Heterogeneity and ‘memory’ in stem cell populations

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Abstract
Modern single cell experiments have revealed unexpected heterogeneity in apparently functionally ‘pure’ cell populations. However, we are still lacking a conceptual framework to understand this heterogeneity. Here, we propose that cellular memories—changes in the molecular status of a cell in response to a stimulus, that modify the ability of the cell to respond to future stimuli—are an essential ingredient in any such theory. We illustrate this idea by considering a simple age-structured model of stem cell proliferation that takes account of mitotic memories. Using this model we argue that asynchronous mitosis generates heterogeneity that is central to stem cell population function. This model naturally explains why stem cell numbers increase through life, yet regenerative potency simultaneously declines.

1. Introduction
Advances in experimental methods are allowing us to profile the molecular state of hundreds of thousands of individual cells in a single experiment, and explore cellular identities in unprecedented detail (Shapiro et al 2013 and Stuart and Satija 2019). Accordingly, a number of efforts to catalogue the cellular diversity in multicellular organisms are now well under way, including the human cell atlas (Regev et al 2017), the mouse cell atlas (Han et al 2018), and maps of various model organisms including D. rerio (Wagner et al 2018), D. melanogaster (Karaikos et al 2017), C. elegans (Cao et al 2017), the planarian S. mediterranea (Plass et al 2018 and Fincher et al 2018) and the cnidarian N. vectensis (Seb-Pedrós et al 2018). These efforts are important not only to build our understanding of healthy biology, but also to determine how different cellular identities, and the balance of cell types present in tissues and organs, change with disease and age (Regev et al 2017).

Central to these efforts is the desire to properly understand the mapping from cellular molecular ‘states’ (i.e. patterns of gene and protein expression, or other regulatory molecules, within the cell) to cell ‘types’, ‘fates’ or functions (Casey et al 2020). However, despite tremendous progress in this area, the regulatory principles that underpin this mapping are still unclear. Indeed, despite its centrality to our understanding of multicellular life, we still do not have a clear notion for what we mean by a cell ‘type’ (Clevers et al 2017 and Greulich et al 2020).

It is worth noting that until the pioneering work of John Gurdon and co-workers in the 1970s it was not even apparent if each cell in the adult organism possessed the same genome, or only the portion of the genome it needed to perform its particular function (Gurdon et al 1975). We now know that most adult cells possess essentially the same complement of genetic information (Allis et al 2015), yet vastly different patterns of gene expression are observed between cells. This is a remarkable fact—and one that allows for the possibility that individual cells can be reprogrammed in a myriad of ways, thus paving the way for personalized regenerative medicine (Rackham et al 2016)—the consequences of which we are still exploring.

Typically, cell types are defined informally based on experimentally observable features such as cell morphology, gene and protein expression patterns and the ability to perform specialised functions. Yet, both cellular form and function are complex issues that depend on the cell’s environmental context and its developmental history (Clevers et al 2017). Fur-
thermore, any practical definition of form, function or ‘type’ is contingent on what is experimentally observable, and some aspects of cellular character, such as developmental history, are hard to observe and harder still to quantify.

To deconvolute these issues, developmental biologists have traditionally mapped adult cell identities to cellular genealogies, since much of the cellular heterogeneity observed in the adult organism is established during development (Davidson and Levin 2005). For example, distinct cell types are commonly associated with their germ layer of origin (Remak 1855). In eutelic organisms such as C. elegans (which contain a fixed number of cells at adulthood) we can go further, and whole organism lineage trees have long been used to trace the developmental histories of diverse adult cell types (Sulston et al. 1983).

Similar lineage trees have also been inferred in complex organisms such as zebra fish and mouse, using cell lineage tracing by imaging (Keller et al. 2010 and McDole et al. 2018) and, increasingly, CRISPR-CAS9 induced genetic scar sequencing (Alemany et al. 2018, McKenna et al. 2016 and Kalhor et al. 2018), among other methods (Kretzschmar and Watt 2012 and Woodward et al. 2017 and Kester and van Oudenaarden 2018). These lineage maps help show how distinct cell types are produced during development in a regular and reproducible way. Lineage tracing studies using viral barcoding or genetic reporters have also helped elucidate the regenerative potential of individual stem cells and hence the mechanisms by which the numbers of cells under continuous turnover in the adult organism are maintained (Lemischka et al. 1986, Lu et al. 2019, Nguyen et al. 2014 and Snippert et al. 2010).

From these studies it is now becoming increasingly clear that many functional cell types are highly heterogeneous in the sense that cells in a variety of different molecular states (i.e. expressing different patterns of genes and proteins) may all perform the same function. Mathematically, the mapping from cell states to molecular states (i.e. expressing different patterns of genes and proteins) may all perform the same function. Mathematically, the mapping from cell states to molecular states (i.e. expressing different patterns of genes and proteins) may all perform the same function. However, if the accumulation of prior cell divisions confers distinct biases to individual stem cells then mitotic history may be an important regulator of HSC population function.

There is emerging evidence that this is the case. A number of recent reports have found that repopulation activity is concentrated in the most quiescent HSC subpopulations (Wilson et al. 2008, Foudi et al. 2009, Takizawa et al. 2011 and Qiu et al. 2014) and, furthermore, the accumulation of cell divisions is directly associated with loss of stem cell potency both during native hematopoiesis and under conditions of stress (Bernitz et al. 2016 and Säwén et al. 2016, Qiu et al. 2014, Wilson et al. 2008, Nygren and Bryder 2008, Foudi et al. 2009 and Araï et al. 2019). Although the precise reasons for this association are unclear, it is likely that HSCs are sensitive to the DNA damage that commonly occurs during cell division (Walter et al. 2015). Cells naturally acquire genetic alterations on cell division, and proliferation can thereby give rise to complex mosaics (Ju et al. 2017) that can significantly affect HSC population function (Haas et al. 2018). General mechanisms, such as telomere shortening, may also define a ‘mitotic clock’ that places a fundamental limit on HSC proliferative capacity in vivo (Harley et al. 1990, Notaro et al. 1997, Hills et al. 2009, Allsopp et al. 2003, Allsopp et al. 2003 and Werner et al. 2015). Remarkably, there is evidence that—however it is recorded—such a mitotic clock is encoded reliably enough that individual HSCs are able to ‘count’ cell divisions and thus have a ‘memory’ of their mitotic history (Bernitz et al. 2016).

Collectively these results indicate that mitotic memories play an important part in regulating stem cell population function. However, the precise role of mitotic memories is unclear. To explore putative roles for such memories further, here we will consider a simple model of stem cell proliferation as an age-structured process. To make ideas concrete, we will frame our model in the context of hematopoiesis, but note that comparable age-structured processes may play a central part in regulating stem cell dynamics in other contexts.
2. An age-structured model of stem cell proliferation

Age-structured processes arise whenever ageing affects the behaviour of individuals within a population, and age-structured models have accordingly been used to model a wide-range of biological and epidemiological processes (Li and Brauer 2008 and Auger et al 2008). Here we will model stem cell proliferation as an age-structured process. At the beginning we should stress that our model is not intended as a detailed molecular description of stem cell proliferation, but rather as a simple conceptual framework in which the implications of the connections between mitotic asynchrony and population heterogeneity can be explored.

To start we consider a population of HSCs proliferating under normal conditions in vivo and assume that stem cell divisions can be of three types: (1) symmetric S–S divisions, which produce two daughter stem cells; (2) asymmetric S–D divisions which produce one daughter stem cell and one differentiated daughter cell; and (3) symmetric D–D divisions, which produce two differentiated daughter cells. This is a common and long-standing simplifying assumption for models of stem cell proliferation in numerous contexts (McCulloch and Till 1960, Simons and Clevers 2011, Alcolea et al 2014, Frede et al 2016, Clayton et al 2007, Klein and Simons 2011, Greulich and Simons 2016, Lopez-Garcia et al 2010, Snippert et al 2010 and Klein et al 2010).

To encode mitotic memory we allow the propensities for S–S, S–D and D–D divisions to depend on the number $n$ of prior divisions that the stem cell has undergone. For simplicity, we will assume that all adult HSCs arise from a common population of founder cells during development—for example, hemogenic endothelial precursor cells (Bertrand et al 2010)—and begin counting divisions at the onset of adulthood. To allow for niche regulation of stem cell divisions we additionally assume that the local micro-environment of the cell is described by a single variable $m$ that also affects cell divisions. Without loss of generality we can assume that $0 \leq m \leq 1$ such that $m = 1$ implies a ‘strong’ niche that maximally stimulates self-renewal and $m = 0$ implies a ‘weak’ niche that does not support self-renewal. Thus, the variable $m$ provides a simple way to take account of the numerous niche factors that collectively regulate stem cell divisions.

To account for evolving division patterns, let $p_{11}(n, m), p_{10}(n, m)$ and $p_{00}(n, m)$ be the probabilities that a stem cell that has divided $n$ times previously will undergo S–S, S–D or D–D division respectively in the niche $m$. Thus, division probabilities are governed by a bivariate Bernoulli distribution, which may be written in general form as

\begin{align*}
p_{11}(n, m) &= p_n^2 + \sigma_n, \\
p_{10}(n, m) &= 2p_n(1 - p_n) - 2\sigma_n, \\
p_{00}(n, m) &= (1 - p_n)^2 + \sigma_n,
\end{align*}

where $p_n = p_n(m)$ is the probability that each daughter cell will adopt a stem cell identity in niche $m$ and $\sigma_n = \sigma_n(m)$ is the covariance between the daughter identities in niche $m$. If $\sigma_n = 0$ then daughter cell
identities are adopted independently of each other; while if \( \sigma_n \neq 0 \) then daughter cells are produced in regulated pairs.

Under this model stem cell numbers are described by the following set of ordinary differential equations

\[
\frac{dx_i}{dt} = -kx_0, \quad (1)
\]

\[
\frac{dx_i}{dt} = k(1 + \Delta_{i-1})x_{i-1} - kx_i \quad \text{for } i > 0, \quad (2)
\]

where \( x_i = x_i(m, t) \) is the expected number of stem cells that have divided \( i = 1, 2, \ldots \) times previously in niche \( m \), \( \Delta_i = \Delta_i(m) = p_{11}(n, m) - p_{00}(n, m) \) and \( \ln(2)/k \) is the expected cell cycle time. Note that for simplicity we do not explicitly include cell death, although loss from the stem cell pool is accounted for via D–D divisions. Note also that equations (1) and (2) implicitly assume that cell cycle times are exponentially distributed. However, it is known that cycle times are better described by an Erlang (or modified Erlang) distribution, that reflects the multi-cycle nature of the cell cycle (Yates et al 2017). Here, for mathematical convenience we will make this simplifying assumption, but note that future work could account more precisely for cell cycle time variation.

From these equations it is clear that the ability of a stem cell that has divided \( n \) times previously to self-renew in the niche \( m \) is quantified by the factor \( \Delta_n(m) \). In particular if \( \Delta_n(m) < 0 \) then D–D divisions predominate over S–S divisions and the niche is not strong enough to stimulate net self-renewal in the sub-population of cells that have divided \( n \) times previously (i.e. each cell division will produce less than one stem cell daughter on average). Conversely, if \( \Delta_n(m) > 0 \) then S–S divisions predominate over D–D divisions and the niche is able to net support self-renewal.

The total expected stem cell number \( X(t) = \sum_i x_i(t) \) is governed by

\[
\frac{dX}{dt} = k \sum_i \Delta_i(m)x_i. \quad (3)
\]

Thus, if \( \Delta_i(m) = 1 \) for all \( i \) then all stem cells in the pool undergo S–S divisions and the pool grows exponentially with time; if \( \Delta_i(m) = 0 \) for all \( i \) then \( dX/dt = 0 \) and the stem cell pool maintains homeostasis; and if \( \Delta_i(m) = -1 \) for all \( i \) then all stem cells in the pool undergo D–D divisions and the pool declines exponentially with time. These three special cases are not representative however, and in general the stem cell pool may expand or decline depending on the magnitude of

\[
S(m, t) = \sum_i \Delta_i(m)x_i(t), \quad (4)
\]

which represents the self-renewal ability of the population as a whole in niche \( m \), taking into account its age-structure and the effect of mitotic history on each individual cell’s self-renewal ability. In particular, if \( S > 0 \) then the stem cell pool will be expanding, while if \( S < 0 \) then the pool will be in decline.

Notably, both stochastic and deterministic (or instructive) dynamics are allowed in this framework, depending on the way that division history \( n \) and niche instruction \( m \) interact to co-regulate \( p \) and \( \sigma \). For example, if \( \sigma = p(1 - p) - 1/2 \) then the niche unambiguously instructs all divisions to be asymmetric (of type S–D); if \( \sigma = p^2 - 1/2 \) then the niche unambiguously instructs all divisions to be of type S–S; and if \( \sigma = (1 - p)^2 - 1/2 \) then the niche unambiguously instructs all divisions to be of type D–D.

To make this idea more apparent, an explicit model for the interplay between division history and niche instruction is needed. From a mathematical perspective, functional forms for \( p_n(m) \) and \( \sigma_n(m) \) are required to close the model. In principle there many ways that the interplay with division history and niche instruction could affect cell divisions. Very little is currently known about this interplay, except that division history diminishes self-renewal ability (Bernitz et al 2016, Arai et al 2019 and Säwén et al 2016). Since a simple model suffices to illustrate our point, we will therefore assume that daughter cells are produced independently of each other (i.e. \( \sigma_n(m) = 0 \) for all \( n, m \)) and the likelihood that daughter cells adopt a stem cell identity declines with division history (i.e. \( p_n(m) \) is monotonic decreasing in \( n \)). For illustrative purposes we will assume an exponential loss of niche sensitivity with mitotic history and set \( p_n(m) = m^p \). A schematic of this model is given in figure 2(A).

2.1. Model implications

Although clearly a simplification, this model illustrates how heterogeneity can arise in an age-structured proliferating stem cell population. It has a number of implications.

Firstly, within a defined niche (i.e. for \( 0 < m < 1 \) fixed), stem cell self-renewal ability will decline with number of divisions (see figure 2(B)). Juvenile stem cells will have a strong preference for symmetric S–S divisions that expand the stem cell pool. However, as individual stem cells accumulate cell divisions there will be a shift away from the S–S divisions that are characteristic of young cells and towards asymmetric S–D divisions that maintain but do not expand the stem cell pool. Finally, into old age it becomes increasingly difficult for the niche to maintain self-renewing divisions, and a bias towards D–D divisions begins to emerge (see figures 2(B) and (C)).

Secondly, the degree of stochasticity of stem cell divisions also changes with mitotic history. Because juvenile stem cells have a strong preference for S–S divisions, yet the balance between divisions shifts through mid-life and towards D–D divisions in later...
life, divisions of young and old stem cells are primarily instructive (i.e. unambiguously or strongly determined by the niche), while those through the middle of life are highly stochastic. The Shannon entropy is a simple information-theoretic way to quantify how stochastic an event is (Cover and Thomas 2012), and so can be used to quantify this shift (see figure 2(D)). In this case, the division entropy $H_n(m)$ in niche $m$ for a stem cell that has divided $n$ times previously is

$$H_n(m) = -\sum_{j=0}^{m} p_{ij}(n,m) \log_2 p_{ij}(n,m),$$

for $i,j = 0,1$.

Thirdly, while stem cell division propensities and self-renewal abilities naturally change with mitotic history, these age-related changes can be continuously re-set by the niche. Particularly, aged cells can be ‘reactivated’ by transient guidance from a more strongly instructive niche. For instance, if be ‘reactivated’ by transient guidance from a more strongly instructive niche, for example under conditions that promote emergency hematopoiesis, then individual cells can be rejuvenated in their self-renewal ability. Here $m = 0.95$ is taken as a representative ‘strong’ niche.
The essential characteristics of stem cell ageing suggest that despite its simplicity it may capture the dynamics described by equations (1) and (2) naturally give rise to an instructive mathematical model has only one free parameter, it provides a remarkable good fit to the data (see figure 3(A)), suggesting that despite its simplicity it may capture the essential characteristics of stem cell ageing.

In the context of ageing, the dynamics described by equations (1) and (2) naturally give rise to an evolving age-structured population in which each stem cell in the pool exhibits a different propensity for self-renewal depending on its particular mitotic history (see figures 2(B), 3(B) and (C)). Importantly, this means that the potency of the population as a whole depends on the collective dynamics of an inherently heterogeneous mix of cells with different innate regenerative abilities (see figures 3(B)–(D)).

Furthermore, in this age-structured setting, the distinction between stochastic and instructive divisions becomes opaque. Again the Shannon entropy can be used to assess division stochasticity. In particular, the weighted average

$$H(t) = \sum_n P_n(t) H_n,$$

where $P_n(t)$ is the proportion of cells in the population that have divided $n$ times previously, determines the stochasticity of divisions in the population as a whole, taking into account its mitotic heterogeneity. Analysis of this quantity indicates that generally more instructive dynamics will be observed in young individuals, with increasing stochasticity of divisions occurring with age (see figure 3(E)). Note that these population dynamics are qualitatively different to the dynamics at the single cell level, in which the entropy reaches a maximum for an intermediate number of divisions (see figure 3(E) cf figure 2(D)). Taken together this results highlight that changes in individual cell dynamics with age are highly nuanced: depending on mitotic history some cells will respond unambiguously to niche instruction while others will behave more or less stochastically, depending on the particular niche within which they are located. Under these conditions it may be practically impossible, nor preferable, to attempt to dissect this complexity since it is an inherent part of how hematopoiesis is regulated. In this sense, stem cell proliferation is highly contextual, and both stochastic and instructive.

Finally, because the propensity for symmetric divisions that deplete the stem cell pool increases with age, in this model it is expected that the pool will exhaust on ageing if the stem cell population is too proliferative. However, this does not appear to happen. Rather, it has been widely observed that the immunophenotypic stem cell pool increases with age, yet this increase is associated with loss of regenerative potential and increase in lineage bias (Pang et al. 2011, Sudo et al. 2000, Rossi et al. 2005, Morrison et al. 2005).
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1996 and Geiger et al 2013). Thus, maintenance of a viable stem cell pool with age requires that stem cells must either: (1) divide very rarely, or (2) enter into a state of protective dormancy once their self-renewal ability has been sufficiently compromised (e.g. once $\Delta_n < 0$). Thus, the notion that there must be a slowly cycling or dormant stem cell population arises naturally as a logical consequence of proliferation within an age-structured population. Both these options are possibilities. As already noted, it is well-known that the most potent HSCs divide only very rarely in vivo (Wilson et al 2008). Similarly, it has been proposed that the most potent LT-HSCs divide only 4 times before entering a state of permanent protective dormancy (Bernitz et al 2016 and Wilson et al 2008).

It is currently unclear if this is a hard limit or simply reflects the fact that most stem cells do not have time to divide more than four times within a single lifetime. However, it is notable that our model estimates that $\Delta_n < 0$ for $n \geq 5$, suggesting that the limit of four divisions may represent an important critical threshold above which the stem cell population loses net self-renewal ability. (Note that this does not exclude the possibility of self-renewal divisions at the individual cell level, see figure 2(B), or the possibility of stem cell rejuvenation under conditions of stress, see figure 2(E)).

3. Conclusions

We have argued that mitotic memories have an important role in regulating stem cell population function with age. More generally, cellular memories—broadly defined as changes in the molecular status of a cell in response to a stimulus, that modify the ability of the cell to respond to future stimuli—may have a central role in fate regulation in numerous other contexts (Yu et al 2016). Epigenetic mechanisms such as DNA methylation and histone modifications, are well-known to allow historical events to be encoded in stable, heritable modifications to the DNA and chromatin (Holliday and Pugh 1975, Razin and Riggs 1980, Bird 2002, Schübel 2005, Allis and Jenuwein 2016 and Gaydos et al 2014).

Remarkably, this memory storage can be sufficiently robust that it allows developmental programs to be reinitated in cells from epigenetic memory, thereby providing a ‘fossil record’ of development (Jadhav et al 2019). Such epigenetic records may play an important role in cell fate maintenance, regulation of differentiation, healthy ageing, and malignant transformation in many situations (Yu et al 2016, Hu and Shilatifard 2016, Haas et al 2018, Challen et al 2014 and Trowbridge et al 2009). Furthermore, it is also well-known that epigenetic alterations can confer substantial molecular and functional heterogeneity to otherwise ‘pure’ cell populations (Angermueller et al 2016 and Cheow et al 2016). In light of this evidence, it is likely that individual cells commonly carry complex personal sets of epigenetic alterations that collectively confer subtle biases to their behaviour that are hard to quantify. Consequently, the traditional strategy of organizing cells into discrete functional ‘types’ while undoubtedly practically useful, may ultimately be limited in its explanatory power since it does not fully acknowledge the subtle fecundity of cell biology.

Because they may be encoded in subtle, combinatorial ways, epigenetic memories are hard to measure and dissecting their effect on cell fates is a significant technical challenge. Advances in experimental methods that track the mitotic history (or more generally the epigenetic history) of individual cells in vivo will help elucidate the mechanisms by which functional memories are formed and propagated. We anticipate that live-cell imaging, genetic barcoding and fluorescent label retention strategies (Kester and van Oudenaarden 2018 and Schaniel and Moore 2009), in combination with mathematical, physical and statistical models will be particularly important in this regard.

4. Methods

4.1. Mice

Evi1-GFP knock-in mice were provided by Dr Kurokawa (The University of Tokyo, Japan). The gene recombination experiment safety and animal experiment committees at Kyushu University approved this study, and all experiments were carried out in accordance with the guidelines for animal and recombinant DNA experiments.

4.2. Flow cytometry and antibodies

The following monoclonal antibodies (Abs) were used for flow cytometry and cell sorting: anti-c-Kit (2B8, BD Biosciences, 1:100), -Sca-1 (E13-161.7, BD Biosciences, 1:100), -CD41 antibody (MWReg30, eBioscience, 1:100), -CD48 (HM48-1, Biolegend, 1:100), -CD150 (TC15-12F12.2, Biolegend, 1:100), and -CD34 (RAM34, BD Biosciences, 1:20). The following antibodies were used in combination for the depletion of lineage-positive cells: anti-CD4 (RM4-5, BD Biosciences, 1:100), -CD8 (53-6.72, BD Biosciences, 1:100), -CD117 (8C5, BD Biosciences, 1:100), and -Mac-1 (RB6-8C5, BD Biosciences, 1:100), and -Mac-1 (M1/70, BioLegend, 1:100).

4.3. Cell isolation

LT-HSCs were identified as Evi1-GFP + LSKCD41-CD48-CD150+CD34-cells in 4, 8, and 60 week-old mice. To isolate Evi1-GFP + LT-HSCs, Evi1-GFP mouse bone marrow cells were purified using a two-step protocol. First, LT-HSCs were enriched by positive selection for c-Kit expression using anti-CD117 immunomagnetic beads (130-091-224, Miltenyi Biotec Inc. 1:5 dilution).
Second, c-Kit + enriched cells were stained with a set of fluorophore-conjugated antibodies (lineage mix, anti-Scal, anti-c-Kit, anti-CD41, anti-CD48, anti-CD150, and anti-CD34). Estimates of total LT-HSC numbers were then calculated based on the total number of bone marrow mononuclear cells, frequency of LT-HSCs, and frequency of Evi1-GFP + cells.

5. Model fitting

Fitting of LT-HSC numbers to equation (3) was performed by minimizing the residual sum of squares between the experimental data and the model. Optimization was performed using the Nelder–Mead simplex algorithm, implemented in MATLAB® version 2018b (The MathWorks Inc., Natick, MA).

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