Cell Stem Cell
Review

Stem Cell States, Fates, and the Rules of Attraction

Tariq Enver,[1] Martin Pera,[2] Carsten Peterson,[3,4] and Peter W. Andrews[5,*]
[1]The Molecular Haematology Unit, The Weatherall Institute of Molecular Medicine, Oxford OX3 9DS, UK
[2]Eli and Edythe Broad Center for Regenerative Medicine and Stem Cell Research, Keck School of Medicine, University of Southern California, Los Angeles, CA 90033, USA
[3]Computational Biology and Biological Physics, Department of Theoretical Physics, Lund University, Lund SE-223 62, Sweden
[4]Lund Strategic Research Center for Stem Cell Biology and Cell Therapy, Lund University, Lund SE-221 84, Sweden
[5]Centre for Stem Cell Biology and Department of Biomedical Science, The University of Sheffield, Sheffield S10 2TN, UK
*Correspondence: p.w.andrews@sheffield.ac.uk
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Understanding cell-fate decisions in stem cell populations is a major goal of modern biology. Stem and progenitor cell populations are often heterogeneous, which may reflect stem cell subsets that express subtly different properties, including different propensities for lineage selection upon differentiation, yet remain able to interconvert. We discuss these properties with examples both from the hematopoietic and embryonic stem cell (ESC) systems. The nature of the stem cell substates and their relationship to commitment to differentiate and lineage selection can be elucidated in terms of a landscape picture in which stable states can be defined mathematically as attractors.

Introduction

The biological properties and clinical potential of stem cells elicit continued scientific, commercial, and public interest. The cell-fate options that confront stem cells include self-renewal, differentiation and lineage-specification, programmed cell death, and quiescence. To varying degrees, these fates also extend to the immediate progeny of stem cells, known as progenitor or transit-amplifying cells. A key challenge is to understand how the different cell-fate options confronting stem and progenitor cells are selected and coordinated such that adoption of a given cell fate is coupled to appropriate regulation of the alternative pathways. Understanding the mechanisms involved in guiding the fate of stem cells has broad ramifications for biomedical science from elucidating the causes of cancer to the use of stem cells in regenerative medicine.

While much still remains to be understood about the nature of the molecular pathways involved in the regulation of stem and progenitor cell fate, it is generally accepted that transcription factors are key intrinsic regulators of these fate decisions and that fate choice involves modulating networks of transcription factors. With roughly 30,000 genes in mammalian genomes, there are a staggering $2^{30,000}$ potential combinations of gene expression values even if, for simplicity, we assume only "on" and "off" states for each gene. To visualize this combinatorial explosion further, consider that a fictitious small genome with 260 genes would host the same number of combinations as the number of atoms in the visible universe! In reality, gene expression is graded, making the potential gene expression space available even larger. While the vast majority of theoretically possible combinations are inevitably not compatible with life, it might yet appear difficult to understand how such a large repertoire of potential states can be constrained so that only a few hundred cell types that together comprise an organism are generated. However, biochemical interaction dynamics between the genes must provide robustness with respect to varying intrinsic and extrinsic signals: that is, the cell states that are generated must not be unduly sensitive to small fluctuations in these signals (see Table 1 for a glossary of terms). This requirement strongly limits the number of solutions or "states" for the system.

Such state stability is required in stem and progenitor cells to support self-renewal and maintenance of the uncommitted state, but must also afford flexibility in cell-fate choice to permit cell-type diversification and differentiation in response to intrinsic cues or extrinsic signals. In terminally differentiated cells, transcriptional networks must be stable and irreversible, at least under homeostatic or physiological conditions. Evidence suggests that during development or differentiation, cells make very precise transitions between apparently stable "network states" or gene expression patterns. Moreover, it seems that alteration of the level of a single transcription factor within a network may be sufficient to alter cell fate (Gurdon and Melton, 2008). Historically, this concept is highlighted by the experimental phenomenon of lineage reprogramming, for example, by the conversion of fibroblasts to muscles cells following transfection with a vector encoding MyoD (Tapscott et al., 1988). Similarly, GATA-1 has been shown to induce lineage switching of committed cells in hematopoiesis, first in cell lines (Kulessa et al., 1995) and subsequently in primary cells (Heyworth et al., 2002) More recently and more dramatically, the potential for cell state conversions is exemplified by the reprogramming of somatic cells to a pluripotent cell state by a handful of transcription factors (Takahashi and Yamanaka, 2006). Similar considerations apply to pathological states, such as metaplasia, induced by misexpression or mutation of transcriptional regulators or signaling molecules (Slack, 2007).

From a metaphorical perspective, it is intuitive to think of cell states and state-transitions in the context of landscape models. Indeed, Waddington, in his "canalization of development," did precisely that, depicting cells as rolling down different bifurcating

Table 1. Glossary of Terms

Term	Definition
Genetic Regulatory Network (GRN) Architecture	Comprises the genes and proteins that can interact with each other within a system, or cell.
	Includes the underlying regulatory logic that governs the interactions between genes and proteins.
Rate Equations	Mathematical representations of how the concentrations of interacting gene transcripts, proteins and other molecules (variables) in a GRN evolve in time.
Attractor	The equilibrium solutions of the rate equations are called attractors, which represent observable cell phenotypes and can be visualized as wells, or depressions, in a landscape.
Basin of Attraction	The set of initial conditions of the rate equations that describe how a system moves to a particular attractor.
	Corresponds to the interior of the "rim" of a depression, based on the image of a ball rolling into a well, and also represents a particular cell state, such as a specific lineage, developmental stage, or a fate-primed subset of a given population.
Noise	Random, or unpredictable, fluctuations in the levels of specific cues.
	Extrinsic signals and intrinsic factors can be noisy due to, e.g., low molecule numbers.
	May influence the outcome of how modeling equations define specific attractors.
Deterministic Transition	Case when the rate equations defining a system specifies how it will move from one attractor to another when appropriate initial conditions—for example, altered external signals—are defined.
Stochastic Transition	Case when the rate equations defining a system do not specify how it will move from one attractor to another because the presence of noise blurs the landscape contours.
	Movement from one attractor to another is thus influenced by random factors in addition to the forces defined by the rate equations.
Bistable Switch	A state at which rate equations may define one of two potential solutions, or attractors.
	Reaching the basin of attraction for one of the two attractors, or states, precludes movement in the alternate direction.
Robustness	The capacity of a system to withstand modulating factors that may perturb defined equations, such as noise

channels on a hillside, thereby acquiring divergent and irreversible cell fates (Waddington, 1957). However, the smooth valleys of Waddington's figure missed the scope for relatively stable, if transitory, intermediate cell types observed during the differentiation of a given lineage, which suggests a rather more craggy landscape (Andrews, 2002). Currently, a landscape view also emerges from network biology in which cell fates are defined by stable states, known as attractors (Table 1), in which the expression of a large number of genes is stationary (Kauffman, 1967, 1993). Attractor properties have been in focus for a long time to describe the behavior of different nonlinear dynamical systems ranging from weather patterns to animal population dynamics (Wright, 1988), associative memories (Hopfield, 1982), and protein folding (Bryngelson et al., 1995), to name a few examples. A major challenge then that faces regulatory biology in the postgenomic era is to map out in detail the core transcription factor subnetworks associated with different cells types, including the underlying regulatory logic that governs their behavior. Establishing such a map will provide insights into the rules that define cellular states and how transitions between stable cell states are achieved, the latter by finding solutions (attractors) to dynamical models.

Attractors and Basins of Attraction

An attractor is a stable solution to the set of mathematical equations that describe a dynamical system: that is, it represents the state of equilibrium to which a dynamical system will tend to move. Dynamical systems often have more than one solution, or attractor. The imagery of a landscape is often used to illustrate the concepts of an attractor, which is envisaged as a depression (bowls or valleys) in the landscape so that, for example, a ball (representing the "system") which is rolling around in the landscape will eventually enter into the depression (Figure 1). Within the depression, if the ball settles at the bottom, then, in mathematical terms, this represents a stationary solution or fixed point for the system. Alternatively, the ball may circulate at different levels around the walls of the depression. Such solutions correspond to oscillatory states. Finally, stochastic solutions are also possible in the presence of noise. While these solutions represent subtly different system states, they nevertheless fall within the same attractor. In the context of cells, an attractor would be a stable solution after a system of genes and proteins has settled from some initial activity values: effectively, an attractor would represent a particular cell state, which is defined by the constellation of genes that it expresses. Such cell states would correspond to the different cell types that we observe. However, the precise position of an individual cell within the attractor space may encode a degree of functional heterogeneity in that the cell can transition from one position to another without exiting from the attractor, or depression, within the landscape.

A basin of attraction represents the set of initial conditions from which a system will tend to move to the equilibrium conditions defined by a particular attractor. In the imagery of a ball rolling in a landscape, the basin of attraction represents those regions in the landscape from which the ball would eventually roll into a particular depression. The qualitative long-term behavior of a system will vary depending upon the basin of attraction to which the initial condition belongs. With regard to cells, where each attractor corresponds to a particular cell state, the size of its basin of attraction reflects its robustness: that is, the larger the basin of attraction, the more difficult it will be to move a cell out of that state and into another state, so that it is

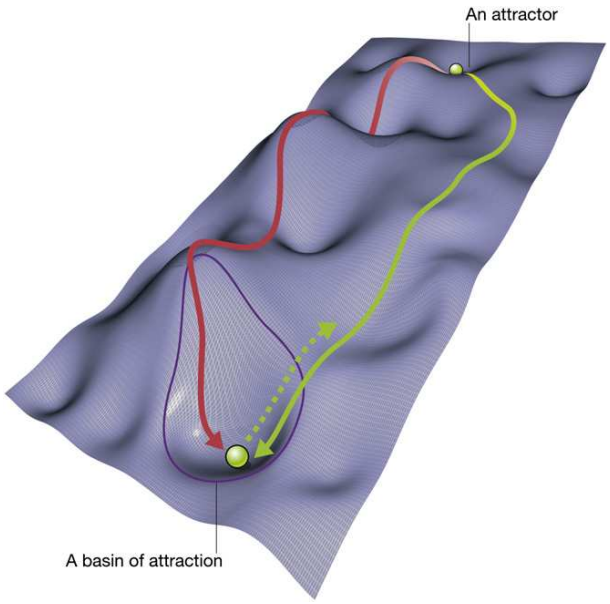

Figure 1. Landscape Pictures of Cell Differentiation

Landscape pictures of cell differentiation attempt to depict different states of a cell by different positions projected onto a two dimensional plane. A third dimension depicts the "energy" of a cell adopting a particular state. The result is an undulating landscape with low lying hollows representing those states that are most stable and, thus, those that cells are most likely to adopt. States depicted by each depression correspond to the cell phenotypes that we observe. In thermodynamic terms, the third dimension represents the free energy of the system in different states, such that the depressions depict low-energy states and, as such, are relatively stable. Mathematically, the depressions are "attractors" representing stable solutions to the set of mathematical equations that describe a dynamical system, such as that represented by the whole landscape: that is, it represents the state of equilibrium to which a dynamical system will tend to move. A "basin of attraction," depicted by the rim of a depression in the landscape (purple line), represents the specific states from which a system will tend to move toward the equilibrium conditions, as defined by a particular attractor. Using the imagery of a ball rolling on a landscape, the basin of attraction would represent the depressions in the landscape into which the ball would eventually roll. By overexpressing certain genes, modifying interaction strengths, or changing externals signals, a cell state could be "lifted up" from its attractor and moved to another attractor. In normal development, we can envisage that the nature of the landscape is such that the cell states would most probably "move" in a single direction (in this case, top to bottom; green solid arrow), but the possibility must also exist of moving in the reverse or alternative directions (green dotted arrow), resulting in the reversal of a cell-fate decision, or transdetermination and transdifferentiation. Note that cells could, in principle, move from one attractor to another by different routes (pink solid arrow), though one path may be more likely than another, described by the height of the landscape between two basins of attraction.

more likely to survive large perturbations, or noise, than cells in attractors with small basins of attraction.

As early as the 1960s, Stuart Kauffman employed the metaphor of attractors when studying Boolean (on/off) genetic regulatory networks (Kauffman, 1967). This pioneering model was considered too much of a simplification at the time, but it contained interesting generic results with regard to stability and the evolution of random networks (Kauffman, 1993). In brief, it was conjectured that even if the solutions or attractors are stable, they should be close enough to instability in order to allow evolution of the system. If the attractors are overly stable, perturbations would not alter the mathematical solutions and so, for example, in animal populations, mutations would have nominal effects and evolution would thus be minimal. In the context of cell differentiation, cell states corresponding to very stable attractors would be impervious to changes in the environment (e.g., signaling molecules), so stem cells and progenitor cells would be expected to correspond to considerably less stable attractors than terminally differentiated cells.

In physics, a complementary description of a dynamical system is often its energy. The energy of a system at different states defines a landscape with respect to the variables (or coordinates), in which the height of the landscape represents the energy of particular states of the system. Low energy regions will be bowls or valleys corresponding to attractors. The movement of a system from one attractor to another is given by deterministic rate equations. However, the process of transiting between states is sometimes influenced by the presence of noise, which implies a probabilistic description. Such a probabilistic view of cell differentiation was developed by Goodwin, a student of Waddington (Goodwin, 1963). Although a landscape picture describes the stable states of cells defined by different attractors, it does not define the routes by which a cell may transit from one attractor to another in the presence of noise; it is conceivable that multiple routes may be taken (Figure 1). Such landscapes also afford the possibility of less orthodox routes, such as those encountered during transdetermination or transdifferentiation, or in experimentally induced reprogramming, such as in the generation of iPS cells from somatic cells.

Definition of Cellular States by Gene Regulatory Networks

A Genetic Regulatory Network (GRN) architecture specifies the genes and proteins that can interact with each other. It also specifies the underlying regulatory logic that governs the interactions that connect the individual variables. A network architecture, including the interaction rules, can be modeled mathematically by a set of differential equations, the solutions of which will indicate whether a particular gene is active, or not, under a particular set of conditions. These equations are examples of dynamical systems that often occur in other contexts in physics and engineering, e.g., the swinging of a clock pendulum, fluid flows, and weather development. They describe the time evolution of variables that are subject to fixed rules. In the case of a GRN, the variables are protein levels, and the rules are the interactions between proteins and between proteins and DNA. GRNs depict the regulatory interactions between individual genes, representing the available information in an accessible form and providing insights into the molecular mechanisms underlying developmental decisions and processes. They may often be influenced by external signals from the niche on which many adult stem cells have long been considered to depend (Schofield, 1978; Scadden, 2006) and which has also been suggested recently in the context of ESCs in culture (Bendall et al., 2007). Relatedly, the influence of external signals in terms of interacting cell populations have also been subject to dynamical modeling (Nakajima and Kaneko, 2009).

There has been substantial progress in defining the transcriptomes and, to some extent the proteomes, of different cell types, and recent years have also witnessed progress in using this information to generate partial or provisional GRNs for some

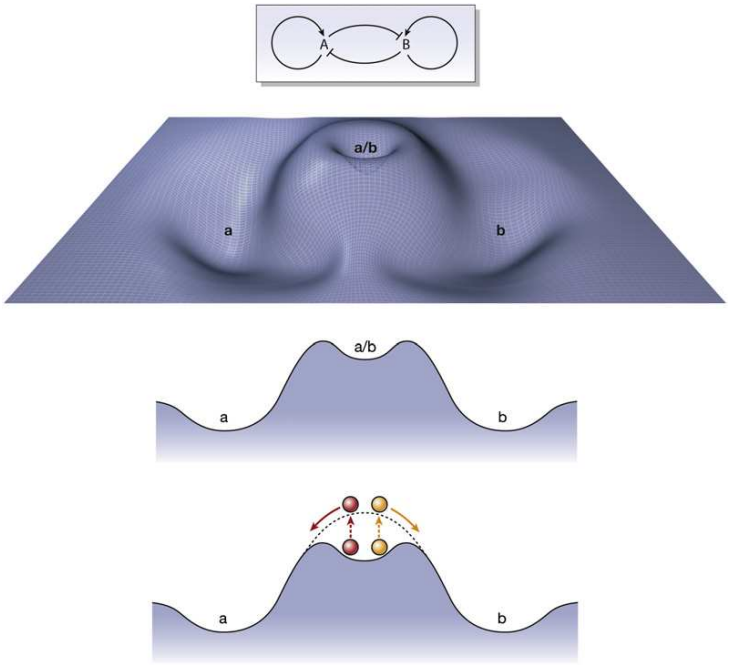

Figure 2. A Landscape with Three Attractors Is Generated by Mathematical Modeling of a Simple Genetic Regulatory Network

In this GRN, illustrated at the top, two transcription factors, A and B, mutually inhibit expression of each other but autoactivate expression of themselves. Mathematical modeling of this GRN generates a landscape (depicted in three dimensions and in cross-section, middle panels) comprising three attractors: two stable states, a and b, in which factors A or B are exclusively expressed, and a metastable state, a/b, characterized by low coexpression of both factors A and B. Considering a bipotential cell occupying the metastable state, commitment and lineage specification to either the a or b pathway is proposed to involve a two-step process. First, the metastable state is destabilized (step 1, broken arrows, bottom panel) producing, in effect, a "hilltop" (dotted black line) where a "bowl" or valley once existed. From this raised position, a relatively small cue or fluctuation will be sufficient to induce rolling (step 2, solid arrows, bottom panel) toward a lineage-committed territory, a or b. If cells were to fluctuate within the metastable attractor from one side to the other in a manner that would reflect lineage bias (red versus yellow cells), then at the point of destabilization, a given cell may already be prone to "flow" in one direction. This model equally allows roles for intrinsic programs, external cues, or "noise"-based fluctuations in the lineage commitment process.

model organisms and mammalian cell systems (Davidson et al., 2002; Loose and Patient, 2004). Detailed analysis of the topology of the GRNs of *E. coli* and yeast has resulted in the identification of a number of different regulatory modules, or "network motifs," which have also been found in metazoans (Lee et al., 2002; Shen-Orr et al., 2002). These modules can be considered as small subnetworks of a particular structure that together define the transcriptional program of a cell. Some such motifs are relatively simple, such as an autoregulation motif in which a transcription factor directly regulates its own expression. Cross-antagonism of factors is also frequently encountered (Laiosa et al., 2006). Other modules are more complex, involving several transcription factors cross-regulating multiple targets. For example, coherent feed-forward motifs, such as the positive control of hemoglobin expression by GATA-1 directly and via FOG-1 (Swiers et al., 2006), govern temporal control, whereas incoherent motifs in which one element acts negatively rather than positively, can act as concentration detectors. Examples of a core GRN for different hematopoietic compartments and processes are provided by Swiers et al. (2006). The authors collated and reviewed the currently available literature on the activities of transcription factors based upon expression profiles, transcription factor perturbations, chromatin immunoprecipitation, and prior knowledge of *cis*-regulatory elements. From this information, they proposed a series of GRNs underlying the initial specification of the hematopoietic stem cell and its subsequent derivatives. Of course, the quality of the GRNs identified is limited by both the quality and quantity of the available experimental data. Nevertheless, these GRNs present a first step toward predicting the mechanisms underlying hematopoietic cell-fate decisions. In many cases, these predictions support the current experimental observations, but novel predictions can also be made that are open to experimental verification. One prediction, for example, was the cross-antagonistic pairing of Fli-1 and EKLF functioning at the erythroid megakaryocyte lineage branch point. Experimental data in support of this relationship was recently reported by James Bieker and colleagues (Frontelo et al., 2007).

Gene Activity and Cell States

GRNs provide a static view or snap shot of a cell state. However, knowledge of network architecture alone cannot describe the dynamics of these networks, which describe how gene expression patterns change over time and, hence, ultimately map to cell fate during differentiation. One also needs to know the nature of the interactions and, very importantly, insight into the crucial dynamic aspect must come from mathematical modeling based on the formulation and solving of, for example, ordinary differential equations that capture the regulatory relationships between the network components (i.e., the genes), as well as the detailed quantification of gene activity. In instances with substantial extrinsic noise, or with relatively few molecules involved (intrinsic noise), these equations have to be replaced or augmented by stochastic procedures, which result in probabilities rather than fixed solutions. A well-examined network module is the interaction between the transcription factors GATA-1 and PU.1 in a myeloid progenitor cell. These two transcription factors mutually inhibit each other and, in doing so, establish an either/or decision situation for the progenitor cell as it chooses between erythroid/megakaryocyte or myeloid-monocytic fates (Laiosa et al., 2006). This example provides a useful paradigm for modeling the process of lineage specification in hematopoietic and other stem and progenitor cells, generating a landscape picture that illustrates the relationship between the stem cell and two alternative fates (Figure 2). These lineage-specific transcription factors also promote the expression of genes that implement the erythroid/megakaryocytic and myelomonocytic programs, respectively. Interestingly, in addition to cross-inhibition both factors are able to autoregulate their own gene

expression. Attractors were generated when the GATA-1/PU.1 interaction was modeled as a simple gene circuit involving autostimulation and mutual inhibition (Roeder and Glauche, 2006; Huang et al., 2007; Chickarmane et al., 2009). The stable attractors correspond to the erythroid and myelomonocytic fates, but a metastable attractor was located between them, characterized by coexpression of both transcription factors and corresponding to the bipotent progenitor state (Huang et al., 2007; Chickarmane et al., 2009). This prediction of simultaneous expression at low levels of the two lineage-associated transcription factors in uncommitted progenitor cells provides a formal explanation for the phenomenon of multilineage gene priming, initially documented in hematopoietic stem and progenitor cells (Hu et al., 1997; Delassus et al., 1999; Enver and Greaves, 1998; Månsson et al., 2007) but now also reported in populations of self-renewing ESCs (Laslett et al., 2007; Hayashi et al., 2008). This property appears—according to the mathematical models—to stabilize the progenitor state and maintain a bipotential property. This new insight that comes from mathematical modeling is in contrast to the traditional model of a graded "stochiometric balance" between these two factors, which does not explain how the progenitor state is stabilized or how discrete cell-fate decisions can be made and implemented. In effect, the GATA-1/Pu.1 regulatory motif functions as a so-called bistable switch, orchestrating mutually exclusive outcomes. The nature of this bistable switch has implications for robustness as it exhibits a property akin to memory. Thus, while the switch is thrown upon achieving a given threshold value of the required signal, once thrown, reversing the switch—and hence, the fate choice—involves lowering the same signal far below the threshold value that first engaged the switch. In a sense, this arrangement provides memory in the system, as cell fates can be preserved without maintaining the high level of the signals required to initiate them. This arrangement is not unique to GATA-1/Pu.1 interactions nor to the blood system, as examples of similar switches have been documented and modeled for the early lineage choice of ESCs (Chambers et al., 2003; Boyer et al., 2005; Chickarmane et al., 2006; Chickarmane and Peterson, 2008).

Studies along these lines suggest that relatively small transcriptional circuits or motifs can generate attractor-like behavior and raise the question as to how these circuits play out on a global scale with respect to gene expression. If one returns to the model that depicts cell types, or states, as stable attractors in a landscape of gene expression space, then certain questions immediately follow. For example, how does a cell transit from one attractor corresponding, for example, to cell type, or cell state, A, to another corresponding to cell type, or cell state, B? The beginning and end of the transit path are fixed by the attractors in question, but in the absence of additional constraints, there are, in principle, multiple theoretical unstable trajectories that link the two stable positions (Figure 1).

Attractors in Hematopoiesis

In the example of myeloid differentiation involving GATA-1 and Pu.1 discussed above, paths between attractors have been assessed by sampling global gene expression as human leukemia cells (HL-60) differentiate down a myelomonocytic path in response to two different inductive signals. The multidimensional gene expression data are collapsed as gene expression trajectories, and the results show that cells traverse different trajectories under the two conditions before converging on the same final myelomonocytic attractor (Huang et al., 2005).

A key question is what happens within the attractor basins themselves and at the boundary of the basin where cells finally exit the stable attractor state. The latter has been examined in murine multipotential progenitor cells undergoing differentiation to erythroid, or myelomonocytic, cell fates. Sampling of global gene expression profiles show that cells initially embark on similar paths and then undergo a critical bifurcation at which point gene expression trajectories rapidly diverge toward the erythroid or myelomonocytic cell-fate attractors. These data have been interpreted as indicating that the establishment of lineage committed cells from a multipotent state may comprise a two-step process (Figure 2). Step one involves destabilization, or minimization, of the multipotent attractor, and step two consists of the cascade toward the differentiated cell type attractors, which ensues as a result of modest factor-induced, or cell intrinsic, asymmetries within individual cells that position each one on either side of the separatrix that divides the erythroid and myelomonocytic gene expression domains (Huang et al., 2007). Such a view has the advantage of uniting cell intrinsic and cell extrinsic modes of lineage commitment and differentiation.

This notion has been further developed in recent studies from Huang and colleagues (Chang et al., 2008). They demonstrated that the multipotent cell line EML was heterogeneous for expression of the cell surface antigen Sca-1. Intriguingly, sorted Sca-1^{lo} cells reconstituted a heterogeneous culture containing both Sca-1^{lo} and Sca-1^{hi} subpopulations, and vice versa, suggesting interconvertibility between Sca-1 states. Furthermore, Sca-1 expression appeared to be correlated with lineage bias. That is, Sca-1^{lo} cells expressed more GATA-1 than Pu.1, and Sca-1^{hi} cells expressed more Pu.1 than GATA-1. These differences in GATA-1/Pu.1 ratios appeared to have functional significance, with GATA-1-high and Pu.1-high cells exhibiting propensities for erythroid and myelomoncoytic differentiation, respectively. If confirmed on a single cell level, these results would provide evidence for lineage primed subsets of the EML stem cells.

Interestingly and relatedly, such cell type heterogeneity has also been discussed in the context of stochastic transitions between cell types in bacteria. For example, *E. coli* cells appear to behave as if they are hedging their bets against future antibiotic treatment by keeping a cell mixture of both quiescent and growing cells, since antibiotics act on the latter (Losick and Desplan, 2008). In other words, external noise appears to be needed to sustain a mixed population that coexists with bistability.

The concept that cells present within a single multipotential or uncommitted cell-fate attractor may exhibit intrinsic differences that can be revealed by destabilization of the attractor is an intriguing one, and this idea resonates with previous observations of heterogeneity in blood stem and progenitor cell compartments (reviewed by Delassus et al., 1999). In these studies, single cell RT-PCR analysis revealed that cells within the multipotent compartment coexpressed lineage-affiliated genes and were, therefore, primed for multilineage differentiation. Intriguingly, individual cells in the population were to an extent heterogeneous with respect to lineage affiliated gene expression. On

the basis of these gene expression studies, we proposed that, under conditions of self renewal, cells within the multipotent compartment fluctuated between different lineage-biased states (Enver et al., 1998).

It is important to distinguish between the heterogeneity discussed above, as detected by functional interconvertibility and other instances of reported heterogeneity that reflects a lack of precision in cell purification. For example, as purification protocols for blood stem cells have been progressively refined, observed functional heterogeneity in isolated stem cell populations has been reduced. Murine bone marrow stem cells purified on the basis of an immunophenotype, (KLS: c-Kit+, Lineage-, Sca-1+) were initially shown to be heterogeneous in terms of stem cell function, with only 10% or so of cells displaying stem cell activity in transplant assays. Further refinement of cell purification strategies has revealed that the KLS population contains at least three distinct compartments corresponding to long-term reconstituting stem cells (KLS, $CD34^-$, $Flt3^-$), short-term reconstituting stem cells (KLS, $CD34^+$, $Flt3^-$), and a set of progenitors termed LMPPs that are restricted to lymphoid and granulomonocytic lineages (KLS, $CD34^+$, $Flt3^+$) (Adolfsson et al., 2005). Thus, the KLS population contains a mixture of different cell types representing distinct attractor states rather than a pure population of heterogeneously functioning stem cells coexisting within a single attractor. However, in this review, we are focusing upon the latter situation in which cell heterogeneity reflects a dynamic relationship between different network substates within a single attractor. Evidence for such dynamic behavior within single cell compartments has recently been obtained in both blood and ESC populations, and a variety of other existing data may profit from being interpreted in such a context (e.g., Enver et al., 1998, 2005; Chang et al., 2008; Laslett et al., 2007; Chambers et al., 2007; Hayashi et al., 2008).

The Stem Cell Compartment in ESC Cultures

ESCs are typified by the property of pluripotency—the ability of a cell to give rise to all the tissues of the body. Many studies have attempted to elucidate and define the molecular nature of this pluripotent state. Indeed, roles for the STAT3 and ERK pathways—controlled in part by the cytokine LIF—and for the key transcription factors Oct4, Nanog, and Sox2 have emerged from studies of murine ESCs (Silva and Smith, 2008). Remarkably, recent studies have culminated in the suggestion that the pluripotent stem cell state represents a "ground state" and that maintenance of pluripotency and self renewal involves prevention of cells leaving that ground state rather than an active process of maintaining it (Ying et al., 2008). Such a ground state represents one attractor in a landscape depicting embryogenesis, surrounded by other depressions that correspond to the different cell types that emerge during the differentiation of pluripotent cells.

Although a similar network of transcription factors appears to regulate pluripotency in both mouse and human ESCs, the extrinsic signals that drive stem cell renewal vary in the two species. For example, human ESCs fail to respond to LIF (Daheron et al., 2004; Humphrey et al., 2004; Sumi et al., 2004), an external factor that can be used to maintain the pluripotency of mouse ESCs. In addition, the roles of activin/TGFβ and BMP signaling appear to be reversed in the two species: BMP signaling appears to promote the pluripotent state of mouse ESCs (Ying et al., 2003), whereas activin/TGFβ signaling promotes maintenance of undifferentiated human ESCs and BMPs induce their differentiation (Pera et al., 2004; Vallier et al., 2005). These differences have been used as evidence that mouse and human ESCs correspond to different stages of the early embryo, with mouse ESCs representing the late inner cell mass (ICM) stage whereas human ESCs appear to possess properties of the later epiblast. Indeed, pluripotent mouse cells, known as Epiblast Stem Cells (EpiSCs), corresponding to this stage of development have also been produced by explanting later mouse embryos (Brons et al., 2007; Peerani et al., 2007; Pera et al., 2004; Tesar et al., 2007). Such mouse EpiSCs more closely resemble human ESCs with respect to those characteristics that differ between human and mouse ESCs.

One particular difficulty in elucidating the control mechanisms of human ESCs is that they grow extremely poorly in clonogenic assays, and cultures are typically heterogeneous, containing both stem cells and their differentiated progeny. These differentiated derivatives may feed back signals that affect growth of the parent population of stem cells. For example, the extraembryonic endoderm that commonly arises from ESCs in culture is a source of BMP that might tend to drive their differentiation (Peerani et al., 2007). Indeed, in microarray studies, decreased expression of some endoderm markers was evident in "culture-adapted" human ESCs that had acquired a capacity for more robust growth (Enver et al., 2005): a reduced tendency for endoderm differentiation could well present such variant cells with a selective growth advantage. More recently, it was reported that fibroblast-like cells differentiating from human ESCs produce IGF1, which drives proliferation of the undifferentiated ESCs (Bendall et al., 2007). These authors suggested that the ESCs produce a "niche" that supports their own self-renewal and further suggested that the FGF2 response of human ESCs results not from a direct action of FGF2 on the undifferentiated ESCs, but rather from the action of FGF on the derivative fibroblasts, driving their production of IGF1.

However, there is also the possibility of a more subtle form of heterogeneity, within the stem cell compartment itself, which may indeed be an aspect of stem cells of all types and species. In the context of the landscape picture of attractors, and as discussed above, a single population of stem cells may in fact include cells with varied growth factor responsiveness and/or protein expression within a given attractor. Mounting evidence suggests that, in particular situations, cells with the properties of stem cells may not all be identical but may exist in different, interconvertible substates that have significant consequences for their behavior and ability to differentiate. Although detailed studies of the dynamics of OCT4 and NANOG expression in human ESCs have yet to be carried out, the patterns of cell surface marker expression present on these human pluripotent cells also points to the existence of interconvertible substates of the stem cell compartment (Figure 3) as is now apparent in adult stem cell systems (e.g., Hu et al., 1997; Booth and Potten, 2000; Jones et al., 2007) and in early mouse embryos and ESCs (e.g., Chambers et al., 2007; Hayashi et al., 2008).

In a study of early passage, "normal," and late-passage, "culture-adapted" human ESCs from the H7 line, the properties of cells isolated based upon their expression of the surface

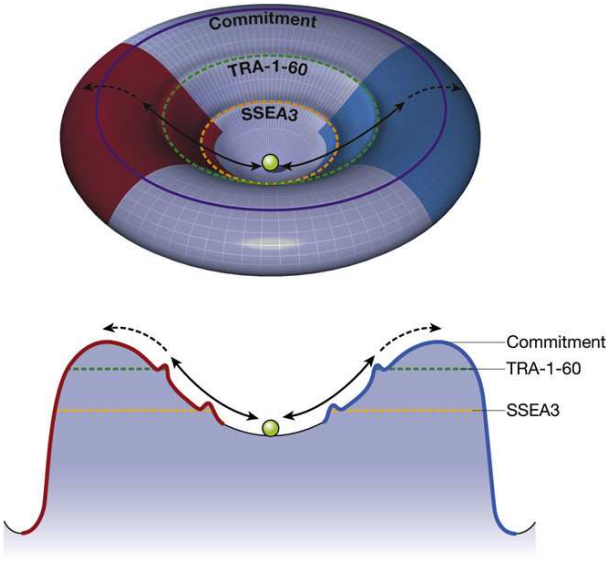

Figure 3. Substates and Prepatterning, or Lineage Priming, within the Stem Cell Attractor

The pluripotent state of human ESCs might be described by one "global" attractor that hosts all possible states that the cell could adopt. However, within this attractor, there might be metastable "substates." From our previous study of SSEA3 expression by human ESCs (Enver et al., 2005), we postulated that human ESCs could exist in SSEA3(+) or SSEA3(−) states. In this example, the SSEA3(+) cells would occupy the more "stable" lower energy region at the bottom of the attractor (below the dotted yellow line), whereas the SSEA3(−) cells would occupy less stable regions toward the top of the attractor. While within the attractor, these SSEA3(−) cells have a significant probability of returning to lower energy SSEA3(+) states at the bottom, although perhaps a "ridge" in the slope might tend to keep the cells temporarily in the metastable SSEA3(−) state. Cells that moved to states beyond the top of the attractor (purple line) would now have a higher probability of moving to another attractor, corresponding to another cell type, than of returning to the stem cell attractor—they would be "committed" to differentiate. Thus, the top of the attractor could be construed as a "commitment barrier." Within the stem cell attractor, we may identify possible other substates. For example, another human ESC marker, TRA-1-60, is lost after SSEA3 expression is lost (Fenderson et al., 1987; Draper et al., 2002). Thus, loss of TRA-1-60 expression might allow us to describe further substates (green dotted line) as the ESCs progress from the more "pristine" SSEA3(+)/TRA-1-60(+) stem cell state to eventual commitment to differentiate at the top of the attractor. The discovery of Nanog(+) and Nanog(−) mouse ESCs (Chambers et al., 2007) may also denote substates within the attractor defined by Nanog expression, and cells within these or other subregions in a "ground state" attractor may be predisposed to eventual differentiated derivatives, without having lost their pluripotentiality. For example, cells in one subregion (red) may have a higher probability of exiting the stem cell attractor toward a more differentiated attractor corresponding to a differentiated cell type defined by one marker than to an attractor corresponding to another differentiated cell type defined by a different protein (blue). Such "prepatterned" substates within the ESC attractor could correspond to the lineage priming observed in hematopoietic stem cell systems (Hu et al., 1997).

antigen SSEA3 were compared (Enver et al., 2005). In previous experiments, SSEA3 had proved to be the human ESC marker that is most rapidly downregulated upon differentiation (Draper et al., 2002; Fenderson et al., 1987). Both the gene expression patterns and the clonogenic potential of the SSEA3(+) and (−) normal H7 ESCs indicated the populations represented different subsets, and the observed traits were consistent with the SSEA3(−) cells having exited the stem cell compartment, or attractor. However, in the culture-adapted pool of the same cell line, the SSEA3(+) and (−) subpopulations exhibited similar global gene expression profiles to one another and to the normal SSEA3(+) cells. Also, clonogenic cells were found in both subsets, but only when isolated from the adapted subline; few clonogenic cells were present in the SSEA3(−) normal subline. From these data, we suggested that culture adaptation traps cells within the stem cell compartment such that subsets of undifferentiated stem cells with both SSEA3(+) and (−) phenotypes can be observed. By contrast, we proposed that most SSEA3(−) cells in cultures of the normal H7 cells had already passed a "commitment barrier" and left the stem cell compartment. In this model, we envisaged that SSEA3(+) and (−) cells within the stem cell compartment could interconvert and that the SSEA3(−) were closer to committing to differentiation than the SSEA3(+) cells. Similarly, Stewart et al. (2006) reported distinct SSEA3(+) and (−) subsets of self-renewing stem cells in cultures of other human ESC lines. In another study, it was also reported that human ESCs can be subdivided into different substates based upon the levels of two other surface antigens, CD9 and GCTM2 (Laslett et al., 2007). This study suggested that within the stem cell compartment, there was a continuous gradient of expression of pluripotency genes. The results indicated that lineage specific transcription factors were coexpressed alongside the pluripotency genes in cells that still maintained expression of the stem cell markers. In line with the landscape picture, this situation would correspond to different positions within an attractor (Figure 3).

Heterogeneity in Embryonic Systems

It might be supposed that the observed heterogeneity of human ESC populations is a consequence of their forced extended proliferation under less than optimal conditions in the abnormal environment of cell culture. But, in fact, it is increasingly apparent that pluripotent mouse ESCs maintained under conditions that support highly efficient clonal self-renewal also show a significant degree of heterogeneity and plasticity in the expression of key regulators of pluripotency and lineage determination. The same trend appears to be true of pluripotent stem cells in the mouse embryo itself, and this plasticity in gene expression may reflect the capacity of the mammalian embryo to respond robustly to experimental or physiological perturbations. Whatever role plasticity might play in regulative development, the emerging picture is that, while pluripotent mouse cells inside or outside the early embryo are quite consistent in their expression of some key regulators like Oct-4 and Sox-2, they do show apparently random variable expression of other pluripotency genes and of genes characteristic of specific differentiated lineages.

Nanog was originally identified as an essential component of the molecular circuitry regulating pluripotency. Chazaud et al. (2006) first made the surprising observation that, unlike Oct-4, Nanog expression in the inner cell mass (ICM) of the E3.5 mouse embryo is not uniform but is found in a subset of randomly distributed cells. Transcripts for Nanog were expressed in a pattern of cells mutually exclusive to those encoding the transcription factor GATA-6, which plays a key role in the formation of the primitive endoderm, the first differentiated lineage to emerge from the ICM. From lineage tracing and chimera analysis, it appeared that even at E3.5, the majority of the

blastomeres of the ICM were specified to form either primitive endoderm or epiblast. An underlying assumption in the interpretation of these studies was that Nanog, being essential for pluripotency, marked the cells that were fated to contribute to the ICM and that the GATA-6-positive cells were specified to form primitive endoderm.

However, further investigation of the role of Nanog in early mouse development has shown that, although embryos lacking Nanog expression fail to develop and die, it is not, in fact, essential for pluripotency of ESCs in vitro (Chambers et al., 2007). Cultures of mouse ESCs also show heterogeneity for Nanog expression, but it is clear, in vitro at least, that Nanog-negative cells can interconvert with Nanog-positive cells. Also, ESCs in which Nanog is genetically deleted are capable of extensive contribution to fetal tissues, with the exception of the germ line. Importantly, although Nanog null cells are still pluripotent, they differentiate much more readily than normal ESCs and exhibit a bias toward the formation of primitive endoderm. These findings led to a picture in which Nanog levels act to control the probability of cells exiting the pluripotent compartment. The model stresses the concept that fluctuating Nanog levels may render a cell susceptible to loss of pluripotency, conditional on other inputs (Figure 3). Thus, Nanog is not essential for pluripotency, and it remains possible that Nanog-negative cells in the unperturbed ICM of the E3.5 embryo might retain pluripotency.

Similar results have been obtained for Rex-1 expression in mouse ESCs (Toyooka et al., 2008). Rex-1 is a transcription factor expressed in the ICM but downregulated in the primitive ectoderm. Most ESCs express Rex-1 in vitro under conditions that support stem cell maintenance, but a minority population does not express this marker. The Rex-1-negative cells population, like Nanog-negative cells, can convert back to a Rex-1-positive state. The Rex-1-negative cells share many features in common with primitive ectoderm cells, including a bias toward somatic rather than extraembryonic differentiation. The balance of the two cell populations in vivo is a function of the cellular environment: different growth factors control the proportion of cells in either compartment. The notion that ESCs can undergo reversible conversion to a primitive ectoderm-like state upon changes in culture conditions was first shown years ago by Rathjen et al. (1999), who isolated pluripotent ''epiblast-like'' stem cells, or EPL cells. These cells were marked by expression of FGF5 and downregulation of Rex-1 but could revert back to FGF5-negative, Rex-1-positive ESCs, depending upon culture conditions. Recently, Hayashi et al. (2008) have tracked the changing expression of Stella to demonstrate that mouse ESCs can also interconvert between a Stella-positive, ICM-like state and a Stella-negative, epiblast-like state.

Cdx-2 is a transcription factor expressed in trophectoderm and considered to be an early marker for that lineage. However, a recent study showed expression of this marker in all blastomeres of the mouse embryo at 10.5 hpc, though the specific levels of Cdx-2 expressed varied widely in individual blastomeres at this stage (Dietrich and Hiiragi, 2007). Contrary to previous reports suggesting that trophectoderm fate is regulated by an interplay of the levels of Cdx-2 and Oct-4, no correlation was observed between the levels of the two proteins, neither was there a consistent relationship between the levels of Nanog and Cdx-2 in early blastomeres. Only later in development, when cells had segregated to the inside and the outside of the embryo, did patterns of Cdx-2 and Oct-4 expression show a reciprocal relationship.

Another lineage-specific gene that shows apparently random localization within the early mouse ICM is Lefty, a Nodal antagonist that later marks the distal visceral endoderm (Takaoka et al., 2006). Lefty is randomly expressed in a subset of inner cell mass blastomeres and, as with GATA-6, it is hypothesized that these cells may be fated to become the primitive endoderm. However, lineage tracing has not yet established this to be the case.

From these studies of the mouse embryo, it is now evident that multiple lineage specific markers are coexpressed with pluripotency markers like Oct-4 in some cells, and heterogeneity is even observed in the expression of some pluripotency genes, such as Nanog. Cultured mouse ESCs also show heterogeneity in the expression of the pluripotency marker Nanog and the ICM marker Rex-1, and it appears that the cells can reversibly convert back and forth into Nanog-positive and -negative, and Rex-1-positive and -negative, compartments while retaining pluripotency. What these studies imply is that the interconversion between two pluripotent states with different developmental potential and different patterns of gene expression is facile and reversible. They also suggest more of a probabilistic rather than a strictly determinative role for those genes in pluripotency and commitment to differentiation during development.

Concluding Remarks

It is becoming increasingly evident that, in different stem cell and progenitor cell systems, whether representing adult, cancer, or embryonic cells, there is considerable heterogeneity within sets of cells initially conceived as representing uniform populations. This heterogeneity can be described as comprising substates of cells that appear able to fluctuate between one substate and another. The concepts of attractors and the associated landscape pictures provide a useful conceptual framework in considering the nature of these substates and their relevance to cell differentiation. In some examples, such as the regulation of erythroid and myeloid differentiation from a hematopoietic progenitor by the cross-regulation of two transcription factors, it has been possible to describe mathematically the relevant attractors and to make predictions about the paths that differentiating cells follow from the undifferentiated, undetermined stem cell state. Similar approaches have been used for ESCs and, given that pluripotent cells also appear to be controlled by relatively few interacting transcription factors, such as OCT4 and NANOG, it may be possible to develop precise models based on the equations defined by the relationships between these factors, or variables. However, in other stem cell contexts, the number of interacting factors may well be increased beyond two or three, which will require additional experimental searches in which novel experiments, database searches, and dynamical modeling proceed seamlessly to find the appropriate GRN. During this process, one should also consistently scan for the multimerization properties of the interactions as these determine key switch properties (Chickarmane et al., 2009).

In the context of stem cell systems involving complex multiple interacting factors, intuitive benefits may be gained by considering landscape or attractor metaphors in terms of their

thermodynamic properties. Integrating these model analogies requires proper computational treatment of the inputs that generate noise (see Table 1) and, thus, will incur significant, albeit feasible, costs for computer time. In this thermodynamic view, different potential states of a cell are associated with particular levels of free energy: stable states corresponding to the cells we observe are low-energy regions of the landscape. Other higher-energy regions would then be unstable and correspond to cell states or types that we do not observe. Differentiation would involve cells transiting from one low-energy state to another, and whether they make such transitions could be described in terms of probabilities that are inversely related to the energy barriers between the stable states, which correspond again to attractors in the landscape. Considering cell states and differentiation in this way leads inevitably to considering differentiation and lineage selection in terms of probability functions. Thus, inducing differentiation involves changing the energy landscape and changing the probabilities of particular differentiation steps. In such a system, the probabilities of particular transitions may be low or high, but they will never be 1.0 or 0.0. This then allows for transdetermination, such as described, for example, by Hadorn (1968), with respect to *Drosophila* imaginal discs, or transdifferentiation as occurs in the various pathological conditions of metaplasia and, indeed, for reprogramming, such as in the generation of iPS cells.

An important question that arises from this line of thinking is whether such fluctuating substates normally exist in vivo, and if so, whether they elicit consequences for the likelihood of self-renewal, differentiation, or lineage selection of particular stem cell types. The results from recent embryo studies discussed above suggest that, indeed, such fluctuations do occur naturally and do have important consequences for embryonic development as well as tissue homeostasis in the adult (Silva and Smith, 2008; Hayashi et al., 2008). Some transitional states may normally experience only a fleeting existence but may be sufficient to provide a basis for regulative development in the embryo. These results underscore that the existence of such fluctuations must be incorporated into existing models in order to understand the processes of early embryonic development, as presaged by theoretical analyses carried out by Goodwin, a student of Waddington, over 40 years ago (Goodwin, 1963).

Fluctuations or noise, whether extrinsic or intrinsic, may occur for many reasons and will likely mediate a greater impact in relatively simple systems that involve few molecules. No matter what the origin of the noise, its presence will enhance the probability of a cell's switching from one attractor to the other. A computational approach must, therefore, take the entropy of the system into account and by doing so will generate a thermodynamic picture that incorporates the probabilities of being in one attractor versus another, resulting in a means to characterize the system as a whole. The concept of taking noise into account can also be depicted as landscapes that become fuzzy, in which the attractors have less precise edges. Similar to the deterministic case (i.e., in the absence of noise), the attractor picture in a blurred landscape offers its own pedagogical and intuitive merits. However, the power of this model does not extend beyond intuition—one must still explicitly formulate the computational models and solve the equations and, in the case of high noise levels, perform the tedious stochastic Monte Carlo estimates of the probabilities. Down the road, computational models will also have to include multicellular environments to account for compartmental heterogeneity and niche influences.

Much of the analysis to date that examines the control of cell differentiation or, indeed, the reverse processes of transdetermination and reprogramming has relied upon studies of cell populations. Such studies provide details of many of the parameters relating to signaling, both internal and external to the differentiating cells, and allow an initial modeling of the underlying networks that control cell behavior. However, if we accept the existence of multiple substates of particular stem cells, for example, such analyses inevitably present an average view. A full analysis will require monitoring the behavior of individual cells in real time. The success of this endeavor will, therefore, require the development of suitable reporters that not only track the expression patterns of individual key genes and transcription factors, but that also can integrate information about the expression patterns of multiple genes that combine to define the state of a cell at a particular point in time. The carbohydrate surface marker antigens of human ESCs, such as SSEA3, are of interest in this respect, since their cell surface expression depends upon the activities of multiple genes; unfortunately, their expression patterns are not easily amenable to real-time monitoring. A number of groups have begun to make use of real-time imaging to follow cell differentiation (Chambers et al., 2007; Eilken et al., 2008; Ravin et al., 2008), but the technology is still in its infancy. Even so, ultimately, it may not be possible to observe cells directly at the points when they commit to differentiate or select a particular lineage to follow. At these decision points, cells may well occupy the unstable transition states in the high ground of a landscape, outside a particular basin of attraction, for a fleeting period and, as such, may be difficult to capture experimentally. It may be necessary to infer the rules that govern such transitions from indirect assessments. For example, the nature and topography of a landscape might be inferred by measuring the probability that a cell may transit from one attractor to another and then comparing these estimates under different environmental conditions that may, in turn, influence the shape of the landscape itself.

The importance of pursuing computational models for pluripotency and lineage-commitment issues in stem cell and progenitor systems cannot be overemphasized. In addition to predicting dynamical behavior, such models will provide insight into switch and reprogramming properties and, very importantly, also reveal specific fluctuating behaviors. Not only are computational models useful for deriving GRN putative architectures and interactions, they can also pinpoint missing components and interactions from functionality requirements that can be confirmed in the laboratory (Chickarmane et al., 2009). Collectively, an iterative procedure emerges in which modeling will play a pivotal role in designing new experimental strategies. The computational toolbox for this modern approach to scientific inquiry is already in place. All that remains is for the proper embedding of these approaches to be undertaken in existing experimental laboratories.

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