NORMAL AND PATHOLOGIC ERYTHROPOIESIS

Molecular and cellular mechanisms that regulate human erythropoiesis

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To enable effective oxygen transport, ~200 billion red blood cells (RBCs) need to be produced every day in the bone marrow through the fine-tuned process of erythropoiesis. Erythropoiesis is regulated at multiple levels to ensure that defective RBC maturation or overproduction can be avoided. Here, we provide an overview of different layers of this control, ranging from cytokine signaling mechanisms that enable extrinsic regulation of RBC production to intrinsic transcriptional pathways necessary for effective erythropoiesis. Recent studies have also elucidated the importance of posttranscriptional regulation and highlighted additional gatekeeping mechanisms necessary for effective erythropoiesis. We additionally discuss the insights gained by studying human genetic variation affecting erythropoiesis and highlight the discovery of BCL11A as a regulator of hemoglobin switching through genetic studies. Finally, we provide an outlook of how our ability to measure multiple facets of this process at single-cell resolution, while accounting for the impact of human variation, will continue to refine our knowledge of erythropoiesis and how this process is perturbed in disease. As we learn more about this intricate and important process, additional opportunities to modulate erythropoiesis for therapeutic purposes will undoubtedly emerge.

Introduction to physiological erythropoiesis

Each day, the process of red blood cell (RBC) production or erythropoiesis is crucial to maintain steady-state hemoglobin levels that allow for effective oxygen transport.1 This multistep process occurs in the bone marrow of adults in dedicated areas that cluster differentiating erythroid precursors around a central macrophage, a subcompartment in the bone marrow that has been termed the erythroblastic island.2 The erythroblastic islands enhance erythropoiesis by allowing cell-cell contacts to promote survival and proliferation, and the central macrophages engulf the extruded nuclei from erythroblasts. Erythropoiesis lasts ~14 days in humans and includes 7 to 8 steps over 2 major phases (Figure 1). The earlier phase originates with multipotential hematopoietic stem and progenitor cells (HSPCs) that give rise to erythroid-committed progenitors, and the latter phase is characterized by maturation of erythroid precursors into enucleated reticulocytes that undergo terminal maturation into RBCs in the circulation. In the first phase, an initial group of erythroid-committed progenitors termed burst forming unit erythroid (BFU-E) cells are produced from multipotential HSPCs and subsequently differentiate into colony forming unit erythroid (CFU-E) cells. These progenitors are not morphologically identifiable but can be enumerated when they are cultured in semisolid media such as methylcellulose, allowing their identification a posteriori. Although BFU-Es and CFU-Es were once thought to represent distinct stages of progenitor cells, recent studies reveal heterogeneity in these populations and suggest a continuum of progenitor states.3 Subsequently, proerythroblasts are the first morphologically distinguishable cells and characterize the onset of the second phase of erythroid maturation. These cells then advance through multiple stages of differentiation distinguishable by morphology, including basophilic erythroblasts, polychromatophilic erythroblasts, and orthochromatic erythroblasts (Figure 1). Finally, the nucleus condenses and extrudes, giving rise to reticulocytes released into the peripheral blood where they fully mature into functional RBCs. A classical view of erythropoiesis involves differentiation and maturation through discrete and distinct stages as described; however, recent studies at single-cell resolution and using genetic approaches suggest a more continuous model of differentiation.4,7

As erythroid cells differentiate, the expression of many membrane surface antigens changes over time, allowing differentiation to be tracked and quantified by flow cytometry. It is possible to enrich for different subsets of erythroid progenitors based on CD34/CD36/CD71 and CD105 sequential expression in CD123 (IL-3 receptor) negative cells.8 Combining expression of GYPA (CD235A) and CD105, or alternatively band 3/SLC4A1 acquisition (CD233) and CD49d loss, can allow tracking of erythroid maturation (Figure 1).9 This cellular tracking can be helpful to elucidate the mechanisms of ineffective erythropoiesis.8,10 Importantly, although these surface markers enable enrichment of distinct stages of this differentiation process, sorted populations are still heterogeneous, and some subsets with
Extrinsic regulation of erythropoiesis

A number of extrinsic factors control the differentiation, proliferation, and survival of erythroid cells by activating key downstream signaling pathways (Figure 1). Although not specific to erythroid differentiation, IL-3 has been shown to enhance the proliferation of early progenitors, including BFU-Es. Stem cell factor/KIT ligand binds the KIT receptor (KIT/CD117) and promotes the proliferation and survival of BFU-Es and CFU-Es, as well as pro-erythroblasts. The cytokine EPO is crucial for erythroid differentiation, allowing the survival and proliferation of maturing erythroid cells beginning with CFU-E progenitors through the later stages of maturation, although its receptor (EPOR) is weakly expressed on erythroid cells and quickly decreases with terminal maturation. In addition, EPO may also favor erythroid commitment from HSCs, and EPOR may be more broadly expressed in progenitors than was once thought, supporting evolving models that reveal how erythroid lineage commitment may occur earlier in the hematopoietic hierarchy than initially envisioned. Identification and cloning of EPO have enabled the production of recombinant protein, which has significantly improved the treatment of anemic patients, particularly those with kidney or marrow failure. Other cytokines or soluble factors, such as insulin and insulin-like growth factor, transforming growth factor β superfamily members, and glucocorticoids, have been shown to modulate erythropoiesis as well as iron metabolism through the erythroferrone-hepcidin-ferroportin axis. We will not detail these pathways any further in this review, given prior extensive coverage of this topic.

All of the major cytokines and growth factors, including EPO, stem cell factor, and IL-3, stimulate a number of downstream effectors, including the JAK/STAT, MAPK, and phosphatidylinositol 3-kinase signaling pathways, which have been studied extensively, to promote survival and/or proliferation. For instance, EPO binding leads to EPOR dimerization that activates JAK2 through phosphorylation. In turn, JAK2 phosphorylates STAT5, which promotes its nuclear translocation and helps shape the erythroid transcriptional program. Hyperactivation of these signaling pathways has been shown to promote erythropoiesis, most often through constitutive activation of JAK2 as a result of somatic mutations or less commonly through germline EPOR mutations. Older studies have portrayed these cytokines and growth factors as stimulating a fixed set of pathways. However, this canonical model has been expanded by newer studies using engineered molecules and naturally occurring genetic variation, revealing intricate and variable regulation of these downstream signaling pathways through modulation of receptor activity and downstream effectors. Better knowledge of these mechanisms is important to understand the control of erythropoiesis and illuminate how these pathways may be co-opted in diseases states, as exemplified through the insights gained from new genes and pathways involved in erythrocytosis that can modulate these signaling mechanisms. These insights might lead to the development of targeted therapies to improve ineffective and defective erythropoiesis, as recently illustrated by the inhibitors of the transforming growth factor β receptor superfamily and modulators of the hypoxia-inducible factor pathway.

Transcriptional control of erythropoiesis

These extrinsic factors facilitate effective erythropoiesis by further activating downstream, intracellular pathways. These pathways converge in the nucleus, as exemplified by the activation of STAT5 downstream of JAK2 and EPO, as discussed in the previous section. Moreover, the complex gene expression programs that characterize erythropoiesis are coordinated by a powerful set of intrinsically acting transcription factors (TFs), the potency of which is illuminated by their sufficiency to program nonerythroid cells into an erythroid fate. Key among these TFs is GATA1, which is critical to erythroid lineage commitment and differentiation and the expression of which is modulated throughout this process. Indeed, loss of GATA1 activity in mice and humans results in major impairments in erythropoiesis. GATA1 is highly expressed in erythroid cells from early progenitor stages, and its activity represses GATA2 during erythroblastic maturation. Although GATA1 expression slightly decreases after the proerythroblast stage proportionally to the global decrease in protein content throughout erythroblastic maturation, GATA2 expression is more dramatically reduced following this stage. This switch results in a strong increase in GATA1 activity, which in turn regulates numerous target genes that favor terminal erythroblast maturation. The importance of direct competition between GATA2 and GATA1 is unclear; however, their binding sites widely overlap during erythroid differentiation, and cofactors including FOG-1/ZFP1 and the nucleosome remodeling and deacetylase (NuRD) complex facilitate GATA1 binding to regulatory regions at the expense of GATA2. KLF1 is another key TF highly expressed in erythroid cells, the disruption of which leads to severe anemia when mutated in mice and causes a range of erythroid phenotypes in humans. KLF1 plays both an early role by favoring erythroid differentiation at the expense of megakaryocytic differentiation and a role in terminal maturation by triggering cell cycle exit and chromatin condensation before enucleation. In addition, the roles of a number of other key TFs in erythropoiesis have been studied, although some of these factors, such as TAL1, LMO2, LDB1, and GFI1B, have a variety of roles in hematopoiesis.

Although many TFs play vital roles in erythropoiesis, the work of single, isolated factors is insufficient to explain the complex changes that occur throughout this process. A more nuanced view of erythropoietic regulation recognizes these TFs function in complex networks, recruit and bind coactivators or corepressors to modulate their target gene
expression, and also have altered interactions at different stages of this process (Figure 2).50,80 In the specific case of erythropoiesis, GATA1 binds coactivators, such as LMO2, LDB1, and TAL1/SCL, to activate the transcription of erythroid-associated genes.50 Conversely, GATA1 can recruit corepressors, including the polycomb repressor complex 2, which involves EZH2 and EED, the GFI1B/LSD1 complex, or the FOG-1/ZFPM1 and NuRD complex, to repress target genes that prevent erythroid differentiation.50,81-86 Moreover, corepressors seem to be more abundant and stable than coactivators, making the coactivators limiting factors in promoting erythroid differentiation.60 Each TF generally binds to distinct cis-regulatory elements, which are often enriched for TF-specific binding motifs and exhibit altered accessibility and other epigenetic changes during human erythropoiesis.63 The complexity of this regulation arises as a result of combinatorial activity at each cis-regulatory element for different TFs and for different cis-regulatory elements that synergistically (or at least additively) regulate gene expression, as exemplified by distinct regulation of key erythroid genes, such as the α- or β-globin gene clusters.7 Further complexity arises from the long-range chromatin interactions that underlie this process and that are essential for gene activation.87-89 Some TFs, including GATA1 and KLF1, primarily bind distal regulatory regions,68,90 but the precise regulatory logic involved remains poorly understood. For example, GATA1 is thought to bind proximal regions of induced genes, whereas it binds more distal regions of repressed genes, but these rules often vary at different genetic loci.91 Additionally, these TFs frequently recruit histone-modifying enzymes to specific regulatory elements that enable epigenetic alterations, which play critical roles in erythropoiesis.92-94

Genomic approaches have significantly advanced our knowledge of transcriptional regulation in a range of settings through chromatin occupancy analyses, assessment of accessible chromatin, and long-range interaction data, including in the context of erythropoiesis,95 but the development of improved technologies that enable such insights at single-cell resolution and with increasing precision (often at single-nucleotide resolution) is likely to further advance our knowledge of the precise mechanisms involved in TF regulation during erythropoiesis.96,97 Beyond the technologies that assess TF and chromatin alterations at increasingly higher resolution, the ability to more precisely manipulate this process, particularly using genome-editing tools, such as CRISPR/Cas9, is advancing our ability to define the functional roles of specific elements and even their subcomponents.98 This opportunity to manipulate specific cis-regulatory elements is likely to teach us a tremendous amount about the intricacies of gene regulation, as nicely exemplified by detailed mechanistic studies involving the regulation of the α-globin locus.99

Control of the erythroid cell cycle
Progression and variation in the cell cycle are crucial for erythropoiesis, because they balance proliferation and differentiation. Cell-cycle regulation is essential to determine the fate of early erythroid progenitors. Megakaryocytic-erythroid progenitors preferentially differentiate into erythroid progenitors as a result
of a more rapid cell cycle arising from the activity of MYC, TP53, and the CDK4–cyclin D1 and CDK2–cyclin E complexes.100 Subsequently, there is a distinct transition involving a shortening of S-phase induced by the downregulation of p57KIP2 after the progenitor stages, allowing for the transition into proerythroblasts.101,102 Effective terminal erythropoiesis then requires cell-cycle exit, which requires many key factors, including RB1, E2F4, and CCND3 (cyclin D3).103-106 Interestingly, KLF1 deficiency in erythroid cells impairs enucleation after nuclear condensation, and orthochromatic erythroblasts continue to proliferate, which has been attributed to low levels of CDKN2C (p18) and CDKN1A (p21), which are required for cell-cycle exit.74

**Posttranscriptional control of erythropoiesis**

Although the major focus of studies on intrinsic regulatory mechanisms underlying erythropoiesis has been on transcriptional regulation, posttranscriptional mechanisms are also clearly important. It is striking that many of the key discoveries of these posttranscriptional processes arose from studies conducted in erythroid cells, including the discovery of ubiquitin-mediated protein degradation107 and early work examining the fundamentals of translational regulation by the ribosome.108 Despite limitations in routinely surveying these processes using genomic tools, which have resulted in a focus on transcriptional mechanisms over the past couple of decades, several important findings have emerged on the role of these posttranscriptional processes in erythropoiesis. It is clear that intricate regulation of ribosome levels is critical for altering protein production during hematopoiesis, including to enable erythroid lineage commitment through effective translation of GATA1 with higher ribosome levels in progenitors.109-111 These fundamental findings arose from studies of Diamond-Blackfan anemia, a rare bone marrow failure disorder that is characterized by a paucity of erythroid progenitors, with a majority of genetic mutations occurring in ribosomal proteins.112 Specific RNA binding proteins can also affect translation in erythroid cells, as exemplified by studies of the heme-regulated eIF2alpha HRI/EIF2AK1 kinase that plays critical roles in erythropoiesis by modulating messenger RNA translation.113,114

In addition to the intricate regulation of protein production, there are notable and dynamic changes that occur in the proteome during human erythropoiesis,61 including tremendous

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**Figure 2. Models for control of erythroid gene expression by transcription factors and cis-regulatory elements.** (A-B) The same cis-regulatory element can be bound by different TFs that exert antagonistic activities, such as GATA1 and GATA2. (C) The same TF, such as GATA1, can either repress or activate gene expression, and this may depend, at least in part, on the distance of the cis-regulatory element to which it binds. (D) Different TFs, such as GATA2 and GATA1, can bind distinct cis-regulatory elements and control the same gene in an antagonistic manner. (E-F) The same TF, as exemplified by GATA1, can bind the same cis-regulatory element, but can display activating effects or inhibitory effects based on the recruitment of coactivators, such as the LDB1 complex, or corepressors, such as the GFI1B/LSD1 complex. These complexes help to activate or repress gene expression through epigenetic changes. (G-H) TFs can be modulated by long-range spatial interactions, bridging 2 distal enhancers through homo- or heterotypic interactions (with CTCF for instance) in a synergistic way. Professional illustration by Somersault18:24.
variation in protein levels at distinct stages of this process and dynamic alterations in posttranslational protein modifications, such as phosphorylation. Finally, the regulation of protein degradation has critical roles in enabling erythropoiesis. For instance, heat shock protein 70 (HSP70) promotes the survival of maturing human erythroblasts by protecting GATA1 from caspase-3–induced degradation, which is critical for erythroid maturation. Other heat shock proteins and chaperones help to maintain the conformation of nascent globin chains to facilitate their association and prevent aggregation in erythroblasts, which would lead to toxicity and ineffective erythropoiesis. Finally, there are a number of examples of how ubiquitin-dependent degradation through the proteasome can fine tune erythropoiesis. For instance, FBXO11-mediated degradation of the heterochromatin-associated protein BAH1D is required to enhance erythroid transcription and enable effective erythropoiesis. Importantly, it is likely that current studies on translational and posttranslational protein regulation will significantly advance in the coming years, with improved methods for surveying translation, such as ribosome profiling, and through higher-resolution proteomic approaches. Importantly, integration of such studies with more holistic studies of erythroid cell biology is likely to reveal key changes during the process of erythropoiesis, such as the necessity of autophagy in enabling effective erythropoiesis to occur.

How human genetic variation enables a deeper understanding of erythropoiesis

The regulatory mechanisms discussed in this article paint a stereotyped picture of human erythropoiesis; however, there is considerable interindividual variation in this process. Studies of this variation can provide key insights, both in the context of diseased states and in the context of healthy variation. Recent studies have revealed how common genetic variation found in the population can explain ~15% to 25% of overall interindividual variation, and >8000 genetic variants have been associated with RBC traits. Importantly, this variation not only plays a role in healthy states, but also critically points to many genes that have crucial roles in erythropoiesis and diseases affecting this process, as exemplified by focused studies on specific loci harboring such variation. Large studies focusing on RBC indices have identified variation in many genes involved in monogenic RBC membrane defects including PIEZO1, ANK1, SLCA4A1, monogenic enzymopathies including HK1 or PKLR, components involved in cytokine signaling, and regulators of iron metabolism. Some of these targets have been then functionally validated in vitro, as exemplified by SH2B3, the silencing of which resulted in increased RBC production, mimicking human erythrocytosis. Moreover, this common genetic variation underlies variable penetrance and expressivity among presumed monogenic blood disorders, and the combination of multiple common polymorphisms together might explain some RBC diseases that have been largely recalcitrant to monogenic mapping approaches, such as some forms of polycythemia, demonstrating how a spectrum of genetic variation likely underlies most, if not all, blood diseases.

Perhaps the most important opportunity lies in the ability of genetic variation to shed light on critical biological mechanisms that can in turn enable more effective therapies in individuals with blood disorders. Much of the common genetic variation occurs in the noncoding genome and is likely to affect cis-regulatory elements that alter erythropoiesis. Although it is challenging to study such noncoding elements, there are important opportunities to gain functional insights into these putative

Figure 3. A model of fetal hemoglobin (Hb) regulation. The upstream enhancer locus control region (LCR) preferentially interacts with the γ-globin genes to produce the γ-globin chains that assemble with α-globin chains to constitute the heterotetrametric fetal Hb (left). At baseline, ZBTB7A represses the γ-globin genes and helps maintain globin chain balance. To switch from fetal to adult Hb, BCL11A and ZBTB7A independently repress the transcription of the β-globin genes and help to maintain the conformation of nascent globin chains to facilitate their association and prevent aggregation in erythroblasts, which would lead to toxicity and ineffective erythropoiesis.117 BCL11A requires both proximal promoter and long-range interactions to carry out its activity. Professional illustration by Somersault.
At the same time, another area that is likely to see significant advances in the coming years is the study of clonal dynamics and its impact on erythropoiesis. Our understanding of erythropoiesis continues to evolve through single-cell genomic and functional assays. These newer approaches are beginning to provide fruitful insights into the pathogenesis of a variety of erythroid diseases. However, the clonal contributions to erythropoiesis are poorly understood. Bulk studies have shown that even oligoclonal hematopoiesis, as occurs in the premalignant state clonal hematopoiesis of indeterminate potential, can occur with ostensibly normal erythropoiesis. As improved tools to measure clonal dynamics and contributions in unmodified cells are developed, such as the assessment of nuclear or mitochondrial somatic mutations for phylogenetic inference, there will be many opportunities to dissect these clonal contributions at single-cell resolution. Our ability to discern whether pathological erythropoiesis can alter clonal contributions and increase the risk for clonal blood diseases, as illuminated by the myeloid malignancy predisposition that may occur in sickle cell disease, could be further enhanced with these advances. In many ways, although our understanding of human erythropoiesis can seem extremely advanced, as we learn more about this process, we are finding that there is just as much, if not more, that remains to be learned.

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Footnote

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