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Epigenetic and Epitranscriptomic Factors Make a Mark on Hematopoietic Stem Cell Development

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Abstract

Purpose of the Review—Blood specification is a highly dynamic process, whereby committed hemogenic endothelial cells (ECs) progressively transdifferentiate into multipotent, self-renewing hematopoietic stem cells (HSCs). Massive changes in gene expression must occur to switch cell identity, however the factors that mediate such an effect were a mystery until recently. This review summarizes the higher-order mechanisms involved in endothelial to hematopoietic reprogramming identified thus far.

Recent Findings—Accumulating evidence from mouse and zebrafish studies reveal that numerous chromatin-modifying (epigenetic) and RNA-modifying (epitranscriptomic) factors are required for the formation of HSCs from hemogenic endothelium. These genes function throughout the endothelial-hematopoietic transition, suggesting a dynamic interplay between ‘epi’-machineries.

Summary—Epigenetic and epitranscriptomic regulation are key mechanisms for reshaping global EC gene expression patterns to those that support HSC production. Future studies that capture modification dynamics should bring us closer to a complete understanding of how HSCs transition from hemogenic endothelium at the molecular level.

Keywords

hemogenic endothelium; HSC production; endothelial to hematopoietic transition; chromatin modification; m⁶A methylation; transdifferentiation

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Compliance with Ethical Standards

Conflict of Interest

Dionna M. Kasper and Stefania Nicoli declare that they have no conflict of interest.

Human and Animal Rights and Informed Consent

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Introduction

The creation of HSCs during a narrow window in embryonic development is crucial for the constitution and lifetime maintenance of all blood cell lineages. HSCs arise from an endothelial to hematopoietic transition (EHT). During this process, a committed subset of ECs lining the ventral dorsal aorta (VDA) in the aorta-gonad-mesonephros (AGM) region, called hemogenic ECs, acquire multi-lineage and long-term repopulating potential and bud as HSCs into the circulatory system [1–6]. This transdifferentiation process is incredibly dynamic, involving progressive silencing of the endothelial expression program with gradual activation of multipotency and hematopoietic genes (Figure 1) [7–9]. How EHT unfolds at the molecular level is not fully understood, but is under intense investigation given the therapeutic potential of HSC-inducing factors for the understanding and treatment of blood disorders [10].

Studies in mouse and zebrafish developmental models have been instrumental in pinpointing a conserved genetic hierarchy of transcription factors and signaling pathways driving HSC formation from endothelium. Key nodes in this cascade include Notch signaling and Gata2 transcription factor as upstream regulators, followed functionally by Runx1 and cMyb transcription factors. Expression of these genes is often used as early and late EHT markers, respectively. The detailed functions of these central pathway members, as well as other genetically-encoded regulators are covered in a number of excellent reviews [11–14].

Recently, the application of sophisticated genomic approaches and large-scale screens has revealed some of the complexity of EHT regulation. Specifically, epigenetic and epitranscriptomic factors are emerging as central mechanisms to direct or reinforce endothelial-hematopoietic reprogramming (Figure 1, Table 1) [11, 15, 16]. These newly identified EHT genes represent all the major classes of high-level information, including DNA and RNA methylation, histone modification, and chromatin remodeling, suggesting that these mechanisms have a significant contribution to reshaping gene expression patterns to support HSC production. In this concise review, we cover the epigenetic and epitranscriptomic players regulating HSC formation from endothelium in mouse and zebrafish to date, and outline their molecular function in relation to the classical EHT genetic hierarchy. We conclude with an outlook on the potential dynamic interplay of ‘epi’-machineries and the contribution of non-coding RNAs (ncRNAs) to these higher-order EHT mechanisms.

Epigenetic Regulation of EHT

DNA is wrapped around nucleosomes composed of histone proteins, and further organized into higher-order chromatin structures. Epigenetic mechanisms regulate the degree of chromatin compaction, and thus permit or prevent accessibility of transcription factors to promoters and other DNA regulatory elements. These include chemical modification of DNA directly or to histone proteins, and the sliding or disassembly/reassembly of whole nucleosomes. Epigenetic marks can reversibly change the chromatin landscape, and thus provide a means to activate and/or suppress transcriptional programs globally. Importantly,

the plasticity offered by epigenetic regulation often characterizes cell fate transitions, like EHT [17].

DNA methylation

DNA methylation is an important epigenetic mark driving various cellular states, and accumulating evidence points to a role for it in HSC development (Table 1, Figure 1) [16, 18]. DNA methylation refers to the covalent attachment of a methyl group to the carbon-5 position of a cytosine residue, forming 5-methylcytosine (5mC). DNA methyltransferases (DNMTs) catalyze 5mC reactions on single cytosine-phosphate-guanine (CpG) dinucleotides or CpG clusters, also known as CpG islands [19]. Two classes of DNMTs exist in vertebrate genomes; the maintenance DNMT, Dnmt1, propagates established methylation patterns onto newly replicated DNA, and the *de novo* DNMTs belonging to the Dnmt3 family, methylate unmodified genomic regions [19]. Classically, DNA methylation induces transcriptional silencing, especially when 5mC occurs within promoters or enhancers, such as in the cases of genomic imprinting and X-chromosome inactivation [19]. This epigenetic mark can also promote gene expression via Dnmt3-induced methylation of gene bodies [20, 21]. The Ten-Eleven Translocation (Tet) family of cytosine dioxygenases (Tet1, Tet2, Tet3) can reverse 5mC-mediated effects by converting 5mC to 5-hydroxymethylcytosine (5hmC) and other derivatives. Hydroxylation of 5mC facilitates DNA demethylation through active excision of methyl groups or through progressive dilution of these oxidized bases with each round of cell division [22].

Recent studies performed in zebrafish identified a requirement for specific Tet enzymes and DNMTs in early and late steps of the EHT hierarchy, respectively. Within the Tet family, only Tet2 and Tet3 contribute to HSC formation from the endothelium [23, 24]. *Tet2* and *tet3*, but not *tet1*, transcripts localize to the AGM in the developing zebrafish embryo where HSCs are born [23]. Generation of a Tet family mutant allelic series, comprised of single, double, and triple mutant combinations of the three Tets, identified an overlapping function for Tet2 and Tet3 in EHT. *Tet2/3* double mutant embryos have decreased numbers of HSCs emerging from the VDA and exhibit larval lethality, whereas Tet single mutants and both *tet1*-deficient double mutants are viable as adults [24].

Consistent with the HSC defects in *tet2/3* mutant embryos and Tet molecular function, 5hmC levels were drastically reduced upon combined loss of Tet2 and Tet3 with lesser effects in single mutant and other double mutant combinations [24]. Importantly, 5hmC levels were similarly decreased between *tet2/3* and the *tet1/2/3* mutants; thus, suggesting that Tet1 does not play a role in HSC formation [24]. A second study using bisulfite sequencing and hMeDIP qPCR (hydroxymethylated DNA immunoprecipitation-quantitative polymerase chain reaction) reported similar results; 5hmC was diminished with a concomitant increase in 5mC at CpGs in promoters of key EHT genes, *scf* and *cmyb* in *tet2* morphant zebrafish embryos [23]. While the change in methylation status at hematopoietic genes likely accounts for the HSC defects in *tet2/3* mutants, it remains unclear how Tet2/3 directly mediates 5mC to 5hmC conversion at these loci, as it appears to function at the very top of the EHT genetic pathway upstream of Notch signaling, and thus prior to *scf* and *cmyb* [24]. In *tet2/3* mutants, Tg(TP1:GFP), a well-established transgenic reporter harboring

Notch signaling responsive elements, has decreased Notch activity specifically within the VDA and not other portions of the dorsal aorta. In mouse, TET2 activity is present in HSCs and regulates stem cell self-renewal [18], however it has not yet been implicated in EHT. It is also unknown whether TET3 controls mammalian HSC ontogeny.

DNMTs follow Tet activity to further shape the DNA methylome during zebrafish HSC specification. The Dnmt1 maintenance methyltransferase was identified from an ENU-based forward genetic screen for hematopoiesis phenotypes [25]. *Dnmt1*-deficient zebrafish embryos exhibited diminished proliferation of Tg(*cmyb*:GFP)⁺ HSCs in the caudal hematopoietic tissue (CHT), a transient hematopoietic site analogous to the mammalian fetal liver. This defect was subsequently followed by loss of differentiated myeloid and lymphoid blood lineages. Molecularly, HSC loss stemmed from decreased methylation of four CpG islands within the *cebpa* promoter, which led to its de-repression [25]. *C/ebpa* is a well-studied transcription factor that inhibits self-renewal and proliferation of fetal and adult mouse HSCs via cell cycle regulation [26]. Interestingly, *scl*, *runx1*, and *cmyb* expression were diminished in the AGM at the peak of EHT, before the HSC proliferation phenotype, suggesting that Dnmt1 activity in the hemogenic endothelium is required for HSC specification and/or maintenance in zebrafish [25]. In mouse, embryonic lethality at gastrulation in global *Dnmt1* knockouts [27], and a lack of EC-specific conditional knockout *Dnmt1* alleles has precluded the investigation of maintenance methylation in EHT regulation.

Along with Tet proteins and Dnmt1, the de novo methyltransferase Dnmt3bb.1 comprises the DNA methylation epigenetic machinery in zebrafish HSC ontogeny. At the height of EHT, *dnmt3bb.1* was localized to the AGM and this pattern was dependent on Notch and Runx1 signaling [28]. Zebrafish *dnmt3bb.1* mutants had diminished *cmyb*⁺ HSCs due to increased apoptosis, which subsequently led to reduced differentiated blood cell populations. Surprisingly, the HSC reduction was first detected in the CHT after EHT, despite prior expression of *dnmt3bb.1* in hemogenic endothelium. Genome-wide DNA methylome and transcriptome profiling revealed *cmyb* as the primary target of Dnmt3bb.1 in hemogenic ECs and/or emerging HSCs [28]. 5mC modification of the *cmyb* gene body by Dnmt3bb.1 resulted in its transcriptional activation. While these results are consistent with a role for Dnmt3bb.1 in HSC maintenance, it likely also has an earlier function in HSC induction. Ectopic *dnmt3bb.1* expression is sufficient to activate transcription of *cmyb* and other hematopoietic genes even in the absence of Runx1 and in non-endothelial cells [28]. Careful analysis of EHT dynamics in *dnmt3bb.1* mutants is necessary to confirm whether *dnmt3bb.1* plays a role in HSC specification. Of the six *de novo* DNMTs in zebrafish, Dnmt3bb.1 is the closest ortholog to one of two *de novo* DNMTs in mouse, DNMT3B. DNMT3B is expressed in HSCs during mammalian development [29], and thus could have a conserved function in HSC formation from endothelium. The second type of *de novo* DNMT, DNMT3A in mouse and Dnmt3aa and Dnmt3abb in zebrafish, has yet to be implicated in embryonic hematopoiesis.

Histone modification

Posttranslational changes to core histone proteins in nucleosomes provide additional means to regulate chromatin accessibility [17]. Histone modifications that establish a repressive chromatin landscape are emerging as a common mechanism to manage endothelial cell identity during HSC development (Table 1, Figure 1).

Acetylation and methylation of lysine (K) residues on N-terminal histone tails are among the best-studied histone modifications. Addition of single acetyl groups on histone tails is catalyzed by histone acetyltransferases (HATs), and promotes open, transcriptionally-active chromatin states. Removal of acetyl groups by histone deacetylase (HDACs) opposes this activity and causes gene inactivation through chromatin compaction. Histone methylation has more complex effects on gene regulation. Histones can be mono- di- or tri- methylated through the activities of histone methyltransferases (HMTs) and histone demethylases (HDMs), which append or detach methyl groups on specific residues, respectively. The extent to which HMTs and HDMs act on specific histone residues dictates transcriptional activation or repression [16, 18]. Histone modifying enzymes, including those described above, often function within multi-protein complexes to mediate their epigenetic effect. One such example is the Polycomb-group complexes (PcG), in which the activities of two HMTs (Ezh1 and Ezh2) in the Polycomb repressive complex (PRC)1 and a Ring-type E3 ubiquitin transferase (Ring1b) in PRC2 cooperatively work to repress genomic loci via histone H3K27 methylation and histone H2AK119 ubiquitination (H2AK119Ub), respectively [16].

Hemogenic endothelium specification is an essential requirement for the production of HSCs from endothelium during development [11]. How some progenitors acquire hemogenic potential, while others retain their endothelial identity is a fundamental question in the field. While the genetically-encoded regulators of hemogenic EC specification have been established for some time [11], the contribution that epigenetic regulators have to this process has only recently been realized.

PRC1 is a key inhibitor of hemogenic EC specification and one of the earliest known epigenetic regulators of HSC formation during development [30]. In a clonogenic assay for hematopoietic progenitors, sorted populations of mouse EC progenitors (ETV2⁺FLK1⁺) exhibited a dramatic decline in hemogenic potential between embryonic day (e) 7.5 and e8.5 due to *Runx1* repression at this timepoint. PRC1 is responsible for silencing the *Runx1* genomic locus as PRC1 core component BMI1 was abundant in e8.5, but not e7.5 EC progenitors, and BMI1 inhibition was sufficient to derepress *Runx1* expression and provide hemogenic potential to otherwise incompetent e8.5 endothelial progenitors in culture. Thus, *Runx1* silencing by PRC1 is required for maintaining EC identity of mouse e8.5 progenitors [30]. Although these studies show that ectopic expression of Runx1 at this time point can confer hemogenic potential in cell culture [30], hemogenic ECs still form upon deletion of *Runx1* later in development in vivo [31]. Together, these results suggest that RUNX1 is not required, but may only be sufficient, for hemogenic EC specification. Additional studies are needed to better understand the spatiotemporal requirement for RUNX1 during HSC formation from hemogenic EC endothelium, and to determine whether PRC1 silences the *Runx1* locus directly by H2AK119Ub or by an indirect mechanism. Studies in zebrafish indicate that PRC1 has an additional role in HSC ontogeny later in development [32, 33].

Morpholino knockdown of PRC1 core members *bmi1/bmi1b*, *ring1b* [32], *cbx6b*, and *cbx8b* [33] reduced *runx1/cmyb* expression within the AGM, suggesting that PRC1 in this context promotes HSC formation. Epigenetic silencing of specific subsets of target genes could explain the functional differences of PRC1 activity on hematopoietic output, however this question remains to be investigated.

Later in HSC development, EC identity is regulated by different epigenetic machinery, namely the CoREST repressive complex [34]. CoREST complex members include Hdac1 and Hdac2 deacetylases, and Lsd1 demethylase, which silence loci by removing activating H3K56 and H4K16 acetylation (H3K56Ac, H4K16Ac) and H3K4 mono- or di- methylation (H3K4me1, H3K4me2), respectively [35]. Upon treatment of mouse AGM explants with an LSD1 antagonist, hemogenic ECs failed to decrease expression of endothelial gene *Cdh5* and emerge as HSCs from the VDA wall. Likewise, embryonic stem cells (ESCs) lacking LSD1 activity formed hemogenic ECs, but failed to differentiate into blood cells. Profiling of these LSD1-deficient ESC-derived hemogenic ECs revealed expression patterns indicative of EHT arrest, in which upregulated genes were enriched for vascular gene ontology terms, whereas downregulated genes were attributed to hematopoiesis [34]. Hematopoietic phenotypes caused by LSD1 inhibition were similar to those in double knockout mutant mice for *Gfi1* and *Gfi1b* [34, 36], transcriptional repressors that direct CoREST silencing in murine erythroleukemia cells [35]; hemogenic ECs in *Gfi1/Gfi1b*-knockout embryos were arrested in EHT, remaining imbedded in the VDA and failed to express the hematopoietic gene *cKit* [34]. Interestingly, mapping of GFI1 and GFI1b binding sites by DamID (DNA adenine methyltransferase identification) revealed that many GFI-occupied genes were upregulated when LSD1 was blocked in ESC-derived hemogenic ECs [34], suggesting a cooperative function for these proteins. While evidence demonstrating an interaction between *Lsd1* and *Gfi1/Gfi1b* is still lacking, these data put forth a model whereby GFI proteins mark endothelial genes for CoREST-mediated repression to promote the acquisition of the hematopoietic fate during EHT. LSD1 can also function in a complex with Jumonji C domain-containing protein (JMJD2C) to remove repressive H3K9 methyl marks and consequently activate transcription [37]. Thus, hematopoietic genes downregulated upon LSD1 inhibition may be targeted by LSD1/JMJD2C machinery during EHT. Further investigation, including the analysis of H3K4 and H3K9 methylation status at hematovascular genes, is required to fully delineate LSD1 function during HSC specification.

In zebrafish, Hdac1, a component of CoREST and other epigenetic machinery, is required downstream of Notch signaling and upstream or in parallel to Runx1 to promote HSC production [33, 38]. Conversely, valproic acid, an HDAC inhibitor that primarily targets class I HDACs, was discovered to increase the number of *runx1/cmyb*⁺ HSCs arising from endothelium in a chemical screen [39]. These data indicate that CoREST function in the AGM may be conserved across vertebrates.

A reverse genetic screen of zebrafish embryos targeted with morpholinos against 425 human chromatin factor orthologs further expanded the repertoire of epigenetic factors involved in developmental hematopoiesis [33]. Twenty genes had altered *runx1/cmyb* expression in the AGM when knocked down, and corresponded mainly to components of histone modifying and chromatin remodeling complexes. Histone modifiers belonged to the PRC1/2 class, as

well as additional number of complexes not previously implicated in EHT. These include activating SET1 (histone methylation) complex, and repressive complexes NuA4, P300/CBP, HBO1 (histone acetylation). Interestingly, loss of both positive and negative regulators of chromatin (e.g. HATs and HDACs) produced the same HSC defect, suggesting that opposing epigenetic activities are required for HSC specification, although likely on different target genes and/or at different steps in the genetic pathway [33].

Chromatin remodeling

Chromatin remodeling represents the third type of epigenetic mechanism regulating HSC development (Table 1, Figure 1). During this process, multi-subunit complexes reorganize the genomic landscape by utilizing ATP to change nucleosome position via sliding or to alter nucleosome composition through eviction, followed by reassembly with histone variants. The resulting nucleosome expansion or compaction promotes or restricts transcription factor accessibility to regulatory regions, respectively [40]. The four major classes of ATP-dependent chromatin remodeling families are grouped based on similarities within their ATPase domain. These include the CHD (chromodomain helicase DNA-binding), SWI/SNF (switch/sucrose non-fermentable), ISWI (imitation SWI), and INO80 (SWI2/SNF2 related) subfamilies. While ATPase subunits provide the energy to displace or reconstruct nucleosomes, accessory subunits in remodeling complexes provide target specificity and modulate ATPase catalytic activity [40]. Furthermore, histone-specific chaperone proteins often work together with chromatin remodelers, particularly during nucleosome assembly [41].

CHD1 was the first chromatin remodeling ATPase that regulates HSC formation from hemogenic endothelium in mouse [42]. In mouse embryonic fibroblasts, CHD1 removes promoter-proximal nucleosomes to allow RNA Polymerase II (POL II) engagement and elongation [43]. Endothelial-specific deletion of *Chd1* results in EHT arrest, in which hematopoietic progenitors form, but fail to release from the AGM and instead undergo apoptosis. In contrast, hematopoietic cell-specific *Chd1* deletion did not impair hematopoiesis, suggesting CHD1 functions in a narrow window of time during EHT. Analysis of nascent RNA levels by 5-ethynyl-uridine incorporation in hematopoietic progenitors showed an increase in global transcription compared to structural non-hemogenic endothelium, which was abolished upon loss of endothelial CHD1. Interestingly, downregulated transcripts in *Chd1* mutant ECs were enriched for genes related to hematopoiesis and immune function [42]. Together, these findings suggest that CHD1 promotes maturation of developing HSCs by increasing global transcriptional output, particularly at pro-hematopoietic genes. Whether CHD1 remodels promoter proximal nucleosomes in ECs and/or hematopoietic progenitors to establish this permissive chromatin state during EHT requires further validation.

Chromatin accessibility at hematopoietic genes also involves Pol II promoter-proximal regulation in zebrafish, specifically by a Pol II pausing mechanism. Pol II pausing occurs when negative elongation factors, including DRB sensitivity-inducing factor (DSIF) and the negative elongation factor (NELF) complexes, stalls Pol II ~30-50 nucleotides (nt) downstream of the transcription start site [44]. Pausing is overcome by pTEFb (positive

transcription elongation factor b)-mediated phosphorylation of DSIF, NELF and Pol II c-terminal tail, allowing for productive Pol II elongation. Disruption of DSIF and NELF activity led to diminished *runx1*⁺ cells in the AGM of zebrafish embryos [45]. Known pathways involved in HSC formation, TGFβ and IFNγ signaling, were misregulated upon loss of Pol II pausing. While Pol II pausing inhibition led to premature elongation and upregulation of TGFβ genes, it also resulted in decreased chromatin accessibility and diminished expression of IFNγ genes [45]. Thus, paused Pol II acts in part as an epigenetic mechanism to prevent nucleosome assembly and to ensure transcriptional activation at genes that promote HSC formation in zebrafish.

In addition to nucleosome positioning, nucleosome composition with specific histone variants is important for HSC specification. Mouse ESC-derived hemogenic ECs depleted of histone chaperone HIRA were unable to differentiate into blood progenitors [46]. Gene expression analysis revealed normal expression of *Flk1* and *Sox17*, while *Runx1* and downstream regulators were diminished in HIRA knockdown hemogenic ECs, suggestive of EHT arrest. Analysis of the *Runx1* enhancer revealed that incorporation of histone H3.3 variant was dramatically reduced upon HIRA depletion. Thus, HIRA-dependent incorporation of H3.3 at the *Runx1* enhancer is required for its expression, and consequently EHT.

Additional chromatin remodeling complexes that regulate HSC formation from endothelium were identified from a reverse genetic morpholino screen in zebrafish [33], but have yet to be studied in detail. These include members of the BAF/PBAF complex of the SWI/SNF family and ISWI family related factors.

Epitranscriptomic Regulation of EHT

In a manner analogous to epigenetic transcriptional regulation, covalent modifications to RNA, collectively termed the epitranscriptome, provide additional gene regulatory information at the post-transcriptional level [47]. Over a hundred RNA modifications exist, and have the capacity to alter RNA structure, splicing, translation and stability. Dedicated epitranscriptomic machinery can catalyze, read, or metabolize the mark, providing a way to rapidly and reversibly modulate gene expression programs [48, 49].

m⁶A mRNA methylation

In a seminal study, nitrogen-6 methyladenosine (m⁶A) modification of messenger RNAs (mRNA) was identified as a novel mechanism regulating EHT in zebrafish and mouse (Table 1, Figure 1) [15]. m⁶A is the most prevalent modification on mRNAs, and is catalyzed by the methyltransferase like 3 (Mettl3) subunit, typically on adenosines within DRACH (where D = G, A, or U; R = G or A; and H = C, A, or U) motifs. Such epitranscriptomic marks can either positively or negatively affect gene expression depending on the recruitment of specific YTH domain-containing proteins [48, 49]. For example, Ythdf1 binding of m⁶A promotes protein expression through interaction with initiation factor eIF3 and other translational machinery [50]. In contrast, Ythdf2 traffics m⁶A transcripts to cytoplasmic processing bodies for degradation [51]. These m⁶A regulatory effects can be reversed primarily by the activity of Alkbh5 RNA demethylase [52, 53].

During zebrafish EHT, *mettl3* is enriched in hemogenic ECs, and reduction of *mettl3* prevents hemogenic ECs from transiting into HSCs, ultimately leading to a loss of differentiated blood cells [15]. Sophisticated m⁶A-specific methylated RNA immunoprecipitation (MeRIP)-seq technology revealed that the failure in HSC emergence stemmed from decreased m⁶A methylation of *notch1a* and other arterial endothelial genes, which prevented their decay. Similar to *mettl3*, the *ythdf2* m⁶A binding protein is abundant in hemogenic endothelium, and is required for HSC production and *notch1a* downregulation [15]. Expression of wildtype *ythdf2* mRNAs, but not mutant transcripts lacking m⁶A recognition sites, restored HSC production in *ythdf2* zebrafish morphants, suggesting that this particular YTH protein is responsible for the degradation of m⁶A-modified endothelial genes during EHT. m⁶A marks appear to have a conserved function in mammalian developmental hematopoiesis. siRNA knockdown of *Mettl3* in the mouse AGM inhibited hematopoiesis based on decreased numbers of granulocyte-macrophage and erythroid progenitors in colony forming unit assays, and increased expression of endothelial genes *EphrinB2*, *Notch1*, and Notch effectors [15]. Additionally, *Notch1* transcripts were modified with m⁶A at peak EHT, however whether murine YTHDF2 induces the decay of m⁶A-modified *Notch1* and other endothelial mRNAs remains unknown. Taken together, epitranscriptomic regulation by m⁶A mRNA methylation provides a mechanism to silence the endothelial program to promote the transition to the HSC cell fate. Importantly, this work introduces the possibility for the involvement of additional RNA modifications in EHT regulation.

Conclusion & Outlook

Higher-order mechanisms play a crucial role in the reprogramming of ECs to HSCs during development. Studies in zebrafish and mouse have revealed a compendium of epigenetic and epitranscriptomic factors that either erase endothelial transcriptional programs or institute hematopoietic gene expression by altering global DNA or RNA methylation, histone modification, and nucleosome organization patterns (Figure 1, Table 1). While we have a basic understanding of the types of epigenetic and epitranscriptomic mechanisms that are involved in HSC specification, several fundamental questions remain as to how this machinery dynamically orchestrates the gene expression changes required to promote EC to HSC transition. Addressing these questions should bring us closer to a fuller mechanistic understanding of EHT, and provide a better set of instructions to mass-produce long-term reconstituting HSCs from pluripotent cells for the treatment of hematologic disorders.

How do epigenetic and epitranscriptomic marks evolve during EHT?

Research thus far has provided a rich inventory of epigenetic and epitranscriptomic factors that are required for the formation of HSCs from hemogenic endothelium; however, little is known about which genes are marked by such machineries and/or how these marks change during each step of the transition. For example, determination of genes modified with 5hmC and 5mC genome-wide during hemogenic EC specification and EHT could help reveal how the opposing activities of Tet DNA demethylases and DNMT proteins coordinate endothelial and hematopoietic expression patterns throughout HSC formation. While it is already known that differential methylation of zebrafish *cmyb* at its promoter with 5hmC early in EHT, and

later at its gene body with 5mC, leads to its transcriptional repression or activation, respectively [23, 28]; this type of analysis would elucidate when 5hmC is removed in relation to when 5mC is added, and how potential overlap of these methyl marks effects its transcriptional status. It is also likely that other genes or sets of genes in addition to *cmyb* are similarly regulated by DNA methylation to facilitate this developmental cell fate change, and would be identified from such a study. Temporal characterization of global DNA methylation, histone modifications, nucleosome occupancy, and m⁶A methylation patterns would provide a complete systems overview of how EHT unfolds at the molecular level. Although a heroic task, this effort should prove to be beneficial as the catalog of epigenetic marks for different ESC pluripotency states largely mirrors the epigenetic dynamics in embryonic development [17].

What factors provide specificity to EHT epigenetic and epitranscriptomic factors?

Transcriptome profiling of ECs deficient for epigenetic and epitranscriptomic factors during EHT typically reveal enrichment for hemato-vascular terms amongst differentially expressed genes [15, 34]. How these ‘epi’-machineries achieve endothelial and hematopoietic target gene selectivity is currently unknown for HSC specification. Enzymes that catalyze epigenetic modification lack DNA-binding sequence specificity [17]; thus, other mechanisms must be required to deposit marks in a tissue-specific manner. Interaction with transcription factors could provide this selectively by directing the placement of the initial modification. Runx1 may play this role as it induces de novo H3K9 acetylation upon binding to weakly-modified hematopoietic target gene loci in mouse [54].

NcRNA-based mechanisms could also direct epigenetic and epitranscriptomic mark placement through base pairing interactions with DNA or RNA, respectively. Long ncRNAs (lncRNAs) are a versatile class of >200 nt RNAs that have essential roles in HSC self-renewal and other cell fate decisions by scaffolding repressive epigenetic machinery to chromatin [55, 56]. One such example is the lncRNA, Xist, which silences key transcription factors involved in myeloid-erythroid fate determination and other genes on the X chromosome through the progressive binding of chromatin factors that recruit HDACs and PRC1/2 complexes [55]. While lncRNAs are ideal candidates for directing epigenetic machinery specifically to EHT genomic loci, they have not yet been investigated in the context of HSC specification in either zebrafish or mouse models.

Small, ~22 nt, microRNAs (miRNAs) have a similar potential as lncRNAs to guide epigenetic and epitranscriptomic changes to particular target loci during HSC development. Although miRNAs typically associate with AGO2/RISC (Argonaute2/RNA-induced silencing complexes) to posttranscriptionally repress target mRNAs in the cytoplasm, several miRNAs have additional nuclear roles [57]. For example, miR-223/AGO1 targeting of NFIA, a transcription factor involved in the erythroid/granulocytic lineage decision, leads to its silencing through the recruitment of PRC1 machinery in human cell lines [58]. Recently, our group has discovered that miR-223 also functions to regulate HSC production in zebrafish by limiting the number of HSC that emerge from VDA [59]. Potentially, miR-223 could act as an epigenetic regulator in the context of EHT, however direct miR-223 target genes have not yet been characterized. miRNAs have also been implicated in the selective

deposition of m⁶A methylation in at least three different murine stem cell types [60]. miRNAs regulate m⁶A levels by directing METTL3 binding to mRNAs in a sequence-dependent and AGO2-independent manner. In addition to miR-223, miR-142 and let-7 are also required for HSC formation [61, 62], and numerous miRNAs are differentially expressed during EHT in mouse [63], suggesting that this class of small RNAs will likely have a central role in modulating EHT gene expression levels, be it through epigenetic, epitranscriptomic or posttranscriptional gene regulation.

Is there interplay between ‘epi’- machineries during HSC specification?

It is evident that numerous epigenetic and epitranscriptomic mechanisms are at play to reprogram existing endothelial gene expression patterns to new programs that support HSC production. It is very likely that there is interplay between different types of epigenetic mechanisms and between epigenetic and epitranscriptomic machineries during this cell fate transition. Crosstalk amongst epigenetic regulators is well characterized in ESCs and in early development [17]. For example, marking of promoters with both H3K4me3 and H3K27me3, poises genes to become fully activated or repressed upon differentiation, respectively. Additionally, DNA methylation can synergize with H3K9 di- and tri- methyl marks, or antagonize H3K27me3 and nucleosome occupancy [17]. Interplay between epigenetic machinery can be revealed through the characterization and comparison of DNA and histone modifications on EHT genes and identification of genetic interactions between chromatin-modifying enzymes. New evidence suggests that epitranscriptomic marks can mediate epigenetic silencing. Two of the YTH m⁶A binding proteins, YTHDC1 and YTHDC2, are localized to the nucleus, and recognition of m⁶A-modified *Xist* lncRNA by YTHDC1 is required for *Xist*-mediated repression during X-inactivation [64]. Thus, any lncRNAs involved in EHT could require m⁶A methylation to carry out its epigenetic effects.

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Fig 1. During the formation of HSCs from hemogenic endothelium, EC genes are progressively silenced (*red gradient*) at the same time HSC genes are gradually activated (*green gradient*). Epigenetic and epitranscriptomic machinery facilitate the transition of these expression programs by modifying chromatin or mRNA, respectively. EC genes will become repressed within silenced chromatin in the nucleus or m⁶A-modified EC transcripts will be degraded in the cytoplasm (**left**). Conversely, open chromatin will promote Pol II-mediated transcription (*brown*) of HSC genes (**right**). Well-characterized EHT epigenetic and epitranscriptomic factors (*ovals*) are schematized here with their respective modifying-activity as described in the text and in Table 1. Modifications are defined in the key.

Table 1

Epigenetic and epitranscriptomic factors involved in HSC formation from hemogenic endothelium

	Gene(s)	Class/ Complex	Associated modification	activating/ silencing	EHT Localization	Function in HSC development	Organism	Ref.
DNA methylation	<i>tet2/tet3</i>	DNA demethylase	5hmC and derivatives	activating	AGM	Cooperate upstream of Notch to promote HSC emergence	zebrafish	23, 24
	<i>dnmt1</i>	DNA methyltransferase	5mC	silencing	N.D.	Required for <i>scl</i> , <i>runx1</i> , <i>cmyb</i> AGM expression; methylates <i>cepba</i>	zebrafish	25
	<i>dnmt3bb.1</i>	DNA methyltransferase	5mC	activating	AGM	Methylates <i>cmyb</i> gene body to promote HSC maintenance	zebrafish	28
Histone modification	<i>Bmi1</i>	Polycomb Repressive Complex 1	H2AK119Ub	silencing	e8.5 Eiv2+ Flk1+ ECs	Represses RUNX1 to maintain endothelial cell identity	mouse	30
	<i>bmi1/1b, ring1b, cbx6b, cbx8b</i>	Polycomb Repressive Complex 1	H2AK119Ub	silencing	N.D.	Required for <i>runx1/cmyb</i> AGM expression; promotes HSC emergence	zebrafish	32, 33
	<i>Lsd1</i>	histone demethylase/CoREST complex	H3K4me1, H3K4me2	silencing	AGM	Functions with GF11/1b proteins to silence EC expression and promote HSC maturation and emergence	mouse	34
	<i>hdac1</i>	histone deacetylase/CoREST & others	H3K56Ac, H4K16Ac	silencing	N.D.	Required upstream or in parallel to Runx1 to promote HSC production	zebrafish	33, 38
	<i>ash2l, cxxc1l, setd1ba</i>	histone methyltransferase/Set1 complex	H3K4me3	activating	N.D.	Required for <i>runx1/cmyb</i> AGM expression	zebrafish	33
	<i>prdm12, prdm16</i>	histone methyltransferases	H3K9me2/3, H3K9me1	silencing	N.D	Required for <i>runx1/cmyb</i> AGM expression	zebrafish	33
	<i>hdac6, hdac9, sir7</i>	histone deacetylases	various	silencing	N.D.	Required for <i>runx1/cmyb</i> AGM expression	zebrafish	33
	<i>ep300, crebbpa</i>	histone acetylation/P300/CBP complex	H3K18Ac, H3K27Ac, chromatin remodeling	activating	N.D.	Required or limits <i>runx1/cmyb</i> AGM expression, respectively	zebrafish	33
	<i>Chd1</i>	ATP-dependent chromatin remodeling	H3K4me2/3 nucleosomes	activating	ECs	CHD1 promotes HSC maturation by increasing global transcriptional output	mouse	42
	<i>spt5, nellb</i>	DSIF & NELF Pol II pausing factors	N.A.	both	N.D.	Pol II pausing provides chromatin accessibility at some HSC genes	zebrafish	45
Chromatin remodeling	<i>Hira</i>	histone chaperone	histone H3.3	activating	ESC-derived hemEC	Promotes RUNX1 expression through incorporation of H3.3 histone variant	mouse	46
	<i>brd8a, nap14a, smarc1/d2</i>	various remodeling activities	N.A.	N.A.	N.D.	Required for <i>runx1/cmyb</i> AGM expression	zebrafish	33
	<i>ccc2, chd7</i>	various remodeling activities	N.A.	N.A.	N.D.	Limits <i>runx1/cmyb</i> AGM expression	zebrafish	33
m ⁶ A RNA methylation	<i>mettl3</i>	RNA methyltransferase	m ⁶ A	both	AGM	Marks <i>notch1a</i> with m ⁶ A methylation to promote HSC formation	zebrafish, mouse	15
	<i>ythdf2</i>	m ⁶ A reader that promotes decay	m ⁶ A	silencing	N.D.	Reads m ⁶ A marks on <i>notch1a</i>	zebrafish	15