Epigenetic Regulation in Mesenchymal Stem Cell Aging and Differentiation and Osteoporosis

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Mesenchymal stem cells (MSCs) are a reliable source for cell-based regenerative medicine owing to their multipotency and biological functions. However, aging-induced systemic homeostasis disorders in vivo and cell culture passaging in vitro induce a functional decline of MSCs, switching MSCs to a senescent status with impaired self-renewal capacity and biased differentiation tendency. MSC functional decline accounts for the pathogenesis of many diseases and, more importantly, limits the large-scale applications of MSCs in regenerative medicine. Growing evidence implies that epigenetic mechanisms are a critical regulator of the differentiation programs for cell fate and are subject to changes during aging. Thus, we here review epigenetic dysregulations that contribute to MSC aging and osteoporosis. Comprehending detailed epigenetic mechanisms could provide us with a novel horizon for dissecting MSC-related pathogenesis and further optimizing MSC-mediated regenerative therapies.

1. Introduction

1.1. Mesenchymal Stem Cells (MSCs). MSCs are adult stem cells distributed in various mesenchymal tissues, which are derived from the mesoderm in the embryonic stage. MSCs exist in diverse tissues, such as bone marrow, umbilical cord blood, placenta, and adipose tissue [1]. Since MSCs are firstly isolated and defined from bone marrow, it has been traditionally accepted that bone marrow is the prevailing source of MSCs in humans [2, 3]. MSCs from different origins possess unique self-renewal capacities and can differentiate into multilineage cell types, including osteocytes, adipocytes, chondrocytes, and even endothelial cells or hepatocytes under certain given culture medium [4–6]. Apart from the aforementioned two basic characteristics, MSCs also exhibit various positive effects through paracrine action and immunomodulation during tissue repair, including regulating angiogenesis and osteoclastogenesis and guiding immune communication [7–9]. These properties signify that MSCs could perform extensive and active interactions with tissue-specific stem cell niches and represent an ideal and promising tool for tissue regeneration.

Although tentative therapeutic applications of MSCs have been carried out in the past years, disadvantages such as poor cell sources from diseased or aged hosts and in vitro passaging-induced senescent hypofunction both impair their therapeutic efficacy in tissue regeneration and hinder their large-scale clinical trials. MSC senescence manifests as division arrest, reflected by impaired proliferation and biased differentiation from osteoblasts towards adipocytes. Therein, biased differentiation can be induced by the imbalance between runt-related transcription factor 2 (Runx2) and peroxisome proliferator-activated receptor γ (PPARγ) pathway. These changes during senescence underlie bone mass loss and fat accumulation in aged or diseased skeletal tissues [10–13]. MSC aging is molecularly characterized by upregulated expression of senescence-associated genes such as p53, p21, p16INK4a, and β-galactosidase genes [14].
Notably, epigenetic regulation has emerged as a vital contributor to MSC aging and hypofunction, thus perturbing stem cell niche homeostasis and harming tissue health. Intriguingly, epigenetic alterations have also been demonstrated to modulate canonical senescence-associated genes directly or indirectly. Accordingly, therapeutic strategies based on epigenetic regulation may remedy tissue disorder in aging and diseases and further maximize the advantages of MSC-mediated tissue regeneration. In this review, we mainly focus on epigenetic marks and modifiers in regulating MSC aging in vitro or in vivo, in order to clarify the interactive link between epigenetic regulation and aging-related tissue diseases such as osteoporosis, and offer some clues for future utilization of epigenetics mediated tissue regeneration [15].

1.2. Epigenetic Regulation. Epigenetic regulation refers to altering phenotype through gene differential expression without changing DNA sequence and is a characteristic of heritability, reversibility, and no gene changes [11, 16]. Epigenetic alterations in cells happen in response to extrinsic environmental stimuli and cellular intrinsic inheritance to maintain cell and niche homeostasis. Accordingly, MSC aging or senescence in vivo or in vitro is also influenced by its own intrinsic dysregulation and microenvironmental factors from MSC niche, in the process of which typical epigenetic marks could be detected. In MSCs, epigenetic profile reflects dynamically transforming chromatin structure and corresponding transcriptional activity of genes; the major epigenetic mechanisms include DNA methylation, histone modifications, and chromatin remodeling [17]. In addition, posttranscriptional processing through mRNA and noncoding RNAs (ncRNAs) also takes part in epigenetic regulation of MSCs [18] (Figure 1). It has been widely documented that these epigenetic marks all have profound influences on MSC fate at multiple levels. Hence, further rationalizing and understanding the function mechanism of different epigenetic marks and modifiers occurring in MSC aging are of instructive importance to analyze the pathogenesis of aged and diseased tissue disorders and explore more effective therapeutic or regenerative strategies.

2. Epigenetic Regulation in MSC Aging and Osteoporosis

2.1. DNA Methylation in MSC Aging and Osteoporosis. DNA methylation refers to the covalent binding of methyl to the 5th carbon of cytosine at CpG dinucleotide to form 5-methylcytosine (5-mC) under the catalysis of DNA methyltransferase (DNMT) [19]. This process can be reversed by demethylation relying on the catalysis of ten-eleven translocation protein (TET), which catalyzes the transformation of the 5th carbon of cytosine into 5-hydroxymethylcytosine (5-hmC) [20]. In most cases, methylation of gene promoters or enhancers represents repressed expression (Table 1).

No matter in vitro or in vivo, alteration of DNA methylation profile gradually emerges as a close connection to MSC aging. Recently, researchers have successfully detected gene sites with methylation changes in the aging process by BeadChip microarray and found that alteration of methylation overlaps in aged MSCs in vivo and in vitro [21]. Afterward, in 2015, more than 10000 hypermethylation CpG sites and 40000 hypomethylation CpG sites were uncovered, many of which are associated with homeobox genes related to cell differentiation. For example, Hox and Runx2, as key transcription factors for osteogenesis, are hypermethylated in aged MSCs [21–23]. Moreover, in 2017, enhanced reduced representation bisulfite sequencing was used to depict a more precise DNA methylation profile, which finds that transcription factor binding sites (TFBS) for silent regulator 6 (Sirt6), E2F transcription factor 6 (E2F6), JunB proto-oncogene, and signal transducer and activator of transcription (Stat5) genes were hypermethylated along with the culture process, while TFBS for Stat3 gene were hypomethylated. Besides, transcription factors influencing chromatin structure, such as SMARCs and SIN3A, are also differently methylated [24]. In general, the degree of methylation generally decreases in the process of aging [22]. On the other hand, these DNA methylation sites have also been discovered to be related to repressive and promotive histone modification, respectively. In MSCs, a large number of hypomethylated CpG sites are enriched in the region of active histone mark methylation of lysine 4 on histone H3 (H3K4me) indicating that H3K4 methylation is accompanied by DNA hypomethylation, and both of them are signs of increased transcription activity. By contrast, the hypermethylation CpG DNA region mainly overlaps with the repressive chromatin marks trimethylation of lysine 27 on histone H3 (H3K27me3), H3K9me3, and enhancer of zeste2 (EZH2), all of which play synergistic roles in inhibiting transcription activity [22, 25]. Particularly, H3K9me-promoted DNA hypermethylation of gene p16INK4a has also been proved to serve as a significant signature for epigenetic senescence in human MSCs (hMSCs) [26]. In MSCs isolated from aged individuals, 5-hmc also appears in specific CpG sites and, coincidentally, corresponds mostly to the hypomethylation region in the aged MSCs [27].

In the complicated regulatory network of DNA methylation, some regulatory factors and sites have been identified to have a direct relationship with MSC aging. For instance, 5-azacytidine (5-AzaC), as an inhibitor of DNMT1 and DNMT3b, exacerbates cell senescence by downregulating polycomb group proteins (PcGs) including B cell-specific Moloney murine leukemia virus integration site 1 (BMI1) and EZH2 through miRNAs. Since these PcGs are responsible for repressive mark H3K27me3 formation at the p16 gene promoter, 5-AzaC finally facilitates p16’s activation in cell senescence. Moreover, inhibition of DNMT also directly demethylates cyclin-dependent kinase (CDK) inhibitor genes p16 and p21 [28]. However, 5-AzaC and another DNMT inhibitor RG108 were also reported to alleviate senescence by preventing reactive oxygen species (ROS) accumulation and maintaining telomerase reverse transcriptase (Tert) activity in aged human bone marrow MSCs (hBMSCs) [29, 30]. The methylation status of DNA is also responsible for differentiation potential and further influences the process of skeletal diseases. For example, in MSCs with high stemness, stemness-related transcription factors Octamer-binding transcription factor (OCT4) and NANOG protein
both directly bind to the promoter region of DNMT1 and then transcriptionally activate it, followed by methylation and silencing of senescence-related genes [31]. On the contrary, when MSCs are treated with a DNMT1 inhibitor 5-AzaC, downregulation of DNMT1 is accompanied by hypomethylation of genomic DNA and increased expression of osteogenic genes such as Runx2, Osteocalcin (Ocn), distal-less homeobox 5, and Osterix (Osx), which is more visually presented by enhanced alkaline phosphatase (ALP) activity and mineralization [32]. Clinically, it has been reported that DNMT1 dysfunction also influences skeletal metabolic homeostasis. Aberrant CpG hypermethylation at gene ATP-binding cassette subfamily B member 1 (ABCB1, the encoding gene of P-glycoprotein) leads to osteonecrosis of the femoral head (ONFH) [33]. Besides, depletion of demethylase TET1 and TET2 causes osteopenia phenotype in mice by impeding demethylation of P2rX7 promoter; P2rX7 deficiency further leads to MSC incapability of exosome release, which results in intracellular accumulated miR-297 targeting Runx2 signaling pathway [34].

2.2. Histone Modification in MSC Aging and Osteoporosis.
Histone modification is closely related to transcriptional activities of genes surrounding it. There are many different covalent modification types of the N-terminal amino acids of histone lysine, including acetylation, methylation,
phosphorylation, ubiquitylation, and SUMO modification. Generally speaking, the first two are related to transcription activation, while the latter three often dominate transcription inhibition, with the exception of H3K4me3 [35]. In cases of aging and diseases related to MSCs, histone acetylation levels depending on the balance between histone deacetylases (HDACs) and histone acetyltransferases (HATs) and methylation levels relying on histone methyltransferases (HMTs) and histone demethylases (HDMTs) both make a critical difference [36, 37] (Table 2).

Histone modification regulates senescence by affecting the transcription activity of surrounding DNA related to cell cycle. For instance, the most direct regulators of repressive H3K27me3 can be divided into two categories: one is HMT such as G9a and PcGs including MLL1, EZH2, and SUZ12, and the other is HDMT such as lysine-specific demethylase 1 (LSD1) and jumonji domain-containing protein 3 (JMJD3). Both upregulation of Jmjd3 gene and downregulation of PcG genes suppress H3K27me3 at the promoter of p14 and p16, which triggers the activation of corresponding proteins and then exacerbates MSC aging [38]. On the other hand, HDAC and Twist1 exert their influence in MSC aging at the upstream of PcGs and JMJD3. Downregulation of Twist1 gene expression in aged MSCs is followed by EzH2 suppression and E47 promotion. Then, the upregulated E47 protein directly binds to the p16 promoter to increase its transcriptional activity, producing a synergistic action with insufficient EZH2 protein [39]. In comparison to Twist1, the regulatory mechanism of HDAC is more specific. In normal cells, HDAC restrains Jmjd3 expression through deacetylating histone near its promoter region and oppositely promotes PcG proteins and c-MYC activity via the RB/E2F pathway. However, in aging MSCs, HDAC deficiency induces hypophosphorylation of retinoblastoma-like protein (RB). This prompts RB to bind to E2F and further compromises the overall expression of PcGs genes. Finally, alterations of JMJD3 and PcGs level bring about cell cycle arrest by demethylating H3K27 at the p16\(^{INK4A}\) promoter [38]. Lee et al. also reported that HDAC inhibitors valproic acid and sodium butyrate both promote the acetylation of histone H3 and H4 to activate the transcription of p21\(^{CDKI/NAF1}\), but the p16 expression level remains unchanged unexpectedly [40]. By contrast, another group found that low concentrations of HDAC inhibitor largazole or trichostatin A induce improved proliferation, suppressed differentiation, and delayed aging of hUCSCs. The underlying mechanism is based on histone H3 acetylation and methylation around Tert, Nanog, Oct4, Alp, Ocn, and Cxcr4 genes [41]. SIRT6 protein is another regulator with HDAC activity. As an NAD-dependent H3K9 and H3K56-specific deacetylase, SIRT6 deficiency causes acetylated H3K56 accumulation and compromised recruitment of RNA polymerase II (RNAS II) complex to heme oxygenase 1 (Hmox-1) gene promoter. More importantly, without the cooperation of SIRT6, expression of RNAS II, Nrf2, and Hmox-1 genes declines, resulting in impaired cellular redox homeostasis [42].

In terms of diseases, an imbalance between histone modifications of osteogenic and lipogenic genes is a possible mechanism. Intriguingly, as for histone modification, the regulatory effects of the same factor on osteogenic differentiation or adipogenic differentiation are not necessarily opposite. In other words, factors that promote adipogenesis may either inhibit or promote the biological osteogenic process [43]. For instance, mixed lineage leukemia protein (MLL), general control non-derepressible5 (GCNs, namely, KAT2A), and P300/CBP-associated factor (PCAF, namely, KAT2B) can promote both osteogenesis and lipogenesis, while HDAC1 can inhibit both. Moreover, SET domain bifurcated 1 protein, lysine-specific demethylase 4B/6B (KDM4B/6B), and HDAC3 all promote osteogenesis but inhibit lipogenesis; oppositely, EZH2 and HDAC6 promote lipogenesis but inhibit osteogenesis [43, 44]. Thus, homeostasis of bone tissues relies largely on coordinating and orderly expression in spatial-temporal dimensions. And histone modification dysregulation in osteoporosis is closely related to the break of balance among associated regulatory factors. For instance, in osteoporosis, the upregulated EZH2 and KDM5A and downregulated absent, small, or homeotic 1-like (ASH1L) genes suppress Wnt and Runx2 pathways by altering H3K4me3 and H3K27me3 levels [45–47]. Similarly, H3K9 acetyltransferase GCN5 and PCAF (namely, KAT2A and KAT2B) gliding deacetylates H3K9 on the promoter of Wnt, BMP, and Runx2 genes [48–50]. As to the mechanism of oculoacardiocardiodental (OFCD) syndrome characterized by extremely long dental roots and craniofacial defects, recruitment restriction of KDM mediated by BCL-6 corepressor increases the H3K4me3 level and promotes upregulation of AP-2a, whose osteogenesis-fortifying function is overactivated, leading to osteogenic hyperfunction in OFCD syndrome [51].

2.3. Chromatin Remodeling in MSC Aging and Osteoporosis. In a narrow sense, chromatin remodeling is an ATP-dependent process catalyzed by chromatin-remodeling complexes. The core component of the complexes is an ATPase subunit from the SNF2 family including SWI/SNF (switch/sucrose nonfermentable) and INO80 [SWI2/SNF2 related (SWR)] subfamilies [52]. In a broad sense, all factors that bring about chromatin structural alterations, including the relaxing or packing of chromatin by histone modification, contribute to chromatin remodeling (Table 3).

During the process of aging, the protein encoded by Brahma-related gene 1 (Brg1), the ATPase subunit of SWI/SNF chromatin remodeling complex, has been regarded as an essential factor in global modulation. Both the upregulation and downregulation lead to acceleration of cell senescence. On the one hand, when Brg1 is silenced, the chromatin compaction cannot be completed. This facilitates DNMT recruitment and methylation at Nanog promoter and eventually induces transcription inhibition [53]. Besides, Brg1 insufficiency-induced senescence is also linked to γ isoforms of heterochromatin formation and p53 activation-induced cell cycle arrest [53, 54]. On the other hand, overexpression of Brg1 also induces an increasing portion of programmed cell death, despite the fact that the specific mechanism is not clarified [55].

Apart from chromatin remodeling complexes, factors directly related to the chromatin structure, such as condensin
and KRAB-associated protein 1 (KAP1), also contribute to chromatin remodeling. It has been reported that condensin I/II can alleviate DNA damage by chromatin reorganization in normal cells. However, hypermethylation around NCAPD2/NCAPG2, the encoding genes of the core components of condensin, leads to condensin shortage and DNA damage accumulation during aging [56]. Loss of heterochromatin is proved to be a potential cause of MSC aging [57].

For example, abnormality of the heterochromatin component KAP1 promotes MSC aging via a chromobox4-dependent manner. When CBX4 declines in aged hMSCs, fibrillarin (FBL) and KAP1 cannot be recruited at nucleolar rDNA, leading to excessive expression of rRNAs, then trigger detrimental ribosome biogenesis and destabilize nucleolar heterochromatin [58].

As to osteoporosis, the INO80 chromatin remodeling complex interacts with WD repeat-containing protein 5 (Wdr5) protein that catalyzes H3K4me3 formation to

| Epigenetic changes | Factors | Mechanism | In vivo or in vitro | Consequence | Material | Ref. |
|-------------------|---------|-----------|-------------------|-------------|----------|------|
| Senescence and aging | Histone acetylation | HDAC1 | Directly upregulates JMJ3 and indirectly downregulates PcGs through Rb/E2F pathway to inhibit H3K27me3 at p16NKX6 | In vitro | Aging | hADSCs, hUCSCs [38] |
| | Histone acetylation | HDAC1 | Promotes the transcription of p21CIP1/WAF1 through increasing H3 and H4 acetylation | In vitro | Aging: decreased differentiation ability and proliferation rate | hADSCs, hUCSCs [40] |
| | Histone acetylation | SIRT6 | Insufficient SIRT6 causes increased H3K56ac and compromised recruitment of RNAPII complex to Hmox-1 gene promoter, leading to decrease in Hmox-1 expression and impaired cellular redox homeostasis | Both | Senescence, dysregulated redox metabolism, and increased sensitivity to oxidative stress | Human embryoid bodies MSC, mouse model [42] |
| | Histone methylation | TWIST1 | Insufficient to prevent senescence by recruiting EZH2 and form repressive H3K27me3 at p16/p14 promoters; upregulates E47 that binds to p16 promoter and promotes transcription activity | In vitro | Senescence | hBMSCs [39] |
| | Histone methylation | BMI1 | Fails to recruit and stabilize PRC2 which protects H3K27me3 of p16NKX6 | In vitro | Aging | hADSCs, hUCSCs [38] |
| | Histone methylation | EZH2 | Fails to methylate H3K27 as catalytic subunit of PRC2; insufficient H3K27me3 cannot suppress p16 and p14 expression | In vitro | Aging | hADSCs, hUCSCs [38] |
| | Histone methylation | G9a | (Unclear) | In vitro | Aging: decreased differentiation ability and proliferation rate | Rat BMSCs [149] |
| Skeletal diseases | Histone methylation | Ezh2 | Promotes H3K27me3 on Wnt1, Wnt6, and Wnt10a promoters to silence Wnt signaling pathway | Both | Osteoporosis | hBMSCs, mouse BMSCs, mouse model [47] |
| | Histone methylation | KDM5A | Increases H3K4me3 levels on promoters of Runx2 | Both | Osteoporosis | hBMSCs, mouse BMSCs, mouse model [45] |
| | Histone methylation | ASH1L | Fails to mediate H3K4me3 recruitment at the transcription start sites of Otx, Runx2, Sox9, and Creb genes | Both | Osteoporosis | hBMSCs, mouse BMSCs, mouse model [46] |
| | Histone methylation | KDM2B | Unable to be recruited to the promoter of AP-2α and inhibit AP-2α expression via removing H3K4me3 | Both | Oculofaciocardiodental (OFCD) syndrome | hBMSCs, mouse BMSCs, mouse model [51] |
| | Histone acetylation | GCN5 (KAT2A) | Insufficient to increase H3K9 acetylation on the promoters of Wnt genes | Both | Osteoporosis | hBMSCs, mouse BMSCs, mouse model [48] |
| | Histone acetylation | PCAF (KAT2B) | Insufficient to acetylate H3K9 at promoters of BMP2, BMP4, BMPR2B, and Runx2 | Both | Osteoporosis | hBMSCs, mouse BMSCs, mouse model [49] |
positively regulate the canonical Wnt pathway. Correspondingly, after INO80 gene is knocked down, the osteogenic potential of MSCs decreases both in in vitro and in ectopic transplantation models, reproducing a similar phenotype as osteoporosis [59].

In general, chromatin remodeling is intertwined with DNA methylation and histone modification; the DNA and histone modification status directly determines the accessibility and structure of chromatin. Histone acetylation may “open” chromatin by neutralizing the positive charges of lysine to increase site exposure of the surrounding negatively charged DNA [60]. Meanwhile, histone modification modulates the synthesis of chromatin remodeling-related proteins [56]. Thus, the functional importance of the interactive mechanism is to realize orderly integration and feedback of all three processes.

2.4. mRNA Modification in MSC Aging and Osteoporosis. There are only a few studies about mRNA modification of MSCs during aging. Even so, the biological activity of MSCs in a normal bone is closely related to RNA N6-methyladenosine (m6A) modification. Osteogenesis induced by methyltransferase like 3 (METTL3) is counterbalanced with demethyltransferase fat mass and obesity-associated protein (FTO) [61]. During the aging process, expression of Fto gene increases and inhibits m6A formation on Ppary mRNA, which results in lipogenesis through the GDF11-FTO-PPARγ axis [62]. However, the adipogenesis and osteoporosis process could be prevented by METTL3 application [63].

2.5. ncRNA in MSC Aging and Osteoporosis

2.5.1. ncRNA in MSC Aging. Alterations of miRNA and long noncoding RNA (lncRNA) abundances are closely associated with the aging process of MSCs either in vivo or in vitro. Researchers compared miRNA profiles in aged MSCs with younger generations to figure out up- or downregulated miRNA types (Table 4). Data from different organizations varied widely, probably because of different sources of MSCs or the experimental conditions [64–66]. Notably, the variation among MSC miRNA expression profiles from different tissues indicates that their regulatory mechanisms are relatively tissue-specific [67, 68].

Some upregulated miRNAs maintain senescent cells in a proliferation-disabled state by binding to the transcripts of genes related to the cell cycle. For instance, miR-22 and miR-485-5p directly target the cyclin-dependent kinase regulatory subunit 1 gene, thus impeding synthesis and function of CDK and cyclin B and cause G2/M phase arrest [69]. miR-34a also targets CDK2, CDK4, CDK6, cyclin D, cyclin E, and RBP2 to hinder self-renewal ability [70]. In addition, miR-31a-5p can bind to the 3′ UTR of E2F2 mRNA and bring about senescence-associated heterochromatin foci formation in aged rat BMSCs [71]. Besides, CNOT6 encodes deadenylase subunits of the Ccr4-Not complex; miR-29c-3p-induced CNOT6 downregulation can induce responsive elevation of p53, p21, and p16 expression followed by arrest of cell cycle [72, 73].

Correspondingly, with replicative pressure due to serial passages, miRNAs that play roles in repressing senescence-inducing proteins are downregulated. Downregulation of these miRNAs initially leads to dysregulation of global gene regulatory network and eventually fosters the aging process. For example, downregulation of miR-10a makes it insufficient to suppress senescence-inducing function of Krüppel-like factor 4 (KLF4) [74, 75]. And intriguingly, downregulation of miR-17 family (including miR-17, miR-20b-5p, and miR-106a-5p) regulates the activities of various genes, including Smad ubiquitination regulatory factor-1 (Smurf1), p21, CCND1, and E2F1 genes in aging [76, 77]. Besides, other downregulated miRNAs including miR-543, miR-590-3p, and miR-24a have been discovered to modulate p18/p21 and p16 activity separately [78, 79]. In addition, downregulation of miR-199b-5p also promotes cell cycle arrest indirectly via laminin gamma 1 [80].

Accumulation of reactive oxygen species (ROS) is another important mechanism of stem cell aging. Several miRNAs influence the production or elimination of ROS epigenetically. In
| Regulatory factors | Mechanism | In vivo or in vitro | Consequence | Material | Ref. |
|-------------------|-----------|-------------------|-------------|----------|------|
| **Senescence and aging** | | | | | |
| miR-10a↓ | Insufficient to target Kit/4 and repress its function | Both | Senescence, decreased differentiation | hBMSCs | [74, 75] |
| miR-20b-5p and miR-106a-5p↓ | Insufficient to inhibit Smads/p21/CDK/E2F pathway, which alleviates suspension of DNA synthesis during oxidative stress-induced premature senescence | In vitro | Premature senescence | hBMSCs | [77] |
| miR-22 and miR-485-5p↑ | Targets CKS1 to downregulate CDK1 and cyclin B | In vitro | Senescence | SHED | [69] |
| miR-31a-5p↑ | Targets E2F2 and promotes SAHF formation | Both | Senescence | Rat BMSCs | [71] |
| miR-27b↑ | Upregulates p16 expression and MAPK pathway activation | In vitro | Senescence | hBMSCs | [72, 73] |
| miR-34a↑ | Targets CKS1 to downregulate CDK1 and cyclin B | Both | Senescence and intrinsic apoptosis | Mouse BMSCs, rat BMSCs | [89, 90] |
| miR-485-5p↑ | Targets CNOT6 thus inducing senescence via p53/p21 and p16/pRB pathways | In vitro | Senescence | hBMSCs | [72, 73] |
| miR-29c-3p↑ | Targets CNOT6 thus inducing senescence via p53/p21 and p16/pRB pathways | Both | Senescence | hBMSCs | [72, 73] |
| miR-34a↑ | Targets Sirt1 to induce senescence via Sirt1/FoxO3a pathway, induces mitochondrial dysfunction | In vitro | Senescence and intrinsic apoptosis | Mouse BMSCs, rat BMSCs | [89, 90] |
| miR-188↑ | Targets Zmpste24 transcripts, causing prelamin A accumulation in nuclear envelope and intracellular DNA damage | In vitro | Senescence | hUCSCs | [92] |
| miR-141-3p↑ | Targets YAP to inhibit proliferation and accelerate senescence | In vitro | Senescence | Human papilla apical stem cells | [93] |
| miR-142↑ | Targets Epsas to downregulate pexophagic activity and induce ROS accumulation | In vitro | Aging | Mouse BMSCs | [81] |
| miR-155-5p↑ | Targets Cab39 and then reduces mitochondrial fission and increases mitochondrial fusion via the Cab39/AMPK signaling pathway | Both | Aging | Mouse model, hBMSCs | [86] |
| miR-182↑ | Targets FoxO1, which is critical to protecting cells from ROS | In vitro | Aging, decreased proliferation, and osteogenesis | hBMSCs | [83] |
| miR-183-5p↑ | Targets Hmox-1 to impair response to oxidative stress | In vitro | Senescence | Mouse BMSCs | [84] |
| miR-188↑ | Targets HDAC9 and RICTOR | Both | Aging, decreased proliferation | Mouse BMSCs, mouse model | [94, 95] |
| miR-195↑ | Targets Tert and prevents TERT to repair the shortened telomeres with replication | In vitro | Aging | Mouse BMSCs | [91] |
| miR-199b-5p↑ | Insufficient to repress LAMC1 | In vitro | Senescence | hBMSCs | [80] |
| miR-206↑ | Targets Alp1, which is essential for the intracellular ATP level and AMPK pathway | Both | Premature senescence | Rat BMSCs, rat model | [96] |
| miR-363-3p↑ | Targets TRAF3, which inhibits adipogenic differentiation and senescence | In vitro | Senescence, upregulated adipogenesis | Rat BMSCs | [99] |
| miR-486-5p↑ | Targets Sirt1 | In vitro | Senescence | hADSCs | [85] |
| miR-543 and miR-590-3p↑ | Insufficient to target AIMP3/p18 to inhibit expression, inducing an increase in CDK inhibitors p16INK4A and p21CIP1/WAF1 | In vitro | Senescence | hUCSCs | [78] |
| miR-1292↑ | Targets Wnt receptor FZD4, thus hindering the Wnt/β-catenin/TCF/LEF1 pathway | In vitro | Senescence, downregulated osteogenesis | hADSCs | [100] |

Both [103]
younger cells, the amount of peroxisome is restricted by pexophagy. However, in aged cells, upregulated miR-142 targets the endothelial PAS domain protein (Epas1) gene, a positive regulator of pexophagy, inducing ROS accumulation [81].

Moreover, miR-155-5p can repress antioxidant genes by targeting the common transcription factor CCAAT/enhancer-binding protein β (C/EBP-β) gene and then induces ROS generation [82]. miR-182 antagonizes osteoblast proliferation and differentiation by targeting FoxO1 gene, which protects hBMSCs from ROS-induced harm [83]. Besides, miR-183-5p, which belongs to the same cluster to miR-182, also increases in extracellular vesicles (EVs) derived from the bone marrow of aged mice. It accelerates cell senescence by impairing HMOX-1 protein’s responsive capacity to oxidative stress [84].

**miRNA also affects cell aging through mitochondrial or telomere mechanisms.** miR-34a targeting Sirt1 and miR-

**Table 4: Continued.**

| Regulatory factors | Mechanism | In vivo or in vitro | Consequence | Material | Ref. |
|--------------------|-----------|---------------------|-------------|----------|-----|
| lncRNA-Bmnncr↓     | Insufficient to serve as a scaffold to facilitate the interaction of ABL and transcriptional coactivator with TAZ, hindering the assembly of the TAZ and RUNX2/PPARγ transcriptional complex | Aging, transition from osteogenesis to adipogenesis | hBMSCs, mouse BMSCs |  | |
| lncRNA-HOTAIR↑     | Modulates senescence-associated changes in gene expression and DNA methylation via triple helix DNA-DNA-RNA formation | In vitro Senescence | hBMSCs | [102] |

**Skeletal diseases**

| miR-21↓            | Insufficient to target Spry1, which negatively regulates osteogenesis via FGF and MAPK | Both | Osteoporosis | hBMSCs, mouse model | [112] |
| miR-21a↓           | Insufficient to target Pten and PTEN downregulates AKT pathway to induce osteocyte apoptosis | Both | Glucocorticoid-induced osteonecrosis | hUCSCs, mouse model | [116] |
| miR-23b↑           | Targets Runx2 | Both | Osteoporosis | hBMSCs, mouse model | [106] |
| miR-27↓            | Insufficient to target Mef2c, which facilitates the adipogenic differentiation | Both | Osteoporosis | hBMSCs, mouse model | [113] |
| miR-181a↓          | Leads to the accumulation of Fasl from BMSCs, followed by CD4+ T cell apoptosis | Both | Osteoporosis | Mouse BMSCs, mouse model | [115] |
| miR-212 and miR-384↑ | Targets Runx2 | Both | Osteoporosis | Mouse BMSCs, mouse model | [108] |
| miR-542-3p↓        | Insufficient to inhibit sfrp1 expression, which is a negative regulator of Wnt pathway | Both | Osteoporosis | HEK293T cells, rat BMSCs, rat model | [111] |
| miR-596↑           | Targets Smad3 to inhibit Runx2 expression and osteogenesis | In vitro | ONFH | hBMSCs | [109] |
| miR-705 and miR-3077-5p↑ | Respectively, targets HOXA10 and Runx2 mRNA, leading to MSC lineage commitment transition to adipocytes | In vitro | Osteoporosis | hBMSCs | [107] |
| miR-708↑           | Targets Smad3 to inhibit Runx2 expression | In vitro | ONFH | hBMSCs | [110] |
| miR-1263↓          | Insufficient to suppress Mob-1/YAP/ Hippo signaling pathway-induced apoptosis | Both | Disused osteoporosis | hUCSCs, rat model | [114] |
| lncRNA-MALAT1↓     | Insufficient to inhibit miR-143, whose target is Orox | In vitro | Osteoporosis | hBMSCs | [117] |
| lncRNA-ORLNC1↑     | Endogenously competes with miR-296 and eliminates miR-296’s suppression of Pten, which is a negative regulator of osteogenesis | Both | Osteoporosis | hBMSCs, mouse model | [119] |
| lncRNA-DEPTOR↑     | Binds to ncRNA-MEG3’s promoter and reduces its function to activate BMP4 pathway | Both | Osteoporosis | Mouse BMSCs, mouse model | [120] |
| lncRNA-H19↓        | Insufficient to inhibit MSC proliferation and osteogenic differentiation via suppressing miR-19b-3p | In vitro | Inhibits osteoporosis | hBMSCs | [121] |
| lncRNA-HOTAIR↑     | Suppresses miR-17-5p to elevate Smad7 pathway | In vitro | Inhibits ONFH | hBMSCs | [122] |

ABL1: oncogene homolog 1; AIMP3: aminoacyl-tRNA synthetase-interacting multifunctional protein-3; CKS1: cyclin-dependent kinase regulatory subunit 1; Mef2c: myocyte enhancer factor 2c; LAMC1: laminin gamma 1; PRC2: polycomb repressive complex 2; SAHF: senescence-associated heterochromatin foci; sFRP1: secreted Frizzled-related protein-1; Spry1: sprouty homolog 1.
targeting calcium-binding protein 39 (Cab39) are all contributors in mitochondrial mechanisms [85–90]. When it comes to the telomere hypothesis of aging, miR-195 binds to the 3′ UTR of Tert mRNA, thus preventing TERT protein from repairing the shortened telomeres due to replicative senescence [91].

miRNAs such as miR-27, miR-141-3p, and miR-1292 can also induce aging through multiple cellular pathways involved in cell differentiation and metabolism. The more detailed information is listed in Table 4 [92–100].

As for IncRNAs, although they are regarded as crucial modulators of MSC-mediated ectopic tissue regeneration, only a few studies have reported the influence of IncRNAs on aging. IncRNAs regulate gene expression in diverse manners, such as serving as scaffolds to facilitate the assembly of specific transcriptional complexes or acting as sponges to reduce the availability of targeted miRNAs [101]. For instance, upregulated IncRNA-HOTAIR has been found to bring about senescence-associated changes, including differential expression of specific genes and abnormal DNA methylation by facilitating triple-helix DNA-DNA-RNA formation [102]. Besides, IncRNA-Bmncr serves as a scaffold to facilitate the interaction of Abelson murine leukemia viral oncogene homolog 1 protein and transcriptional coactivator with PDZ-binding motif (TAZ), guaranteeing the assembly of the TAZ and Runx2/Ppary transcriptional complex to inhibit MSC adipogenic differentiation. When IncRNA-Bmncr expression decreases in aging MSCs, the tendency of lipogenesis increases [103].

2.5.2. ncRNAs in Osteoporosis. It has been widely reported that miRNAs and IncRNAs play significant roles in maintaining the balance between osteogenesis and adipogenesis. Scholars have summarized that miRNAs were involved in osteogenic regulation mainly through two patterns: affecting Runx2 expression via canonical Wnt pathway, TGF-β pathway, and BMP pathway or directly targeting Runx2 or Oxs genes. On the other hand, ncRNAs influence adipogenesis through PPARγ and C/EBP-α [104, 105]. When these miRNA expression levels change and differentiation balance is broken, bone diseases such as osteoporosis will occur. For instance, it has been reported that in osteoporosis, upregulated miR-23b, miR-3077-5p, miR-212, and miR-384 inhibit osteogenesis by directly targeting Runx2 gene [106–108], while in ONFH, upregulated miR-596 and miR-708 hinder osteogenesis by binding to Smad3 transcripts to suppress Runx2 gene expression [109, 110]. In addition, miRNAs such as miR-542-3p, miR-21, and miR-27 target negative regulators in osteogenesis-related pathways; thus, their downregulation also leads to MSC lineage commitment transition from osteocytes to adipocytes [111–113]. In addition to modulating MSC differentiation, miR-181a and miR-1263 also influence skeletal homeostasis by regulating cell apoptosis through Fasl accumulation and Mob-1/YAP/Hippo, respectively, [114, 115]. Besides, downregulated miR-21a is insufficient to inhibit phosphatase and tension homolog (Pten) gene expression, and abundant PTEN protein downregulates the AKT pathway to induce osteocyte apoptosis in glucocorticoid-induced osteonecrosis [116].

IncRNAs often exert their pathogenic effect via miRNAs or other ncRNAs. For example, miR-143, a direct inhibitor of Oxs gene, is suppressed by IncRNA-MALAT1 in normal MSCs. However, in osteoporosis patients, downregulation of IncRNA-MALAT1 leads to decreased Oxs gene expression and loss of bone mass [117]. Another team also proved that IncRNA-MALAT1 acts as a sponge of miR-34c to increase the expression of special AT-rich sequence binding protein 2 (SARTB2), which is conducive to restoring osteogenesis in osteoporosis conditions [118]. Besides, elevated IncRNA-OBRLNC1 endogenously competes with miR-296 and eliminates miR-296’s suppression of Pten gene, which is a negative regulator of osteogenesis [119]. Moreover, in ONFH, upregulated IncRNA-DEPTOR binds to IncRNA-MEG3 promoter and prevents it from activating BMP4 pathway [120]. It is worth mentioning that in the process of skeletal diseases, alteration of IncRNA expression may work against bone destruction. For example, IncRNA-H19 inhibits MSC proliferation and osteogenic differentiation via suppressing miR-196-3p when estrogen exists. However, in postmenopausal osteoporosis, H19 expression level decreases [121]. Similarly, in ONFH, IncRNA-HOTAIR is upregulated and promotes osteogenesis via the miR-17-5p/Smad7 pathway [122].

In general, existing research reveals that in terms of mechanism, ncRNAs primarily regulate cell cycle or affect aging-related factors including ROS, telomere, and mitochondria to induce MSC aging. In the process of skeletal diseases, epigenetic factors promote disease progression through biased differentiation and cell apoptosis. Specifically speaking, the dysregulation of miRNA and IncRNA forecasts the dysfunction of MSCs, and notably, modulatory effects of ncRNAs are not directly realized in a unidirectional manner.

2.6. The Interplay of Different Epigenetic Factors in MSC Aging. As we mentioned in the previous parts, MSC aging is epigenetically marked by heterochromatin loss, altered DNA methylation profile, and organized histone modification. Actually, it should be particularly noted that epigenetic factors do not perform their functions in a parallel and independent fashion. It has been extensively reported that a multitude of them mutually intertwine and influence. Firstly, DNA methylation and histone modification are closely related in many aspects. In aged MSCs, hypomethylated CpG sites always come together with H3K4me, and both of them are signs of increased transcription activity, while DNA regions enriched in hypermethylated CpG sites mainly overlap with the repressive chromatin marks H3K27me3 and H3K9me3, synergistically inhibiting transcription activity [22, 25]. Moreover, DNA demethylation-induced PcG downregulation can regulate H3K27 methylation [28]. Secondly, histone modifications can alter chromatin structure not only by influencing histone-DNA, histone-histone interactions, and chaperone-histone binding [60] but also through modulating the synthesis of chromatin remodeling-related proteins such as condensin [56]. Last, DNA methylation also influences chromatin structure by regulating transcription factors, such as SMARCs and S1N3A [24]. Besides, it has been discovered that considerable ncRNAs interplay with complicated molecular networks and pretranscriptional epigenetic marks.
3. Application of Epigenetic Regulation in Skeletal Diseases and Engineering Regeneration

MSCs are an essential source for cell-based bone regeneration. The premise of bone regeneration is to maintain MSC stemness and promote their osteogenic differentiation. As mentioned above, it has been demonstrated that epigenetic markers and modifiers play fundamental roles in aging and diseases through modulating MSC function. Thus, MSC-mediated therapeutic or regenerative strategies based on epigenetic principle possess enormous potential in the treatment of aging-related bone disorders and defects. Despite the lack of experiments that mediate bone regeneration by means of epigenetic regulation in aging models, great efforts have been made in corresponding explorations with normal animal models. In turn, tentative application of epigenetic therapies in vivo further reinforces our understanding of the intrinsic mechanisms and makes it possible to realize our clinic utilization in the future. So far, there are three kinds of commonly used epigenetic interventions of tissue diseases or regeneration in vivo: exogenous inhibition of negative epigenetic regulators, exogenous supplement of positive regulators, and direct gene manipulation (Figure 2).

3.1. Exogenous Blocking. One common method is to block epigenetic factor-induced MSC aging and diseases by using exogenous inhibitors. For bone tissue regeneration in non-aging animal models, a combination of collagen sponge and HDAC1/4 inhibitor MS-275 exerts a promotive effect in rat critical-sized calvarial defect healing [123]. Moreover, intraperitoneal injection of MS-275 avoids delayed cranial suture closure in Runx2-null mice [124]. Similarly, when vorinostat, another HDAC1 inhibitor, was intraperitoneally injected into mice, the number of osteoblasts in endocortical bone increased and OCN level in serum rose [125]. Except for that, miRNA-mediated bone regeneration usually proceeds with specific biomaterials [126]. For example, when hMSCs transfected with anti-miR-34a, anti-miR-138, or anti-miR-222 by lipofectamine are loaded on a hydroxyapatite/tricalcium phosphate ceramic powder, the ectopic bone formation on complex scaffolds increases compared to the untransfected group [127, 128]. Moreover, anti-miR-222 also manifests a promotive effect toward bone defect healing when directly loaded on the atelocollagen scaffold [129].

3.2. Exogenous Supplement. ncRNAs or their mimics can be exogenously supplemented to delay or reverse disease progression. It has been extensively reported that loading MSCs transfected by proosteogenic miRNAs (such as miR-26a, miR-148b, miR-5106, miR-335-5p, or their mimics) on biomaterials is an effective strategy to promote bone regeneration [133–136]. With regard to skeletal disease treatment, agomiR-130a intravenously injection reduces bone loss in elderly mice by targeting Smurf2 and Ppary genes [137, 138], while collagen-based hydrogel containing agomir-34a elevates bone volume in mouse radiational bone injury area by downregulating Notch1 expression in BMSCs [139]. In addition, miR-328 is the antagonist of Axin1 gene, whose product AXIN1 protein is an inhibitor of Wnt signaling pathway. Thus, application of apoptotic bodies containing miR-328 significantly ameliorates osteopenia in OVX mice [140].

3.3. Gene Manipulation. In addition to the above two methods, virus transfection and CRISPR/Cas9 are applied to realize direct gene manipulation. Junomji AT-rich interactive domain 1A (JARID1A) protein is a KDM5A component participating in Runx2-related H3K4 demethylation. Compared with the control group, scaffolds containing BMSCs transduced with si-Jarid1a increase bone volume and mineral density during the process of calvarial defect healing [141]. Besides, SATB2 protein is a nuclear matrix protein involved
in chromatin remodeling, and the Satb2 gene overexpression by lipofectamine transfection enhances skeletal tissue regeneration and mineralization in mouse mandibular bone defects [142]. Moreover, anti-miR-31-expressing BMSCs/poly (glycerol sebacate) complex and miRNA-21-modified BMSCs/β-tricalcium phosphate composite both bring higher bone regeneration rate in rat bone defects [143, 144]. Similarly, knockdown IncRNA MIR31HG or MIAT with lentivirus significantly enhances ADSCs’ bone formation capacity when implanted subcutaneously with biomaterials [145, 146].

As to therapeutic application in diseases, injection of lentiviruses encoding CBX4 protein into the joint capsules leads to upregulation of proliferation, bone growth-associated
genes, and downregulation of inflammation and cell death-related genes [58]. Similarly, mammalian brahma (BRM) protein is a component of SWI/SNF complex with ATPase activity. Knockdown of Brm gene in mice helps it resist aging-related osteoporosis and reduces adiposity in bone marrow [147]. Lentivirus is also used to alter histone acetylation and methylation level in osteoporosis. In OVX mice, injection of lentiviruses expressing Gcn5 gene restores endogenous BMSC osteogenic potential by increasing H3K9ac on the promoters of Wnt genes [48]. Except for that, knockdown of Ezh2 gene by lentivirus-expressing shRNA decreases H3K27me3 on Wnt genes, reversing the abnormal MSC adipogenic lineage commitment in osteoporosis [47]. Moreover, when RNA N6-methyltransferase Mettl3 gene is knocked in transplanted MSCs with CRISPR/Cas9 and Cre/LoxP, mice are protected from OVX-induced osteoporosis [63]. When it comes to ncRNAs, bone defects completely healed with transplantation of BMSCs expressing miR-214 sponges transduced by baculovirus [148].

4. Conclusion

Epigenetic regulation of MSCs occurs in several steps of transcription, including chromatin remodeling, DNA methylation, and histone modification, and posttranscription, including mRNA processing and ncRNA regulation. Epigenetic markers and modifiers have been proved to play indispensable roles in MSC aging and fundamental homeostasis in vivo, both of which are related to the pathogenesis of tissue disorders in aging and diseases. Initial experimental attempts, roughly according to epigenetic clues, have been carried out to delay MSC aging or rejuvenate senescent MSCs, which is aimed at enhancing their self-renewal capacity and correct biased differentiation lineage. However, there remain several obstacles for translational application, including lack of sequential identification of spatiotemporal epigenetic alteration, and difficulties in precise translational intervention in vivo. Hopefully, many revolutionary technological progresses emerged just in the past years, including single-cell epigenomic analysis and CRISPR/Cas9, cell transplantation, and regenerative biomaterials. In this context, therapeutic or regenerative strategies based on epigenetic regulation of MSC aging stand a tremendous chance to restore MSC homeostasis in vivo and even boost translational application in tissue regeneration, especially among the elderly or people with bone diseases.

Conflicts of Interest

The authors declare no conflict of interest.

Authors’ Contributions

Ruoxi Wang and Yu Wang contributed equally to this work.

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