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Research Article

Epigenetic modifiers in normal and aberrent erythropoeisis ☆

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A B S T R A C T

Erythroid differentiation program is comprised of lineage commitment, erythroid progenitor proliferation, and termination differentiation. Each stage of the differentiation program is heavily influenced by epigenetic modifiers that alter the epigenome in a dynamic fashion influenced by cytokines/humeral factors and are amicable to target by drugs. The epigenetic modifiers can be classified as DNA modifiers (DNMT, TET), mRNA modifiers (RNA methylases and demethylases) and histone protein modifiers (methyltransferases, acetyltransferases, demethylases, and deacetylases). Here we describe mechanisms by which these epigenetic modifiers influence and guide erythroid-lineage differentiation during normal and malignant erythropoiesis and also benign diseases that arise from their altered structure or function.

Introduction

Erythropoiesis is a remarkably high volume yet finely regulated process, in which ~200 billion red blood cells are produced daily, with turnover of ~1% per day. Several humeral and non-humeral factors regulate this complex and elegant process. Erythropoiesis absolutely depends on the coordinated expression of specific erythroid-lineage specific master transcription factors, concurrent with suppression of hematopoietic stem cell, and early myeloid specific master transcription factors: master transcription factors are transcription factors that are essential for and dictate cell fates and functions. The coordinated suppression of hematopoietic stem cell or early myeloid progenitor master transcription factors and upregulation of erythroid-lineage specific master transcription factors requires changes in epigenetic landscape, that is, changes in accessibility of erythroid lineage gene promoter DNA to binding by transcription factors [1,2]. Early cell-fate decisions depend also on dynamic modifications in epigenetic marks on DNA, RNA and histones. Here we discuss the most important and widely studied epigenetic alterations, their functional impact on erythroid-lineage differentiation, and disorders arising due to aberrations in epigenetic modifiers. The translational importance is the increasing numbers of candidate therapeutics that target epigenetic modifiers, that being enzymes, are eminently druggable.

Some of the earliest work examining epigenetic marks, for example, DNA methylation, utilized purified primary erythroid cells from fetal tissue, with a focus on the β-globin locus, motivated by trying to understand how red blood cells controlled the switch from fetal to adult hemoglobin production during development [3]. The productive nature and translational significance of this work and availability of recombinant erythropoietin motivated subsequent development of several human and mouse *in vitro* primary erythroid culture systems, that have enabled profiling of epigenetic modifications and gene expression through every stage of erythroid lineage differentiation [4,5]. We developed a human liquid culture system in the 1990s that permitted our contributions to this area of basic and translational investigation [6,7]. Our model, including its subsequent refinements, allowed us to isolate and characterize in detail various stages of erythroid-lineage maturation, either defined functionally (Burst-forming Unit-Erythroid [BFU-E], Colony-forming Unit-Erythroid [CFU-E]) or morphologically/histochemically. Over the years, these models have yielded a wealth of knowledge regarding regulatory networks that exquisitely control the numbers of red blood cells produced from a single blood stem cell. Epigenetic reconfiguration occurs throughout erythropoiesis influenced by humeral factors (Fig. 1). Also throughout the differentiation program, the expression profile of the DNA, mRNA and histone epigenetic modifiers are altered (Fig. 1). We focus our review on these epigenetic modifiers, because of their druggable nature and hence their translational relevance.

Epigenetic modifiers of DNA

Although modifications of specific bases in DNA was discovered in the 1970s [8], the functional importance of such mod-

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Figure 1

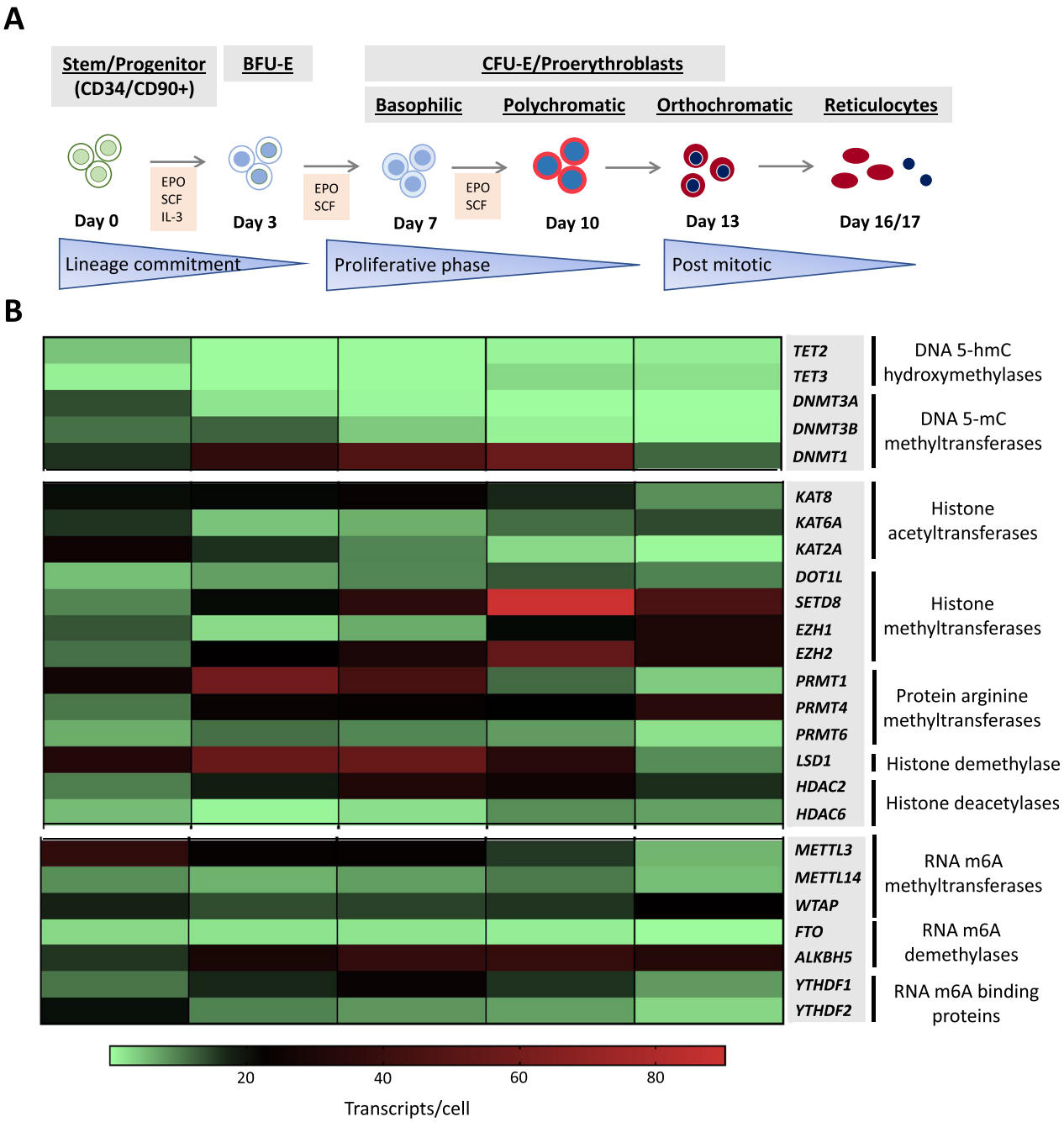

Fig. 1. Expression of epigenetic modifiers during human erythropoiesis. (A) Schematic model of the human erythroid differentiation program. The diagram shows temporal stages during differentiation and cytokine requirements for recapitulating the program *ex vivo*. (B) Heat map showing mRNA expression patterns of key epigenetic modifiers during various stages of the differentiation program [19]. BFU-E; Burst forming unit – erythroid, CFU-E; Colony forming unit – erythroid, EPO; erythropoietin, SCF; stem cell factor, IL-3; interleukin 3.

ifications has only come to light in the preceding 2 decades. These reversible, dynamic changes on DNA regulate transcription factor-binding and gene expression, to thereby impact fundamental cell fate/function processes such as proliferation, cell survival, and terminal-differentiation. DNA 5-methylcytosine (5-mC) is the first and most extensively characterized DNA modification. Recent technological advancements, however, enabled the identification of several additional DNA cytosine modifications: 5-hydroxymethylcytosine (5-hmC), 5-formylcytosine, and 5-carboxycytosine [9-11], and the enzymes which catalyze these modifications. Deposition of the 5-mC is catalyzed by the DNA methyltransferases (DNMT1, DNMT3a and DNMT3b proteins), whereas the oxidation of 5-mC to 5-hmC is catalyzed by ten-eleven translocases (TET1, TET2 and TET3 methyl cytosine dioxygenases) that depend on the isocitrate dehydrogenase family (IDH1 and IDH2) enzymes that generate their mandatory cofactor alpha- ketoglutarate (AKG) [12-14]. DNMT1 is the maintenance methyltransferase which recapitulates cytosine methylation of the parental

Table 1
DNA modifiers and their function during hematopoiesis/erythropoiesis.

Protein/Enzyme	Role in DNA modification	Functions in hematopoiesis/erythropoiesis	References
DNMT3A	Enzyme responsible for *de novo* methylation of cytosines on DNA	Essential for stem cell commitment and proper differentiation of erythroid cells. Loss of Dnmt3a expands MEP populations and Cd71+Ter119+ erythroid cells in bone marrow leading to anemia and erythroid dysplasia	[23,24,29,30]
DNMT3B	Enzyme responsible for *de novo* methylation of cytosines on DNA	Dispensable for normal hematopoiesis.	[16]
DNMT1	Enzyme responsible for maintenance methylation of cytosines on DNA	Indispensable for HSC self-renewal and differentiation. Loss of Dnmt1 results in decreased frequencies of MEPs.	[17,18]
TET2	Enzyme responsible for oxidizing 5-methylcytosine to 5-hydroxymethylcytosine on DNA	Important for stem cell commitment into erythroid lineage. Loss or impaired of function of TET2 prevent erythroid lineage commitment	[19,35–37,40]
TET3	Enzyme responsible for oxidizing 5-methylcytosine to 5-hydroxymethylcytosine on DNA	Essential during the terminal phase differentiation of erythroblasts. Reduced levels of TET3 in differentiating erythroblasts lead to defects in nuclear condensation and enucleation	[39]
IDH1/2	Enzyme involved in the generation of a substrate utilized by TET proteins	Mutations in IDH family proteins impair erythroid differentiation. Stage specific functions of IDH1/2 are unknown	[125]

MEP – Megakaryocyte-Erythroid progenitors.

strand onto the daughter strand during cell division. DNMT1 is also a corepressor recruited by transcription factors, and may participate in methylating unmethylated DNA *de novo*. DNMT3A and DNMT3B are, however, the conventionally recognized *de novo* methyltransferases that methylate unmethylated cytosine [12,15-18]. TET family proteins oxidize 5-mC to 5-hmC in the presence of AKG, a metabolite generated by IDH1/2 enzyme activity [13,14]. TET2 and TET3 are predominantly expressed in hematopoietic cells, whereas TET1 is expressed in embryonic stem cells [19-22]. We and others have studied how the activity of these epigenetic modifiers is regulated during hematopoiesis in general and during erythroid-lineage differentiation. In Table 1, we summarize the most widely studied DNA modifying enzymes and their functions during stem cell lineage commitment and erythropoiesis. It is particularly useful to consider the activities and functions of these enzymes according to the various developmental stages of erythroid lineage-differentiation: lineage commitment, early-stage progenitor proliferation, pre and most mitotic differentiation.

Lineage commitment

Concerted actions of paracrine/extracellular factors, membrane receptors, signaling networks and cis-modifying enzymes direct the activation of transcription factors that regulate hundreds of genes defining erythroid lineage commitment and subsequent terminal differentiation within the lineage. The first postlineage commitment stage consists of cells termed as BFU-E. We and others have focused on studying how 5-mC and 5-hmC influence commitment of hematopoietic stem cells (HSC) into the erythroid lineage as well as subsequent maturation. These studies have demonstrated that DNA 5-mC is dynamically regulated throughout HSC commitment and erythroid differentiation [23,24]. Using genome-wide DNA methylation analyses approaches such as HpaII fragment enrichment by ligation-mediated PCR assay and reduced representation bisulfite sequencing, demonstrated a progressive global DNA hypomethylation during adult and fetal erythropoiesis beginning from the early stem/progenitor stage [23,24]. Demethylation was preferentially observed at gene bodies, intergenic regions, CpG shores and transcription factor binding sites. This global demethylation, noted specifically at gene promoters, correlated with transcriptional activation and exit from stem/progenitor stage and commit into erythroid lineage. For instance, the promoter region of erythroid master transcription factor *GATA1* is hypermethylated during the stem cell stage and upon stem cell commitment to erythroid lineage, the *GATA1* promoter becomes demethylated with concomitant increased expression of *GATA1* [23]. Ectopic expression of Dnmt3a, Dnmt3b, or Dnmt1 in fetal liver cells did not prevent global demethylation during erythroid lineage commitment, indicating that expression levels of these enzymes was not the sole controlling factor [24]. In addition, knockdown of highly expressed DNA demethylation regulators Gadd45a or Mbd4 also did not prevent global demethylation, reinforcing the notion that additional regulatory circuits are in play [24]. DNMT3A mRNA levels do decrease significantly during commitment of HSC into the erythroid lineage while *DNMT3B* levels stay relatively unchanged. DNMT1 mRNA levels increase throughout the process before decreasing during terminal-differentiation (the postmitotic phase) (Fig. 1) [19,20].

Contrasting with the consistent trend in the 5-mC mark, 5-hmC marks vary dynamically throughout erythroid differentiation, beginning with a dramatic increase during HSC commitment into the erythroid lineage in both human and mice [19]. In fact, 5-hmC levels peak in BFU-E stage, correlating with a simultaneous marked increase in TET2 protein in committed BFU-Es [19]. We mapped the genome-wide 5-hmC distribution during erythropoiesis using chemical conjugation and affinity purification of 5-hmC-enriched sequences followed by next-generation sequencing (hMe-Seal). 5-hmC marks were detected across almost at all genomic loci with frequent occurrences in gene body and transcription factor binding sites [19]. In a broader sense, gain of 5-hmC is associated with increased gene expression. Consistent with this idea one can now reasonably conclude DNA hydroxymethylation not only acts as an intermediary step in the oxidation process of methyl cytosine but also garner the specific function in enabling gene activation. For instance, the promoters of the erythroid master transcription factors *GATA1* and *KLF1* gain markedly in 5-hmC during lineage commitment, as these genes are then upregulated to drive erythroid-lineage commitment and maturation. Meanwhile, stem cell genes such as *CD34*, *CD90* and *CD133* exhibited 5-hmC loss concurrent with their downregulation [19]. These up- and down-regulations were reflected at the mRNA and protein levels as well. Considering the observations of 5-mC and 5-hmC marks together, therefore we can see how increases in 5-hmC link to decreases in 5-mC at the loci of key genes that drive lineage commitment and differentiation of the erythroid-lineage. It is noteworthy the remaining oxidation states (-CHO and –COOH) of methyl cytosine only exist transiently and in very low densities and it is currently assumed that these states do not garner a specific function other than completing the oxidation process for removal of the methyl group.

A striking finding is that mutations in DNA epigenetic modifiers DNMT3A and TET2 characterize patients with myeloid malignancies but are also in healthy individuals––a finding described as clonal hematopoiesis of indeterminate significance [25,26]. These clinical findings have underscored the relevance and significance of studies into the enzymes that create 5-mC and 5-hmC epigenetic marks, especially in the context of hematopoiesis/erythropoiesis. It is worth noting that loss-of-function in DNMT3A or TET2 are causative in clonal myelopoiesis, including myelodysplastic syndromes (MDS) and acute myeloid leukemia (AML), despite having antagonistic biochemical functions [27,28]. There is conflicting data over whether loss of DNMT3A impairs erythroid differentiation with some studies reporting erythroid defects while others do not observe such defects [29-32]. However, loss of TET2 in HSC impairs erythroid differentiation, specifically impeding differentiation after the commitment stage *in vitro* and *in vivo* [33,34]: loss of Tet2 *in vivo* results in mild anemia with bone marrow progenitors that are incapable of upregulating Cd71 and Ter119 erythroid markers [35–38]. Primary CD34+ HSC and myeloid progenitors derived from MDS or chronic myelomonocytic patients with TET2 mutations are impeded in their ability to differentiate along the erythroid lineage, seen also in CD34+ HSCs and progenitors from healthy donors engineered artificially to have reduced levels of TET2 [19,39]. Since the types of mutations detected in TET are quite heterogeneous and spans from point mutations, frame shift mutations, or deletion mutations throughout multiple exons of the gene, it is likely that phenotypic outcomes of such diverse group of mutations are not just limited to a general inactivation of TET2. Therefore, carrying out mutational modeling studies will allow one to precisely uncover functional significance of each specific mutation, and distinguishing between passenger mutations from disease causing mutations.

Decades of observations have demonstrated that extracellular cytokines play pivotal roles in erythropoiesis, but until recently, it was unknown how these cues were imparted into the epigenetic network to regulate hematopoiesis/erythropoiesis. We specifically examined whether TET2 possesses the ability to respond to extracellular cytokine cues. These studies revealed that erythropoietin, stem cell factor, and/or FLT3- ligand cause TET2 to be phosphorylated by JAK2 kinase, a post-translational modification that increased TET2 enzyme function [40]: JAK2 binds TET2 and phosphorylated tyrosine residues 1939 and 1964 that are within the catalytic core. Furthermore, the TET2 phosphorylation increased its interaction with the erythroid-lineage master transcription factor KLF1, thereby influencing expression of large numbers of erythroid lineage genes (Fig. 2). Interestingly, these 2 tyrosine residues are not present in TET1 or TET3, suggesting a nonredundant function for TET2 in the erythroid lineage. Others have identified that TET2 is also phosphorylated, on other residues, by the kinase AMPK, a post-translational modification not linked with altered enzyme activity [41,42]. DNMT3A has been shown to be phosphorylated by Casein kinase 2 (CK2) and extracellular signal-regulated kinase 1/2 (ERK1/2) [43,44]; the precise functional roles of these modifications in erythropoiesis are unknown.

Proliferative phase

During the proliferative stage of erythropoiesis, committed erythroid progenitors (BFU-E) divide, to generate sufficient numbers of red blood cells, as they advance through a series of morphologically distinct stages namely basophilic, polychromatic and orthochromatic stages (Fig. 1). These stages are characterized by progressive decrease in cell size, nuclear condensation, and increases in hemoglobin expression [45]. We and others have found progressive demethylation throughout the proliferative phase as erythroblasts transition from basophilic to orthochromatic stages [23,24].

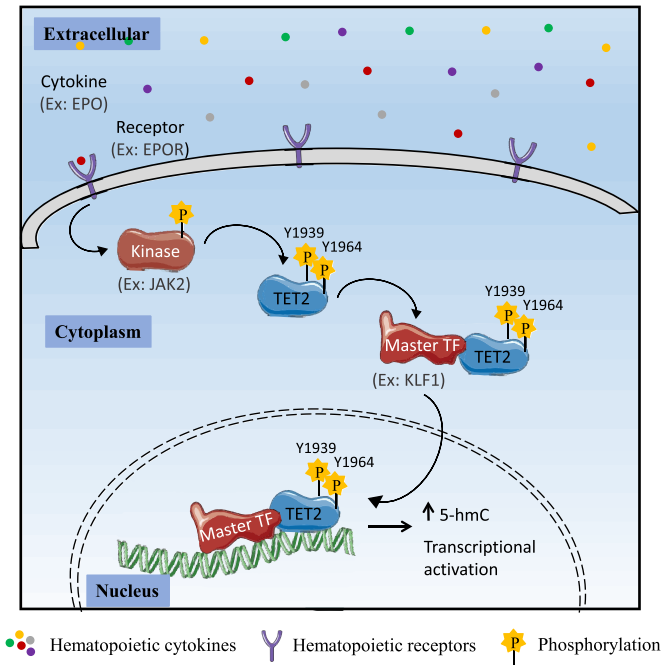

Fig. 2. A simplified schematic model illustrating TET2 mediated signaling in hematopoietic cells. Transmission of cytokine mediated extracellular signals via JAK2 leads to tyrosine phosphorylation and activation of TET2 resulting in recruitment of transcription factors to TET2 and hydroxymethylation of DNA.

DNA 5-hmC levels decrease after the initial increase during commitment and then remain relatively stable [19]. Although more work is needed, but early work indicates a role for TET3 during the transition from proerythroblasts to post mitotic stages of erythroid maturation [39].

Postmitotic differentiation

Hallmarks of the postmitotic (terminal-differentiation) phase of erythroid-lineage differentiation are dramatic reduction in transcription, nuclear condensation (significant increase of cytoplasmic/nuclear ratio), greatly accumulated levels of hemoglobin and initiation of erythroblast enucleation [6,46-48]. It is postulated that hypermethylation might contribute to the global gene repression that happens during the terminal phases of erythropoiesis. Supporting this notion, we have found an increase in 5-mC levels during the terminal-differentiation phase, albeit modest [19]. In addition, we have also observed remethylation of *GATA1* intergenic loci during this phase, that correlated with *GATA1* repression [23]. The absence of global hypermethylation during the terminal stage, except at a handful of genes, suggest a role for other epigenetic mechanisms, for example, histone modifications, nucleosome positioning, in the gene expression program changes of this phase.

Experimental demonstration for a role of DNA epigenetic modifiers contribute to the terminal erythroid differentiation phase include that loss of Dnmt3a *in vivo* causes macrocytic anemia and persistence of nucleated red blood cells, indicative of terminal maturation defects [29]. In fact, these mice exhibited increased levels of early Cd71+Ter119+ erythroblasts even as late Cd71-Ter119+ reticulocytes were decreased, indicating that erythroblasts were incapable of exiting the proliferative phase to undergo terminal maturation [29]. TET3 transcripts are upregulated during the terminal stages of erythroid differentiation, and HSC which were knocked-down for TET3 could commit into erythroid lineage but were unable to complete terminal erythroid differentiation [39]. Moreover, TET3-knockdown erythroblasts exhibited reductions in enucleated

Table 2
Histone modifiers implicated in hematopoiesis/erythropoiesis.

Protein/Enzyme	Role in histone modification	Functions in hematopoiesis/erythropoiesis	References
MOF (KAT8)	Histone acetyltransferase responsible for the acetylation of histone 4 lysine 16	Essential for stem cell commitment into erythroid lineage.	[53]
MOZ (KAT6A)	Histone acetyltransferase responsible for the acetylation	Precise function in erythroid lineage commitment unknown. However, Moz knockout resulted in accumulation of Cd71+Ter119+ erythroblasts and reduction in Cd71-Ter119+ erythrocytes.	[54,55,58]
BRD1	A Protein in the MOZ acetyltransferase complex	Brd1 knockout resulted in accumulation of early phase (Ter119-) erythroblasts accompanied by a reduction in Ter119+ late-stage erythroblasts in fetal liver.	[56]
KAT2A	Histone acetyltransferase responsible for the acetylation of histone 3 lysine 9	Essential for stem cell commitment into erythroid lineage and post commitment erythroid differentiation *in vitro*.	[57]
DOT1L	Histone methyltransferase responsible for the methylation of histone 3 lysine 79	Essential for proliferative stage of erythropoiesis. Dot1l knockout results in impeded erythropoiesis at the proliferative phase. Dot1l knockout results in decreased levels of MEPs suggesting a role during erythroid lineage commitment.	[59,60]
SETD8	Histone methyltransferase responsible for mono-methylation of histone 4 lysine 20	Essential for exiting proliferative phase and progress to terminal differentiation. Setd8 knockout results in accumulation of erythroblasts and harbor defects in enucleation.	[61–64]
PRMT1/4	Arginine methyltransferase responsible for di-methylation of histone 4 arginine 3	Prmt1 knockout bone marrow cells exhibit proliferative defects in erythroblasts. Prmt4 impeded erythroid differentiation *in vitro*.	[65–68]
PRMT6	Arginine methyltransferase responsible for di-methylation of histone 3 arginine 2	Prmt6 knockdown resulted in accelerated erythropoiesis *in vitro*.	[69]
LSD1	Histone demethylase responsible for demethylating mono-and di-methylation of histone 3 lysine 4/9	Essential for proerythroblast to basophilic erythroblast transition and during the late stages of the differentiation program.	[70-75]
HDAC2	Histone deacetylase responsible for deacetylating lysine on core histones	Essential for chromatin condensation and enucleation during terminal stages of the differentiation program.	[78,79]
HDAC6	Histone deacetylase responsible for deacetylating lysine on core histones	Important for enucleation during terminal stages of the differentiation program. Knockdown of HDAC6 results in impeded enucleation *in vitro*.	[80]
EZH2	Histone methyltransferase responsible for di-or tri-methylation of histone 3 lysine 27	Essential for fetal erythropoiesis and dispensable for adult erythropoiesis.	[82,83]
ASXL1	Member of polycomb repressor complex 2 (PRC2)	Essential for terminal erythroid differentiation. Asxl1 is critical for orthochromatic erythroblast to reticulocyte transition and important for chromatin condensation and enucleation.	[84]

MEP, Megakaryocyte-Erythroid Progenitors.

erythroblasts and reduced ability to condense their nuclei. The knockdown also reduced proliferation and increased apoptosis of late stage erythroblasts [39].

Concluding remarks

Thus, the natural genetic experiment of clonal hematopoiesis has demonstrated central roles of DNA epigenetic modifiers in regulating hematopoietic, including erythroid-lineage, commitment and/or maturation [25,26]. The translational significance of studies into these factors cannot be understated, since these are druggable enzymes. Moreover, we and others have demonstrated that other druggable and modifiable factors, cytokine signaling, inflammatory signaling, and gut microbiota, also impact the functions of epigenetic modifiers such as TET2 [40,49-51]. Other significant influences could include stromal matrix tension within the bone marrow, and basic metabolites such as glucose and glutathione levels.

Epigenetic modifiers of histones

Eukaryotic DNA is packaged around histone proteins resulting in tightly packed chromatin. Dynamic post-translational modifications of histones by acetylation, methylation, phosphorylation and other changes govern this packaging to in turn regulate cell fates and functions. Along these lines, several histone marks namely histone 3 (H3) lysine 4 (K4) di-methylation (H3K4me2), H3K9 acetylation (H3K9Ac), and H4K16Ac are linked with the erythroid differentiation program [52]. Table 2 depicts some of the widely studied histone modifying proteins during erythropoiesis. In a broader sense, histone acetyltransferases exert crucial functions during lineage commitment by opening up the chromatin, whereas histone deacetylases perform important functions during the terminal stages of erythropoiesis possibly enabling transcriptional silencing prior to nuclear condensation. Histone methyltransferases mostly seem to function during the proliferative stages of erythroid differentiation (Table 2). Within this section, we discuss the important functions of histone modifiers, which regulate different stages of erythropoiesis.

Lineage commitment

Histone modifiers that participate in stem cell commitment into the erythroid lineage are not well defined due to limitations that exist in obtaining large numbers of homogenous stage-specific cells at a reasonable cost for such studies. However, recent efforts have begun to uncover multiple histone modifiers that play a critical role in erythroid specification. Global gene profiling studies have identified H4K16Ac mark to positively correlate with erythroid specific gene expression programs [52]. Supporting this observation, a recent study identified lysine acetyltransferase Mof (also known as KAT8) to be dynamically expressed during erythropoiesis. Mof belongs to the MYST (Moz, Ybf2, Sas2, and Tip60) family of histone acetyltransferases (HATs) and not surprisingly, reduced levels of Mof led to a decrease in global H4K16Ac levels. Most importantly, ablation of Mof in HSCs resulted in impaired erythroid differentiation *in vitro* and *in vivo* [53]. In contrast, inducible knockout of Mof using Cag-Cre in lineage

primed megakaryocyte-erythroid progenitors (MEPs) did not impact erythroid differentiation thereby highlighting that Mof has a role during erythroid lineage commitment [53]. With respect to other members of the MYST family of proteins, studies have shown Moz (also known as KAT6A) has important functions during erythropoiesis at postlineage commitment phase although its role in lineage commitment remains to be determined [54,55]. Furthermore, knockout of Brd1, a subunit of the Moz HAT complex, also resulted in disruptions in fetal erythropoiesis reinforcing the importance of regulation of histone acetylation during erythropoiesis [56]. Additionally, KAT2A, a H3K9 acetyltransferase has also been demonstrated to be important for erythroid lineage specification *in vitro* although KAT2A might have additional post commitment functions as well [57]. Taken together, these studies underscore the importance of histone regulators, mainly histone acetyltransferases in erythroid lineage commitment. Therefore, it is likely other histone modifying enzymes also may influence stem/progenitor cell commitment to the erythroid lineage and warrants investigation.

Proliferative phase

Histone acetyltransferases are involved not only during erythroid lineage commitment but also during the postcommitment proliferative stages. In contrast to Mof, loss of MYST family HAT Moz led to deregulation of normal erythropoiesis postlineage commitment. Cells from fetal liver of Moz knockout mice when plated on colony assays gave rise to decreased numbers of erythroid colonies. Consistent with the *in vitro* data, Moz knockout embryos accumulated Cd71+Ter119+ erythroblasts without progression into Cd71-Ter119+ erythroblasts/reticulocytes [54]. While the functional role of Moz in regulating the proliferative phase of fetal erythropoiesis is evident, its role in adult erythropoiesis is less clear. At least in 1 recent study, where hematopoietic specific knockout of Moz was carried out in the setting of adult hematopoiesis no impact was observed in the numbers of erythrocytes [58].

In addition to HATs, multiple other histone modifying enzymes have been reported to perform crucial functions during the proliferative stage of erythropoiesis. DOT1L, a H3K79 methyltransferase has been found to regulate the proliferation and cell cycle in lineage committed erythroid progenitors. Not surprisingly, the loss of DOT1L specifically ablates H3K79me (monomethylation), H3K79me2 (di-methylation) and H3K79me3 (trimethylation) without impacting H3K4me2, H3K9me2, H3K27me2, or H3K36me2 levels. As a result, Dot1l knockout yolk sac shows defective erythropoiesis with a significant reduction in the numbers and size of the BFU-Es without significant differences in hemoglobinization suggesting that an impact on cell cycle [59]. Consistent with this observation, the Dot1l ablated erythroid cells exhibited impeded cell cycle progression with accumulation of erythroid cells in G0/G1 stage with a concurrent increase in apoptosis [59]. Although the authors of this study did not observe any significant differences within the progenitor populations, a later study using Dot1l hematopoietic specific knockout mice demonstrated that these mice harbored significant reductions in MEP (Megakaryocyte-Erythroid Progenitor) populations in the bone marrow indicating a potential involvement of Dot1l in erythroid lineage commitment in postnatal erythropoiesis [60].

Another histone methyltransferase that has been studied extensively in the context of erythroid differentiation is SetD8, the sole enzyme which is known to mono-methylate H4K20. Two independent groups using different *in vitro* murine erythropoiesis models have demonstrated that SetD8 is essential for the differentiating erythroblasts to exit the proliferative phase and complete terminal maturation. RNAi mediated reduction of SetD8 in differentiating erythroblasts resulted in accumulation of cells in the late erythroblast stage (R2/R3 stage) which were incapable of extruding their nucleus [61,62]. In addition, erythroblasts lacking Setd8 exhibited cell cycle defects and increased apoptosis, which explained the anemia observed in the Setd8 knockout mice [63]. Mechanistically, Setd8 impairs erythropoiesis by preferentially upregulating Gata2 levels by reducing the H4K20me mark on the Gata2 promoters [61,62,64]. Although this mechanism explains the accumulation of erythroblasts in the R2/R3 stages, it does not explain why the Setd8 ablated cells are devoid of enucleated cells. Furthermore Setd8 mutant erythroid cells not only expressed *Gata2* and its target genes but also other stem cell genes such as *Hhex*, *Hlx,* and *Cebpa* were upregulated [64] suggesting that Setd8 might play role in erythroid lineage commitment as well. Future detailed investigations are needed in order to understand the role/s of Setd8 in (1) erythroid lineage commitment, (2) nuclear condensation and enucleation, and (3) during human erythropoiesis.

Recent studies have uncovered another class of methyltransferases, protein arginine methyltransferases (PRMTs), which methylate arginine residues on multiple proteins including histones to play important functions during the erythroid proliferative stage. PRMT1 accounts for about 85% of all protein arginine methylations and facilitates di-methylation of H4R3, which is associated with transcription activation [65]. Hematopoietic specific loss of Prmt1 in mice results anemia *in vivo* and formed reduced erythroid colonies *in vitro*. Analysis of various erythroid populations using canonical erythroid markers Cd71-Ter119 showed a significant increase in early erythroid (Cd71+Ter119-) and significant decrease in late erythroid (Cd71-Ter119+) populations in Prmt1 knockout bone marrow compared to the controls indicating proliferative defects during erythropoiesis [66]. Furthermore, independent reports have shown the requirement of Prmt1 for efficient transcription of β-globin gene [65,67] underscoring the fact that Prmt1 might have multiple functions during erythropoiesis. In addition to Prmt1 both Prmt4 and Prmt6 have also been implicated in erythropoiesis. Similar to Prmt1, reduced levels of Prmt4 impeded erythroid differentiation *in vitro* [68]. In contrast, reduction of H3R2 methyltransferase Prmt6, which is associated with transcriptional repression, resulted in increased erythroid differentiation *in vitro* [69].

Histone demethylases are also implicated in regulating the proliferative phase of erythropoiesis. LSD1 (also known as KDM1A), a H3K4/9 demethylase, which specifically demethylates mono- and di- H3K4/9 methylation, have garnered significant interest in the recent years. Multiple groups have demonstrated the requirement of Lsd1 in normal erythropoiesis using a variety of *in vitro* and *in vivo* model systems [70-74]. Reducing the levels of Lsd1 in murine erythroleukemia cells resulted in decreased numbers of hemoglobinized cells suggesting both lineage commitment and subsequent differentiation are impacted [70,71]. Erythroid specific knockout of Lsd1 using Epor- Cre, resulted in expansion of early erythroid R2 populations and decrease in terminal R3, R4, and R5 stages due to (1) defects in pro to basophilic erythroblast transition and (2) increased apoptosis [72]. Interestingly, reintroduction of either wild-type or catalytically inactive Lsd1 in Lsd1 mutant zebrafish was able to rescue the observed erythropoietic defects suggesting that nonenzymatic functions of Lsd1 might also be essential for normal erythropoiesis [74,75]. Future lines of investigations are necessary to identify epigenetic *vs* nonepigenetic functions of Lsd1.

Postmitotic differentiation

It is well known that extensive nuclear remodeling occurs during erythroid terminal differentiation and recent studies have highlighted the role/s of histones and their modifiers as crucial regulators of this phase Specifically, histones undergo various modifications during chromatin condensation and the histones

seemed to be released from the nucleus into the cytoplasm through transient but repeated nuclear openings mostly prominent in mouse [76,77]. One of the histone marks that has been studied widely during the nuclear condensation phase is histone acetylation because several members of the histone deacetylase family such as HDAC1,2,3 and 5 are expressed at high levels [78]. The treatment of erythroblasts with HDAC inhibitors trichostatin A or valproic acid abrogated nuclear condensation and subsequent enucleation without impacting proliferation or terminal differentiation implying histone deacetylases play a selective role in this unique process that is exclusive to most but not all mammals [78,79]. Most importantly, knockdown of HDAC2, but not HDAC1, 3, 5 in erythroblasts phenocopied the effects observed due to treatment with HDAC inhibitors implying HDAC2 as a critical regulator of chromatin condensation and enucleation. In addition to HDAC2, a recent report has shown HDAC6 to play a critical role during terminal erythropoiesis [80]. Targeting HDAC6 through genetic or pharmacological approaches resulted in significant reduction of enucleation [80]. Interestingly, the same study did not find any obvious defects in chromatin condensation during HDAC6 ablation. However, it should be noted that pharmacological inhibition of HDAC6 results in impaired terminal differentiation specifically at the pro to basophilic erythroblast transition stage as well [80]. Although reduction of HDAC2 and HDAC6 results in overlapping as well as contrasting phenotypes, in both cases, the formation of contractile actin ring was blocked, which is a necessary step prior to nuclear extrusion. However, the mechanism of how HDAC6 regulates terminal erythropoiesis will require detailed investigation using genetic knockout/knockdown model systems. Taken together, these studies highlight the complex roles of specific histone deacetylases in regulating the terminal differentiation and enucleation during erythropoiesis.

Although the function/s of the histone demethylase KDM1A (LSD1) during the proliferative phases of erythropoiesis is known, the precise roles of LSD1 during the terminal erythroid differentiation phase remains less clear. Knock-down of Lsd1 *in vivo* resulted in significant decreases in reticulocytes and mature RBCs [72,73]. Morphological analysis of peripheral blood from Lsd1 ablated mice depicted immature erythrocytes with nuclei that did not show chromatin condensation: uncovering the precise roles of Lsd1 during chromatin condensation and enucleation are especially significant because several LSD1 inhibitors are in clinical evaluation to treat cancers including of myeloid lineages [81].

Members of the polycomb repressor complex 2 (PRC2) have also been reported to be essential for erythroid differentiation. EZH2, a member of the PRC2 complex, is a methyltransferase that di- or trimethylates H2K27 to induce gene repression. Ezh2 has been shown to be essential for fetal erythropoiesis but dispensable for adult erythropoiesis [82], which may be due to a Ezh2 to Ezh1 switch that occurs during terminal murine erythropoiesis [83]. However, there are gradual increases in both EZH1 and EZH2 transcripts during adult human erythropoiesis (Fig. 1) [19,20]. ASXL1 is a member of the PRC2 complex which is essential for normal adult erythropoiesis even though it lacks enzyme activity. Ablation of ASXL1 *in vitro* or *in vivo* impaired erythroid differentiation by interfering with the orthochromatic erythroblast to reticulocyte transition [84]. Thus, *Asxl1* knockout mice exhibit significant decreases in reticulocyte and RBC populations accompanied by increases in pro-, basophilic-, polychromatic-, and orthochromatic erythroblasts [84]. Furthermore, enucleation was significantly decreased with a concurrent increase in apoptosis. *ASXL1* ablated human and mouse erythroid cells exhibited nuclear dysplasia and absence of chromatin condensation [84]. Reducing Asxl1 levels in Ter119+ erythroid cells globally decreased repressive H3K27me3 and increased H3K4me3.

Concluding remarks

Thus, much has been found regarding the role and function of histone modifiers in erythropoiesis, however, several knowledge gaps exist, understandably given the complexity and context specific functions of histone marks. From a translational perspective, particularly lacking is our knowledge about how these epigenetic modifiers are integrated into signaling circuits. In particular, the details of signaling cues that regulate the repression of the global transcriptome during nuclear condensation but enable selective expression of genes critical for enucleation is still a mystery. Recognition that JAK2, traditionally known to engage and operate on hematopoietic cytokine receptors is capable of phosphorylating histone H3, is a glimpse into a field of unexplored possibilities [85]. Also new are several novel histone modifications, identified recently as a result of advances in mass spectrometry methods [86]: histone propionylation, butyrylation, 2-hydroxybutyrylation, and lactylation (histone acylations) [87]. It is quite likely that these epigenetic modifications are also present in differentiating erythroblasts since the enzymes responsible for catalyzing these reactions, such as p300 and sirtuins, are highly expressed in this lineage at the mRNA level [19]. Almost all of the histone acylations are metabolically regulated by the tricarboxylic acid cycle (TCA) and lead to changes in transcription essential for proper cell functioning [88], serving as another link between metabolism, epigenetics, and the regulation of lineage proliferation and differentiation [89], and again highlighting potential intervention points for therapy.

Epigenetic modifiers of RNA

Although methylated mRNA was first observed in the 1970s, functional studies are a new area of investigation, contrasting with well-established study of DNA modifications [90,91]. N^6-methyladenosine (m^6A), which is the methylation on N6 position of adenosine, is the most common RNA modification of more than a hundred documented modifications. Emerging studies have suggested that m^6A RNA modification plays an essential role in tissue metabolism/homeostasis, by directing RNA to processing, translation and degradation [92]. Three key classes of RNA modifying enzymes have been identified: adenosine methyl transferases (m^6A writers), binding proteins (m^6A readers), and demethylases (m^6A erasers). Here we focus on m^6A RNA modifying and/or binding proteins during hematopoietic lineage commitment and terminal erythroid differentiation.

Accumulated evidence from studies using animal models and primary human cells suggest that m^6A is essential for hematopoietic stem cell (HSC) maintenance and differentiation. Several groups have shown that knockout of key essential m^6A pathway regulators disrupts normal hematopoiesis [93-97]. For example, knockout of a key m^6A writer protein Mettl3, resulted in accumulation of HSCs leading to a block their differentiation in part by downregulating Myc activity [98]. Table 3 depicts most widely studied m^6A molecular machinery during hematopoiesis [96-104] and highlights the dynamic nature of expression of m^6A modifying proteins during erythropoiesis (Fig 1). Collectively, these studies have contributed to our understanding of the role of m^6A pathway during HSC homeostasis and lineage commitment.

While the roles of m^6A in HSC maintenance have been clearly established by several studies, stage specific functions of m^6A during terminal erythropoiesis is poorly understood. Analysis of previously published RNA-sequencing data of distinct stages of human erythroid differentiation by our group and others [19,20], indicates a dynamic regulation of every member of m^6A modifiers suggesting potential stage specific role/s for m^6A during erythropoiesis (Fig. 1). For example, a recent study [105] using genetic

Table 3
RNA modifiers implicated in hematopoiesis/erythropoiesis.

Protein/Enzyme	Role in RNA modification	Functions in hematopoiesis/erythropoiesis	References
METTL3 (writer)	Enzyme responsible for the deposition of m6A mark on mRNA	Essential for stem cell maintenance and differentiation. Not essential for myeloid cell function. Facilitates translation of erythroid genes during erythropoiesis.	[93–96,98,105]
METTL14	A cofactor in the METTL3 m6A transferase complex	Essential for stem cell maintenance and myeloid lineage commitment and differentiation. Facilitates translation of erythroid genes during erythropoiesis.	[97,101,105]
WTAP	A cofactor in the METTL3 m6A transferase complex	Facilitates translation of erythroid genes during erythropoiesis.	[105]
FTO (eraser)	Enzyme involved in the removal of m6A mark on mRNA	Although overexpressed in acute myeloid leukemia, its functions are unknown in normal hematopoiesis and erythropoiesis	[100]
ALKBH5 (eraser)	Enzyme involved in the removal of m6A mark on mRNA	Not essential for hematopoiesis. Knockout of ALKBK5 in mice doesn't impact hematopoiesis. Functions unknown in the context of erythropoiesis.	[102,103]
YTHDF1 (reader)	Protein Involved in recognizing m6A mark on mRNA	YTHDF1 enhances translation of m6A marked transcripts; however, its functions are unknown in the context of erythropoiesis/hematopoiesis.	[99]
YTHDF2 (reader)	Protein Involved in recognizing m6A mark on mRNA	Not essential for hematopoiesis. Conditional knockout of YTHDF2 in mice leads to expansion of the HSC compartment. Unknown in the context of erythropoiesis.	[104]

screening on human erythroleukemia cells has demonstrated that m^6A methyltransferase complex proteins (METTL3, METTL14, and WTAP) are critical for expression of glycophorin A, a key erythroid specific gene. Moreover, the same study demonstrated KLF1, GATA1 transcription factors and SETD1 methyltransferase expression are also dependent on proper levels of m^6A methyltransferases (METTL3, METTL14, or WTAP). Interestingly, suppression of m^6A levels selectively impeded erythroid differentiation without any impact on the megakaryocyte and myeloid lineages [105]. These findings together with observations demonstrating dynamic changes in transcript levels of m^6A modifiers during erythropoiesis (Fig. 1) strongly suggests a need to further study this emerging field in order to understand the implications of aberrant RNA modifications on hematologic malignancies and other benign conditions. One of the few mammalian species of mRNA that have an extremely long half-life are mRNAs for globin genes (10-24 hours *vs* 5h median half-life for most other transcripts) [106]. It is thus conceivable that RNA modifying proteins play a selective role in protecting these transcripts from degradation [107]. Therefore, elucidation of metabolic pathways regulating RNA modifying enzymes and binding proteins may pave the pathway for identifying intervention points to regulate hemoglobin levels, for example, to treat anemia, or to increase fetal hemoglobin transcripts and "mark" beta globin transcripts for degradation in sickle cell or in β-thalassemia patients. Altogether, recent development of sensitive chemical methods to identify m^6A-containing mRNAs together with CRISPR approaches for silencing gene expression have provided a fertile environment to study this relatively new field in cellular physiology and metabolism.

Epigenetic modifiers and therapy of erythroid lineage diseases

Aberrant expression or function of epigenetic modifiers involved in erythropoiesis is causative to major diseases of the erythroid lineage such as MDS and erythroleukemia. The aberrant expression or function is in most cases from mutations in the genes encoding for the modifiers. Alternatively, signaling proteins, binding partners, and other proteins regulating epigenetic modifiers may be genetically altered, and mediate clinical consequences via their impact on the epigenetic modifiers. The gene alterations may originate in the germline or in HSC (or anywhere in between) but the most prominent phenotypic consequences, and clinical manifestations, are seen in post erythroid-lineage commitment stages. DNA methylases and DNA hydroxymethylases are prominent classes of epigenetic modifiers that are recurrently altered as causes of anemia, erythroid dysplasia, and clonal expansion leading to erythroleukemia.

DNA methyl transferase family of proteins (DNMT1, DNMT3A, and DNMT3B)

Mutations in these methyltransferases lead to development of MDS, AML, and erythroleukemia. Although mutations in these genes are quite prevalent in stem/progenitor cells their impact in silencing of key erythroid-lineage differentiation-driving transcription factors is manifest in the postlineage commitment phase as differentiation block, causing clonal expansion concurrent with peripheral anemia. *DNMT3A* mutations can occur decades prior to diagnosis of overt malignancy [108,109]. The application of high-throughput DNA methylome analysis of primary MDS specimens, including single-cell sequencing, has uncovered several genes involved in pathways critical for MDS onset. These include genes associated with cell cycle, DNA repair, differentiation, signaling, adhesion and motility [110-112]. Most importantly, these studies have revealed novel genes contributing to malignant erythropoiesis and erythroid dysplasia. An earlier study identified 5′ CpG islands of p15INK4B (CDKN2B) to be hypermethylated in about 50% of the MDS patients [113]. Later, it was demonstrated that normal levels of p15INK4B are essential for proper erythroid commitment and terminal differentiation. Hematopoietic cells from p15ink4b KO mice give rise to less numerous, smaller size erythroid colonies [114]. Similarly, the erythroid master transcription factor *GATA-1* has also been reported to be hypermethylated in MDS [115]. We uncovered aberrant promoter methylation and reduced expression of *DOCK4* (a signaling intermediate in the Rac GTPase pathway) as contributing to erythroid-lineage dysplasia by disrupting the F-actin network [116,117]. Altogether, disruption of DNA methylation patterns impacts commitment into the erythroid-lineage and retards maturation after commitment, a decoupling of progenitor proliferation from maturation that underlies malignant clonal expansions. Recent studies have shown approximately 33% of erythroleukemia patients possess mutations in both DNMT3A as well as TET2 [118-120].

Ten–eleven translocation proteins (TET1, TET2, TET3)

MDS and AML are characterized by recurrent mutations specifically in *TET2,* although *TET3* is also expressed during the latter stages of erythroid maturation [39,121]. *TET2* mutations are

mostly frame-shift and point mutations, and span the entire *TET2*, although most patients with MDS and/or AML show mutations within the 2 longest exons (exons 3a and 10) [122,123]. A block in erythroid differentiation is observed when TET2 levels are depleted [19,33,39], with expansion of early hematopoietic precursors, culminating in leukemia [35]. A global reduction in the DNA hydroxymethylation content in patients harboring *TET2* mutations have led to the realization that mutations in *TET2* lead to loss of function and therefore it can be considered as a tumor suppressive gene. Carrying out mutational modeling studies *in vitro* and *in vivo* or when possible analyzing patient material devoid of mutations in other known epigenetic modifiers will allow a better understanding of precise impact of various *TET2* mutations on cellular function without being obscured by comutations in other genes. Such studies will not only allow one to ascertain whether *TET2* mutations result in only a reduction in 5-hmC density within the genome or concurrent redistribution of 5-hmC marks occur as well resulting in adversely impacting new regions within the genome but also identify *TET2* mutations, which are indeed pathogenic. An example of such skewing of hydroxymethylation landscape was seen in 1 recent study when hMe-Seal approach was employed to profile DNA hydroxymethylation during the erythroid differentiation program [19]. Although by far TET2 aberrations are central to the pathogenesis of MDS, an *in vitro* study had demonstrated a role for TET3 in promoting erythroid differentiation during the terminal phase of erythroid differentiation [39]. Moreover, the presence of *TET2* (and also *DNMT3A/ASXL1*) mutations in healthy individuals with clonal hematopoiesis argues that the presence of these mutations alone most likely is insufficient to cause MDS or AML. Recent studies have identified potential cell extrinsic factors associated with malignant transformation in individuals harboring mutations in *TET2/DNMT3A/ASXL1* genes but our knowledge in this regard is at its infancy [26].

Indirect influencers of epigenetic modifiers

The IDH1/2 proteins are critical components of the tricarboxylic acid cycle, and mediate the conversion of isocitrate to α-ketoglutarate (AKG). AKG is a mandatory cofactor for TET enzyme family activity. Approximately 5% of MDS patients harbor mutations in IDH1/2 genes. Mutant IDH1/2 proteins produce an oncometabolite 2-hydroxyglutarate instead of AKG, thus inhibiting TET2 activity (and that of other AKG-dependent demethylases) [124]. Specifically, HSC harboring the recurrent IDH2^{R140Q} mutation have diminished ability to generate erythroid colonies [125]. Remarkably, leukemia patients treated with a mutant IDH2 specific inhibitor, enasidenib exhibited improvements in erythropoiesis and achieved transfusion independence [126].

We and others have found that JAK2 post-translationally modifies TET2 to increase its activity and function. In the context of myeloproliferative disorder, polycythemia vera, where JAK2 is constitutively active, data from sequencing studies show some patients also harbor *TET2* mutations as well. We found global decreases in DNA hydroxymethylation and increases in DNA methylation in these cases, suggesting that the dominant phenotypic impact was from the *TET2* mutations [40].

Histone modifiers

Histone methyltransferases and demethylases, and their key co-factors, are also recurrently altered in myeloid malignancies. Here, we only focus on alterations specifically linked to abnormalities of erythropoiesis. *ASXL1* loss of function mutations are observed in about 15% of MDS patients [127,128]. MDS patients with *ASXL1* mutations exhibited significantly lower RBC counts and hemoglobin levels compared to MDS patients with wild type *ASXL1*. Furthermore, morphological analysis of bone marrow specimens from MDS patients with *ASXL1* mutations depicted an increase in immature erythroblasts and decrease in mature erythroblasts which is consistent with the phenotypes associated with *Asxl1* loss in mice [84].

The histone methyltransferases *EZH2* and *MLL3* reside on the q arm of chromosome 7, which is deleted in about 10% of MDS patients, a structural alteration linked to erythroid dysplasia [129], although the precise roles of losses of *EZH2* and *MLL3* to this phenotype is poorly understood.

Therapies directed at epigenetic modifiers

Thus, epigenetic modifiers clearly regulate commitment into, and differentiation within, the erythroid lineage. Neoplastic evolution selects for mutations in epigenetic modifiers to contribute to clonal expansions, including erythroid-lineage cells. The translational importance is that epigenetic modifiers, or the signaling networks that regulate them, for example, JAK2, are druggable. Indeed, drugs that target and deplete DNA methyltransferase 1 (DNMT1) from cells such as decitabine and 5-azacytidine, are mainstays for treating myeloid malignancies and for relieving red cell transfusion dependence [130]. Targeting DNMT1 is also a scientifically and clinically validated approach, although not FDA-approved, for preventing an erythroid maturational switch from fetal to adult hemoglobin production, to thereby treat the β-hemoglobinopathies, diseases in which the adult β-globin gene is mutated. Additional reversible and selective DNMT1 inhibitors are currently being tested [131]. Mutant-IDH1/2 targeted therapies, ivosidenib, and enasidenib, are approved to treat *IDH* mutated AML, and can produce transfusion independence in ~45% of these patients [132,133]. A recent study demonstrated that enasidenib can drive erythroid differentiation [126] suggesting potential use of enasidenib to alleviate anemia in other disease settings also. Multiple other epigenetic treatment modalities are under clinical trials and are reviewed elsewhere [134,135].

Conclusion

Epigenetics is an important, druggable regulatory layer that controls red blood cell production, evidenced by the mainstay role of drugs that target a DNA methyltransferase in treating myeloid malignancies, and their promising role to treat β-hemoglobinopathies and potentially other anemias [130,136]. Our recent work in shedding light into how TET2 catalytic activity is regulated by phosphorylation in developing blood cells can be an incentive for developing drugs that activate TET2, to counter loss-of-function alterations that contribute to myeloid oncogenesis, including transformations of the erythroid lineage. The study of epigenetic modifiers, and of modifiers of epigenetic modifiers, have already yielded extremely useful information for designing treatment strategies and are bound to yield even more significant results in the near future.

Conflict of Interest Statement

Sriram Sundaravel has nothing to disclose. Ulrich Steidl reports research funding from GSK, Bayer Healthcare, Aileron Therapeutics; honoraria for consultancy services from Bayer Healthcare, Celgene, Aileron Therapeutics, Stelexis Therapeutics, and Pieris Pharmaceuticals; equity ownership in Stelexis Therapeutics. Amittha Wickrema is a Scientific advisory board member Celligenics, Ltd.

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