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Review article

The marrow cell continuum: stochastic determinism

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Abstract: Traditional models of hematopoiesis have been hierarchical in nature. Over the past 10 years, we have developed data indicating that hematopoiesis is regulated in a continuum with deterministic and stochastic components. We have shown that the most primitive stem cells, as represented by lineage negative rhodamine^{low} Hoechst^{low} murine marrow cells are continuously or intermittently cycling as determined by *in vivo* BrdU labeling. When marrow stem cells are induced to transit cell cycle by *in vitro* exposure to cytokines, either IL-3, IL-6, IL-11, and steel factor or thrombopoietin, FLT3 ligand, and steel factor, they progress through cycle in a highly synchronized fashion. We have determined that when the stem cells progress through a cytokine stimulated cell cycle the homing, engraftment, adhesion protein, global gene expression, and hematopoietic differentiation phenotypes all change in a reversible fashion. This has led to the continuum model, in which, with cycle transit, chromatin is continually changing altering open transcription areas and providing a continually changing landscape of transcriptional opportunity. More recently, we have extended the changing differentiation profiles to differentiation into lung cells and found that non-hematopoietic differentiation also shows cycle related reversibly modulation. These observations all together support a continuum model of stem cell regulation in which the phenotype of the marrow stem cells is continually and reversibly changing over time.

Key words: Hierarchy - Continuum - Stem cell - Cell cycle - Progenitor

Engraftment studies

A growing body of data has indicated that marrow progenitor/stem cells are regulated on a continuum, rather than in a hierarchy. This indicates that the phenotype of the marrow "stem cell" is labile and ever changing. These classes of cells can only be defined on a population basis, not at a single cell level, since the single cell will continuously and reversibly be changing its functional, cell surface and gene expression phenotype.

Over the past 10 years, we have reported studies showing that the marrow stem cell capacity varies with phase of cell cycle and that this variation is not unidirectional, rather it is reversible. Initial studies showed that *in vitro* exposure to the cytokines interleukin-3 (IL-3), IL-6, IL-11, and steel factor resulted in a loss of engraftment after 48 hours of culture [10, 11]. When highly purified lineage negative rhodamine^{low} Hoechst^{low} (LRH) marrow cells were mapped through cell cycle, it was shown that it took approximately 18 hours to reach S-phase, and that the first cell cycle was completed by 36-38 hours [12]. Subsequent cell cycles were quite rapid, being completed at 12 hour intervals for up to 5 cycles. More recent studies have shown that this population, which is tightly synchronized by the cell separative procedure, shows up to 98% cells in S phase at one point during the first cell cycle. A very prominent decrease in 8 week or 6 month engraftment was seen at 48 hours of cytokine culture. Subsequent studies showed that there were reversible decreases in engraftment capacity which could occur at 2 to 4 hour intervals; a prominent and reproducible decrease in engraftment was seen to occur between 28-36 hours of culture, a time representing late S/early G2 [7]. These studies were particularly important, because they indicated that the observed changes in the engraftment phenotype did not represent an irreversible unidirectional differentiation

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step, but rather was a reversible variation which occurred as the stem cells transited cell cycle.

Adhesion proteins and marrow homing

There followed studies on different adhesion proteins showing reversible fluctuations of adhesion protein expression in both LRH and lineage negative Sca-1+ (Lin-SCA-1+) marrow stem cells as they transited cell cycle. Changes in VLA-4 showed the tightest correlation with alterations in engraftment capacity [1, 2]. Further studies indicated that marrow cell homing was markedly depressed after 48 hours in IL-3, IL-6, IL-11 and steel factor [4]. These studies indicated that marrow stem cells exposed to cytokines *in vitro* modulated their adhesion protein expression which led to alterations in homing to marrow which in turn led to changes in short and long-term engraftment.

Progenitors and stem cell/progenitor inversions

Further studies showed that marrow progenitor cells, as represented by colony-forming unit culture (CFU-c) or multifactor responsive high-proliferative potential colony forming cells (HPP-CFC), also showed reversible variations with cycle transit [6]. These studies were carried out in culture with thrombopoietin, FLT3 ligand and steel factor (TFS) as the cytokines which stimulated cell cycle transit. These progenitors generally showed increases when engraftment capacity was decreased. We term these progenitor/stem cell inversions. These studies suggested, although they did not prove, that progenitors, as conventionally assayed, and engraftable stem cells may simply be the same cell showing different phenotypes at different points in a cell cycle transit.

Cell cycle status of stem cells

The changes in stem cell phenotype with cell cycle passage under cytokine stimulation, while of intrinsic interest, would have less general biologic meaning if marrow stem cells were truly quiescent non-cycling cells. However, they are not. Important work by Bradford and colleagues [3], confirmed by two other groups [5, 9] showed that *in vivo* the most primitive "dormant" stem cell class is in fact cycling. These investigators fed mice BrdU in their drinking water and then isolated the lineage-negative, rhodamine^{low} Hoechst^{low} (LRH) marrow stem cells at different times in the feeding schedule and determined BrdU labeling of these stem cells. BrdU is incorporated into DNA during DNA synthesis and, thus, labeling with this agent is an accurate measure of whether a cell has progressed through S-phase while transiting the cell cycle. They found that 60% of these primitive stem cells were labeled after 4 weeks and showed a time to 50% labeling of 19 days. Cheshire and colleagues [5] using a different mouse strain and stem cell separation confirmed these data, showing more rapid labeling kinetics. Our group investigated whether DNA damage and repair might explain the labeling, but after extensive studies came to the conclusion that BrdU incorporation did probably indicate proliferation of these primitive stem cells over time [9]. Thus, the most primitive stem cells are proliferating *in vivo* over time and are presumably continuously and reversibly changing their functional phenotype over time.

Table 1. Phenotypic characteristics which reversibly modulate in stem/progenitor cell with cell cycle transit

Six week and 6 month competitive engraftment capacity
Homing at 3 hours to marrow
Expression of surface adhesion proteins and genes coding for these proteins
Stem cell progenitor inversions. Reversible increases in progenitors tied to decreases in stem cells with cell cycle transit
Global gene expression as determined by 3"end gene display or real-time PCR

Gene expression

Global gene expression was also evaluated in LRH marrow stem cells at isolation and after 48 hours of culture in IL-3, IL-6, IL-11, and steel factor, a time when engraftment is reproducibly and reversibly depressed [8]. To assess gene expression, we utilized a 3' end-gene display approach, which detects all sequences. Initially we compared lineage positive cells to LRH stem cells. We found 637 stem cell specific genes, of which 411 were unknown. While interpretation of individual gene expression was difficult, especially given the number of unknowns, we observed a dramatic inversion of gene expression in the LRH cells at 48 hours. Genes which were highly expressed at time 0 in LRH cells were minimally expressed at 48 hours. Conversely, many lowly expressed genes at time 0 were highly expressed at 48 hours. In more recent studies, evaluating gene expression by real-time PCR in LRH cells progressing through a cytokine (TPO, FLT3L, and steel factor) stimulated cell cycle, we observed marked fluctuations in expression of certain cell surface markers, cytokine receptors, and transcription factors. Genes which were reversibly modulated included those coding for c-kit, CD4, Sca-1, and SDF-1.

All of these observations weighed against a hierarchical model of stem/progenitor regulation and for a continuum model. These studies are summarized in Table 1.

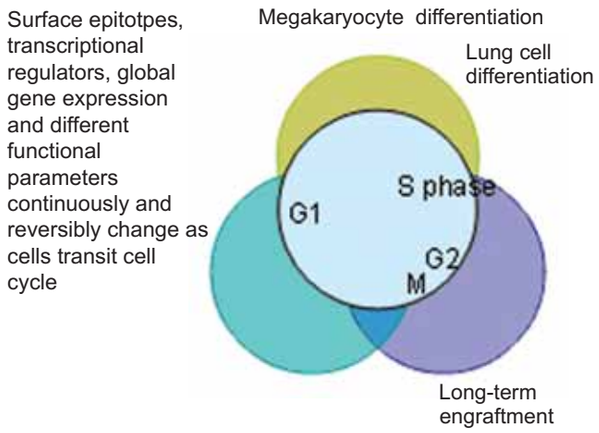

Fig. 1. The continuum model of stem cell biology

Differentiation into hematopoietic and non-hematopoietic lineages

The studies cited above indicate that the stem cell changes phenotype with cell cycle passage. Cycling cells continually change chromatin coverage and this, in turn, will lead to different transcriptional control areas being open to interaction with transcription complexes. This is what presumably determines whether the cell renews, proliferates, and/or differentiates. The cell would in essence be continually presenting different windows of transcriptional opportunity. Thus, a stem cell transiting cell cycle and exposed to a constant cytokine induction would show different outcomes at different points in cycle. In order to test this hypothesis, we structured experiments in which highly purified marrow LRH cells were driven through cycle by exposure to TPO, FLT3L, and steel factor and then subcultured in GM-CSF, G-CSF, and steel factor at different times in cycle. The final differentiation outcome was determined 14 days later. In these experiments, there was marked induction of megakaryocyte differentiation in early S phase. In additional experiments, using green-fluorescent protein positive congenic marrow cells and IL-3, IL-6, IL-11, and steel factor as a stimulus for cycle transit, the capacity of marrow cells to differentiate into lung cells was assessed. In these experiments, GFP+ marrow cells engrafted into irradiated mice and then later G-CSF mobilized mice showed a marked increase in GFP+CD45 negative pulmonary events in mid-S phase. Thus, differentiation in both the hematopoietic and non-hematopoietic pathways showed reversible cell cycle related modulation. The concept of a stochastically deterministic continuum model is presented in Figure 1.

Heterogeneity of a stem cell population

The continuum model of stem cell regulation implies pronounced heterogeneity within the stem cell compartment. This would be so because no matter how tightly synchronized the stem cells may appear to be, using tritiated thymidine or propidium studies, the synchrony is not exact and, probably on a micro-time scale, most freshly isolated purified stem cells will not be in exactly the same phase of cycle, although most may be in early G1 phase. When highly purified LRH marrow cells are investigated for any functional attribute, these cells are almost totally heterogeneous. For instance, when single cell colony formation is evaluated in the presence of 7 cytokines, virtually every colony is different in gross colony morphology or in the type or quantity of different lineages within a colony. Other parameters of stem cell function show similar heterogeneity. This is to be expected but highlights the meaninglessness of calls for carrying out stem cell studies on a clonal basis. This has been particularly prominent in plasticity studies. In fact, due to the noted heterogeneity and the lability of these cells, the most meaningful studies will, of necessity, be on a population basis, not on a single cell basis. The latter mainly provide information about the intrinsic heterogeneity of the population under study.

Implications of the continuum model

The continuum model is compatible with the great bulk of published literature on stem/progenitor cells, but it puts the system in a different context. The model strongly implies that reductionist approaches to defining the stem cell will be misleading. Rather, stem/progenitor cells will have to be studied on a population basis in order to understand their regulation.

The myriad of different stem cell types which have been reported are probably explained by the continually changing surface phenotype of these cells. Thus, one may see Sca-1+ mpl+ cells at one point in time, endoglin+ CD34- cells at another or even CD4+ CD34+, c-kit positive at another point in time while, in fact, these are all the same cell, simply changing reversibly their surface phenotype.

In a similar vein, a highly purified population of marrow stem cells such as LRH may at one certain phase of cell cycle be predominantly engraftable stem cells, while at another time point the phenotype may be predominantly that of progenitors. In fact, the progenitors and the apparently more primitive engraftable stem cell may be the same cell in different functional states.

There have been elegant and highly ordered transcriptional profiles for different levels of the stem/progenitor cell hierarchy. The continuum proposes that these do not exist, but that the profiles are continuously

changing and represent fluctuating windows of transcriptional opportunity.

These considerations, backed by the data presented above, indicate that the potential to differentiate into different tissues is continuously changing and, with a synchronized stem cell population, one can discern differentiation hot spots for specific hematopoietic or non-hematopoietic lineages. The implications of this for effective tissue restoration in a variety of diseases are apparent and exciting.

Lastly, these deliberations indicate that mathematical modeling will become more and more important in our understanding of stem cell biology and that effective collaboration with our computer and mathematically conversant friends will become necessary for continued progress in this fascinating field.

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