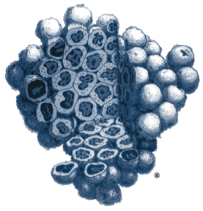

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Concise Review: Roles of Polycomb Group Proteins in Development and Disease: A Stem Cell Perspective

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Concise Review: Roles of Polycomb Group Proteins in Development and Disease: A Stem Cell Perspective

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ABSTRACT

The acquisition and maintenance of cell fate are essential for metazoan growth and development. A strict coordination between genetic and epigenetic programs regulates cell fate determination and maintenance. Polycomb group (PcG) genes are identified as essential in these epigenetic developmental processes. These genes encode components of multimeric transcriptional repressor complexes that are crucial in maintaining cell fate. PcG proteins have also been shown to play a central role in stem cell maintenance and lineage specification. PcG proteins, together with a battery of components including sequence-specific DNA binding/accessory factors, chromatin remodeling factors, signaling pathway intermediates, noncoding small RNAs, and RNA interference machinery, generally define a dynamic cellular identity through tight regulation of specific gene expression patterns. Epigenetic modification of chromatin structure that results in expression silencing of specific genes is now emerging as an important molecular mechanism in this process. In embryonic stem (ES) cells and adult stem cells, such specific genes represent those associated with differentiation and development, and silencing of these genes in a PcG protein-dependent manner confers stemness. ES cells also contain novel chromatin motifs enriched in epigenetic modifications associated with both activation and repression of genes, suggesting that certain genes are poised for activation or repression. Interestingly, these chromatin domains are highly coincident with the promoters of developmental regulators, which are also found to be occupied by PcG proteins. The epigenetic integrity is compromised, however, by mutations or other alterations that affect the function of PcG proteins in stem cells leading to aberrant cell proliferation and tissue transformation, a hallmark of cancer. STEM CELLS *2007;25: 2498–2510*

Disclosure of potential conflicts of interest is found at the end of this article.

INTRODUCTION

Even before the identification of DNA as genetic material, Conrad Waddington coined the term "epigenetics" to define the phenotypic execution of developmental programs [1]. Epigenetics now broadly describes the additional information superimposed on the genome that contributes to the heritable establishment and maintenance of transcriptional states and cellular identity [2–6] (see [5] for a recent commentary on the use of the term epigenetic). In molecular terms, epigenetic phenomena can be traced to the process of switching between lysis and lysogeny in lambda bacteriophage and to the inheritance of transcriptional repressors in bacterial progeny [7, 8]. As such, a single genome may be modified to produce multiple epigenomes allowing for cellular diversity, a necessary criterion for the development of metazoans [3]. Currently, research efforts are moving toward elucidating the mammalian epigenetic mechanisms that regulate cell identity and fate, particularly with reference to stem cells because of their emerging roles in development and disease [9–18].

Studies over the last decade have established a role for chromatin in regulating gene expression beyond the primary DNA sequence [19]. Except in mammalian spermatozoa, the basic unit of chromatin is generally a nucleosome, which is comprised of a histone octamer (a histone H3-H4 tetramer and two H2A-H2B dimers) around which DNA, 147 base pairs in length, is wrapped in 1.75 superhelical turns [20]. The nucleosomes are connected by the so-called linker DNA and the histone H1. The overall histone-dependent spatial organization of chromatin into higher-order structures has important implications for DNA-mediated transactions such as the regulation of DNA accessibility, which is essential for DNA repair, DNA replication, and gene transcription [21, 22]. Various known post-translational histone modifications are recently shown to be the part of epigenetic mechanisms regulating selective gene expression patterns to maintain cell identity and/or cell lineage specifications [23–25] (supplemental online Text Box 1).

Similarly, DNA methylation is recognized as an additional mechanism that can influence DNA accessibility. Although methylation occurs exclusively in the CpG dinucleotides of the vertebrate genome [26], nearly 40% of human genes do not manifest bona fide CpG islands in their promoters [27]. Non-CpG methylation (such as CNG and CNN, where N is any nucleotide) has also been observed significantly in embryonic stem (ES) cells as compared with somatic cells [28]. In contrast

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to somatic cells [29] and germ cells [30], the differential methylation pattern of only some pluripotency related genes correlates with their expression levels in human ES cell lines [31]. More recently, using mutant mouse ES cells that completely lack DNA methylation, it was shown that DNA methylation affects nuclear reorganization, histone modifications, and histone H1 binding but not the compaction of chromatin [32]. However, a functional link between histone methylation and DNA methylation has long been known [33, 34].

A variety of epigenetic regulatory factors are identified to play an important role in this process (supplemental online Text Box 1). For example, Polycomb group (PcG) and Trithorax group (TrxG) proteins have emerged as key players in gene regulation and are thought to function coordinately to orchestrate DNA accessibility throughout the organism's development [2, 35–37]. These epigenetic regulators act antagonistically to either promote (TrxG) or repress (PcG) transcription through regulation of specific amino acid modifications in histones. It is not known how the PcG and TrxG proteins switch and balance between transcriptionally silenced heterochromatin (for example, enriched in histone H3 lysine 27 trimethylation, H3K27me3) and transcriptionally competent euchromatin (for example, enriched in histone H3 lysine 4 trimethylation, H3K4me3), respectively, during development [36, 38]. Interestingly, recent high resolution profiling of histone methylations in the human genome revealed that monomethylations of at least H3K9, H3K27, and H3K79 are associated with gene activation, whereas their trimethylations are linked to repression [39]. In *Drosophila melanogaster*, the PcG and TrxG proteins are recruited to distinct DNA regions called Polycomb response elements and Trithorax responsive elements; it is not known whether vertebrates have similar cis-acting elements [40]. However, H3K4me3 is generally detected surrounding the promoter regions of transcriptionally active genes in mice and humans [41–43].

Additionally, the PcG proteins have been implicated in regulation of other processes such as cell cycle control [44], actin polymerization [45], cellular senescence [46], X-inactivation [47], genomic imprinting [48], and hematopoiesis [49, 50]. Interestingly, noncoding RNAs (including Piwi-interacting RNAs) and components of the RNA interference (RNAi) machinery colocalize with [51–53] or bind to the PcG proteins [54] and regulate heterochromatin formation and thus mediate transcriptional gene silencing [55–57]. As an example, Argonaute proteins, known to be the core components of effector complexes facilitating RNAi, have been demonstrated to induce the epigenetic silencing at the specific gene promoters [58]. Particularly, the human Argonaute-1 homolog directs the small interfering RNA-mediated transcriptional gene silencing by colocalizing with other RNA binding proteins and the PcG proteins on the promoter of a tumor suppressor gene *RASSF1A* and by inducing the formation of transcriptionally repressive histone methylations. Also, given the functional importance of microRNAs (miRNAs) in maintaining cell fate [59] and the binding of PRC2 components (SUZ12) to the miRNA genes cluster [60], it is just beginning to emerge that additional layers of control by the PcG proteins may exist during development and disease [61].

Due to the concise nature of this review, however, we will focus here on recent advances in PcG protein-mediated epigenetic control of stem cell self-renewal and maintenance of stem cell identity in metazoan development. How dysregulation of such epigenetic control systems in some stem-like cells may contribute to aberrations in development such as those that occur in cancer types of diseases will also be addressed.

Polycomb Group Proteins as Epigenetic Regulators

PcG proteins were first identified in *Drosophila* as regulators of the expression of Hox genes [62, 63]. PcG proteins are evolutionarily conserved and known to maintain specific repressive states of homeotic (Hox) gene expression patterns within body segments from flies to humans [38, 64]. Mechanisms by which this repression takes place at the level of transcription are not known. Moreover, the PcG gene families have been found to be increasingly diverse in vertebrates as compared with *Drosophila*, in which each type of PcG protein is encoded by a single gene [12, 37]. During every cell cycle transition, the PcG proteins maintain cellular identity by preserving chromatin states against chromatin disrupting processes such as DNA replication and transcription [35, 65]. In mammals, mutant embryos lacking the PcG proteins die during implantation stages [66–68]. Genome-wide analysis of *Drosophila* PcG protein targets recently identified genes associated with the transcriptional repression of developmentally important genes, including those implicated in cell proliferation and growth-factor-receptor signaling pathways [69, 70].

Polycomb Repressive Complexes

Primarily, the PcG proteins comprise two functionally and biochemically distinct multimeric Polycomb repressive complexes (PRCs), 2–5 MDa in size, called PRC1 and PRC2. Biochemical purification of PRC1 from human cells has revealed the presence of a number of subunits including BMI1/MEL18 (vertebrate ortholog of posterior sex combs), RING1A/RING1B/RNF2 (ring finger protein), hPC 1–3 (Polycomb), hPH1–3 (Polyhomeotic), and YY1 (Pleiohomeotic) among others [64]. PRC2 comprises the core components enhancer of zeste-2 (EZH2), suppressor of zeste-12 (SUZ12), and embryonic ectoderm development (Eed) [38]. Both the SUZ12 and the Eed are required for complex stability and for the methyltransferase activity of the EZH2 [66]. The EZH2-mediated transcriptional silencing depends upon the evolutionarily conserved catalytic SET (Su[VAR]3–9, Ezh2, Trithorax) domain, which imparts histone methyltransferase activity to the complex [71]. Components of PRC1 and PRC2 contain intrinsic histone modifying activities specific for ubiquitination of lysine 119 of histone H2A (H2AK119ub) and trimethylation of lysine residue 27 of histone H3 (denoted as H3K27me3), respectively [72]. Moreover, PRC2 has additional activity in lysine 26 of Histone H1 under certain conditions [73].

A general contention of the current models is that PRC2 initiates transcriptional repression by inhibiting transcription initiation, whereas the PRC1 maintains the repressive conditions (Fig. 1). Consistent with this model, Ezh2-mediated H3K27 methylation is required for function of PRC1 and Hox gene silencing [72]. It is important to note that functional mutations in the components of PRCs eliminate the ability of ES cells to be pluripotent [17, 74] and result in embryonic lethality [67, 75], although there are moderate phenotypes arising from the functional redundancy of PRC components (for additional references see [76]). Ring1b knockout ES cells or Eed-deficient ES cells fail to repress the expression of differentiation-specific transcription factors (for example, Msx1, HoxA7, and Gata4 [77]), supporting the contention of transcriptional repression by functional collaboration between the components of PRC1 and PRC2. However, Math1-like target genes are derepressed specifically in Eed mutant ES cells, suggesting the idea that the components of PRC1 and PRC2 do not share all target genes [78]. Interestingly, Suz12 has been found to be required for

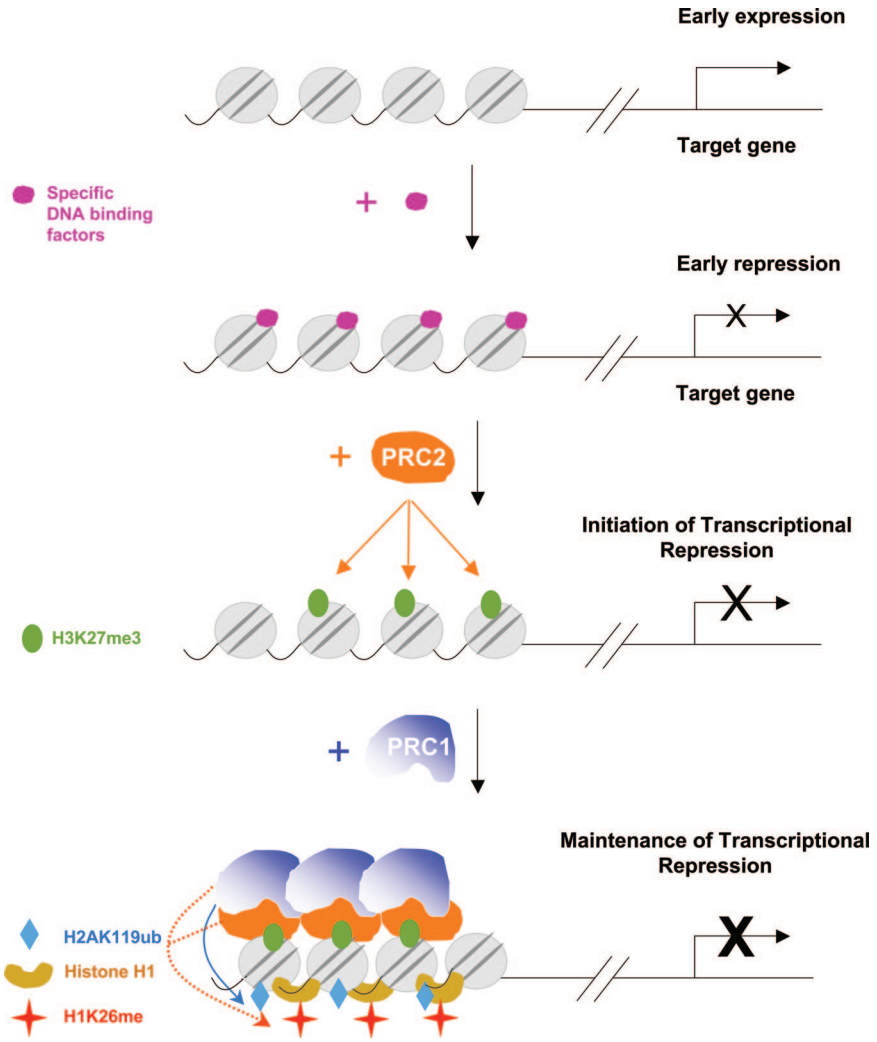

Figure 1. Schematic representation of hierarchical chromatin binding of polycomb group proteins. PRC1 and PRC2 represent multiprotein complexes and their cooperative recruitment to nucleosomes. Early binding of some DNA binding factors facilitates the recruitment of PRC2. Enhancer of zeste-2 (EZH2), the PRC2 component, forms a trimethylation mark on H3K27 (H3K27me3) that serves as a mark for PRC1 recruitment. PRC1 is thereby recruited via additional cooperative effects with the DNA, DNA binding factors, and PRC2. Monoubiquitination of histone H2A is accomplished by the PRC1 component RING. Differential EZH2-containing PRCs may exhibit H1K26 methyltransferase activities. Cumulatively, PRC2 and PRC1 may induce chromatin compaction, as the former initiates transcriptional repression and the latter maintains the repression. (Modified from [210] with permission.) Abbreviation: PRC, Polycomb repressive complex.

H3K9me3 formation only in differentiated, but not in undifferentiated, mouse ES cells. Furthermore, human SUZ12 regulates the formation of H3K9me3 independent of EZH2 [79], implying that the components even within the PRC2 may not share all the targets. Recent studies in *Drosophila* have shown that the PCG-protein-silenced chromatin is not overlapped with HP1/histone H3K9me3 [80]. These observations suggest that multiple types of heterochromatin may exist, yet further studies are needed to verify this possibility.

PRC Diversity

Although several functionally redundant subunits of PRC1 have evolved in vertebrates [12], core components of the PRC2 and the related PRC3 or PRC4 are evolutionarily conserved [75]. Diversity of the PRCs may also be conferred by additional proteins (for example, TAFs [TATA box-binding protein-associated factors] [81] or Histone deacetylases [82]). These complexes are developmentally regulated and often become heterogeneous even within the same cell [83, 84]. For example, different Ezh2 complexes have been identified that contain one of at least four Eed isoforms [71, 73]. The largest isoform, Eed1, is involved in formation of the PRC2, whereas the two shortest forms, Eed3 and Eed4, are associated with the PRC3 complex. Eed2 is specifically present in undifferentiated ES cells as well as in cancer cells as part of the PRC4 complex. The accumulation of PRC4 is also associated with overexpression of Ezh2 [85], observed during the late stages of prostate cancer [86]. Isoform-specific interactions and developmental stage-specific functions of PRC components may influence the eventual epigenetic outcome. For example, H3K27 or H1K26 specificity depends on which isoform of Eed associates with the Ezh2 [85]. PRC3-mediated H3K27 methylation is not favored in the presence of histone H1, whereas the PRC2 methylates both H3K27 and H1K26 in the presence of Histone H1 [85]. It remains to be determined what role the histone H1 plays in this context. As another example, the Polycomb homolog Chromobox 7 (Cbx7) was recently identified and found to immortalize various cell types by transcriptional repression similar to that of Bmi1 [87]. The Bmi1 and the Cbx7 associate biochemically with distinct repressive complexes; however, the Cbx7 cannot induce telomerase activity like that of Bmi1 to enhance cellular lifespan. Thus, the composition of PRCs, histone substrate specificity, and interaction with chromatin may be distinct and highly dynamic in a cell type-, cell cycle-, signaling pathway-, and gene-dependent manner [85, 88, 89–91] (supplemental online Text Box 2).

From Epigenetic to Genetic Modifications

A physical interaction among the PcG protein, EZH2, and DNA methyltransferases (DNMTs, which attach methyl groups to DNA and thereby establish chromatin silencing) has been suggested to be essential for transcriptional repression in somatic

cells [92]. EZH2 is also suggested as a recruitment platform for DNMTs. Although the precise signal that triggers PRC2-mediated recruitment of DNMTs remains to be understood [37], the EZH2-dependent formation of H3K27me3 has been considered a requirement for DNA methylation of regulatory regions of the genes. However, it is unclear whether the DNMTs directly or indirectly recognize the H3K27me3 through EZH2 or some other unknown interaction partners conferring specificity to gene-specific promoter DNA for methylation [93]. It has also been suggested that DNMT1 facilitates the proper localization of PRC1 components such as BMI1 [94]. Thus, establishment and maintenance of cellular memory in somatic cells appear to be regulated by both the histone and DNA methylation-dependent epigenetic mechanisms [92].

The Role of PcG Proteins in Stem Cell Maintenance

Epigenetic differences in chromatin have been detected as early as the 4-cell stage of the mouse preimplantation embryo [95]. These cells, referred to as blastomeres, are transiently totipotent (each cell can develop into a complete organism). ES cells are derived from the inner cell mass (ICM) of the blastocyst stage of developing mammalian embryo and are maintained as in vitro cell cultures, whereas adult stem cells are found in differentiated tissues of the adult organs that maintain organ tissue homeostasis [96, 97]. ES cells can differentiate into all cell types of an adult organism (pluripotency), but adult stem cells can form only the cell types of their parental organ (multipotency). Primordial germ cells and organ tissue adult stem cells that appear during later stages of embryo development maintain multipotency for relatively prolonged periods of life. In vitro cultures of primordial germ cell layers can also generate pluripotent embryonic germ cells [98].

PcG proteins are highly abundant in ICM that forms the embryo proper and also in both ES and adult stem cells. These high levels of PcG proteins decline as stem cells begin to differentiate [99]. Consistent with its early embryonic expression pattern, Ezh2 is highly expressed in ES cells and is required for the derivation of ES cells [67]. It is interesting to note that, although Ezh2 and Eed levels decline during stem cell differentiation, Suz12 levels remain constant, suggesting that stem cell maintenance may require a particular composition of PRCs [100]. Recently, Suz12 was also found to be required for ES cell differentiation-specific gene expression programs [101]. Using a conditional knockout strategy, Ezh2 was also shown to be required for H3K27me3 formation in the pluripotent epiblast cells that ultimately give rise to derivatives of the three germ layers [102]. Several recent studies have highlighted roles for PcG proteins in the maintenance of pluripotency in both ES cells and adult stem cells by silencing key lineage specific transcription factor genes, thereby preventing differentiation [74, 77].

ES cells manifest largely the euchromatin associated with activating histone marks, whereas heterochromatin accumulates by associating with silencing histone marks during differentiation [103]. Global analysis of PcG protein binding in ES cells identified promoters of a large number of genes associated with differentiation, suggesting that PcG proteins help to maintain the silencing of these genes in undifferentiated ES cells. Expression of these cognate genes is mostly repressed at the transcriptional level in ES cells. Consistent with this contention, the PcG gene mutants also lose the ability to maintain ES cells in the undifferentiated state [60, 104]. PcG-protein-mediated stem cell maintenance is a biologically dynamic process and not a consequence of permanent silencing. For example, during the onset of stem cell differentiation, PcG protein binding as well as H3K27me3-like chromatin repressive marks decline at the target gene loci. This is coincident with a release from PcG-mediated transcriptional repression.

Moreover, some of the PcG protein targets are associated with activating transcription factors but remain silent in expression (poised for activation) or active in stem cells but become repressed upon differentiation (poised for repression) [105] (more details on this aspect are in the novel chromatin domain section). For example, crucial transcription factors involved in lineage specification, such as Msx1, Nkx2–2, Pax3, and Sox1, are not expressed in ES cells but are associated with both active (H3K4me3) and repressive (H3K27me3) histone modifications. It is interesting to note that, in ES cells, the active promoters of pluripotency genes such as octamer-binding transcription factor 4 (OCT4) and NANOG are associated with transcriptionally active histone modifications [106]. Particularly, these ES cell pluripotency regulators co-occupy a significant subset of PcG protein targets that are the key developmental regulators and must be silenced to maintain ES cell identity [60, 107, 108]. Moreover, knockdown of the OCT4 levels also reduced PcG protein binding at the target genes [109]. Together, these results suggest a possible functional interaction between the ES cell pluripotency transcription factors and PcG proteins in maintaining stem cell identity exists, although their physical associations have not been firmly established. It has also been shown that levels of Oct3/4 were inversely correlated with the association of Ezh2, H3K27me3, and mPC3 (a mouse homolog of *Drosophila* Polycomb) on the Oct3/4 promoter during retinoic acid-induced differentiation of mouse embryonic carcinoma (P19) cells [71].

In addition to their role in early embryonic development, PcG proteins have also been implicated as essential regulators of adult stem cell maintenance, including stem cell aging [110–112]. For example, hematopoietic stem cell (HSC) proliferation appears to be regulated by a balance between Eed and Bmi1 levels, whereas overexpression of Ezh2 preserves the long-term (memory) repopulating potential of the HSCs over serial transplantations [99, 112–115]. Although Bmi1 is important for neural stem cell renewal [116–118], Ezh2 is necessary for maintaining muscle cell precursors called myoblasts [119]. Together, these studies suggest a direct role for PcG proteins in regulating self-renewal and the maintenance of stem cell identity.

On the other hand, DNA methylation profiles of various human ES cells are distinct from other human cell types including normal tissues, embryonal carcinoma cell lines, adult stem cell populations, lymphoblastoid cell lines, and cancer cell lines [120]. However, this epigenetic signature is dispensable for ES cell propagation [121–125]. Consistent with this, DNA methylation levels are extremely low in the preimplantation embryo and increase dramatically upon implantation and gastrulation, the stage at which the pluripotent ICM gives rise to all three primary germ cell layers [103]. Thus, it appears that PcG-mediated gene repression is a dynamic process in early embryos and in ES cells in accommodating global changes in gene expression that accompany differentiation.

Genome-wide demethylations occurring in preimplantation embryos are considered essential for pluripotency development in epiblast cells, whereas those in primordial germ cells (PGCs) are considered to ensure that aberrant epigenetic modifications are not transmitted to the progeny and maintain equivalent epigenetic state in the germ line of male and female embryos [126]. There is a strict developmental regulation in the sequential conversion of one entire X chromosome from a euchromatin state to a heterochromatin state. This is triggered by the expression and cis-localization of a large noncoding RNA (Xist

RNA—the chromosome X inactive specific transcript, encoded by the Xist gene) as well as mediated by its recruitment of PRC2 [127–129]. For example, Suz12 levels are found to be enriched during the early stages of X chromosome inactivation (Xi) in a Xist RNA-dependent manner [100]. Although Eed is dispensable for initiation of random Xi [130], it protects the inactive X-chromosomes from differentiation-induced reactivation [131]. It has also been shown that there is an imprinted, Xist RNA-dependent Eed-mediated inactivation of the paternally inherited X chromosome in trophectoderm and primitive endoderm lineages of mouse embryos [127]. The trophectoderm is the first differentiating extraembryonic lineage of the ectoderm of mammalian embryos at the blastocyst stage, and it differentiates into a trophoblast that mediates the implantation of fetus into placenta. The mitotically stable association of PcG proteins with the inactive X chromosome has been subsequently identified in trophoblast stem cells [132]. Furthermore, PRC1 components link ubiquitylation of histone H2A to heritable gene silencing and Xi by differentially utilizing the PRC1 components in trophoblast versus ES cells [133]. Although the X inactivation does not occur in undifferentiated mouse ES cells, it is not known how an X chromosome is chosen to be inactivated as the ES cells begin to differentiate and what epigenetic role, if any, the PcG proteins play in this important developmental program (Mary F. Lyon, personal communication). An extensive erasure of the previous epigenetic modifications followed by a fresh epigenetic reprograming during development of the PGCs from proximal epiblast cells also takes place [134]. Particularly during migration of the PGCs to genital ridges [135] and before their entry into meiosis, one of the 2X chromosomes is randomly inactivated in the PGCs, like that in XX somatic cells [136]. It remains unclear how PcG proteins maintain the silent state of Xi once it is established and how they facilitate the Xi inheritance over subsequent cell generations throughout the life of an organism.

Genomic Maps of PcG Protein Localization in Embryonic Stem Cells

Given the important role of PcG proteins in cellular identity and stem cell maintenance, it is essential to identify their molecular targets. Several groups have utilized mouse ES [104] and human ES cells [60] to identify the global targets of PcG proteins and the epigenetic events associated with these cells. Interestingly, similar studies with other comparable ES cells [105, 109, 137], human fibroblasts [78], *Drosophila* cells [36, 69, 138], and other distinct cell types such as mouse F9 teratocarcinoma cells, human testicular germ cell carcinoma NTERA2 cells, human MCF7 breast cancer cells, and human SW480 colon cancer cells [109] have revealed a highly conserved role for PcG proteins throughout evolution.

The components of PRC1 (Phc1, Rnf2) and PRC2 (Suz12, Eed) as well as the repressive histone modification H3K27me3 colocalize at promoter regions of hundreds of target genes in mouse ES cells [104]. Nearly all of the PcG-protein-bound regions are detected proximal to transcriptional start sites, and most of the target gene transcripts are lower in abundance in mouse ES cells compared with differentiating embryoid bodies. Mutations in PRC2 components such as Eed [104] and Suz12 [78] resulted in the derepression of a large pool of target genes and rapid differentiation. These data are consistent with a direct role for PcG proteins in gene repression associated with transition from cellular pluripotency to their lineage commitment. The genes occupied by PcG proteins are found to encode regulatory factors with known roles in developmental processes such as organogenesis, morphogenesis, pattern specification, neurogenesis, cell differentiation, embryonic development, and cell fate commitment. Upon closer examination, it is determined that many of the developmental regulators could be identified as lineage-specific transcription factors. Similar conclusions are drawn by mapping SUZ12 binding throughout the human ES cell genome [60]. Except for the overexpression of a small percent, most of the genes occupied by SUZ12 in human ES cells are transcriptionally repressed and also represent a wide range of key developmental regulators similar to those observed with mouse ES cells. These studies indicate that PcG proteins maintain pluripotency by targeting a large cohort of developmentally important regulators in ES cells, whose expression would otherwise lead to differentiation.

According to this model, the PcG-mediated gene repression would have to be dynamic in order to allow for the subsequent activation of these genes during differentiation. Consistent with this contention, Boyer and colleagues [104] observed markedly reduced levels of PcG binding and H3K27me3 at neural-specific genes upon directed differentiation of ES cells into neural precursors. The authors also reported that an increase in H3K4me3 levels and RNA Polymerase II occupancy at these genes coincides with their expression. Conversely, the H3K27me3 is maintained at genes that are expected to be permanently silent in these lineage-committed cells. Similar results are observed when human ES cells are compared with differentiated muscle cells [60]. These results indicate that PcG protein target genes maintain the potential to become activated or stably silenced during lineage specification.

Genome-wide studies also have been performed in human embryonic teratocarcinoma cell line NTERA2 (NT2/D1), an adult progenitor cell model that has neural progenitor cell properties and the potential for directed differentiation into neural lineages [78]. These studies revealed similar roles for the PcG protein target genes, where a loss in PcG protein binding correlated with a decrease in H3K27me3 levels and the activation of differentiation genes. Remarkably, the PcG protein targets (such as OLIG2, NEUROG2, MT1G, and HOXA7–13 locus genes) that become repressed during differentiation are found to be occupied by the PcG proteins in the undifferentiated NTERA2 cells [78]. Moreover, there has been a marginal increase in further PcG protein binding at the target DNA upon induction of neuronal differentiation, suggesting that developmental genes are marked for activation during ES cell differentiation and that stem cell maintenance genes may also be cued for subsequent repression. These results also point out that PcG protein binding alone does not strictly correlate with transcriptional repression and are consistent with the idea that transcriptional states result from a balance between both activating and repressive activities.

Thus, it is likely that cell type-specific accessory factors function with PcG and TrxG proteins to fine-tune gene expression. This contention also suggests that changes in the overall composition of PcG complexes, such as those that occur during development, would result in the alteration of gene expression patterns and cellular identity. Although it has been firmly established in *Drosophila* that PcG proteins are directed to target genes through interactions with site-specific DNA binding factors [119, 139, 140] (for a review see [38]), it is not known how PcG complexes are recruited to genes in mammals. The observation that OCT4, SOX2, and NANOG localize to approximately one-third of PRC2-occupied regions in human ES cells suggests that these transcription factors may direct the repression of target genes through interaction with PcG proteins [60]. On the other hand, these same transcription factors are also associated with RNA polymerase II-occupied regions that are transcriptionally active in human ES cells [60]. Thus, the OCT4, SOX2, and NANOG proteins may play a role in directing both PcG and TrxG proteins to target sites in ES cells, but it is likely

that other factors are also required. However, the molecular mechanisms behind the initiation of these processes and how they are maintained still remain to be determined.

Novel Chromatin Domains in ES Cells and Their Association with PcG Proteins

Elucidating the epigenome across all cell types is important, particularly in view of their contributions to maintaining transcriptional states over many generations. ES cell chromatin is characterized by less condensed and transcriptionally active euchromatin, whereas lineage commitment is marked by an increase in highly condensed and transcriptionally inactive heterochromatin [9, 141]. Recent genome-wide studies describing an initial epigenetic profile of ES cells have yielded surprising results [137]. In order to explore regulatory elements in the genome, Bernstein and colleagues utilized ChIP combined with oligonucleotide tiling arrays containing representatives of mouse loci enriched for highly conserved noncoding elements (HCNEs). Interestingly, these HCNEs cluster near genes that encode developmentally important transcription factors. The majority of these genes are also reported to be targets of PcG-mediated transcriptional repression.

These authors identified a novel chromatin modification pattern consisting of both repressive H3K27me3 and activating H3K4me3 histone modifications called "bivalent domains." As has been initially identified in mouse ES cells, the majority of bivalent domains are resolved into either transcriptionally repressive H3K27me3 or transcriptionally permissive H3K4me3 in the differentiated cell types. Many of the bivalent domains in the HCNE regions overlap with transcription start sites, and it has been hypothesized that the bivalent domains repress these genes while maintaining them in a state poised for subsequent activation upon differentiation. These authors also raised the possibility that the HCNEs may promote chromosome conformations such that epigenetic switching is facilitated [137]. An independent study showed a similar coexistence of histone marks for both the active and the repressive chromatin in ES cells that maintain pluripotency [105]. This study also reported that genes associated with dual histone marks exhibited characteristics of euchromatin such as early DNA replication during S-phase, indicating that these silent genes are maintained in a transcriptionally permissive state.

These bivalent chromatin domains appear to be enriched in ES cells. Initial studies did not identify them in multipotent neural and hematopoietic cells [6, 105, 137], but a later study provided evidence that such structures exist in the primary human T cells [43]. Genetic code and epigenetic landscape are thought to cooperatively direct the recruitment of transcriptional regulators to promoters [142]. Bernstein and associates have also identified a significant correlation between epigenetic modification and the underlying DNA sequence, suggesting that histone modifications rest on a particular genetic landscape [137]. For example, 95% of the transcription start sites with H3K4me3 sites are associated with dense CpG islands and often devoid of transposons [137]. Teleologically, these bivalent domains are comparable with the bithorax locus during early stages of *Drosophila* development, where both PcG and TrxG proteins are present [137, 143]. However, the mechanisms that establish the initial conditions by which the PcG proteins are recruited to such specific sites, as well as maintain the particular chromatin landscape necessary for ES cell pluripotency, represent a gap in our understanding of stem cell identity and maintenance.

The Role of PcG Proteins in Oncogenesis

Maintaining prolonged quiescence (to remain without undergoing cell division), self-renewal capacity, and multipotency are the hallmarks of adult stem cells. Self-renewal in adult stem cells occurs predominantly by extensive cell divisions that occur symmetrically or asymmetrically. Asymmetrical cell divisions preserve the maintenance of stem cell number and cellular homeostasis, whereas symmetrical cell divisions may lead to aberrant cellular differentiation [144]. Oncogenesis is an aberrant differentiation in cells that involves the inappropriate regulation of developmental genes and cellular signaling pathways leading to the onset of cancer [145]. Besides the known genetic changes that occur in cancer, such as deletion of tumor suppressor genes, amplification of oncogenes, and loss of heterozygosity or gene mutations in tumor associated genes [145], epigenetic lesions such as altered DNA methylation, misregulation of chromatin remodeling by histone modifications, and aberrant expression of PcG proteins have emerged as common hallmarks of many cancers [109, 146–150] (supplemental online Text Box 3).

Moreover, epigenetic abnormalities cooperate with genetic alterations in manifesting various cancer phenotypes [16, 92]. For example, genome-wide histone modifications are common hallmarks of human cancers [151, 152]. Similarly, there are aberrant methylations of large DNA regions in cancer genomes [148, 153]. Approximately 50% of the genes associated with familial cancers represent mutations leading to methylation-correlative gene silencing in various sporadic forms of tumors [147]. Not surprisingly, aberrant expression of PcG and TrxG proteins has also been implicated in tumorigenesis [10, 154, 155]. Components of PRC1 (such as BMI1 [10]) and PRC2 (such as EZH2 [156]) are amplified and/or overexpressed in a broad spectrum of cancers (Table 1). Dysregulation in the expression of PRC components is associated with alterations in PcG protein compositions [85] as well as with target gene affinities [109]. Moreover, pharmacological disruption of PRC2-dependent gene repression induced apoptosis only in cancer cells and not in normal cells [157].

It is now understood that a strict functional equilibrium between oncogenes and tumor suppressor genes regulates stem cell function throughout life [60, 78, 104, 158, 159]. Any dysregulation in such networks, such as those that normally occur with aging [160, 161] or inflammation [162, 163], may result in the development of cancers, depending on the degree of aberrations found in the levels and function of PcG proteins. Thus, the epigenetic aberrations are thought to play a central role in early neoplastic transformation based on the contention that stem cells in tumor tissues constitute a substrate for these early epigenetic alterations [14, 16, 164].

Cancer Stem Cells—Epigenetic Control

Cancer is largely a heterogeneous disease; therefore, epigenetic variations in progenitor cells may contribute to tumor heterogeneity. Non-neoplastic but epigenetically abnormal stem and progenitor cells are therefore suggested as cancer-risk target cells [14]. Analogous to the role of adult stem cells in tissue homeostasis, tumors from several cancer types are also found to contain a minor population of tumor-initiating cells, called tumor-initiating cells, stem-like cancer cells, or cancer stem cells [165, 166]. Similar to adult stem cells, the cancer stem cells are able to maintain quiescence for prolonged periods and self-renew while maintaining the ability to differentiate into multiple cell types. Cancer stem cells are thought to originate from

Table 1. Polycomb group (PcG) proteins and associated human cancers

Drosophila homolog	Human homolog	Protein domain/function	Expression	Cancer type	Reference
PRC2 initiation complex					
Enhancer of zeste, E(z)	EZH2	SET/Histone methyl transferase	Gene amplification and/or overexpression	B-cell non-Hodgkin lymphoma	[216]
				Bladder	[156, 217–219]
				Breast	[156, 186, 220–222]
				Colon	[156, 186, 220–223]
				Glioblastoma	[156]
				Hodgkin lymphoma	[224]
				Larynx	[156]
				Liver	[225]
				Lung	[156]
				Mantle cell lymphoma	[226]
				Melanoma	[156, 221]
				Prostate	[86, 211, 214, 221, 227–229]
				Sarcoma	[156]
				Stomach	[156, 230]
				Testis	[156]
				Uterus	[221]
Suppressor of zeste, Su(z)	SUZ12	Zinc-finger domain	Overexpression	Breast	[231]
				Colon	[153, 231]
				Liver	[231]
PRC1 maintenance complex					
RING	RING1/RNF1/RING1A/ RNF2/RING1B	RING-finger domain/ ubiquitin ligase	Overexpression	Prostate	[227]
				Bladder	[212]
				Breast	[212]
				Cervix	[212]
				Colon	[212]
				Kidney	[212]
				Larynx	[212]
				Liver	[212]
				Lymphoma	[212]
				Non-small cell lung cancer	[212]
				Ovary	[212]
				Pancreas	[212]
				Parathyroid	[212]
				Prostate	[212]
				Thymus	[212]
				Thyroid	[212]
				Uterus	[212]
Posterior sex combs, Psc	BMI1	RING-finger domain/ ubiquitylation	Gene amplification and/or overexpression	B-cell non-Hodgkin lymphoma	[216]
				Bronchial squamous cell cancer	[232]
				Cervix	[212]
				Colon	[212, 233]
				Ependymoma	[212]
				Head and neck squamous cell cancer	[234]
				Hodgkin	[235]
				Leukemia	[236]
				Liver	[212]
				Mantle cell lymphoma	[212, 213]
				Medulloblastoma	[116]
				Meningeoma	[212]
				Nasopharyngeal carcinoma	[237]
				Neuroblastoma	[238]
				Non-small cell lung cancer	[239, 240]
				Oral cancer	[241]
				Pituitary	[212]
				Prostate	[211, 227]
Pleiohomeotic, Pho	YY1	Zinc finger/sequence-specific DNA binding	Overexpression	Prostate	[152]
				Retinoblastoma	[242]
Polycomblike, Pcl	PCL3	PHD	Overexpression	Cervix	[243]
				Colon	[243]
				Liver	[243]
				Lung	[243]
				Rectal	[243]
				Skin	[243]
				Uterus	[243]
Polyhomeotic, Ph	RAE28	Not known	Loss of heterozygosity	Acute lymphoblastic leukemia	[215]

Besides aberrant overexpression in various cancers, genes encoding the PcG proteins are also amplified. For example, the BMI1 is found to be amplified in prostate cancer [211], mantle cell lymphoma [212, 213], and pituitary adenoma [212]. Similarly, EZH2 is amplified in prostate [211, 214], breast [156, 186], and also many other organ cancers [156]. To date, RAE28 is the only PcG protein found to be associated with loss of heterozygosity in cancers such as hematologic malignancy (acute lymphoblastic leukemia) and consequent loss of its expression [215].
Abbreviations: BMI1, B-cell-specific Moloney murine leukemia virus insertion site 1; EZH2, enhancer of zeste-2; PHD, plant homeodomain protein; PRC, Polycomb repressive complex; RAE28, polyhomeotic gene product isolated from retinoic acid differentiated murine embryonal carcinoma cells; SET, Su(var)3-9, Enhancer-of-zeste, Trithorax.

mutations in adult stem cells that have sustained specific genetic alterations or by dedifferentiation of mature somatic cells to reacquire stem cell characteristics [167, 168]. The opposing effects of different PcG proteins, such as between Cbx2 and Bmi1, Mel18 and Bmi1, or Eed and Bmi1, may disrupt the delicate balance between self-renewal and differentiation, leading to cancer development [87, 115, 169]. Consistent with the latter concept, embryonic stem cell maintenance genes such as OCT4 and NANOG are also found to be inappropriately expressed in many human tumors [170–172]. Particularly, Gidekel et al. demonstrated that Oct4 endures oncogenic potential to ES cells in a dose-dependent manner [172]. Activation of Oct4 results in dysplastic growth in epithelial tissues and is dependent on continuous Oct4 expression [173]. Based on the observation that Oct4 expression causes dysplasia by inhibiting cellular differentiation in the intestine, it has been proposed that adult progenitors can integrate the key embryonic signals to drive tumorigenesis [174].

Because of the fundamental roles of PcG genes and histone modifications in stem cell maintenance and tumor development, it is suggested that the oncogenic potential of PcG genes such as BMI1 and EZH2 may also facilitate the maintenance of cancer stem cells [158, 165]. It is also known that the isoform-2 of Eed is expressed in cancer and undifferentiated ES cells but not in differentiated ES cells [85]. Similarly, high levels of Suz12 are found in adult tumor cells, whereas they are expressed at low levels in normal adult cells [109]. It is therefore possible that overexpression of PRC components may affect the delicate balance in the normal stoichiometry of PcG proteins. In fact, it has been found that Suz12+/Eed+ and Suz12+/Eed− targets represent different functional groups in tumor cells and that Oct4-like factors may facilitate the recruitment of Suz12 to certain subsets of gene promoters characterized as Oct4+/Suz12+/RNA polymerase II− [109]. This may not only alter the histone methylation profile but could also induce transcriptional aberrations required for cellular transformation [85, 175].

PcG protein target genes have been found to display a greater likelihood of acquiring specific promoter DNA hypermethylation during cancer progression than nontarget genes [104, 112–114]. In principle, the reversible epigenetic transcriptional silencing pattern in stem cells is replaced by a putative PRC-DNMT crosstalk-mediated aberrant DNA methylation. This could potentially predispose the affected stem cells to become cancer precursor cells, suggestive of a stem cell origin of cancer [113]. A large percentage of gene promoters that are methylated in cancers (colon cancers) appears to be premarked with the H3K27me3 pattern and by upregulated PRC components [176]. The PcG protein-bound genes with hypermethylated promoter DNAs in adult human cancer cells are not methylated in both the normal ES cells and their malignant counterparts, namely the embryonal carcinoma (EC) cells [168]. Unlike cancer cells, the EC cells maintain multilineage potential. During regular embryonic development, this chromatin pattern permits only normal gene activation by developmental cues. However, EC cells are found to have acquired two additional key repressive marks, such as H3K9me3 and H3K9me2 as an epigenetic transition state. The combination of both the H3K27me3 and the H3K9me3/2 marks is thought to foster a shift from the transient silencing of these crucial tumor suppressor gene promoters in stem and/or progenitor cells to a heritable gene silencing by acquiring the aberrant PcG levels, which induce de novo hypermethylation of CpG islands of the promoter DNAs in cancer stem cells [104, 153, 176]. Thus, it appears that there exists a fine control of epigenetic gene silencing mechanism in adult tumors (Fig. 2) [177].

Of interest, the p16*INK4a*-ARF locus represents one of the above identified candidates with hypermethylated promoter DNAs in cancer cells but not in normal cells. This locus encodes two alternatively spliced gene products, the tumor suppressor

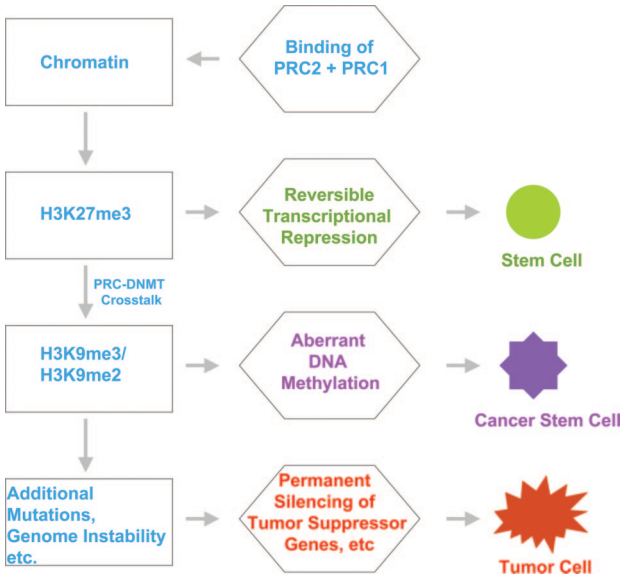

Figure 2. Schematic model of epigenetic changes in the onset of cancer. Graded progression of epigenetic marks from reversible gene repression in stem cells (including embryonic stem cells) via transiently aberrant promoter DNA hypermethylation in cancer stem/precursor cells (embryonal carcinoma cells) to the "locked-in state" of gene silencing in cancers (e.g., tumor cells). (Drawn after [153, 168, 176].) Abbreviations: DNMT, DNA methyltransferase; PRC, Polycomb repressive complex.

protein p16*INK*4a (an inhibitor of cell cycle progression) and ARF (a regulator of p53) [178]. BMI1 is a well-known repressor of p16*INK4*a and, in some cases, such as in mammalian cells, ARF genes [179, 180]. Bmi1 and Ezh2 promote the self-renewal of adult stem cells as well as aberrant proliferation of cancer stem cells by repressing the p16*INK4a*-ARF locus [117, 154, 155, 181]. Importantly, this repression has been shown to require a direct association of BMI1 and depends on the formation of H3K27me3 by the EZH2 containing PRC2 [182, 183]. The presence of EZH2 on the *INK4A-ARF locus* [183] and the fact that EZH2 can act as a platform for DNMTs [92] are consistent with the observations of a crosstalk between the PcG proteins and the DNMTs during cancer progression.

Alternatively, during the induction of senescence by cellular aging and stress, the activation of the p16*INK*4a-ARF locus is shown to depend on an associated decrease in the EZH2 level, the concomitant loss of the H3K27me3 mark, and the consequent displacement of BMI1 [180, 183–185]. Expression of EZH2 has long been known as the functional marker that distinguishes indolent prostate cancers from those at risk for lethal progression [86]. Similarly, elevated EZH2 expression detected preneoplastic lesions in morphologically normal in vivo breast epithelium and distinguished histologically normal breast tissues at elevated risk for transformation [186]. Small interfering RNA-mediated knockdown of EZH2 also resulted in a reduction of the H3K27me3 mark as well as in the loss of clonogenic survival of acute myelogenous leukemia cells [187]. Thus, it may be interesting to investigate whether the overexpression of EZH2 in stem cells could not only confer a resistance to stress and aging but also permit these cells with a time frame for acquiring additional genetic lesions that would perpetuate them with malignant phenotypes [183].

Although the p16*INK*4a locus is enriched by H3K27me3 and bound by the PcG proteins, the silencing of p16*INK*4a is found to strictly depend on functional pRB family proteins that bind to and negatively regulate E2F transcription factors in mouse and human primary cells [182]. This represents the first evidence that pRB

proteins function upstream of the PcGs and also for the link between the function of recruited PcG proteins and the formation of H3K27me3. Some of these regulatory processes appear to manifest regional differences among tissue types with which the stem or the progenitor cells are associated [180]. Cell intrinsic and/or cell extrinsic signals may integrate into the epigenetic regulation of gene expression [9], possibly by facilitating a selective discrimination of CpG islands in the promoter DNAs that are potentially vulnerable to aberrant hypermethylations [188]. Which cells are chosen, when and how they are targeted to be cancer cell progenitors, and the nature of molecular signals, epigenetic or otherwise, involved still remain to be understood.

The stem cell microenvironment in adult tissues is known to form a physiological niche for their maintenance [96, 189, 190]. Similarly, the tumor microenvironment is required for the maintenance of cancer stem cells [191, 192]. This raises an important question with regard to the functional role of such niches in discriminating normal adult stem cells from cancer stem cells. Preliminary evidence is reported for cell type-specific epigenetic changes at the level of DNA methylation in the stromal cells, such as epithelial and stromal fibroblasts from normal breast and breast carcinomas [193]. The molecular mechanisms and biological significance of such epigenetic changes in stromal cells of the tumor remain unknown.

Perspectives and Outlook

PcG proteins are known to function as epigenetic switches on chromatin that delineate both repressive and derepressive gene signals, thereby maintaining a particular cell identity or allowing differentiation. These epigenetic processes are also shown to modulate the properties of stem cells, namely self-renewal, maintenance of pluripotency, lineage specification, and neoplastic transformation. In ES cells, the pluripotent transcription factors have been suggested to associate with the PcG proteins and exert both positive and negative control on the expression of factors related to stem cell maintenance and differentiation, respectively [104]. How chromatin architecture remains stable over many cell generations by propagation of its prior active and inactive chromatin states, however, is an important yet unresolved question in genome biology. How these molecular states are changed upon lineage specification also remains an enigma. To date, there is no evidence that PcG protein-mediated histone modifications directly define the cellular epigenetic state, although such modifications correlate with the different epigenetic states of cells. What regulates the expression of PcG proteins, such that PcG protein homeostasis is maintained as a function of cell identity and/or cell fate determination, also remains to be understood (supplemental online Text Box 3).

It is likely that DNA sequence and associated PcG proteins contribute to the overall epigenetic landscape in stem cells [137]. Cell-type-specific novel PcG transcriptional repressors such as Nspc1, identified in the nervous system with homology to Bmi1 [194], and other still unknown chromatin accessory factors/effectors [195–198] may regulate stem cell identity. For example, physical and genetic interaction with an inhibitor protein of cell proliferation, namely E4F1, determines the function of Bmi1 in the regulation of HSC maintenance [199]. Despite conservation in the methylated histone (H3) binding chromodomains, different PcG homologs are found to display varied binding preferences depending on combination of histone modifications and RNA molecules enriched at the target sites [200]. Thus, unveiling the epigenetic mechanisms that regulate genome function in its native topography will be essential to our molecular understanding of development. Binding of PcG proteins is recently suggested to alter the topography of the DNA [200]. With recent developments in the isolation and characterization of adult stem cells [201], the three-dimensional structural determination of derepressed and repressed chromatin [202], and quantitative proteomic analysis of human histone modifications [203], the epigenetic molecular signature of stem cells may soon be uncovered. More recent advances in the high resolution mapping of chromatin modifications in the human genome [39, 204], the genome-wide mapping of in vivo target loci of chromatin proteins and transcription factors (novel "DamID" technique) [80], the carrier ChIP protocol [205], and the induction of stem-like cells from adult fibroblasts [206–209] are expected to advance the studies toward epigenetic reprogramming of mammalian adult cells into therapeutically compatible pluripotent cells. Understanding how epigenetic dysregulation contributes to cancer offers great promise for the development of precise oncotherapeutics in the clinical front.

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The authors indicate no potential conflicts of interest.

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