Cell Stem Cell
Minireview

Heterogeneity of Embryonic and Adult Stem Cells

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New studies suggest that stem cells of embryonic, neural, and hematopoietic origin are heterogeneous, with cells moving between two or more metastable states. These cell states show a bias in their differentiation potential and correlate with specific patterns of transcription factor expression and chromatin modifications.

How stem cells balance their self-renewal capacity and their ability to differentiate are central questions in stem cell research. Here we review recent findings supporting the notion that heterogeneity is a hallmark of both embryonic and adult stem cells. This heterogeneity might have evolved as a mechanism that enables stem cells to respond to differentiation-inducing signals while retaining their self-renewal potential.

Regulation of Embryonic Stem Cell Self-Renewal and Differentiation

The first differentiation event during mammalian development is the segregation of trophectoderm and inner cell mass (ICM) at the late morula state. The ICM goes on to form the primitive endoderm and the epiblast, which during gastrulation gives rise to the three primordial germ layers and ultimately to all cell types of the embryo proper. Embryonic stem cells (ESCs) are immortal cell lines derived from the ICM of mouse and human blastocysts. Similarly, pluripotent epiblast stem cells (EpiSCs) and embryonic germ cells (EGCs) are cell lines derived from epiblast stage embryos and primordial germ cells, respectively (Yu and Thomson, 2008). The two most defining features of ESCs are their unlimited in vitro self-renewal capacity combined with their ability to differentiate into all somatic cell types. A number of transcription factors, most prominently Oct4, Nanog, Klf4, and Sox2, have been identified as positive regulators that induce and maintain self-renewal and the undifferentiated state of ESCs (Jaenisch and Young, 2008). The power and importance of these genetic regulators was dramatically demonstrated by their induction of pluripotency in fibroblasts (Takahashi and Yamanaka, 2006).

Several studies reported that transcription factors associated with pluripotency are expressed in a heterogeneous fashion in ESC cultures. For example, approximately 80% of ESCs express Nanog, while 10%–20% do not (Chambers et al., 2007; Singh et al., 2007). In addition, Gata6, a transcription factor governing primitive endoderm formation, is predominantly expressed in Nanog^{neg} cells (Singh et al., 2007). ESCs also display heterogeneity with regard to expression of the transcription factor Rex1 (Toyooka et al., 2008). This heterogeneity is not due to the coexistence of independent cell populations, since culturing of isolated marker positive and negative fractions restored cells with the original expression pattern, implying that the two populations can convert into each other. What is the biological significance of this heterogeneity? It turns out that the different Nanog subpopulations exhibit distinct differentiation biases: Nanog^{pos} ESCs generate undifferentiated cell colonies at high frequencies, while Nanog^{neg} cells show a higher propensity for differentiation (Chambers et al., 2007). ESCs are therefore able to switch between one state biased toward self-renewal and another biased toward differentiation. Interestingly, cellular heterogeneity can also be seen in vivo. That is, in the ICM, Gata6 and Nanog are expressed in an apparently random but mutually exclusive "salt-and-pepper" fashion (Chazaud et al., 2006). The expression of these factors is probably subject to extracellular signaling, since abrogation of Grb2, a member of the MAP kinase pathway, induces expression of Nanog in all cells of the ICM at the expense of Gata6 expression and primitive endoderm formation. Similarly, treatment of ESCs with an antagonist of the MAP kinase pathway leads to Nanog repression and induces differentiation (Toyooka et al., 2008). These and other experiments suggested that self-renewal represents the ESC ground state. In support of this idea, murine ESCs can be maintained in a self-renewing state in the absence of leukemia inhibitory factor (LIF) or other extrinsic signals when the MAP kinase pathway is blocked (Ying et al., 2008). However, LIF signaling might nevertheless be necessary for the initial establishment of pluripotency, as was suggested by recent work with facultative pluripotent cell lines established from the mouse ICM (Chou et al., 2008). In conclusion, these data suggest that ESCs, and possibly also other pluripotent cells, can move between different metastable cell states that are accompanied by fluctuations in transcription factor expression. These states differ in their responsiveness to differentiation-inducing extracellular stimuli.

A recent paper by Surani and colleagues (Hayashi et al., 2008) sheds new light on the molecular mechanisms underlying heterogeneity of gene expression in ESCs. The authors noted that *Stella* is expressed in 20%–30% of ESCs, using a cell line with a GFP reporter gene driven from promoter elements of the stella gene. Stella, also known as PGC7 or Dppa3, has been implicated in the maintenance of gene-specific DNA methylation in the early embryo (Nakamura et al., 2007). Phenotypically, Stella-GFP^{pos} ESCs resemble ICM cells, since they express Nanog and Rex1 at high levels. By contrast, Stella-GFP^{neg} ESCs are more epiblast-like, as they express *Fgf5* and *Gbx2* at levels intermediate between Stella-GFP^{pos} ESCs and epiblast-derived stem cells. When Stella-GFP^{pos} and Stella-GFP^{neg} ESC fractions were isolated and placed in separate cultures, the original distribution of marker gene expression was restored. In spite of their interchangeability, the two populations exhibited distinct

differentiation biases, since Stella-GFP^{neg} ESCs differentiated more readily into cells expressing somatic as well as trophectodermal markers than did Stella-GFP^{pos} cells, which were more prone to give rise to embryoid bodies. The relatively low abundance of Stella-GFP^{pos} ESCs (~30%) compared to Nanog^{pos} ESCs (~80%) suggests that the Nanog-positive subset is heterogeneous or, alternatively, that Stella fluctuations have a longer off phase.

How are the Stella-positive and negative ESC subpopulations established? Insight into this question came from the analysis of histone modifications at the endogenous *stella* locus. Thus, H3K4me3 and H3K9ac, histone modifications associated with gene activation, were more prevalent in Stella-GFP^{pos} than in Stella-GFP^{neg} ESCs and lowest in EpiSCs. In addition, the *stella* promoter was largely unmethylated in Stella-GFP^{pos} and Stella-GFP^{neg} ESCs, indicating an active state, whereas it was heavily methylated in EpiSCs, indicating an inactive state. Treatment of ESCs with the histone deacetylase inhibitor Trichostatin A increased the proportion of Stella-GFP^{pos} cells, while the DNA methylation inhibitor 5-azacytidine had no effect. These observations suggest that histone modifications can transiently stabilize the oscillatory expression of transcription factors involved in self-renewal and differentiation of ESCs. Subsequently, when ESCs commit to become EpiSCs, DNA methylation irreversibly silences inappropriate gene expression, thus demarcating a developmental boundary between ESCs and EpiSCs. Figure 1 shows a summary of these findings and their interpretation.

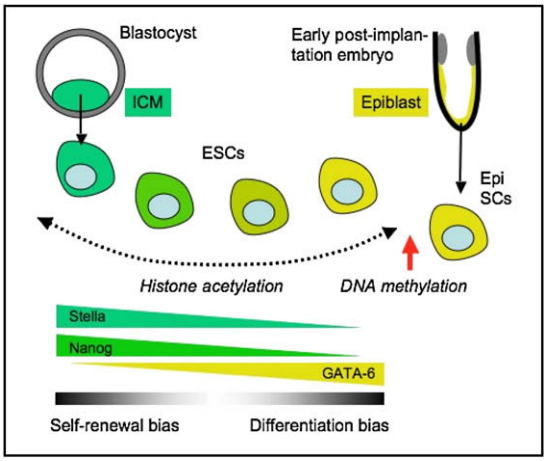

Figure 1. Heterogeneity of Embryonic Stem Cells
ESCs consist of various cell subsets that express different levels of specific markers (such as Stella and the transcription factors Nanog and GATA-6) and that continuously convert into each other. These subsets grossly recapitulate different stages between the ICM and epiblast-like cells. The oscillations between the subsets (indicated by the broken arrow) involve changes in histone modifications. In contrast, the developmental transition to EpiSCs is irreversible and involves methylation of ESC-specific promoters, such as Stella. Cells expressing Stella and Nanog are biased toward self-renewal, where cells at the other end of the spectrum are biased toward differentiation (Hayashi et al., 2008).

Heterogeneity of Adult Stem Cells

Can subpopulations with distinct biological properties also be observed in adult stem cells, or are they limited to embryonic stem cells? Adult stem cells are present in numerous tissues, such as bone marrow, gut, and skin, where they serve to replace cells lost to injury, attrition, or natural turnover. They are rare, mostly quiescent cells that are contained within specialized niches in the body. These properties, together with the limited self-renewal potential of most adult stem cells in culture, complicate their study at the molecular level. Nevertheless, a growing body of evidence indicates that at least some adult stem cell types are heterogenous. Tracking the expression of the G protein-coupled receptor Lgr5 identified an intestinal stem cell (ISC) population located at the base of the crypt (Barker et al., 2007). More recently, Capecchi and colleagues identified another ISC population (Sangiorgi and Capecchi, 2008) using the polycomb repressor complex protein Bmi1 as an indicator. The Bmi1^{pos} ISC is located at a higher position within the crypt, thus occupying a different niche. Both Lgr5^{pos} and Bmi1^{pos} ISCs are capable of generating all epithelial cell types of the small intestine, but the latter commit to differentiation more slowly, indicating that they are more quiescent. This observation suggests that Bmi1^{pos} ISCs are precursors of Lgr5^{pos} ISCs. Nevertheless, it is still possible that the two ISCs represent two lineages with distinct developmental origins, or that they can convert into each other.

Several lines of evidence indicate that hematopoietic stem cells (HSCs), the best-studied adult stem cell type, also consist of distinct subpopulations. HSCs are functionally defined by their multilineage, long-term reconstitution potential when transplanted into irradiated mice and constitute approximately 1 in 10,000 nucleated cells in the bone marrow. Various protocols, most of them using combinations of antibodies against cell-surface markers, permit enrichment of HSCs to a high degree of purity. In a systematic study to resolve the question whether individual HSCs differ in their self-renewal and differentiation potential, single HSCs were transplanted into mice analyzed for the presence of donor-derived cells at different times in both primary as well as in secondary and tertiary recipients (Dykstra et al., 2007). Of about 100 mice with long-term reconstitution examined, four distinct patterns of reconstitution were observed, which were used to retrospectively define the transplanted cells. Two HSC types with long-term reconstitution potential were identified: α type HSCs, which produced a substantially higher proportion of myeloid cells (macrophages and granulocytes) compared to lymphoid progeny (B and T cells); and β type HSCs, which showed a much more balanced distribution of lineage output. When bone marrow cells from primary recipients cells were transplanted into secondary and even tertiary hosts, these patterns remained reproducible, suggesting that the two types of HSCs are stabilized by epigenetic mechanisms. Notably, however, approximately half of the HSCs that generated an α cell type repopulation pattern in primary recipients switched to a β cell repopulation pattern when serially transplanted, while the reverse was not observed. Together, these observations suggest that HSCs fall into two (or more) subpopulations that exhibit distinct self-renewal and differentiation biases. The fact that conversions between these subpopulations appear to be unidirectional, at least under the somewhat artificial conditions of transplantation, raises the possibility that the mechanisms that generate heterogeneity in embryonic stem cells and HSCs differ. To unravel the molecular mechanisms involved in the establishment of the two HSC subsets it will now be necessary to identify

markers that allow the prospective isolation of α type and β type HSCs.

Using antibody based cell separation techniques, HSC subsets with distinct phenotypic and functional properties were recently described (Haug et al., 2008). These authors separated HSCs based on N-cadherin expression. A first subset termed "reserved" HSCs expresses N-cadherin at intermediate levels and has poor repopulation potential and low cell-cycle entry rate; a second subset, termed "primed" HSCs is N-cadherin low, has robust repopulation potential, and expresses genes that might prime them for mobilization. Reserve HSCs acquire both phenotypic and functional characteristics of primed HSCs upon overnight culture. N-cadherin is an adhesion molecule thought to help anchoring HSCs into the osteogenic niche, although the expression of N-cadherin by HSCs and their requirement for it have recently been called into question (Kiel et al., 2007). Nonetheless, the findings from Haug and colleagues suggest that the bone marrow contains two partially interconvertible HSC populations, at least in the context of in vitro culture. Whether these subsets correspond to the two types of HSCs identified in the study of Eaves and colleagues (Dykstra et al., 2007) remains unclear.

Oscillatory Gene Expression in Adult Progenitor and Stem Cells

Whether biological "noise," such as the stochastic fluctuations of transcriptional regulators, contributes to cell lineage or fate decisions has been intensively debated for some time. As early as 15 years ago, it was shown that cytokine withdrawal induces multilineage differentiation in a hematopoietic progenitor cell line in which Bcl2 was overexpressed to prevent apoptosis (Fairbairn et al., 1993). This finding is consistent with the idea that hematopoietic cells possess an intrinsic mechanism that generates a spectrum of progeny with different differentiation biases, resulting in commitment either spontaneously or as a consequence of extracellular cues. In support of this concept is the observation that HSCs express a variety of lineage-restricted genes at low levels (Miyamoto et al., 2002). The conflicting coexpression of various lineage-associated programs within individual cells becomes resolved once progenitors commit and lineage-specific genes are selectively upregulated (Miyamoto et al., 2002).

A recent study by Huang and colleagues (Chang et al., 2008) directly demonstrated that stochastic-oscillatory expression of lineage-associated genes can drive cell-fate commitment (see also Figure 2). Using the myeloid-erythroid precursor EML cell line as a model, they showed that Sca-1, a cell surface marker of HSCs and some early progenitors, is expressed in a broad, bell-shaped pattern. Culturing either Sca-1^{pos} and Sca-1^{neg} cell fractions regenerated the original antigen distribution after ~12 population doublings with no obvious differences in timing between the two subfractions. Mathematical modeling suggested that the observed slow fluctuation of Sca-1 expression resulted from a process involving stochastic transitions between multiple metastable states. Most strikingly, the cells at the extremes of the spectrum differed in their differentiation potential: Sca-1^{pos} cells were strongly biased toward myeloid differentiation, Sca-1^{neg} cells toward erythroid differentiation. This correlated with the expression of the transcription factors PU.1 and GATA-1, which are known to play antagonistic roles in the specification of myeloid and erythroid fates (Graf, 2002). The data thus indicate that multipotent progenitors express lineage-instructive transcription factors in a mutually exclusive fashion and in an oscillatory manner, continuously generating cells that exhibit distinct differentiation biases. A theoretical model of lineage commitment controlled by antagonistic cross-interaction between PU.1 and GATA-1 was recently proposed by Huang and Enver (Huang et al., 2007). Of note, primary multipotent blood progenitor cells in the bone marrow can also be subdivided into a myeloid-primed fraction and an erythroid-primed fraction based on the expression of PU.1 and GATA-1 (Arinobu et al., 2007). This raises the possibility that different states of multipotent hematopoietic progenitors also oscillate in vivo.

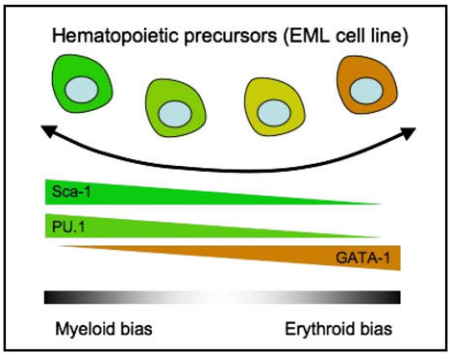

Figure 2. Heterogeneity of Hematopoietic Progenitor Cells
The scheme summarizes data obtained with the EML cell line, which can be induced toward myeloid and erythroid differentiation using different cytokines (Chang et al., 2008). EML cells exhibit a broad spectrum of Sca-1 expression, and cells with different levels of Sca-1 restore the original spectrum when cultured (arrow). Sca-1^{high} cells exhibit a high ratio of PU.1 versus GATA-1 expression and are biased toward myeloid differentiation; Sca-1^{neg} cells show a high ratio of GATA-1 versus PU.1 expression and are biased toward erythroid differentiation.

Direct evidence that oscillations of gene expression presage commitment in normal multipotent precursors comes from the study of neural progenitors (Shimojo et al., 2008). The cell surface receptor/transcription factor Notch has long been known to be a key determinant of neural cell fate. In neural progenitors, activation of Notch signaling represses neural fate through ligand binding, and progenitors expressing elevated levels of ligand prevent neighboring cells from becoming neurons. The effects of Notch are mediated by upregulation of the helix-loop-helix transcription factor (HLH) Hes1. Performing real-time live imaging of cultured neural precursors and in vivo, Kageyama and colleagues observed that Hes1 expression oscillates with a wavelength of 2–3 hr. Overexpression of Hes1 in neural precursors induced the downregulation of the neural genes Delta-like1 and Neurogenin2. Importantly, Delta-like1 and Neurogenin2 also oscillate, with peaks corresponding to the valleys of Hes1 expression, suggesting that Hes1 oscillations drive Delta-like1 and Neurogenin2 oscillations. Experiments using the Notch inhibitor gamma secretase indicates that Notch signaling is also required for the induction of Hes1 oscillations under physiological conditions. In conclusion, antagonistic oscillations of neural and nonneural genes preceding cell commitment appear to be necessary for commitment while maintaining a stem cell state. What sets Hes1 oscillations in motion? It is unlikely that the

induction of these oscillations is linked to the circadian clock because their wavelength is considerably shorter than 1 day. Instead, Shimojo et al. suggest that the oscillations are regulated by Jak/Stat signaling (Shimojo et al., 2008). However, even if substantiated, the chicken and egg question remains. Perhaps the oscillations result from an amplification of noisy expression of specific regulatory factors and are modulated by positive and negative feedback loops, as has been shown for genes in yeast (reviewed by Arias and Hayward, 2006).

In conclusion, both embryonic and adult stem cells display a surprising degree of heterogeneity caused by the oscillatory expression of synergistically and antagonistically acting transcription factors and stabilized by epigenetic modifications. These fluctuations may have evolved to allow stem cells to self-renew while also offering "windows of opportunity" to respond to environmental signals that can trigger specific differentiation. What generates stochastic fluctuations of transcription factors in the first place and how the crosstalk between stem cells and the niche translates into changes in transcriptional networks and chromatin modifications remain hot questions in stem cell research.

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