Polycomb group proteins and their roles in carcinogenesis

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In the cell nucleus, DNA is wound around histone proteins, which are then packed together to form chromatin. Histones can be chemically tagged by methyl and acetyl groups. Polycomb group (PcG) proteins attach methyl groups to genes, which block their activity. This is similar to the attachment of methyl groups to gene promoters by DNA methyltransferases (DNMTs). This action is directly linked with tumor initiation and metastasis via the promotion of anti-senescence and anti-apoptosis pathways, and by facilitating epithelial mesenchymal transition (EMT). Cell fate transcriptional factors (CFTFs) and long non-coding RNAs (long ncRNAs) recruit PcG proteins to the promoters of tumor suppressor genes, resulting in epigenetic gene silencing by influencing chromatin structure and DNA accessibility. Thus, PcG proteins are potential diagnostic markers and targets for new chemoprevention and therapeutic strategies.

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Tumorigenesis has traditionally been considered to be driven by the sequential acquisition of mutations, leading to the activation of oncogenes and to the loss of function of tumor suppressor genes. An increasingly large amount of evidence suggests that carcinogenesis also involves “epigenetic changes”, which are mediated by mechanisms that do not affect the primary DNA sequence. Epigenetic changes include both genome-wide loss and regional gain of DNA methylation, and aberrant post-translation modifications of histones. Both DNA methylation and histone modification have key functions in governing gene expression by influencing chromatin structure and DNA accessibility. Abnormal chromatin structure can cause inappropriate gene expression and genomic instability, which results in cellular transformation and malignant growth. Therefore, proteins that control chromatin structure are important in carcino-

genesis. Polycomb group proteins (PcG proteins), which regulate chromatin remodeling, are one such class of proteins that regulate chromatin structure. PcG proteins form multiprotein repressive complexes, called polycomb repressive complexes (PRCs). As repressors of transcription, PRCs silence specific genes by altering chromatin modification. Here we review the biological functions and the molecular mechanisms of PcG proteins in oncogenesis. We also consider the potential role of PcG proteins in the diagnosis and treatment of cancer.

1 What are polycomb group proteins?

PcG proteins were first described in the fruit fly as repressors of *hox* genes, based on mutant phenotypes involving posterior transformation of body segments. PcGs act by maintaining chromatin in a transcriptionally inactive or

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“OFF” state [1]. PcG proteins work to regulate chromatin dynamics and gene expression cascades to preserve the developmental program of a cell. Since aberrations of the normal development program can result in cancer, the abnormal function of PcG proteins is associated with an increased risk of cancer. At the molecular level, as shown in Table 1, PcG proteins are classified into two groups, polycomb repressive complex 1 (PRC1) and polycomb repressive complex 2 (PRC2), based on their association with distinct classes of multimeric complexes [2].

PRC2 establishes the histone code and PRC1 interprets this code. The mammalian PRC2 complex contains enhancer of zeste homologue 2 (EZH2), embryonic ectoderm development (EED), suppressor of zeste homologue 12 (SUZ12) and retinoblastoma-binding protein 4 (RBBP4) or RBBP7 [3]. EZH2 contains a SET domain and is a histone methyltransferase (HMT). EZH2 attaches methyl groups to histone H3 at lysine 27, which is characteristic of PRCs that mediated inactive chromatin. Methylation of H3 at lysine 9 is another repressive mark generated by PRCs. EED and SUZ12 do not possess HMT activity, but are required for HMT activity of EZH2. After PRC2 establishes the histone code (H3K27 tri-methylation and H3K9 tri-methylation), the repressive histone marks are recognized by PRC1. PRC1 contains chromobox homologue 2 (CBX2), either CBX4 or CBX8, polyhomeotic homologue 1 (PHC), PHC2 or PH3, BMI1 and ring finger protein 1 (RING1) or ring finger protein 2 (RNF2). A working model of how PcG

complexes modulate chromatin is as follows: Transcription factors and their associated machinery dictate loci destined for silencing, and PRC2 tags these target loci by methylating histone H3 at lysine 27. The H3K27me mark is recognized and bound by the chromodomain of CBX2, CBX4 or CBX8 within PRC1, which mediates transcriptional silencing by binding methylated histones in the vicinity and by ubiquitinating histone H2A. Although *in vitro* assays have suggested that PRC complexes mediate silencing by physically blocking the binding of transcriptional machinery at promoters, PRCs are more likely to function by impeding RNA polymerase II (RNA Pol II) initiation or elongation [4,5]. In addition, the fact that EZH2 and CBX7 can physically associate with DNA methyltransferases (DNMTs) suggests that PcG proteins directly contribute to altered DNA methylation profiles [6]. Therefore, PcG proteins can integrate histone modifications, DNA methylation and chromatin remodeling in tumorigenesis to promote carcinogenesis.

2 What are the biological functions of PcG proteins?

Upregulation of PcG proteins is commonly observed in human cancer, demonstrating the oncogenic potential of PcG proteins. For example, BMI1 and EZH2 are oncogenes. PRCs function reciprocally to regulate transcriptional

Table 1 The components of polycomb repressive complexes (PRCs)

PcG complex	Name (<i>Homo sapiens</i>)	Function
PRC2	EZH2	SET domain histone methyltransferase, specific for H3K27
	EZH1	
	EED	EED is required for the histone methyltransferase activity of EZH2 within the PRC2 complex
	SUZ12	Like EED, SUZ12 is a critical minimal component required for enzymatic activity of the PRC2 complexes. In <i>Drosophila</i> , Su(z)12 drives nucleosome binding by PRC2
	RBBP4/RbpAp48 RBBP7/RbAp46	Form the minimal nucleosome binding module of PRC2 <i>in vitro</i>
PRC1	RING1/RING1A/RNF1	RING domain protein that is essential for the H2A ubiquitylation function of RING1B within the hPRC1L complex
	RNF2/RING1B/RING2	RING1B is the catalytic subunit responsible for the H2A ubiquitylation within the hPRC1L complex
	CBX2/HPC1/M33 CBX4/HPC2 CBX8/HPC3/PC3	Chromo domain protein, reader of the H3K27me3 mark
	PHC1/EDR1/HPH1 PHC2/EDR2/HPH2 PHC3/EDR3/HPH3	Polyhomeotic proteins are SAM and Zn-finger proteins that are stoichiometric components of PRC1 and required for silencing
	BMI1	RING domain protein that is essential for the H2A ubiquitylation function of RING1B. BMI1 is the central component of the hPRC1L complex that contains RING1A, RING1B, HPH2 and PC3

programs that affect apoptosis, cellular senescence and epithelial mesenchymal transitions. In addition, the co-operation between oncogenic virus and PcG proteins can transform host cells and lead to tumor formation.

2.1 Apoptosis

Apoptosis is the process of programmed cell death. Generally PcG proteins inhibit apoptosis of cancer cells. BMI1, the first PcG protein to be implicated in cancer, inhibits apoptosis of cancer cells by binding to the *CDKN2A* locus. The *CDKN2A* locus encodes two structurally distinct tumor suppressor genes, *p16^{INK4A}* and *p14^{ARF}*. *p16^{INK4A}* is a cyclin-dependent kinase inhibitor that activates the retinoblastoma (RB) pathway; *p14^{ARF}* facilitates apoptosis by inhibiting MDM2-mediated ubiquitination and degradation of p53. *bmi1^{-/-}* mice have increased apoptosis in lymphoid tissues, a phenotype that is rescued by depleting *p16^{INK4A}* and *p14^{ARF}* or by over-expressing the apoptosis inhibitor BCL2 [7]. Loss-of-function analysis of BMI1 using RNA interference (RNAi) in both normal and malignant human cells showed that loss of BMI1 promoted cancer specific cell death, as well as an increased production of cleaved caspase-3 and the increased expression of proapoptotic gene Bim [8]. On the other hand, an *S*-adenosylhomocysteine hydrolase inhibitor, 3-deazaneplanocin A (DZNep), induces efficient apoptotic cell death in cancer cells but not in normal cells. This is because DZNep can effectively deplete cellular levels of PRC2 components EZH2, SUZ12 and EED, reduce tri-methylation of H3K27 and activate the expression of the apoptosis effector F-box-only protein 32 (FBXO32) [9]. To summarize, loss of PcG-mediated repression causes apoptosis; increased PcG-mediated activity contributes to the inhibition of apoptosis in cancer cells.

2.2 Cellular senescence

Cellular senescence is the phenomenon by which normal diploid cells lose the ability to divide (normally after about 50 cell divisions *in vitro*). It is widely believed that cellular senescence evolved as a way of preventing the onset and spread of cancer. BMI1, co-operating with MYC, facilitates tumorigenesis by mediating escape from cellular senescence, which is a state of withdrawal from the cell cycle that provides a potent tumor suppressive mechanism *in vivo* [10]. As mentioned above, *CDKN2A* and *CDKN2B* loci encode three cellular senescence inducers, *p14^{ARF}*, *p15^{INK4B}* and *p16^{INK4A}*. BMI1 represses both *CDKN2A* and *CDKN2B* loci simultaneously by mediating the loading of CDC6 onto a cis-acting replication origin several thousand nucleotides upstream of the transcriptional start site. Recently, studies have shown that chromobox 7 (CBX7) within PRC1 binds to a long non-coding RNA, ANRIL, and both CBX7 and ANRIL are found at elevated levels in prostate cancer tissues. In concert with H3K27me recognition, binding to

RNA contributes to CBX7 function and disruption of either interaction impacts the ability of CBX7 to repress the *CDKN2A-CDKN2B* locus and to control senescence [11]. These observations provide the new insight that PcG proteins play a crucial role in the cellular senescence process.

2.3 Epithelial mesenchymal transition

The epithelial mesenchymal transition (EMT) is a developmental process in which epithelial cells lose their polarity and acquire the migratory properties of mesenchymal cells. A recent breakthrough has shown that EMT plays a very important role in promoting the metastasis of cancer and in generating cancer cells with some properties that are similar to those of stem-cells [12]. These findings provide a crucial link between the acquisition of metastatic traits and tumor initiating capability in cancer cells undergoing EMT. EZH2 has a master regulatory function in controlling certain processes, such as stem cell differentiation, cell proliferation, early embryogenesis and X chromosome inactivation [13]. Epithelial cells show very low levels of EZH2, but increased levels of EZH2 have been observed in aggressive solid tumors, such as those observed in prostate, breast and bladder cancer. Repression of E-cadherin is a molecular marker of EMT. Studies have demonstrated that EZH2 and Suz12 mediate transcriptional silencing of the tumor suppressor gene *E-cadherin* by trimethylation of histone H3 lysine 27 [14,15], which promotes the metastasis of cancer. These observations provide a functional link between the dysregulation of PRC2 and the repression of E-cadherin during cancer progression. Recent research suggests that PRC1 is also involved in the metastasis of cancer. Up-regulation of BMI1 induced EMT and enhanced the motility and invasiveness of human nasopharyngeal epithelial cells, whereas silencing endogenous BMI1 expression reversed EMT and reduced motility. Furthermore, up-regulation of BMI1 led to the stabilization of Snail, a transcriptional repressor associated with EMT, via the binding of BMI1 to the *pten* loci [16]. In head and neck squamous cell carcinoma (HNSCC), Twist1 and BMI1 act together to repress the expression of both E-cadherin and *p16^{INK4A}*, which are correlated with the worst prognosis of HNSCC [17]. Together, those studies imply that PRC-induced EMT and metastasis of cancer via chromatin remodeling leads to unfavorable clinical outcomes and that targeting PRCs may be useful for the development of therapeutic treatments.

2.4 Transformation of host cells

The WHO International Agency for Research on Cancer estimated that in 2002, 17.8% of human cancers worldwide were caused by infection, with 11.9% being caused by one of seven different viruses [18]. Analyses of tumor viruses have been instrumental in understanding cancer biology.

Several studies have reported how oncogenic virus-encoded proteins drive tumorigenesis via the deregulation of PcG proteins. The malignant phenotype of human papilloma virus (HPV)-positive cancer cells is maintained by the activity of viral E6 and E7 genes. *EZH2* is an E7 target gene that is important in the proliferation of HPV-positive cancer cells, and contributes to their apoptotic resistance [19]. Epstein–Barr virus (EBV) is another tumor virus, which is associated with the development of several lymphoid and epithelial malignancies. Latent membrane protein 1 (LMP1) is the principal oncoprotein encoded by EBV. LMP1 mediates the disruption of several important cell signaling pathways, including the AP-1, NF- κ B, STAT3 and EGFR pathways [20]. In Hodgkin lymphoma cells, BMI1 is up-regulated by LMP1, and this up-regulation is mediated by NF- κ B signaling. High levels of BMI1 contribute to LMP1-induced oncogenesis in Hodgkin lymphoma [21]. On the other hand, numerous tumor suppressor genes have also been identified as the cellular targets of tumor-causing viruses. Adenoviruses produce a protein called E1B-55k, which binds to p53, inhibiting its transcription-promoting activity and directing its degradation by the proteasome. For many years, this was believed to be the central adenovirus mechanism of carcinogenesis. Recently, it has been observed that infection with an adenoviral mutant that lacks E1B-55k results in high levels of p53, as expected, but the target genes of p53 are still deactivated. Furthermore, researchers have identified another virus-encoded gene, E4-ORF3, as a second repressor of p53. Crucially E4-ORF3 selectively silences the activation of p53 target genes by mediating the tri-methylation of histone H3 at lysine 9 at the promoters of the target genes. E4-ORF3 does this through its effect on members of the cellular methylase enzyme family, SUV39 [22]. Although SUV39 is not a PcG protein, it is very similar to EZH2 because SUV39 has a SET domain. We propose that tumor viruses can inactivate tumor suppressor genes, not only by cooperating with PcG proteins, but also by increasing the expression of PcG proteins.

3 How are PcG proteins recruited to target genes during oncogenesis?

An increasingly large amount of work has established a functional link between PRCs and tumorigenesis. However, the mechanisms by which PcG proteins bind to target genes are still unclear. Analysis of the structure of PcG proteins has revealed that they do not have the ability to bind to specific DNA motifs (as DNMTs do) [23]. In *Drosophila melanogaster*, one transcriptional factor, encoded by Yy1, has been shown to co-occupy most PREs along with PRC1 and PRC2 components. More interestingly, some PcG proteins contain an RNA binding motif [24]. These observations suggest that many different mammalian transcription factors

or RNAs are required to recruit PcG proteins to polycomb repressive elements (PREs). In addition to this, recent studies demonstrate that JARID2 (an ARID-domain-containing protein) regulates binding of PRC2 to target genes in ES cells [25].

3.1 Cell fate transcription factors (CFTFs)

CFTFs are transcription factors that function to regulate cell fate decisions during embryogenesis or adult cell differentiation. CFTFs include Hox, Sox, OCT, Fox, Pax and Gata transcriptional factors. There is also strong evidence that at least 30 CFTFs, when altered, can contribute to cancer in a tissue-specific manner. CFTFs are strong candidates for the regulation of PcG protein recruitment to and dissociation from target genes [23]. OCT4 is normally expressed in pluripotent cells of the early embryo and in ES cells, and it is also required for producing iPS cells. A potential oncogenic activity of OCT4 was discovered when it was found to be highly expressed in human germ cell tumors and was shown to be required for the continued growth of the aforementioned tumors. In addition, the ectopic expression of OCT4 blocks progenitor cell differentiation and causes dysplasia in epithelial tissues [26]. Importantly, OCT4 can occupy several hundred PcG protein target genes and it is thought to contribute to the sustained recruitment of PcG proteins to the promoters of target genes [27]. Some other CFTFs can form fusion proteins, such as the promyelocytic leukemia (PML)—RAR α fusion protein, which aberrantly recruits PcG proteins to target genes during cancer development [28].

3.2 Long non-coding RNAs (long ncRNAs)

In addition to well-characterized protein coding mRNAs and microRNAs, long ncRNAs are also emerging as important regulatory molecules in tumor-suppressor and oncogenic pathways. Long ncRNAs are over 200 nucleotides in length, and are often transcribed from gene loci that are interspersed with and overlap coding genes. Recent studies have found that long ncRNAs regulate key cancer pathways at transcriptional, post-transcriptional and epigenetic levels by serving as a regulator of PRC2. Long ncRNAs help to recruit PcG proteins to the promoters of target genes [29]. HOX transcript antisense RNA (HOTAIR), a long ncRNA located among the *hox* loci, becomes systematically dysregulated during breast cancer progression. The expression of HOTAIR is higher in metastatic breast cancer than in primary breast epithelial cells. Enforced expression of HOTAIR in epithelial cancer cells induces genome-wide re-targeting of PRC2 onto numerous tumor suppressor genes and leads to alteration of the tri-methylation of histone H3 at lysine 27 of target gene promoters. Tumor suppressors, such as JAM2, PCDH10 and PCDHB5, are transcriptionally repressed after HOTAIR expression and de-repressed after concomitant PRC2 depletion [30]. The

long ncRNA, ANRIL, (described above) interacts with CBX7, a component of PRC1, to epigenetically silence *p14^{ARF}*, *p15^{INK4B}* and *p16^{INK4A}* [11].

4 Diagnostic and therapeutic implications of PcG proteins

Numerous studies have discovered a functional link between PcG proteins and carcinogenesis, which means that PcG proteins can serve as useful diagnostic markers, or as prognostic markers for predicting clinical behavior. The expression of BMI1 and EZH2 indicates high cancer cell proliferation rates and poor outcome for the patient. BMI1 and EZH2 have been identified as potential diagnostic and prognostic markers for several cancer types. Targeting PcG proteins is a valid therapeutic strategy because decreased levels of BMI1 and EZH2 induce cancer cell specific apoptosis and inhibit chemoresistance in tumors undergoing PcG protein-induced EMT [8,9]. MicroRNAs (miRNAs) are short ribonucleic acid (RNA) molecules; they are post-transcriptional regulators that bind to complementary sequences on target mRNAs, usually resulting in translational repression and gene silencing. Recent studies have revealed that a subset of miRNAs, including miR26a and miR101, control the expression of polycomb group genes. *In vitro* and *in vivo* experiments indicate that therapeutic delivery of these miRNAs inhibits cancer cell proliferation and induces tumor-specific apoptosis [31,32]. Dietary omega-3 polyunsaturated fatty acids suppress the expression of EZH2 in breast cancer cells [33]. Similarly to PcG proteins, CFTFs and long ncRNAs could also be possible diagnostic and prognostic markers, or targets for cancer treatment. There have been multiple investigations into using the DNA methylation status of HOXA9 genes as biomarkers. In breast cancer, a two-gene test using qPCR to determine the ratio of HOXB13 expression to IL17RB expression that enables the prediction of tumor recurrence in patients with ER-positive tumors taking tamoxifen has already been developed. Some Hox tumor suppressor genes are epigenetically silenced, and, therefore, the use of HDAC or DNMT inhibitors together with conventional chemotherapies may make the tumors more susceptible to drugs and result in fewer metastases [34]. In conclusion, further research on PcG proteins and their co-factors will generate new possibilities for using PcG proteins as diagnostic markers and as targets for new intervention strategies.

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