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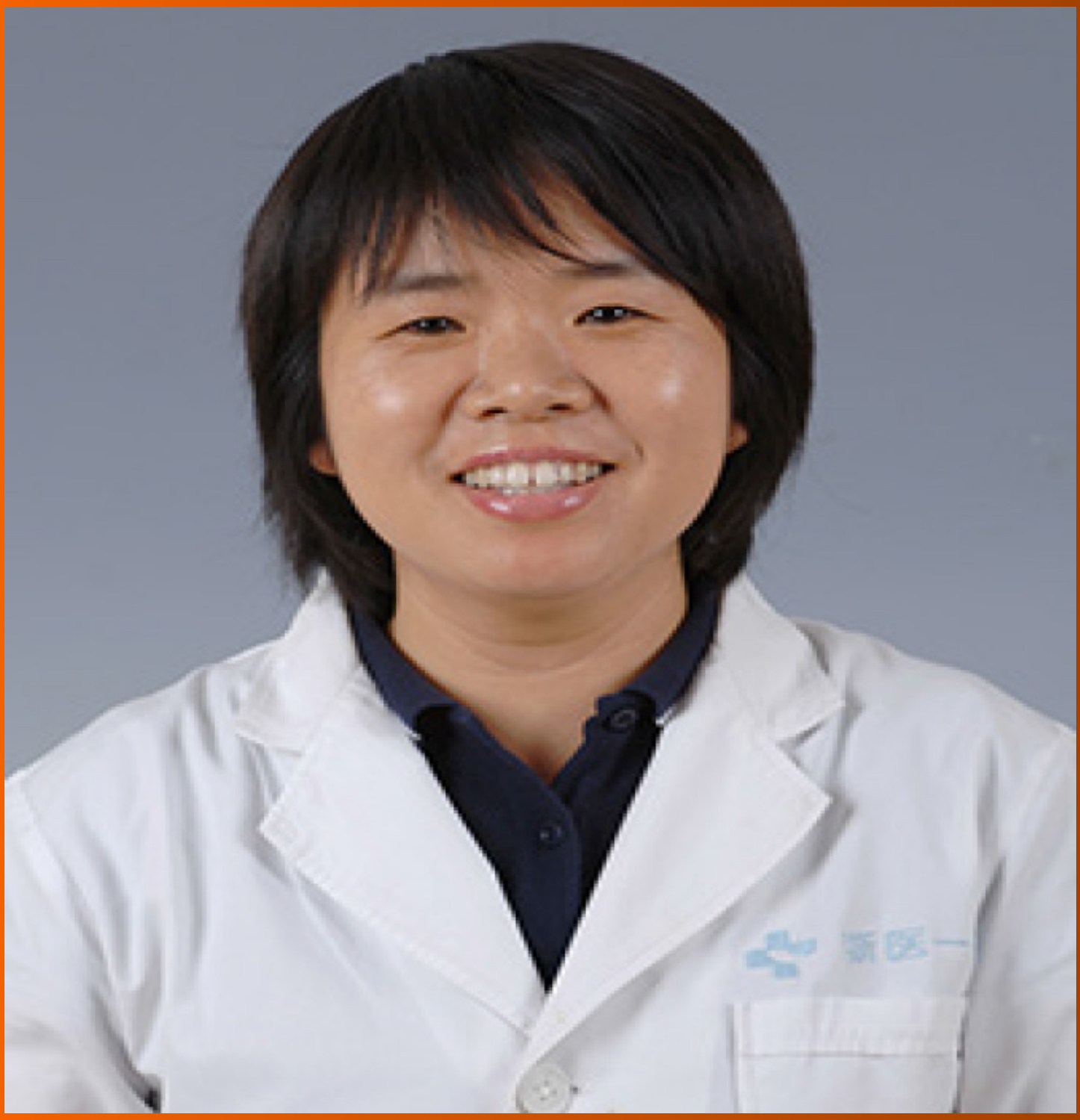

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MINIREVIEWS

# DNA methylation and demethylation link the properties of mesenchymal stem cells: Regeneration and immunomodulation

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## Abstract

Mesenchymal stem cells (MSCs) are a heterogeneous population that can be isolated from various tissues, including bone marrow, adipose tissue, umbilical cord blood, and craniofacial tissue. MSCs have attracted increasingly more attention over the years due to their regenerative capacity and function in immunomodulation. The foundation of tissue regeneration is the potential of cells to differentiate into multiple cell lineages and give rise to multiple tissue types. In addition,the immunoregulatory function of MSCs has provided insights into therapeutic treatments for immune-mediated diseases. DNA methylation and demethylation are important epigenetic mechanisms that have been shown to modulate embryonic stem cell maintenance, proliferation, differentiation and apoptosis by activating or suppressing a number of genes. In most studies, DNA hypermethylation is associated with gene suppression, while hypomethylation or demethylation is associated with gene activation. The dynamic balance of DNA methylation and demethylation is required for normal mammalian development and inhibits the onset of abnormal phenotypes. However, the exact role of DNA methylation and demethylation in MSC-based tissue regeneration and immunomodulation requires further investigation. In this review, we discuss how DNA methylation and demethylation function in multi-lineage cell differentiation and immunomodulation of MSCs based on previously published work. Furthermore, we discuss the implications of the role of DNA methylation and demethylation in MSCs for the treatment of metabolic or immune-related diseases.

**Key words:** Mesenchymal stem cells; DNA methylation and demethylation; Multi-lineage differentiation; Regeneration; Immunomodulation; Immune disease

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**Core tip:** Mesenchymal stem cells (MSCs) harbor the capacity to regenerate diverse tissues and can also perform key immunomodulatory functions. DNA methylation and demethylation are known to modulate stem cell maintenance and differentiation in embryonic stem cells. However, the role of DNA methylation and demethylation in MSC-based tissue regeneration and immunomodulation requires further investigation. In this review, we discuss how DNA methylation and demethylation function in multi-lineage cell differentiation and immunomodulation of MSCs based on previously published work. In addition, we discuss the implications of the role of DNA methylation and demethylation in MSCs for the treatment of metabolic or immune-related diseases.

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## INTRODUCTION

DNA methylation and demethylation are two vital epigenetic regulatory mechanisms for gene expression. DNA cytosine methylation is a frequently occurring process that is orchestrated by DNA methyltransferases (DNMTs), which generate 5-methylcytosine (5mC)[1]. The methylation process at the 5$^{th}$ cytosine can be reversible, which is called DNA demethylation. This process has received increased attention over recent years. Increasingly more researchers began to identify enzymes that could generate 5-hydroxymethylcytosine (5hmC) from 5mC in mammalian cells. For the first time, in 2009, TET1 was shown to convert 5mC into 5-hmC[2]. Thereafter, all three of the TET family proteins (TET1, TET2, and TET3) were demonstrated to catalyze a similar hydroxymethylation reaction[3]. TET family proteins are also receiving increased attention because of their function in DNA demethylation.

In addition to their function in multi-lineage differentiation and tissue regeneration[4], mesenchymal stem cells (MSCs) also display profound immunomodulation capacity *via* a sophisticated molecular network[5]. DNA methylation and demethylation are known to modulate stem cell maintenance and differentiation by activating or suppressing an array of genes[6]. Previous research on DNA methylation and demethylation has primarily focused on embryonic stem cells and neural systems. Nevertheless, how DNA methylation and demethylation impact MSC function remains elusive. Here, we discuss recent studies concerning the effect of DNA methylation and demethylation on MSC-based regeneration and immunomodulation.

## OSTEOGENIC DIFFERENTIATION OF MSCS IS REGULATED BY DNA METHYLATION AND DEMETHYLATION

MSCs hold promising potential for regenerative medicine due to their capacity for self-renewal and multi-lineage differentiation into tissue-specific cells, which include osteoblasts, chondrocytes, and adipocytes. During osteogenic differentiation of MSCs, osteogenic-specific genes such as *RUNX2*, *OPN*, *COX2*, *ALP*, and *OCN*[7-11], which are regulated by DNA methylation, showed increased expression and decreased DNA methylation. Demethylation was observed at specific CpG regions in the promoters of osteogenic lineage-specific genes, including *Runx2, Dlx5, Bglap*, and *Osterix*, during osteogenic differentiation in adipose-derived MSCs (Ad-MSCs). Upon demethylation inhibition, osteogenic gene expression became down-regulated[12]. On the other hand, Daniunaite *et al*[13] found that genes encoding the main pluripotency factors, such as Nanog and Sox2, showed decreased gene expression along with decreased 5hmC levels during the osteogenic differentiation of Ad-MSCs.

In another study on Ad-MSCs, an age-related decline in proliferation was observed. Ad-MSCs isolated from old donors showed significantly impaired osteogenic differentiation capacity compared to young donors. Furthermore, decreased expression of *Nanog, Oct4*, and *Lin28A* and increased expression of *Sox2* were observed. A simultaneous decrease of global 5hmC in Ad-MSCs from old donors also

occurred. When 5-azacytidine (5-Aza), a DNMT inhibitor, was used to treat Ad-MSCs from old donors, increased global 5hmC and increased TET2 and TET3 expression were observed, which was accompanied by an increase in osteogenic differentiation capacity[14]. These results suggest that global DNA demethylation levels correlate with the osteogenesis capacity of MSCs, and that DNMT inhibitors could down-regulate DNA methylation to improve osteogenesis. Notably, an additional study by Kornicka *et al*[15] drew similar conclusions.

Bone marrow MSCs (BMMSCs) are a population of multipotent stem cells isolated from bone marrow that harbor the capacity for self-renewal and multi-lineage differentiation. The osteogenic differentiation of BMMSCs is also regulated by dynamic changes, as well as a balance of DNA methylation and demethylation. Bone loss caused by mechanical unloading is partially due to the impaired regeneration capacity of BMMSCs[16]. When mechanical stimuli were rescued, Dnmt3b was released from the *Shh* gene promoter, thus leading to promoter demethylation and up-regulated gene expression. Hedgehog signal was then activated by Shh, promoting BMMSCs to differentiate into osteoblasts[17]. Yang *et al*[18] found that in *Tet1* and *Tet2* double knockout mice, 5hmC levels of the *P2rX7* promoter were down-regulated, leading to miR-293a-5p, miR-293b-5p, and miR-293c-5p accumulation, and a decrease in BMMSC osteogenic differentiation capacity. Upon re-activating P2rX7, microRNA secretion from *Tet* double knockout BMMSCs was increased, thus partly rescuing both the osteopenia phenotype and BMMSC function.

Mechanisms of TET-mediated DNA demethylation in distinct MSCs vary due to their diverse sources. When small hairpin RNA lentiviral vectors were transfected to knock down TET1, the proliferation rate and odontogenic differentiation capacity of human dental pulp stem cells were significantly suppressed. This indicated that TET1 plays an important role in dental pulp repair and regeneration[19]. In another study focusing on human BMMSCs, TET1 recruited other epigenetic modifiers, including SIN3A and EZH2, to inhibit the osteogenic differentiation of BMMSCs in an indirect manner. On the other hand, TET2 was found to directly promote the osteogenic differentiation of BMMSCs[20]. The underlying mechanisms of how the TET family proteins regulate MSC function from distinct sources require further investigation.

## ADIPOGENIC DIFFERENTIATION OF MSCS IS RELATED TO DNA METHYLATION AND DEMETHYLATION

Noer *et al*[21] reported that in undifferentiated Ad-MSCs, the promoters of adipogenic genes, including *LEP*, *PPARγ2*, *FABP4* and *LPL*, are hypomethylated, in contrast to myogenic or endothelial genes. During adipogenic differentiation, although specific CpG sites of the *LEP* promoter undergo demethylation, the global methylation status of *LEP, PPARG2, FABP4*, and *LPL* promoters across different Ad-MSC clones remains stable. Yang *et al*[18] showed that *Tet*1 and *Tet*2 small interfering RNA treatment does not alter the adipogenic differentiation capacity of BMMSCs.

Barrand *et al*[22] showed that in adipose MSCs, the promoter of *OCT4* was hypermethylated consistent with its repression. Melzner *et al*[23] found that the promoter of leptin underwent extreme demethylation (9.4% ± 4.4%) during the maturation of human preadipocytes toward terminally differentiated adipocytes. What's more, methyl-CpG binding proteins could bind to specific sites in the promoter and repressed leptin expression. Fujiki *et al*[24] reported that during the differentiation of 3T3-L1 preadipocytes to adipocytes, the hypermethylated *PPARγ2* promoter was progressively demethylated, while 5-Aza could increase the expression of *PPARγ2*, indicating that the methylation of its promoter inhibited the gene expression.

Overall, additional research on the dynamics of DNA methylation and demethylation during adipogenesis from different MSC sources is necessary.

## CHONDROGENIC DIFFERENTIATION IS REGULATED BY DNA METHYLATION AND DEMETHYLATION

DNA methylation and demethylation status also change during MSC differentiation into chondrocytes. Chondrogenic differentiation of Ad-MSCs and BMMSCs was associated with a < 50% reduction in methylation rates at two specific CpG sites in the *COL10A1* gene, and transcription of this gene was strongly induced[25]. Ito *et al*[26] discovered that 5hmC increased during chondrogenic differentiation of C3H10T1/2, a MSC line, and that *TET1* expression was significantly up-regulated. Furthermore, *Tet1*

knockdown resulted in a marked decrease in the expression of chondrogenesis markers such as Col2 and Col10. In addition, 5hmC in the *Igf1* promoter is a preferable binding site for TET proteins in chondrocytes. Additional targets of *Tet* genes, as well as other enzymes that function in DNA methylation and demethylation, need to be identified in order to reveal the underlying mechanisms of chondrogenic differentiation of MSCs.

Lin *et al*[27] found that stepwise preconditioning–manipulated BMMSCs showed improved cell proliferation and chondrogenic differentiation potential *in vitro* and enhanced therapeutic effect on the progression of osteoarthritis *in vivo*, and one mechanism of that is the reduction in CpG methylation at the promoters of *Nanog* and *Oct4*. Pollock *et al*[28] demonstrated an experimental DMSO-free formulation which could improve post-thaw function of MSCs including chondrogenesis, as DMSO is a strong inducer of demethylation which may affect the potential of MSCs for therapeutic use in treatment of human diseases. These studies reminded us that epigenetic modification of MSCs could be a promising approach to improve their therapeutic effects.

These results regarding DNA methylation and demethylation indicate that hypomethylation of specific genes, such as *Runx2*, *Opn*, *Dlx5*, *Osterix*, *Col2*, and *Col10*, play important roles in multi-lineage differentiation of and tissue regeneration by MSCs (Figure 1).

## MYOGENIC DIFFERENTIATION ASSOCIATED WITH DNA DEMETHYLATION

Cardiogenic differentiation is another important property of MSCs, and stem cell therapy for cardiovascular diseases is now in clinical trial[29]. Bhuvanalakshmi *et al*[30] found that in differentiated cardiomyocytes from MSCs, six out of the ten CpG islands of the promoter regions of *Nkx2.5*, the early cardiac gene, underwent demethylation. What's more, the CpG promoter demethylation of *sFRP4*, a Wnt antagonist, was also observed. This result is consistent with the previous findings that 5-Aza treatment of BMMSCs inhibited the ventricular scar from thinning and expanding, minimized left ventricular chamber dilatation, and thus improved myocardial function[31]. Antonitsis *et al*[32] treated hBMMSCs with 5-Aza *in vitro* to induce them to differentiate towards a cardiomyogenic lineage. Nakatsuka *et al*[33] also used 5-Aza to investigate the myogenic differentiation potential of mouse dental pulp stem cells. DNA demethylation induced by 5-Aza and forced expression of Myod1 upregulated the muscle-specific transcriptional factors such as Myogenin and Pax7.

## IMMUNOMODULATION OF MSCS ASSOCIATED WITH DNA METHYLATION AND DEMETHYLATION

Aside from tissue regeneration, MSCs play an important role in immunomodulation, which may prove critical for treating a variety of immune diseases such as colitis, arthritis, and systemic lupus erythematosus[34-36]. Immunomodulation by MSCs relates to the secretion of extracellular matrix proteins[37] as well as a variety of cytokines including IL-2, IL-4, IL-10, TNF-α, and INF-γ[38-40]. MSC immunoregulation can also occur through cellular contacts[40-42]. B cell proliferation was found to be inhibited by human MSCs, not through the induction of apoptosis but through G0/G1 cell cycle arrest[43]. MSCs may suppress T cell proliferation, cytokine release, cytotoxicity, and Th1/Th2 balance[44,45].

Of late, how DNA methylation and demethylation regulate MSC-induced immunomodulation has received increasingly greater attention. Yang *et al*[46] found that *Tet*1- and *Tet*2-mediated *Foxp3* demethylation plays a significant role in the differentiation of regulatory T cells as well as the maintenance of immune homeostasis. Khosravi *et al*[47] reported that MSCs could enhance the demethylation of the Treg-specific demethylated region upon cell-cell contact, and MSC-based induction of regulatory T cells is associated with direct modifications of the RUNX complex genes (*RUNX1*, *RUNX3*, and *CBFB*). Yu *et al*[48] found that the down-regulation of both *TET1* and *TET2* leads to hypermethylation of the *DKK-1* promoter, which leads to activation of the Wnt/β-catenin signaling pathway and thus up-regulates Fas ligand (the *FasL* gene) expression in periodontal ligament stem cells. This in turn enhances their immunomodulatory ability, which is demonstrated by their elevated capacity to induce T cell apoptosis. Taken together, these results demonstrate a significant role for TET-mediated DNA demethylation in MSC-based

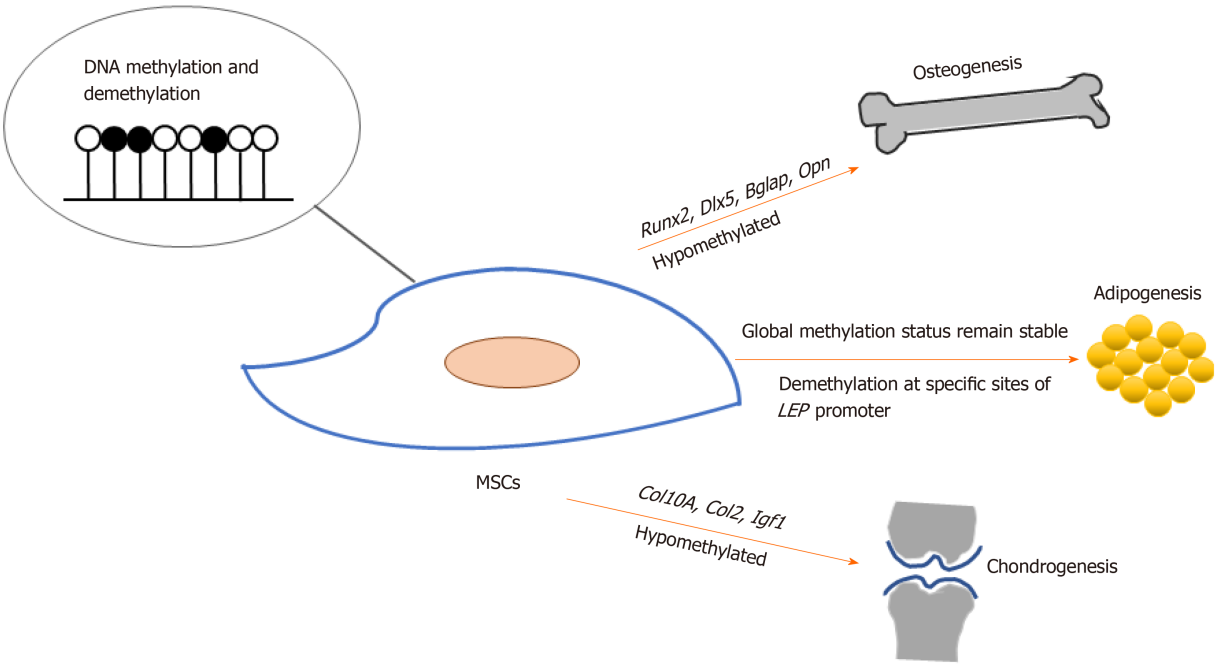


**Figure 1 Hypomethylation of specific genes in mesenchymal stem cells drives multi-lineage differentiation and tissue regeneration.** MSCs: Mesenchymal stem cells.

immunomodulation (Figure 2). Nevertheless, further investigations are required to reveal whether the methylation of MSCs is involved in regulation of other immune cells such as macrophages and natural killer cells and the underlying mechanisms.

## IMPLICATIONS FOR DISEASE TREATMENT

As previously mentioned, DNA methylation participates in the regulation of gene expression, which may contribute to metabolic diseases when there is an imbalance in DNA methylation *vs* demethylation. García-Ibarbia *et al*[49] compared bone tissue samples from patients with osteoporotic hip fractures and osteoarthritis. Their results showed that Wnt pathway activity is reduced in patients with hip fractures compared with those with osteoarthritis. Additionally, six genes, including *FZD10*, *TBL1X*, *CSNK1E*, *SFRP4*, *CSNK1A1L*, and *WNT8A*, showed significantly different methylation rates between both groups. *FZD10*, *CSNK1E*, *TBL1X*, and *SFRP4* are hypermethylated in osteoarthritis, while *WNT8A* and *CSNK1A1L* are hypomethylated compared with fractures. This result may help explain the distinctions in Wnt pathway activity between the two groups. MSCs from spinal ligaments with ectopic ossification largely differentiated into osteogenic lineage. Chiba *et al*[50] found that MSCs isolated from the spinal ligaments of ossification from yellow ligament patients showed higher expression of *GDNF* and *Wnt5a*, which are hypomethylated compared with the control group. This result indicates that osteogenic features of MSCs from patients with ossification of the yellow ligaments are promoted by *GDNF* and *Wnt5a* demethylation.

In 2002, Bartholomew *et al*[51] first reported that MSCs harbored immunosuppressive functions by demonstrating their ability to inhibit a mixed lymphocyte response *in vitro* as well as prevent rejection in a baboon skin allograft model *in vivo*. The immunosuppressive capacities of MSCs have therein provided new therapeutic insights into immune-mediated disease treatments. Centeno *et al*[52] reported that autologous MSCs and physiologic doses of dexamethasone could increase meniscus volume of the human knee. In addition, MSCs can relieve symptoms of multiple sclerosis, multiple system atrophy, and amyotrophic lateral sclerosis in varying degrees[53,54]. How DNA methylation and demethylation function in MSC therapy for immunological diseases necessitates further exploration.

## CONCLUSION AND PERSPECTIVE

Although a wealth of research has investigated MSC therapy, including hundreds of

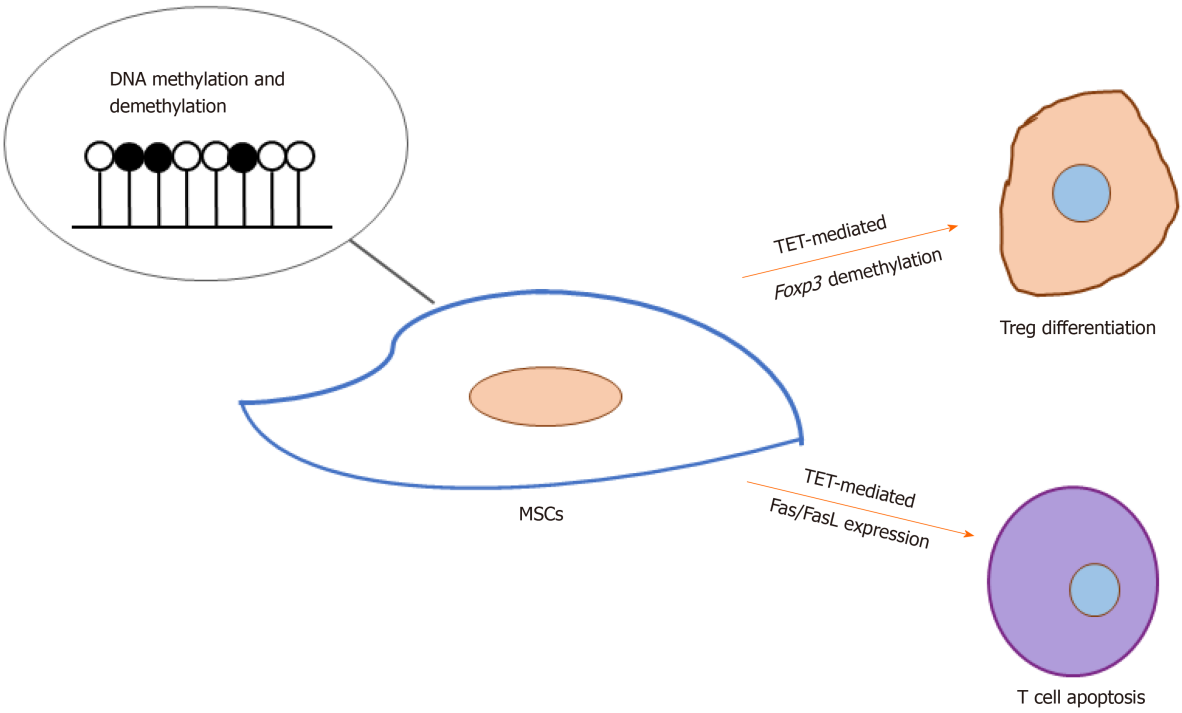


**Figure 2 TET-mediated demethylation functions in regulatory T cell differentiation and mesenchymal stem cell-induced T cell apoptosis.** MSCs: Mesenchymal stem cells; Treg: Regulatory T cells.

MSC-based clinical trials that have been administered, the mechanisms that underlie the multiple distinct MSC functions remain elusive. This review sheds light on the roles that DNA methylation and demethylation play in regulating MSC-based regeneration and immunomodulation, although it is possible that we overlooked a few studies due to our literature resource limitations. However, the precise function of DNA methylation and demethylation in different MSC types, as well as the associated underlying mechanisms, remain to be thoroughly investigated. This knowledge would inform the development of novel approaches for enhancing MSC-based tissue regenerative and immune therapies.

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