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From stem cell to erythroblast: Regulation of red cell production at multiple levels by multiple hormones

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Abstract

This article reviews the regulation of production of red blood cells at several levels: (1) the ability of erythropoietin and adhesion to a fibronectin matrix to stimulate the rapid production of red cells by inducing terminal proliferation and differentiation of committed erythroid CFU-E progenitors; (2) the regulated expansion of the pool of earlier BFU-E erythroid progenitors by glucocorticoids and other factors that occurs during chronic anemia or inflammation; and (3) the expansion of the hematopoietic cell pool to produce more progenitors of all hematopoietic lineages.

Red blood cells are continuously replenished; their half-life in humans is 120 days. In mammals, definitive erythropoiesis first occurs in the fetal liver with progenitor cells from the yolk sac (1). Within the fetal liver and the adult bone marrow, hematopoietic cells are formed continuously from a small population of pluripotent stem cells that generate progenitors committed to one or a few hematopoietic lineages (Figure 1). In the erythroid lineage, the earliest committed progenitors identified ex vivo are the slowly proliferating burst-forming unit–erythroids (BFU-E). Early BFU-E cells divide and further differentiate through the mature BFU-E stage into rapidly dividing colony-forming unit–erythroids (CFU-E) (2). CFU-E progenitors divide three to five times over 2–3 days as they differentiate and undergo many substantial changes, including a decrease in cell size, chromatin condensation, and hemoglobinization, leading up to their enucleation and expulsion of other organelles (3).

Each branch of the cell lineage tree has different cytokine regulators, allowing exquisite control of the production of specific cells types, and here we summarize the ways in which red cell production is regulated. Short-term production of red cells is controlled largely by erythropoietin (Epo), a kidney-derived hormone that is induced under hypoxic conditions and that stimulates terminal proliferation and differentiation of CFU-E progenitors (4). BFU-E cells respond to many hormones additional to Epo, including SCF, IGF-1, corticosteroids, IL-3, and IL-6. It is not known which cells in the fetal liver or adult bone marrow produce these cytokines, or how they interact to regulate the division of BFU-E cells and control their self-renewal and their ability to differentiate into more mature CFU-E progenitors. In cases of chronic erythroid stress, such as during hemolysis, the number of CFU-E progenitors is insufficient to produce the needed red cells even under high Epo levels, and the body responds by producing more of these progenitors (5). Here we discuss recent evidence that glucocorticoids and other stress hormones stimulate limited self-renewal of early BFU-E erythroid progenitors and thus increase the output of CFU-Es.
Finally, we discuss factors that stimulate self-renewal of hematopoietic cells to produce more progenitors of all hematopoietic lineages.

Because of space limitations, and since this review is meant to summarize a talk by HL at the IUBMB meeting in Melbourne in October 2010, we focus on work from our own laboratory; our heartfelt apologies go to those whose important work we are unable to cite.

Terminal proliferation and differentiation of CFU-E erythroid progenitors

Erythropoietin has long been understood to be the major factor governing erythropoiesis and its role in regulating the expansion, differentiation, apoptosis, and activation of erythroid specific genes is well characterized (4). The first phase of CFU-E erythroid differentiation is highly Epo dependent, whereas later stages are no longer dependent on Epo (6). Consistent with this, Epo receptors are lost as erythroid progenitors undergo terminal proliferation and differentiation (7). This raises the question of what other signals, if any, these differentiating erythroblasts require to support terminal proliferation, differentiation, and enucleation.

Recently we demonstrated the importance of the extracellular matrix protein fibronectin in erythropoiesis (8). By culturing fetal liver CFU-E erythroid progenitors we showed that fibronectin and Epo regulate erythroid proliferation in temporally distinct steps. During the first day in culture CFU-E erythroid progenitor cells undergo two divisions, upregulate the transferrin receptor, and begin expression of Ter119 and several hundred other erythroid-important genes. This stage requires Epo but is independent of fibronectin. During the second day there are 2–3 rapid cell divisions with short or absent G1 and G2 stages; most cells are in S or M. There is complete repression of all gene transcription, chromatin condensation, nuclear condensation, and enucleation. Adhesion to fibronectin, but not the presence of Epo, is essential for these last 2 – 3 terminal cell divisions. In each phase, Epo and fibronectin promote expansion by preventing apoptosis, in part through inducing bcl-xL expression.

We showed that \( \alpha_4, \alpha_5, \) and \( \beta_1 \) are the principal integrins expressed on erythroid progenitors; their downregulation during erythropoiesis parallels the loss of cell adhesion to fibronectin and likely accounts for the release of mature cells from the marrow into the circulation. Culturing erythroid progenitors on recombinant fibronectin fragments revealed that only substrates that engage \( \alpha_4\beta_1 \) integrin support normal terminal proliferation. Taken together, these data suggest a two-phase model for growth factor and extracellular matrix regulation of erythropoiesis, with an early Epo-dependent, integrin-independent phase followed by an Epo-independent, \( \alpha_4\beta_1 \) integrin-dependent phase.

Binding of Epo to Epo receptors (EpoRs) on the surface of erythroid progenitors triggers activation of multiple intracellular signal transduction pathways, including the Stat5 (Signal transducer and activator of transcription 5), Phosphoinositide -3 kinase/Akt, and Shc/Ras/MITogen-Activated Kinase (MAPK) pathways. Elimination of either of the first two pathways leads to significant apoptosis of early progenitors and reduced output of erythrocytes (9). In contrast, blocking the Ras/MAPK pathway has only subtle effects on terminal erythropoiesis (10).

Our studies on Stat5a\(^{-/-}\)/ Stat5b\(^{-/-}\) mice were very revealing in uncovering not only the cellular role of this factor in erythropoiesis, but also the regulation of red cell production by expansion of earlier progenitor and stem cell populations. Stat5 is a latent cytoplasmic transcription factor activated by EpoR as well as many other hematopoietic and non-hematopoietic cytokine receptors. Following EpoR activation, Stat5 binds to phosphorylated tyrosines on EpoR, and itself becomes tyrosine-phosphorylated. This results in its dimerization and translocation to the nucleus where it initiates transcription of target genes.
Known Stat5 targets include tissue-specific genes as well as genes regulating cell growth. We showed in a number of erythroid and hematopoietic cell lines that Stat5 induces the immediate early expression of the anti-apoptotic gene bcl-xL by directly binding to Stat5 consensus sites in the bcl-x gene. Consistent with this, Stat5 has an anti-apoptotic effect in erythroid cell lines, and dominant negative Stat5 molecules increased apoptosis and inhibited growth of cultured fetal liver erythroid progenitors (11).

However, many adult Stat5a−/−Stat5b−/− mice have a normal or near-normal steady-state hematocrit (12). How then could this factor be crucial for red cell production? To resolve this dilemma, we showed that Stat5 is essential for the high erythropoietic rate during fetal development. Stat5a−/−Stat5b−/− embryos are severely anemic; erythroid progenitors are present in low numbers, show higher levels of apoptosis, and are less responsive to Epo. This is mediated in part by reduced accumulation of the bcl-xL protein (11).

Subsequently we confirmed that some adult Stat5a−/−Stat5b−/− mice have a near-normal hematocrit but showed that they are deficient in generating high erythropoietic rates in response to stress and have very high endogenous levels of Epo in the blood. Further, many adult Stat5a−/−Stat5b−/− mice have persistent anemia in spite of a marked compensatory expansion in their erythropoietic tissue leading to gross expansion of the spleen and its conversion largely to a erythropoietic organ. Analysis of erythroblast maturation in Stat5a−/−Stat5b−/− hematopoietic tissue showed a dramatic increase in early erythroblast numbers, but these fail to progress in differentiation and undergo apoptosis. Decreased expression of bcl-xL and increased apoptosis in Stat5a−/−Stat5b−/− early erythroblasts correlate with the degree of anemia (13). Hence, Stat5 indeed controls a rate-determining step regulating early erythroblast survival, leading to induction or repression of multiple genes in a defined temporal order.

Thus the reason that many adult Stat5a−/−Stat5b−/− mice have normal or near normal red cell levels relates to their ability to greatly increase the numbers of CFU-E progenitor cells. Despite the fact that many undergo apoptosis in spite of the extremely high level of Epo, enough are able to proliferate and differentiate to generate near normal levels of red cells. This response to the chronic stress of anemia is termed stress erythropoiesis.

Stress erythropoiesis: regulated expansion of erythroid progenitors

In situations of severe loss of red blood cells mammals and birds respond by the process known as stress erythropoiesis (SE). Unlike steady-state erythropoiesis, which is largely regulated by Epo, SE also requires stem cell factor (SCF) and glucocorticoids (GCs) (14–16). Cortisol is the most abundant natural GC, a steroid stress hormone that regulates inflammatory responses and glucose metabolism. It is well documented that release of cortisol from the adrenal glands is increased during conditions of stress erythropoiesis, such as sepsis or severe trauma (17). Direct effects of cortisol on erythropoiesis are demonstrated, by polycythemia as an early clinical manifestation of Cushing’s syndrome, (18) and by the therapeutic effect of prednisone in patients with the red cell progenitor disorder Diamond-Blackfan Anemia (DBA) (19). Although the molecular events following glucocorticoid receptor activation in inflammation and metabolism are well understood (20), relatively little is known of how GCs promote red cell formation during SE.

The process of SE can be replicated and studied in vitro, by culturing erythroid progenitors in medium containing SCF, Dexamethasone (Dex) and Epo. Both in vitro proliferation of FL erythroblasts and in vivo SE are severely decreased by a mutation in the glucocorticoid receptor that disrupts dimerization. (14,21) The stimulatory effects of GCs on red cell production therefore likely requires glucocorticoid receptor dimerization, which is required for efficient transactivation of promoters with glucocorticoid receptor element (GRE) full-
sites but which may also be necessary for repression at negative GRE full-sites (20).
Although SE has been extensively studied, the target cell and the physiological effects of
GCs have been elusive. In work under review (Flygare, J. and H. Lodish), we established
two FACS-based methods to separate and purify BFU-E and CFU-E cells from mouse fetal
liver. By culturing these cells in the presence of SCF and IGF-1, we demonstrated that GCs
induce limited self-renewal of BFU-E cells, and not of CFU-E cells or erythroblasts. GCs
thereby protect BFU-E cells from exhaustion, and in parallel over time they increase the
number of CFU-E cells formed from each BFU-E greater than 10-fold. Thus we propose a
physiological model of stress erythropoiesis where increased levels of GCs help maintain
the earliest erythroid progenitors, increase CFU-E output, and at the same time stimulate
terminal differentiation, thus promoting both a rapid and long-lasting increase in red blood
cell production. Identification of BFU-Es as the target cell of GCs in SE, together with our
novel method to isolate BFU-Es, will allow studies towards development of novel
erythropoiesis stimulating agents that act by promoting SE by the same mechanisms
employed by GCs.

**Regulation of hematopoietic stem cell expansion**

Hematopoietic stem cells (HSCs) are defined by their ability to self-renew and to
differentiate into all blood cell types. *In vivo* these cells have at least four fates: self-renewal,
cell death (apoptosis), differentiation, and mobilization. They reside in specialized niches
and receive signals - both via secreted and cell surface proteins – from surrounding stromal
cells, but the identity of most of these signals and the effects they have on HSCs is largely
unknown (22,23). In adults HSCs are largely quiescent and reside in two well-characterized
bone marrow niches – one apposed to osteoblasts on the endosteal surface, and the other
adjacent to or near the sinusoids (24–26). Except for osteoblasts, the identity of other cells in
the niche is not known, and the identity of most of the signals produced by these cells is also
unknown (27–29).

Our work focuses on defining the niche in fetal liver, where HSCs are rapidly expanding to
produce the large numbers of hematopoietic progenitor and differentiated cells. To begin we
identified a novel cell population that supports *ex vivo* expansion of bone marrow HSCs -
CD3+ cells isolated from Embryonic Day 15 (E15) fetal livers (30). DNA array experiments
showed that, among other proteins, insulin-like growth factor 2 (IGF-2) and Angiopoietin-like
proteins 2 (Angptl2) and 3 (Angptl3) are specifically expressed in these cells but not in
several cell types that do not support HSC expansion. We then developed a simple serum-
free culture system for bone marrow HSCs using low levels of SCF, TPO, FGF-1, IGF-2
and Angptl2 or Angptl3. As measured by competitive repopulation analyses, there was a 24
to 30 fold increase in numbers of long-term repopulating HSCs (LT-HSC) after 10 days of
culture (31). A similar cocktail of growth factors supported a ~20 fold expansion *ex vivo* of
human cord blood HSCs (32).

In trying to characterize the ~2% of fetal liver cells reactive with an anti-CD3ε monoclonal
antibody and that support *ex vivo* expansion of both fetal liver and bone marrow HSCs, we
realized that these cells actually do not express any CD3 protein. Neither do they express
any other proteins characteristic of T cells. These are a defined subset of fetal liver cells and
we do not know what protein(s) made by them react with the supposedly specific anti-CD3ε
monoclonal antibody.

Recently we were able to purify these same HSC-supportive stromal cells based on the
surface phenotype of SCF+DLK+ (Chou, S. and H. Lodish, unpublished). These are the
principal fetal liver cells that express not only Angiopoietin-like 3, and IGF-2, but also SCF
and Thrombopoietin, two other growth factors important for HSC expansion. They are also

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the principal fetal liver cells that express CXCL12, a factor required for HSC homing, and also α-fetoprotein (AFP), indicating they are fetal hepatoblasts. Immunocytochemistry shows that >93% of the SCF+ cells express DLK and Angptl3, and about a third of the SCF+ cells also expresses CXCL12. Thus SCF+DLK+ cells are a highly homogenous population that express a complete set of factors for HSC expansion and are the hepatoblast stromal cells that support HSC expansion in the fetal liver. Although we have not yet determined whether these SCF+DLK+ stromal cells physically interact with HSCs, the fact they express membrane-bound forms of DLK and SCF on their surface does suggest they are able to interact with HSCs via the binding between the SCF receptor c-kit and the still unknown receptor for DLK on HSCs.

We do not know whether these SCF+DLK+ cells are present in adult bone marrow. Nor do we know the identity of the factors that stimulate self-renewal of HSCs in bone marrow. Several of the factors we showed support ex vivo expansion of HSCs – Tpo and Angptl3 – continue to be made by adult liver at levels similar to that in fetal liver (Chou, S. and H. Lodish, unpublished). But we do not know whether these hormones function systemically to regulate HSCs in the bone marrow.

Thus our work showed that four key growth factors - SCF, TPO, IGF-2 and Angptl3 – are made by the same fetal liver cell, and synergize to support maximum expansion of HSCs. But we do not know the function of each of these individual growth factors on hematopoietic stem cells. Do they stimulate division, prevent apoptosis, or prevent differentiation of daughter cells? We need to discover the signal transduction pathways induced in hematopoietic stem cells by each of these growth factors and elucidate how these signaling pathways interact to support HSC self renewal. Finally, we need to understand how hematopoietic stem cells interpret these signals and decide whether to divide, differentiate, undergo apoptosis, or simply remain quiescent.

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FIGURE 1. Formation of blood cells from hematopoietic stem cells in the bone marrow

Pluripotent long-term reconstituting stem cells may divide symmetrically to self-renew or divide asymmetrically to form a myeloid or lymphoid progenitor cell and a daughter cell that is pluripotent like the parental stem cell. Depending on the types and amounts of cytokines present, the myeloid and lymphoid progenitor cells undergo rapid rounds of cell division and generate different types of unipotent precursor cells, which are incapable of self-renewal. Unipotent precursor cells respond to one or a few specific cytokines, and are detected by their ability to form colonies containing the differentiated cell types shown at right, measured as colony-forming units (CFU-s). (Adapted from (33)).