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Hematopoiesis during development, aging, and disease

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Hematopoietic stem cells were once considered identical. However, in the mid-1990s, it became apparent that stem cells from a person's early developmental phases are superior to those from adults, and aged stem cells are defective compared with young stem cells. It has since become clear that polycomb group proteins are important regulators of stem cell functioning. Polycomb group proteins are chromatin-associated proteins involved in writing or reading epigenetic histone modifications. Polycomb group proteins are involved in normal blood cell formation, in cancer, and possibly in aging. In this review, we describe how the different phases of hematopoietic stem cells—birth, maintenance, functional decline, derailment, and death—are continuous processes that may be controlled by polycomb group proteins.

When hematopoietic stem cells were first "discovered" and methods to purify these cells were developed, it was generally assumed that all hematopoietic stem cells were functionally identical and contributed equally to blood cell formation during an organism's lifetime [1]. In a series of elegant experiments, partly published in *Experimental Hematology*, Gary Van Zant and his co-workers reported that, in fact, hematopoietic stem cells do age [2]; the rate of aging is related to their (strain-dependent) proliferative activity [3]; and stem cell quiescence is reversible [4]. Collectively, these and other studies led to a model that proposed that any time a hematopoietic stem cell divides, its two daughter cells inherit a somewhat lower stem cell potential, directly linking hematopoietic stem cell turnover with loss of stem cell quality [5]. Enhanced cell turnover, caused by (serial) transplantation, repeated rounds of chemotherapy, or normal aging, leads to loss of stem cell functioning. At the time, it was postulated that telomere shortening could provide the molecular clock that would restrict stem activity.

In recent years, it has become evident that the classic divisions between development, normal aging, and malignant degeneration of the hematopoietic system may not be very distinct and may constitute a continuum. Although the molecular mechanisms that specify the birth of the first hematopoietic stem cells during development are likely different from those required for the maintenance of blood cell formation, key genes have been found to be important for both. Similarly, the gradual decrease of blood cell production during aging is often not distinguishable from preclinical conditions that may culminate in hematologic malignancies.

The fact that (pre-)hematologic malignancy mutations have been found in genes encoding for proteins involved in epigenetic regulation underscores the relevance of this process in normal blood cell development and blood cell aging and their causal role in hematologic malignancies. Here, we postulate that the mitotic clock that restricts hematopoietic stem cell functioning may be governed by the correct deposition of epigenetic modifications at hundreds of loci in stem cell daughter cells. A key class of epigenetic regulators is the polycomb group (PcG) proteins, which play essential roles in embryogenesis, adult life, possibly aging, and the development of malignancies. We provide a brief overview of PcG proteins, the composition of the complexes in which they occur, and their functioning in stem cells, with a special focus on benign, aging, and malignant hematopoiesis.

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Polycomb group proteins: Composition and function

Polycomb group proteins are chromatin-associated proteins that were first discovered in *Drosophila melanogaster*, as

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repressors of HOX genes to control body segmentation along the anterior–posterior axis during development [6]. The function of PcG proteins in mammals as repressors of developmental genes is highly conserved [7].

Polycomb group proteins are involved in essential cellular processes, like senescence [8,9], cancer [10,11], cell cycle control [11,12], and stem cell self-renewal [13]. PcG proteins assemble in *Drosophila* and in humans in multiprotein complexes that are involved in altering chromatin compaction, thereby regulating transcription of genes. During evolution, the number of genes coding for the various PcG proteins increased from 15 in *Drosophila* to 37 in mammals [14], establishing substantial functional diversity.

The best-characterized complexes are the canonical polycomb repressive complex 1 and 2 (PRC1 and PRC2) (**Fig. 1**) [15]. The PRC2 complex, which is highly conserved from flies to mammals, consists of four components, referred to as SUZ12, EED, EZH1/2, and RbAp 46/48 in mammals [16,17]. In mammals, there are two orthologues of the enhancer of Zeste subunit (EZH1 and EZH2), which are mutually exclusively present in the complex and are both able to methylate H3K27. Although the two proteins share substantial similarities (ca. 65%) and can assemble with the same PRC2 components, they seem to harbor different methyltransferase activities via their SET domains and are often differentially expressed [16]. Whereas EZH2 is more abundant in dividing cells and its knockdown leads to a global loss of H3K27me2 and H3K27me3, EZH1 is expressed in dividing and nondividing cells, and its knockdown leads to only marginal changes in the methylation pattern of H3K27 [18].

These findings suggest a model in which EZH2-containing PRC2 is more important for de novo methylation of H3K27, and EZH1-containing PRC2 plays a role in maintenance and restoration of H3K27 methylation [17]. Interestingly, EZH2 is overexpressed in many cancer cell lines, and mutations of EZH2 can be found in myeloid neoplasms [19,20].

The assembly of all subunits is important for proper functioning of PRC2 [21–24]. Next to the four core components of PRC2, there are additional proteins, which can integrate into PRC2, either facilitating the recruitment of PRC2 to its target genes or increasing the enzymatic activity of EZH1 or EZH2. For further details on the PRC2 complex, we recommend the review by Margueron and Reinberg [17].

Whereas the composition of PRC2 is constant, the PcG genes that encode for proteins that assemble into PRC1 experienced much more diversification so that in humans, every PRC1 subunit can be assembled by many homologues, leading to more than 180 theoretical permutations of canonical PRC1 [25]. In fact, it is probably more appropriate to refer to the PRC1 complex as a family of different PRC1 complexes [20]. Nevertheless, in all different types of PRC1, the enzymatically active subunit RING1A or RING1B is present, and this protein promotes the ubiquitination of the histone tail of H2A on lysine 119 [25].

It has become common to distinguish between canonical and noncanonical PRC1 complexes [26]. In mammals, canonical complexes are characterized by the presence of one of the five chromobox domain proteins (Cbx2, Cbx4, Cbx6, Cbx7, and Cbx8), which are able to recognize the H3K27me3 mark set by PRC2. In addition, canonical PRC1 contains one of the six members of the PCGF family (PCGF1-6), one of the three members of the HPH-family (HPH1-3), and the E3-ligase RING1A or RING1B [14,27,28].

Noncanonical PRC1 complexes contain either RYBP, the demethylase Kdm2b, or E2F6/L3MBTL [25,29,30]. The exact biological function of all the different PRC1 complexes so far remains elusive (Fig. 1).

Because Cbx proteins can recognize H3K27me3 with their chromobox domain [31] and because PRC1 and PRC2 have largely overlapping target sites, it was originally postulated that transcriptional repression is achieved by the initial trimethylation of H3K27 by PRC2 with subsequent ubiquitination of lysine 119 of H2A through PRC1 [26,28,32]. However, this classic model had to be revised after the discovery of noncanonical PRC1 complexes that contain proteins, like RYBP, Kdm2b, or E2F6/L3MBTL, that bear no chromodomain at all [26]. Currently, it is unclear how collectively PRC1 and PRC2 are achieving gene repression. For further reading on this topic, we recommend the review from Blackledge et al. [33].

Polycomb proteins in development and their role in stem cells

As mentioned, polycomb proteins were initially discovered as repressors of Hox genes during early embryonic development in *D. melanogaster* [6]. Mice lacking one of the three PRC2 components—Ezh2 [34], Eed [35], or Suz12 [22]—are not viable and manifest severe defects during gastrulation, emphasizing their role in embryonic development.

The majority of the PRC1 proteins also seem to have important roles in mouse development but at later stages. The exception is Ring1B; deletion of Ring1B in murine embryonic stem cells leads to a lethal phenotype caused by impaired gastrulation. Although Ring1A and Ring1B proteins are homologous, Ring1A is not able to compensate for the loss of Ring1B in embryonic stem cells [36].

Bmi1-deficient mice are viable, but have a shortened life span because of various defects in the nervous and hematopoietic systems. For instance, bone marrow, spleen, and the thymic cortex of $Bmi1^{-/-}$ mice manifest signs of severe hypoplasia, which is associated with reduced absolute cell count, especially of B-lymphoid and myeloid cells [37]. Deletion of chromobox proteins in murine embryonic stem cells seems not to impair embryogenesis, but rather

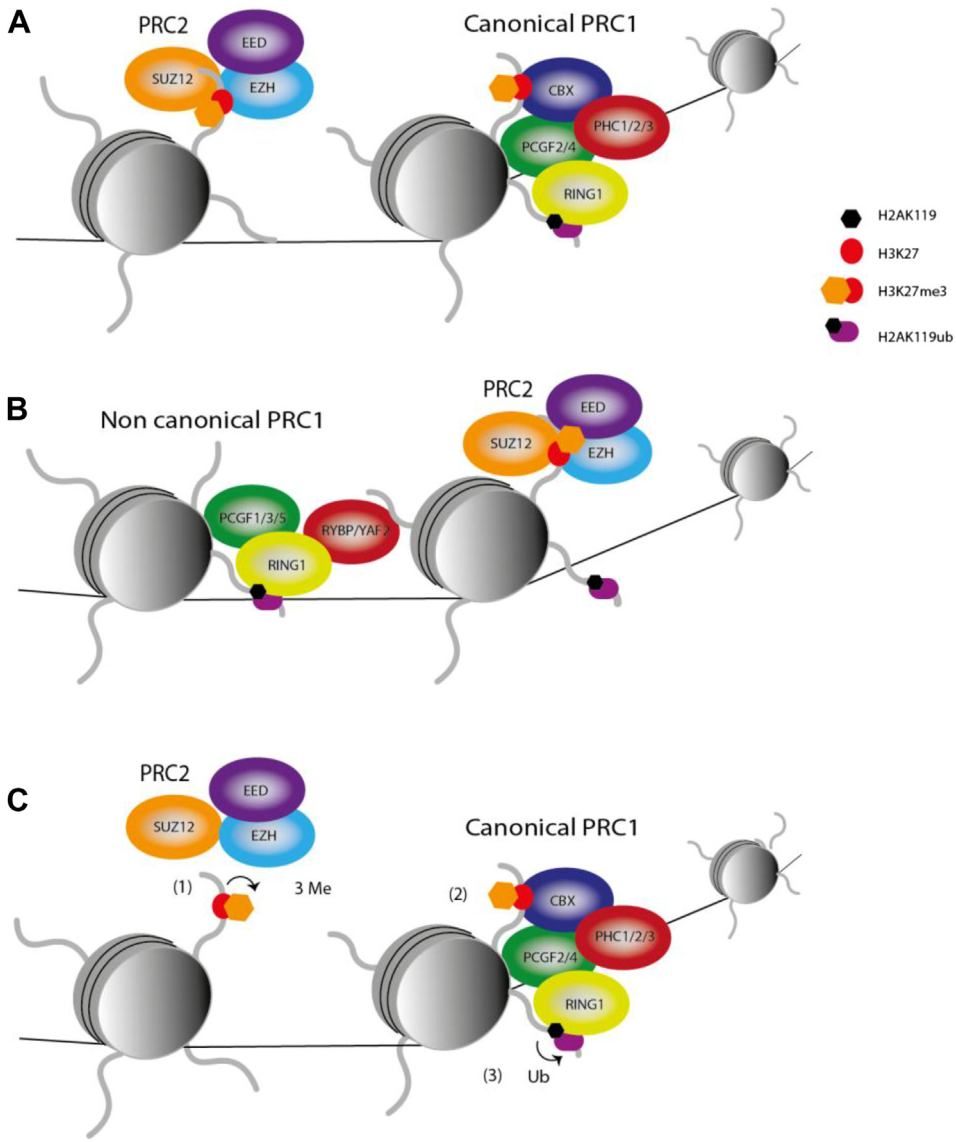

Figure 1. Composition of the (**A**) canonical and (**B**) non-canonical polycomb repressive complex 1 (PRC1). (**B**) Noncanonical PRC1 variants can be recruited to chromatin by, for instance, KDM2B, which leads to ubiquitination of lysine 119 on histone 2A, which results in formation of PRC2 with subsequent trimethylation of lysine 27 on histone 3A. (**C**) According to the hierarchical model, the enzymatic subunit of PRC2, EZH1, or EZH2, mediates the methylation of lysine 27 on histone 3A (1), H3K27me3. This mark is recognized by the chromodomain of the CBX proteins of PRC1 (2) and leads to mono-ubiquination of lysine 119 on histone 2A (3), H2AK119.

affects later stages of development. For instance, knockout of Cbx2 results in retarded growth, homeotic transformations, malformations, and reduced expansion of lymphocytes and fibroblasts in vitro [38]. In contrast to Cbx2, deletion of Cbx7 results in an increased body length and a shortened life span caused by the development of liver and lung carcinomas [39]. Expression studies suggest that Cbx6 and Cbx7 are the most abundant Cbx proteins in self-renewing murine embryonic stem cells. Chromatin immunoprecipitation- sequencing (ChIP-seq) experiments indicated that the promoters of Cbx4 and Cbx8 were decorated with the repressive epigenetic mark H3K27me3. During differentiation to embryoid bodies, the Cbx7 locus was repressed by H3K27me3, which coincided with a release of the repression of Cbx4 and Cbx8. These data indicate that Cbx proteins balance self-renewal and differentiation in embryonic stem cells and form an intricate feedback regulatory loop [40].

Epigenetic proteins in aging

One hallmark of aging is the declining function and physiologic integrity of tissue [41]. Because normal tissue function is characterized by a proper balance of self-renewal and differentiation of adult stem cells, stem cell functioning is often impaired in aged tissues. In contrast to what was

reported in the early days, when hematopoietic stem cell assays did not allow single-cell analyses, the aged murine and human hematopoietic systems are characterized by increased numbers of stem cells, but these are impaired in their differentiation toward the lymphoid lineage, resulting in a shift toward the myeloid lineage [42–44]. As described earlier, normal hematopoietic stem cell function is strongly controlled by epigenetic mechanisms, and therefore, dysregulation of epigenetic writers or erasers might contribute to the aged phenotype of stem cells. It seems plausible that the deposition of key epigenetic stem cell modifications in the two daughter stem cells is compromised on (repeated) cell division, in such a way that daughter cells epigenetically and transcriptionally drift away from the pristine ground state, which specifies optimal stem cell functioning (Fig. 2). Indeed, epigenomic profiling of aged murine hematopoietic stem cells revealed broader H3K4me3 peaks and hypomethylation of transcription factor-binding sites of genes important for hematopoietic stem cell self-renewal or maintenance. Simultaneously, transcription factor-binding sites of genes important for differentiation were hypermethylated. In addition, the PRC2-mediated H3K27me3 mark exhibited an increased length of coverage and an increased intensity at many promoters of genes [45]. The expression levels of epigenetic proteins were changed in old hematopoietic stem cells compared with their young counterparts. Although expression of EZH1 was increased, expression of EZH2 and Cbx2 was decreased [45].

During aging, the DNA methylome in human cells also changes. One of the key enzymes promoting de novo methylation of DNA is the methyltransferase DNMT3A. This gene is one of the most mutated genes in cancer patients, notably in hematologic malignancies, like acute myeloid leukemia (AML), myelodysplastic syndrome, and T-cell acute lymphoblastic leukemia [46–49]. Hematopoietic stem cells bearing DNMT3A mutations have a proliferative advantage over "healthy" hematopoietic stem cells in xenotransplantation studies, indicating that mutant DNMT3A might transform healthy hematopoietic stem cells into preleukemic ones, which still contribute to hematopoiesis but lead to clonal hematopoiesis. Sequencing studies of patient samples from diagnosis, remission, and relapse indicate that DNMT3A-mutated preleukemic stem cells are relatively chemoresistant and contribute to hematopoiesis after remission, but also represent a pool of preleukemic stem cells for relapse [50]. These findings reinforce the notion that normal aging and overt disease are tightly linked and controlled by epigenetic mechanisms.

Polycomb proteins in cancer

The first link between polycomb proteins and the development of cancer was the discovery that Bmi1 collaborates with c-myc in murine lymphomagenesis [51]. Although enforced overexpression of BMI1 in murine and human hematopoietic stem cells led to increased self-renewal activity, the development of hematologic malignancies was not observed, indicating that increased BMI1 expression in hematopoietic stem and progenitor cells, as a single event, is not sufficient for malignant transformation [52,53]. Yet, BmI1 is crucial for the maintenance of malignant hematopoietic stem cells in vivo, as only leukemic cells derived from BmI1 wild-type mice are able to generate leukemia in secondary recipients [54]. In human myeloid leukemia, downregulation of BMI1 resulted in reduced self-renewal activity of leukemic stem cells in vitro and in vivo [55].

Such a proliferative advantage of BMI1-overexpressing hematopoietic stem cells may lead to a clonal expansion of premalignant stem cells, which can acquire additional genetic or epigenetic changes that may result in an overt malignancy [56]. Indeed, BMI1 expression in $CD34^+$ cells of patients in the accelerated phase of chronic myeloid leukemia is higher in comparison to that of patients in the chronic phase [57]. Also, in patients with myelodysplastic syndrome, higher BMI1 levels correlated positively with disease progression [58]. In a subset of chronic lymphocytic leukemia and mantle cell lymphoma, higher BMI1 levels were detected [59,60]. Interestingly, high expression of BMI1 can also be observed in nonhematologic cancers, like non-small cell lung cancer [61] and breast cancer [62], indicating that BMI1 may play a universal role in different cancers. The mechanism of high BMI1 expression levels is not clear, and BMI1 is not frequently mutated in cancer patients.

Unlike those of BMI, mutations of EZH2 are found in patients with various lymphoid (diffuse large B-cell lymphoma, follicular lymphoma) [63] and myeloid (myelodysplastic/myeloproliferative overlap syndrome, myelofibrosis) malignancies [19]. EZH2 is also frequently overexpressed in breast, bladder, and prostate cancer, and its expression levels correlate with higher proliferation rates and affect prognosis [64]. Interestingly, EZH2 mutations in hematologic malignancies can lead to loss or gain of function of the methyltransferase activity, which indicates that EZH2 can act either as a tumor suppressor or as an oncogene, depending on the cellular context and type of alteration [19].

Such bimodal behavior is also observed for the PRC1 member Cbx7. Cbx7 can act as an oncogene in the hematopoietic compartment and as a tumor suppressor in epithelial cancers. Mice transplanted with bone marrow cells overexpressing Cbx7 developed different types of leukemia [65]. Similarly, in human follicular lymphoma, overexpression of CBX7 can be detected [66]. On the other hand, loss of CBX7 expression in some epithelial cancers was associated with a more aggressive phenotype [67]. In general, polycomb proteins are involved in crucial pathways involved in both stem cell regulation and carcinogenesis.

In recent years, it has become evident that there is ample crosstalk between the polycomb protein system and DNA

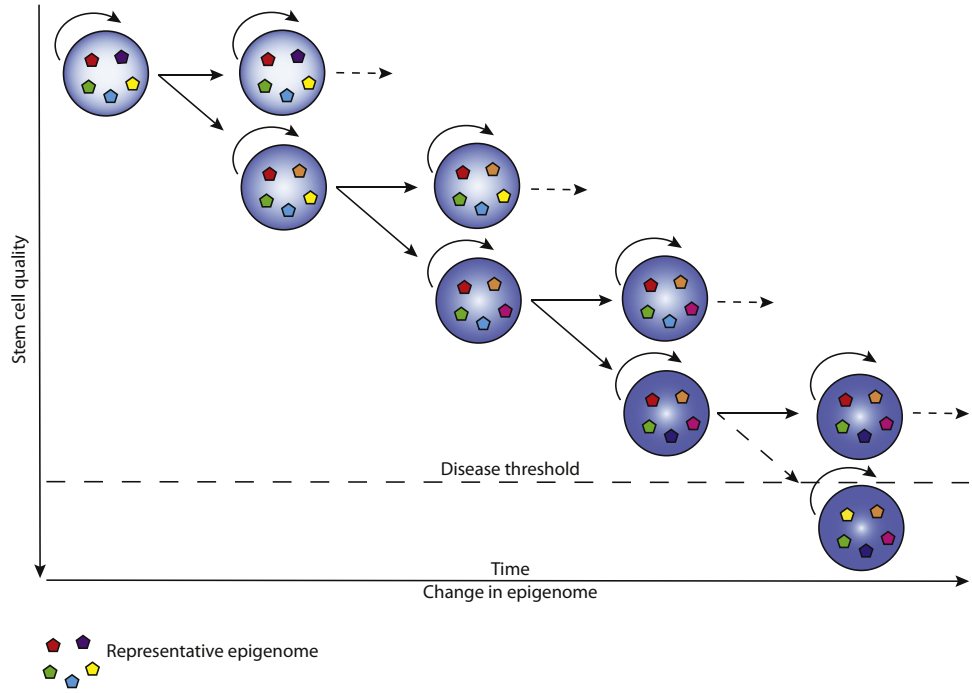

Figure 2. Young "high-quality" stem cells give rise to high-quality stem cells and differentiate into high-quality progeny. This process is at least partly regulated by the proper establishment of key epigenetic modifications. Changes in epigenome occur with each cell division, which can lead to a decline of stem and progenitor cell quality. Such potentially stochastic changes in the epigenome accompany the normal aging process. However, when epigenomic aberrations accumulate and proper stem cell potential is below a certain level (the disease threshold), hematologic disease ensues. It is important to note that not all cells cross the disease threshold with time, and conversely, young stem cells can also give rise to disease.

methylation. In embryonic stem cells, loci enriched for the EZH2 mark H3K27me3 exhibit nearly no overlap with those enriched for DNA methylation. In contrast, in somatic cells and cancer cells, there is substantial overlap between these two epigenetic marks [68–70]. Furthermore, embryonic stem cell promotors, which are enriched for H3K27me3, exhibit increased gain of DNA methylation during differentiation and carcinogenesis [71,72]. There are some indications that DNA methylating enzymes can be recruited by PRC2, as it was reported that EZH2 is able to interact with all three DNMTs and thereby recruit them to H3K27me3 loci [73].

Polycomb group proteins as therapeutic targets in cancer

Because epigenetic changes, unlike genetic lesions, are in principle reversible, pharmaceutical perturbation of the activity or composition of polycomb complexes might be a promising therapeutic approach. Such an approach may liberate cancer stem cells from excessive self-renewal and, in fact, introduce differentiation or apoptosis of cancer stem cells. Recent data indicate that pharmacologic inhibition of polycomb proteins may be a viable therapeutic strategy. However, these potentially drugable chromatin-modifying enzymes also occur in healthy cells. In addition, these enzymes may also display functions beyond their histone-modifying role, and thus, intervention in their functioning may result in off-target side effects [74].

In the near future, an increased molecular understanding of epigenetic mechanisms and their role in oncogenesis is likely to result in better and more targeted therapies in cancer patients. Eventually, this knowledge may also lead us to reconsider the aging process. If aging of the hematopoietic system can be explained in part by potentially reversible epigenetic changes, the aging process might be amenable to interventions aimed at slowing some of its deleterious effects. This could offer an option for preventing or treating some of the initial stages of a process that may ultimately lead to hematologic malignancy.

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