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REVIEW

Mechanisms of erythrocyte development and regeneration: implications for regenerative medicine and beyond

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ABSTRACT

Hemoglobin-expressing erythrocytes (red blood cells) act as fundamental metabolic regulators by providing oxygen to cells and tissues throughout the body. Whereas the vital requirement for oxygen to support metabolically active cells and tissues is well established, almost nothing is known regarding how erythrocyte development and function impact regeneration. Furthermore, many questions remain unanswered relating to how insults to hematopoietic stem/progenitor cells and erythrocytes can trigger a massive regenerative process termed 'stress erythropoiesis' to produce billions of erythrocytes. Here, we review the cellular and molecular mechanisms governing erythrocyte development and regeneration, and discuss the potential links between these events and other regenerative processes.

KEY WORDS: Erythroblast, Erythrocyte, Regeneration, Stress, Transcription

Introduction

The erythrocyte – the red blood cell – serves as a master regulator of metabolism and life by unremittingly delivering oxygen to the cells and tissues of an organism in developmental, physiological and regenerative contexts. In the context of regenerative medicine strategies, such as those aiming to engineer organs and organoids that evade immune destruction, oxygen delivery may be the crucial lynchpin for transitioning experimental tissue into promising clinical material. Thus, although the effective and durable vascularization of bio-engineered tissues is a prerequisite for ensuring tissue vitality, erythrocyte-dependent oxygen delivery may be rate limiting to achieve a successful clinical outcome. Though considerable efforts have been made to understand how erythrocytes develop and function, many questions remain unanswered regarding how these mechanisms relate to those controlling erythrocyte regeneration. Furthermore, the potential interconnectivity between erythrocyte regeneration and other regenerative processes is largely unexplored.

A number of diverse stresses can impede erythrocyte development and function, thereby causing anemia. However, knowledge of the underlying cellular and molecular mechanisms is incomplete and there is, therefore, immense interest in gaining a better understanding of how stress can influence erythropoiesis, both during development and in a regenerative context. In this Review, we focus on cell-autonomous and non-cell-autonomous mechanisms governing erythrocyte development, the applicability of these mechanisms to stress-instigated erythropoiesis (erythrocyte regeneration) and their implications for other regenerative processes. We begin by considering the principles that govern the cell fate transitions that produce the diverse complement of blood cells, including erythrocytes. It is not our intent, however, to comprehensively address this topic, as it has been heavily reviewed elsewhere (Crisan and Dzierzak, 2016; Dzierzak and de Pater, 2016; Orkin and Zon, 2008; Tober et al., 2016).

An overview of the stem and progenitor cell transitions that generate erythrocytes

Blood cell development (hematopoiesis) from stem and progenitor cells takes place at multiple locations in the embryo and adult (Dzierzak and de Pater, 2016; Orkin and Zon, 2008). The hematopoiesis site varies dynamically in developmental, physiological and pathological contexts. This geographic diversity imparts mechanisms involving regulatory signals emanating from different microenvironments (niches) (Mendelson and Frenette, 2014; Morrison and Scadden, 2014) that seamlessly interface with cell-intrinsic mechanisms to regulate the self-renewal, lineage-commitment, differentiation and terminal maturation of hematopoietic stem and progenitor cells (HSPCs).

Developmental studies using chicken and *Xenopus* embryos, and the *in vitro* differentiation of mouse (Chung et al., 2002) and human (Choi et al., 2012) embryonic stem cells and induced-pluripotent cells, indicate that endothelial and blood cells emerge from a common cellular ancestor, the hemangioblast, that has dual vascular and hematopoietic potential (Choi et al., 1998; Faloon et al., 2000; Lancrin et al., 2009). During mammalian embryogenesis, yolk sac-derived hematopoietic precursors generate embryonic or 'primitive' erythroid cells and macrophages (Barminko et al., 2016). In the mouse, primitive hematopoiesis is followed by a blood-producing process involving an 'endothelial to hematopoietic transition'. During this event, hemogenic endothelial cells in the aorta gonad mesonephros (AGM) region of the embryo proper generate hematopoietic cell clusters harboring adult or 'definitive' hematopoietic stem cells (HSCs) (Bertrand et al., 2010; Boisset et al., 2010; de Bruijn et al., 2002, 2000; Lancrin et al., 2009). AGM-derived HSCs then produce multipotent progenitors that differentiate into lineage-committed progenitors and precursors that generate the full complement of blood cells; a comparable AGM-dependent stem cell-generating mechanism also exists in humans (Ivanovs et al., 2017, 2011; Ng et al., 2016). The resulting HSCs populate the fetal liver, which serves as the major hematopoietic site in the mouse from approximately embryonic day (E) 12-E16 (Ema and Nakauchi, 2000; Medvinsky and Dzierzak, 1996; Morrison

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et al., 1995; Müller et al., 1994; Sánchez et al., 1996). Thereafter, fetal liver hematopoietic potential declines, concomitant with establishment of the bone marrow as the predominant site of hematopoiesis in the developing newborn and adult. There is also evidence for a yolk-sac origin of a component of the definitive hematopoietic system; in effect, a second wave of hematopoiesis that bridges the gap between primitive and AGM-dependent definitive hematopoiesis (Inlay et al., 2014; Lee et al., 2016; McGrath et al., 2015). However, the mechanisms underlying yolk sac-dependent definitive hematopoiesis are not as thoroughly deconvoluted as those involving the AGM HSC generator. Taken together, these analyses revealed crucial junctures during development in which new pathways of erythropoiesis emerge to accommodate the oxygen needs of the developing embryo.

In the fetal liver and bone marrow of mice, HSC-derived progenitors differentiate into megakaryocyte-erythrocyte progenitors (MEPs), a common precursor to both erythrocytes and megakaryocytes (Akashi et al., 2000). Single-cell transcriptomic and functional analyses have revealed that MEPs are heterogeneous (see Box 1), which is not surprising for a cell population defined with a limited set of molecular markers. It has also been reported that human MEPs yield predominantly single-lineage, with less frequent bi-lineage, developmental outputs (Miyawaki et al., 2017; Psaila et al., 2016).

The erythroid progeny of MEPs are termed burst-forming unit-erythroid (BFU-E) cells, based on their activity during the formation of diagnostic cell colonies in a methylcellulose-based culture system (Heath et al., 1976). Gene expression signatures and colony attributes suggest that BFU-E cells mature from an early BFU-E to a late BFU-E population, which form large and small colonies, respectively. BFU-Es, in turn, generate an erythroid precursor population with distinct colony attributes termed the colony-forming unit-erythroid (CFU-E) (Stephenson et al., 1971). CFU-E cells differentiate into proerythroblasts, which progressively mature into basophilic, chromatophilic and orthochromatic erythroblasts, followed by reticulocytes and erythrocytes (Koury, 2016). This final stage of erythroblast maturation involves nuclear condensation as a prelude to nuclear polarization, followed by enucleation to yield the reticulocyte and additional cellular remodeling, including organelle loss, to produce the erythrocyte (Fig. 1).

Box 1. Heterogeneity

Populations of seemingly homogenous cells can exhibit stochastic changes in gene expression at the single-cell level, including bursts in the expression of transgenes (Feng et al., 1999) and of functionally important genes (Vera et al., 2016). Despite offering the extraordinary potential to address previously intractable problems, such heterogeneity can be difficult to interpret, both mechanistically and biologically. Removing cells from their microenvironment terminates non-cell-autonomous regulatory inputs, thus corrupting the circuits that establish and/or maintain phenotypes. Dismantling the intricate interconnections between non-cell-autonomous and cell-autonomous regulatory machinery may also create non-physiological cell-to-cell differences in signaling, transcription and differentiation potential; such differences are commonly detected in single-cell transcriptomic and functional analyses. It is also often difficult to relate observed heterogeneities to functional outputs within a normal microenvironment *in vivo*. However, this new avenue of highly promising investigation is still in its infancy, and a major challenge is to differentiate between physiologically relevant single-cell phenotypic heterogeneity versus experimentally measured phenotypes, reflecting corruption of mechanistic circuits that endow cells with unique identities.

General mechanisms of regenerative or 'stress' erythropoiesis

To date, the majority of studies on erythrocyte biogenesis and function have focused on developmental and steady-state mechanisms, rather than stress-instigated alterations in erythropoiesis. Nonetheless, these mechanistic foundations have provided a solid rubric for deciphering how acute or chronic environmental influences can impact the complex developmental steps occurring in distinct microenvironments and the levels/activities of specific blood cell types.

The induction and implications of regenerative erythropoiesis

Stress erythropoiesis can be induced by an acute crisis, such as trauma-induced severe bleeding, which must be swiftly rectified to ensure cell and tissue oxygenation and survival. Alternatively, stress erythropoiesis can be an adaptive response to prolonged hypoxia, e.g. in individuals residing in high-altitude zones or those with a sustained impediment to erythrocyte generation and/or function. Like most stress responses, stress erythropoiesis is designed to be short-term, producing ample erythrocytes to maintain homeostasis until physiological erythropoiesis resumes. This attribute constitutes a weakness, however, when long-term obstacles to erythropoiesis result from infection, chronic inflammation, chronic kidney disease, chemotherapy, environmental toxins, nutritional deficiencies (e.g. iron), aging or genetically based ineffective erythropoiesis syndromes (Sankaran and Weiss, 2015). For example, in the case of myelodysplastic syndrome (MDS), defective HSCs fail to generate adequate levels of erythroid precursors and erythrocytes. In myelofibrosis (a type of myeloproliferative neoplasm), bone marrow cells, including aberrant megakaryocytes (Gilles et al., 2017), release factors that generate fibrotic bone marrow with a reduced capacity to support hematopoiesis, thus impairing erythrocyte output (Vainchenker and Kralovics, 2017). These conditions can overwhelm the stress response, leading to chronic anemia.

Cellular mechanisms of stress erythropoiesis

Acute or chronic anemia create a vital need to extend the limits of physiological erythropoietic capacity to regenerate normal erythrocyte levels. Considering the mechanistic underpinnings of this increased erythropoiesis, stress erythropoiesis might reflect increased erythropoietic output through mechanisms identical to those controlling developmental and steady-state erythropoiesis. Indeed, early studies in the mouse suggested that recovery from acute hemolytic anemia caused by phenylhydrazine (an agent that lyses red blood cells) is regulated principally by anemia-dependent increases in serum erythropoietin (Epo) (Hara and Ogawa, 1976, 1977), which enters the bone marrow and increases erythroid progenitor proliferation such that excess progenitors migrate to the spleen to complete their differentiation. This model assumes that murine stress erythropoiesis is accomplished via 'extramedullary hematopoiesis' at sites outside of the bone marrow, predominantly in the spleen, but also in the liver (Ploemacher et al., 1977). Extramedullary hematopoiesis is also observed in humans and involves a dedicated set of progenitors and signals that respond to anemic stress, e.g. in genetically caused anemia (Korsten et al., 1970) and myeloproliferative neoplasms (Laszlo, 1975). Furthermore, in bone marrow transplant patients, the bone marrow is a major hematopoiesis site, but hematopoiesis also occurs at extramedullary sites, including the spleen and liver (Arnold et al., 1985).

Alternatively, stress erythropoiesis may resemble the repair and regeneration mechanisms observed in the mammalian

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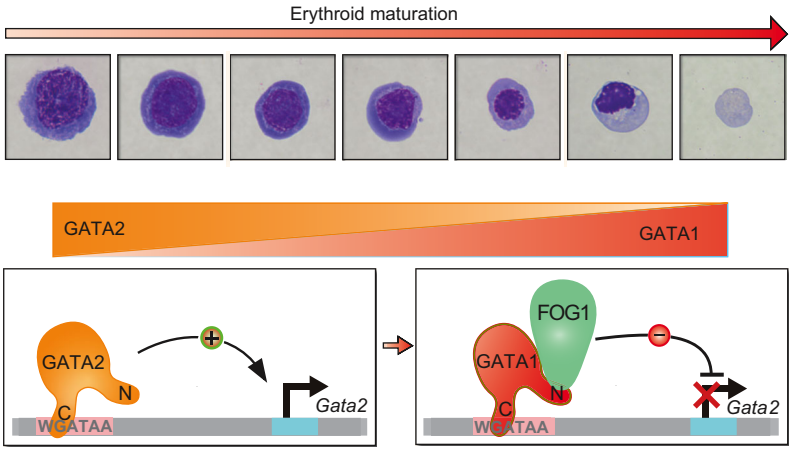

Fig. 1. An overview of cellular and molecular transitions during erythropoiesis. (Top) The photomicrographs depict the progressive maturation of erythroid precursors (erythroblasts), which involves extreme chromatin condensation, organelle remodeling and enucleation. (Bottom) GATA1 directly represses *Gata2* transcription, thus yielding largely mutually exclusive GATA2 and GATA1 expression patterns. The GATA1 co-regulator Friend of GATA1 (FOG1) is essential for the 'GATA switch' mechanism that represses genes (e.g. *Gata2* and *Kit*) required for maturation. In other contexts, GATA switches are linked to transcriptional activation or maintenance of a pre-existing transcriptional state (Bresnick et al., 2012, 2010, 2005; DeVilbiss et al., 2014; Katsumura et al., 2017).

intestinal epithelium, in which stem cells distinct from those involved in homeostasis can be activated to regenerate damaged epithelium. In support of this, the analysis of *flexed-tail* (*f*) mutant mice demonstrated that stress erythroid progenitors in the spleen are mobilized by anemic stress through increased bone morphogenetic protein 4 (BMP4) expression in the spleen (Lenox et al., 2005). These stress erythroid progenitors express unique markers relative to those of bone marrow physiological erythroid progenitors and require different factors and niche conditions to stimulate their expansion and differentiation (Harandi et al., 2010; Lenox et al., 2005; Paulson et al., 2011; Perry et al., 2007, 2009).

The stress erythroid progenitor

Stress erythropoiesis appears to use a distinct hematopoietic precursor, the stress progenitor, in splenic erythropoiesis (Xiang et al., 2015). Splenic stress erythroid progenitors express unique cell-surface markers (e.g. CD34, CD133 and Sca1) that are normally associated with stem cells, which likely reflects the immediate stem cell origin of these progenitors. Yet they also express the hematopoietic marker Kit and the erythroid marker transferrin receptor CD71, but are negative for the erythroid antigen Ter119. In a stress response that demands accelerated reticulocyte generation, stem cell factor (SCF), the activating ligand for Kit (Chabot et al., 1988; Huang et al., 1990; Zsebo et al., 1990a,b), BMP4 and hedgehog signals expand the stress progenitors.

The relationship between these stress erythroid progenitors in the mouse spleen and the cognate cells that mediate stress erythropoiesis in humans is incompletely understood. *Ex vivo* studies have revealed that, similar to cultures of mouse bone marrow, culturing human bone marrow generates stress erythroid progenitors that express fetal γ-globin and adult β-globin, and resemble murine splenic stress erythroid progenitors (Xiang et al., 2015). Given the structurally distinct splenic and bone marrow microenvironments, and the unique molecular and cellular considerations vis-à-vis stress versus steady-state erythropoiesis, it is particularly informative to compare and contrast the respective mechanisms. At a rudimentary level, it appears that the growth, differentiation and survival factors Epo and SCF are vital determinants of erythropoiesis in both contexts. Below, we discuss how these and other signaling factors, including glucocorticoids and thyroid hormone, as well as other cell types, function during erythropoiesis.

Signaling networks and circuitry in developmental and regenerative erythropoiesis

Erythropoietin synthesis and signaling mechanisms

Anemia creates hypoxic microenvironments that impact a multitude of biochemical and cellular processes. The quintessential cellular response to hypoxia involves activation of hypoxia-sensing transcription factors (hypoxia inducible factors; HIFs) (Semenza, 2012; Wang et al., 1995). In normoxia, prolyl hydroxylase (PHD) enzymes catalyze the hydroxylation of proline residues within the α subunit of heterodimeric HIFs (Jaakkola et al., 2001), which facilitates binding of the von Hippel-Lindau (VHL) E3 ubiquitin ligase, α subunit poly-ubiquitylation and proteasome-mediated degradation (Maxwell et al., 1999). This PHD-dependent mechanism is instrumental in suppressing HIF levels. In hypoxia, prolyl hydroxylation of the α subunit is blocked, thus decreasing VHL binding and increasing HIF levels. HIF2 then directly induces transcription of the gene encoding Epo and is responsible for generating the bulk of the circulating Epo. This mechanism operates in specialized renal cells termed renal Epo-producing (REP) cells (Obara et al., 2008; Pan et al., 2011) or peritubular interstitial fibroblast-like cells (Farsijani et al., 2016; Kobayashi et al., 2016).

Hypoxic induction of HIF2 activity in REP cells appears to be only one component of the mechanism. Metabolic changes that increase glycolysis in non-REP cells in the kidney increase pO2 levels in the kidney, thereby stimulating HIF2 degradation, reducing Epo expression and causing hypo-proliferative anemia (Farsijani et al., 2016). The opposite metabolic changes, involving increased oxidative phosphorylation, stabilize HIF2 and promote Epo expression. Furthermore, the mutation of different PHD-encoding genes in REP cells has revealed that Epo production in REP cells increases as more PHD genes are mutated, suggesting that the degree of hypoxia fine-tunes this process (Tojo et al., 2015). As the PHD-dependent mechanism dictates renal Epo production, PHD inhibitors are being evaluated as potential anemia therapeutics (Maxwell and Eckardt, 2016).

Epo binds a plasma membrane receptor (EpoR) that is highly expressed in CFU-E erythroid precursors and erythroblast progeny (D'Andrea et al., 1989; Jones et al., 1990; Lodish et al., 1995). EpoR signaling stimulates definitive erythroid precursor proliferation, differentiation and survival (Lin et al., 1996), but is not required for BFU-E and CFU-E generation (Wu et al., 1995b). A single administration of Epo to mice increases splenic erythroblast survival and increases regenerative erythropoiesis (Liu et al., 2006). Although EpoR is also expressed in select non-

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erythroid cells, e.g. endothelial cells (Anagnostou et al., 1994; Beleslin-Cokic et al., 2004), much of what is known about EpoR signaling derives from mechanistic dissection in erythroid cell systems (Kuhrt and Wojchowski, 2015). These studies have shown that EpoR activation in erythroid cells induces a molecular cascade that activates multiple downstream signaling effectors, with a major effector being janus-activated kinase 2 (JAK2). JAK2 stimulates phosphorylation of the signal transducer and activator of transcription 5 (Stat5) transcription factor (Damen et al., 1995; Klingmuller et al., 1996; Witthuhn et al., 1993), increasing its nuclear localization, DNA binding and target gene transcriptional regulation (Kuhrt and Wojchowski, 2015; Penta and Sawyer, 1995; Zhu et al., 2008). EpoR signaling also increases mitogen-activated protein kinase (MAPK) (Miura et al., 1994) and phosphoinositide 3 kinase (PI3K)/Akt (Bao et al., 1999) activities/signaling. The internalization of EpoR represents an important mode of downregulating signaling; in addition to mediating signaling, the Epo signaling circuitry components JAK2 and the p85 subunit of PI3K promote EpoR internalization (Sulahian et al., 2009). Internalization also requires EpoR K256 ubiquitylation, and an EpoR mutant that cannot be ubiquitylated exhibits reduced signaling and mitogenic activity (Bulut et al., 2011).

As EpoR signaling impacts multiple intracellular factors (Kuhrt and Wojchowski, 2015; Verma et al., 2014), deciphering the contribution of individual factors to specific Epo-dependent biological outputs is challenging. One component of the survival response involves Epo-dependent induction of the anti-apoptotic factor Bcl-xL (Socolovsky et al., 1999). Transcription of *Bcl2l1*, which encodes Bcl-xL, is directly activated by GATA1 (Gregory et al., 1999), a master transcriptional regulator of erythroid genes that drives erythroid cell development and maturation (Evans and Felsenfeld, 1989; Katsumura et al., 2017; Tsai et al., 1989). Besides its role in developmental and steady-state erythropoiesis, GATA1 promotes stress erythropoiesis (Gutierrez et al., 2008). Stat5, an important determinant of fetal liver, but not steady-state, erythropoiesis in adult mice, also activates *Bcl2l1* transcription (Socolovsky et al., 1999). Epo signaling can stimulate GATA1 phosphorylation (Kadri et al., 2015; Zhao et al., 2006), which may contribute to the Epo-mediated increase in Bcl-xL expression. Increased Epo signaling in stress erythroid progenitors also downregulates the pro-apoptotic factors Fas/FasL (Liu et al., 2006) and Bim (Koulnis et al., 2012) that promote erythroblast survival and expansion.

Studies of human genetics have also informed Epo-mediated signaling mechanisms. For example, an individual with an anemia-inducing Epo mutation (R150Q) provided insights into the contribution of downstream effectors of EpoR signaling (Kim et al., 2017). Although the Epo R150Q mutation did not affect Stat5 activation, it impaired receptor binding kinetics, receptor dimerization and JAK2-dependent Stat1 and Stat3 phosphorylation. Furthermore, mathematical modeling and signaling analyses in cell lines have suggested that the relative abundance of Epo-regulated signaling components is a key parameter for dictating signaling dynamics and cellular response (Adlung et al., 2017). Knowledge of the EpoR signaling circuits that are essential in specific developmental, physiological and stress contexts remains incomplete.

Stem cell factor synthesis and mechanisms of action

Stem cell factor (SCF), which exists in soluble and membrane-bound forms, functions as a dimer that binds the Kit tyrosine kinase receptor in the plasma membrane of a target cell, promoting Kit dimerization and kinase activation (Anderson et al., 1990; Huang et al., 1990; Jiang et al., 2000; Martin et al., 1990; Philo et al., 1996). Genetic evidence indicates the membrane-bound configuration of SCF is important for Kit-dependent cell signaling (Brannan et al., 1991; Kapur et al., 1998). Considerable progress has been made in elucidating the cellular origin of SCF that activates the Kit receptor tyrosine kinase expressed by HSPCs and erythroid precursors in different microenvironments. Bone marrow perivascular cells produce most of the SCF in the mouse bone marrow compartment (Ding et al., 2012). Conditional deletion of SCF in murine endothelial cells or leptin receptor-expressing perivascular cells depletes the majority of bone marrow HSCs (Ding et al., 2012). A similar approach deployed in the spleen revealed that splenic endothelial cells and Tcf21^{+} stromal cells generate SCF that mediates stress erythropoiesis in response to myeloablation, bleeding or pregnancy (Inra et al., 2015). The expression data are supported by ample biological evidence demonstrating the essential role of SCF/Kit signaling in erythropoiesis. Mice carrying mutations in the *dominant white spotting* (*W*) or *Steel* (*Sl*) loci, which encode Kit and SCF, respectively, are exquisitely sensitive to phenylhydrazine. The stress erythropoiesis defect in *W* mutant mice involves failure to expand stress progenitors or stress progenitor deficiency in the spleen (Harrison and Russell, 1972). Accordingly, SCF stimulates the expansion of *ex vivo*-cultured stress progenitors from mouse and human bone marrow (Perry et al., 2007; Xiang et al., 2015).

Cis-regulatory elements and transcriptional mechanisms responsible for controlling the transcription of *KITLG*, which encodes SCF, in hematopoietic cells have not been reported. By contrast, major progress has been made in understanding the mechanisms that control *Kit* expression. GATA2, which is expressed prior to GATA1 in HSPCs and in early erythroid precursors (Leonard et al., 1993; Weiss et al., 1994), directly activates *Kit* transcription (Gao et al., 2013; Jing et al., 2008; Lecuyer et al., 2002) and, as is often the case with GATA2, functions at chromatin sites with the basic helix-loop-helix transcription factor stem cell leukemia (Scl)/T-cell acute lymphocytic leukemia 1 (TAL1) and its associated factors, including the LIM domain proteins Ldb1 and Lmo2 (Hewitt et al., 2016; Hoang et al., 2016; Lecuyer et al., 2002; Wadman et al., 1997; Wozniak et al., 2008) (Fig. 2). GATA2 is essential for HSC emergence from hemogenic endothelium (de Pater et al., 2013; Gao et al., 2013; Johnson et al., 2012), for HSC function (Lim et al., 2012; Ling et al., 2004; Rodrigues et al., 2005) and for myelo-erythroid progenitor differentiation (Johnson et al., 2015; Rodrigues et al., 2008; Mehta et al. 2017); it is also implicated in controlling endothelial cell (Dorfman et al., 1992; Hartmann et al., 2016; Johnson et al., 2012; Linnemann et al., 2011) and neuronal (Craven et al., 2004; El Wakil et al., 2006; Kala et al., 2009; Lahti et al., 2016) functions. As proerythroblasts begin to mature into erythrocytes, GATA1 replaces GATA2 at *Kit* chromatin sites (Fig. 1), disrupting the three-dimensional locus conformation (Jing et al., 2008) and instigating transcriptional repression (Munugalavadla et al., 2005).

Interconnected and independent Epo and SCF signaling circuits

Resembling EpoR signaling, Kit signaling activates multiple downstream pathways, including MAPK and PI3K/Akt (Lennartsson and Ronnstrand, 2012). In certain contexts, Epo functions in concert with SCF to regulate the same cell (Menon et al., 2006; Sui et al., 1998; Wu et al., 1997), and it is also known that erythroid precursors can express both EpoR and Kit (Watowich et al., 1996). However, there are many unanswered questions regarding how combinatorial signaling may yield quantitatively and/or qualitatively distinct consequences.

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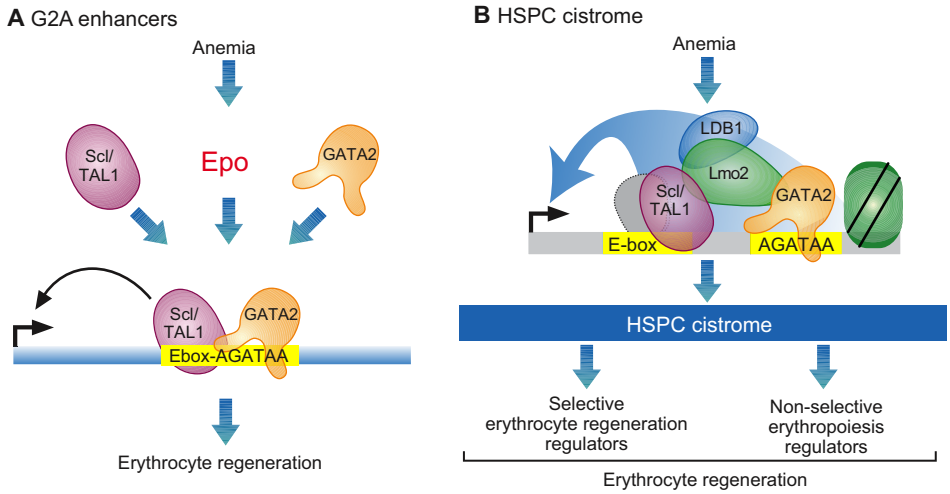

Fig. 2. GATA2- and anemia-activated enhancers control expression of selective erythrocyte regeneration regulators and non-selective erythropoiesis regulators. (A) Anemia induces Epo production and increases Scl/TAL1 and GATA2 levels, all of which contribute to GATA2- and anemia-activated (G2A) enhancer activation (Hewitt et al., 2017). Although the precise mechanism whereby Epo-EpoR signaling contributes to enhancer activation has not been established, it is likely that this involves direct actions on transcription factors and chromatin constituents. GATA2 and Scl/TAL1 occupy chromatin at G2A enhancers, which harbor E-box and GATA motifs separated by an 8 bp spacer. This *cis*-element configuration is necessary, but not sufficient, for enhancer activity. Many identical elements in the genome are not GATA factor occupied, lack molecular attributes of enhancers and do not mediate GATA2-dependent regulation of a neighboring gene. G2A enhancers reside at genes controlling vital molecular and cellular processes, which constitute a genetic network that drives erythrocyte regeneration. (B) G2A enhancers are important components of an ensemble of *cis*-elements that make up the HSPC cistrome. G2A enhancer-regulated factors parse into two groups: (1) selective erythrocyte regeneration regulators (SERRs), exemplified by Samd14, exert essential functions to control regeneration, but not developmental and steady-state erythropoiesis (at least not non-redundantly); and (2) non-selective erythropoiesis regulators, exemplified by GATA2, exert essential functions to control developmental, steady-state and regenerative erythropoiesis.

An important component of combinatorial signaling mechanisms that give rise to distinct outcomes involves the GATA factors. The process whereby GATA1 represses *Gata2*, *Kit* and additional genes is deemed a GATA switch mechanism (Fig. 1), in which GATA1 replaces GATA2 at *cis*-elements, frequently inducing a qualitatively or quantitatively distinct transcriptional output (Bresnick et al., 2010, 2005; Grass et al., 2003). This switch requires GATA1 to engage its co-regulator Friend of GATA1 (FOG1) (Pal et al., 2004), which mediates both activation and repression (including *Kit* repression) (Crispino et al., 1999; Tsang et al., 1997). FOG1 binds tightly to the nucleosome remodeling and deacetylase (NuRD) chromatin remodeling complex (Hong et al., 2005). Despite this GATA1-FOG1 partnership, a cohort of GATA1 target genes are activated or repressed normally in cells lacking FOG1 or expressing a GATA1 mutant defective in FOG1 binding (Johnson et al., 2007; Kim et al., 2007). GATA1 also has a considerably longer half-life than GATA2, which is rapidly degraded by the ubiquitin-proteasome system; proteasome inhibition stabilizes GATA2 and attenuates GATA switching (Lurie et al., 2008).

The mechanisms responsible for triggering GATA2 and GATA1 expression are not fully understood. In certain contexts, BMP4 induces GATA2 expression (Friedle and Knöchel, 2002; Lugus et al., 2007). GATA2 itself and its interacting factors (e.g. Scl/TAL1) co-occupy *Gata2* enhancers 9.5 kb downstream and 77 kb upstream of the transcription start site (Fig. 3) (Grass et al., 2006; Sanalkumar et al., 2014; Wozniak et al., 2008). These *Gata2* enhancers are vital for conferring hematopoietic cell type-specific *Gata2* transcription (Gao et al., 2013; Johnson et al., 2012, 2015; Mehta et al. 2017) and almost certainly mediate GATA2-dependent positive autoregulation. GATA2 occupancy thus likely reflects the positive autoregulation of *Gata2* expression (Grass et al., 2003, 2006; Martowicz et al., 2005). The analysis of mutant mouse strains lacking these enhancers has been particularly informative. Deletion of the +9.5 kb enhancer inactivates the HSC generator in the AGM and depletes HSPCs in the fetal liver, yielding lethality at E13.5 (Gao et al., 2013; Johnson et al., 2012), whereas deletion of the −77 kb enhancer impairs the myelo-erythroid differentiation capacity of progenitor cells, yielding lethality after E15.5 (Johnson et al., 2015). Accordingly, mutations in these enhancers in humans have adverse effects; mutations in the +9.5 enhancer cause immunodeficiency, MDS and AML (Hsu et al., 2013; Johnson et al., 2012; Katsumura et al., 2017), while disruption of the −77 enhancer via chromosomal inversion re-positions it adjacent to *MECOM*, which encodes the EVI1 oncogene, upregulating its expression and inducing AML (Gröschel et al., 2014; Yamazaki et al., 2014). As the −77 enhancer confers *Gata2* expression in myelo-erythroid progenitor cells (Johnson et al., 2015), the leukemogenic mechanism is proposed to involve *GATA2* downregulation (and corruption of its genetic network, including *Kit*) concomitant with *MECOM* upregulation.

Analogous to *Gata2*, GATA1 appears to positively autoregulate *Gata1* expression (Tsai et al., 1991), and potential *cis*-elements have been described (Onodera et al., 1997; Suzuki et al., 2009). GATA1 is also implicated in increasing EpoR expression (Zon et al., 1991), but whether it is essential for expression is not known. Furthermore, although GATA1 and GATA2 are commonly expressed in distinct cell types (Fujiwara et al., 2004), GATA1 can co-occupy chromatin with the GATA2-associated factors noted above (DeVilbiss et al., 2016). In a context-dependent manner, GATA1 co-occupies chromatin with the forkhead transcription factor FoxO3 (Kang et al., 2012), an important component of oxidative stress pathways and facilitator of stress erythropoiesis (Marinkovic et al., 2007). GATA2 also occupies chromatin sites at many GATA1-activated genes prior to GATA1 expression and chromatin occupancy; however, there are no reports of a mechanism in which GATA2 actively represses a gene, e.g. EpoR, prior to GATA1-mediated activation. Thus, the GATA2 occupancy might reflect chromatin 'priming' to generate a site poised for subsequent GATA1 entry,

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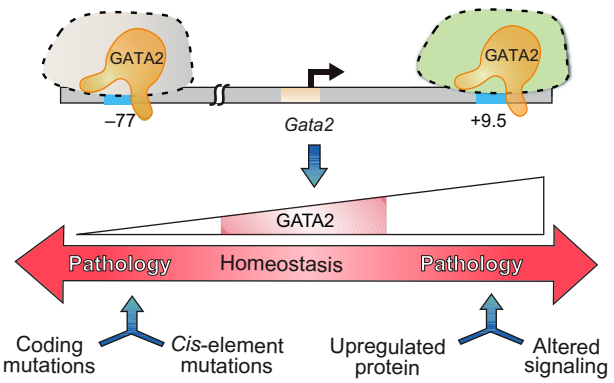

Fig. 3. *Gata2* +9.5 and −77 enhancers confer physiological levels of GATA2. The +9.5 and −77 enhancers are positioned +9.5 and −77 kb, respectively, from the *Gata2* transcription start site. These enhancers function in a cell type-specific manner to ensure that GATA2 expression remains within a restricted physiological window (Gao et al., 2013; Johnson et al., 2012, 2015; Mehta et al., 2017). Both decreases (e.g. resulting from coding or *cis*-element mutations) and increases (e.g. caused by increased protein expression or increased signal-dependent GATA2 activation) in GATA2 expression and/or activity cause, or are linked to, the development of malignant and non-malignant hematological pathologies (Bresnick et al., 2012; Dickinson et al., 2014; Katsumura et al., 2017; Spinner et al., 2014).

active repression or some degree of activation (Katsumura et al., 2017; Sanalkumar et al., 2014).

Another example of differential regulation of Kit and EpoR involves the exosome complex. This multi-subunit complex, which functions as an RNA-degrading and -processing machine (Kilchert et al., 2016), creates a barricade to murine fetal liver erythroblast maturation (McIver et al., 2014) and increases the number of BFU-Es (McIver et al., 2016). Mechanistically, the exosome complex confers Kit, but not EpoR, transcription, expression and signaling (McIver et al., 2016). Accordingly, exosome complex dismantling downregulates Kit in primary erythroid precursors, concomitant with precocious acquisition of EpoR expression and function (McIver et al., 2016).

Although Epo and SCF signals can be integrated by certain erythroid precursors, as noted above, their receptors can be differentially regulated. Once GATA1 represses *Kit* expression, depriving erythroblasts of the SCF receptor, Epo signaling is crucial for stimulating erythroid maturation. In certain contexts, therefore, the signaling systems function independently but, presumably, they remain tightly interlinked with other hematopoietic and broadly operating signaling pathways. This mechanism assumes that Epo signaling provides instructions to the erythroblast that are unique from those imparted by SCF, and the functional consequences of the differential directives are almost certainly dictated by the signaling milieu. However, Epo overexpression (~800 fold) using a platelet-derived growth factor-B chain promoter-driven Epo transgene rescues the lethality of $Kit^{W/W}$ mutant mice, which normally die before 10 days of age (Waskow et al., 2004). Epo overexpression also partially rescues hematopoietic phenotypes, including SCF-responsive CFU-E cells; BFU-E cells are not reduced in number in $Kit^{W/W}$ mice. As exceptionally high Epo levels can overcome life-threatening signaling defects, this might reflect activation of very low levels of receptor on precursors that are not normally activated by physiological Epo concentrations. Alternatively, a small number of *Kit* mutant precursors that survive the loss of SCF signaling might retain the capacity to inefficiently generate progeny that can be greatly expanded above a critical threshold. As SCF and Epo signaling can occur in the same cell in certain contexts (Wu et al., 1995a) and the dual signaling can be synergistic (Joneja et al., 1997), the exceptionally high Epo concentration that yields rescue might also reflect inefficient Epo signaling due to loss of physiological Epo-SCF synergism.

The dynamics of Kit and SCF signaling may also influence their biological outcome. For example, although Kit activity is essential to stimulate erythroid precursor proliferation and/or survival (Watowich et al., 1996), sustained Kit signaling suppresses erythroid maturation (Haas et al., 2015; McIver et al., 2016). This common theme in hematopoiesis and broader biological contexts is also exemplified by GATA2, which is required to promote hematopoiesis (Katsumura et al., 2017) but when overexpressed suppresses and/or corrupts hematopoiesis (Persons et al., 1999) and can correlate with adverse prognosis of AML (Vicente et al., 2012). Moreover, constitutively active *KIT* mutations occur in cancers, including hematological malignancies (Lennartsson and Ronnstrand, 2012; Valent et al., 2017), which powerfully reinforces the concept that unopposed activity of GATA2 or constituents of its genetic network (e.g. Kit) can corrupt HSPC transitions and cause cancer. Constitutively active *Kit* mutations drive uncontrolled mast cell proliferation to yield systemic or cutaneous mastocytosis and the often fatal mast cell leukemia (Valent et al., 2017).

Cellular context also appears to play a key role in determining the outcome of Kit/SCF signaling. Although Kit signaling is vital for the physiological regulation of HSCs, progenitors, erythroid precursors and mast cells, the specific signaling requirements can differ in distinct contexts. Insights into this have recently been provided by a structure-based engineering approach that interfered with SCF dimerization, yielding an SCF F63A mutant that attenuates Kit dimerization, an important step in Kit signaling, and decreases the magnitude of Kit signaling. This dimerization-defective F63A mutant retains the competence to regulate HSPCs while having considerably reduced activity to regulate mast cells (Ho et al., 2017). It has also been shown that mice with a knock-in mutation of the Kit juxtamembrane phosphorylation site (Y567) have normal steady-state erythropoiesis but exhibit defective stress erythropoiesis (Agosti et al., 2009), and that the mutation of Kit juxtamembrane tyrosines affects mast cells and melanocytes, but not erythroid or intestinal Kit phenotypes (Kimura et al., 2004).

The pivotal importance of Kit signaling magnitude and dynamics is further supported by the finding that cells within LT-HSC populations isolated using conventional strategies have activities that differ depending on their level of Kit expression. An intermediate level of Kit expression characterizes quiescent HSCs *in situ* with high multilineage repopulation activity in a murine transplant assay Grinenko et al., (2014). By contrast, cells expressing the highest Kit levels exhibit greater metabolic activity *in situ* and reduced activity upon transplantation; the differential activity of the two cell populations persists in secondary and tertiary transplants. Shin et al., (2014) demonstrated that Kit-low cells exhibit the highest self-renewal and long-term repopulating activities, while the Kit-high cells have a megakaryocytic lineage bias. The negative consequences of dysregulated Kit signaling may therefore not be restricted to scenarios involving grossly unopposed Kit signaling, as is the case with constitutively active Kit disease mutants. Subtler perturbations may corrupt the fine-tuned Kit mechanisms that endow hematopoietic precursors with their essential activities and ensure normal hematopoiesis.

Finally, it is likely that multiple feedback and feed-forward loops also contribute to the biological outcome of Kit/SCF signaling. For example, it is known that GATA2 activates *Kit* transcription, which

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increases MAPK signaling (Katsumura et al., 2017). Intriguingly, GATA2 also activates *Il1b* transcription, and IL1β functions through its receptor to also increase MAPK signaling (Katsumura et al., 2016). This dual regulation of Kit and IL1β by GATA2, coupled with the IL1-mediated increase in Kit expression, constitutes a coherent type I feed-forward loop that predicts the circuit is relatively resistant to short initiating pulses of active GATA2. A sustained stimulus would be required to effectively deploy the circuit; reversibility should be rapid upon shortening the duration of the initiating signal (Alon, 2007). Given that IL1β stimulates primary AML cell proliferation, that IL1β levels are elevated in patient bone marrow and that downregulating the IL1 receptor (IL1R1) reduces proliferation (Carey et al., 2017), such GATA2 feed-forward loops may have important implications for human AML.

Nuclear receptor-based stress erythropoiesis circuits: roles for glucocorticoid and thyroid hormone receptors

Glucocorticoids exert diverse activities that allow organisms to contend with severe stress. The major endogenous glucocorticoid cortisol functions by binding an intracellular ligand-activated transcription factor and member of the nuclear receptor superfamily: the glucocorticoid receptor (GR). In the inactive state, the GR exists in a complex with heat shock protein 90 and multiple components of the chaperone machinery (Pratt, 1993). This complex confers high-affinity ligand binding (Bresnick et al., 1989) and opposes gene regulatory activity in the absence of an agonist (Picard et al., 1990; Pratt et al., 1988). Ligand binding promotes dissociation of the GR from these components, leading to chromatin occupancy, co-regulator recruitment and transcriptional activation or repression of target genes (Pratt, 1993; Weikum et al., 2017).

Unlike Kit and EpoR signaling, which mediate developmental, steady-state and stress erythropoiesis, glucocorticoid signaling is selectively required for stress erythropoiesis *in vivo*, as revealed using *ex vivo* studies of GR-null mice that die at birth and using $GR^{dim/dim}$ mice expressing a defective DNA-binding receptor mutant (Bauer et al., 1999). These studies showed that GR-null fetal liver erythroblasts are not competent to expand into mature hemoglobinized erythroblasts *ex vivo*. Furthermore, in phenylhydrazine-induced hemolytic anemia and hypoxia-treated mouse models, the $GR^{dim/dim}$ mutation abrogates the accumulation of CFU-Es and other erythroid precursors in the spleen. Transplantation analyses also provide evidence for a cell-intrinsic GR requirement in hematopoietic cells. In *ex vivo* culture systems, glucocorticoid receptor agonists stimulate erythroid progenitor self-renewal at the expense of differentiation (von Lindern et al., 1999; Wessely et al., 1997). In humans, a glucocorticoid receptor gene polymorphism (A3669G) has been reported to be a susceptibility allele for primary myelofibrosis and to stabilize the transcript of a dominant-negative GR isoform (GRβ) (Poletto et al., 2012).

GR synergizes with another nuclear receptor, peroxisome proliferator-activated receptor α (PPARα), to promote BFU-E self-renewal *ex vivo* and to increase the generation of differentiated erythroid cells (Lee et al., 2015). GR and PPARα co-occupy chromatin and may collectively recruit an ensemble of co-regulators, with one or more co-regulators being limiting when only GR or PPARα is present. These mechanistic insights led to the proposal that the simultaneous use of GR and PPARα agonists to treat Epo-resistant anemias (e.g. Diamond-Blackfan anemia) could allow for a reduced glucocorticoid dose, thereby decreasing deleterious side-effects (Lee et al., 2015).

Thyroid hormone receptor α (TRα), another nuclear receptor superfamily member, is also implicated in stimulating erythropoiesis. Adult TRα-null mice exhibit a slight, but significant, reduction in hematocrit (Kendrick et al., 2008). Furthermore, in the phenylhydrazine-induced stress erythropoiesis model, BFU-E are much lower in TRα-null mice than in controls (Kendrick et al., 2008). Another study revealed that TRα promotes erythroid maturation selectively in the neonatal mouse spleen (Angelin-Duclos et al., 2005), at a time when stress erythropoiesis is induced. More recently, the erythroid maturation defects of mice expressing a dominant-negative TRα1 that mimics a human disease mutation (TRα1PV) were attributed to failure of TRα1 to transcriptionally activate *Gata1* expression (Park et al., 2017). These results are consistent with prior work demonstrating that the avian erythroblastosis virus encodes a mutated TR1α (v-ErbA) that induces erythroleukemia (Graf and Beug, 1983). Finally, humans with heterozygous TRα mutations are commonly anemic (Demir et al., 2016; van Gucht et al., 2017).

Further investigation is required to elucidate the factors functioning downstream of GR and TRα signaling that may interface with SCF- and Epo-dependent mechanisms to promote erythrocyte regeneration. Initial progress has identified the RNA-binding protein ZFP36L2, which plays a key role in hematopoiesis, as being important in mediating GR signaling. $Zfp36l2^{-/-}$ mice display pan-cytopenia and die within 2 weeks of birth; the mutant mice are profoundly anemic and lack BFU-E in their spleen (Stumpo et al., 2009). ZFP36L2 is a glucocorticoid-induced protein that promotes BFU-E self-renewal and erythrocyte development (Zhang et al., 2013). The diverse RNAs bound by ZFP36L2 in an RNA-immunoprecipitation assay extend the complexity of the regulatory processes governing erythrocyte development, function and regeneration.

Other stress erythropoiesis circuits: deciphering the GATA factor-regulated transcriptome

A recent genomic analysis of *cis*-regulatory elements resembling the +9.5 enhancer mediating GATA2 function at the *Gata2* locus has described new components of the GATA2-regulated HSPC cistrome (Hewitt et al., 2016, 2015). A subset of these *cis*-elements constitutes GATA2- and anemia-activated (G2A) enhancers (Hewitt et al., 2017). One of these G2A enhancers (*Samd14 Enh*) mediates GATA2-dependent activation of *Samd14*, which is predicted to encode an unstudied member of a large protein family (Kim and Bowie, 2003) with the common attribute of harboring a sterile α motif (SAM) domain. SAM domains are implicated in protein, RNA and lipid interactions, but the function of any particular SAM domain cannot be predicted from its sequence (Kim and Bowie, 2003). Loss-of-function studies with mouse fetal liver hematopoietic progenitors have provided evidence that Samd14 increases progenitors and facilitates Kit signaling (Hewitt et al., 2015). The targeted deletion of *Samd14 Enh* in mice abrogates Samd14 expression in fetal liver and in bone marrow hematopoietic progenitors and erythroid precursors without affecting its expression in brain (Hewitt et al., 2017). Although the resultant mutant mice have no detectable developmental or steady-state hematopoietic defects, they die in response to severe phenylhydrazine-induced anemia. Phenylhydrazine, phlebotomy and transplant-induced anemia activate the enhancer and induce *Samd14* expression in splenic erythroid precursors, which confers protection from the lethal anemia (Fig. 2A). During stress erythropoiesis in the spleen, Samd14 facilitates Kit signaling, consistent with the *ex vivo* analyses (Hewitt et al., 2015). Thus, whereas Kit signaling is important for developmental, steady-state and regenerative erythropoiesis, the Kit signaling

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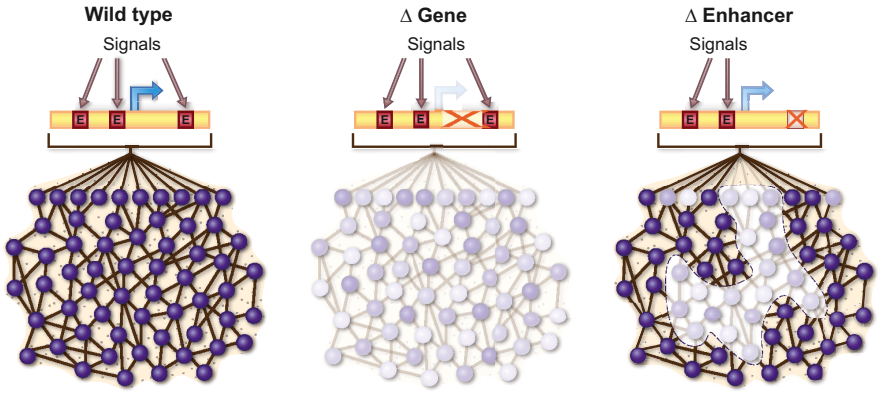

Fig. 4. The targeted deletion of enhancers reveals unique sectors of complex transcriptomes. Enhancers integrate diverse signals, thus serving vital functions in establishing and maintaining genetic networks. The targeted disruption of a gene encoding a master regulator, e.g. GATA2, eliminates the regulatory potential of all signals converging upon its enhancers, thus yielding a catastrophic collapse of the genetic network. By contrast, removing individual enhancers from loci containing multiple enhancers ablates only a subset of the signal-dependent enhancer mechanisms, in effect unveiling a unique sector of the genetic network. Targeted deletions of *cis*-regulatory elements individually and in combinations, e.g. those constituting enhancers, provide a uniquely powerful approach to dissect these mechanisms.

facilitator Samd14 appears to selectively promote erythrocyte regeneration.

Considering the depth and diversity of the GATA factor-regulated transcriptome, it is likely that many other GATA2- and/or GATA1-regulated factors intermesh with known signaling systems such as Kit and EpoR. These factors may function as selective erythrocyte regeneration regulators (SERRs; Fig. 2B) or may be components of more broadly deployed systems that also control developmental and steady-state erythropoiesis. The targeted deletion of cell type-specific enhancers and specific factors may help to tease apart these functions (Fig. 4). There is also much to be learned regarding mechanisms that function upstream of GATA2 and GATA1 and control their expression at the transcriptional level, as well as the translational and post-translational modes of regulating their activities. GATA2 not only regulates Kit expression, but also facilitates Kit signaling through induction of Samd14 expression (Hewitt et al., 2015, 2016, 2017). This circuit can be integrated into that of BMP4 signaling, in which BMP4 induces *Gata2* expression (Friedle and Knöchel, 2002; Lugus et al., 2007). During stress erythropoiesis, defective BMP4 signaling impairs *Gata2* expression and delays recovery from anemia. Retroviral-mediated expression of *Gata2* rescues this defect, underscoring the pivotal function of GATA2 in stress erythropoiesis (Harandi et al., 2010), in addition to its vital functions in developmental and steady-state hematopoiesis.

GATA2 multi-site phosphorylation, which is catalyzed by p38 and ERKs, also enhances GATA2 chromatin occupancy at select loci and regulates target gene transcription (Katsumura et al., 2016, 2014). GATA2 increases expression of genes encoding chemokines and cytokines (Katsumura et al., 2016; Linnemann et al., 2011; Mehta et al. 2017)), which, in turn, act on plasma membrane receptors to activate MAPK-dependent pathways. These signal-dependent positive autoregulatory loops are almost certainly crucial for erythrocyte regeneration. Discovering the key steps involved in mediating establishment versus maintenance of GATA2 and GATA1 levels and their specific activities will provide a new perspective into erythrocyte regeneration.

Input from other cell types

The diverse cells constituting the microenvironment of HSPCs also contribute to integrative regulation. For example, well-established functional links exist between macrophages and erythroblasts. In certain contexts, erythroblasts encircle a macrophage to generate a structure termed an erythroblastic island (Klei et al., 2017). Macrophages adopt multiple functional or activation states, the extremes of which are termed classical or M1, which produce high levels of inflammatory cytokines, and alternative or M2, which are linked to tissue repair/remodeling and resistance to infection (Murray et al., 2014). Depletion of a specialized macrophage population ($CD169^{+}$) reduces bone marrow erythroblasts without inducing anemia (Chow et al., 2013). In the context of hemolytic anemia, acute blood loss and myeloablation, macrophage depletion reduces erythrocyte regeneration. Moreover, in the case of JAK2 (V617F)-induced polycythemia vera, macrophage depletion attenuates disease phenotypes that result from excessive erythropoiesis. In another study, macrophage depletion attenuated erythrocyte regeneration in anemia, and it was also shown that macrophages contributed to disease phenotypes in polycythemia vera and β-thalassemia (Ramos et al., 2013). Thus, the functional plasticity of macrophages, including their roles in cytokine/chemokine sensing and elaboration, impacts diverse steps in the erythrocyte life cycle.

Dendritic cells, another vital component of the innate immune system, also promote stress erythropoiesis. The administration of anti-CD24 monoclonal antibodies to mice induces transient splenomegaly, which reflects a classic stress erythropoiesis response involving a major accumulation of $Ter119^{+}$ erythroblasts in the spleen. It has been shown that the depletion of $CD11c^{high}$ dendritic cells using a diphtheria toxin-based cell ablation approach can abrogate this response (Kim et al., 2015). Moreover, this response is not elicited in *Batf3*$^{-/-}$ mice lacking type I conventional dendritic cells. Analysis of the window in which diphtheria toxin-mediated ablation of $CD11c^{high}$ cells abrogates stress erythropoiesis revealed an early requirement at the time of stress erythropoiesis induction. Consistent with the role of Kit signaling in stress erythropoiesis, the Kit kinase inhibitor imatinib (Gleevec) inhibits stress erythropoiesis induced by CD24 antibody engagement. The study by Kim et al. provides a new perspective on the intercellular interactions that signal stress erythroid progenitors to mount a regenerative response.

Is erythrocyte regeneration coupled to other regenerative processes?

Considering the cell-intrinsic and non-cell-autonomous determinants of erythrocyte regeneration, abundant opportunities exist for linking this process with cell and tissue protection, repair and regeneration in broader contexts. In addition to genetic disorders such as sickle cell disease and β-thalassemia, anemia is a common attribute of diseases including cancer, kidney disease, chronic inflammatory disorders and infections, and can also result from pharmacological interventions. As erythrocyte regeneration ensures the oxygenation of vital cells and tissues, any impediments in this process may impact the integrity of diverse organ systems, including the heart and brain. Although it is instructive to contemplate the degree of erythrocyte regeneration required to

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negate or attenuate potentially lethal organ damage, there is little to no foundation to address this problem. One can envision a vicious cycle in which inadequate erythrocytes cause hypoxia, which initiates and/or exacerbates cell and tissue damage, and the damage further increases the demand for erythrocytes and oxygen. Thus, the rate and extent of damage may accelerate and considerably exceed the repair capacity. As mentioned above, hypoxia stimulates cytoprotective and reparative pathways, including HIFs (Cai et al., 2003; Karhausen et al., 2004) and NF-E2-related factor 2 (NRF2) (Chan and Kan, 1999; Itoh et al., 1999). Although many factors dictate the balance between damage and repair, erythrocyte-delivered oxygen is perhaps the most fundamental component required for maintenance of cell and tissue integrity.

Can the mechanisms governing erythrocyte regeneration and novel SERRs be leveraged to promote expansion and/or regeneration of distinct cell types and tissues? In a scenario with an unusually high metabolic demand, erythrocyte regeneration may be rate-limiting and therefore a prerequisite for the unimpeded function of cell- and/or organ-specific mechanisms. Indeed, hypoxia is permissive for cardiomyocyte regeneration; in the adult mouse heart, oxygen confers cardiomyocyte cell cycle arrest and maintains quiescence (Puente et al., 2014), whereas gradual systemic hypoxia in 3-month-old mice induces the proliferation of quiescent cardiomyocytes, which renders the cells competent to regenerate myocardium (Nakada et al., 2017). It is also known that vascular organization promotes neuronal regeneration. In the peripheral nervous system, for example, Schwann cell-dependent neuronal regeneration requires normal vascularization, and disruption of vascular organization attenuates this process (Cattin et al., 2015). Though the roles of hypoxia-induced erythrocyte regeneration and vascular delivery of erythrocytes/oxygen were not described in these studies, one can envision vital contributions to these regenerative processes.

Conclusions

As we have reviewed here, considerable progress has been made in elucidating the mechanisms governing erythrocyte development and regeneration. Major unanswered questions remain, however, at the molecular, cellular and systems levels. Much remains to be learned regarding how microenvironment-dependent signaling instructs the intracellular circuitry of the erythroblast to dictate the decision to undergo massive expansion or terminal differentiation. Integrating such signals with the complex machinery mediating transcriptional, post-transcriptional, translational and post-translational control of the maturing erythroblast represents a major challenge. Further studies on the profound nuclear transitions that prepare the erythroblast for enucleation are essentially guaranteed to yield important discoveries that inform nuclear cell biology, development and regeneration. The machinery mediating terminal differentiation-linked organelle remodeling and enucleation is also incompletely understood, and comparative analyses of the mechanisms operating in physiological versus regenerative contexts will be highly instructive. This point highlights a theme that emerges from this review: although there is some overlap between developmental and regenerative mechanisms in red cell biology, developmental- and regeneration-specific mechanisms also exist; current work has only scratched the surface vis-à-vis conducting the requisite comparisons and elucidating mechanistic relationships.

Given the vital role of tissue oxygenation in regenerative processes, are the erythrocyte developmental and regenerative mechanisms intricately intermeshed with those mediating tissue regeneration and repair? We expect that the process of erythrocyte regeneration will be tightly linked to diverse regenerative processes and therefore will have crucial involvement in broad sectors of regenerative biology and medicine. Further dissecting the mechanisms underlying erythrocyte regeneration is thus likely to unveil innovative strategies to promote regeneration *in vivo* and/or new opportunities to generate and implement effective and durable bioengineered systems.

Competing interests

The authors declare no competing or financial interests.

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