthermore, many of the genes in a metazoan genome may function to coordinate cellular cooperation by constraining cellular competition [9]. This implies that many loss-of-function mutations may provide a competitive advantage for the mutant cell [17]. Accumulation of such somatic mutations through cell-level selection

could lead to two general classes of pathology: Firstly, diversion of cell resources into cell survival and replication and away from organismal function could impair a wide range of organismal functions, leading to general senescence [18]. Secondly, the shedding of restrictions on cell division and survival, if unchecked, ultimately leads to uncontrolled cell proliferation and cancer [19,50].

The hypothesis: Serial differentiation as a defense against somatic evolution

Somatic evolution is inevitable given the cumulative Darwinian selection that occurs in any self-renewing population of dividing cells, together with a supply of variation in cell fitness from somatic mutation. In multicellular organisms with substantial cell turnover, both self-renewal and cell proliferation are necessary, and the complete suppression of all mutation may not be achievable. However, it may be quite feasible through serial differentiation to almost entirely suppress somatic selection, without which the appearance of somatic mutations is quite harmless to the organism. If, as a result, somatic evolution is greatly retarded, we can expect to see the pathologies associated with it to persist to some degree, but primarily as ailments of old age.

Any self-renewing cell population with high replicative activity is subject to strong somatic selection. In order for a tissue to sustain healing or substantial cell turnover, it must include both self-renewal and high replicative activity. However, if these two properties are segregated into different cell compartments (e.g., stem and non-stem respectively), no one cell compartment combines all the ingredients for somatic evolution.

We hypothesize that stem compartments are subject to little somatic evolution because they are small and quiescent, with little replicative activity, while populations of transient amplifying cells (TACs) are not subject to somatic evolution because they are not self-renewing, so that mutations conferring increased survival and replication do not persist and go to fixation.

Here, we use a simplified computational model of cell population dynamics and differentiation to test our hypothesis. We also study factors influencing the rate of somatic evolution under serial differentiation, including symmetric versus asymmetric differentiation, the number of TAC stages, and loss-of-function mutations in differentiation pathways. The model is not designed to faithfully replicate the details of any one tissue, but rather to capture the essential dynamics relevant to our hypothesis.

Methods

The Model

To investigate the role of cell differentiation in somatic evolution, we developed a simplified model of the evolutionary dynamics of cells within a tissue of an adult organism. This model consists of a set of assumptions about the behavior and population dynamics of cells within tissues, which we embodied in an agent-based computer simulation. This was a discrete-time stochastic simulation, with somatic cells as the only agents. The source code for the computational model is freely available from the authors upon request. A description of the model's assumptions and algorithms follows.

Representation of cells

Each cell was represented by three heritable characteristics: intrinsic replication rate, (*r*) intrinsic mortality rate (*d*), and whether or not it was capable of differentiation upon division. A fourth cell characteristic was the cell's current differentiation stage. When a cell underwent mitosis, it ceased to exist, and was replaced by two daughter cells. Each daughter cell inherited the first three of the intrinsic characteristics listed above. The current differentiation stage was normally incremented from that of the parent cell. (Instead, the current differentiation stage was directly inherited without being incremented in various scenarios of self-renewing compartments described below, including stem cells, self-renewing TACs, and mutant TACs with differentiation knock-outs.) Intrinsic growth and mortality rates were modeled as quantitative traits representing the cell's genetic tendency to divide, and to die through apoptosis or other causes. Capacity to differentiate was represented by a binary variable designating either the functionality of differentiation pathways in the cell, or their disruption by mutation. Current differentiation stage was an integer (*i*) ranging from 0 (for a stem cell) to *n* (a terminally differentiated cell). A model parameter determined the total number of non-stem cell stages (*n)*. At the start of a simulation run, all cells had the same intrinsic growth rate (*r*) and mortality rate (*d*), set by parameters (Table 1). All initial cells were also assumed to be capable of differentiation until a somatic mutation disrupted that capacity.

During each time step, every cell had the opportunity to divide or to die, with the stochastic probability of each determined by its values of *r* and *d*, respectively. If a cell was capable of differentiation, then immediately upon dividing, its daughter cells had the opportunity to advance to the next differentiation stage. The control of cell division and differentiation is further described below under the heading, "Tissue homeostasis".

The cell population

The model represented a population of cells constituting one or more proliferative units. This population included cells in a series of differentiation stages, indexed by *i*, ranging from stem cells ($i = 0$), continuing through zero or more

transient amplifying stages ($0 < i < n$), and ending with terminally differentiated cells ($i = n$). (Figure 1)

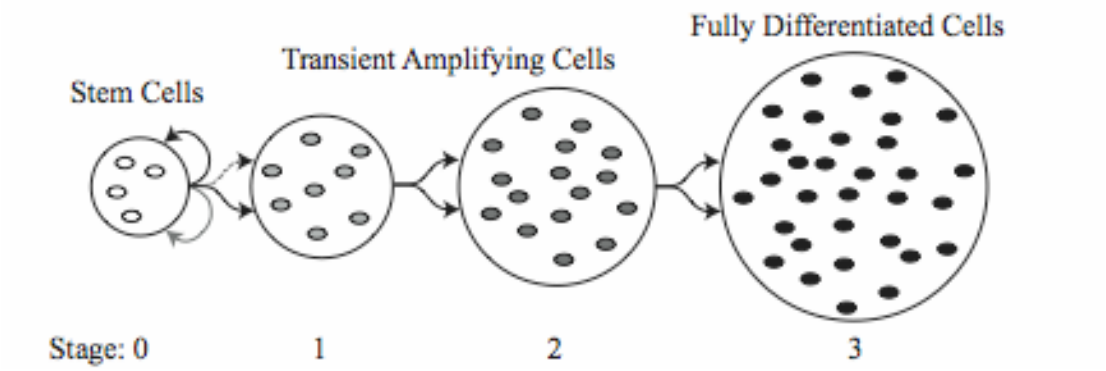

Figure 1. A diagram of serial symmetric differentiation. The series includes stem cells (Stage 0, in white), transient amplifying cells (TACs) (in grey), and finally, terminally differentiated cells (Stage 3, in black). Stem cells divide asymmetrically with one daughter rejoining the stem cell compartment and one daughter differentiating (black arrows), unless the stem cell population is below homeostatic levels, in which case both daughter cells become stem cells (grey arrow). If there is an over-abundance of stem cells, both daughter cells will differentiate (dotted arrow). Transient amplifying cells divide symmetrically, so that both daughter cells advance to the next differentiation stage. Thus, every cell division outside the stem cell compartment entails differentiation into the next downstream stage, eventually ending in the terminally differentiated cells.

The initial number of cells in each differentiation stage i (K_i) increased from one stage to the next by a factor of t (for 'tapering ratio'), where ($t = K_{i+1} / K_i$). Thus, the initial number of cells in stage i (K_i) was determined by a combination of the parameters for the initial number of terminally differentiated cells (K_n) and the tapering ratio (t) such that:

$$K_i = \frac{K_n}{t^{(n-i)}} \tag{1}$$

Thus, summing across all stages, the total number of cells in the modeled cell population was:

$$K_{tot} = K_n \sum_{i=0}^{n} t^{-i} . \tag{2}$$

Undifferentiated tissues

For comparison to tissues organized by serial differentiation, we also modeled hypothetical undifferentiated tissues, in which all cells had equal (and unlimited) replicative potential. These cells were also capable of performing the work of the organ for the benefit of the organism. For valid comparisons, it was important to use the same number of functional cells for differentiated versus undifferentiated tissues. We assumed that all cells were functional in the hypothetical undifferentiated tissues, but that under serial differentiation only the terminally differentiated cells were functional. We therefore compared an undifferentiated tissue containing a given total initial number of cells (K) against a serial differentiation series ending with that same number of terminally differentiated cells ($K_n = K$), but containing a greater number of cells in total (K_{tot} from Eqn. (2) above).

Tissue homeostasis

Presumably, homeostatic mechanisms maintain the appropriate size of cell populations in the various tissues of metazoans. Cell proliferation must be stimulated when needed, and suppressed when not needed. Little is known about these homeostatic mechanisms in most tissues [5]. In our model, we assumed that cell division was regulated by extrinsic micro-environmental signals such as competition for limited growth factors [8,67] or by end-product inhibition, with end-products generated by the terminally differentiated cells (as has been shown in the hematopoietic system [68]), so that cell division is responsive to the number of terminally differentiated cells. In our model, the probability of division (p) for a TAC cell followed a logistic function:

$$p = r\left(\frac{K_n - N_n}{K_n}\right), \tag{3}$$

where r was the cell's intrinsic growth rate and K_n and N_n were the initial and current number of terminally differentiated cells, respectively. The probability of division (p) was truncated at the limits of 0 and 1. The effect was to maintain the number of terminally differentiated cells close to the initial number. These feedback loops are a simplified representation of the roles of stromal cells, cytokines, morphostats [46] and cell-to-cell contact in regulating cell proliferation to maintain tissue homeostasis. The result of implementing these rules in our model was that initial cell numbers were maintained as an equilibrium between cell production and cell loss to terminal differentiation and death (Figure 11).

For any TAC stage i, ($0 < i <$ n), cells entered the stage through division and differentiation from stage i-1, and exited the stage through both cell mortality and division with differentiation to stage i+1. When division was symmetrical such that both daughter cells differentiated then the expected change per time step in the population size of stage i was:

$$\begin{aligned}\Delta N_i &= 2pN_{i-1} - dN_i - pN_i \\ &= \left(r\frac{K_n - N_n}{K_n}\right)2N_{i-1} - \left(d + r\frac{K_n - N_n}{K_n}\right)N_i \,.\end{aligned} \tag{4}$$

(When individual cells were allowed to vary due to somatic mutation, then p and d in this equation represent mean values.)

In addition to non-stem cells, the number of stem cells must also be regulated [5] in order to replenish stem cell losses due to apoptosis and extrinsic damage and to thereby preserve the integrity of the entire proliferative unit. We modeled the probability of stem cell division (p_0) as the sum of stimulation from both the stem and the terminally differentiated compartments:

$$p_0 = Max\left(0, r\left(\frac{K_0 - N_0}{K_0}\right)\right) + Max\left(0, r\left(\frac{K_n - N_n}{K_n}\right)\right) \tag{5}$$

where K_0 was the initial number of stem cells and N_0 was the current number of stem cells. The use of the maximum function here prevented suppression of cell division due to an overabundance of one cell type from interfering with the replenishment of the other cell type.

When a stem cell divided, each daughter cell differentiated into the next stage (TAC stage 1) if and only if the stem cell population was at or above its initial population size ($N_0 \geq K_0$).

In our simulation, cells differentiated only immediately subsequent to mitosis (in the same time step), though this rule could represent differentiation at any time between mitosis and the next cell cycle. When a stem cell divided, both daughter cells remained stem cells if the stem cells were below their initial number ($N_0 < K_0$). Otherwise one daughter cell differentiated into the first transient amplifying stage (stage 1), while the other became a stem cell (stage 0) [69-71].

The differentiation of transient amplifying cells (TAC) was modeled in two different ways for comparison. Under symmetric differentiation (Experiments 1a & 1b), when transient amplifying cells divided, both daughter cells differentiated into the next stage in the series. Under asymmetric differentiation in TAC stages (Experiment 1c) one daughter cell remained in the same cell stage as the parent and the other daughter cell progressed to the next cell stage. To simulate symmetric self-renewal by TACs (Part 2), all differentiation stages behaved like stem cells, in that daughter cells from each stage differentiated into the next stage if and only if their own stage was at or above its initial population size ($N_i \geq K_i$). Unless otherwise noted, parameter values for all simulation runs approximated values from the GI crypt literature [5] (Table 1). Parameters were varied in some experiments, but except where otherwise noted, the standard values were used. Under these parameter settings, the initial number of stem cells, as specified by Eqn (1) was $K_0 = 16$, and the total number of cells in the simulation, as specified by Eqn. (2), was $K_{tot} = 496$.

Table 1. Standard parameter values.

Parameter	Symbol	Value
Number of non-stem differentiation stages	n	4
Tapering ratio	t	2
Initial number of terminally differentiated cells	K_n	256
Initial intrinsic mortality rate	d	0.1
Initial intrinsic growth rate	r	1

Experiments

In our experiments we introduced somatic mutations of the three heritable cell characters: intrinsic replication rate (r), intrinsic mortality rate (d), and capacity for differentiation upon division. The evolutionary outcomes we measured were the average values of quantitative traits (intrinsic replication or mortality rate), the frequencies of discrete mutant alleles for differentiation ability, or changes in total cell population size.

To study the effects of mutations affecting rates of replication and mortality, we carried out two types of experiments, using either a controlled mutation of a single cell at a time, or stochastic mutation of all dividing cells:

Controlled mutations

In controlled mutation experiments, we turned off stochastic mutation After creating the cell population, we let the model equilibrate for 500 time steps and then introduced a single mutant cell with an altered rate of intrinsic replication or mortality, or (in Part 4), with heritable loss of normal differentiation ability. We then ran the model for 10,000 time steps, or stopped it sooner if and when the mutant clone either went extinct or to fixation. To reduce the extreme stochasticity of time to complete fixation, we used as a proxy for fixation the criterion of a mutant allele reaching a frequency of > 90% of the cell population. Because our model did not include any frequency-dependent fitness effects, it was safe to assume that any mutation that increased from an initial low frequency to >90% would eventually have gone to fixation given sufficient time.

We introduced mutations of varying magnitudes into different differentiation stages and tested each case multiple times with different random number seeds.

Stochastic mutations

In experiments with stochastic mutation, we let the intrinsic growth or mortality rates mutate as follows: Upon each cell division, the growth or mortality rates of the daughter cells were changed to represent the quantitative effects of mutations caused by DNA replication errors during cell division. At cell division, each daughter cell inherited the parental cell's quantitative trait multiplied by a normally distributed random variable with mean 1 and standard deviation 0.05. Thus, stochastic mutations could either increase or decrease these traits, with equal probability.

In experiments with stochastic mutation, we stopped each simulation run after a two-fold change, (i.e. when either the average intrinsic growth rate doubled or the mortality rate was halved from the initial rates), or after 10,000 time steps, whichever came first. We varied the number of cell stages in the model from a single cell stage (no distinction between stem and differentiated cells) to 7 cell stages (stem, terminally differentiated, and 5 transient amplifying stages).

We also used stochastic mutation experiments to study the effects of differentiation knock-out mutations (see Part 4 of Results). Here, we allowed stochastic mutations to disrupt differentiation pathways, at a rate of 10^{-4} mutations per

cell division. This mutation was heritable upon cell division, so that it prevented further differentiation in the entire resulting clone. We stopped the simulation run when either the clock reached 10,000 time steps, or the total cell number increased 10-fold, which we interpreted as the initiation of a tumor. Each experiment was replicated multiple times.

Results

Part 1: Effect of number of differentiation states

Our first set of experiments was set in the context of serial cell differentiation, under the assumption that TAC division was symmetric, with both daughter cells acquiring the same differentiation stage (Fig.1). We allowed somatic mutations that either increased the cell's intrinsic division rate (r), or reduced its probability of death (d), and observed the results of somatic selection.

Experiment 1a: Fate of single selfish-cell mutations

Our first experiment used the introduction of controlled mutations to examine the probability of a single mutation sweeping to fixation within the cell population, as a function of the cell-level fitness advantage it conferred.

Because our model included stochastic cell mortality and replication, when a novel mutation was first introduced into a population as a single cell, it was in danger of going extinct through drift even if its intrinsic fitness was superior to its competitors. Those that survived long enough to reproduce and establish a clone could potentially spread to fixation. In undifferentiated cell populations, mutations increasing the division rate by even a moderate degree went to fixation with high frequency (Figure 2). Under serial differentiation, such mutations sometimes spread to fixation when introduced into stem cells, although stochastic effects were also important. Mutations introduced into non-stem cells *never* reached fixation, but instead were *always* lost from the population, regardless of the mutant's replicative advantage (Figure 2). When mutations were introduced that increased the cell's intrinsic survival rate, the results were similar. Again, the mutations often went to fixation when introduced into stem cells, but *never* when introduced into TACs (Figure 3).

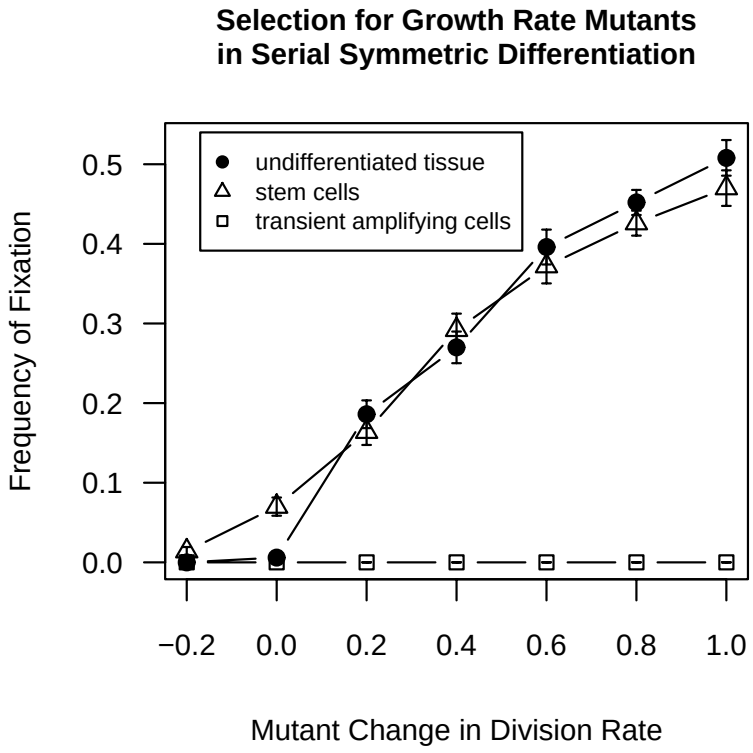

Figure 2. The frequency of fixation of mutations altering division rate when introduced into cells in undifferentiated tissues (filled circles), or into stem cells (triangles), or transient amplifying cells (squares).Values on horizontal axis show change in cell's value of *r* relative to initial value of 1. Mutant values of *r* thus ranged from (1- 20% = 0.8) to (1 + 100% = 2.0).

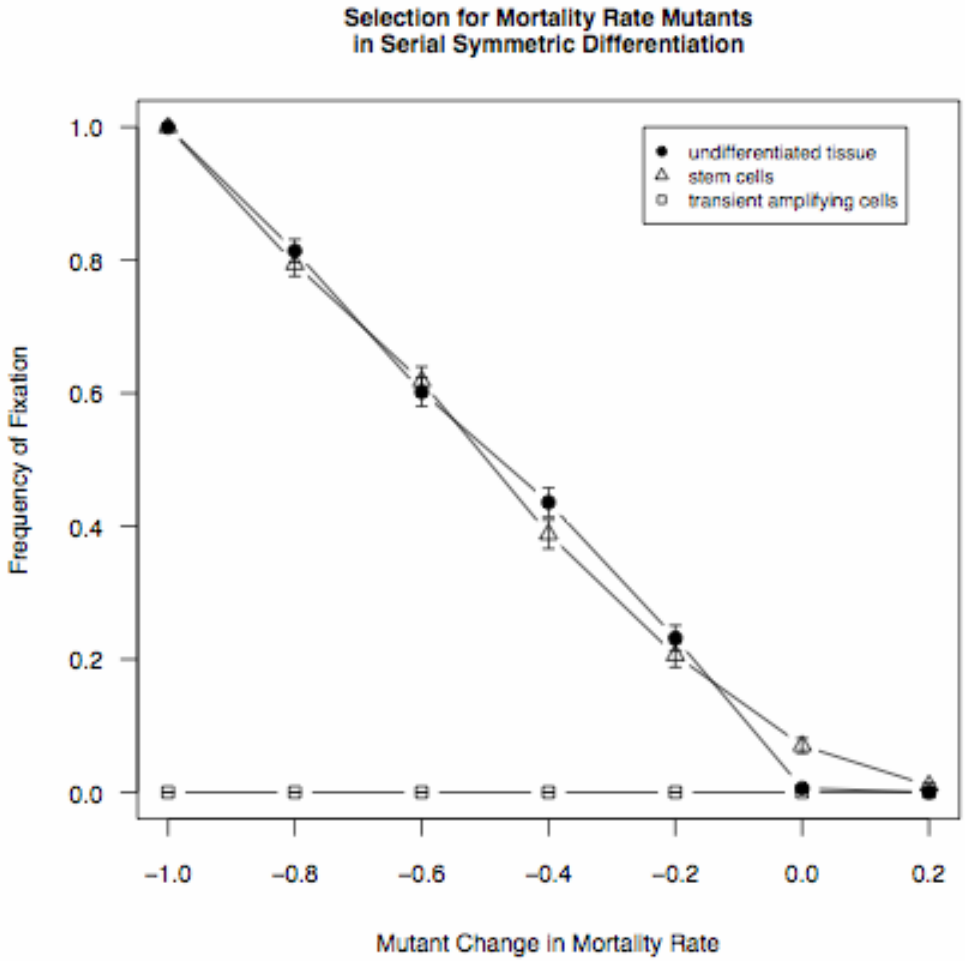

Figure 3. Frequency of fixation (mutant reaches >90% of cell population) for mutations affecting cell mortality rate when introduced into undifferentiated populations (filled circles), stem cells (triangles), and transient amplifying cells (squares). Bars show standard errors. Because they did not vary, results for all three transient amplifying stages are pooled. Values on horizontal axis show mutant cell's value of *d* relative to initial value of 0.05. Mutant values of *d* thus ranged from (0.05 -100% = 0) to (0.05+ 20% = 0.06).

Experiment 1b: Accumulation of quantitative selfish-cell mutations

Experiment 1a showed that under serial differentiation, only stem cells were vulnerable to accumulating selfish-cell mutations that increased cell survival and replication. This suggests that somatic evolution could be suppressed by making stem cell populations small so that stem mutations are rare, and selection on stem cells is weak relative to drift. If we assume that the sustained number of terminally differentiated cells (K_n) is dictated by the needs of the organism, then Eqn (1) implies that the required number of stem cells (K_0) can be reduced in a sustainable tissue either by increasing the number of non-stem differentiation stages (n), or by increasing the 'tapering ratio' (t), according to the expression $K_0 = K_n / t^n$. Here we assume that cell numbers double with each stage of differentiation ($t = 2$), and we focus on the number of non-stem differentiation stages (n).

Using the protocol of stochastic mutation (see Methods), we allowed the quantitative cell trait of either intrinsic replication rate (r) or mortality rate (d) to mutate at each cell division, and measured the time until somatic evolution caused the average replication rate to double or mortality rate to fall by half. As the number of differentiation stages increased, so did the total number of cells, the number of cell divisions per time step, and the number of novel mutations arising per time step. All else being equal, these factors would increase the rate of somatic evolution. Nonetheless, as the number of differentiation stages increased, so did the waiting time before we recorded a two-fold evolutionary change in the trait under study. For every differentiation stage added to the model the relative risk (RR) of the cell population doubling its mean intrinsic replication rate was cut in half (Cox proportional hazard RR = 0.498, 95% CI: 0.476-0.522, $p < 0.001$). The same was true for the effect of number of cell stages on waiting time until intrinsic mortality rates were halved (RR = 0.689, 95% CI: 0.667-0.712, $p < 0.001$). The Cox regression takes into account both the time until the two-fold change, and the fact that some simulation runs were censored at 10,000 time steps [20]. Kaplan-Meier curves illustrate this pattern for both the doubling of intrinsic replication rate (Figure 4) and the halving of intrinsic mortality rate (Figure 5).

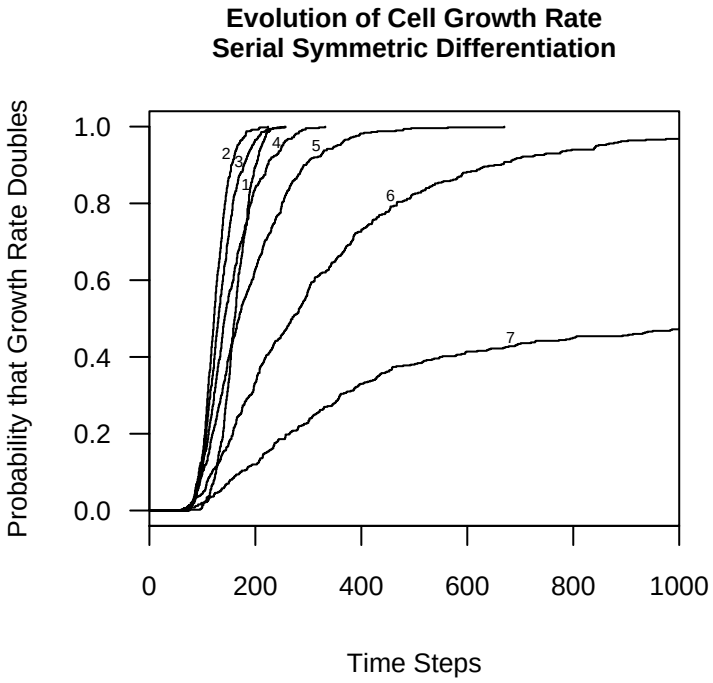

Figure 4. Kaplan-Meier curves show, as a function of time, the probability that a population of cells doubled its average division rate (*r*). Each curve is labeled with the number of differentiation stages in the model (1-7); (1 = no distinction between stem and non-stem cells). With more differentiation stages, somatic evolution was less likely to produce a two-fold change within any given time period.

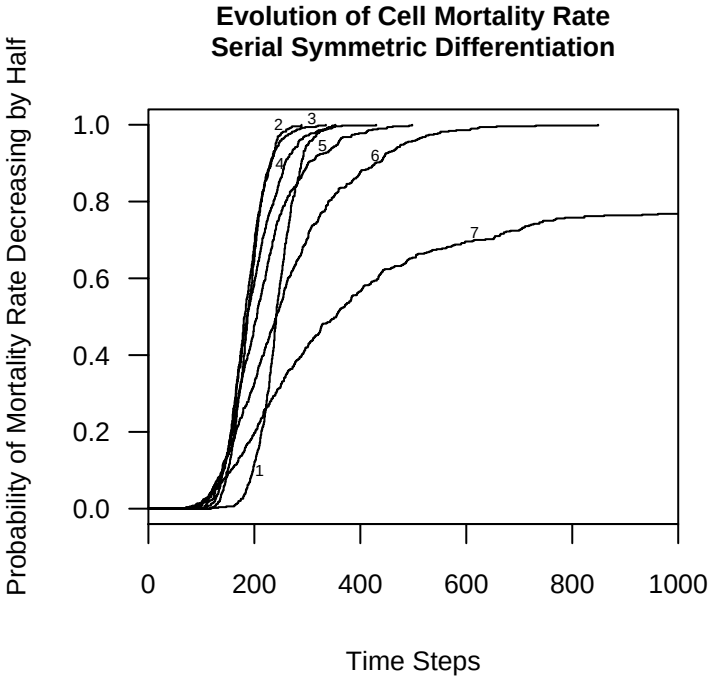

Figure 5. Kaplan-Meier curves show, as a function of time, the probability that a population of cells halved its average mortality rate. Each curve is labeled with the number of differentiation stages in the model. With more differentiation stages, somatic evolution was less likely to produce a two-fold change.

The response to varying *n* was not linear. It may seem surprising that somatic evolution was more rapid with two cell stages (stem and fully differentiated), than it was with a single self-renewing cell stage. This is because the two-stage situation required stem cell divisions to supply replacement cells to both the stem cell compartment and the differentiated cell compartment. Thus, even though this situation involved half as many stem cells as the situation with a single self-renewing cell stage, it involved more total stem cell divisions and therefore produced more stem cell turnover and faster somatic evolution than the single-stage situation (Figs. 4 and 5). Because both the number of stem cells and their replicative activity were important, the most effective suppression of somatic selection occurred with more than 4 non-stem stages ($n > 4$).

Part 2: Effect of asymmetric differentiation

For some cells, differentiation may be asymmetric, with one daughter cell remaining in the parental cell stage and the other further differentiating [36]. Because such asymmetric division represents a form of self-renewal, we hypothesized that allowing it in TACs would increase the rate of somatic evolution. To test this hypothesis, we repeated Experiment 1a including one treatment with asymmetric instead of symmetric differentiation in all cell stages including TACs:

Experiment 2: Effect of asymmetric differentiation on somatic evolution

Compared to symmetric differentiation, asymmetric differentiation resulted in more rapid somatic evolution. In a multivariate Cox regression controlling for number of differentiation stages, asymmetric differentiation increased the risk of mean intrinsic replication rate doubling (Figure 6) (relative risk = 1.56 (95% CI: 1.41-1.73, $p < 0.001$).

In addition, as we observed with symmetric differentiation in Experiment 1a, under asymmetric differentiation somatic evolution slowed dramatically as the number of differentiation stages increased This was true both for cell replication rate (Cox regression: RR = 0.545, 95% CI: 0.530-0.560, $p < 0.001$), and for cell mortality rate (Fig. 6; RR = 0.704, 95% CI: 0.688-0.719, $p < 0.001$).

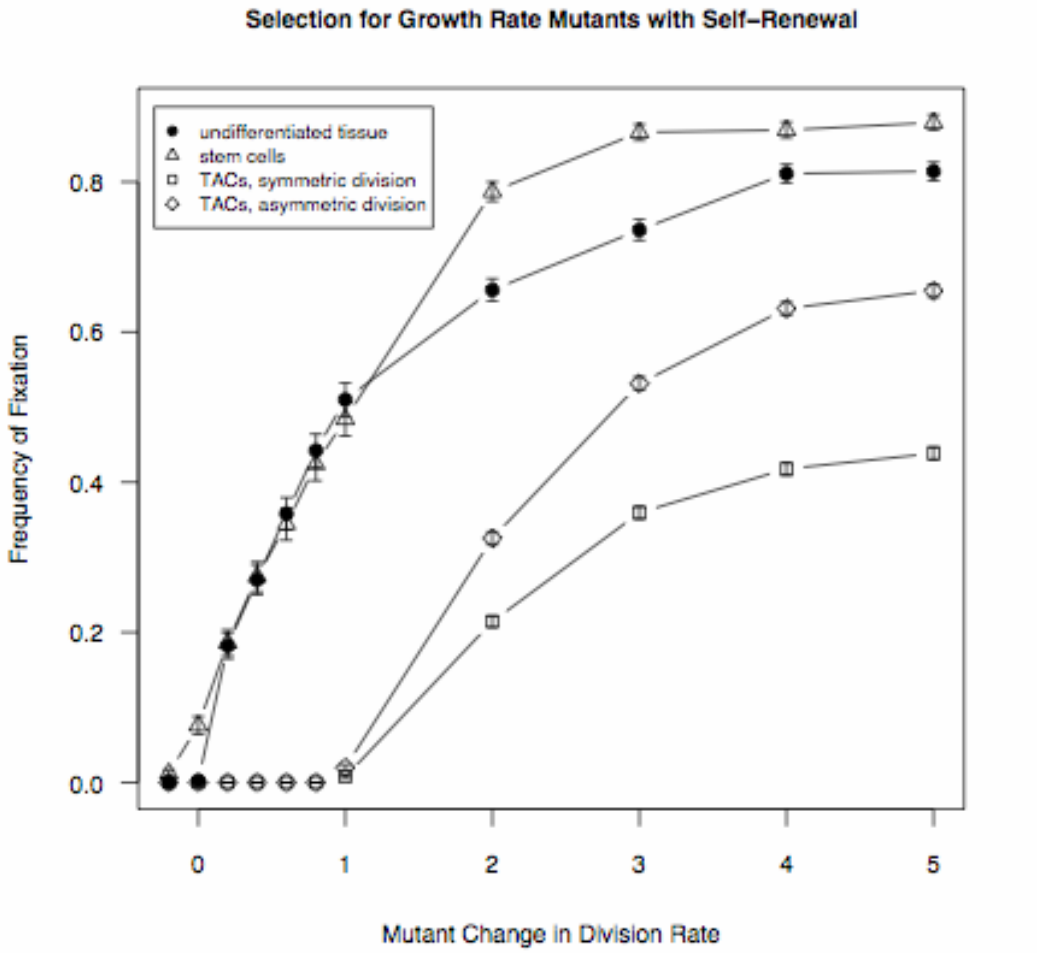

Figure 6. Frequency of fixation by mutations affecting cell division rate in undifferentiated populations (filled circles), and under serial differentiation when introduced into stem cells (triangles), transient amplifying stages dividing symmetrically (squares), and transient amplifying stages dividing asymmetrically (diamonds). Each data point represents at least 500 trials with standard error bars. Values on the horizontal axis indicate the fold change in the mutant value of *r* relative to the original value of 1. Rapid-replication mutations were more likely to go to fixation in TACs dividing asymmetrically vs. symmetrically (diamonds vs. squares).

Part 3: Symmetric self-renewal in transient amplifying cell stages

Some published models of cell differentiation assume that non-stem cell populations are partly self-renewing (e.g., [4,21]). To study this scenario, we assumed transient amplifying cells were like stem cells in that both of their daughter cells could either remain in the parental differentiation stage or proceed to the subsequent stage, depending on homeostatic signaling mechanisms that maintain an equilibrium number of cells in each stage. According to our hypothesis, such self-renewing cell populations would be more vulnerable to somatic evolution than would tissues following strict serial differentiation (Figure 1).

Experiment 3a: Effect of symmetric self-renewal on somatic evolution

To test the hypothesis that self-renewal by TACs would accelerate somatic evolution independently of whether division was asymmetric or symmetric, we repeated Experiment 1a under the scenario of symmetric self-renewal by TACs. We observed that symmetric differentiation with self-renewal was more prone to somatic evolution than was symmetric differentiation without self-renewal (Figure 7); (for doubling of intrinsic replication rate, RR = 1.86, 95% CI: 1.66-2.09, $p < 0.001$); (for halving of mortality rate, RR = 1.39, 95% CI: 1.25-1.54, $p < 0.001$,).

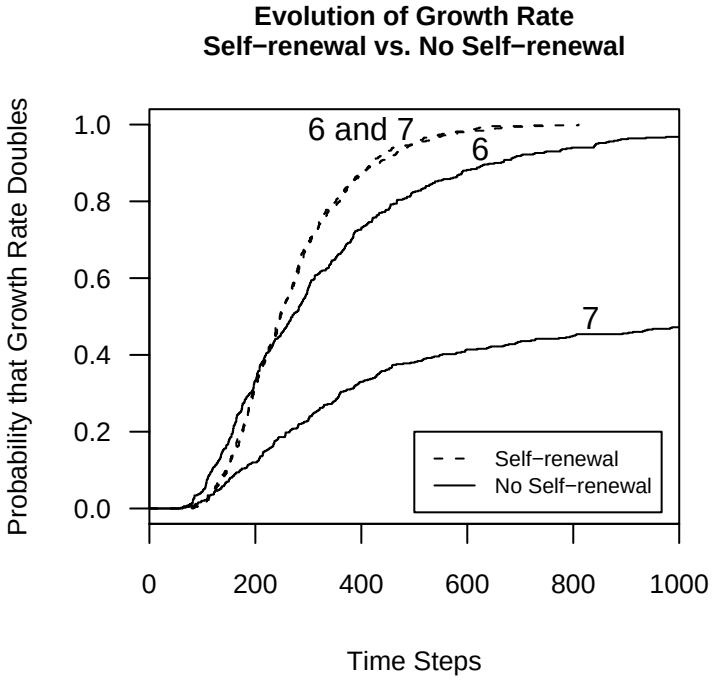

Figure 7. Kaplan-Meier plot showing probability, as a function of time, of the average cell replication rate (*r*) doubling when TACs underwent self-renewal (dashed lines) vs. serial differentiation (solid lines). Lines are labeled with the total number of differentiation stages (6 or 7). TAC self-renewal increased the rate of somatic evolution.

Experiment 3b: Fate of discrete selfish-cell mutations in self-renewing cell compartments

To clarify the reasons for the results of experiment 3a, we again examined the fate of single selfish-cell mutations, using our controlled mutation protocol, this time including a treatment of symmetric self-renewal by TACs.

As predicted, allowing self-renewal by the transient amplifying stages did increase fixation rates for selfish-cell mutations introduced into TACs. For example, a mutation conferring a 5-fold increase in division rate was more likely to spread to fixation when TACs underwent self-renewal then when they did not (Figure 8). This was true whether the mutation arose in a cell at stage 1: (multivariate logistic regression: odds ratio OR 1.45, 95% CI 1.41 - 1.49); at stage 2 (OR 1.24, 95% CI 1.21 - 1.28); or at stage 3: (OR 1.06, 95% CI 1.03 - 1.10) ; ($p<0.001$ in all cases). Similarly, a mutation conferring a 5-fold decrease in mortality rate was more likely spread to fixation when TACs underwent self-renewal then when they did not (Figure 9). This was true whether the mutation arose in stage 1 (OR 1.54, 95% CI 1.46-1.61), stage 2 (OR 1.49, 95% CI 1.42-1.57), or in stage 3 (OR 1.17, 95% CI 1.12-1.23); ($p<0.001$ in all cases). For both types of selfish-cell mutation, the only condition that entirely prevented them from ever spreading to fixation in TACs was TAC symmetric division without self-renewal (Figs. 8 & 9).

As one would expect, allowing self-renewal by the transient amplifying stages did not change fixation rates for selfish-cell mutations introduced into stem cells (multivariate logistic regression odds ratio OR = 1.01, 95% CI: 0.99-1.03).

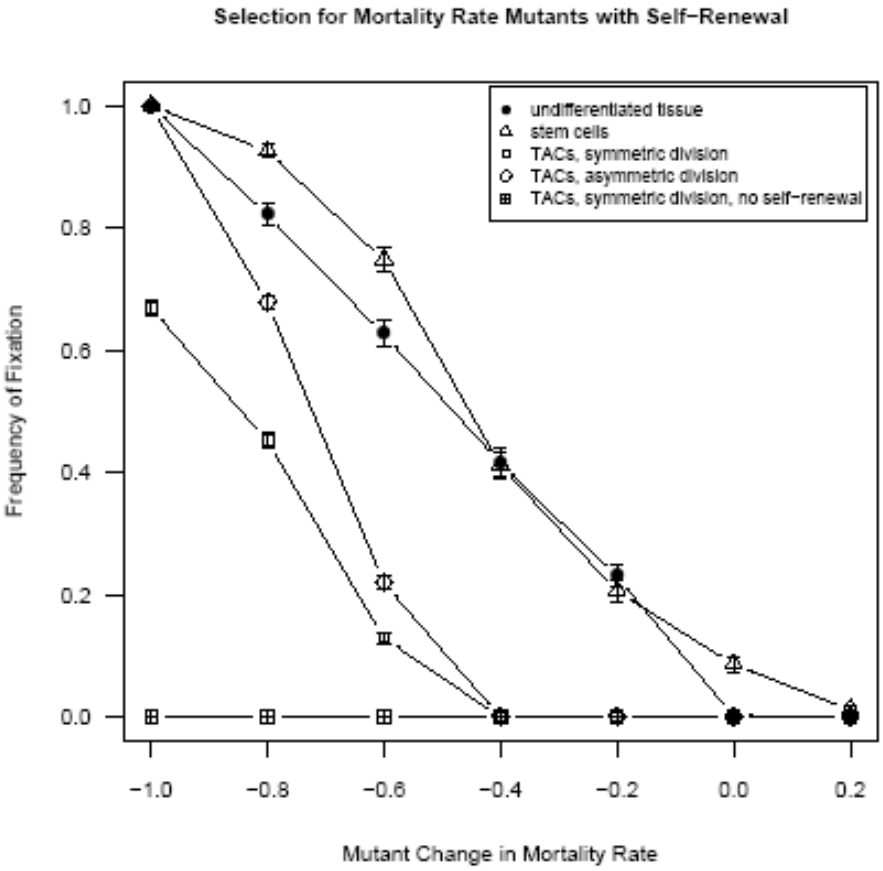

Figure 8. Frequency of fixation by mutations affecting cell division in undifferentiated tissues (filled circles), and under serial differentiation with TAC self-renewal for mutations introduced into stem cells (triangles), transient amplifying stages dividing symmetrically (squares), and transient amplifying stages dividing asymmetrically (diamonds). Bars show standard errors.

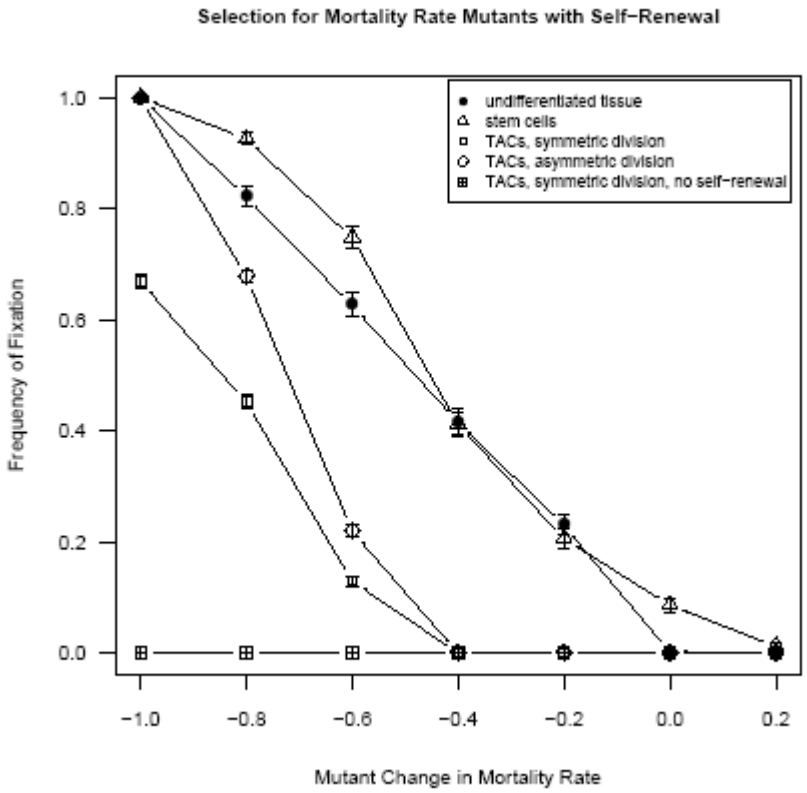

Figure 9. Frequency of fixation by mutations reducing cell mortality when introduced into undifferentiated cell populations (filled circles), or under serial differentiation into stem cells (triangles), transient amplifying stages dividing symmetrically (squares), and transient amplifying stages dividing asymmetrically (diamonds). Each data point represents 500 trials with standard error bars. Clones of cells that cannot apoptose (mortality change = -1.0) may still be cleared when they differentiate into the fully differentiated stage and stop dividing.

Part 4: Mutations that disrupt differentiation

The foregoing experiments show that serial differentiation can effectively block the spread of selfish-cell mutations that increase cell survival and replication. However, loss-of-function mutations can also affect cell differentiation itself. In this section we investigate whether differentiation knock-out mutations are positively selected, and how they affect the dynamics of cellular evolution and proliferation. Differentiation knock-out mutations were positively selected within the cell compartment they arose in because they caused both daughter cells to remain in the parental stage. Thy also had striking effects on cell proliferation.

Experiment 4a: Fate of controlled differentiation knockouts

Our first experiment in Part 4 used our 'controlled mutation' protocol to follow the fate of a knock-out mutation introduced into a single cell in a TAC stage. When a single mutation was introduced, it tended to spread to fixation, except in the instances where it was lost through stochastic drift while still very rare. If the mutant clone did not go extinct within approximately 100 time steps, it expanded exponentially to fixation.

These clonal expansions occurred despite the fact that the cells remained subject to normal feedback controls on division rate. This is because the differentiation knock-out mutation interacted with the negative feedback mechanism of tissue homeostasis to eliminate normal growth inhibition. Because the non-differentiating clone did not provide any cells to the down-stream differentiation stages, the terminally differentiated cell compartment tended to fall below its equilibrium population size. Because of the negative-feedback control of proliferation (Methods, Eqn. 3), this caused proliferative stimulation of all the TACs and the stem cells, including the non-differentiating mutant cells. Differentiation knockouts were selectively favored in any cell still capable of division. Despite the fact that stem cells were normally capable of foregoing differentiation, differentiation knockouts were selectively favored even in this compartment (Figure 10)

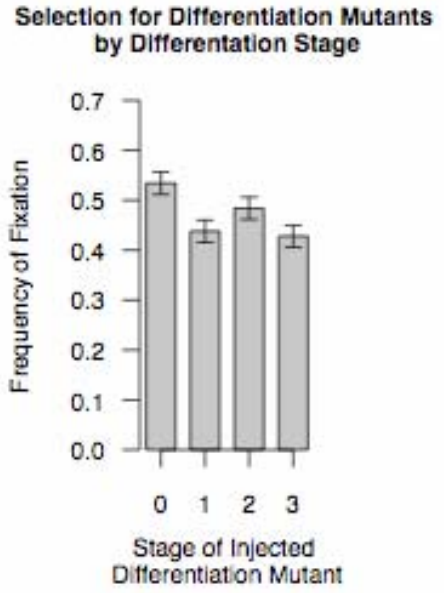

Figure 10. Fixation frequency of a differentiation knock-out mutant as a function of stage the mutation arose in.

Experiment 4b: Effect of stochastic selfish-cell mutations

Our second experiment in Part 4 used our 'stochastic mutation' protocol to impose a risk of differentiation knock-out accompanying each cell division, at a rate of 10^{-4} per daughter cell. Under these conditions, exponential growth in the cell population (interpreted as neoplasia) developed quickly (Figure 11). Traces of cell population sizes from individual runs show that even though differentiation knock-out mutations frequently occurred, most quickly went extinct due to stochastic drift (Figure 12). If they survived long enough, they eventually triggered exponential growth due to interaction with the homeostatic feedback mechanisms (Figure 12).

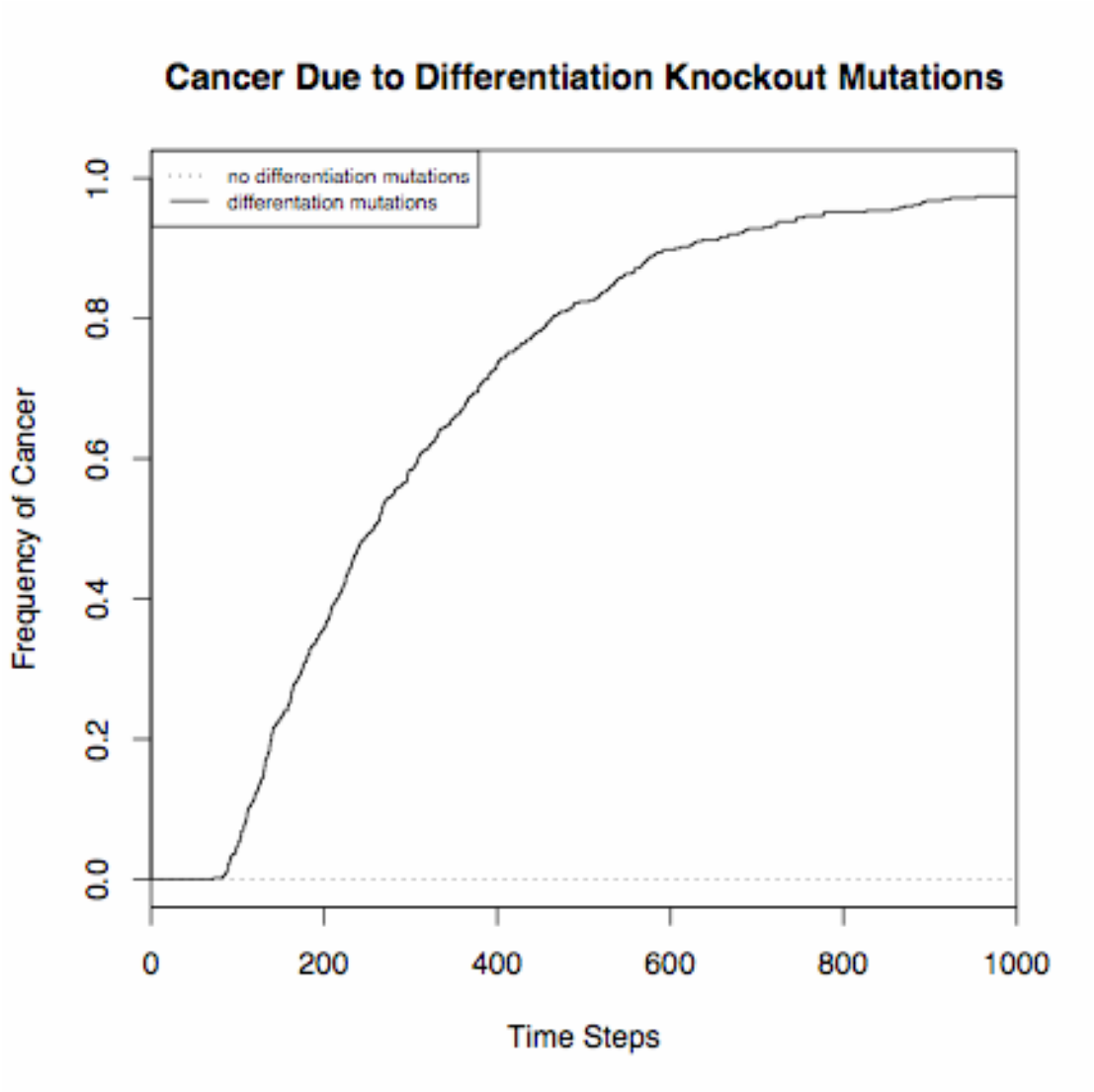

Figure 11. Kaplan-Meier curves showing the effect of stochastic differentiation knock-out mutation on the time until exponential cell growth (cancer). The solid line represents 500 trials during which differentiation loss-of-function mutations occurred at a rate of 10^{-4} per cell division. The dotted line represents 500 trials in which differentiation mutations were not allowed.

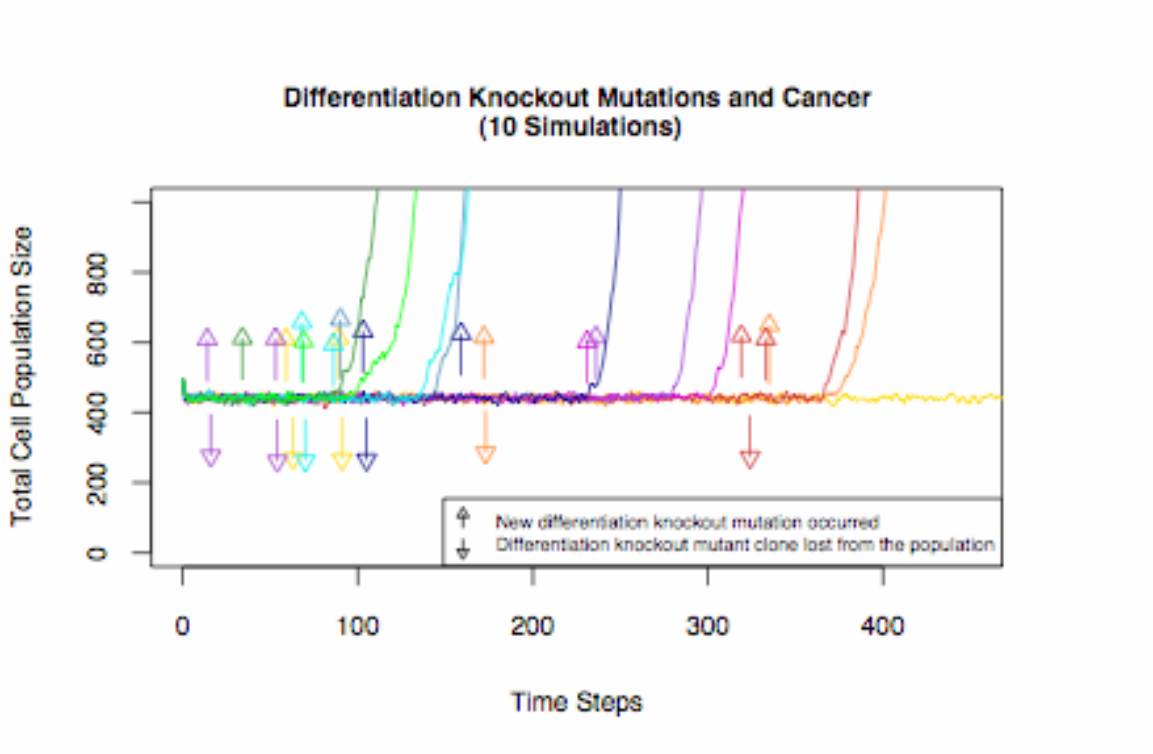

Figure 12. Cell population size in response to stochastic occurrence of differentiation knock-out mutations. The total population size for over 400 time steps is pictured here for 10 different runs in different colors. Color-matched arrows indicate when a new differentiation knock-out mutation occurred, and when the resulting mutant clone went to extinction. Note that the new differentiation knock-out mutations that are not lost from the population develop into cancer with a lag time of between 70 and 150 time steps. One run (in yellow) never progressed to cancer even though it acquired and lost several differentiation knock-out mutations. In each run, homeostasis of cell numbers can be seen up until the appearance of a differentiation knock-out mutation.

Discussion

Summary of key findings

We have shown that, in principle, ongoing serial cell differentiation in mature tissues can suppress cell level selection and somatic evolution. We suggest that this pattern of cell differentiation was a critical step in the evolution of large long-lived metazoans with extensive cell turnover. Serial cell differentiation makes it possible to segregate replicative activity and population self-renewal into different cell compartments, so that no compartment possesses all the attributes necessary for somatic evolution.

Our simulation experiments confirmed our a priori prediction that self-renewal in transient amplifying cells would allow rapid somatic evolution. In addition, they revealed that asymmetric cell divisions are one of the forms of population self-renewal that can increase the rate of somatic evolution. Symmetric cell division without self-renewal suppresses somatic evolution by constantly flushing non-stem mutant cells from the tissue. Any form of self-renewal, including asymmetric cell division, disrupts this flushing dynamic, allowing the sequential accumulation of multiple selfish-cell mutations.

Implications for the evolutionary transition to multicellularity

The evolution of multicellularity from preexisting unicellular life is one example of historical events during evolution, in which new kinds of biological ‘individuals’ have emerged from collections of previously existing entities. In each of these ‘transitions in individuality’, selection at the level of the newly emergent individual is thought to have created mechanisms to suppress internal selection among its subunits, which would otherwise disrupt its integrity and lower its fitness [9-12]. Several such mechanisms have been proposed for the evolutionary transition to multicellular life. Buss (1987) argued for the central role of germ-line segregation [9], while Queller [22] emphasized the importance of a single-celled stage in the life cycle. Both of these mechanisms may be important to mitigating long-term somatic evolution across many organismal generations.

Here we propose another mechanism that has long been known to exist, but the functional significance of which has not previously been examined. Like the mechanisms mentioned above, serial differentiation suppresses somatic evolution across generations of multicellular organisms. In addition, it also suppresses the short-term somatic evolution that can have significant consequences within the life span of a single organism that is large and long-lived [56].

Testing our conclusions against empirical data

The main purpose of this study was to test a hypothesis for the evolutionary origin and function of an already-familiar pattern of cell differentiation in large metazoans. Given that the existence of this pattern is not in doubt, what testable predictions follow from our hypothesis that could be used to reject or support it? Several predictions concerning the architecture of normal, healthy tissues in long-lived metazoans can be tested experimentally or even by careful observation. One such prediction is that large and proliferating (e.g., non-stem) cell populations are not expected to be self-renewing. Another prediction is that when non-stem cells divide, both daughter cells must be committed to further differentiation. Thus, cell division should be intimately tied to differentiation. Dividing without committing to differentiation is the definition of self-renewal and we have shown that this is a risk for somatic evolution and associated pathologies.

Opportunities for testing our hypothesis arise where our predictions appear to conflict with the prevailing view of cell differentiation in certain tissues. For example, it has been suggested that mouse pancreas ß-cells self-renew without contribution from stem cells [2]. If confirmed, this would either cast doubt on our conclusions, or raise questions about how these cell populations avoid rampant somatic selection and accompanying pathologies. Other interpretations of the evidence are possible, however. The presence of stem cells may have been overlooked because, contrary to expectations, they also produce insulin, and thus are not easily distinguished from non-stem cells. One hypothesis proposed by Dor et al. and consistent with our results is that not only terminally differentiated ß-cells, but also unipotent ß-stem cells and TACs produce insulin [2]. If this is true, then serial differentiation in a cryptic form may be present even in pancreas ß-cells, as our hypothesis would predict.

As a second example, the hematopoietic system is commonly assumed to involve self-renewing TACs [4,23]. We predict that closer study will reveal these morphologically indistinguishable cells to be functionally stratified into a series of non-self-renewing TAC stages. Our prediction is that when such a TAC divides, its daughter cells are one division closer to terminal differentiation than the parental cell. This must involve some form of ‘counter’ for mitoses such that cells that are more generations removed from their ancestral stem cell in the tissue are closer to terminal differentiation than are cells that are fewer generations removed from their ancestral stem cell. This prediction is supported by the observation that even among hematopoietic cells that appear similar to self-renewing stem cells based on cell surface markers, some have limited self-renewal capacity, as is typical of TACs [24]. In solid tissues such as intestinal epithelium, the mitosis ‘counter’ might be implemented by position in the crypt, as long as proliferating cells consistently move in one direction as they divide and differentiate [25]. However, for the principles we have elucidated to apply, the functional sequence of differentiation stages need not always correspond directly to physical location.

Perhaps the most important counterexample to our hypothesis is the adaptive immune system, which contains large cell populations that are both self-renewing and actively dividing throughout life. The apparent reason for this exception to the general rule of serial differentiation is that the adaptive immune system depends on somatic evolution though clonal selection for its effectiveness [26, p.15]. According

to our hypothesis, serial differentiation greatly slows somatic evolution in most tissues, forestalling its pathogenic consequences into old age. Because this is not true of the adaptive immune system, it would be reasonable to expect pathologies arising through somatic evolution to manifest at much earlier ages in these cell populations than in other tissues. Indeed leukemia and lymphoma are unusual cancers in being relatively common at younger ages. Whether the adaptive immune system is also vulnerable to accelerated senescence relative to the rest of the body has not been closely investigated to our knowledge, but there are intriguing clues. Thymectomized adult mice that received a transplanted thymus enjoyed improved immune function. However, the improvement was significantly greater when the donor was newborn versus 33 months old [27, p. 45].

Whenever evolution has scaled organisms up from small and short-lived to larger and longer-lived, the potential for somatic evolution has increased [28]. Our model suggests that because somatic stem cells normally form a self-renewing cell compartment, they pose the highest tumorigenesis risk on a per-cell basis of any cells in a tissue. It is possible to increase the number of cells and the amount of cell turnover per organism without increasing the number or replicative activity of somatic stem cells, simply by increasing the number of TAC stages. This is what we would expect to see in comparisons between species with different body size. This prediction is consistent with previous theory [12,28,29] and also with data showing a lower ratio of stem to fully differentiated cells in the feline hematopoietic system relative to that of the mouse [30].

Medical implications in general

If the hypothesis we propose for the control of somatic evolution is correct, it may have important medical implications. Both diseases involving uncontrolled cell proliferation (cancers), and those involving generalized loss of normal tissue function are candidates for conditions arising through the expression of unrestrained somatic selection. For this reason, research into the etiology of these diseases should include a focus on post-embryonic patterns of cell differentiation.

If our hypothesis is correct, it may help in understanding why senescence and general loss of tissue function tends to be a typical part of aging [18]. It may also be highly relevant to conditions of accelerated senescence, or progeria syndromes. In this regard, it is significant that patients with Hutchinson-Gilford Progeria Syndrome appear normal at birth, while the disease is usually diagnosed near the end of the second year of life [31]. This pattern suggests that fetal, but not post-natal, development is normal.

Implications for cancer

The conventional wisdom is that cancer results from genetic lesions causing excessive cell proliferation. The results presented here suggest a more nuanced picture of the dynamics of carcinogenesis. We have shown that in the context of normal serial differentiation, a genetic lesion causing excessive division by its host non-stem cell will not result in uncontrolled cell proliferation. On the other hand a

genetic lesion that has no direct effect on division rate, but that disrupts normal cell differentiation, may quickly lead to uncontrolled cell proliferation.

For purposes of prevention and early detection, it is critical to understand the earliest stages in carcinogenesis. Our results may be useful in this regard. It is clear that any self-renewing cell population is at high risk of somatic evolution and thus of carcinogenesis. It is also clear however that there are two distinct ways this situation may arise. One is that a normally self-renewing population of stem cells may acquire mutations that increase replication or reduce apoptosis. Such 'selfish-cell' mutations will immediately be favored by selection among cells, and may rapidly go to fixation within the cell compartment if not lost by genetic drift. This route is facilitated by any factors that increase the rate of cell replication, including factors that are normal in themselves, such as healing [32], or cyclic growth of female breast and reproductive tissues [33,34].

The second basic route to tumorigenesis begins with non-stem cells such as TACs. These compartments are normally large and proliferative but not self-renewing. Here somatic selection among cells will not favor increased replication or survival unless preceded by an initiating mutation that blocks normal differentiation and thereby converts the clone carrying it into a self-renewing population. After this initiating step, all further selfish-cell mutations will spread and accumulate through somatic selection. In contrast to the stem cell pathway, this non-stem pathway is not facilitated simply by higher rates of cell turnover and replication. Instead it is highly dependent on a specific class of mutations, and thus may be more stochastic and unpredictable, but also more sensitive to specific mutagens that tend to target genes involved in cell differentiation.

It seems likely that distinguishing between these two distinctive pathways in early carcinogenesis may reconcile what could otherwise appear to be conflicting evidence about the earliest steps of tumorigenesis. Moreover, tumors resulting from these two different early pathways may retain persistent differences that are relevant to medical strategies for their prevention, detection, and treatment. The potential importance of stem cells in tumorigenesis has received considerable attention recently, partly due to the recognition of cancer stem cells [35]. Some research models have therefore focused on the role of somatic stem cells and their differentiation patterns [36], Our results, however, emphasize that neoplastic cells with a stem-like capacity for unlimited self-renewal can potentially arise from mutations in either stem or non-stem normal cells.

The role in tumorigenesis of normal mechanisms for tissue homeostasis has received little attention, but our results suggest this might be a fruitful avenue of research. One intriguing implication of our results is that the structure of the feedback controls that maintain tissue homeostasis can have a dramatic impact on the probability that the tissue will develop cancer. Without any redundant checks on cell proliferation in this simplified model, a differentiation knock-out mutation generated uncontrolled growth if the mutant cell was not quickly cleared by stochastic background mortality (Fig. 9). This single mutation generated uncontrolled proliferation though an interaction with the negative feedback controlling the production of terminally differentiated cells. When a differentiation knock-out mutant arose, the mutant cell's progeny no longer contributed to the terminally differentiated

compartment. Thus, when the terminally differentiated compartment dropped below its equilibrium level, stem cells and TACs were stimulated to proliferate, including mutant cells. The mutant clone grew, taking over more of its compartment and thereby further reducing the tissue's capacity to replenish the terminally differentiated cells. A vicious cycle ensued in which the more the mutant clone grew, the more the terminally differentiated compartment signaled the need for more proliferation. The result was an exponential expansion of the mutant clone. These results are similar to those of a computational model of skin in which differentiation was based on distance from the basal membrane mediated through mechanical and adhesive forces in the tissue [37]. Rashbass et al. (1996) found that disruption of the differentiation responses of the cells could lead to exponential cell growth. Of course, in a real tissue, the growth of the mutant clone could be limited by additional proliferative repression and by nutrient availability. Our model highlights the importance of the relatively understudied mechanisms of tissue homeostasis.

Based on our results, we predict that genetic lesions disrupting differentiation are often critical to tumor initiation. Because of the small size and low activity of somatic stem compartments, it seems unlikely that any tissue with serial differentiation could accumulate the mutations necessary for cancer unless differentiation is disrupted very early in the process. Considerable evidence supports this view. For example, blocked differentiation is a frequent theme in the development of hematopoietic malignancies [38, p. 470]. Similarly, lesions in APC, a gene involved in differentiation in crypts of the intestine [39], are considered "gatekeeper" lesions that initiate colonic adenomatous polyps and are necessary for the future development of colorectal cancer [40]. In another tissue, recent genome-wide analyses have shown that most alterations in acute lymphoblastic leukemia target the B cell differentiation pathways [41].

It is worth noting that many of the cell characteristics considered to be hallmarks of cancer are examples of 'selfish-cell' traits that are favored by somatic selection when it is operating These include traits that reduce intrinsic mortality rate, such as evasion of apoptosis; as well as traits that increase intrinsic replication rate, such as self-sufficiency in growth signals, and insensitivity to anti-growth signals [42]. When we understand that somatic selection is the underlying process driving carcinogenesis, it is clearly no coincidence that, "most if not all cancers have acquired the same set of functional capabilities during their development, albeit though various mechanistic strategies" [42, p. 58]. Furthermore, when we understand the central role that disruption of normal differentiation plays in allowing somatic selection, this suggests that early loss of differentiation may deserve to be recognized as one of the most fundamental, and earliest to appear, of the hallmarks of cancer. If true, this could point toward important directions in using genetic tests to screen for cancer, or even sporadic cancer risk, before the first directly observable symptoms appear.

One active area of cancer research involves the use of chemotherapeutic agents that act by promoting cell differentiation [43]. The feedback loops that maintain tissue homeostasis are likely to modulate the efficacy of these differentiation agents. It may be important to interrupt those feedback loops so as to prevent the cancer cells from increasing their proliferation rate to compensate for cells lost to differentiation [19,21,23,43-56].

Some of the ideas we have explored in this study have been raised previously in the specific context of carcinogenesis. Cairns proposed that the elaborate system of somatic stem cells, transient amplifying cells (TACs) and terminally differentiated cells in a gastrointestinal (GI) crypt is an adaptation to suppress cancer [19]. Mutations that occur in the TACs of a crypt are destined to be sloughed off in a matter of days [5]. Only the self-renewing stem cell population, or mutant cells that no longer differentiate properly, are vulnerable to mutations that may establish an expanding clone and become fixed in a compartment. In adults, stem cells are typically quiescent and few in number [5,57]. These traits may be organismal adaptations that both reduce the frequency of somatic mutations and limit the role of somatic selection relative to genetic drift in this compartment [57]. We suggest that serial differentiation may be a general principle for the suppression of somatic evolution and thus neoplasia not just in GI crypts but in all tissues with extensive cell proliferation. Even in the less physically structured hematopoietic system, serial differentiation may serve to limit somatic cell selection.

Previous agent-based models of carcinogenesis have been used to explore theories of the clonal evolution that drives neoplastic progression [58-62]. Mathematical models have also been used (e.g., [36,63]). While these previous studies have all highlighted the detrimental effects of somatic evolution, they have not focused on the question of what normally suppresses somatic evolution, and thus have not completely explained what key turning points cause healthy tissue to become neoplastic.

Kirkland has developed a model of differentiation in the hematopoietic system that is conceptually similar to ours. In Kirkland's model, cell stages are not compartmentalized, but rather, "stemness" is represented as a continuous variable [64]. A probability density function determines the stemness of daughter cells. Although "stemness" was represented as a continuous variable in Kirkland's model, and as a discrete variable in our model, the same principles apply in both, and both lead to similar conclusions: If daughter cells can be as undifferentiated as the parent cell, then self-renewal occurs and the tissue is vulnerable to somatic evolution. Only tissues in which the daughter cells are more differentiated than the parental cell are protected from somatic evolution. The same concept also applies to theories of stem cell niches where extrinsic properties of the microenvironment determine the differentiation state of cells [65]. In this case, differentiation is determined by migration and we would predict that non-stem cells should migrate out of but not into the stem cell niche.

Tomlinson and Bodmer also developed a similar model with self-renewing compartments of stem cells, along with TACs and differentiated cells [63] and homeostatic feedback mechanisms [63,66]. Several authors have extended this model [42, 43]. They found that failures of apoptosis or differentiation led either to clonal expansion to higher equilibrium levels (benign tumors) or to extended exponential growth of the cell population (cancer). Nowak et al. showed that a linear model of a crypt, with a single stem cell and asymmetric division at all cell stages limits somatic evolution and slows progression to cancer [57]. Frank et al. also analyzed a model of a crypt in which stem cells and TACs could have different mutation and division rates [29]. They found that differences in mutation rates between the cell compartments

changed the optimal distribution of cell divisions among the compartments to minimize somatic evolution [29].

Several previous authors have proposed that the failure of cell differentiation plays an important role in tumorigenesis [19,21,43-46]. We have expanded on this idea by showing how cell differentiation prevents the onset of neoplasia by controlling cells' selective environment and thereby suppressing somatic evolution. Similarly, several previous authors have recognized that somatic evolution occurs and is probably central to neoplastic progression [23,47-51] and that trade-offs in evolution and the selective pressure of cancer may have shaped multicellular genomes and bodies [52-56]. Here we have shown how somatic evolution is normally controlled, and how that control breaks down during the events preceding tumorigenesis.

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References cited

1. Jablonka E, Lamb MJ (1998) Epigenetic Inheritance in Evolution. *J Evol Biol* 11: 159-183.

2. Dor Y, Brown J, Martinez OI, Melton DA (2004) Adult pancreatic beta-cells are formed by self-duplication rather than stem-cell differentiation. *Nature* 428: 41-46.

3. Potten CS, Schofield R, Lajtha LG (1979) A Comparison of Cell Replacement in Bone Marrow, Testis and Three Regions of Surface Epithelium. *Biochimica et Biophysica Acta* 560: 281-289.

4. Fuchs E, Segre JA (2000) Stem Cells: A New Lease on Life. *Cell* 100: 143-155.

5. Potten CS (1998) Stem Cells in Gastrointestinal Epithelium: Numbers, Characteristics and Death. *Philosophical Transactions of the Royal Society of London - Series B* 353: 821-830.

6. Wright NA (2000) Epithelial stem cell repertoire in the gut: clues to the origin of cell lineages, proliferative units and cancer. *Int J Exp Pathol* 81: 117-143.

7. Watt FM (1998) Epidermal Stem Cells: Markers, Patterning and the Control of Stem Cell Fate. *Philosophical Transactions of the Royal Society of London - Series B* 353: 831-837.

8. van den Brink GR, Offerhaus GJ (2007) The morphogenetic code and colon cancer development. *Cancer Cell* 11: 109-117.

9. Buss LW (1987) *The Evolution of Individuality*. Princeton, NJ: Princeton University Press.

10. Maynard Smith J, Szathmary E (1995) *The Major Transitions in Evolution*. Oxford, UK: Oxford University Press.

11. Klekowski EJ (1988) *Mutation, Developmental Selection and Plant Evolution*. New York: Columbia University Press.

12. Michod RE (1997) Cooperation and Conflict in the Evolution of Individuality. I. Multilevel Selection of the Organism. *American Naturalist* 149: 607-645.

13. Kavanagh KD (2003) Embedded molecular switches, anticancer selection, and effects on ontogenetic rates: A hypothesis of developmental constraint on morphogenesis and evolution. *Evolution* 57: 939-948.

14. Loeb LA (1991) Mutator phenotype may be required for multistage carcinogenesis. *Cancer Research* 51: 3075-3079.

15. Bullen TF, Forrest S, Campbell F, Dodson AR, Hershman MJ, et al. (2006) Characterization of epithelial cell shedding from human small intestine. *Lab Invest* 86: 1052-1063.

16. Jandl JH (1996) *Blood*. Boston: Little, Brown and Co.

17. Rajagopalan H, Nowak MA, Vogelstein B, Lengauer C (2003) The significance of unstable chromosomes in colorectal cancer. *Nature Reviews Cancer* 3: 695-701.

18. Charlton BG (1996) Endogenous Parasitism: A Biological Process with Implications for Senescence. *Evolutionary Theory* 11: 119-124.

19. Cairns J (1975) Mutation Selection and the Natural History of Cancer. *Nature* 255: 197-200.

20. Cox DR, Oakes D (1984) *Analysis of Survival Data*. London; New York: Chapman and Hall. pp201 p.

21. Tomlinson IPM, Bodmer WF (1995) Failure of Programmed Cell Death and Differentiation as Causes of Tumors: Some Simple Mathematical Models. *Proceedings of the National Academy of Sciences of the United States of America* 92: 11130-11134.

22. Queller DC (2000) Relatedness and the fraternal major transitions. *Philos Trans R Soc Lond B Biol Sci* 355: 1647-1655.

23. Weissman IL (2000) Stem Cells: Units of Development, Units of Regeneration, and Units in Evolution. *Cell* 100: 157-168.

24. McKenzie JL, Gan OI, Doedens M, Wang JC, Dick JE (2006) Individual stem cells with highly variable proliferation and self-renewal properties comprise the human hematopoietic stem cell compartment. *Nat Immunol* 7: 1225-1233.

25. Batlle E, Henderson JT, Beghtel H, van den Born MM, Sancho E, et al. (2002) Beta-catenin and TCF mediate cell positioning in the intestinal epithelium by controlling the expression of EphB/ephrinB. *Cell* 111: 251-263.

26. Janeway, C.A., P. Travers, W. Walport & M. Shlomchik (2001) Immunobiology. Garland Publishing, New York.

27. Goldsby, R.A., T.J. Kindt, B.A. Osborne, & J. Kuby. 2003. Immunology. 5th edn. W.H. Freeman & Co., New York.

28. Leroi AM, Koufopanou V, Burt A (2003) Cancer selection. *Nat Rev Cancer* 3: 226-231.

29. Frank SA, Iwasa Y, Nowak MA (2003) Patterns of cell division and the risk of cancer. *Genetics* 163: 1527-1532.

30. Abkowitz JL, Golinelli D, Harrison DE, Guttorp P (2000) In Vivo Kinetics of Murine Hemopoietic Stem Cells. *Hematopoiesis* 96: 3399-3405.

31. DeBusk FL (1972) The Hutchinson-Gilford progeria syndrome. Report of 4 cases and review of the literature. *J. Pediatr* 80: 697-724.

32. Maley, C. C. (2007). Multistage carcinogenesis in Barrett's esophagus. *Cancer Letters* 245(1-2): 22-32.

33. Henderson BE, Ross RK, Judd HL, Krailo MD, Pike MC (1985) Do regular ovulatory cycles increase breast cancer risk? *Cancer* 56: 1206-1208.

34. Eaton, SB & Eaton, SB (1999) Breast Cancer in Evolutionary Context. Pp. 429-442 in: Trevathan WR, Smith EO, McKenna JJ, editors. *Evolutionary Medicine*. Oxford: Oxford University Press.

35. Reya T, Morrison SJ, Clarke MF, Weissman IL (2001) Stem cells, cancer, and cancer stem cells. *Nature* 414: 105-111.

36. Dingli D, Traulsen A, Michor F (2007) (A)symmetric stem cell replication and cancer. *PLoS Comput Biol* 3: e53.

37. Rashbass J, Stekel D, Williams ED (1996) The use of a computer model to simulate epithelial pathologies. *J Pathol* 179: 333-339.

38. Weinberg, R. A. (2007) The Biology of Cancer. New York: Garland Science.

39. Stappenbeck TS, Wong MH, Saam JR, Mysorekar IU, Gordon JI (1998) Notes from some crypt watchers: regulation of renewal in the mouse intestinal epithelium. *Curr Opin Cell Biol* 10: 702-709.

40. Kinzler KW, Vogelstein B (1997) Cancer-susceptibility genes. Gatekeepers and caretakers. *Nature* 386: 761, 763.

41. Mullighan CG, Goorha S, Radtke I, Miller CB, Coustan-Smith E, et al. (2007) Genome-wide analysis of genetic alterations in acute lymphoblastic leukaemia. *Nature* 446: 758-764.

42. Hanahan D, Weinberg RA (2000) The hallmarks of cancer. *Cell* 100: 56-70.

43. von Wangenheim K-H, Peterson H-P (1998) Control of Cell Proliferation by Progress in Differentiation: Clues to Mechanisms of Aging, Cancer Causation and Therapy. *Journal of Theoretical Biology* 193: 663-678.

44. Tzen C-Y, Estervig DN, Minoo P, Filipak M, Maercklein PB, et al. (1988) Differentiation, Cancer, and Anticancer Activity. *Biochemical Cell Biology* 66: 478-489.

45. Hedrick L, Cho KR, Fearon ER, Wu TC, Kinzler KW, et al. (1994) The DCC gene product in cellular differentiation and colorectal tumorigenesis. *Genes Dev* 8: 1174-1183.

46. Potter JD (2001) Morphostats: a missing concept in cancer biology. *Cancer Epidemiol Biomarkers Prev* 10: 161-170.

47. Nowell PC (1976) The clonal evolution of tumor cell populations. *Science* 194: 23-28.

48. Nederbragt H (1997) Hierarchical organization of biological systems and the structure of adaptation in evolution and tumorigenesis. *J Theor Biol* 184: 149-156.

49. Heppner G, Miller F (1998) The Cellular Basis of Tumor Progression. *International Review of Cytology* 177: 1-56.

50. Merlo LM, Pepper JW, Reid BJ, Maley CC (2006) Cancer as an evolutionary and ecological process. *Nat Rev Cancer* 6: 924-935.

51. Schwab ED, Pienta KJ (1996) Cancer as a complex adaptive system. *Med Hypotheses* 47: 235-241.

52. Greaves M (2007) Darwinian medicine: a case for cancer. *Nat Rev Cancer* 7: 213-221.

53. Crespi B, Summers K (2005) Evolutionary Biology of Cancer. *Trends in Ecology and Evolution* 20: 545-552.

54. Crespi BJ, Summers K (2006) Positive selection in the evolution of cancer. *Biol Rev Camb Philos Soc* 81: 407-424.

55. Summers K, da Silva J, Farwell M (2002) Intragenomic conflict and cancer. *Med Hypotheses* 59: 170-179.

56. Zimmer C (2007) Evolved for cancer? *Sci Am* 286: 68-74, 75A.

57. Nowak MA, Michor F, Iwasa Y (2003) The linear process of somatic evolution. *Proc Natl Acad Sci U S A* 100: 14966-14969.

58. Gonzalez-Garcia I, Sole RV, Costa J (2002) Metapopulation dynamics and spatial heterogeneity in cancer. *Proc Natl Acad Sci U S A* 99: 13085-13089.

59. Spencer SL, Gerety RA, Pienta KJ, Forrest S (2006) Modeling somatic evolution in tumorigenesis. *PLoS Comput Biol* 2: e108.

60. Maley CC, Forrest S (2000) Exploring the relationship between neutral and selective mutations in cancer. *Artificial Life* 6: 325-345.H57

61. Maley CC, Reid BJ, Forrest S (2004) Cancer prevention strategies that address the evolutionary dynamics of neoplastic cells: simulating benign cell boosters and selection for chemosensitivity. *Cancer Epidemiol Biomarkers Prev* 13: 1375-1384.

62. Schwab ED, Pienta KJ (1997) Modeling signal transduction in normal and cancer cells using complex adaptive systems. *Med Hypotheses* 48: 111-123.

63. Johnston MD, Edwards CM, Bodmer WF, Maini PK, Chapman SJ (2007) Mathematical modeling of cell population dynamics in the colonic crypt and in colorectal cancer. *Proc Natl Acad Sci U S A* 104: 4008-4013.

64. Kirkland MA (2004) A phase space model of hemopoiesis and the concept of stem cell renewal. *Exp Hematol* 32: 511-519.

65. Arai, F. & Suda, T. (2007) Maintenance of quiescent hematopoietic stem cells in the osteoblastic niche. *Ann N Y Acad Sci*.

66. d'Onofrio A, Tomlinson IP (2007) A nonlinear mathematical model of cell turnover, differentiation and tumorigenesis in the intestinal crypt. *J Theor Biol* 244: 367-374.

67. Hafen E, Stocker H (2003) How are the sizes of cells, organs, and bodies controlled? *PLoS Biol* 1: E86.

68. Fisher JW (2003) Erythropoietin: physiology and pharmacology update. *Exp Biol Med* (Maywood) 228: 1-14.

69. Morrison SJ, Kimble J (2006) Asymmetric and symmetric stem-cell divisions in development and cancer. *Nature* 441: 1068-1074.

70. Booth C, Potten CS (2000) Gut Instincts: Thoughts on Intestinal Epithelial Stem Cells. *The Journal of Clinical Investigation* 105: 1493-1499.

71. Kim KM, Shibata D (2002) Methylation reveals a niche: stem cell succession in human colon crypts. *Oncogene* 21: 5441-5449.