




# Bivalent Regulation and Related Mechanisms of H3K4/27/9me3 in Stem Cells

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## Abstract

The "bivalent domain" is a unique histone modification region consisting of two histone tri-methylation modifications. Over the years, it has been revealed that the maintenance and dynamic changes of the bivalent domains play a vital regulatory role in the differentiation of various stem cell systems, as well as in other cells, such as immunomodulation. Tri-methylation modifications involved in the formation of the bivalent domains are interrelated and mutually regulated, thus regulating many life processes of cells. Tri-methylation of histone H3 at lysine 4 (H3K4me3), tri-methylation of histone H3 at lysine 9 (H3K9me3) and tri-methylation of histone H3 at lysine 27 (H3K27me3) are the main tri-methylation modifications involved in the formation of bivalent domains. The three form different bivalent domains in pairs. Furthermore, it is equally clear that H3K4me3 is a positive regulator of transcription and that H3K9me3/H3K27me3 are negative regulators. Enzymes related to the regulation of histone methylation play a significant role in the "homeostasis" and "breaking homeostasis" of the bivalent domains. Bivalent domains regulate target genes, upstream transcription, downstream targeting regulation and related cytokines during the establishment and breakdown of homeostasis, and exert the specific regulation of stem cells. Indeed, a unified mechanism to explain the bivalent modification in all stem cells has been difficult to define, and whether the bivalent modification is antagonistic in inducing the differentiation of homologous stem cells is controversial. In this review, we focus on the different bivalent modifications in several key stem cells and explore the main mechanisms and effects of these modifications involved. Finally, we discussed the close relationship between bivalent domains and immune cells, and put forward the prospect of the application of bivalent domains in the field of stem cells.

**Keywords** Bivalent domain · H3K4me3 · H3K9me3 · H3K27me3 · Stem cells · Immune cells

## Introduction

Stem cells are cells with strong self-renewal capacity, proliferation ability and multilineage differentiation potential [1]. Embryonic stem cells (ESCs) come from blastocysts in the inner cell mass in the process of embryonic development, and induced pluripotent stem cells (iPSCs) are a class of pluripotent stem cells derived from reprogramming terminally differentiated somatic cells by introducing specific transcription factors [2, 3]. Both of them have the ability to produce almost all types of cells in terms of differentiation [4, 5]. Mesenchymal stem cells (MSCs) are fibroblast-like cells originating from the mesoderm [6]. MSCs can form bone, cartilage, muscle, or adipose tissue under the influence of growth factor pathways and downstream transcription factors with normal physiological conditions [7]. Under the condition of induction, MSCs can also regenerate the myocardium and neurons [8]. In addition to differentiation

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potential, MSCs also have obvious ability of immunosuppression and tissue regeneration [6]. Therefore, taking stem cells as the research object has extensive guiding significance for the exploration of bivalent domains.

In recent years, with the deepening of the researches on stem cells, more and more evidences show that epigenetic modification is a key control factor in regulating stem cells (Table 1). As a unique epigenetic modification, bivalent domains regulate many life processes of stem cells. The bivalent domain was first identified in the study of methylation in mouse ESCs [20], and the bivalent domain composed of H3K4me3 and H3K27me3 was observed [20]. Thereinto, H3K4me3 is a very common chromatin modification at the transcription start site of active genes in eukaryotes [21]. H3K4me3 plays a guiding role in gene transcription, primarily promoting transcription in a positive way, and is often described as "active" histone modification [21]. The enrichment of H3K27me3 was associated with gene silencing [22]. Oppositely, H3K27me3 maintains gene inhibition by recruiting other regulatory factors and may indirectly regulate transcription by spatially keeping proteins from binding to chromatin [23]. With the further research, bivalent regulation is not limited to H3K4me3 and H3K27me3, but H3K9me3 can also form bivalent domains with H3K4me3 or H3K27me3, respectively [12, 24–27]. Similar to H3K27me3, H3K9me3 also has inhibition effects on gene transcription [28, 29].

In this review, we mainly discuss the structure and function of the three bivalent domains composed of H3K4me3, H3K27me3 and H3K9me3, as well as the regulatory roles and related mechanisms of bivalent domains in stem cells.

## Function and Regulation of Bivalent Domains

Bivalent domains are characterized by both activating and inhibiting histone modifications [30]. In stem cells, multiple methyl modifications coexist on promoters of developmental regulatory genes, which can be divided into two main functional groups: one connected with transcriptional activation effects such as H3K4me3, and one connected with transcriptional inhibition effects such as H3K9me3 and H3K27me3 [31]. Methyl modifications with different functions are expressed at different levels in life activities such as cell differentiation, and there is also a correlation between different modifications [32]. This special modified state, consisting of two interrelated methylation modifications, is called "bivalent domain" [33]. The subtle balance between the three methylation modifications causes a variety of changes in chromatin structures, leading to various transcriptional states of downstream genes: "balanced", "activated", or "inhibited" [34, 35].

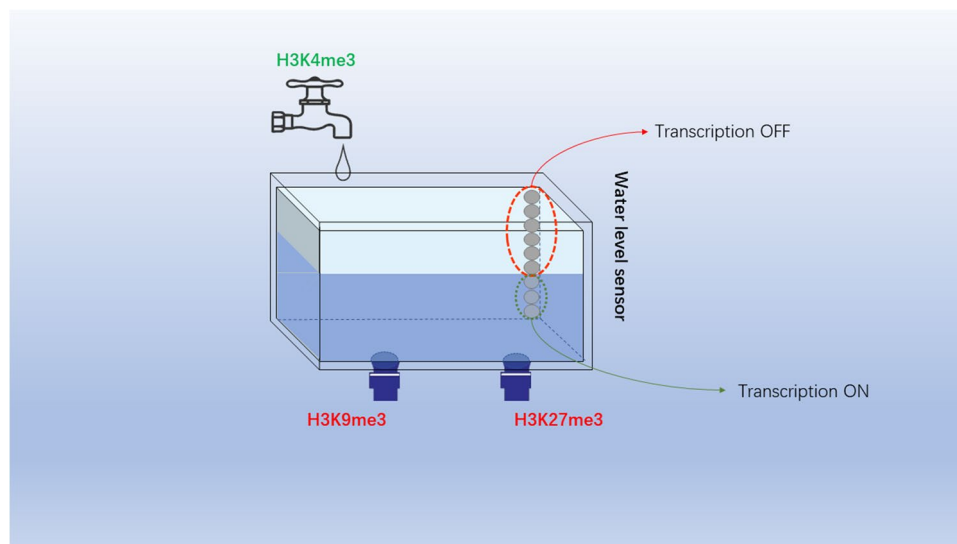
**Table 1** Histone Methyltransferases and Demethylases Involved in Regulating Stem Cell Fate

Source cell type	Developmental regulatory genes	Methyl modification enzymes	Methylation sites	Cell fate	References
Embryonic stem cells and induced pluripotent stem cells	Sox2, Sox4, Foxd3, Pax3, Pax6, Pax7, Nanog and Ebf1, etc	KDM5B, SETDB1, SUV39H1 and SUV39H2	Demethylation of H3K4 and methylation of H3K9	Maintaining the self-renewal and differentiation ability	[9, 10]
		KDM6B	Demethylation of H3K27	Inducing the differentiation	[11]
Mesenchymal stem cells	ZBTB16, MX1, FHL-1, and CEBP $\alpha$ , etc	Ezh2 and SETDB1	Methylation of H3K27 and H3K9	Promoting adipogenic differentiation	[12, 13]
		SET1A, SET1B, MLL3 and MLL4	Methylation of H3K4	Promoting osteogenic differentiation	[14]
Hepatic stem/progenitor cells	Ink4a / Arf and Sox4	Ezh2	Methylation of H3K27	Inducing the differentiation	[15]
Neural stem cells	Sox10	H3K4 methyltransferase	Methylation of H3K4	Differentiation of neural stem cells into oligodendrocyte progenitor cells and newly formed oligodendrocytes	[16]
Cancer stem cells	Rhox5 and HOX	UTX and WDR5	Demethylation of H3K27	Inhibiting cancer	[17, 18]
		PCGF1	Increasing expression of the H3K27me3 demethylase KDM6A and the H3K4me3 methyltransferases KMT2A	Sustaining the stem cell-like phenotype of colorectal cancer	[19]

The regulation of genes by the above three methylation modifications was visualized in Fig. 1. And the regulatory role of the bivalent domains is mainly influenced by lysine methyltransferases and demethylases. H3K4me3 and H3K27me3 modifications are catalyzed by lysine methyltransferases, which normally exist in two noteworthy chromatin modification systems: Trithorax group (TrxG) and Polycomb group (PcG) proteins complexes [33, 36]. The Trithorax (Trx) protein is the histone H3K4 methyltransferase. The direct homologs of Trx protein in mammals are SET1A, SET1B, MLL1, MLL2, MLL3 and MLL4 [14, 37, 38], which specifically regulate H3K4me3 [39]. In mammals, the complex formed by PcG proteins mainly includes the Polycomb Repressive Complex 1 and 2 (PRC1 and PRC2) [40]. Ezh2 participates in H3K27me3 as a component of PRC2 [41]. The PcG and TrxG proteins that control the tri-methylation levels of H3K4 and H3K27 respectively are not completely independent protein families, but are correlated [40]. TrxG protein has been suggested to inhibit the activity of PcG protein [40]. When TrxG and PcG proteins are affected, the methylation would also be affected. So TrxG and PcG proteins are also a factor that regulates the variation of the bivalent domains. Likewise, the demethylation of H3K4me3 and H3K27me3 is mediated by histone demethylases. The lysine-specific demethylase 5 (KDM5/JARID1) family catalyzes demethylation of H3K4 [42]. Historically, Lysine-specific demethylase 6B (KDM6B/JMJD3)

and lysine-specific demethylase 6A (KDM6A/UTX) have been used to mediate the demethylation of H3K27 [11, 43, 44]. SET domain bifurcated histone lysine methyltransferase 1 (SETDB1) is the most critical modification enzyme among various H3K9 methyltransferases, which methylates H3K9 [9]. In addition to SETDB1, related enzymes SUV39H1 and SUV39H2 are also involved in the modification of H3K9me3 [45, 46]. SETDB1 plays a crucial role in early development [47]. SETDB1 is also an essential factor in germ cell development [48], neurogenesis [49], maintenance of hematopoietic progenitor cell [50], T and B cell development [51–53], osteoblastic differentiation [54], and limiting the differentiation potential of preadipocytes [12]. In other words, SETDB1-mediated H3K9me3 plays an important role in cell development and differentiation through regulating genes and transcriptional silencing [9]. A variety of enzymes regulate methylation to form or destroy bivalent domains at different stages of cellular activity.

In summary, the bivalent domains balance the expression of genes by regulating the levels of methylation of two histone proteins that have antagonistic effects, allowing it to remain in an inhibited state and ready for activation in the absence of differentiation signals [36]. Additionally, two methyl modifications with the same effect can also form a synergistic bivalent domain, which plays a regulatory role together.



**Fig. 1** Regulation of H3K4/27/9me3 on genes. The regulation of genes by H3K4/27/9me3 is like the rise and fall of the water level in a reservoir. As shown in the figure, the activation effects of H3K4me3 act as a faucet for releasing water into a reservoir, while the inhibition effects of H3K27me3 and H3K9me3 act as two channels for releasing water. It is known that different genes were activated by different levels of methylation, which is reflected in the water level at different heights in the reservoir. On the wall of the pool are a series of

water level sensors, each of which corresponds to a different gene. The height of the water level sensor represents the "threshold" at which different genes are activated, namely the methylation level of H3K4/27/9. When a water level rises high enough to be detected by a receptor, the gene for that level is activated and transcription is switched on. When the water level is not detectable by the receptors, the gene is relatively silent and transcription cannot be activated

## Regulation of Bivalent Domains on Stem Cells

### Developmental Regulatory Genes Affecting Embryonic Stem Cells and Induced Pluripotent Stem Cells

The genes that regulate the development of ESCs and iPSCs are related to methyl modification with activation (H3K4me3) and inhibition (H3K9me3 and H3K27me3) effects, which can form bivalent domain in pairwise. This bivalent structural modification leaves the development regulatory genes in an activated state with low-level expression and prepares for activation [12, 55, 56]. After receiving the development signals, the balance between activation and inhibition of the genes is broken. The genes start to be transcribed when H3K4me3 modification level is increased, or stop the transcription when H3K27me3/H3K9me3 modification level is increased, thus initiating the spontaneous differentiation process, which is irreversible [34]. Compared with ESCs, the number of bivalent domains found in differentiated cells were significantly reduced [20]. This means that when ESCs begin to differentiate, the bivalent chromatin decomposes into univalent [57]. Next, we will further explain the relationship between the bivalent domains and the vital activities of ESCs and iPSCs.

Firstly, for the bivalent domains consisting of H3K4me3 and H3K27me3, the bivalent domains mainly regulate the expression of related genes through a theory called "winner-take-all" [58], and on this basis, gene regulation can be achieved through different approaches.

"Winner-take-all" is a theory describing the relationship between the histone modification levels of the bivalent domains and the expression levels of genes. According to this theory, when the methylation level of H3K4me3 is high in the bivalent structural domain, the bivalent genes show high expression level, which promotes the genes forward transcription by recruiting nucleosome remodeling enzymes and histone acetylase [59–63]. Similarly, high level of H3K4me3 relaxes tight heterochromatin and promotes the binding of transcription factors to "open" chromatin to achieve the same goal [64, 65]. When the methylation level of H3K27me3 is high, the expression level of bivalent genes is low, leading to negative transcription by promoting the compact chromatin structure [66, 67]. The bivalent domain sites often coincided with the transcription factor genes which express at low levels, but genes at low levels have the ability to be activated [20]. For example, Sox2 is involved in neural differentiation and development of the visual system, which leads to the differentiation of ESCs into multipotent neural precursor cells [20, 68]. Sox4 is involved in the formation of hematopoietic system and causes the

differentiation of ESCs into vascular cells [20, 68]. Foxd3 is involved in promoting the self-renewal and survival of ESCs and prevents the differentiation of ESCs [20, 68]. Pax6 promotes the development of the visual system, while Pax3 and Pax7 promote the differentiation of ESCs into muscle progenitor cells, which then differentiate into muscle cells and finally develop into skeletal muscle cells [20, 68, 69].

In addition to the above, we have learned that the Ebf1 gene seemed to be associated with the bivalent domains in controlling ESCs differentiation [20]. When the level of H3K4me3 was high, Ebf1 gene expression level was high, and ESCs differentiated into fibroblasts and myoblasts [20]. The Ebf1 gene was also associated with H3K27me3, and the high level of H3K27me3 led to the low expression level of Ebf1 gene, which induced ESCs differentiation towards neural cells [20]. Although the relationship between the bivalent domains and the differentiation direction had not been clarified in their study, we can reasonably speculate from the results of various studies that the bivalent domains can regulate the related cells by regulating the Ebf1 gene [20]. Moreover, when ESCs differentiated along the neural pathway, the methylation level of H3K4me3 significantly induced the genes Nkx2.2, Sox2.1, and Zfp2, while the action of H3K27me3 inhibited the genes Pax5, Lbx1h, and Evx1. Under the regulation of the bivalent domain, ESCs differentiated into pluripotent neural precursor cells [20]. In addition, the bivalent domains are related to the self-renewal ability of ESCs. Studies had found that the consumption of KDM5B led to the prolongation of self-renewal duration of ESCs [10, 70]. KDM5B can concentrate H3K4 methylation on promoters and enhancers of active genes in ESCs, that is to say, KDM5B regulated H3K4 methylation of bivalent genes during differentiation, thus maintaining self-renewal ability and controlling differentiation ability of ESCs [10, 71, 72].

Secondly, H3K9me3 can also form the bivalent domain with H3K27me3 or H3K4me3 respectively. When H3K9me3 and H3K27me3 form a bivalent domain, they have a certain synergistic effect, and they can up-regulate the expression of Nanog gene in different ways to maintain the pluripotency of ESCs [56, 73, 74]. For example, H3K9me3 can inhibit trophoblast-specific factors through histone H3K9 methyltransferase SETDB1, resulting in Nanog gene expression [73–75]. When H3K9me3 and H3K4me3 form a bivalent domain, their interaction type tends to be antagonistic [56]. The absence of SETDB1 makes H3K9me3 affect stem cell development and differentiation significantly, and down-regulate genes controlling pluripotency such as Sox2 and Oct4, thus promoting ESCs differentiation [73, 76].

In summary, the bivalent structure can be combined in many ways, and regulate the self-renewal capacity, pluripotency and differentiation direction of ESCs through different

sites or target genes. At the same time, with the differentiation process, the bivalent modification will change into a monovalent modification gradually, showing different differentiation results (Fig. 2).

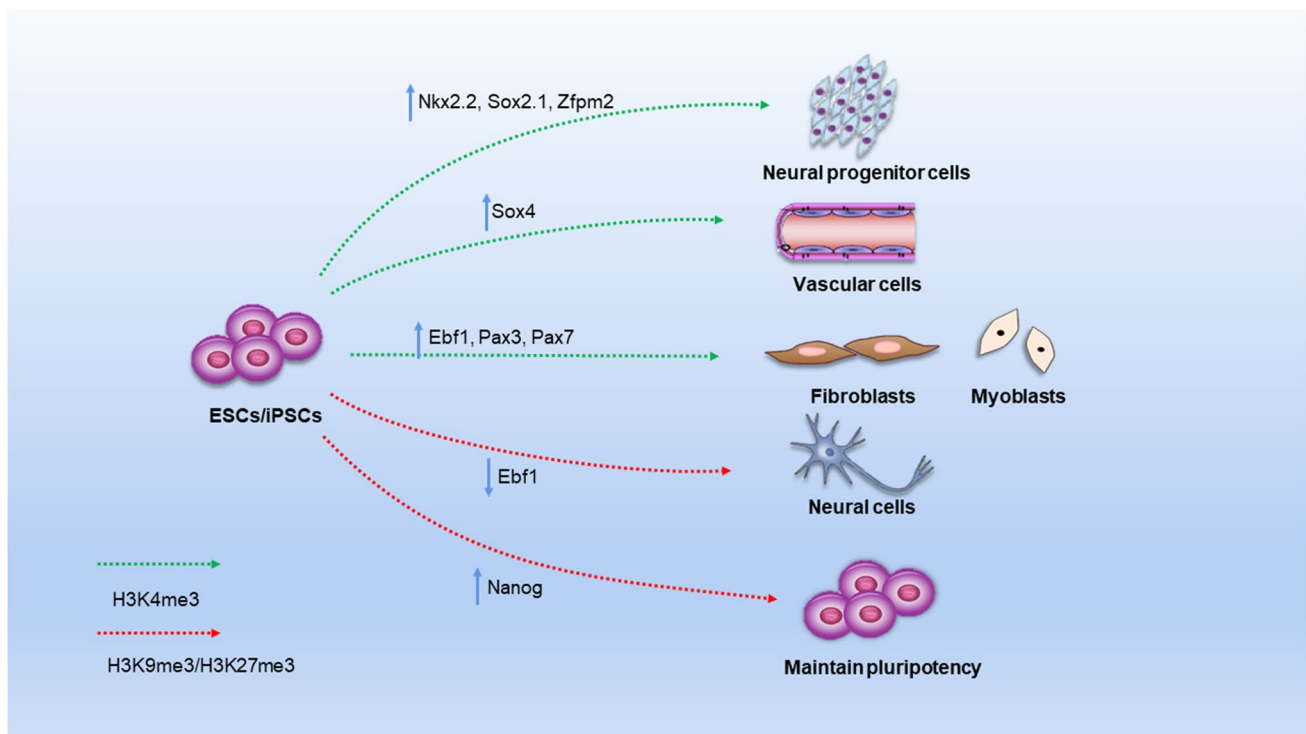
### Regulate the Proliferation and Differentiation of Mesenchymal Stem Cells

The osteogenic differentiation and adipogenic differentiation of MSCs are mutually regulated [77–79], and this mutual regulation is closely related to H3K4me3, H3K9me3, H3K27me3 and the bivalent domains formed by them.

Firstly, gene activation of H3K4me3 is related to osteogenic differentiation [80]. On the one hand, H3K4me3 forms a loose chromatin structure, inducing the transcription of key transcription factors for osteogenesis Osterix (OSX) and runt-related transcription factor 2 (RUNX2), and increases the downstream osteoblast markers osteocalcin (OCN), osteopontin (OPN) and alkaline phosphatase (ALP) expression [81, 82]. On the other hand, H3K4me3 also plays a role in the activation of high expression genes

during osteogenesis [83]. H3K4me3 can not only directly act on factors related to the process of osteogenic differentiation, but also indirectly regulate some regulatory factors [84]. For example, H3K4me3 affects the expression of the suppressor of cytokine signaling 1 (SOCS1), which in turn affects the inhibition effects of SOCS1 on the process of osteogenic differentiation [84]. In addition to the aforementioned role of H3K4me3 in the process of osteogenic differentiation, studies have confirmed that H3K4me3 is also involved in the regulation of fat metabolism, and it has been found that if the process of H3K4me3 is inhibited, it will lead to excessive fat accumulation [85].

Secondly, H3K27me3 is related to promoting fat formation and inhibiting osteogenic differentiation. H3K27 methyltransferase Ezh2 is an active regulator of adipogenesis, but it also has the function of inhibiting osteogenic differentiation [13, 86]. Ezh2 can promote adipogenesis by up-regulating the key transcription factor for adipogenesis, peroxisome proliferator-activated receptor  $\gamma$  (PPAR $\gamma$ ) [86, 87]. In addition, Ezh2 directly inhibits osteogenic genes ZBTB16, MX1, FHL-1, WNT through H3K27me3, as well as the key



**Fig. 2** Dynamic changes of bivalent domain in ESCs/iPSCs differentiation. The high level of H3K4me3 significantly induces Nkx2.2, Sox2.1, Zfp2 and other genes, and leads to the differentiation of ESCs along the neural pathway and the formation of neural progenitor cells. The high level of H3K4me3 affects Sox4 differentiation into vascular cells. The high level of H3K4me3 leads to the high expression level of Ebf1, which promotes the differentiation of ESCs into fibroblasts and myoblasts and affects the expression of Pax3 and

Pax7, causing the development of ESCs towards myoblasts. The high level of H3K27me3 leads to low expression level of Ebf1 gene, and Ebf1 can hardly be detected. At this time, ESCs differentiate into nerve cells. H3K27me3 and H3K9me3 have certain synergistic effects, and when they are at a high level, they can up-regulate the expression level of Nanog gene in different ways, thus maintaining the pluripotency of ESCs

osteogenic transcription factor RUNX2 and its downstream targets (such as OCN and OPN) to inhibit osteogenesis [88, 89]. The H3K27 demethylase KDM6A, corresponding to Ezh2, has the opposite effect to Ezh2 [86]. That is, it can improve osteogenic differentiation in vitro and in vivo, and inhibit adipogenesis in vitro [86]. In addition, H3K27me3 also silences ATOH8, the key promoter of chondrogenic differentiation, and inhibited the chondrogenic differentiation of MSCs [90].

Finally, H3K9me3 can promote both adipogenic differentiation and osteogenic differentiation. Studies have shown that H3K4me3 and H3K9me3 can constitute a bivalent chromatin domain to suspend adipocyte differentiation [12]. When the H3K9 methyltransferase SETDB1 is activated, H3K9 tri-methylation begins, and H3K4me3 is further silenced, causing RNA polymerase II to stop, thereby limiting the expression of CEBP $\alpha$  gene and increasing lipogenesis [12]. In addition, H3K9me3 can also inhibit the transactivation function of PPAR $\gamma$ , and make cells differentiate towards osteogenic direction [91]. The mechanism is that phosphorylation of nemo-like kinase forms a complex with SETDB1. This complex inhibits H3K9 methylation and thereby inhibits the activation of PPAR $\gamma$ . Promote osteogenic differentiation of MSCs [91]. Interestingly, studies have found that in the bivalent H3K9me3 and H3K27me3 chromatin domains, the methylation levels of both increases and the activity of adipogenesis increases [92].

It is worth noting that during the differentiation of MSCs, these three methylation modifications are in a state of dynamic change along with the life course of the cells [93]. Methylation levels have been measured in three stages of adipocyte formation: proliferation, differentiation, and maturation [93]. It was found that the level of H3K4me3 decreased after cell differentiation, while the level of H3K9me3 increased, especially for the ADIPOQ gene (encoding adiponectin), while the methylation levels of both increased before differentiation [93].

These results indicate that when MSCs differentiate, methylation of H3K4 and H3K27 plays a major decisive role, while methylation of H3K9 plays a synergistic role. The bivalent domains formed by these methylations regulate each other internally and determine the direction of cell differentiation (Fig. 3). In the H3K4me3/H3K27me3 bivalent domain, when the level of H3K4me3 is high and the H3K27me3 is inhibited, the cells develop towards osteogenesis. On the contrary, they develop towards the direction of lipogenesis. In the H3K4me3/H3K9me3 bivalent domain, when H3K9me3 inhibits H3K4me3, the cells develop towards adipogenic direction. Otherwise, H3K9me3 can cooperate with H3K4me3 to promote the development of osteogenesis. In the H3K9me3/H3K27me3 bivalent domain, the two synergistically promote adipogenesis.

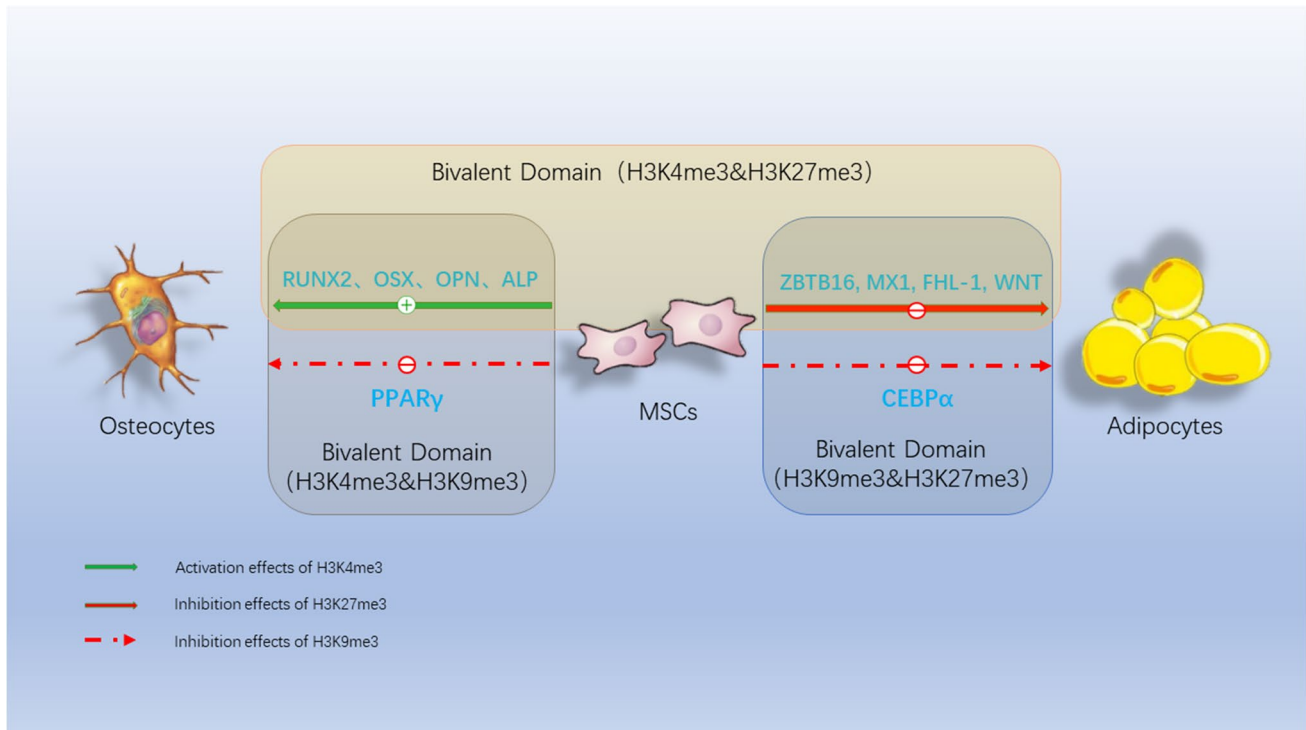
However, the process of cell differentiation is a dynamic process, and the methylation situation cannot be generalized for cells of different states. So, we need to learn more about stem cells and expand the influence of the bivalent domains.

## The Effect of Bivalent Domains on Other Stem Cells

### Regulate the Differentiation of Cancer Stem Cells

It has become clear that epigenetic dysregulation of chromatin plays a major role in the formation of cancer stem cells (CSCs) [94]. Previous research has established that some cancer types have shown bivalent domain modifications [95], which may ultimately influence tumor genesis or progression by affecting the ability of CSCs to maintain their stem-cell characteristics [94]. However, due to the differences of the bivalent domains between CSCs and other stem cells, it is necessary to have a more in-depth understanding of the explanation [96].

CSCs carry various pro-oncogenic mutations [94, 97]. For CSCs, the proliferation and cell-cycle transitions cannot be strictly regulated, and affects the process of epithelial-mesenchymal transition (EMT), which is related to malignant phenotype [94, 98]. Therefore, we focus on the bivalent domain changes in CSCs to illustrate the key role of the bivalent domains in cancer [94]. Bivalent domains regulate many key genes in stem cell differentiation, including Rhox5 in CSCs [99]. Some studies have demonstrated the regulatory effect of partial histone methylation on Rhox5 gene [99]. Since Rhox5 gene expression promotes cancer growth, it also indicates that histone methylation plays an important regulatory role in tumor growth [99]. During the growth of cancer, the induction of EMT endows stationary carcinoma cells with the migratory and invasive potential, as well as the stemness properties [100, 101]. There is a study that confirmed the presence of the H3K27 demethylase UTX deficiency in adenocarcinoma, bladder cancer, kidney cancer and leukemia [102–104]. Subsequent studies confirmed that UTX inhibited the regulatory factors SNIL, ZEB1 and ZEB2 of EMT through controlling H3K27me3, as well as negatively regulated CSCs properties [17]. Moreover, the depletion of H3K27me3 also promotes the expression of HOX, a key gene in the development of many cancers [18]. As for H3K9me3, an experimental study showed that the reduction of DNA methyltransferase 1 (DNMT1) promoted the inhibition effects of H3K9me3 and H3K27me3 in the promoter regions of prostate cancer cells and induced the occurrence of EMT [105]. The transient induction of EMT produces a self-renewal state that allows for redifferentiation and migration to distant sites [100]. The role of H3K27me3 and H3K9me3 in CSCs is not quite clear and needs to be analyzed on a case-by-case basis.



**Fig. 3** Regulation of three bivalent domains in MSCs. MSCs have multidirectional differentiation potential, and here we briefly describe the osteogenic and adipogenic differentiation of MSCs. H3K4me3 activates genes and makes MSCs differentiate toward osteogenesis by acting on RUNX2, OSX, OPN, ALP and other key factors of osteogenic differentiation; H3K27me3 has an inhibitory effect on genes and can induce the differentiation of MSCs towards adipogenic direction by acting on ZBTB16, MX1, FHL-1, WNT and other sites; on

the one hand, H3K9me3 inhibited the key adipogenic factor PPAR $\gamma$  to induce the differentiation of MSCs towards osteogenesis and on the other hand it inhibited the CEBP $\alpha$  gene to induce the differentiation towards adipogenic direction. Three methyl modifications constitute three bivalent domains, which regulate gene expression and achieve the goal of controlling the balance of osteogenic and adipogenic differentiation of MSCs

In the study of colorectal cancer, WDR5 (an important component of histone methyltransferase complex) triggers EMT process, and this phenomenon is associated with H3K4me3 [106, 107]. WDR5 and H3K4me3 directly bind to the promoter region of miR-21 (a representative oncogenic miRNA), and then promote EMT progression in colorectal cancer cells [107]. Interestingly, WDR5 and H3K4me3 also promote the proliferation and self-renewal of neuroblastoma and glioblastoma cells [108]. Other studies have found changes in other methyl modifications in colorectal cancer [19]. Polycomb group ring finger 1 (PCGF1), by increasing expression of the H3K27me3 demethylase KDM6A and the H3K4me3 methyltransferases KMT2A, decreases the H3K27me3 marks and increases the H3K4me3 marks on their promoters, and thus binds to labeled promoter of colorectal CSCs and activates their transcription [19]. Moreover, PCGF1 is dysregulated in a variety types of CSCs, such as oral squamous cell carcinoma stem cells [109]. However, in different CSCs,

the specific mechanisms of EMT induction are different, and the action sites of H3K4me3 are also different. For example, H3K4me3 is enriched at the ZEB1 promoter [110]. Transcription factor ZEB1 is one of the most efficient EMT-activators and is associated with invasion and metastasis in different cell types [110]. H3K4me3 acts on ZEB1, eliciting the induction of EMT and conversion to the CSCs state [110]. In other words, for the stimulation and maintenance of CSCs state and the induction of EMT, it is not a single effect of any methyl modification, but a combination effects of multiple methyl modifications. Moreover, due to the influence of the same mechanism in different CSCs, the effects of methyl and bivalent modification on CSCs are both extensive and special.

In summary, the regulatory effect of bivalent domains on CSCs is reflected in the promotion and inhibition of cancer cell development. CSCs showed such a general trend that suppressive modifications are globally reduced, and stimulative modifications are generally increased.

## The Role of Bivalent Domains in Hepatic Stem/Progenitor Cells

In addition to CSCs, bivalent domain modifications have been described as extremely important in developmental processes in both the liver and pancreas [111, 112]. Differentiation of hepatic stem/progenitor cells is precisely controlled by bivalent domains [111]. In this part, we mainly introduce the regulation and related mechanism of bivalent modification in hepatic stem/progenitor cells.

It has been demonstrated from various studies that in hepatic stem/progenitor cells, Ezh2 depletion eliminates their ability to self-renew, causing them to exhibit abnormal differentiation toward the hepatocyte lineage [15]. The mechanism is that transcriptional inhibition of the target genes such as *Ink4a/Arf*, which PcG acts on, is essential for self-renewal of hepatic stem/progenitor cells [113]. Related experimental studies also proved that *Ink4a/Arf* was up-regulated and H3K27me3 level was decreased in mature hepatocytes and bile duct cells, while H3K4me3 level was not decreased [111]. *Sox4* was also upregulated in mature hepatocytes and bile duct cells, and increased levels of H3K4me3 at the *Sox4* locus were detected in terminally differentiated cells [111].

In other words, Ezh2-mediated H3K27me3 plays an important role in inhibiting hepatic stem/progenitor cells differentiation [114, 115]. The tri-methylation level of H3K27 decreased with the differentiation of the cells into hepatocytes and bile duct cells, while the tri-methylation level of H3K4 increased [111]. Above all, this shows that the modification enzymes regulate the differentiation direction of the hepatic stem/progenitor cells by affecting the bivalent domains, which reflect the important role of the bivalent domains in cell differentiation.

## Regulation of Bivalent Domains in Neural Stem Cells

Histone variation plays an indispensable role in regulating gene expression [116], and because of this, the dynamic change of the bivalent domains plays a regulatory role in the differentiation of neural stem cells (NSCs) [16].

The development of oligodendrocytes goes through three stages: NSCs stage, oligodendrocyte progenitor cells (OPCs) stage and newly formed oligodendrocytes (NFOs) stage [16]. It has been found that *Sox10* gene, which induces NSCs into oligodendrocyte lineage, only has H3K4 marker in OPCs and NFOs [16]. In addition, the cells of the three stages all have their own unique genes, which show “H3K4 only” and “H3K27 only” markers [16]. The number of genes modified by H3K4me3 decreased gradually during the differentiation and development of NSCs into OPCs and NFOs, while the number of genes modified by H3K27me3 decreased first

and then increased, indicating that the bivalent domain is a dynamic domain [16].

That is, the dynamic changes in the bivalent domains determine which genes are expressed and which are inhibited, and these regulated genes influence the differentiation and development of the NSCs into the other two stages.

## The Role of Bivalent Domains in Immunity

In the previous article, we described the important regulatory role of bivalent domains on stem cells in detail, but the role of bivalent domains is not only reflected in stem cells, nor limited to regulating development and differentiation. Here, we will briefly introduce Th17 cells and invariant natural killer T cells (iNKT).

### Bivalent Domains Affect the Immune Function of Th17 Cells

Th17 cells are one of the important cells in immune regulation, and a kind of pro-inflammatory cells [117]. We have already understood the interactions in the bivalent domains, which affect the levels of STAT1, STAT3 and ROR $\gamma$ t promoters, which are the key promoters that determine the fate of Th17 cells [118].

H3K27me3 inhibits critical transcription factors in Th17 differentiation process, such as ROR $\gamma$ t [119], and inhibits Th17 differentiation [120]. H3K9me3 also inhibits the differentiation of Th17 cells. Studies have shown that the *Jarid2* can regulate the levels of H3K27me3 and H3K9me3 to regulate the differentiation of Th17 cells [121]. In addition, the costimulatory receptor OX40 inhibits Th17 cells by accumulating H3K9 methyltransferase SETDB1 [122, 123]. These indicate that the bivalent domain composed of H3K9me3 and H3K27me3 inhibits Th17 cell differentiation and has the effect of regulating immune function. On the contrary, H3K4me3 has positive effects on Th17 cells, that is, it promotes Th17 cells to exert immune function. For example, H3K4me3 can activate STAT1 and STAT3 [124, 125]. Several studies have revealed that the regulation of Th17 cell differentiation by JMJD3 is also achieved by mediating the methylation status of H3K27 and/or H3K4 on target genes [126]. The study helps us to understand the stability and mutual change of H3K4me3 and H3K27me3 at key promoters such as STAT1, STAT3 and ROR $\gamma$ t determine the fate of Th17 cells [127].

Therefore, we believe that the bivalent domains also have a unique regulatory role in Th17 cells, and affect the immune function of Th17 cells by affecting key transcription factors. Although the exact mechanisms underlying each of these bivalent domains are yet to be investigated, the effects of H3K4me3, H3K9me3, and H3K27me3 on Th17 cells are



well established and are also shown in other immune cells such as iNKT cells.

### Bivalent Domains Influence the Generation and Differentiation of iNKT Cells

It is well known that iNKT cells, like Th17 cells, are critical in immune regulation [128]. With further research, more and more findings indicate that the bivalent domains also play an important role in iNKT cells [129].

Bivalent modification of H3K4me3 and H3K27me3 was found in the promoter of PLZF gene (which drives iNKT cell differentiation), and the change from a bivalent modification state to H3K4me3 only modification is a prerequisite for iNKT cell differentiation [129]. Therefore, we speculate that the presence of H3K27me3 will hinder the differentiation of iNKT cells. Of course, there are other results that can be used as evidence [129]. For example, the H3K27me3 demethylases Utx and Jmjd3 are critical for iNKT cell generation, and lack of Utx or Jmjd3 caused decreased expression of PLZF mRNA [129]. However, the deletion of Ezh2 promoted the development and differentiation of iNKT cells [129]. H3K9me3 also has a regulatory effect on PLZF gene, and this process is driven by Jarid2 [121]. The bivalent modification of H3K9me3 and H3K4me3 or H3K27me3 has not been investigated, but it is clear that H3K9me3 also shows the same inhibition effects on differentiation as H3K27me3 [121].

In other words, bivalent modification affects the PLZF gene. Once expressed, the PLZF proteins are further responsible for the differentiation and maintenance of iNKT cells by binding to its target gene.

### Conclusion

It has been more than a decade since the discovery of bivalent domains. Enormous progress has also been made in understanding how bivalent domains function. The broad role of bivalent domains in transcriptional regulation and maintenance of pluripotency in stem cells is becoming clearer. It also plays a role in various types of cells. There is also an increasing body of data on changes in bivalent domains methylation levels in various pathologies. Although it is difficult to explain all the regulatory effects of the bivalent domains in a single mechanism, it is fundamentally due to the activation effects of H3K4me3 on genes or regulatory factors and the inhibition effects of H3K9me3/H3K27me3. Any two of them can form a bivalent domain, which can act synergistically or in an inversely proportional manner. The three methylation modifications show different levels of change in different life activities, and the bivalent modification states formed by them also tend to be univalent modification,

which eventually leads to different changes in cells. There is also a connection between three kinds of methylation, not fully independent. However, there are still some issues that need to be addressed in order to have a more complete understanding of bivalent domains. The exact contribution of various factors affecting the methylation level of the bivalent domains, whether there is a relationship between the influencing factors at different levels, and many other factors that have not been explored. During the evolution from invertebrates to mammals, the complexity of bivalent domains and related regulatory factors has also increased. The emergence of a large number of paragenetic homologues also raises many questions: Can the composition and characteristics of the bivalent domains be representative of all cell types? What are the differences in different processes of life development? Are the related factors regulating the bivalent domains specific? Much work needs to be done to further clarify the application of bivalent domains in the medical field, but it is undeniable that bivalent domains provide a new direction for stem cells therapy, and have great research value in many diseases related to stem cells, such as ovarian cancer, breast cancer, osteoporosis, obesity and so on. At the same time, it also provides a reliable mechanism and research direction for other types of cells to play their respective functions.

The emergence of many biological methods provides technical support for the further study of bivalent domains. What is clear, however, is that the long road to explore bivalent domains is far from being completed.

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**Availability of Data and Material** The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

### Declarations

**Ethical Approval** Not applicable.

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**Competing Interests** The authors declare that they have no conflict of interest.

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