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Advances in Understanding Erythropoiesis: Evolving Perspectives

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Abstract

Red blood cells (RBCs) are generated from haematopoietic stem and progenitor cells (HSPCs) through the step-wise process of differentiation known as erythropoiesis. In this review, we discuss our current understanding of erythropoiesis and highlight recent advances in this field. During embryonic development, erythropoiesis occurs in three distinct waves comprising first, the yolk sac-derived primitive RBCs, followed sequentially by the erythro-myeloid progenitor (EMP) and HSPC-derived definitive RBCs. Recent work has highlighted the complexity and variability that may exist in the hierarchical arrangement of progenitors responsible for erythropoiesis. Using recently defined cell surface markers, it is now possible to enrich the erythroid progenitors and precursors to a much greater extent than has been possible before. While a great deal of knowledge has been gained on erythropoiesis from model organisms, our knowledge of this process is being refined through human genetic studies. Genes mutated in erythroid disorders can now be identified more rapidly by the use of next-generation sequencing techniques. Genome-wide association studies on erythroid traits in healthy populations have also revealed new modulators of erythropoiesis. All of these recent developments have significant promise not only for increasing our understanding of erythropoiesis, but also for improving our ability to intervene when RBC production is perturbed in disease.

Keywords

erythropoiesis; haemopoiesis; haemopoietic progenitors; red cell disorders; red cells

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Developmental and Hierarchical Origins for Erythropoiesis

Three Developmental Waves of Erythropoiesis

During embryonic development, mammalian haematopoiesis emerges in three distinct waves (**Figure 1A**). Most of our understanding of this process comes from extensive mouse studies (Palis, 2014a; Yoder, 2014). The first wave of haematopoiesis emerges from the yolk sac at embryonic day 7.5 (E7.5) in mice. This first wave, commonly known as the primitive wave, is characterized by the production of nucleated megaloblastic erythroblasts that are referred to as primitive erythroblasts (PEs), along with diploid platelet progenitor cells and macrophages (Potts et al., 2014). PEs are characterized by the predominant expression of embryonic globin genes. While it was initially assumed that PEs could not enucleate, studies have shown that PEs eventually enucleate in the circulation in both mice and humans (Kingsley et al., 2004; Van Handel et al., 2010). The close proximity of the blood and endothelial cells in the yolk sac led to the hypothesis nearly 100 years ago that haematopoietic cells and blood vessels originate from a common precursor. This precursor, referred to as the haemangioblast, has been suggested to arise from a unique population of primitive streak mesodermal cells (Palis, 2014a).

The second wave of haematopoiesis is characterized by the emergence of erythro-myeloid progenitors (EMPs) in the yolk sac at approximately E8.25 in mice (Palis, 2014a). Yolk sac EMPs can generate erythroid colonies similar to those derived from adult bone marrow (BM) and these cells have also been shown to have at least the capacity to produce multiple other myeloid lineages (Gomez Perdiguero et al., 2015; McGrath et al., 2015). Some work has suggested that specific lymphocyte populations may also be produced from the EMP or a related lineage transiently produced in the yolk sac (Boiers et al., 2013). The EMPs eventually migrate to the fetal liver and produce definitive erythroblasts, which predominantly express mouse adult globins (McGrath et al., 2011). Recent work has shown that EMP-derived erythroblasts can self-renew, at least *in vitro,* in the presence of dexamethasone, stem cell factor (SCF, also termed KITLG) and erythropoietin (EPO) (Kim et al., 2015). To what extent this self-renewal occurs *in vivo* is currently unknown.

The third wave of haematopoiesis is characterized by the emergence of haematopoietic stem cells (HSCs) from a unique population of endothelial cells termed the haemogenic endothelium, which can be found in the dorsal aorta of the aorta-gonad-mesonephros (AGM) region at approximately E10.5 in mice. In parallel, HSCs may also emerge from other haemogenic endothelial cells within the arteries found in the umbilical, vitelline, cranial, yolk sac and placental regions (Yoder, 2014). These HSCs migrate to the fetal liver where they undergo an expansion period before reaching their final destination in the BM, where the HSCs primarily remain in a quiescent state (Kiel et al., 2007). The relative contribution from HSC-derived cells during embryonic development and when the EMP-derived contribution wanes is currently unknown and is confounded by the similarity between EMP- and HSC-derived differentiated definitive haematopoietic cells. In mutant mice lacking HSCs, EMPs are able to maintain embryonic viability until birth, suggesting broad functions for this lineage throughout much of gestation (Chen et al., 2011). In

addition, the number of HSCs that seed the BM to give rise to mature adult haematopoiesis remains unknown.

Erythropoiesis in the Haematopoietic Hierarchy

In humans, after birth, HSCs reside in the BM and are thought to give rise to all mature haematopoietic cells, including the erythroid lineage, through a series of intermediate progenitors (**Figure 1B**). Although there is a classical model of haematopoietic differentiation that involves differentiation into increasingly more lineage-restricted progenitors, recent work suggests that there may be alternate pathways that could be used in specific circumstances or concomitant with differentiation through the classical pathway. Using functional transplantation studies and lineage tracing in mice, it was shown that differentiation into erythroid- and megakaryocyte-committed progenitors occurs early in the haematopoietic hierarchy from HSCs or their immediate downstream progenitors (Sanjuan-Pla et al., 2013; Yamamoto et al., 2013). Single cell gene expression analysis of various mouse stem and progenitor populations has shown that the phenotypically defined megakaryocyte and erythroid progenitors (MEPs) cluster closely with HSCs and segregate distinctly from lymphomyeloid lineages (Guo et al., 2013). Recent studies in human haematopoietic cells have also demonstrated that lineage specification of the erythroid and megakaryocyte lineages appears to occur early in the hierarchy at either the stem or multipotential progenitor stage (Notta et al., 2015). These data suggest a more complex model in which committed erythroid progenitors can directly differentiate from the HSC or immediate downstream multipotential progenitors, although *in vivo* haematopoiesis may be more complicated.

The committed erythroid progenitors are the burst-forming unit erythroid (BFU-E) and colony-forming unit erythroid (CFU-E) cells, which are named for their ability to form colonies in semisolid media. The BFU-E is the earliest committed erythroid progenitor and can form significantly larger colonies than the CFU-E. BFU-Es can be found in the circulation, whereas CFU-Es cannot except under pathological conditions (Clarke and Housman, 1977). While BFU-Es and CFU-Es can be functionally distinguished and enriched using surface marker expression, their morphology cannot be distinguished from other blast cells of different lineages. BFU-Es and CFU-Es further differentiate into erythroid precursor cells with distinct morphologies. The earliest morphologically identifiable erythroid precursor is the proerythroblast, which differentiates sequentially into the basophilic, polychromatophilic and orthochromatic erythroblast, which can then enucleate to form a reticulocyte. The precursors show a gradual reduction in cell and nuclear size, while a robust increase in the accumulation of haemoglobin occurs.

The prospective enrichment of erythroid progenitor and precursor cells has enabled an improved understanding of the molecular basis of erythroid differentiation and its disorders (**Figure 2**). Several groups have suggested strategies by which human MEPs can be enriched from the fraction of progenitor cells that express CD34 and CD38 in the absence of lineage markers (Doulatov et al., 2010; Edvardsson et al., 2006; Manz et al., 2002). However, it is clear that these MEP populations are extremely heterogeneous and probably contain committed erythroid progenitors. CD105 (endoglin) expression has been suggested to mark

one of the first steps of erythroid commitment and may help to further enrich for BFU-Es found using MEP enrichment approaches (Mori et al., 2015). Independent work has allowed significant enrichment of BFU-Es and CFU-Es based on CD34, CD36, CD71 and CD45RA marker expression from subfractions of cord blood, BM and peripheral blood (Li et al., 2014). More differentiated human erythroid precursors can be isolated by surface expression of the transferrin receptor (CD71) and glycophorin A (CD235a). More recently used markers, including α4-integrin (CD49d) and band 3, have been shown to improve the resolution of the various stages of erythroid precursor differentiation (Hu et al., 2013). Similarly, mouse erythroid progenitors and precursors can be enriched using various combinations of cell surface markers including CD71, glycophorin A (Ter119), CD44 and KIT (Chen et al., 2009; Flygare et al., 2011). As multiple and sometimes conflicting strategies exist to isolate erythroid progenitor and precursor populations, future research comparing the overlap of the proposed markers will allow for isolation and characterization of increasingly pure populations from a variety of sources (**Figure 2**).

Developmental Switching of Haemoglobin

Haemoglobin is a tetramer of 2 α-like globin polypeptide chains and 2 β-like globin chains with an iron containing porphyrin ring called haem bound to each subunit that allows for effective oxygen transport. The sequential expression of embryonic, fetal and adult globin genes during development occurs through a series of two haemoglobin switches, mediated primarily through changes in expression of the β-like globin genes expressed, which have been extensively studied and reviewed in detail elsewhere (Sankaran and Orkin, 2013). Here, we provide a brief overview of this process. In humans, there are five functional β-like globin genes: embryonic (*HBE1*), fetal (*HBG1*, *HBG2*), and adult (*HBD* and *HBB*). There is initially a switch from predominant expression of the embryonic globin (*HBE1*) in primitive erythroid cells to the fetal globins (*HBG1*, *HBG2*) that coincides with the onset of definitive erythropoiesis. Shortly after birth, fetal globin expression in the definitive erythroid cells is gradually replaced by the adult globins. There are three major functional α-like globin genes: *HBZ*, *HBA1* and *HBA2*. The embryonic HBZ globin is expressed almost exclusively in primitive erythroid cells, while the adult globins (HBA1, HBA2) are expressed at all stages of erythropoiesis.

Understanding the molecular basis of the fetal-to-adult haemoglobin switch has been of long-standing interest in the field due to its therapeutic relevance. There is substantial clinical evidence that elevated fetal haemoglobin (HbF) production reduces disease severity in β-thalassaemia and sickle cell disease (SCD) (Sankaran and Weiss, 2015). Individuals with hereditary persistence of fetal haemoglobin (HPFH) concomitantly with β-thalassaemia or SCD have reduced disease severity and are often clinically asymptomatic (Galanello et al., 2009). Modest induction of HbF by hydroxycarbamide can alleviate symptoms, organ damage and mortality in SCD and in some patients with β-thalassaemia (Musallam et al., 2013).

The molecular regulation of the haemoglobin switches has been extensively covered in other reviews (Sankaran and Orkin, 2013) and will only be briefly discussed here. By using insight from genome-wide association study (GWAS) examining common variation in HbF

levels, the multi-zinc finger containing transcriptional regulator, BCL11A, was identified as a key regulator of the fetal-to-adult haemoglobin switch and HbF silencing (Sankaran et al., 2008). BCL11A binds to the β-globin enhancer and also to an intergenic region between the *HBG1* and *HBD* genes that is often deleted in individuals with HPFH (Sankaran et al., 2011a). The exact mechanisms by which BCL11A acts to silence HbF remain unclear and may either involve direct modification of chromatin and/or alteration of long-range interactions. Erythroid-specific enhancers of BCL11A have also been identified (Bauer et al., 2013). *BCL11A* appears to be a promising target for therapeutic HbF induction and recent work has shown that humans with haploinsufficiency of this gene have substantial persistence of HbF, although there appear to be adverse neurodevelopmental phenotypes found in such patients, which emphasizes the need to develop targeted approaches to suppress *BCL11A* (Basak et al., 2015). GWAS studies have also implicated the transcription factor, MYB, as a regulator of HbF levels and reduction of this factor does appear to robustly induce HbF production, although its mechanism of action remains unknown (Sankaran et al., 2013; Stadhouders et al., 2014). Additionally, rare mutations in *KLF1* have been associated with increased HbF levels in some patients, but the effects are variable and certain mutations in this factor have been associated with both increased HbF and congenital anaemias (Sankaran and Orkin, 2013). A number of other factors have also been suggested to be involved in this process, but *in vivo* evidence for most of these other factors is currently limited and further work is necessary (Sankaran and Orkin, 2013; Sankaran and Weiss, 2015).

Model Systems and Organisms Used for Understanding Erythropoiesis

Mouse and Zebrafish Models

Much of our knowledge regarding the process of erythropoiesis comes from studies of valuable model organisms, including mice and zebrafish (**Figure 3**) (Carroll and North, 2014; Schmitt et al., 2014). Morphologically and at the molecular level, erythropoiesis is largely conserved between mice and humans. Studies of erythropoiesis have been facilitated through the availability of well-established tools, including the ability to readily manipulate the mouse genome with reverse genetic engineering approaches and the extensive availability of antibodies for stage-specific enrichment of haematopoietic progenitors (Orkin and Zon, 2008). Erythroid disorders, including SCD and β-thalassaemia, have been modelled in mice and are actively used in preclinical studies (Nienhuis and Persons, 2012; Xu et al., 2011). Mouse haematopoietic cytokines are readily available, leading to development of *in vitro* culture and colony assays.

Despite their evolutionary distance from humans, many haematopoietic genes are conserved in zebrafish, making it a valuable model system. The ability to economically screen a large number of zebrafish has facilitated extensive forward genetic screens that have identified numerous genes involved in erythropoiesis (Jing and Zon, 2011). Moreover, zebrafish undergo external fertilization, allowing for *in vivo* imaging of embryonic development. Thousands of mutants have been generated by these forward genetic screens and screened for haematopoietic phenotypes. Analysis of many of these mutant fish has led to advances in our understanding of erythropoiesis, including the identification of key molecules involved

in iron metabolism from hypochromic zebrafish mutants, such as the key plasma membrane iron transporter SLC40A1/ferroportin 1 (Donovan et al., 2000).

Cellular Models

One of the unique aspects of erythropoiesis, compared with many other examples of physiological differentiation, has been the long-standing availability of both primary and immortalized *in vitro* cellular models. Currently, the gold standard is the primary culture of haematopoietic stem and progenitor cells (HSPCs) obtained from the peripheral blood (either mobilized or normal mononuclear cells), BM, cord blood or fetal liver of human donors or mice. In general, the culture of primary cells has involved multiple phases that promote either the expansion of progenitor cells or the differentiation toward precursors that can eventually enucleate. It should be noted that enucleation rates in such systems tend to be modest and can vary considerably (Hu et al., 2013). Thus, primary cell systems may not fully recapitulate the efficient expansion and differentiation observed *in vivo*. The use of cells differentiated from immortalized pluripotent stem cells also offers the prospect of modelling specific erythroid disorders (Mills et al., 2014), but at the current time, these cells can only be differentiated toward erythroid cells resembling those that arise in the initial haematopoietic waves from the yolk sac (Yoder, 2014).

A number of cell lines have proven incredibly valuable in improving our understanding of erythropoiesis and its molecular regulation. One of the most useful models is the mouse erythroblast cell line G1E and its derivative, G1E-ER4. These cell lines are derived from GATA1-null mouse embryonic stem (ES) cells and are arrested at a stage that resembles the proerythroblast (Welch et al., 2004). These cells can be rescued by the addition of GATA1 to semi-synchronously complete erythroid differentiation, which has provided important insight into erythroid transcriptional regulation. Similar cell lines with both megakaryocyte/erythroid potential have revealed important biology about the megakaryocyte/erythroid lineage decision (Noh et al., 2015). Mouse erythroleukaemia cells have been particularly valuable in broadening our understanding of certain aspects of gene regulation, particularly given their adult globin gene expression pattern (Bauer et al., 2013). Human erythroleukaemia cell lines, including TF-1, which is cytokine responsive, and K562 cells, which are cytokine independent, have also proven to be valuable models (Sankaran et al., 2012a; Stadhouders et al., 2014; Ulirsch et al., 2014). More recently, immortalized human erythroid cell lines have been created through a variety of methods, and some of these lines may be valuable in future studies (Kurita et al., 2013). Some of these newer cell lines have the distinct advantage of showing an adult-type haemoglobin expression pattern as well as allowing for increased terminal differentiation, compared with more traditional erythroid cell lines, such as TF-1 or K562 cells. With the advent of precise and efficient genome editing through the use of CRISPR/Cas9 (clustered regularly interspaced short palindromic repeats-associated protein 9 nuclease) or other related approaches, these cell models have become increasingly utilized.

Xenograft Models

Humanized mouse models allow engraftment of human haematopoietic cells and are thus excellent tools to study human haematopoiesis in conditions that can mimic normal

physiology. The NOD/SCID IL2Rγ^{null} (NSG) and BALB/c Rag$^{-/-}$ IL2Rγ^{null} (Brg) immunodeficient mice lacking T cells, B cells and NK cells show robust engraftment of human haematopoietic cells (Ishikawa et al., 2005; Rongvaux et al., 2013). Unfortunately, these mice have poor human erythroid differentiation because human erythroid progenitors make up a low and highly variable fraction of the human haematopoietic cells in the BM and circulating human RBCs have not been found in peripheral blood. Interestingly, inactivation of mouse macrophages resulted in a low transient level of circulating human RBCs (Hu et al., 2011). Recently, both NSG and Brg mice carrying a mutant *Kit* gene showed increased engraftment of erythroid progenitors (~30%) and show great promise for the study of human erythropoiesis (McIntosh et al., 2015). One distinct advantage of these newer models is that they do not rely upon irradiation to allow human cell engraftment. Humanized mouse models that express human cytokines are being developed and may allow for even greater erythroid engraftment (Rongvaux et al., 2013).

Humans as an Emerging Model System

While model organisms are indispensible for the study of erythropoiesis, there are specific aspects of human erythroid biology that do not replicate well. For example, mutations of the human *SEC23B* gene result in congenital dyserythropoietic anaemia type II, but a mouse knockout of this gene does not have a haematological phenotype (probably due to compensation from species-specific expression of the paralog, SEC23A) (Khoriaty et al., 2014; Ulirsch et al., 2014). As another example, mutations in *GLUT1* alter RBC volume in adult humans, but GLUT1 is not expressed in the erythroid lineage of adult mice (Montel-Hagen et al., 2008). Fortunately, advances in both sequencing technology and functional approaches have allowed for the direct study of human erythropoiesis and disorders due to alterations of this process. GWAS coupled with thorough functional follow-up have identified novel roles for BCL11A as an essential HbF silencing factor (Sankaran et al., 2008), CCND3 and CCNA2 as regulators of RBC size and number (Ludwig et al., 2015; Sankaran et al., 2012a), TRIM58 as a regulator of enucleation (Thom et al., 2014), and SMIM1 as a key RBC surface protein encoding the Vel blood antigen (Cvejic et al., 2013). Studies of rare genetic disorders in humans have revealed novel, and sometimes unexpected, findings, such as an erythroid-specific defect due to ribosomal protein haploinsufficiency in Diamond Blackfan anaemia (Ludwig et al., 2014). Importantly, support from human genetics can identify novel therapeutic targets (e.g. BCL11A in β-thalassaemia and SCD) and has the potential to improve the success of therapeutic drug development (Nelson et al., 2015).

Molecular Regulation of Erythropoiesis

Cellular Signaling Pathways

Several cytokines and growth factors have essential roles in erythropoiesis. One of the most well studied cytokines is erythropoietin (EPO) (Kuhrt and Wojchowski, 2015). EPO primarily acts on erythroid progenitors and early precursors. CFU-Es require EPO for survival during *in vitro* cell culture, as well as for forming colonies in methylcellulose media. Some BFU-Es or earlier progenitors may require EPO and some evidence exists to suggest that EPO receptor may be expressed on such progenitor cells, at least in mice

(Singbrant et al., 2011). Even though EPO is required for *in vitro* culture systems, BFU-E and CFU-E populations can be found in *Epo* and *Epor* knockout mice, although they both die at the fetal liver stage with the onset of definitive erythropoiesis (Wu et al., 1995a). Although this initially suggested that this signalling was dispensable for yolk sac primitive erythropoiesis, recent careful analysis of these mutants suggests a critical role for maturation of primitive erythroid cells as well (Malik et al., 2013).

Another well-studied growth factor, stem cell factor (SCF, KITLG), binds to the KIT receptor that functions as a receptor tyrosine kinase (Lemmon and Schlessinger, 2010). SCF can synergize with EPO to promote erythropoiesis (Wu et al., 1995b). *Kitl (Scf)* null (steel mutants) and *Kit* null (W mutants) mice have reduced CFU-E colonies in the fetal liver (Nocka et al., 1989). Moreover, SCF can indirectly phosphorylate EPOR through KIT receptor. Other factors that positively regulate erythropoiesis include insulin, insulin-like growth factor, activin and angiotension II. Transforming growth factor β and a related ligand, growth and differentiation factor 11, inflammatory cytokines, such as γ-interferon, tumour necrosis factor α, and tumour necrosis factor-related apoptosis-inducing ligand all have negative effects on erythropoiesis (Dussiot et al., 2014; Suragani et al., 2014). The precise relevance of these pathways for *in vivo* erythropoiesis remains to be established in most of these cases.

Transcriptional Regulation and non-Coding RNAs in Erythropoiesis

The commitment of multipotent progenitor cells to the erythroid lineage and their subsequent differentiation into mature RBCs is ultimately controlled by master transcription factors (TFs) that tightly regulate erythroid-specific gene expression networks. The importance of a core erythroid network (CEN) of TFs for proper red cell development is increasingly appreciated (**Figure 4**). This CEN is comprised of DNA-binding TFs: GATA1, TAL1, and KLF1 (Xu et al., 2012), as well as those that are non-DNA-binding: LDB1 and LMO2. Knockout mice lacking any TF from the CEN exhibit severe impairments in erythropoiesis and generally do not survive past gestation. In humans, mutations in *GATA1* and *KLF1* result in multiple anaemia phenotypes (Sankaran et al., 2012b). Moreover, these factors have been shown to co-occupy regulatory elements in erythroblasts proximal to key erythroid genes and are more predictive of enhancer activity in comparison with histone modifications (Dogan et al., 2015; Ulirsch et al., 2014; Xu et al., 2012). Impairment of one or more of these TFs often destabilizes the binding of the other factors at co-occupied elements.

Outside the CEN, important roles for other TFs, often requiring cooperativity with the core TFs, have been delineated in erythropoiesis. For example, BCL11A interacts with GATA1 and is the key regulator of the fetal-to-adult haemoglobin switch, as discussed above (Sankaran et al., 2008). Other factors with novel and evolving roles include ZFPM1/FOG1, NFE2, GFI1B, ETO2, TAF10, MYB, and ZNF148/ZBP-89. Other studies have proposed a role for the BMP and Wnt-responsive TFs, SMAD1 and TCF7L2, respectively, in erythropoiesis (Trompouki et al., 2011). Occupancy by SMAD1 and TCF7L2 was shown to shift from GATA2-occupied elements proximal to genes important for multipotency in HSPCs to GATA1-occupied enhancers near key erythroid genes in erythroblasts (**Figure 4**).

Although model organisms, such as mouse and zebrafish, have been instrumental in improving our understanding of the gene expression networks regulated by the CEN, recent studies have surprisingly shown a marked divergence in the global patterns of gene expression between human and mouse erythroblasts (Palis, 2014b). While the overall CEN of TFs governing erythropoiesis is broadly the same for human and mouse, recent studies have shown that occupancy sites for TFs in the CEN are generally poorly conserved at orthologous DNA (Ulirsch et al., 2014), similar to findings in multiple unrelated lineages (Villar et al., 2014). Moreover, species-specific binding by TFs in the CEN has been shown to significantly associate with species-specific expression patterns in erythroblasts (Ulirsch et al., 2014).

The role of non-coding RNAs, including microRNAs and long non-coding RNAs (lncRNAs), in erythropoiesis is increasingly being appreciated. miR-451 (MIR451A) was identified as a microRNA that was dramatically upregulated during erythroid maturation and its role in fine-tuning erythropoiesis has been substantiated by a number of independent studies (Yu et al., 2010). Other microRNAs have also been suggested to have roles in erythropoiesis. For example, miR-24 binds to the 3' UTR of *ALK4* to repress translation, resulting in maturation defects, (Wang et al., 2008), miR-15a (MIR15A) acts through MYB to restrict colony formation and regulate HbF levels (Sankaran et al., 2011b), and miR-221/222 (MIR221/222) regulate KIT translation to potentially alter erythroid differentiation (Gabbianelli et al., 2010). Recently, hundreds of lncRNAs have been shown to have dynamic expression patterns during erythropoiesis, many of which show evidence of regulation by the CEN (Alvarez-Dominguez et al., 2014) (**Figure 4**). Interestingly, the knockdown of multiple lncRNAs in primary cell culture appeared to inhibit erythroid maturation, suggesting that some lncRNAs may serve as key regulators of erythropoiesis. However, lncRNA expression in humans and mice is substantially divergent, and the study of lncRNA function has been an area of considerable controversy that will probably undergo substantial refinement in the coming years (Bassett et al., 2014).

Translational Regulation

Most research into the regulation of erythropoiesis has focused heavily on transcription, but an increasingly important role for mRNA translation has recently begun to be appreciated. Recent studies have shown an important role for alterations in protein synthesis rates during haematopoiesis (Buszczak et al., 2014). Moreover, Diamond-Blackfan anaemia (DBA), an anaemia characterized by a specific reduction in the earliest erythroid-commited progenitors with defective maturation of erythroid precursors, can be caused by autosomal dominant mutations in one of 17 different ribosomal protein genes (Boria et al., 2010). The involvement of ribosomal proteins in DBA suggested that changes in protein translation might cause this disorder. Indeed, rare mutations in *GATA1* have been identified in DBA patients (Sankaran et al., 2012b), which led to the identification of altered translation of *GATA1* mRNA due to ribosomal protein haploinsufficiency as a common pathogenic mechanism for DBA (Ludwig et al., 2014). While further work investigating the underlying mechanisms is necessary, translational regulation appears to have a role in the earliest lineage-commitment decisions, allowing for specification of multipotent progenitors into the erythroid lineage.

During the later stages of erythropoiesis, translational regulation also has a key role. Under specific conditions, such as with iron deficiency, iron responsive proteins (IRPs) bind to stem-loop structured iron responsive elements (IREs) in mRNA UTRs. When IRPs bind to IREs in the 5' UTR of *ALAS2*, *FTH1/FTL* (heavy and light ferritin chains), and *SLC40A1* (ferroportin) mRNAs, their translation is repressed, resulting in decreased haem biosynthesis (Hentze and Kuhn, 1996). However, the binding of an IRP to an IRE in the 3' UTR of *TFRC* (transferrin receptor) actually increases mRNA stability, resulting in increased cellular iron intake (Hentze and Kuhn, 1996). The identification of other novel canonical and non-canonical IREs, such as those in *AHSP* (α-haemoglobin stabilizing protein) mRNA, increases our understanding of the process of cellular iron homeostasis and may inform the treatment of iron deficiency anaemia (dos Santos et al., 2008). In the setting of haem deficiency or oxidative stress, the haem-regulated eIF2α kinase (HRI), an eIF2α kinase that is specific to the erythroid lineage, phosphorylates eukaryotic initiation factor 2 alpha (eIF2α/EIF2A), inhibiting translational initiation and decreasing overall protein synthesis (Chen, 2014). This pathway appears to exist to ensure that globin synthesis remains balanced and can be regulated by the availability of iron and haem. Moreover, recent studies have shown that HRI-dependent eIF2α phosphorylation (eIF2αP) preferentially up-regulates translation of *ATF4* mRNA, which then transcriptionally activates both stress response and erythroid differentiation pathways (Suragani et al., 2012).

An Overview of Disorders Affecting Erythropoiesis

Most intrinsic erythroid disorders can be broadly classified into two categories: disorders where the most significant defects occur during differentiation, such as is observed in conditions characterized by dyserythropoiesis, and those where differentiation proceeds more or less normally, but mature RBCs have defective function or structure. Some intrinsic disorders involve a combination of these defects. Here, we highlight a few representative disorders in each category, focusing on recent advances in our understanding of their underlying molecular mechanisms. More detailed reviews on such disorders have been published elsewhere (Sankaran and Weiss, 2015), and we therefore only briefly discuss the disorders to highlight aspects relevant to our evolving understanding of erythropoiesis.

Defective Differentiation

There is a spectrum of disorders that affect various aspects of erythropoiesis. At one extreme is DBA, which is characterized by a paucity of the earliest erythroid progenitors and defective maturation of the few remaining progenitors into mature erythroid precursors (Nathan et al., 1978). Other aspects of the maturation process can also go awry in certain disorders. For example, excess free α-globin chains in β-thalassaemia – where there is defective production of β-globin due to a variety of different types of mutations – can cause cytotoxicity in erythroid precursors and therefore impair maturation (Arlet et al., 2014). It is for this reason that increasing other β-like globin chains, as occurs with increased γ-globin (which forms HbF), or reducing α-globin (as occurs with concomitant α-thalassaemia) can ameliorate symptoms in patients with β-thalassaemia (Mettananda et al., 2015). RBCs from patients with β-thalassaemia also have a shortened lifespan, which contributes to the anaemia.

Other rare forms of anaemia can impair maturation of erythroid precursors. A rare set of disorders, known as the congenital dyserythropoietic anaemias, are characterized by such defects and can be due to mutations in the *CDAN1*, *SEC23B*, *KIF23*, *C15ORF41*, *LPIN2*, *KLF1* or *GATA1* genes (Iolascon et al., 2013). In addition, we have identified at least one family with features of dyserythropoietic anaemia due to X-linked dominant mutations in the haem biosynthetic enzyme, ALAS2, which appear to act in a cell non-autonomous manner (Sankaran et al., 2015). In most cases, the underlying basis for the dyserythropoiesis resulting from such mutations is unknown. Interestingly, similar features can also be observed in some acquired conditions, in the setting of infections or treatment with specific medications. Of note, this category of diseases can also be characterized by production of RBCs that exhibit defects during circulation.

Faulty Mature Red Cells

The RBC membrane is a specialized phospholipid bilayer comprised of multiple integral membrane proteins that are stabilized by a skeleton of ankyrin and spectrin. As RBCs must be deformable, the RBC membrane skeleton has undergone specialized adaptation to effectively circulate through the narrow capillaries in the bloodstream. This allows the RBC to survive in the circulation for an average of 120 days. A series of disorders disrupt this membrane skeleton and result in anaemia. These disorders include hereditary spherocytosis (HS), hereditary elliptocytosis (HE) and Southeast Asian Ovalocytosis (SAO) (Da Costa et al., 2013). In nearly all cases, a genetic lesion can be identified in one of multiple causal genes encoding membrane and cytoskeletal proteins (Da Costa et al., 2013). In addition, there are disorders that affect volume and ion homeostasis, which are due to mutations in various channel-encoding genes. These include overhydrated stomatocytosis (OHSt) and dehydrated stomatocytosis or hereditary xerocytosis (HX). Recent studies of OHSt have identified genetic lesions in *SLC4A1* (Band 3), *RHAG*, and the glucose transporter *SCL2A1* (previously termed *GLUT1*). Identification of causal genetic lesions in HX has proven more difficult, but recent studies have used modern sequencing approaches to identify heterozygous mutations in *PIEZO1*, a gene encoding a mechanosensitive ion channel, and *KCNN4*, the gene encoding for the potassium selective Gardos channel (Rapetti-Mauss et al., 2015; Zarychanski et al., 2012). For each membranopathy, identification and follow-up of a new causal gene has lead to novel molecular insights that informs both diagnostic and therapeutic approaches, such as the potential use of Gardos channel blockers in HX.

Modifier Alleles in Erythroid Disorders

A major question is whether any two cases of an identical erythroid disorder with identical or similar genetic lesions will share a similar phenotype and disease progression. In many cases the answer is no. Even when the primary genetic lesion is identical between cases, modifier alleles can dramatically alter disease severity. The prototype for this is present in both of the major disorders of β-globin: β-thalassaemia and SCD. There is substantial variation between individuals with similar mutations, but much of this variation can be explained by genetic factors (both common and rare) affecting the expression of HbF (Nuinoon et al., 2010; Sankaran et al., 2011a). Modifier alleles have also been observed in other erythroid disorders, like HS, which exhibits substantial clinical heterogeneity, even within an individual pedigree (Iolascon and Avvisati, 2008). For example, partial pyruvate

kinase deficiency due to a *PKLR* mutation has been observed to aggravate HS disease severity by additively affecting RBC fragility (van Zwieten et al., 2015). However, most modifiers remain to be identified and larger studies are needed to define the extent to which these disorders are simply monogenic, as opposed to being on the spectrum of more complex disorders.

Rare modifier alleles can also inform therapeutic approaches. In congenital erythropoietic porphyria (CEP), an intrinsic erythroid disorder due to mutations in the gene encoding uroporphyrinogen III synthase (*UROS*) - the fourth enzyme in the haem biosynthesis pathway - gain-of-function mutations in the first and rate-limiting enzyme, 5-aminolevulinate synthase 2 (*ALAS2*), were associated with increased disease severity (To-Figueras et al., 2011). This suggested that CEP severity could be modulated by altering ALAS2 activity. Indeed, by restricting iron availability with the iron chelator deferasirox, the translation of *ALAS2* mRNA could be reduced, resulting in a decrease in the toxic coproporphyrin 1 byproducts and an amelioration of CEP severity *in vivo*, at least in one case (Egan et al., 2015).

In DBA, specific modifier alleles have not yet been identified, but variable expressivity and penetrance is observed in DBA cases with similar genetic lesions. In some cases, first-degree relatives with identical genetic lesions are almost entirely asymptomatic (Boria et al., 2010). Although preliminary, some studies have identified non-coding regulatory mutations near RP genes that may affect DBA clinical severity (Cretien et al., 2010). Future studies will identify true modifier alleles and may reveal potential therapeutic targets.

Concluding Remarks

As a result of extensive studies, we have a solid understanding of erythropoiesis and this process has thus long served as a paradigm for cellular differentiation more broadly. Nevertheless, with the advent and availability of many new technologies, such as high-throughput sequencing, our understanding of the complexities of haematopoiesis has grown and novel regulators that govern erythroid differentiation have been uncovered at a rapid pace. Moreover, comprehensive human genetic studies have revealed the underlying molecular aetiologies of many erythroid diseases and, furthermore, have accelerated the identification of therapeutic targets for these disorders. Future studies that leverage human genetics in conjunction with the clever use of model systems, such as through the use of improved humanized mice or by applying genome editing to cellular models, will probably reveal important erythroid biology and inform therapeutic strategies for the numerous and varied disorders that occur in this lineage.

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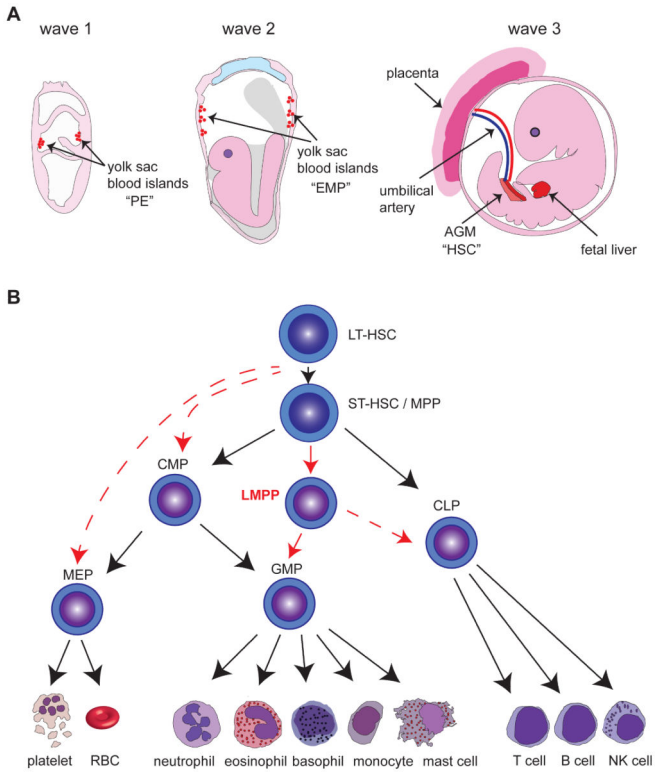

Figure 1.

A) There are three developmental waves of erythropoiesis in mammals. The first wave is marked by the emergence of primitive erythroblasts (PE) that express embryonic globins in yolk sac blood islands. In the second wave, the erythro-myeloid progenitor (EMP) emerges from yolk sac and migrates to the fetal liver, producing definitive erythroblasts expressing predominantly mouse adult globins. In the third wave, the haematopoietic stem cell (HSC) emerges from the haemogenic endothelium in the aorto-gonad mesonephros (AGM) and other sites. The HSC migrates to fetal liver and eventually to the adult bone marrow (BM), producing definitive erythroblasts. **B)** Classical (in black) and alternate (in red) models of the adult haematopoietic hierarchy. In the classical model, the adult HSCs in the BM gives rise to either a common myeloid progenitor (CMP) or common lymphoid progenitor (CLP). The CMP then differentiates into either a granulocyte monocyte progenitor (GMP) or megakaryocyte erythroid progenitor (MEP). These progenitors differentiate into mature cells of distinct lineages. Several alternate pathways have been discovered by recent studies (in red). HSCs were shown to differentiate directly into CMP, MEP and megakaryocytes. HSC can also differentiate into a lymphoid primed multipotent progenitor (LMPP) lacking any megakaryocyte erythroid potential. NK, Natural killer cell; LT-HSC, long-term HSC; ST-HSC; short-term HSC; MPP, multipotent progenitor; RBC, red blood cell.

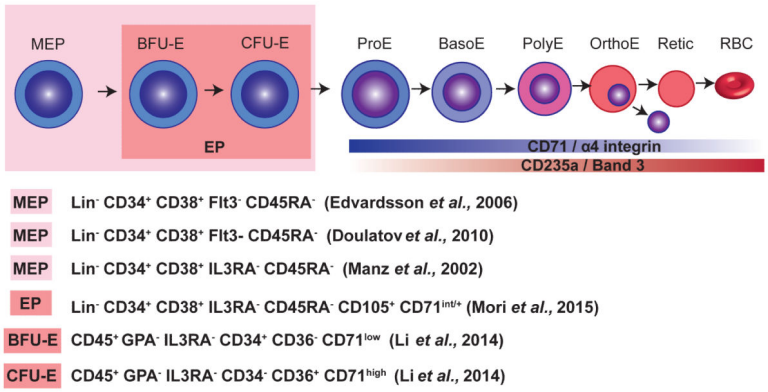

Figure 2.

The differentiation steps from the megakaryocyte erythroid progenitor (MEP) to the mature red blood cell (RBC) are shown. An overview of recent strategies to isolate these cell types based on surface markers is given. Note that the current MEP isolation strategies also appear to contain BFU-Es and CFU-Es. MEP, Megakaryocyte erythroid progenitor; BFU-E, blast colony forming unit - erythroid; CFU-E, colony forming unit - erythroid; ProE, proerythroblast; BasoE, basophilic erythroblast; PolyE, polychromatic erythroblast; OrthoE, orthochromatic erythroblast; Retic, reticulocyte.

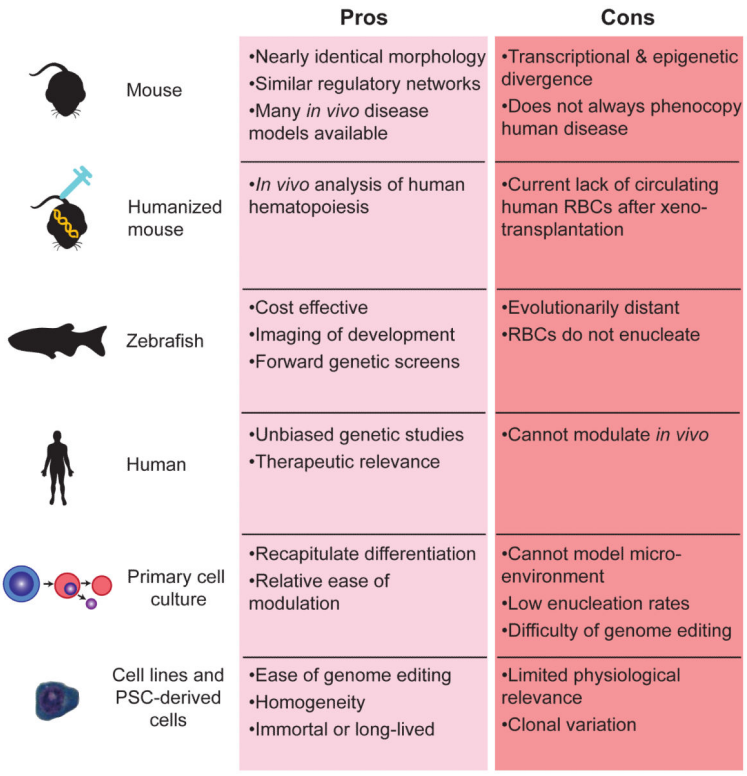

Figure 3.

Brief overview of the advantages and disadvantages of model systems commonly used to understand erythropoiesis. PSC, pluripotent stem cell.

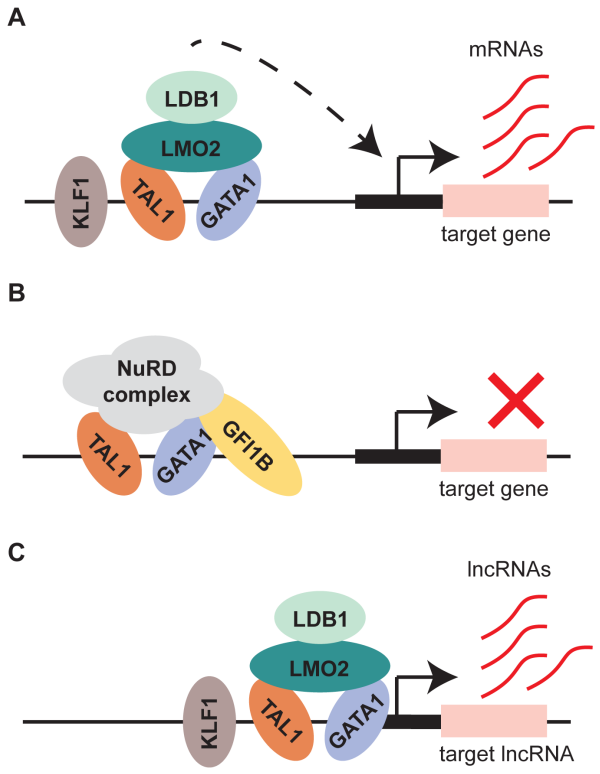

Figure 4.

A) The core erythroid network (CEN) of transcription factors (TFs) is composed of the DNA-binding TFs, GATA1, TAL1 and KLF1, as well as the non-DNA-binding TFs, LDB1 and LMO2. The CEN loops from enhancers to promoters to activate target gene expression. **B)** TFs in the CEN often interact with additional TFs at a subset of regulatory elements. For example, GATA1 and TAL1 interact with GFI1B and the nucleosome remodelling deacetylase (NuRD) complex, resulting in target gene repression. **C)** Long non-coding RNAs (lncRNAs) that are transcribed during erythropoiesis are regulated by the CEN. Future studies will probably refine our understanding of the role of lncRNAs in erythropoiesis.