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Epigenetic regulation of hematopoietic stem cell aging

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Abstract

Aging is invariably associated with alterations of the hematopoietic stem cell (HSC) compartment, including loss of functional capacity, altered clonal composition, and changes in lineage contribution. Although accumulation of DNA damage occurs during HSC aging, it is unlikely such consistent aging phenotypes could be solely attributed to changes in DNA integrity. Another mechanism by which heritable traits could contribute to the changes in the functional potential of aged HSCs is through alterations in the epigenetic landscape of adult stem cells. Indeed, recent studies in hematopoietic stem cells have suggested that altered epigenetic profiles are associated with HSC aging and play a key role in modulating the functional potential of HSCs at different stages during ontogeny. Even small changes of the epigenetic landscape can lead to robustly altered expression patterns, either directly by loss of regulatory control or through indirect, additive effects, ultimately leading to transcriptional changes of the stem cells. Potential drivers of such changes in the epigenetic landscape of aged HSCs include proliferative history, DNA damage, and deregulation of key epigenetic enzymes and complexes. This review will focus largely on the two most characterized epigenetic marks - DNA methylation and histone modifications - but will also discuss the potential role of non-coding RNAs in regulating HSC function during aging.

Introduction

In the hematopoietic system, aging is associated with diminished lymphoid potential, increased auto-immunity, and elevated prevalence of hematological malignancies. Many studies have provided insight into functional changes in the hematopoietic stem cell (HSC) compartment that contribute to age-associated decline. Differences include alterations of lineage-biased clonal composition [1–5], cell polarity changes [6], increased inflammatory

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response [7], elevated levels of ROS [8], and accrual of DNA damage [9–13]. Robust and reproducible differences in the expression of many genes have been observed in aged compared to young HSCs [7, 14–16], suggesting that age-associated differences in transcriptional regulation, potentially via alterations in the epigenetic landscape, may underlie the functional changes associated with HSC aging.

The definition of epigenetic regulation has evolved since it was coined by Waddington [17] and while it is still used to describe how a phenotype is achieved from a genotype, it now broadly encompasses all heritable changes in gene expression that are not due to changes in DNA sequence [18, 19]. Epigenetic modifications allow for every cell in the body to share the same genetic code, yet generate the vast cellular diversity found throughout the body and during development from the embryonic state through adulthood. The two most commonly discussed epigenetic marks are DNA methylation and histone modifications, as these are modifications that affect the structure and accessibility of the DNA, directly impacting the transcriptional state of genetic loci. Non-coding RNA and their effects on gene expression are increasingly being considered to fall within the spectrum of epigenetic regulators given their interactions with both histone modifiers and DNA methyl-transferases. This review will focus largely on the two most characterized epigenetic marks - DNA methylation and histone modifications - but will also discuss the potential role of non-coding RNAs in regulating HSC function during aging.

DNA Methylation

DNA methylation patterns, typically methylated CpGs, are established during early development and DNA methyltransferase enzymes (Dnmt's) are responsible for both the establishment and maintenance of these modifications throughout life. *Dnmt1* is largely responsible for DNA methylation maintenance, while *Dnmt3a* and *Dnmt3b* are *de novo* methyltransferases. These methylases are critical for development, and mice with targeted deficiencies of any of these genes are non-viable [20, 21]. To evaluate their role in hematopoiesis, mice with conditional knockouts of these genes have been generated and demonstrate the importance of DNA methylation in the HSC compartment. Specifically, loss of *Dnmt1* in HSCs leads to dysregulation of lineage output, with a skewing towards myelopoiesis, and defects in self-renewal [22, 23] while a conditional knockout of *Dnmt3a* alone drives a loss in differentiation potential after serial transplant [24], and loss of both *Dnmt3a* and *Dnmt3b* in HSCs leads to an even more severe arrest of HSC differentiation [25]. The genes regulating active DNA demethylation, the ten–eleven translocation (Tet) family enzymes, are also important for HSC function. Loss of expression of *Tet2* in HSCs leads to an increased primitive compartment, encompassing both stem and progenitor cells, suggesting that HSCs deficient in *Tet2* have a competitive advantage [26–28]. Interestingly, Dnmt family members and *Tet2* have been shown to be differentially expressed in aged compared to young HSCs [15, 16] and mice with null alleles of several of these genes share some of the phenotypes associated with aged HSCs including myeloid skewing [27] and predisposition to cancer [27, 28].

To address if aged HSCs have altered methylation patterns that contribute to changes in their functional potential, recent publications have looked at DNA methylation profiles in fetal,

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young and old murine HSCs [15, 16, 29] and in human progenitor cells [30]. These studies have shown locus specific differences in DNA methylation profiles associated with aging of the HSC compartment, with some regions gaining methylation whereas other regions become more hypomethylated [15, 16]. Interestingly, hypermethylated regions are enriched for targets of the Polycomb Repressive Complex 2 (PRC2), which establish the repressive H3K27me3 histone modification [15, 16, 29], suggesting an interplay between these two epigenetic marks. The relationship between H3K27me3 and DNA methylation is complex, with several studies showing that increased DNA methylation prevents PRC2 interaction with chromatin in CpG-rich regions, implicating an antagonistic relationship between H3K27me3 and DNA methylation [31–34]; however, in CpG sparce regions this antagonism is not seen as both H3K27me3 and DNA methylation are found at CpG poor regions of the genome [34]. Furthermore, global loss of DNA methylation leads to deficiencies of H3K27me3 at certain regions [34, 35] suggesting the importance of DNA methylation in ensuring proper localization of histone modifications. Examination of how histone modifications (explicitly H3K27me3) influence DNA methylation patterns is less characterized, but again there appears to be a complex interaction in which PRC2 components are necessary to ensure appropriate DNA methylation during development [31, 36] since loss of H3K27me3 leads to both increases and decreases in DNA methylation at different genomic regions [31]. In HSCs, the age-associated diminution of expression of PRC2 core components *Ezh2*, *Eed* and *Suz12* corresponds with increased DNA methylation at targets of the complex. Though speculative, this observation suggests that loss of repression by PRC2 binding at selected targets allows these regions to become accessible to de-novo DNA methylation associated with HSC aging [15, 16].

Interestingly, whereas increased DNA methylation is generally regarded as a repressive mark associated with diminished gene expression, this correlation is generally not observed during HSC aging. While there are some exceptions, most regions that either gain or lose DNA methylation during HSC aging are not associated with altered gene expression [15, 16]. This raises the possibility that differential DNA methylation may not directly impact expression of genes in stem cell compartments, but instead may have heritable and possibly detrimental effects on the progeny of the stem cells. Indeed, within the blood and skin, it has been demonstrated that DNA methylation is tightly coordinated during differentiation from stem cells to the effector cells [37], and thus dysregulation of the DNA methylation landscape in the stem cell compartment could be inherited by downstream progeny and might thereby affect gene expression and differentiation of these cells. Consistent with this, aged HSCs showed significant increases in methylation of open chromatin regions associated with lymphoid and erythroid cells [15], lineages that diminish during aging, raising the possibility that these altered epigenetic marks poise the aged HSCs towards a more myeloid-restricted lineage output. Furthermore, many of the genes that were differentially methylated in HSCs during aging were genes that normally become expressed only in downstream progeny, and in some cases encode products critical for the function of downstream effector cells [15]. These data suggest that the alterations of the DNA methylome that occur during HSC aging poise them toward a skewed differentiation potential that is manifest in progenitor or effector cells downstream of the stem cell compartment.

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In HSCs, or any adult stem cell that is required for the maintenance and repair of their respective tissue or system throughout life, it is important that there is a mechanism to protect these cells from functional exhaustion. In contrast to most somatic cell types, HSCs possess substantial proliferative potential and can survive and function well beyond the lifespan of an animal [38]. The expansive proliferative capacity of HSCs is thought to be attributed, at least in part, to HSCs' residence in a largely quiescent state during adult life, with estimates ranging from five to twenty cell divisions during a twoyear life span [39, 40]. It is possible that the infrequency of HSC cycling may prevent loss of mC, which is associated with aging in other somatic tissues and cultured primary cells. This loss of methylation in other cell types is potentially associated with increased cell division number and may correlate with loss of proliferative potential. It is of note here that immortalized cells lines, similar to HSCs, also do not exhibit global hypomethylation, suggesting that loss of DNA methylation may be associated with loss of replicative potential. Interestingly, when HSCs are pushed to their functional limits through enforced proliferation *in vivo*, global hypomethylation dependent upon their proliferative history is observed [15]. Thus, like somatic cells, HSCs that reach their proliferative limit also exhibit global hypomethylation suggesting that global loss of methylation may be a universal feature of exhausted cells.

A potential means through which loss of DNA methylation could be driven is via the acquisition of mutations in the DNA. Methylated CpGs (mCpG) are hotspots for mutation accrual [41–43] with CpG substitutions occurring more frequently than other single nucleotide change [44, 45]. This is in part due to deamination, which leads to the transition of cytosine (C) to uracil (U) or methylated cytosine (mC) to thymine (T). These deaminated bases generate a guanine-thymine mismatch, which can which can be resolved by either the correct cytosine or by conversion to a base which is no longer receptive to methylation. Recognition and repair of damaged/mismatched DNA is exquisitely tied to cell cycle, and thus it is not surprising that the loss of mC has been correlated with the cycling history of the cell [43]. These data suggest that accumulation of these mutations are cell cycle dependent and increased cell division could lead to elevated loss of CpG DNA methylation due to mutation accrual at formerly methylated cytosines. Thus, increased proliferation history and associated mutation accrual present a potential rationale for the hypomethylation recurrently observed in functionally exhausted cells, yet whether or not global hypomethylation drives functional exhaustion or is merely a passenger effect is, at this point, unclear.

Chromatin Modifications

Histones are proteins that serve as a core for DNA to wrap around allowing for the DNA to be packaged into higher-order structures. These structures not only compact the DNA but also serve to affect the accessibility of the DNA. Post-translational modifications on the tails of the histones, including acetylation, methylation, phosphorylation, sumoylation, and ubquitination, play a direct role in regulating access to genes and also function in concert with one another to recruit proteins and protein complexes [46].

One of the first insights that histone modifications affect the functional potential of HSCs was the report of high levels of the open chromatic mark H3K4me2 in HSCs associated with

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genes that are highly expressed in committed progenitor populations but have little to no expression in HSCs themselves [47]. Similarly, Maës *et al* assayed a number of histone modifications and showed lineage specification genes appear primed for expression in primitive hematopoietic progenitors, prior to lineage commitment [48]. These studies support the concept that priming at lineage specific loci in HSCs precedes lineage commitment, and is under tight epigenetic control, in a manner very similar to the DNA methylation poising described above.

Expression analysis has demonstrated that multiple genes encoding proteins involved in chromatin remodeling are age-regulated in HSCs [7, 14–16]. This included genes involved in regulating the accessibility of the chromatin, with several lysine-specific demethylases (*Kdm3a–b*, *Kdm5b–d*, and *Kdm6a–b*) with established roles in stem cell biology [49–52] all showing decreased expression with age. One demethylase with high expression in the young primitive hematopoietic compartment, *Kdm5b* or *Jarid1b*, has recently been suggested to promote differentiation and repress genes involved in HSC self-renewal [53]. Thus, it is possible the age-associated decrease of *Jarid1b* [15] expression could contribute to loss of differentiation potential and an expanded HSC compartment in the aged mice. Alternatively, the correlation between gene expression of histone demethylases and changes in HSC potential may not be that straight-forward as expression of *Lsd1*/*Kdm1a* another histone demethylase that, similarly to *Jarid1b*, promotes differentiation by blocking stem and progenitor signatures [54], shows increased expression in aged HSCs.

To gain a more comprehensive understanding of the role of histone modifications in HSC potential, genome-wide analyses of these marks needs to be performed. However, given the large cell numbers required to assay histone modifications, there have been few reports to date directly measuring these epigenetic marks in highly purified HSCs. To circumvent the challenge of cell numbers, Florian *et al* used immuno-staining, together with FACs analysis, to assay global changes of one such histone modification, acetylation of H4H16 (H4K16ac) [6]. Interestingly, while young HSCs express high levels of the H4K16ac activation mark, there is a sub-population of old HSCs that express low levels of H4K16ac. Additionally, the cellular distribution of H4K16ac in the aged HSCs was also altered, displaying a more disperse expression pattern throughout the nucleus compared to the polarized localization seen in young HSCs, though the precise genomic distribution of this histone modification has yet to be defined.

A recent paper has globally queried the location of three other histone modifications in young and old HSCs: methylation of H3K4 (H3K4me3), a mark of active chromatin; methylation of H3K27 (H3K27me3), a mark of repression; and methylation of H3K36 (H3K36me3), present on actively transcribed regions [16]. Though most age-associated shifts in the histone methylation marks were modest, the authors show not only an increased number of H3K4me3 peaks in the aged HSCs, but also an expansion of the activation peaks that were also present in the young HSCs. In contrast to DNA methylation where little direct correlation between methylation and gene expression was seen, Sun *et al* observed a strong positive correlation between the age-associated changes of H3K4me3 and gene expression. Additionally, though there were similar numbers of H3K27me3 peaks in the aged HSCs compared to the young, the distribution of this repression mark was altered, with increased

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H3K27me3 on a substantial number of promoters in the aged HSCs. This finding seems a bit incongruous with documented age-associated loss of expression of key components of the PRC2 complex, (*Ezh2*, *Suz12*, and *Eed*) which is responsible for H3K27me3 [14–16]. It is possible that redundancy or compensatory mechanisms exist in the stem cell compartment. *Ezh2,* which encodes the enzymatic protein, demonstrates the most substantial decrease in gene expression of the three core PRC2 components [15]. However, another member of the histone-lysine methyltransferase family, *Ezh1*, has been shown to interact with the PRC2 complex in embryonic stem cells and HSCs [55–57], and thus may serve as a compensatory component for the loss of *Ezh2*. Conversely, *Ezh1* may replace *Ezh2* in the complex but could target potentially different regions [55, 57, 58]. While this possibility has yet to be examined in aged HSCs, the age-associated increase of *Ezh1* expression in HSCs is suggestive of this possibility [15, 16, 56].

The alterations of histone modifications in aged HSCs and their interactions with DNA methylation are just beginning to be elucidated, but technological advancements and development of protocols that can assay for such marks on small cell numbers will certainly lead to a more comprehensive understanding of how these epigenetic marks contribute to the functional changes associated with HSC aging.

Non-coding RNA

ncRNA's are a class of RNAs that are transcribed but not translated into protein, including, but not limited to, transfer RNA (tRNA), ribosomal RNA (rRNA), piwi-interacting RNA (piRNA), microRNA (miRNAs), and long non-coding RNA (lncRNA). The inclusion of non-coding RNA in our discussion of the epigenetic regulation of aging HSCs is in part due to the expanding definition of epigenetics. Non-coding RNA (ncRNA) play roles in regulating protein expression, and increasing evidence points towards a complex interplay between non-coding RNA and both histone and DNA methylation [59–62].

miRNAs target mRNA leading to their degradation that have been shown to regulate diverse genes including key epigenetic regulators such as histone deacetylases and DNA methyltransferases (reviewed by [59]). The expression of miRNAs is tightly regulated during hematopoietic differentiation, and much like gene expression profiles, different cell types have a unique miRNA signature [63, 64]. miR-125b, which has been show to be regulated by both DNA and histone methylation in cancer settings [65–67], is highly expressed in the primitive compartment where it has been shown to regulate HSC survival [68, 69]. Overexpression of miR-125b affects the composition of the HSC compartment, by conferring a more stress resistant, anti-apoptotic state to the HSCs, [69] leading to increased frequency of the $CD150^{low}$ "lineage balanced", and $CD150^{neg}$ lymphoid-biased HSC subsets [1, 5]. Intriguingly the frequency of these HSC subsets is diminished in aged mice, suggesting that dysregulation of miR-125b might contribute to the changes in frequency of the CD150 compartments, however this has not yet been examined.

miR-126 [70] has also been identified as a key regulator of HSCs. Knockdown of miR-126 resulted in increased HSC cycling that led to a robust expansion of the HSC compartment associated with reduced lymphoid potential [71]. With the exception of total reconstitution

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potential that was maintained, these phenotypes are again evocative of HSC aging: expansion of the HSCs and diminished lymphoid output, and implicate miR-126 as a potential mediator of HSC aging.

Long non-coding RNA's (lncRNA) are defined by their length (>200 nucleotides), and appear to have both activating and repressive functions [72] with roles in imprinting [73], X-chromosome inactivation [74], and in ES cell differentiation and pluripotency [75]. Interestingly, many adult stem cells, including HSCs, maintain high levels of expression of several imprinted lncRNAs that are later repressed in their downstream progeny [37, 76] suggesting an important regulatory role for these ncRNA in the stem cell compartment. One of best characterized lncRNA, Xist, which plays a role in removal of active histone marks and introduction of repressive marks during X-chromosome inactivation, is required for survival of the HSC compartment as loss of Xist leads to hyperproliferation of all blood cells and loss of HSCs [77]. Similarly, another imprinted lncRNA, H19, was recently demonstrated to be crucial for maintaining HSC quiescence as strand specific deletion of H19 led to reduced reconstitution potential, loss of quiescence, and premature functional exhaustion of the HSC compartment [78]. However, the direct impact of ncRNAs in HSCs aging has yet to be examined closely but the fact that several ncRNA's have HSC or HSC and early progenitor restricted expression and that dysregulation of many ncRNAs has been associated with ageassociated cancers suggests that it will be informative to more rigorously examine ncRNAs in HSCs during aging and disease progression, and ultimately the intersection of these two states.

Consequences of Dysregulated Epigenetic Landscapes

Age-associated phenotypes of the hematopoietic system include increased incidence of many hematological malignancies encompassing leukemias (AML, CML, CLL, CMML), myelodysplastic syndromes (MDS), myeloproliferative disorders (CMD), myeloma, and lymphomas (Hodgkins and Non-Hodgkin) (http://seer.cancer.gov). The etiology of many of these conditions indicates profound disruptions in the homeostatic control mechanisms, thus implicating deregulation of hematopoietic stem and progenitor cell activity. Indeed studies in mice have shown that the HSC compartment can serve as a mutation reservoir during aging [9, 13] and recent publications have demonstrated that the HSC compartment indeed does serve as a reservoir for mutation accumulation leading AML and MDS [79–86]. The Majeti group demonstrated mutations arising in the HSC compartment can clonally progress as preleukemic events that can eventually give rise to frank AML [79], findings that were further supported by another recent study that detected initial mutations in the HSC compartment that led to malignant transformation [83]. Interestingly, many of the mutations found in the HSC compartment leading to malignant transformation are genes involved in epigenetic regulation, such as *TET2, DNMT3, and EZH2* [79, 83, 86–88] and reviewed by [89] further implicating the importance of maintaining faithful epigenetic control in HSCs.

Aberrant DNA methylation patterns play an important role in the emergence and progression of MDS, a prototypic disease of the elderly. The aberrant methylation profiles of HSCs isolated from MDS patients [81] together with the many studies showing that key epigenetic regulators are mutated in patients with MDS (reviewed by [90] and [91]),

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implicate methyl-silencing as a dominant mechanism in MDS. The mechanistic importance of DNA methylation in the pathophysiology of MDS been validated by the success of clinical trials with drugs that inhibit DNA methylation [92–95]. With this said, the precise mode of action of these demethylating agents in MDS remains unclear, although it appears to involve epigenetic reprogramming and re-establishment of normal stem cell function triggered by de-repression of genes that had been silenced by DNA hypermethylation and restoration of normal protein expression [96]. Interestingly, while in MDS the loss of epigenetic fidelity is typically manifest as hypermethylation, the progression to AML is typically associated with global hypomethylation [97]. One potential mechanism behind this loss of methylation that is leading to leukemia might be attributed to dysregulation of *DNMT1*. However, loss of *Dnmt1* in a mouse model of MLL-AF9-induced AML led to a reduction in leukemic progression and appears to have little affect on normal hematopoiesis [98]. Alternatively, this global loss of methylation could also be due to overexpression of Ten-Eleven Translocation (Tet) family genes, a group of proteins that convert methylated cytosines to 5-hydroxymethylcytosines (5hmC) ultimately allowing for the removal of DNA methylation. However, a recent publication suggests that outside of embryonic development, Tet1 does not actively remove methylation but instead acts to prevent the spreading of methylation to canyons near highly methylated regions [99]. Additionally the expression of Tet family genes in HSCs decreases with age [15, 16] associated with decreased amount of 5hmC with age [16] suggesting another possible mechanism for loss of methylation. As mentioned earlier, loss of methylation can also occur due to deamination of methylated cytosines. Interestingly, CpG mutations are over-represented in mutations that contribute to disease and many cancers [100, 101], and in a diverse population of cancer types, the C-T transition is frequently the most commonly found mutation [102]. This transition, possibly due to deamination of a methylated cytosine, is also the most frequent mutation seen in aged human HSPCs [103]. In AML, this cytosine transition is also found at a higher frequency when a guanine is directly following, further supporting the theory that many of those mutations arose from deamination of mC. This mutation of methylated cytosines to thymines would correlate well with the suggestion that mutations of epigenetically regulated regions, or loss of epigenetic regulation, contribute to cancers.

That said, global DNA hypomethylation is not the only aberrant methylation profile associated with cancers, as various cancers also demonstrate DNA hypermethylation at specific promoter-associated regions [104]. These regions of increased DNA methylation associated with cancer are typically over-represented for targets of the polycomb group (PcG) [105], similar to what is seen in the aged HSCs. These polycomb targets are largely key regulators of development and are repressed to maintain plasticity in ES cells [106]. Widschwendter *et al* suggest that dysregulation or permanent silencing of typically plastic regions that regulate stem cells' choice between differentiation and self-renewal due to aberrant accumulation of DNA methylation may inhibit differentiation and lead to a self-renewing population that could serve as a pool for mutation accrual [105]. Preliminary support that dysregulation of microRNAs in the primitive compartment could also contribute to AML was demonstrated in two studies whereby overexpressing a microRNA that is normally highly expressed in HSCs (miR-29a and miR-125b) in either the primitive compartment [107] or bone marrow [108] leads to AML in murine models. Additionally

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dyregulation of H19 has also been implicated in myeloproliferative disorders [109, 110], but direct connections between microRNA dysregulation in the HSC compartment and disease have yet to be rigorously assayed for.

Resetting the Epigenetic Clock

There is an expanding base of knowledge shedding light onto the role of epigenetics and stem cell regulation, however the complicated nature of the interactions between these mechanisms (DNA methylation, histone modifications, and ncRNA) and the implications of dysregulation of these interactions is just beginning to be understood. Though there may be some universal facts about the interplay between these epigenetic regulators, there appears to be many unique interactions that may ultimately be cell-type specific. Developments in technology will allow for smaller numbers of cells to be investigated for these epigenetic marks and will provide more information as to steady-state epigenetic regulations of cell populations with limited numbers, such as HSCs. These data will provide a base-line platform for comparison when dysregulation of these epigenetic players occurs – whether it be through aging, cancer, or another means.

Ultimately, epigenetic modifications are not permanent raising the prospect that re-writing the epigenetic marks of aging could be used to alter the potential of HSCs from the elderly. This idea was recently tested in a study by Bryder and colleagues who showed that aged hematopoietic progenitors reprogrammed to induced pluripotent (iPS) cells and then re-differentiated to blood cells no longer exhibited the hallmark functional features of aged HSCs [111]. Indeed, when they examined the hematopoietic system of chimeric mice generated from the iPS derived from either young and old progenitors, they saw no differences in lineage potential or differences in the frequency of the HSCs. Furthermore, competitive transplants of HSCs derived from iPS generated from old progenitors showed robust reconstitution potential, similar to young, freshly purified HSCs [111]. These data demonstrate that resetting epigenetic landscapes via pluripotency induction was sufficient to rejuvenate the functional potential of aged progenitors to yield HSCs with renewed potential.

Recently, committed progenitor and effector cells from young mice were reprogrammed with a set of defined transcription factors to generate cells with the functional and molecular properties of HSCs (termed induced-HSCs (iHSCs)) [112]. Given that myriad epigenetic modifications accompany hematopoietic differentiation from HSCs [37, 113, 114], it seems likely that iHSC derivation is associated with epigenetic reprogramming to allow establishment of HSC potential on otherwise commitment blood cells. Further analysis will however be required to determine if the epigenetic landscape of iHSCs is fully reset or whether the epigenetic memory of the starting cell type is retained in a manner similar to the epigenetic memory associated with early passage iPS cells [115–117]. Nonetheless, the ability of inducing HSC activity onto other cell types raises the prospect that iHSC reprogramming might be used as an effective strategy to restore functional potential to an aged hematopoietic system via resetting the epigenetic clock of aging.

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