REVIEW
The biology and engineering of stem-cell control

Analeah O'Neill and David V. Schaffer[1]

Department of Chemical Engineering and the Helen Wills Neuroscience Institute, University of California, Berkeley, CA 94720-1462, U.S.A.

There is significant interest in studying stem cells, both to elucidate their basic biological functions during development and adulthood as well as to learn how to utilize them as new sources of specialized cells for tissue repair. Whether the motivation is basic biology or biomedical application, however, progress will hinge upon learning how to better control stem-cell function at a quantitative and molecular level. There are several major challenges within the field, including the identification of new signals and conditions that regulate and influence cell function, and the application of this information towards the design of stem-cell bioprocesses and therapies. Both of these efforts can significantly benefit from the synthesis of biological data into quantitative and increasingly mechanistic models that not only describe, but also predict, how a stem cell's environment can control its fate. This review will briefly summarize the history and current state of the stem-cell biology field, but will then focus on the development of predictive models for stem-cell control. Early models formulated on the assumption that cell fate was decided by stochastic, cell-intrinsic processes have gradually evolved into hybrid deterministic–stochastic models with increasingly finer molecular resolution that accounts for environmental regulation. As our understanding of cellular control mechanisms expands from the cell surface and towards the nucleus, these efforts may culminate in the development of a stem-cell culture programme, or a series of signals to provide to the cells as a function of time to guide them along a desired developmental trajectory.

Introduction

A stem cell is defined as a clonal precursor of more identical stem cells, as well as specialized or differentiated progeny cells of one or more defined types. Whether one is considering stem cells that comprise an embryo developing into an adult organism, or cell niches residing in various tissues of the adult, stem cells are subjected to a wide variety of control mechanisms. In particular, a number of their functions or decisions, including survival, death, proliferation, migration, lineage commitment and differentiation, are tightly regulated by processes that are slowly being elucidated [1–4]. In the fields of tissue engineering and regenerative medicine, progress in harnessing these cells to repair tissue damaged by disease or trauma will rely upon gaining a deeper understanding of these regulatory mechanisms. Furthermore, fundamental cell and developmental biology will benefit from elucidating the control mechanisms that translate extracellular information into intracellular decisions. We will first review the history and considerable recent progress in the identification and characterization of various stem-cell classes, but will then focus on the need to gain a deeper understanding of both the cell-extrinsic and -intrinsic mechanisms that control these cells at a molecular and quantitative level.

History and recent progress of 'stemness'

Stem cells were first identified in mouse bone marrow in the 1960s by Till, Becker and colleagues, who observed that single cells could give rise to all haematopoietic lineages *in vivo* [5,6]. Simultaneously, Altman and colleagues presented evidence for the generation of new neurons in the adult brain [7,8], work that presaged the more recent discovery of adult neural stem cells [9–11]. A subsequent set of major advances came with the identification of pluripotent

Key words: deterministic, gene regulation network, mathematical model, signal transduction, stem cells, stochastic.

Abbreviation used: BDNF, brain-derived neurotrophic factor; CNS, central nervous system; EGF, epidermal growth factor; EGFR, EGF receptor; ES cells, embryonic stem cells; G-CSF, granulocyte colony-stimulating factor; G-CSFR, G-CSF receptor; GM-CSF, granulocyte/macrophage colony-stimulating factor; HER, haematopoiesis engendered randomly; HIM, haematopoietic inductive microenvironment; HSC, haematopoietic stem cell; LT-HSC, long-term HSC; MAPK, mitogen-activated protein kinase; M-CSF, macrophage colony-stimulating factor; O-2A, oligodendrocyte type-2 astrocyte; PKC, protein kinase C; RtTA, tetracycline transactivator; Shh, Sonic Hedgehog; SlF, steel factor; TGF-α, transforming growth factor α.

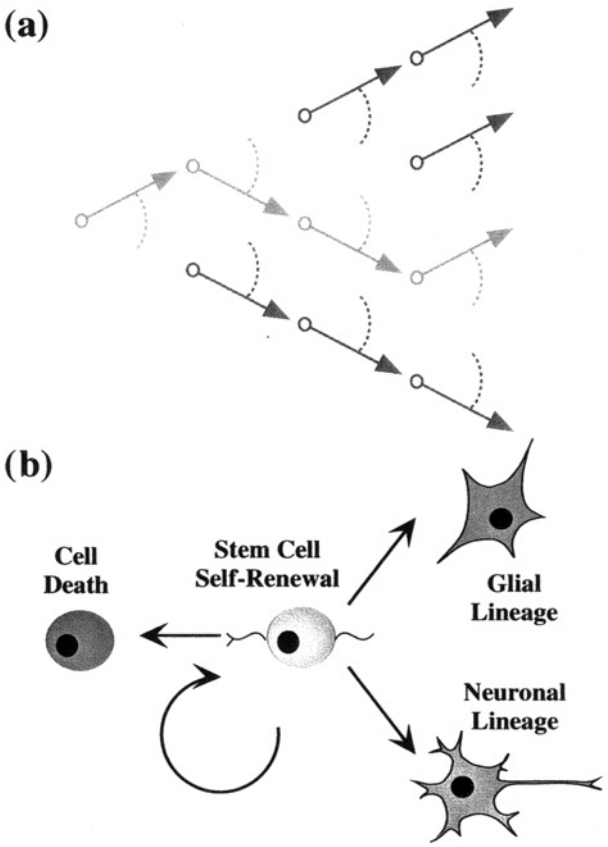
[1] To whom correspondence should addressed, at 201 Gilman Hall, Berkeley, CA 94720-1462, U.S.A. (email schaffer@cchem.berkeley.edu).

stem cells, which give rise to all cell types of the adult organism. In 1974, Evans and colleagues (see, e.g. [11a]) generated embryonic carcinoma cells from reproductive-tissue tumours and found they could develop into cells from each of the embryonic germ layers: ectoderm, mesoderm and endoderm. In addition, in 1981, pluripotent mouse ES (embryonic stem) cells were derived from normal tissue, namely an early-stage embryo [12]. This activity in the stem-cell field over the past several decades has recently escalated into several major advances. These include substantial progress in the identification of numerous signals that control stem-cell fate, the discovery of robust adult neurogenesis, and the derivation of pluripotent human stem cells.

Haematopoietic stem cells

As they were the first stem-cell population discovered, many paradigms of stem-cell biology, as well as techniques for the analysis of cell properties and functions, were originally formulated in HSC (haematopoietic-stem-cell) systems. A stem cell was first defined as a clonal cell capable of both self-renewal and repopulation of a range of terminally differentiated cells of the haematopoietic system. LT-HSCs (long-term HSCs) are capable of permanently reconstituting all the haematopoietic lineages in an organism. These cells can differentiate into shorter-term haematopoietic progenitors, which then develop into the more lineage-restricted myeloid and lymphoid progenitor cells. These multipotent progenitors have the capacity for short term self-renewal, but they eventually differentiate into specific lineages, the former into the myeloid lineages (erythrocyte, megakaryocyte, granulocyte and monocyte) and the latter into lymphoid fates [B-cells, T-cells, NK (natural killer) cells and dendritic cells].

Stem cells are therefore faced with a hierarchical, branching decision tree (Scheme 1a). As discussed in greater detail below, these decisions were initially thought to be controlled stochastically, but more recent advances in molecular and cellular biology have led to the identification of extrinsic signalling factors that influence many of these fate choices. Although LT-HSCs are difficult to culture for long periods of time in their immature state, several factors and cytokines that promote their proliferation or self-renewal have been identified, including SIF (steel factor), IL-3 (interleukin-3), thrombopoietin, Flt3 ligand, Wnts, Notch and Shh (Sonic Hedgehog) [13–15]. Furthermore, factors that influence their conversion from HSCs into common myeloid-versus-lymphoid progenitors [e.g. GM-CSF (granulocyte/macrophage colony-stimulating factor)] and from progenitors towards terminally differentiated cell phenotypes [e.g. G-CSF (granulocyte colony-stimulating factor) and M-CSF (macrophage colony-stimulating factor)]



Scheme 1 Hierarchical branching decision tree (a) and developmental pathways (b)

(**a**) The life of a stem cell can be viewed as a hierarchical branching process where the cell is faced with a series of fate switches. The goal of stem cell bioengineering is to provide the cells with the proper signals as a function of time to guide them down a particular developmental trajectory. (**b**) The developmental pathways available to a neural stem cell include quiescence, death, self-renewal, and differentiation down a number of lineages.

have been identified. Commitment of LT-HSCs to differentiation, however, may be related both to the presence of factors that regulate differentiation as well to the signalling dynamics of the factors that promote self-renewal [16]. In any case, the mechanisms by which all such factors influence and regulate cell behaviour and function are complex, and, as discussed below, a quantitative and mechanistic analysis can yield a deeper understanding of the processes of self-renewal, lineage commitment and differentiation.

In addition to being the first stem cells discovered, HSCs were also the first to successfully be utilized therapeutically. For decades, bone-marrow transplants and peripheral-blood transfusions have been used in the treatment of leukaemias, other cancers and inherited blood disorders [17]. Furthermore, protein drugs such as erythropoietin, GM-CSF, and G-CSF stimulate haematopoietic progenitor cells into repopulating the blood system of patients undergoing chemotherapy and radiotherapy, or for

other conditions resulting in anaemia [18,19]. Further therapeutic progress may result from an improved understanding of the mechanisms by which such factors control HSC self-renewal, differentiation and lineage commitment.

Adult neural stem cells

As discovered earlier for blood, populations of adult stem cells have been discovered in a growing numbers of tissues, including heart [20], skeletal muscle [21], liver [22], skin [23], brain [8–10,24] and spinal cord [25,26]. Since millions suffer from neurodegenerative disorders such as Alzheimer's, Parkinson's and Huntington's diseases, the biology and therapeutic potential of neural stem cells are of significant interest [27,28]. As mentioned above, Altman in the early 1960s, and then Kaplan et al. in the 1970s, found evidence that new neurons are generated in several regions of the adult brain, in particular the hippocampus and a region lining the lateral ventricle of the forebrain, known as the subventricular zone. That is, just as HSCs reside within niches in adult bone marrow, neural-stem-cell niches exist in the CNS (central nervous system). In the early 1990s, proliferating populations of cells were isolated from these regions and were shown to have the hallmarks of stem cells, including self-renewal and differentiation into the three major cell lineages of the nervous system both *in vitro* and *in vivo*: neurons, astrocytes and oligodendrocytes [9–11, 29,30]. As with HSCs, extracellular signalling factors regulate adult neural-stem-cell self-renewal and differentiation fate choices [9,11,31–35].

The therapeutic application of neural stem cells has enjoyed preliminary success. For example, the cerebral implantation of adult neural progenitor cells following their expansion in culture has led to a significant reduction in demyelination and axonal loss in an animal model of multiple sclerosis [36]. Also, grafting an embryonic neural-stem-cell line at the site of spinal-cord damage resulted in impressive functional recovery [37]. Finally, we have used gene delivery of the factor Shh to directly control and stimulate the proliferation of endogenous neural stem cells in the adult brain [38], a result with potential implications for Alzheimer's-disease therapy.

ES cells

While adult stem cells have demonstrated unexpected developmental potential, at the present time, embryonic and germ stem cells are by far the best characterized and validated pluripotent cell population. ES cells are derived from the inner cell mass of the blastocyst stage of the embryo, and these pluropotent cells are capable of differentiating into every cell in the body (with the exception of extraembryonic membranes such as the placenta) [12]. The ES cell is therefore a derived cell line whose actual relationship to endogenous stem cells in a developing embryo is not entirely clear. That said, during the first two decades of their existence they have proved enormously useful in the creation of transgenic mice for basic genetic studies on the roles of specific genes in animal development and physiology [39]. In recent landmark work, pluripotent human embryonic and germ cells have also been derived [40,41]. In parallel with the development of culture protocols and media recipes that promote lineage-specific ES-cell differentiation, a number of studies have begun to explore the broad developmental promise of ES cells for therapeutic efforts.

The previous examples with adult stem cells indicate that numerous tissues in the body contain the signals necessary to guide at least a fraction of these cells towards a needed lineage. However, ES cells often need to be differentiated in culture prior to implantation, particularly if the target cell phenotype is one not ordinarily renewed or repopulated in the adult. Jessell and colleagues recently used a combination of inductive signals, retinoic acid and Shh, to drive ES-cell differentiation into motor neurons [42]. That work has significant promise for the treatment of spinal disorders, such as amyotrophic lateral sclerosis (Lou Gehrig's disease) and spinal injury. In addition, landmark studies by McKay and colleagues have shown that ES cells can be coaxed to differentiate into dopaminergic neurons for potential Parkinson's-disease therapy, insulin-secreting cell clusters similar to islets for diabetes therapy, and oligodendrocytes for remyelination of neurons [43–45]. While this and other groundbreaking work [46–49] is highly promising, the methods used to differentiate the cells are most often determined empirically and are not always highly efficient. Further optimization will therefore be required.

To translate the therapeutic promise of stem cells into clinical practice, two goals must be met. First, we must learn at a fundamental and quantitative level how to manipulate and control these cells. Secondly, processes must be developed to implement stem-cell control at a clinical scale. Several important studies and reviews have already discussed the development of bioreactor processes for stem-cell expansion [50–53]. Therefore, for the remainder of this review, we will discuss how progress towards the understanding of stem-cell control at a quantitative, molecular and mechanistic level can enhance our abilities to harness them for therapeutic endeavours.

The biology and engineering of stem-cell control

Numerous examples above illustrate the principle that stem cells have the potential to differentiate into particular cellular phenotypes; however, precise control must be exerted over

the processes of stem cell proliferation and differentiation for numerous reasons. First, whereas it is highly encouraging to demonstrate that it is possible for a stem cell to develop into a particular target cell type, large numbers of target cells are often necessary for an actual therapeutic effect. Secondly, for both economic and regulatory purposes, highly robust biochemical engineering processes must be developed to expand, bank and, in some cases, partially differentiate stem cells for tissue-engineering applications. The success of these processes will hinge upon the ability to precisely control stem-cell function and fate. Thirdly, it is important to gain tight control over stem-cell differentiation to avoid adverse side effects during stem-cell therapy. For example, implantation of fetal neural tissue into Parkinson's-disease patients ameliorated the symptoms of some patients in recent clinical trials [54]. However, the inability to control this immature tissue actually made the symptoms of several patients worse. As a second example, undifferentiated ES cells generate benign tumours (teratomas) *in vivo*, so certain populations of undifferentiated cells should not be present in a cell graft. Finally, understanding the mechanisms that control stem-cell fate can significantly enhance basic stem-cell biology efforts to understand the functional importance of these cells during development and adulthood.

It is therefore highly desirable to understand how to control stem-cell function. However, initial work in the field supported the view that stem cells could not actually be externally controlled, i.e. that self-renewal, differentiation and lineage commitment were purely cell-intrinsic stochastic processes. Fortunately, the advent of molecular biology and genetics, particularly the discovery of growth factors, cytokines, morphogens and adhesion factors, has enabled numerous studies that have increasingly demonstrated that stem-cell fate is also extrinsically regulated. For example, there are numerous levels of regulation for adult neural stem cells in the brain, indicating that their tight control is important for adult CNS function, a subject we have reviewed recently [55]. Therefore, over the past several decades, mathematical models have evolved in parallel with the biology, from purely stochastic formulations to hybrid models with increasing levels of molecular regulation. The latter models not only describe cell-culture composition, but may also be useful in optimizing culture conditions that achieve desired cell phenotypes for the design of stem-cell bioprocesses. The biological assumptions and mathematical formulations underlying the several major classes of models that have evolved since the initial discovery of stem cells in the 1960s will be discussed. Furthermore, we will propose several future directions in molecular models of stem-cell control, specifically the incorporation of increasing levels of molecular mechanism as our understanding of stem-cell biology gradually progresses from the cell surface to the nucleus.

In addition to their different mathematical formulations, the models attempt to describe and predict different aspects of stem-cell function. First, models can be developed to describe the fate of single cells or of an entire cell population, that is, what fractions of cells within the population adopt alternate fates. The latter models are well suited to studying an expanding cell population and situations in which the cells within the population influence one another's behaviour, i.e. feedback. A second distinction is between models that analyse the decision for stem-cell self-renewal versus differentiation into a specialized phenotype, and models that examine lineage commitment, i.e. the cells' choices among different possible phenotypes once it has chosen to differentiate.

Purely stochastic models

The life of a stem cell can be conceptualized as a hierarchical branching process (Scheme 1a) in which a stem cell faces progressive decision points or 'switches', each of which carries the cell closer to its eventual fate. We would therefore propose that the goal of stem-cell bioengineering is to exert precise and quantitative control over these switches and thereby guide the cells towards a desired outcome. However, the decisions in this branching process may be governed either by environmental, extrinsic cues in a deterministic manner, or by random, cell-intrinsic mechanisms in a stochastic manner. Early work in the field, and even some recent work, favoured the view that stochastic events dominate stem-cell decisions.

Purely stochastic models of stem-cell behaviour assume that there is no environmental influence on stem-cell fate and that cells adopt their fates as a result of unknown random fluctuations intrinsic to the cell. These models were compared with experimental results to derive the intrinsic, fixed probabilities for stem-cell self-renewal, i.e. maintenance in an immature state versus differentiation into a mature phenotype. The earliest such models were developed for HSCs in the 1960s [56–58]. These models were based largely upon early experiments in which animals were sublethally irradiated to eliminate their endogenous haematopoietic system, and exogenous HSCs were subsequently grafted into their spleens to generate cell colonies. These colonies could then be excised, dissociated into single cells and implanted into a second animal to gain information on the composition of the first colony. It was observed that the number of colonies formed [or CFU (colony forming units)] in successive generations was highly variable, but could be fitted with a gamma probability distribution. Till and colleagues [56–58] showed that the observed results of these colony-forming assays could be explained using a stochastic computer simulation in which the HSCs had a self-renewal probability 0.6 and a probability

to differentiate of 0.4. This early model, termed HER (haematopoiesis engendered randomly), did not account for cell death or distinguish between various mature phenotypes or the choice of cell lineage. Interestingly, a steady-state number of HSCs should be reached only if the self-renewal probability is 0.5, so even these very first studies provided evidence against purely stochastic fate choices, as the environment of an irradiated animal apparently modulated and increased the probability for HSC proliferation to 0.6.

That work inspired numerous second-generation stochastic stem-cell models. To analyse HSC function under better controlled conditions, Metcalf and others developed techniques to clonally expand and quantify HSC cell differentiation *in vitro* [59,60], and the resulting data were compared with those obtained using stochastic models. As with Till, McCulloch and Siminovitch's early *in vivo* experiments [56–58], empirical probabilities of self-renewal or differentiation in these cultures closely fit a gamma distribution [61–65]. Nakahata and others showed that applying the stochastic hypothesis yielded an $\approx$60% probability of self-renewal and $\approx$40% chance for differentiation in their particular culture conditions [61,64], the same values measured by Till and colleagues [56–58] *in vivo*. Since the statistical probabilities of differentiation were similar *in vivo* and *in vitro*, the HER hypothesis was strengthened.

In addition to analysing the commitment to differentiation, considerable work has been focused on determining whether lineage selection (i.e. which mature phenotype a cell chooses once it decides to differentiate) is also determined stochastically. Despite similar culture conditions, single HSCs can exhibit wide variations in fate choices. Specifically, numerous studies found that a variety of different lineage combinations could be generated by cells of the same origin, and the actual numbers of cells of each lineage produced from a single cell also showed a random distribution [66–69]. On the basis of these results, it was believed that stochastic processes governed both the decision to self-renew versus differentiate as well as lineage commitment choices.

Deterministic influences on stem-cell-fate choice

HSCs were identified at a time when the general importance of growth factor and cytokine activities had not yet emerged – only a few years after the Nobel-Prize-winning discovery of epidermal and nerve growth factors by Stanley Cohen and Rita Levi-Montalcini [70]. Despite the prominence of the purely stochastic hypothesis of stem-cell fate choice, several pieces of evidence began to weaken it and to favour environmental regulation. First, HSC colonies that formed in the splenic capsule *in vivo* were predominantly white blood cells, whereas those generated in the pulp consisted of red blood cells [71], a result indicating that the cellular microenvironment played a role in their fate choice. Next, it was discovered that treating separated, but clonal, HSC daughter cells with different growth factors significantly impacted the lineage of their progeny [72,73]. These results gave rise to a deterministic hypothesis that, in contrast with HER, was called HIM (haematopoietic inductive microenvironment) [74].

Stem cells have since been shown to exist in regulated microenvironments, or 'niches', throughout the adult organism. Some default to a quiescent resting state, the G_o-phase of the cell cycle, and are activated only by microenvironmental changes, specifically proliferative or differentiative cues [32,75]. Others maintain a low-level, steady rate of self-renewal to maintain the stem-cell population. In either case, the niche microenvironment supports self-renewal and restricts differentiation [76]. These locations include the haematopoietic stroma, the proliferative regions of the adult brain (the subventricular zone and perivascular niches of the dentate gyrus) and the intestinal crypt.

Therefore the processes of self-renewal, differentiation and lineage commitment are regulated by an increasing number of identified growth factors, cytokines, hormones and extracellular-matrix factors. The cell biology of receptor–ligand dynamics and trafficking [77,78], cell adhesion and signal transduction [79] therefore applies to stem-cell function. In addition, there has been early progress in elucidating how these signals elicit intracellular gene regulatory changes. Commitment of a stem cell to a given fate causes the decreased expression of specific genes, some of which are associated with their multipotent nature, and an increase in others that may be associated with a differentiated phenotype. The former include several key stem-cell transcription factors that are known, for example, in the haematopoietic and nervous systems [80–83], but other stem-cell specific factors remain to be identified.

It is also crucial to note that, in addition to specific signalling factors, cell-culture parameters also profoundly influence stem-cell fate. For example, oxygen pressure has been found to be important for the *in vitro* behaviour of haematopoietic [84] and embryonic neural stem-cell cultures [85,86]. Also, important work has shown that cell inoculation conditions and pH can influence neural stem-cell state ([87]; E. Abranches, A. O'Neill, J. M. Cabral and D. V. Schaffer, unpublished work). Future experimental and modelling efforts should further explore the effects of O_2, pH, nutrients and cell density on behaviour.

Instructive versus selective regulation

With an enhanced understanding of stem-cell biology and the increased evidence of extrinsic influences on stem-cell fate choice, the original debate of stochastic effects

versus deterministic (HER versus HIM) effects shifted into a dialogue about the type of influence these environmental factors exert on stem-cell fate choice. With the available evidence, a case can be made for either an instructive or a selective role for extrinsic factors in stem-cell fate choice [74,75,89].

The instructive view proposes that the decision to differentiate, as well as the lineage of the daughter cells are determined by microenvironmental signals (reviewed in [73]). That is, a specific external factor or combination of factors initiates the intracellular events necessary to allow differentiation into a specific cell type. As one illustrative example, Borzillo et al. [90] presented evidence that the ectopic expression of the M-CSF receptor in a pre-B-cell line biased cell lineage outcome from a lymophoid fate to a macrophage fate. In addition, as discussed below, there are also many instructive examples for neural stem cells [91–96].

Alternatively, extrinsic factors may serve only a selective function, in which cells commit to differentiation into various lineages by some rigid statistical distribution, and the environmental factors selectively support the survival or proliferation of only specific phenotypes (reviewed in [89]). As one example supporting this view, Stoffel and colleagues [97] generated a mouse in which the intracellular domain of the thrombopoietin receptor (*mpl*) was replaced with the G-CSFR (G-CSF receptor) intracellular signalling domain. If the G-CSFR actually instructed cell lineage choices, thrombopoietin would be predicted to instruct cells away from a platelet fate and towards the granulocyte phenotypes induced by G-CSF signalling. In contrast, the resulting mice had normal platelet counts, indicating that the G-CSFR domain appeared to support cell survival rather than instruct a specific cell fate.

It appears that growth factors may signal instructively or selectively in different circumstances, and the net result of either mode is qualitatively similar: the stem-cell population is biased towards one or another mature cell type. However, to design predictive models of stem-cell culture control, it will be important to distinguish between these alternatives in the future.

Hybrid stochastic–deterministic models

The acknowledgement that cell extrinsic factors influence commitment to differentiation as well as lineage selection has spurred the development of models with both deterministic and stochastic aspects. In these hybrid models, cellular decisions, in particular exit from the cell cycle and lineage choice, are still determined by a probability distribution. However, the probabilities of each choice are weighted according to environmental factors, such as cell density or the presence of a growth factor. Initial models lacked mechanistic molecular detail of the microenvironmental effects, but were able to capture their empirical influence on the cell population.

Agur et al. [98] recently developed a generic discrete model for stem-cell self-renewal for bone-marrow cells. This model melds a cell-intrinsic 'clock' that stochastically determines the decision to differentiate with a negative-feedback control element to account for effects of the microenvironment. This proposed control system maintains a steady-state HSC level and exhibits robustness when challenged with large perturbations, both hallmarks of homoeostasis. The authors of this model contend that its architecture is sufficiently generic that its conclusions can be extended to all stem-cell populations residing in a heterogeneous environment.

In addition to analysing stem-cell self-renewal versus differentiation, stochastic–deterministic models have been formulated to study lineage commitment. Some are based on the original Till et al. [56] stochastic model, but add deterministic elements [74,99]. Blackett and colleagues [74,99] hypothesized that variability in cell-cycle state is one source of cell-intrinsic stochasticity. An otherwise-homogeneous population of stem cells enters the cell cycle from G_0 at times chosen by a probability distribution, leading to heterogeneity in the experimental outcome. However, they proposed that the G_0 exit probability distribution could be influenced by microenvironmental factors, i.e. a biased probability. In another study, Solberg [100] developed a progressive stochastic model that acknowledged the influence of cytokines on megakaryocyte maturation from progenitor cells. This influence is manifested as a dependence of the probability of transition from progenitor to megakaryocyte on colony size (which is presumably proportional to autocrine/paracrine cytokine signalling). This simulation was able to recapitulate heterogeneity in megakaryocyte maturation stages within a population.

Models have also benefited from recent technological advances that have yielded improved data on stem-cell behaviour [101–104]. Using fluorescent dyes to track individual cells and their progeny, the kinetics of HSC lineage progression was measured [104]. In one model based on such data, cell-cycle progression was partitioned into two compartments. The stochastic compartment described the exit of cells from the G_0 cell-cycle phase according to a probability distribution dependent on the number of cells in the compartment. The deterministic compartment accounts for the effects of extrinsic factors on lineage progression and apoptosis. Together, these two components could recapitulate a heterogeneous cell population response by accounting for each cell individually.

Another cell-differentiation and lineage-commitment stochastic–deterministic simulation was created to study

neural stem cells. O-2A (oligodendrocyte type-2 astrocyte progenitor cells) differentiate into oligodendrocytes in response to certain extracellular stimuli [105]. This oligodendrocyte differentiation appears to be synchronized, but the fraction of oligodendrocytes generated from a single clone is sensitive to environmental cues. Yakovlev, Noble and colleagues [106–111] developed a series of models to describe O-2A behaviour. The first-generation model aimed to determine whether O-2A undergo symmetric or asymmetric division [106,107]. In this model, the cells undergo a critical number of symmetric divisions (which varies from cell to cell by a random distribution) before being allowed to differentiate into oligodendrocytes based on a statistical distribution dependent on environmental factors (namely, thyroid hormone). The model was refined to incorporate cell-cycle history, which improved its fit to experimental data [110]. Additional improvements to the stochastic simulation were made using random-walk partial likelihood functions [108] and other stochastic optimization methods [109] to capture better the heterogeneity observed experimentally in the critical cycle number. The most recent model revisions have replaced the critical cell-division parameter with a gradually increasing probability of differentiation [111].

Signalling thresholds

The above models account for extrinsic signal control over stem-cell fate by empirically expressing the probabilities of alternate cell-fate choices as functions of external signal concentrations or other environmental conditions. The next level of building cell-biological mechanistic information into stem-cell models has been to incorporate the concept of signalling thresholds into cell-fate choices. The idea of threshold responses originated with the observation of spatial pattern formation during development in numerous systems, including *Drosophila* segmentation and wing formation, vertebrate limb patterning and spinal-cord development. Francis Crick [112] first proposed the concept that the diffusion of a soluble signal could establish a concentration gradient, and cells within the gradient could adopt alternate, 'all-or-none' fates at critical threshold levels of the signal.

In support of the threshold concept, there are numerous experimental studies in which manipulating signal concentrations modulates or 'flips' stem-cell fates. For example, the morphogen Shh patterns the developing vertebrate spinal cord and limb bud by establishing a spatial gradient, and stem cells within this gradient switch between alternate fates at critical concentrations [96,113]. Shh controls ventral spinal-cord development, such that floor plate cells form at high Shh concentrations, motor neurons at intermediate concentrations and interneurons at low concentrations. We have recently demonstrated that Shh also regulates adult neural-stem-cell function *in vitro* and *in vivo*, specifically adult neural-stem-cell proliferation [33,38], and below we will discuss a gene-regulation network model that describes the mechanism of threshold responses to this key factor. Threshold levels of signal concentrations also regulate haematopoietic progenitor cells. Zandstra et al. [16] found that moderate cytokine concentrations were sufficient to promote haematopoietic-progenitor-cell survival and proliferation, but high concentrations were necessary for the maintenance of LT-HSC activity.

In addition to manipulating the level of the external signalling factor, modulating the expression of its receptor in stem cells to amplify signalling can also alter cell-fate choices. Lillien [91] used a retroviral vector to overexpress the EGFR [EGF (epidermal-growth-factor) receptor] in retinal progenitor cells *in vitro* and *in vivo*. The transduced cells were sensitized to the ligand TGFα (transforming growth factor-α), and their differentiation was consequently biased away from rod photoreceptors and towards Muller glia. Furthermore, other work has analysed intracellular signal-transduction mechanisms responsible for amplifying signal differences into alternate cell responses. PC12 neural cells normally proliferate in response to EGF, which induces a transient MAPK (mitogen-activated protein kinase) signal, and differentiate into neuron-like cells due to nerve-growth-factor signalling, which stimulates sustained MAPK activation. Overexpression of the EGFR or insulin receptor led to sustained MAPK signalling upon addition of EGF or insulin, resulting in cell differentiation in response to these amplified signals [92,93]. Therefore, shifting the number of cell-surface receptor–ligand complexes through a critical threshold, by changing either the concentration of the ligand or the receptor, can alter cell fate.

This concept of thresholds of cell-surface receptor–ligand complexes in cellular developmental decision-making has recently been incorporated into an elegant mathematical model for ES-cell self-renewal. Viswanathan et al. [3] found that two cytokines that support ES-cell proliferation, namely LIF (leukaemia inhibitory factor) and HIF-6 (hyperinterleukin-6), differ in their ability to maintain stem-cell self-renewal. Mathematical modelling led to the conclusion that this difference in the population probability of self-renewal resulted from different steady-state levels of receptor–ligand signalling complexes in the presence of the two factors.

Systems-biology views of stem-cell signal transduction and gene regulation

Numerous studies have demonstrated the concept that threshold levels of extrinsic signals bias cell-fate decisions, and one example has showed that this key external signal

concentration is correlated to a threshold transcription-factor activation level [114]. We would therefore propose that the next generations of models of stem-cell fate control will likely progress from the cell surface to the nucleus to account for and predict the mechanistic genomic events that underlie critical cell-fate choices.

For stem cells faced with a branching series of very distinct, critical choices (Scheme 1a), there are two important questions. First, which signalling and gene-regulatory structures are capable of amplifying small differences in input signals into critical differences in cell state? Secondly, to what extent are these critical choices governed by extrinsic signals versus intrinsic stochastic effects; that is, do stochastic effects place an inherent limit on our ability to control stem cells?

A number of studies from outside the stem-cell field address the first question and indicate that certain properties in both signal-transduction cascades and gene-regulatory networks can generate a biological switch, i.e. a structure that flips between alternate fates as a function of an input signal level. Non-linearity combined with positive feedback in a cytoplasmic signal-transduction network can yield an ultrasensitive system capable of switching states in response to a small change in an input signal. For example, *Xenopus* oocytes can translate very small differences in progesterone concentration into critical all-or-none fate choices. Ferrell and Machleder [115] showed that MAPK was involved in this switch, as its rapid activation in response to progesterone was equivalent to that of an enzyme with a Hill coefficient (*h*) equal to 35. This ultrasensitivity was due to a protein-synthesis-dependent positive feedback loop containing the MAPK signalling pathway. Similarly, in fibroblasts, Bhalla et al. [115a] demonstrated in fibroblasts that the MAPK cascade can exhibit bistable states. Theoretical analysis indicated that this behaviour could be due to a positive signal-transduction feedback loop in which MAPK led to PKC (protein kinase C) activation, which fed back to yield further MAPK activation.

In addition, moving from the cytoplasm to the nucleus, gene-regulatory networks can also exhibit motifs and structures that amplify small differences in an extrinsic signal into all-or-none state choices, again by employing a combination of non-linearity and positive feedback. For example, theoretical work in the 1970s demonstrated that a hypothetical autoregulatory transcription factor – one that binds to, and transcriptionally up-regulates, its own promoter – could yield a bistable switch [116–118]. This concept was recently experimentally validated in yeast with a synthetic autoregulatory loop composed of an rtTA (tetracycline transactivator) transcription factor, which is activated by the addition of tetracycline, placed in front of a promoter containing rtTA binding sites. The resulting system exhibited switching between two stable states as a function of the tetracycline concentration [119]. Switching behaviour was also observed in *Escherichia coli* with a synthetic gene circuit composed of two mutually repressive transcription factors [120].

Shh control of neural-stem-cell fate

It has been theoretically determined with hypothetical transcription factors, and experimentally demonstrated with a synthetic gene circuit, that autoregulatory transcription factors can yield bistable switches. We have applied this concept to transcription factors that control stem-cell fate to examine the possibility that bistability in a series of gene-regulation networks may underlie the critical cell-fate choices a stem cell traverses as it proceeds from self-renewal through differentiation into a specialized phenotype (Scheme 1). In support of this hypothesis, there are numerous examples of autoregulatory transcription factors that play important roles in cell-fate decisions. These include the Shh-responsive transcription factor Gli1 [121], numerous important transcription factors in *Drosophila* development [122–124], the factor Pax6 crucial for eye development [125], the key developmental homoeodomain factor Hox4a [126], the factor Pit-1 involved in pituitary-gland development [127], members of the GATA transcription-factor family critical in HSC regulation [128,129], the factor EBF (early B-cell factor), which controls B-lymphocyte differentiation [130], and many others [131–133].

We have recently developed a gene-regulation-network model that predicts stem-cell responses to the extracellular concentration of the signalling factor Shh [33]. Two genes lie at the heart of the Shh gene regulation circuit. Gli1 is a transcription factor activated by a Shh signal, and the *gli1* gene contains numerous Gli binding sites within its own promoter, i.e. it is a source of positive feedback in the system. Secondly, Gli1 also binds to, and up-regulates, the expression of Patched, a suppressor of Shh signalling, to yield a negative feedback loop. We used both deterministic and stochastic analysis to determine how this recursive feedback loop structure processes a Shh signal. Bifurcation analysis of deterministic differential equations revealed that the system exhibits bistability; that is, at a key Shh concentration, the circuit flips from a low- to a high-Gli1-concentration state. This behaviour may underlie the ability of Shh to switch stem-cell behaviour at a critical Shh threshold [38,94].

However, recent theoretical and experimental work, mainly in prokaryotes, has demonstrated that a deterministic description is not entirely appropriate for many regulatory circuits [134–137]. Transcription and other regulatory factors are often present at low concentrations, which can translate to fewer than several hundred molecules per cell.

As a result, deterministic chemical-reaction-rate formulations used in differential equation models are inaccurate, and stochastic methods must be employed. Random fluctuations in biochemical reactions can therefore generate noise in the concentrations of key signalling and transcription factors, and the resulting stochastic effects can potentially introduce an element of randomness into the genetic circuits that control stem-cell fate choices. In other words, these stochastic effects may undermine the ability of extrinsic signals to deterministically control stem-cell function.

Furthermore, as discussed above, positive transcriptional feedback loops are ubiquitous in developmentally crucial transcription factors, including Gli1, and positive feedback loops can destabilize systems by amplifying noise. For regulatory circuits to maintain tight control over cell function, it would therefore be reasonable to hypothesize that they contain mechanisms to reduce noise, and one mechanism with the capacity to stabilize control systems is negative feedback [138,139]. We therefore conducted stochastic simulations of the Shh gene-regulatory network and showed that Gli1 up-regulation of Patched, a negative regulator of Shh signalling, dampens noise within the system. Therefore the Gli1 positive feedback loop provides switch-like behaviour to the circuit, while the Patched negative feedback loop counteracts circuit noise to reduce stochastic effects and maintain the ability of Shh to tightly regulate stem-cell function [33].

It is our long-term goal to model the circuits that control each key decision in a stem cell's life (Scheme 1) and to gradually link these modules together and predict how to programme a stem cell into differentiating down a particular pathway by providing it with the correct signals as a function of time. This signal-control programme may be valuable both for basic stem-cell biology as well as for the development of processes to harness stem cells for both tissue-engineering and regenerative-medicine therapies. One major challenge to the development of mechanistic models, however, is that kinetic parameter values, and in some cases even the identities of proteins and genes in the network, are unknown. Until the biology is fully elucidated and quantified, statistical methods such as Bayesian-network approaches may be able to help identify networks and draw causal links from experimental interaction data sets [140,141].

Conclusion

With the discovery that growth factors, cytokines, morphogens, hormones, adhesion factors and cell-culture conditions regulate stem-cell fate both during development and adulthood, the biology and engineering fields have evolved from a view of stem-cell function regulated entirely by cell-intrinsic, stochastic mechanisms to one in which extrinsic factors exert a measure of deterministic control over their fate. It is indeed possible that the apparently random fate choices cells sometimes make in culture are not hardwired by cell-intrinsic stochastic mechanisms, but rather due to our currently incomplete understanding of the factors that control the cells. In the absence of the proper signals *in vitro*, some decisions that are controlled deterministically *in vivo* may be left to chance. Therefore the numerous sources of variability may be amplified, including differences in cell–cell contacts, autocrine signalling [142], cell-cycle state, and heterogeneity in the cell population. Future culture systems may incorporate a combination of soluble factors, synthetic materials [37,143] and bioactive polymeric materials (K. Saha, K. Healy and D. Schaffer, unpublished work) to create synthetic, *in vitro* stem-cell niches with the correct biochemical and mechanical properties. However, future studies will also determine whether stochastic effects due to small numbers of intracellular regulatory molecules could in some situations place an inherent limit on the ability to control stem cells *in vitro* and *in vivo* [33,134–136].

In summary, over the next decade there will be several major challenges for field of stem-cell biology and engineering. First, continuing to identify new extrinsic biological signals that regulate stem cells will significantly aid both efforts to understand the basic mechanisms that control these cells, as well as to harness stem cells for tissue engineering and regenerative medicine. Stem cells appear to reside in niches *in vivo*, and there has been recent progress in elucidating both the identities of the support cells that line these niches [144,145], as well as the signalling factors that these 'chaperone' cells provide to the stem cells [146,147]. Secondly, progress will be made in the development of robust bioprocesses and technology platforms for scaling the findings of basic stem-cell biology into the clinic. Finally, the continuing evolution of increasingly molecular- and genetic-engineering models for stem-cell propagation and control will synergize with basic biology and enhance efforts to translate stem cells from benchtops to bioprocesses.

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