Stem Cells International

Epigenetic Regulation of Stem Cell Fate

Lead Guest Editor: Quan Yuan
Guest Editors: De-Meng Chen, Yang Li, and Shuibin Lin

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Hindawi
Stem Cells International
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https://doi.org/10.1155/2021/8888416

Research Article

Chaetocin Promotes Osteogenic Differentiation via Modulating Wnt/Beta-Catenin Signaling in Mesenchymal Stem Cells

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Received 29 June 2020; Revised 4 September 2020; Accepted 22 January 2021; Published 9 February 2021

Academic Editor: Yang Li

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Mesenchymal stemXin cells (MSCs) are a great cell source for bone regeneration. Although combining MSCs with growth factors and scaffolds provides a useful clinical strategy for bone tissue engineering, the efficiency of MSC osteogenic differentiation remains to be improved. Epigenetic modification is related to the differentiation ability of MSCs during osteogenic induction. In this study, we evaluate the effect of Chaetocin, an inhibitor of lysine-specific histone methyltransferases, on the differentiation of MSCs. We found that MSCs treated with Chaetocin demonstrated increased osteogenic ability and reduced adipogenic ability. The expression of osteogenic markers (Runx2 and OPN) was induced in MSCs by Chaetocin during osteogenic induction. Moveover, treatment of Chaetocin in MSCs improves Wnt/β-catenin signaling pathways and its downstream targets. Finally, we showed increased bone formation of MSC and Wnt/β-catenin signaling activity by treatment of Chaetocin using in vivo bone formation assays. Our data uncovered a critical role of Chaetocin in MSC osteogenic differentiation and provide new insights into bone tissue regeneration and repair.

1. Introduction

Bone healing is a complicated process and not always completely satisfactory. Repair of bone tissue after severe injury has been a great challenge for regenerative medicine. Mounting evidence has shown that mesenchymal stem cells (MSCs) display great ex vivo expansion potential and differentiation properties, making them an attractive tool for bone tissue engineering. [1, 2] Achieving a better osteogenic differentiation efficacy is a central goal for MSCs-based bone regeneration field. The mechanism of MSCs lineage commitment is under control by multiple factors, including growth factors, transcription factors, and epigenetic factors. [3, 4] In particular, most of the epigenetic factors are enzymes, making them suitable targets for drug intervention. DNA methylation and histone modification are the major epigenetic regulation mechanisms. [5]

The activity of Runt-related transcription factor 2 (Runx2) [6], a master regulator of osteogenic differentiation, can be regulated by epigenetic factors. For example, the DNA methylation and acetylation of histones H3 and H4 status in the promoter of osteocalcin (OCN), which harbors binding sites for Runx2, can alter the accessibility of the promoter to Runx2. [7, 8] A study has shown that treatment of DNA-demethylating agent 5-aza-2$'$-deoxycytidine and chromatin-acetylation agent trichostatin A led to adipocyte differentiation in human-derived MSCs. [9]

Histone methylation plays a key role in establishing and maintaining stable gene expression patterns during cellular differentiation and embryonic development. [10] Histone demethylases KDM4B and KDM6B are essential in osteogenic commitment of MSCs via H3K9me3 and H3K27me3 modification, suggesting a promising strategy to improve osteogenic differentiation through manipulation of epigenetic factors. [3]

However, the effect of inhibition of lysine-specific histone methyltransferases on MSCs differentiation has not been explored. Chaetocin is a fungal mycotoxin isolated from *Chaetomium minutum* and an inhibitor of lysine-specific histone methyltransferases, including SUV39H1 and G9a. [11, 12] It has been shown that inhibition H3K9me3-specific methyltransferase by Chaetocin can prevent cell growth in a ROS-dependent manner. [13] However, its potential influences on MSC differentiation are less known.

To evaluate the effect of Chaetocin on the differentiation of MSCs, we treated mouse-derived MSCs with Chaetocin and examined their osteogenic and adipogenic properties. We also checked the expression of osteogenic markers (Runx2 and OPN) and adipogenic markers (Pparg and Fabp4) during differentiation induction. Our results showed that Chaetocin promotes the osteogenic differentiation but inhibits the adipogenic differentiation of MSCs. In addition, treatment of Chaetocin in MSCs increases Wnt/β-catenin activity. Finally, we showed that bone formation of MSC in vivo is enhanced by treatment of Chaetocin. Our data uncovered a critical role of Chaetocin in MSC osteogenic differentiation and provide new insights into bone tissue engineering.

2. Materials and Methods

2.1. Ethics. All experimental protocols and procedures were approved by the Department of Stomatology, The Seventh People's Hospital of Shenzhen (protocol number 2018021001). The animal procedures were conducted in accordance with e-Guidelines for the Care and Use of Laboratory Animals of Department of Stomatology, The Seventh People's Hospital of Shenzhen.

2.2. MSC Isolation and Culture. MSCs were isolated from the femur and tibia bone marrow of adult c57bl/6 mice as described previously. [14] Cells were cultured in α-minimum essential medium (α-MEM; Thermo Fisher Scientific, China) supplemented with 10% fetal bovine serum (FBS; Gibco, China) and 100 μg/ml penicillin-streptomycin (Gibco, China) in a humidified atmosphere containing 5% CO_2 at 37°C. For Chaetocin experiment, 5 μM Chaetocin (Selleck, China) was used. DMSO was used as control.

2.3. Osteoblast Differentiation and Analysis of MSCs. 5×10^5 MSCs were plated onto 6-well plates to reach ~80% confluence. The growth medium was then changed to osteoblast induction medium (OIM). The OIM was composed of high-glucose DMEM with L-glutamine (Thermo Fisher Scientific, China), 10% FBS (Gibco, China), 100 μg/ml penicillin/streptomycin (Gibco, China), β-glycerophosphate 10 mM (Sigma Aldrich, China), 2.5 μg/ml ascorbic acid-2-phosphate (Sigma Aldrich, China), 2.5 μg/ml amphotericin B (Sigma Aldrich, China), and 0.1 μM dexamethasone (Sigma Aldrich, China). For alkaline phosphatase (ALP) staining, MSCs cultured in OIM for 7 days were first fixed in 10% formalin for 1 h at room temperature and then stained by using an ALP staining kit (Sigma Aldrich, China) according to the manufacturer's instructions. ALP activity was assessed using the Alkaline Phosphatase Activity Detection Kit (Yeasen, China) according to the manufacturers protocol. For Alizarin Red S (ARS) staining, MSCs cultured in OIM for 14 days were stained 1% ARS solution for 20 minutes.

2.4. Adipogenic Differentiation and Analysis of MSCs. 5×10^5 MSCs were plated onto 6-well plates to reach ~80% confluence. The growth medium was then changed to adipogenic differentiation medium containing 10 μg/ml insulin (Sigma Aldrich, China),1 μM dexamethasone (Sigma Aldrich, China), and 500 μM IBMX (Sigma Aldrich, China). Differentiated MSCs were first fixed with 10% formalin solution for 30 min and then stained with 0.3% oil red O solution for 10 min.

2.5. MSC-Mediated Ectopic Bone Formation. 2×10^6 MSCs were mixed with 40 μg of tricalcium phosphate/hydroxyapatite (TCP/HA) powder (Sigma-Aldrich, China) and placed subcutaneously into nude mice (Kunming Model Animal Center, China). Mice were separated randomly into two groups of 8 animals each and were administered either with DMSO or Chaetocin (0.5 mg/kg body weight), intraperitoneally every other day. Tissues were then harvested 42 days for histological analysis. The tissue blocks were dehydrated and embedded in paraffin. The embedded tissue blocks were sliced in 5 μm thickness and stained in hematoxylin and eosin staining solution. The sections were dehydrated, permeabilized, and sealed. The sections were observed under the microscope and photographed.

2.6. Real-Time Quantitative Reverse Transcription PCR (qRT-PCR). Total RNA was extracted from cells using Trizol reagent (Invitrogen, China) according to the manufacturer's instructions. Reverse transcription was performed using 1 μg of RNA using a MultiScribe reverse transcriptase kit (Applied Biosystems, China) according to the manufacturer's instructions. For qRT-PCR, a SYBR Green kit (Bio-Rad Laboratories Inc., China) was used according to the manufacturer's instructions. GAPDH was used as an internal control. The primer sequences for qRT-PCR are listed in Table 1.

2.7. Western Blot Analysis. Cells were lysed by using the Nuclear Extraction Kit (Novus Biologicals, China) to collect the nuclear protein. Samples were then used for electrophoresis separation, and then transferred to a nitrocellulose membrane. After blocked with 5% milk at room temperature for 2 hr, the membranes were added with primary antibodies to β-catenin (Abcam, China), Histone H1 (Abcam, China), Axin2 (Abcam, China), Myc (R&D Systems, China), Ccnd1 (Santa Cruz Biotechnology, USA) and GAPDH (Sigma, China) at 4°C overnight. After that, the membranes were incubated with secondary antibody (Abcam, China) at 37°C for 1 hr. The membranes were then completely immersed in the enhanced chemiluminescence (Yeasen, China) to obtain images.

2.8. Statistical Analysis. All data were presented as the mean ± s.d. Student's t-test was used between two groups and a difference was considered statistically significant with $P < 0.05$.

Table 1: Primer information for qRT-PCR.

Primer name	Primer sequence
Runx2 forward	5′-GACTGTGGTTACCGTCATGGC-3′
Runx2 reverse	5′-ACTTGGTTTTTCATAACAGCGGA-3′
OPN forward	5′-CTGCATACTGTAACCGCAGTC-3′
OPN reverse	5′-CTCTCCATCCATAACATGGGC-3′
Pparg forward	5′-TCCTGTAAAAGCCCGGAGTAT-3′
Pparg reverse	5′-GCTCTGGTAGGGGCAGTGA-3′
Fabp4 forward	5′-AAGGTGAAGAGCATCATAACCCT-3′
Pparg reverse	5′-TCACGCCTTTCATAACACATTCC-3′
Ccnd1 forward	5′-GCGTACCCTGACACCAATCTC-3′
Ccnd1 reverse	5′-CTCCTCTTCGCACTTCTGCTC-3′
Myc forward	5′-TTCTACGACTATGACTGCGGA-3′
Myc reverse	5′-TGATGGAAGCATAATTCCTGCC-3′
Axin2 forward	5′-TGACTCTCCTTCCAGATCCCA-3′
Axin2 reverse	5′-TGCCCACACTAGGCTGACA-3′
Dkk1 forward	5′-CTCATCAATTCCAACGCGATCA-3′
Dkk1 reverse	5′-GCCCTCATAGAGAACTCCCG-3′
GAPDH forward	5′-TGGATTTGGACGCATTGGTC-3′
GAPDH reverse	5′-TTTGCACTGGTACGTGTTGAT-3′

All statistical analyses were analyzed by using the SPSS 16.0 software.

3. Results

3.1. Chaetocin Promotes Osteogenic Differentiation of MSCs. The status histone methylation plays a crucial role in regulating chromatin structural changes and determines the accessibility of related gene promoters for transcription factors during MSC differentiation. To determine the effects of the histone methyltransferase inhibitor Chaetocin on osteogenic differentiation, we first examined the expression of the osteogenesis-related genes in MSCs during osteogenic induction (Figures 1(a) and 1(b)). Compared to the control MSCs, the mRNA expression of *Runx2* and *OPN* were upregulated in the cells treated with Chaetocin at day 3, 7 and 14 (Figures 1(a) and 1(b)). We further explored effects of Chaetocin on the osteogenic differentiation by performing the ALP staining and ARS staining to detect ALP activity and mineralization (Figures 1(c) and 1(d)). Compared to the control, ALP activity and mineralization were significantly increased in the MSCs treated with Chaetocin (Figures 1(c) and 1(d)). Thus, treatment of Chaetocin could promote osteogenic differentiation of MSCs.

3.2. Chaetocin Inhibits Adipogenic Differentiation of MSCs. Previous studies have shown that MSCs are associated with multiple lineages, including osteoblast, adipocytes, chondrocytes, and so on. The association of MSCs with adipocytes resulted in the imbalance between bone mass and fat and the increased risk of bone fracture. Hence, we sought to investigate the effects of Chaetocin on adipogenic differentiation of MSCs. Compared to the control MSCs, the mRNA expression of adipogenesis-related genes, *Pparg* and *Fabp4*, was dramatically reduced in the MSCs treated with Chaetocin (Figures 2(a) and 2(b)). Further, oil red O staining results showed that oil droplet formation was significantly inhibited in the MSCs treated with Chaetocin (Figures 2(c) and 2(d)). Thus, treatment of Chaetocin could inhibit adipogenesis of MSCs.

3.3. Chaetocin Activates Wnt/β-Catenin Activity during Osteogenic Induction. As we showed that Chaetocin inhibited adipogenic genes while promoted osteogenic gene expression. It seems that Chaetocin may not necessarily direct regulate the H3K9me3 levels of these particular genes. Since Wnt/β-catenin activity has been well-known for its function in promote osteogenesis and inhibit the adipogenesis of MSCs, we reasoned that Chaetocin might regulates Wnt/β-catenin signaling transduction in MSCs. Indeed, our Western Blot results showed that the level of nuclear β-catenin in MSCs treated with Chaetocin was higher compared with control MSCs (Figure 3(a)). The mRNA expression levels of Wnt/β-catenin target genes (*Ccnd1*, *Axin2*, *Myc*, and *Dkk1*) were also increased in MSCs treated with Chaetocin (Figure 3(b)), indicating that Chaetocin is involved in Wnt/β-catenin signaling transduction.

3.4. Chaetocin Promotes Ectopic Osteogenesis In Vivo. To determine whether Chaetocin could play a role in MSC-mediated bone formation in vivo, MSCs were mixed with TCP/HA and injected into nude mice. Mice were then treated with control vehicle or Chaetocin for 6 weeks and sacrificed for sample collection. HE staining (Figure 4(a)) showed both the control group and the experiment group were found to show osteoblast-like cells. Quantification of the bone area showed that treatment of Chaetocin led to the increase of bone tissue in vivo (Figure 4(b)). In addition, our immunohistochemistry staining of β-catenin results confirm the presence of nuclear β-catenin signal in the sample treated with Chaetocin. Overall, our data demonstrated that Chaetocin could enhance MSC-based bone formation.

4. Discussion

MSCs have multiple differentiation potentials and can be induced to differentiate into two mutually exclusive lineages: osteoblasts or adipocytes. [15] How to induce MSCs into osteoblasts more efficiently has been a major challenge in bone tissue engineering. Previous studies have shown that histone methylation is involved in MSC differentiation. For example, KDM4B and KDM6B played a critical role in MSC cell fate commitment by removing H3K9me3 and H3K27me3 on different sets of lineage-specific genes. [3] In addition, H3K27 methyltransferase EZH2 is required for inhibition of MSC differentiation. [16] Chaetocin was originally identified as an inhibitor of histone methyltransferase SU(VAR)3-9. [11] The role of Chaetocin in anticancer

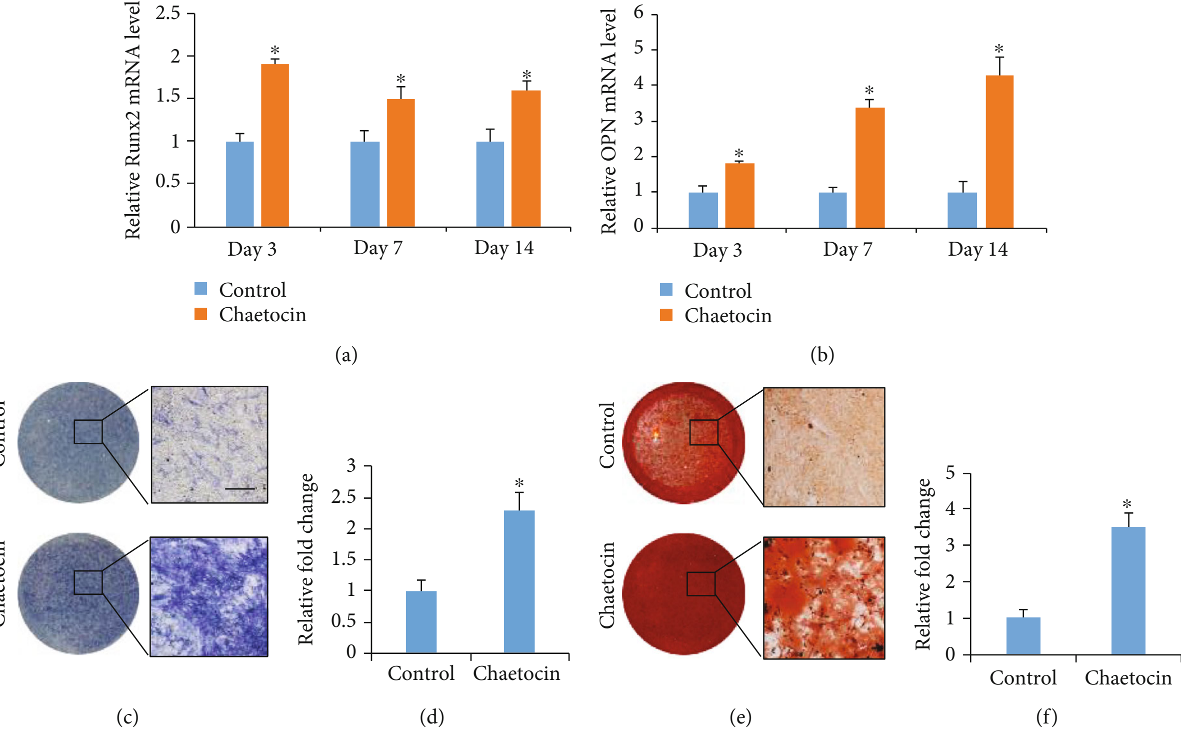

Figure 1: Effect of Chaetocin on MSC osteogenic differentiation. (a, b) Real-time RT-PCR analysis shows that expression of both early and later osteogenic markers (Runx2, OPN) was significantly enhanced by treatment with Chaetocin. (c, d) ALP staining showed a significant increase of ALP activity in MSCs treated with Chaetocin. (e, f) ARS staining showed a significant increase of calcium deposition in MSCs treated with Chaetocin. PCR and quantification data are expressed as means ± s.d. of three independent experiments ($*p < 0.05$). Scale bar: 100 μm.

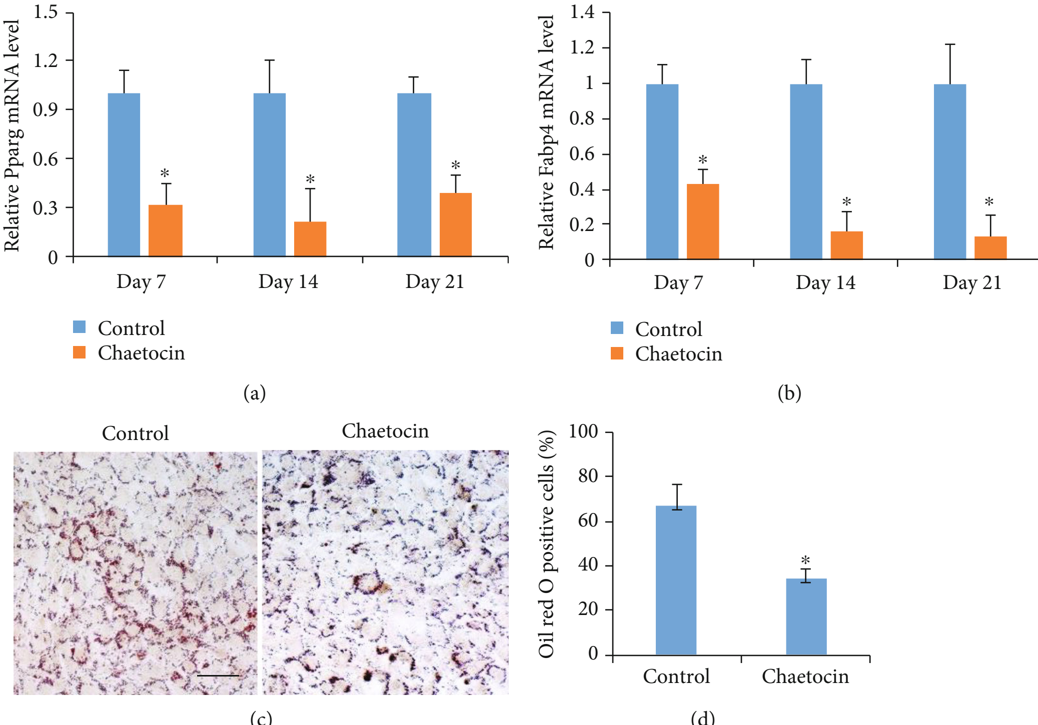

Figure 2: Effect of Chaetocin on MSC adipogenic differentiation. (a, b) Real-time RT-PCR analysis shows that expression adipogenic markers (Pparg, Fabp4) was significantly inhibited by treatment with Chaetocin. (c, d) Oil red O staining showed a significant decrease of oil droplets in MSCs treated with Chaetocin. PCR and quantification data are as expressed as means ± s.d. of three independent experiments ($*p < 0.05$). Scale bar: 500 μm.

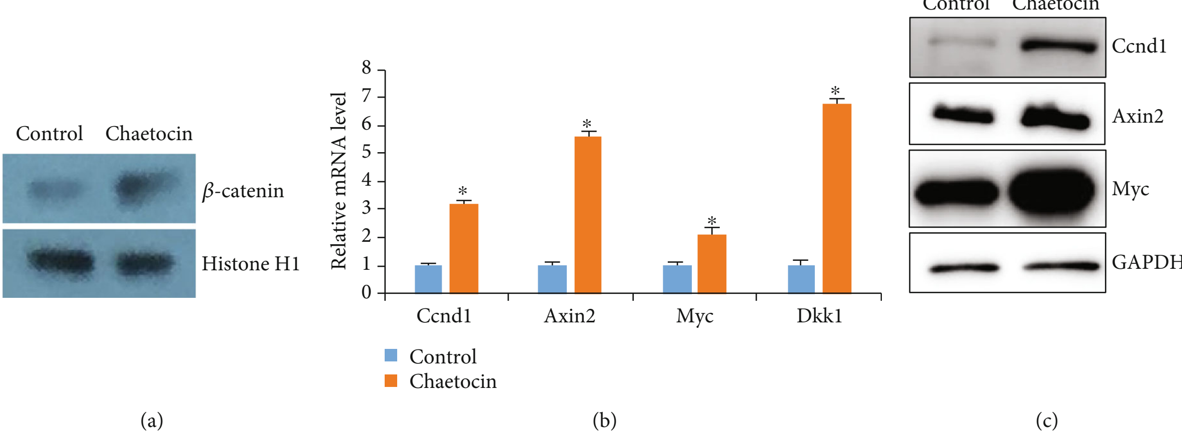

Figure 3: Treatment of Chaetocin increase Wnt/β-catenin activities. (a) Western blot shows nuclear β-catenin protein levels were elevated by Chaetocin treatment in MSC culture at day 7. Histone H1 was used as a loading control. (b) Real-time RT-PCR analysis shows that Wnt/β-catenin target genes (Cnnd1, Axin2, Myc, and Dkk1) were significantly increased by treatment with Chaetocin. PCR data is expressed as means ± s.d. of three independent experiments ($*p < 0.05$). (c) WB results show that Cnnd1, Axin2, and Myc were significantly increased after Chaetocin treatment.

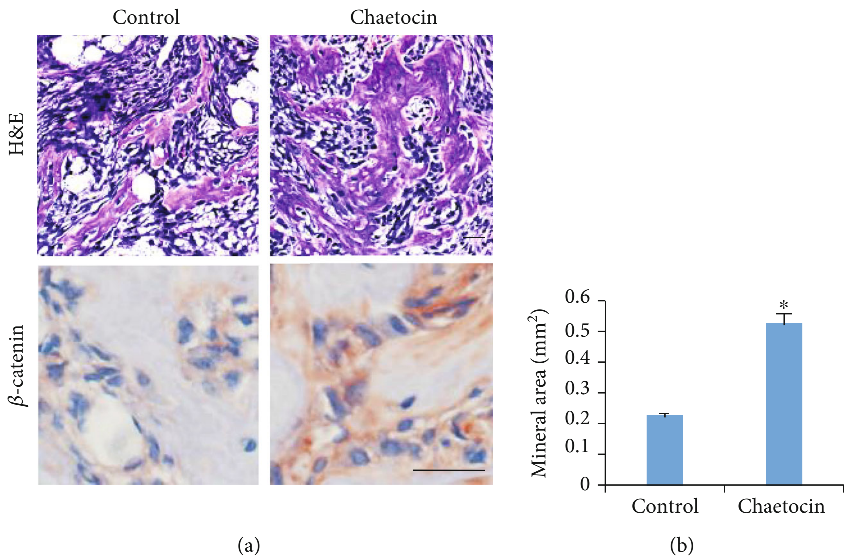

Figure 4: Chaetocin promotes MSC-mediated ectopic bone formation. (a) H&E staining results show more bone tissues from MSCs treated with Chaetocin compared with control. IHC results showed more nuclear β-catenin signal in MSC treated with Chaetocin compared with control. Scale bar: 50 μm. (b) Quantification of the bone area from MSCs treated with Chaetocin and control. Quantification data is expressed as means ± s.d. of three independent experiments ($*p < 0.05$).

treatment has been intensively studied; however, its function in MSCs differentiation has not been explored.

In this study, we showed that Chaetocin can affect the osteogenic and adipogenic abilities of MSCs. Treatment of Chaetocin promoted the osteogenic differentiation of MSCs and induced the expression of osteogenic-related genes. On the contrary, Chaetocin repressed the adipogenic differentiation of MSCs and reduced the number of oil droplets. Chaetocin can inhibit the activity of histone methyltransferase SUV39 family, such as SUV39H1 and G9a, which are required for H3K9 di- to tri-methylation and mono- to dimethylation of H3K9, respectively. [17, 18] Although the specific molecular mechanism by which Chaetocin might affect MSC osteogenesis and adipogenesis is not characterized in the current study, it is highly possible that MSC differentiation regulated by Chaetocin is mediated via alteration of the status of H3K9 methylation. In addition, how Chaetocin can exert a different function in a different context is also elusive, we speculate that Chaetocin can affect the osteo- and adipolineage commitment through Wnt/β-catenin activity instead of regulation of these lineage factors directly.

Our data also showed that treatment of Chaetocin in MSCs promoted Wnt/β-catenin activity. Interestingly, several histone demethylases have been linked to Wnt/β-catenin activity. For instance, KDM7A can regulate adipogenic and osteogenic differentiation via regulation of Wnt/β-catenin signaling. [19] Furthermore, JMJD2D can interact with β-catenin to activate transcription of its target genes. [20]

Hence, it is possible that Chaetocin-mediated inhibition of histone methyltransferase is involved in Wnt/β-catenin signaling. And indeed, both our cell culture assay and in vivo bone formation experiment showed that Wnt/β-catenin is activated when treated with Chaetocin. There is speculation if Chaetocin could be used in patient with osteoporosis to improve the formation of bone mass.

In conclusion, we demonstrate that treatment with Chaetocin can improve the osteogenesis of MSCs via epigenetic regulation. Our study provides useful insights for better exploring the use of Chaetocin in bone tissue engineering.

Data Availability

The data used to support the findings of this study are included within the article.

Conflicts of Interest

The authors declare no conflict of interest.

Acknowledgments

This work was supported by the Science and Technology Project of Shenzhen (JCYJ20180302144621755).

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Hindawi
Stem Cells International
Volume 2020, Article ID 8876265, 16 pages
https://doi.org/10.1155/2020/8876265

Review Article

Epigenetic Regulation of Dental Pulp Stem Cell Fate

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Received 23 July 2020; Revised 21 September 2020; Accepted 24 September 2020; Published 23 October 2020

Academic Editor: De-Meng Chen

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Epigenetic regulation, mainly involving DNA methylation, histone modification, and noncoding RNAs, affects gene expression without modifying the primary DNA sequence and modulates cell fate. Mesenchymal stem cells derived from dental pulp, also called dental pulp stem cells (DPSCs), exhibit multipotent differentiation capacity and can promote various biological processes, including odontogenesis, osteogenesis, angiogenesis, myogenesis, and chondrogenesis. Over the past decades, increased attention has been attracted by the use of DPSCs in the field of regenerative medicine. According to a series of studies, epigenetic regulation is essential for DPSCs to differentiate into specialized cells. In this review, we summarize the mechanisms involved in the epigenetic regulation of the fate of DPSCs.

1. Introduction

Epigenetics, defined as "the study of changes in gene function that are mitotically and/or meiotically heritable and that do not entail a change in DNA sequence" [1], has gradually become a research hotspot in recent decades. Epigenetic regulation can influence gene expression without modifying the primary DNA sequence. Therefore, two cells, containing the same genetic information, can behave totally differently [2]. The principal epigenetic mechanisms, comprising DNA methylation, histone modifications, and those mediated by noncoding RNAs (ncRNAs), have been proved to perform an essential role in the differentiation, proliferation, and behavior of cells [3–5].

Stem cells (SCs) are a unique population of cells which provide progenitor cells via dividing and proliferating throughout postnatal life, which in turn differentiate into specialized cells in most tissues of the body [6]. Mesenchymal stem cells (MSCs), a heterogenic cell population, can be isolated from various tissues and are noted for their multipotency differentiation potential [7]. A group of MSCs, mainly including dental pulp stem cells (DPSCs), periodontal ligament stem cells (PDLSCs), dental follicle progenitor cells (DFPCs), stem cells from exfoliated deciduous teeth (SHED), and stem cells from the apical papilla (SCAP), are derived from dental tissues. Among them, DPSCs, which are originated from dental pulp of permanent teeth, play a critical role in restorative dentin formation and pulp homeostasis. Since DPSCs were first isolated in 2000 by Gronthos et al. [8], numerous studies have identified the self-renewal and multilineage differentiation ability of DPSCs, such as odontogenic, angiogenic, osteogenic, neurogenic, myogenic, adipogenic, and chondrogenic differentiation [9–11]. These unique characteristics make DPSCs applicable in regenerative medicine [12]. Hence, it is vital to investigate the factors that modulate the fate of DPSCs, including their proliferation and differentiation. According to a series of studies, epigenetic regulation is closely related to DPSC fate [11–20]. This literature reviews the general characteristics, immunophenotypes, and multipotential differentiation of DPSCs and current progress on the epigenetic regulation in the determination of DPSC fate.

2. Epigenetics

Epigenetics was first proposed to describe the complicated development process from genotype to phenotype by Conrad Waddington in 1942 [13]. Epigenetic regulation can alter the state of chromatin without changing the DNA sequence, thereby affecting access to genes within the cell [2]. When the environment around the cell changes, the initiator such as ncRNAs receives an epigenator signal and determines the location on the chromosome where the chromatin state needs to be changed, thereby affecting gene expression. The epigenetic maintainers, including DNA methylation and histone modifications, sustain the epigenetic state of chromatin and allow it to be inherited [14, 15].

2.1. DNA Methylation. DNA methylation is a stable and inheritable epigenetic mark that modulates the chromatin structure and gene expression. DNA methyltransferases (DNMTs), including DNMT1, DNMT3A, DNMT3B, and DNMT3L, are the enzymes responsible for DNA methylation. Among them, DNMT1 plays a crucial part in maintaining DNA methylation during the course of DNA replication, while DNMT3A and DNMT3B target unmethylated CpGs and are responsible for *de novo* DNA methylation [16–18]. Moreover, DNMT3L stimulates the DNA methylation activity of DNMT3A and DNMT3B [19–21]. DNA demethylation can be achieved by diluting methylation markers during DNA replication, or independently of DNA replication. The latter requires the involvement of ten-eleven translocation proteins (TET1, TET2, and TET3) and the activation-induced deaminase [22]. DNA methylation leads to gene silencing by arresting the binding of transcriptional factors or by chromatin remodeling, and its dynamics is involved in diverse biological processes [23–25]. Thus, DNA methylation plays a vital part in stem cell differentiation, development, and disease by regulating genes [26–28].

2.2. Histone Modifications. Histone proteins, including H1, H2A, H2B, H3, and H4, along with DNA form nucleosomes, which are referred to as the basic units of chromatin. Studies have confirmed that the lack of histones in the promoter region is essential for transcriptional activation [29]. The posttranscriptional modifications (PTMs) of the amino acids on the histone tails and cores, comprising methylation, acetylation, ubiquitination, phosphorylation, ADP-ribosylation, and glycation, are widely reported to be critical for the chromatin architecture, nucleosome stability, and transcription of genes [30–34].

Among these histone modifications, methylation and acetylation are the most widely studied. Histone methylation, catalyzed by histone methylases and demethylases (HDMs), can occur at multiple sites of histones, mainly on lysine and arginine residues [35]. Histone methylation activates or inhibits transcription depending on the location and methylation status. Similarly, histone acetylation is regulated by histone deacetylases (HDACs) and histone acetyltransferases (HATs) and is always related to active genes [36]. These different histone modifications have crosstalk with each other and constitute a regulatory network that regulates gene transcriptional activity by affecting the chromatin structure, thereby affecting development, diseases, and stem cell fate [30, 34, 37].

2.3. ncRNAs. ncRNAs consist of a group of RNAs that do not encode proteins. They include housekeeping ncRNAs and regulatory ncRNAs. Notably, the regulatory ncRNAs, composed of micro-RNAs (miRNAs), endogenous small interfering RNAs (siRNAs), PIWI interacting RNAs (piRNAs), and long noncoding RNAs (lncRNAs), are critical for epigenetic control [38]. According to their length, the ncRNAs are categorized as lncRNAs and short ncRNAs, which include miRNAs, piRNAs, and siRNAs. lncRNAs, with the length of more than 200 nucleotides (nt), can regulate gene expression through transvection, chromatin modification, and transcriptional and posttranscriptional regulation [39, 40]. miRNA, a sequence of single-stranded RNA about 22 nt in length, can degrade mRNA or repress translation to silence the gene through tie to the $3'$-untranslated regions ($3'$-UTRs) of the particular mRNA [41]. Therefore, ncRNAs can epigenetically regulate gene expression at different levels.

2.4. Epigenetic Network. There are also some crosstalks between these epigenetic mechanisms that modulate the expression of genes and the behavior of cells. DNA methylation can be epigenetically regulated by histone modification. For example, enhancer of zeste homolog 2 (EZH2), a histone methyltransferase, can promote DNA methylation by recruiting DNMTs in the target promoter region and then result in gene silencing [42]. Besides, lncRNAs regulate gene expression through interaction with histone modification enzymes, DNA methyltransferases [43, 44], or miRNA [45]. For example, some lncRNAs such as ANRIL are reported to regulate gene expression by recruiting the polycomb group of proteins, which can lead to heritable gene silencing through di- or trimethylation of lysine 27 of histone 3 (H3K27me2/3) [43, 44, 46]. In addition, lncRNAs can regulate the repression activity of miRNAs on mRNA [47]. Therefore, these regulatory mechanisms together constitute an epigenetic network, which regulates the expression of genes without changing the DNA sequence and affects the fate of cells.

3. DPSCs

3.1. Identification of DPSCs. DPSCs can be obtained from the dental pulp of permanent teeth extracted owing to impaction, orthodontic reason, or periodontitis. Similar to MSCs, DPSCs also express mesenchymal cell markers, like CD29, CD44, CD73, CD90, CD105, CD106, CD146, STRO-1, and aldehyde dehydrogenase 1 [48–52]. Meanwhile, DPSCs display negative or low expression of hematopoietic markers, CD14 or CD11b, CD19, CD34, CD45, and HLA-DR [50, 53, 54], which meets the minimal criteria for defining multipotent mesenchymal stromal cells proposed by the International Society for Cellular Therapy in 2006 [55]. However, DPSCs are a group of heterogeneous cells, and lots of the markers are not expressed in DPSCs consistently. Cells with different surface markers may have different characteristics.

Therefore, purification of DPSCs is important for successful clinical application. Specific cell surface markers can facilitate the isolation of specific subsets of DPSCs, which can subsequently differentiate into specific cell types for clinical use. For example, single $CD271^+$ DPSCs isolated by fluorescence-activated cell sorting have been found to have higher odontogenic potential [56].

3.2. Differentiation and Clinical Potential of DPSCs. The potential application in tissue engineering and regenerative medicine of MSCs has been widely proved. Over the past decades, bone marrow MSCs (BMMSCs), as a kind of MSCs, have become a focus of interest in regenerative medicine because of their multilineage differentiation ability. Recently, due to their easy accessibility, DPSCs have gradually come into the field of regenerative medicine. Compared to BMMSCs, DPSCs have better viability and higher capacity of odontogenic and neurogenic differentiation, but lower capacity to differentiate into chondrocytes [57–60]. When transplanted into immunocompromised mice, DPSCs can form dentin-like tissue, while BMMSCs form lamellar bone [61]. DPSCs can also differentiate into various kinds of cells, including osteoblasts, odontoblasts, adipocytes, endothelial cells, neurons, myocytes, and chondroblasts [9]. In addition, it has been proved that DPSCs can retain their properties even after two years of cryopreservation [62]. Therefore, more and more attention is paid to the differentiation and clinical potential of DPSCs in regenerative medicine (Figure 1).

Since DPSCs were first separated from human impacted third molars' pulp and cultured *in vitro* in 2000 [8], a series of studies demonstrated the self-renewal capability, multilineage differentiation potential, and clonogenic efficiency (colony-forming unit fibroblast) of DPSCs [8, 63, 64]. The osteo/odontogenic differentiation potential of DPSCs is the most widely reported. Both *in vitro* and *in vivo* experiments showed that DPSCs are able to differentiate into osteo/odontoblasts and form bone and dentin tissues [65–67]. When DPSCs are cultured in osteo/odontogenic induction medium, a group of proteins related to mineralization tissues are upregulated. Among them, dentin sialophosphoprotein (DSPP) and dentin matrix phosphoprotein 1 (DMP1) are considered to be specific markers of odontoblasts [68, 69]. While alkaline phosphatase (ALP), type I collagen (Col I), osteopontin (OPN), osteocalcin (OCN), and osterix (OSX) are associated with osteoblastic proliferation and differentiation [69]. As a result of origination from migrating neural crest cells, DPSCs can express some neural crest developmental genes and have the ability to differentiate into neural cells. When cultured in neuronal inductive conditions for an extended period of time, DPSCs exhibit a neuronal morphology and express neuronal-specific markers such as PSA-NCAM, β-III tubulin, neurofilament-M, and nestin, showing the ability to generate a sodium current consistent with functional neuronal cells [70]. Moreover, DPSCs transplanted in vitro can generate functional neurons and improve nerve regeneration [71, 72]. In addition, several studies also showed that DPSCs exhibit the capacity to acquire the phenotype of endothelial cells and generate vascular-like structures [73–76]. When cultured in a 3D fibrin mesh, DPSCs display endothelial cell-like features and form capillary-like structures [77]. After exposure to VEGF, endothelial-specific markers like Flt-l and KDR are increased, together with the occurrence of ICAM-l and the von Willebrand factor-positive cells [77]. After DPSCs have been cultured by using the "pellet culture" technique and chondrogenic medium, the structure of pellets is consistent with the structure of cartilage, and the Alcian blue staining of the extracellular matrix in the center of the pellets indicates the existence of highly sulfated glycosaminoglycans, demonstrating chondrogenic differentiation of DPSCs [9, 50, 78]. After being cultured in specific condition for several weeks, DPSCs elongate and display a myoblast-like phenotype. These DPSCs express specific myocytic immunohistochemical markers such as MyoD1, myosin, and MHC [79, 80]. Other than the myogenic potential, DPSCs also preserve the capability to differentiate into adipocytes [81, 82] and pancreatic cell lineage [83, 84].

Based on the multidirectional differentiation potential of DPSCs and their easy availability, the application of DPSCs in tissue engineering and diseases is increasingly being explored. DPSCs have been shown to form a dentin/pulp-like complex in immunocompromised mice [8]. Moreover, DPSCs in prevascularized, scaffold-free, microtissue spheroids can successfully regenerate vascular dental pulp-like tissue, which provides a new strategy for endodontic treatment and makes dentin-pulp regeneration possible [85]. The clinical application potential of DPSCs is not only in dentistry but also in treatments for other diseases, such as craniofacial bone defects [86], muscle regeneration [87], myocardial infarction [88], Alzheimer's disease [89], nervous system injuries [90], Parkinson's disease, diabetes [91], stress urinary incontinence [80], osteoarthritis [92], and liver diseases [93].

4. Epigenetic Mechanisms in DPSCs

Epigenetic regulation can influence the differentiation potential and proliferation of DPSCs. It is thus vital to understand the epigenetic mechanisms beneficial to the clinical application of DPSCs.

4.1. DNA Methylation. DNA methylation, one of the best-studied epigenetic modifications, is often related to gene silencing and regulation of stem cell fate. A series of studies also reported specific regulatory effects of DNA methylation in DPSCs (Table 1 and Figure 2).

Adult stem cells can be reprogrammed to induce pluripotent stem (iPS) cells and be applied to clinical therapy. During this process, DNA methylation plays a critical part [94]. In a genome-wide DNA methylation analysis, DPSCs exhibited DNA methylation profile closer to human embryonic stem (ES) cells and iPS cells [95]. Among these genes, overexpression of PAX9 and knockdown of HERV-FRD improved the efficacy of iPS generation from DPSCs. These results indicate the reprogramming potential of DPSCs into iPS and the role of epigenetic mechanisms in this process [96].

DPSCs, with multilineage differentiation potential, can differentiate into various kinds of cells under different

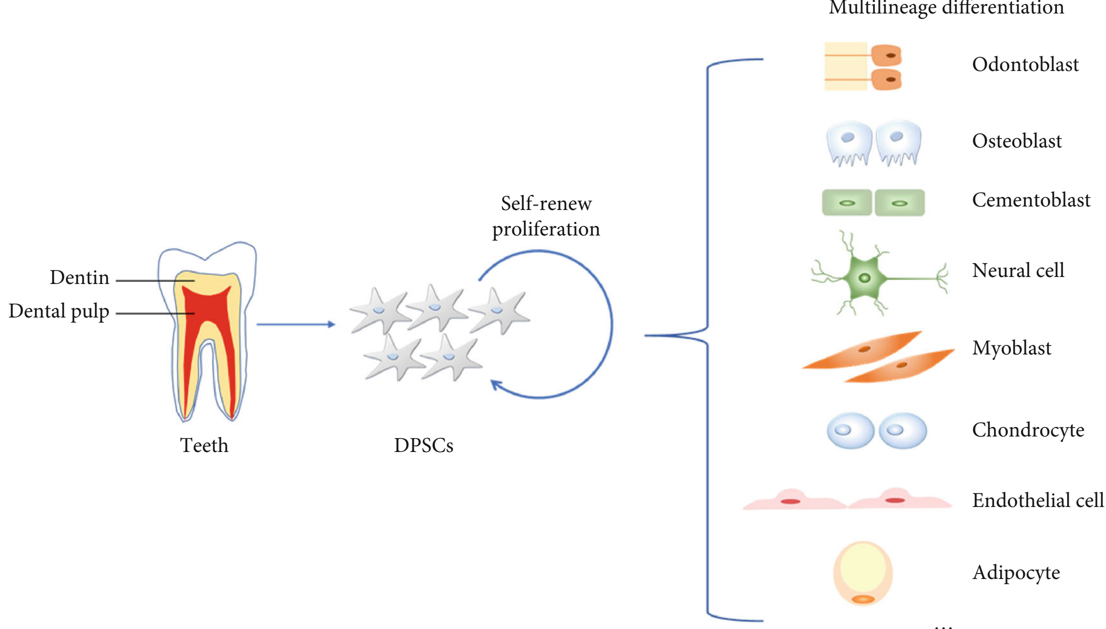

Figure 1: The multilineage differentiation potential of DPSCs. DPSCs can differentiate into odontoblasts, osteoblasts, cementoblasts, neural cells, myoblasts, chondrocytes, endothelial cells, adipocytes, etc.

Table 1: DNA methylation in DPSCs.

Epigenetic modifier	Epigenetic mark	Results
TET1	DNA demethylation	TET1, existing both in the cytoplasm and nuclei of the DPSCs, can improve the proliferation and odontogenic differentiation [102–104].
5-Aza-CdR	DNMT inhibitor	The inhibition of DNA methylation by 5-Aza negatively regulates the proliferation and enhances the myogenic and odontogenic differentiation [98, 100, 101].
RG108	DNMT inhibitor	SP1 can improve the expression of KLF4 through binding to the demethylated promoter region during the odontoblastic differentiation [99].

environments. DNA methylation patterns may affect the process by regulating gene expression in DPSCs. The osteogenic genes with different DNA methylation statuses are associated with osteogenic differentiation potential [97]. Although DPSCs, DFSCs, and PDLSCs share almost similar DNA methylation patterns, some genes related to the development of the skeletal system, like SMAD3 and CD109, exhibit differential methylation profiles leading to variation in osteogenic capacities [97]. The differentiation potential of DPSCs varies with changes in the activity of DNMTs. Upon treatment with 5-Aza-2′-deoxycytidine (5-Aza-CdR), a DNA methyltransferase inhibitor, the proliferation capacity of DPSCs is suppressed. However, 5-Aza-CdR upregulates the odontogenic markers (DSPP and DMP1) and transcription factors (RUNX2, DLX5, and OSX), increases alkaline phosphatase (ALP) activity, and accelerates the formation of calcified nodules, which indicates an enhanced odontogenic differentiation potential [98]. Studies have shown that DNA methylation impacts the transactivation of transcription factor (TF) on its target gene. Inhibition of DNMTs causes demethylation of the *Klf4* promoter region, leading to enhanced binding of SP1, a transcriptional factor that upregulates the expression of *Klf4*. Krüppel like factor 4 (KLF4) has been proved to be vital for odontogenic differentiation [99]. Besides, the myogenic differentiation is also improved after treatment with 5-Aza-CdR [100]. Signs of muscle regeneration can be observed when DPSCs with the pretreatment of 5-Aza-CdR are applied to the muscle injury/regeneration model [101]. DNA demethylation enzymes can also affect the fate of DPSCs, especially TET1. TET1, existing in both the nucleus and the cytoplasm of the DPSCs, is a DNA dioxygenase and can promote DNA demethylation. The expression of TET1 increases during early cell passaging ($<6^{th}$ passages) and then decreases. TET1 is also increased during odontogenic induction [102]. When TET1 is knocked down, the proliferation and odontogenic differentiation are suppressed [103]. Furthermore, TET1 can enhance odontogenic differentiation through the regulation of *FAM20C* demethylation and upregulation of the FAM20C expression [104].

Inflammation can occur in the dental pulp because of bacterial infection or trauma. This leads to the activation of a series of defense responses in DPSCs, including increased expression of inflammatory-related factors, odontogenic

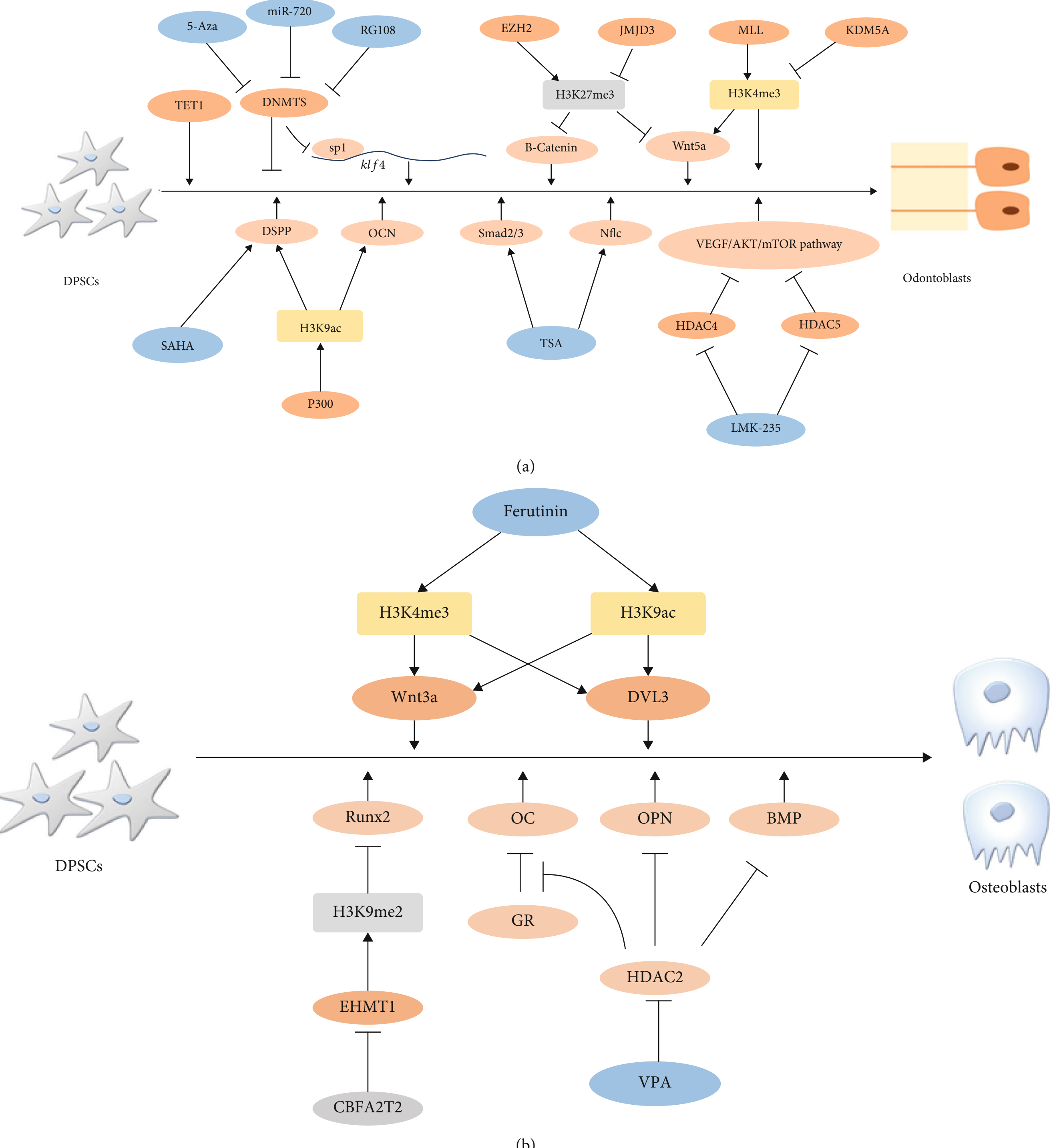

FIGURE 2: The modulation of DNA methylation and histone modifications during the odontogenic and osteogenic differentiation in DPSCs.

differentiation, and formation of restorative dentin. DNA methylation is involved in this process. During lipopolysaccharide- (LPS-) induced inflammation, DNMT1 mRNA and protein levels are reduced in DPSCs. DNMT1 can affect the MyD88 gene promoter methylation and downregulate the miR-146a-5p expression. Further research found that depletion of DNMT1 can enhance the inflammatory response through activation of the NF-κB pathway [105, 106]. Similarly, the expression of proinflammatory cytokines, including GM-CSF, interleukin- (IL-) 6, RANTES, IL-8, and MCP-2, is upregulated, and the MAPK and NF-κB signaling pathways are activated by 5-Aza-CdR in the LPS-treated DPSCs. Furthermore, 5-Aza-CdR decreases the levels of 5mc in the *TRAF6* promoter in DPSCs. These results indicate that 5-Aza-CdR accelerates the inflammatory process by the activation of TRAF6 [107]. Besides, TET2 improves the inflammatory response in the DPSCs by regulating the levels of 5hmC on the MyD88 promoter [108].

Table 2: Histone modifications in DPSCs.

Epigenetic modifiers	Epigenetic marks	Targets	Differentiation
Histone methylation			
EZH2	H3K27me3	Wnt/β-catenin pathway	Inflammation, proliferation, osteogenic [110, 111]
EHMT1	H3K9me2	RUNX2	Osteogenic [113]
MLL	H3K4me3	Wnt5a	Odontogenic [119]
Histone demethylation			
KDM5A	H3K4me3	DMP1, DSPP, OSX, and OCN	Odontogenic [121]
KDM6B	H3K27me3	Wnt5a, BMP2	Osteo/odontogenic [116, 117, 119]
Histone acetylation			
HAT	H3 acetylation	DSPP	Odontogenic [125]
p300	H3K9ac	NANOG, SOX2, DSPP, OCN, Dmp1, and Sp7	Pluripotency, proliferation, odontogenic [126, 127, 132, 134]
Histone deacetylation			
HDAC3	H3K27ac	Dmp1, Sp7	Odontogenic [132, 134]
HDAC6			Odontogenic [131]

The above results indicate the critical role of DNA methylation in the differentiation and proliferation of DPSCs both *in vitro* and *in vivo*. However, further researches are necessary to explore the specific mechanism for the regulation of DPSCs through DNA methylation, so as to apply DPSCs safely and effectively in clinical treatment and tissue engineering.

4.2. Histone Modification. Histone modification often happens on the tail of histones and can turn genes on or off. Here, we conclude the regulation on DPSCs of histone modification (Table 2 and Figure 2).

4.2.1. Histone Methylation. Histone methylation, mainly occurring in lysine or arginine residues located at the histone tails, is widely reported to modulate stem cell maintenance and differentiation. It has also been demonstrated in DPSCs. When comparing the epigenetic states between the DPSCs and the dental follicle cells (DFCs), H3K27me3-mediated repression of odontogenic-related genes, *DSPP* and *DMP1*, can be seen in the DFCs, but not in DPSCs. In accordance with the results, in osteoinductive conditions, DPSCs exhibit higher expression of both DSPP and DMP1, which indicates higher odontogenic ability [109]. EZH2, a histone methyltransferase, is responsible for repressive H3K27me3. During odontogenic differentiation, EZH2 decreases with the level of H3K27me3. Overexpression of EZH2 impairs the odontogenic differentiation; however, overexpression of EZH2 without methyltransferase activity does not affect the odontogenic differentiation of DPSCs. When β-tricalcium phosphate/DPSCs transfected with siEZH2 are transplanted under the skin of nude mice, the formation of mineralized tissue is improved. Further, the results of a CHIP assay suggested that EZH2 downregulates the expression of β-catenin by increasing the levels of H3K27me3 on the promoter region of *β-catenin*, eventually suppressing the Wnt/β-catenin signaling pathway that is critical for odontogenic differentiation [110]. EZH2 is also related to the proliferation, osteogenic differentiation, and inflammatory response of DPSCs. Under the appropriate inflammatory stimulation, DPSCs can differentiate into odontoblasts and migrate to the infected site to generate reparative dentin. In the infected cells, EZH2 and H3K27me3 are decreased. EZH2 inhibition can suppress IL-1b, IL-8, IL-6, and proliferation of DPSCs upon inflammatory irritation but enhances the osteogenic differentiation. These results prove that EZH2 inhibits osteogenic differentiation and enhances the inflammatory response and proliferation [111]. Another repressive histone methylation, H3K9, is also associated with osteogenic differentiation of DPSCs. The euchromatin histone methyltransferases-1 (EHMT1) can repress gene transcription and regulate cell differentiation through H3K9 dimethylation (H3K9me2) [112]. During the BMP-2-induced osteogenic differentiation, the level of H3K9me2 on the promoter of *Runx2* is downregulated by corepressor core-binding factor, runt domain, alpha subunit 2, translocated to, 2 (CBFA2T2). Knockdown of CBFA2T2 upregulates the expression of EHMT1 and increases the level of H3K9me2; however, the osteogenic differentiation is impaired [113]. The osteogenic differentiation of DPSCs is also regulated by the active mark trimethylation of lysine 4 of histone 3 (H3K4me3). Ferutinin, a daucane phytoestrogen, enhances the levels of H3K4me3 and H3K9ac on the promoters of *Wnt3a* and *DVL3* genes in DPSCs and improves the osteogenic differentiation by activating the Wnt/β-catenin signaling pathway [114]. These results demonstrated that histone modifications, such as H3K9me3, H3K27me3, and H3K4me3, are closely related to the differentiation process of DPSCs, especially the osteo/odontogenic differentiation.

Histone methylation is also a reversible process. There are various demethylases that can remove the methyl groups from histone. The Jumonji domain-containing protein D3 (JMJD3), also called lysine-specific demethylase 6B (KDM6B), can specifically demethylate H3K27me2/3 to regulate gene expression and modulate odontogenic differentiation through various mechanisms [115]. Overexpression of JMJD3 can enhance odontogenic differentiation, while the JMJD3 inhibition by alcohol impairs the odontogenic

differentiation [116]. During the odontogenic-induction process, JMJD3 removes silencing H3K27me3 marks on the promoters of *BMP2*, and thus, the expression of transcription protein related to odontogenic differentiation BMP2 is activated [117]. The "bivalent domains," containing both active mark H3K4me3 and repressive mark H3K27me3, are localized at the promoter regions of the *Wnt5a* gene. These modifications maintain the *Wnt5a* gene in a poised state, and under certain stimuli, the gene transcription is activated or repressed by the resolution of these marks [32, 118]. During odontogenic induction in DPSCs, the H3K27me3 on the *Wnt5a* promoter is removed by JMJD3, and Wnt5a is activated. The depletion of JMJD3 upregulates the level of H3K27me3, suppresses the expression of Wnt5a, and impairs the odontogenic differentiation. Besides, JMJD3 is important for H3K4me3 through the interaction with H3K4me3 methylases, mixed-lineage leukemia (MLL) complex [119]. Another HDMs, lysine-specific demethylase 5A (KDM5A), is specific for the active mark H3K4me3 [120]. The depletion of KDM5A can upregulate the level of H3K4me3 on the promoter of the odontogenic marker gene, including *DMP1*, *OSX*, *OCN*, and *DSPP*, and improve the odontogenic differentiation. These results indicate that H3K4me3 is also associated with odontogenic differentiation [121]. As mentioned above, HDMs can regulate gene expression and affect the fate of DPSCs through their specific demethylase activity.

4.2.2. Histone Acetylation. Histone acetylation, which is usually considered to loosen the chromatin structure and facilitate gene transcription, profoundly impacts the differentiation and proliferation of different cells [122–124], including DPSCs. HAT improves the odontogenic differentiation of DPSCs by increasing the histone H3 acetylation of *DSPP* genes [125]. p300, a member of the lysine acetyltransferase 3 family, transfers the acetyl group to lysine residues. p300 upregulates the expression of SOX2 and NANOG in DPSCs, which is critical for maintaining the self-renewal and pluripotency of SCs through the enhancement of the transcriptional activities of the promoter. On the contrary, overexpression of p300 in DPSCs contributes to the reduction of odontogenetic markers, such as DSPP, OCN, DSP, OPN, and DMP1. However, when DPSCs are cultured in the odontoblastic induction medium, overexpression of p300 lacking the HAT domain increases the H3K9ac level on the promoter of *DSPP* and *OCN* and enhances the odontoblastic differentiation. Therefore, p300 is critical for the stemness of DPSCs by regulating the expression of SOX2 and NANOG and acts as a coactivator to upregulate the level of H3K9ac on the promoter of *DSPP* and *OCN* to promote odontogenic differentiation [126]. Consistent with the above results, another study found that when p300 is knocked down in DPSCs, proliferation and odontogenic differentiation are inhibited [127]. Besides, histone acetylation upregulated by photobiomodulation therapy can induce the proliferation of DPSCs [128].

In addition to HATs, histone acetylation levels are also affected by HDACs, which can transfer acetyl groups from histones, resulting in histone hypoacetylation and packed chromatin [123]. HDACs are classified into four categories, of which classes I (HDAC1,2,3,8), II (HDAC4,5,6,7,9,10), and IV (HDAC11) are zinc-dependent enzymes [129], while class III HDACs, often referred to as sirtuins, are nicotinamide adenine dinucleotide-dependent enzymes [130]. HDACs are also closely related to the differentiation of DPSCs. HDAC6 promotes the odontogenic differentiation of DPSCs, and when HDAC6 is knocked down, the odontogenic differentiation is impaired [131]. During the odontoblast differentiation, H3K9ac and H3K27ac are upregulated and p300 is increased, while HDAC3 is decreased [132]. These results indicate that odontoblast differentiation is coregulated by HATs and HDACs. To further explore, it is found that KLF4, a transcriptional factor, has a transactivation domain that binds directly to the target gene promoter and recruits coactivators like p300 or corepressors like HDAC3 [133]. The data of a CHIP analysis revealed that when dental pulp cells are induced into odontoblasts, HDAC3 mainly interacts with KLF4 on the promoter of *Dmp1* and *Sp7* on day 0 of induction, while p300 interacts on day 7 of induction. These results reveal that KLF4 can regulate the odontoblast differentiation by affecting the histone acetylation on the promoter regions of *DMP1* and *Sp7* and by interacting with p300 and HDAC3 [134].

HDAC inhibitors (HDACis) regulate gene expression by modulating the level of histone acetylation and have been widely used in cancer therapy [135]. HDACis can affect the differentiation and proliferation of DPSCs and may have potential applications in dental restoration [136]. Trichostatin A (TSA), a hydroxamic acid, inhibits the activity of all HDACs, except class IIa. TSA affects the proliferation of DPSCs in a dose-dependent manner and promotes the osteo/odontogenic differentiation through the upregulation of Smad2/3 and nuclear factor I-C- (Nfic-) related pathways. The proliferation of DPSCs is increased on exposure to 2 nmol/L and 20 nmol/L of TSA via the activation of the JNK/c-Jun pathway; however, higher concentrations of TSA lead to apoptosis. A 20 nmol/L solution of TSA can promote migration and adhesion of DPSCs [137–140]. Valproic acid (VPA), the short-chain fatty acid, can inhibit class I HDACs. Similar to TSA, the effect of VPA on DPSCs is dose-dependent, and at a certain concentration, it can improve the proliferation, adhesion, and migration of DPSCs [137]. In addition, VPA increases the mineralization and osteo/odontogenic differentiation [138, 139]. VPA increases the expression of OPN and BMP but decreases OCN, a late-stage marker of osteogenic differentiation, via HDAC2, which indicates that VPA promotes early differentiation of osteogenesis but does not promote terminal differentiation. In addition, VPA causes DPSCs to generate a well-organized bone tissue structure *in vivo*. Several studies have reported that glucocorticoid receptor (GR) is critical for this regulation. HDAC2 binds to GR and inhibits its translocation into the nucleus, but when HDAC2 is inhibited by VPA, GR can enter the nucleus and thus affect the expression of the OC [141, 142]. Suberoylanilide hydroxamic acid (SAHA), a pan inhibitor of HDACs, increases the expression of DSPP via the activation of Nfic and enhances odontogenic differentiation in DPSCs [143]. LMK-235, a specific inhibitor to HDAC4 and HDAC5, improves odontogenic differentiation

through the VEGF/AKT/mTOR pathway without affecting the proliferation of DPSCs [144].

Histone acetylation regulates various physiological processes of DPSCs and affects their fate. HDACis may have potential applications in the treatment of mineralized regeneration; however, further research is needed in this context.

4.3. ncRNAs

4.3.1. miRNAs. miRNAs specifically recognize the target mRNA through base complementation and affect its stability by binding to the 3′UTR, which eventually leads to suppression of protein translation. miRNAs are related to stemness, cell reprogramming, and differentiation of various cells, including DPSCs (Table 3).

Table 3: ncRNAs in DPSCs.

ncRNAs	Targets	Differentiation
lncRNAs		
ANCR	p-GSK-3β and β-catenin	Odontogenic [175, 176]
H19	SAHH	Odontogenic [179, 180]
CCAT1	miR-218	Proliferation, osteogenic [183]
G043225	miR-588	Odontogenic [178]
miRNAs		
miR-224	Rac1	Migration, proliferation, apoptosis [148, 149]
miR-152	SIRT7	Senescence [147]
miR-140-5p	TLR-4, Wnt1	Proliferation, odontogenic [153, 171]
miR-720	NANOG	Proliferation, odontogenic [158]
miR-584	TAZ	Proliferation [146]
miR-320b	Foxq1	Proliferation [145]
miR-21	STAT3	Odontogenic [172]
miR-143, miR-143-5p	Runx2, MAPK14, TNF-α	Osteo/odontogenic, myogenic [150–152, 162]
miR-508-5p	GPNMB	Odontogenic [159]
miR-223-3p	Smad3	Odontogenic [165]
miR-506	SIRT1	Inflammation [166]
miR-218	RUNX2	Osteo/odontogenic [156, 157]
miR-215, miR-219a-1-3p	HspB8	Osteogenic differentiation [155]
let-7c, let-7c-5p	IGF-1R, DMP1	Osteo/odontogenic, inflammation [154, 167, 168]
miR-125-3p	Fyn	Odontogenic, inflammation [169]
miR-424	VEGF, KDR	Angiogenic [161]
miR-135		Myogenic [162]
miR-139-5p	Wnt/β-catenin signaling pathway	Myogenic [79]

miRNAs are vital in regulating the proliferative capacity of DPSCs. Foxq1, a transcriptional factor, regulates cell cycle and promotes the stemness and proliferation. When the proliferation of DPSCs is promoted by calcium hydroxide, the expression of Foxq1 is also increased; however, miR-320b, which negatively regulates Foxq1, is decreased. Therefore, miR-320b can mediate the proliferation of DPSCs via Foxq1 [145]. The transcriptional coactivator with PDZ-binding motif (TAZ) is reported to be essential for the proliferation of DPSCs. TAZ can be silenced by miR-584, which binds directly to *TAZ* mRNA and in turn suppresses the proliferation [146].

The senescence of DPSCs is accompanied by a decline in proliferation and differentiation ability, affecting the clinical use of DPSCs. miR-152 is upregulated with the senescence of DPSCs. miR-152 targets sirtuin 7 (SIRT7), which modulates gene expression by regulating histone deacetylase activity, and induces DPSC senescence [147]. Apoptosis is a genetically programmed cell death. miR-224-5p protects DPSCs from apoptosis by silencing Rac family small GTPase 1 (Rac1), which has been proved to induce apoptosis [148]. Besides, miR-224-5p can improve the migration and proliferation of DPSCs [149].

The miR-143 family negatively modulates the odontogenic and osteogenic differentiation of DPSCs. miR-143-5p impairs the odontogenic differentiation by targeting RUNX2 via the OPG/RANKL signaling pathway [150]. It has been reported that miR-143-5p binds to MAPK14 and reduces its expression. Thus, miR-143-5p knockdown increases MAPK14 expression and activates the p38 MAPK signaling pathway, consequently enhancing the odontogenic differentiation [151]. In addition, by directly targeting tumor necrosis factor-α (TNF-α), miR-143 blockades the NF-κB signaling pathway and suppresses the osteogenic differentiation [152]. Some other miRNAs are also associated with the osteo/odontogenic differentiation of DPSCs. miR-140-5p is decreased when DPSCs are induced into odontoblasts, and miR-140-5p mimic can impair the odontogenic differentiation through suppressing the Wnt1/β-catenin signaling pathway by targeting Wnt1 [153]. Insulin-like growth factor 1 has been proved to induce the proliferation and osteo/odontogenic differentiation of DPSCs via activation of the P38 MAPK and JNK pathways. However, overexpression of let-7c can reverse the process but not affect the proliferation by suppressing the insulin-like factor 1 receptor (IGF-1R). During this process, the JNK/P38 MAPK pathway is also repressed [154]. Besides, miR-215 and miR-219a-1-3p negatively modulate the osteogenic differentiation of DPSCs through downregulation of heat shock protein B8 (HspB8) [155]. In an osteoinductive environment, miR-218, which suppresses the osteogenic differentiation of DPSCs by targeting *RUNX2*, is decreased [156]. miR-218 also suppresses the odontogenic differentiation through the MAPK/ERK pathway. On delivering miR-218 inhibitor into DPSCs by a newly designed magnetic nanocarrier, GCC-Fe_3O_4, mineralization nodules are increased, which is a novel application of DPSCs [157]. Compared to the undifferentiated DPSCs, miR-720 is highly expressed in differentiated DPSCs. Further, it has been found that miR-720 decreases the proliferation and enhances the odontogenic differentiation of DPSCs through directly

repressing NANOG and indirectly silencing NANOG by induction of DNMT3A and DNMT3B [158]. In addition, during odontogenesis of DPSCs, miR-508-5p is gradually decreased, while glycoprotein nonmetastatic melanoma protein B (GPNMB), also called osteoactivin, is increased. Further research demonstrated that knockdown of miR-508-5p can promote odontogenesis in DPSCs via upregulation of GPNMB [159]. In conclusion, miRNAs affect the osteo/odontogenic differentiation of DPSCs by regulating various key molecules in the osteo/odontogenesis process.

Growing evidence indicates that miRNAs play a critical role in angiogenic processes [160]. In particular, miR-424 is expressed in a sequential manner during the endothelial differentiation of DPSCs. Overexpression of miR-424 inhibits endothelial differentiation. Thus, miR-424 negatively regulates the endothelial differentiation of DPSCs [161].

Interestingly, the expression of miR-143 and miR-135 is significantly downregulated in myoblast DPSCs induced by 5-Aza. The addition of miR-143 or miR-135 inhibitors to culture medium stimulates the myocytic properties of DPSCs, which eventually fuse to form myotube [162]. Additionally, miR-139-5p regulates the skeletal myogenic differentiation of human DPSCs by interacting with the Wnt/β-catenin signaling pathway [79]. These outcomes reveal that miRNAs are essential for the induction of myogenic differentiation of DPSCs.

The inflammatory microenvironment can interact with DPSCs and affect the fate of DPSCs [163, 164]. A series of studies reported that miRNAs are involved in the interaction between inflammatory microenvironment and DPSCs. By comparing the expression of miRNAs between healthy and inflamed pulp, 79 differentially expressed miRNAs have been identified. Among them, miR-223-3p is significantly upregulated. Furthermore, overexpression of miR-223-3p increases DSPP and DMP1 but suppresses Smad3. According to the dual-luciferase assay, miR-223-3p promotes odontogenic differentiation by targeting Smad3 and enhances pulpal healing [165]. LPS, a major pathogenic factor of Gram-negative bacteria, is closely related to pulpitis caused by caries. In the DPSCs treated by LPS, the proinflammatory cytokines, such as TNF-α and IL-6, are increased, the viability is decreased, and osteo/odontogenic differentiation is impaired. In the LPS-treated DPSCs, the expression of miR-506 is upregulated, and TLR-4 pathway is activated. However, miR-506 knockdown attenuates the inflammatory response and suppresses the TLR-4 pathway by upregulating SIRT1 [166]. These inflammatory responses are reversed in the presence of let-7c-5p overexpression in LPS-induced DPSCs. It has been reported that let-7c-5p protects DPSCs from inflammation by directly repressing DMP1 and promotes the osteogenic differentiation through inhibition of HMGA2/-PI3K/Akt signaling [167, 168]. Besides, DPSCs treated with TNF-α exhibit increased expression of Fyn, a member of the protein tyrosine kinase Src family, which is related to inflammation and odontogenesis; however, the expression of miR-125a-3p is decreased. It has been found that miR-125-3p can reverse the inflammatory response and enhance odontogenic differentiation by repressing Fyn [169]. Moreover, a certain concentration of LPS can improve the proliferation, adhesion, and migration of DPSCs and differentiation of odontoblast through Toll-like receptor (TLR-4), ERK, and P38 MAPK signaling pathways [164, 170]. During the LPS-induced odontoblastic differentiation, the expression of miR-140-5p is downregulated. When miR-140-5p is overexpressed, the differentiation and proliferation of DPSCs are impaired. A luciferase reporter analysis demonstrated that miR-140-5p can bind to the 3′UTRs of the *TLR-4* mRNA, and the inhibition of TLR-4 can reverse the impact on the proliferation and differentiation of DPSCs via inhibition of miR-140-5p. These outcomes indicate that miR-140-5p impairs the differentiation and proliferation of DPSCs induced by LPS [171]. The cytokine TNF-α enhances odontogenic differentiation at low concentrations (1-10 ng/mL) and suppresses the same at high concentrations (50-100 ng/mL). Consistent with the above results, increased expression of miR-21, as well as signal transducer and activator of transcription 3 (STAT3), is observed at low concentrations of TNF-α, while the opposite results are observed at high concentrations. It is noteworthy that miR-21 and STAT3 form a positive feedback loop to regulate odontogenic differentiation [172]. These results reveal that miRNAs are associated with the inflammatory response of DPSCs and also provide a new perspective for the treatment of pulpitis.

4.3.2. lncRNAs. lncRNAs modulate gene expression at different levels and regulate the fate of DPSCs (Table 3).

As the donor's age increases, DPSCs are also gradually senescent. During this process, 389 lncRNAs are downregulated and 172 lncRNAs are upregulated, which also indicates the important role of lncRNAs in the senescence of DPSCs [173]. The lncRNA, antidifferentiation noncoding RNA (ANCR), also called differentiation antagonizing nonprotein coding RNA (DANCR), was first identified in 2012. It suppresses the differentiation and enforces the undifferentiation state of somatic progenitor populations [174]. Similarly, the inhibition of ANCR promotes the osteogenic, neurogenic, and adipogenic differentiation of DPSCs, without affecting the proliferation [175]. It has been reported that ANCR impairs the odontogenic differentiation of DPSCs by repressing of the Wnt/β-catenin signal pathway [176].

lncRNAs are also closely associated with odontogenic ability of stem cells. Through RNA-sequencing analysis, 108 lncRNAs are found to be downregulated and 36 lncRNAs are found to be upregulated in association with the loss of odontogenic differentiation potential [177]. In another research, when DPSCs are induced to differentiate into odontoblasts, the expression of 114 miRNAs and 132 lncRNAs is found to be altered. Through bioinformatics analyses, two lncRNA-associated ceRNA networks centered two odontogenic-related proteins, rhodopsin and Fibrillin 1 (FBN1), are found to be involved in the odontogenic differentiation of DPSCs. Further research reported that lncRNA G043225 improves the odontogenic differentiation by competitively inhibiting the repression activity of miR-588 on FBN1 as an endogenous miRNA sponge [178]. Besides, lncRNA H19 can upregulate S-adenosylhomocysteine (SAH), which is an inhibitor of S-adenosylmethionine-dependent methyltransferase, and downregulate DNA

methylation levels [179]. Therefore, H19 upregulates the expression of distal-less homeobox3 (DLX3) and enhances the odontogenic differentiation of DPSCs through the downregulation of the methylation level on *DLX3* gene [180].

DPSCs are able to differentiate into osteoblasts and form bone tissue, which can be used in bone regeneration therapy. Studies have confirmed that lncRNAs are also associated with the osteogenic differentiation of DPSCs. Examination of the expression of lncRNAs during TNF-α induced osteogenic differentiation revealed 58 upregulated and 19 downregulated lncRNAs on day 7 and 73 upregulated and 60 downregulated lncRNAs on day 14 [181]. lncRNA colon cancer-associated transcript 1 (CCAT1) is initially discovered to participate in metabolic, migratory, and proliferative processes in some cancers [182]. Later on, it was found that CCAT1 can promote the proliferation and odontogenic differentiation of DPSCs. A luciferase assay suggested that CCAT1 directly bind to the miR-218 and negatively regulate the expression of miR-218 [183].

The angiogenic differentiation of DPSCs is important for pulp regeneration. During the angiogenic induction in DPSCs, 376 lncRNAs are significantly upregulated, including SMILR, while 426 lncRNAs are downregulated. These results indicate the critical role of lncRNAs in angiogenic differentiation [184]. Still, further research is warranted to explore specific mechanisms.

5. Conclusion

DPSCs have gained increased attention in the field of regenerative medicine owing to their multilineage differentiation potential and easy accessibility. In this review, we summarize the regulation of epigenetic modifications mainly including DNA methylation, histone modification, and ncRNAs in the differentiation and proliferation of DPSCs. While most studies were mainly conducted *in vitro*, further investigations, including *in vivo* experiments and animal disease models, would be needed to explore the clinical potential in disease treatment and regenerative medicine such as HDACis and DNMT inhibitors related to epigenetic modifications. In addition, some other epigenetic modifications such as RNA modification and chaperones have been shown to be involved in embryo development, cell differentiation, and pluripotency maintenance [185–188]. It would be necessary to reach deeper insights into the role of these epigenetic modifications in the modulation of DPSC fate.

Conflicts of Interest

The authors declare that there is no conflict of interests regarding the publication of this paper.

Acknowledgments

This work was supported by the National Natural Science Foundation of China (NSFC) Grant 81900965 to Wei Du and 81771033 to Liwei Zheng and SCU Postdoctoral Science Foundation 0040304153066 to Wei Du.

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Hindawi
Stem Cells International
Volume 2020, Article ID 8890917, 12 pages
https://doi.org/10.1155/2020/8890917

Review Article

Small Molecule Epigenetic Modulators in Pure Chemical Cell Fate Conversion

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Received 21 July 2020; Revised 16 September 2020; Accepted 3 October 2020; Published 21 October 2020

Academic Editor: Yang Li

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Although innovative technologies for somatic cell reprogramming and transdifferentiation provide new strategies for the research of translational medicine, including disease modeling, drug screening, artificial organ development, and cell therapy, recipient safety remains a concern due to the use of exogenous transcription factors during induction. To resolve this problem, new induction approaches containing clinically applicable small molecules have been explored. Small molecule epigenetic modulators such as DNA methylation writer inhibitors, histone methylation writer inhibitors, histone acylation reader inhibitors, and histone acetylation eraser inhibitors could overcome epigenetic barriers during cell fate conversion. In the past few years, significant progress has been made in reprogramming and transdifferentiation of somatic cells with small molecule approaches. In the present review, we systematically discuss recent achievements of pure chemical reprogramming and transdifferentiation.

1. Introduction

In 1958, Gurdon et al. first reported unknown factors in the oocyte cytoplasm could reprogram differentiated cells to a pluripotent state [1]. The breakthrough suggested that somatic cells are flexible and could be converted to other cell types. In 1987, Davis et al. discovered that a single transcription factor, MyoD, was able to induce fibroblasts directly into myoblasts, which indicated only a few transcription factors could make cell fate decisions [2]. Nearly 20 years later, Yamanaka's team found that pluripotent stem cells (iPSCs) could be obtained from somatic cells using four key transcription factors (Oct4, Sox2, Klf4, and c-Myc, termed OSKM) [3]. One year later, two research groups independently succeeded in creating human iPSCs using a similar method [4, 5]. With this new iPSC technology, the molecular mechanisms of cell fate transition could be investigated and diverse applications, including drug screening, disease modeling, and cell therapy, could be developed [6].

Although the medical applications of iPSCs are promising, transgenic approaches raise safety concerns because of the use of oncogenes and the potential for the integration of exogenous factors. Therefore, several new methods have been developed to resolve these issues, including nonintegrating vectors, nonviral gene delivery methods, miRNAs, cell membrane permeable proteins, and small molecule compounds [7–11]. Compared to other approaches, chemical compounds similar to those employed to treat human diseases for decades have several unique advantages. For example, their structural versatility permits modulation of induction time and concentration [12]. In this review, omitting differentiation, we will focus on pure small molecule inductions for reprogramming or transdifferentiation (Figure 1). The dramatic progress in small molecule induction of cell fate

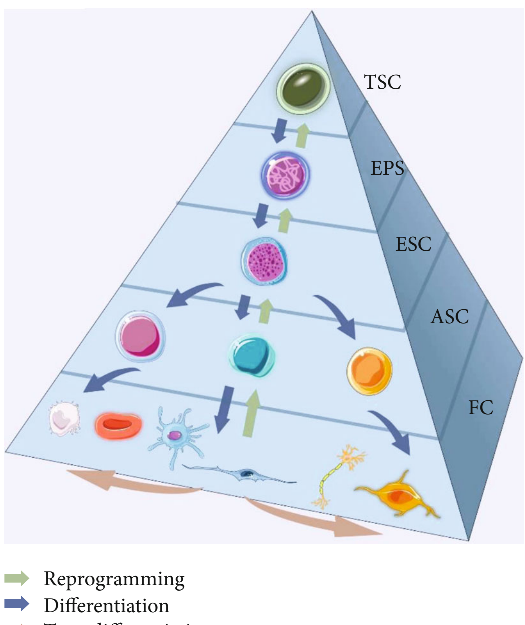

Figure 1: A schematic diagram for differentiation, reprogramming, and transdifferentiation. Cells come down from totipotent stem cells to functional cells in the development process (differentiation) while differentiated cells are able to be reversed back to pluripotent state (reprogramming) by transcription factors or chemical cocktails. Using similar approaches, one type of functional cells can be directly converted to other functional cells (transdifferentiation). TSC: totipotent stem cell; EPS: extended pluripotent stem cell; ESC: embryonic stem cell; ASC: adult stem cell; FC: functional cell.

decisions will undoubtedly accelerate the pace of biomedical studies and clinical translation.

2. Reprogramming *In Vitro*

2.1. Chemical-Induced Pluripotent Stem Cells (CiPSCs). It was demonstrated that small molecules could replace transcription factors for reprogramming of iPSCs. Melton's group firstly revealed that Valproic acid (VPA) was able to promote OS-induced reprogramming of human fibroblasts [13]. Eggan's team discovered that RepSox (also named as E616452), which is an ALK5 inhibitor, could act as a substitute for Sox2 and promote reprogramming via activation of Nanog [14]. Ding's lab found that CHIR99021 and Tranylcypromine (also named Parnate) completed OK reprogramming of human somatic cells [15], and AMI-5 and A83-01 empowered Oct4-induced reprogramming of mouse fibroblasts [16]. Deng's team also found that a chemical cocktail (VPA, CHIR99021, RepSox, and Tranylcypromine) was able to reprogram mouse fibroblasts to iPSCs with Oct4 alone [17]. In 2013, Deng's team reported that mouse fibroblasts could be induced to iPSCs via a combination of seven small molecules (VPA, CHIR99021, RepSox, Tranylcypromine, Forskolin, DZNep, and TTNPB) [18]; however, this induction method has been challenged by other labs [19]. Consequently, Deng's team presented a new induction approach to resolve these problems [20]. They identified two new small molecules (AM580 and EPZ004777) to induce mouse fibroblasts into a stage named "XEN-like cell transition," while three small molecules (5-aza-dC, EPZ004777, and SGC0946) were sufficient to convert these transitional cells to CiPSCs. Compared with the original protocol, the induction efficiency for CiPSCs was raised by 1000-fold via fine-tuning of the factors during these two stages. At the same time, Xie's team discovered that a chemical cocktail including bromodeoxyuridine (BrdU), CHIR99021, RepSox, and Forskolin was able to induce mouse fibroblasts into CiPSCs [19].

In 2016, Deng's team also reported that CiPSCs were reprogrammed from neural stem cells and intestinal epithelial cells [21]. A similar chemical cocktail (VPA, CHIR99021, RepSox, Parnate, Forskolin, AM580, and DZNep) was applied to the reprogramming of MEFs and intestinal epithelial cells. Two extra small molecules Ch55 and EPZ004777 were used in the reprogramming of neural stem cells. In 2018, Pei's team found that three types of mouse cell lineages could be induced to CiPSCs through an epithelial colony stage [22]. A chemical combination containing Vitamin C (VC), bFGF, CHIR99021, BrdU, RepSox, FSK, VPA, AM580, EPZ5676, DZNep, SGC0946, and BMP4 was applied for the induction of epithelial colonies, and then, 2iL (CHIR99021, PD0325901, and LIF) were used to induce full pluripotency in the second stage. In contrast to Deng's and Xin's methods, the induction efficiency and time were dramatically improved in Pei's protocol.

Although mouse CiPSCs have advanced in the last several years, generation of human CiPSCs have remained elusive. Based on different pluripotent signaling pathways in mice and humans [23, 24], a large-scale screening of small molecules may be necessary. Currently, the small molecules involved in induction are classified into three categories, including epigenetics, signaling pathways, and metabolism (Table 1 and Figure 2). As for different starting cells and targeted cells, some clues could be obtained to select small molecules for reprogramming or transdifferentiation from this review.

2.2. Extended Pluripotent Stem Cells (EPSs). In 2017, Deng's team found that ESCs or iPSCs could be reprogrammed into extended pluripotent stem cells (EPSs) that could differentiate into four lineages including trophectoderm, ectoderm, endoderm, and mesoderm via a chemical cocktail consist of LIF, CHIR99021, (S)-(+)-Dimethindene maleate, and Minocycline hydrochloride [25]. After half a year, Liu's team also obtained EPSs using a different small molecule combination containing hLIF, CHIR99021, PD0325901, JNK inhibitor VIII, SB203580, A-419259, and XAV939 [26]. As for a means to create new animal models, EPS cell lines could be applied to explore fundamental questions such as the development of the placenta, yolk sac, and embryo proper.

2.3. Chemical-Induced Neural Stem Cells (CiNSCs). In 2012, we first found a pure small molecule combination (VPA, RG108, VC, BIX01294, A83-01, CHIR99021, and PD032591) was able to induce mouse embryonic and adult tail-tip fibroblasts into neural stem cells [27, 28]. CiNSCs are similar to neural stem cells in morphology, gene

Table 1: Small molecules involved in pure small molecule-induced reprogramming or transdifferentiation.

Name of the compounds	Main mechanism of action	Application in reprogramming or transdifferentiation	References
Signaling pathways			
TGF-β signaling pathways			
A83-01	TGF-beta RI (ALK4/5/7) inhibitor	CiNSCs, CiNs, CiBCs, CiPSCs, CiBLPCs, CiCMs, CiEPCs	[16, 27, 28, 30–34, 46, 55–57, 62, 64]
RepSox (E-616452)	TGF-beta RI (ALK5) inhibitor	CiPSCs, CiNs, CiCMs, CiPCs, CiSMCs, CiCCs	[14, 17–19, 21, 22, 40, 41, 45, 52, 61, 69, 71, 79]
SB431542	Inhibitor of TGF-βRI, ALK4, and ALK7	CiEPCs, CiNs, CiCMs, CiLCs	[35, 43, 55–57, 59, 75]
IDE 1	Activator of TGF-β signaling pathway	CiBCs	[64]
DMH1	Inhibitor of ALK2	CiNs	[41]
BMP signaling pathways			
Dorsomorphin	BMP receptor inhibitor	CiNs	[42]
LDN193189	BMP type I receptor (ALK2/3) inhibitor	CiNSCs, CiNs	[30, 42, 43]
Wnt signaling pathway			
CHIR99021	GSK3 inhibitor	CiPSCs, EPSs, CiNSCs, CiBLPCs, CiNs, CiCMs, CiPCs, CiSMCs	[15, 17–19, 21, 22, 25–28, 30–34, 39–43, 45, 47, 52, 59, 61, 62, 69, 78, 79]
LiCl	GSK3 inhibitor	CiBCs	[64]
XAV939	Wnt/beta-catenin inhibitor	EPSs	[26]
IWR1	Wnt/beta-catenin inhibitor	CiPCs	[52]
CHIR-98014	GSK3 inhibitor		
TWS119	GSK3 inhibitor		
Tideglusib	GSK3 inhibitor		
BIO	GSK3 inhibitor		
AZD2858	GSK3 inhibitor		
TDZD-8	GSK3 inhibitor		
Indirubin	GSK3 inhibitor		
PNU-74654	Wnt/beta-catenin inhibitor		
IWP-2	Wnt/beta-catenin inhibitor		
MAPK/ERK signaling pathway			
PD0325901	Inhibitor of MEK1/2	CiPSCs, CiCMs, CiNs, EPSs	[22, 26, 42, 61]
SC1	ERK1 and RasGAP inhibitor	CiCMs	[62]
Rho signaling pathway			
Thiazovivin	ROCK inhibitor	CiNs	[43]
Y-27632	ROCK inhibitor	CiNs, CiBLPCs, CiCMs	[31–33, 40, 42, 46, 47, 58, 62, 78]
Notch signaling pathway			
DAPT	Gamma-secretase inhibitor	CiNs, CiBCs	[42, 43, 64]
SHH signaling pathway			
Cyclopamine-KAAD	Hedgehog/smoothened inhibitor	CiBCs	[64]
Hh-Ag 1.5	Smoothened agonist	CiNSCs	[30]
Purmorphamine	Smoothened agonist	CiNs	[42, 43, 46]
Other signaling pathways			
A-419259	An inhibitor of Src family kinases (SFK)	EPS	[26]

Table 1: Continued.

Name of the compounds	Main mechanism of action	Application in reprogramming or transdifferentiation	References
dbcAMP	Activates cAMP-dependent protein kinases		[78]
Forskolin	Adenylyl cyclase activator	CiPSCs, CiNs, CiPCs, CiSMCs, CiLCs	[18, 19, 22, 39–42, 45–47, 52, 59, 61, 69, 75, 78, 79]
Gö6983	Inhibitor of protein kinase C (PKC)	CiNs	[40]
Indolactam V	Activator of protein kinase C (PKC)	CiBCs	[64]
JNJ10198409	PDGFR-a and PDGFR-b inhibitor, PDGFR tyrosine kinase inhibitor IV	CiCMs	[62]
SB203580	P38 MAPK inhibitor	EPSs, CiBCs	[26, 64]
SP600125	JNK inhibitor	CiNs	[40, 41]
SU16F	PDGFR-b inhibitor	CiCMs	[62]
Celecoxib	COX inhibitor	CiCCs	[71]
Epigenetic modifications			
DNA methylation inhibitor			
5-Aza-dC	DNMT inhibitor	CiPSCs	[20]
BrdU (bromodeoxyuridine)	Analog of thymidine	CiPSCs	[19, 22]
DZNep	SAH hydrolase inhibitor	CiPSCs	[18, 21, 22]
RG108	DNA methyltransferase inhibitor	CiNSCs, CiEPCs, CiNs	[27, 28, 30, 35, 42]
AMI-5	Protein methyltransferase inhibitor	CiPSCs	[16]
PF-6405761	BET inhibitor		
Histone deacetylation inhibitor			
NaB	HDAC inhibitor	CiNs, CiCMs	[47, 60]
VPA	HDAC inhibitor	CiPSCs, CiNSCs, CiNs, CiCMs, CiPCs, CiSMCs, CiCCs	[13, 17, 18, 21, 22, 27, 28, 40, 41, 43, 45, 47, 52, 61, 69, 71, 79]
I-BET-762	BET inhibitor		
Histone methylation modulator			
AS8351	Inhibitor of histone demethylase	CiCMs	[62]
Bix01294	Histone methyltransferase inhibitor	CiCMs, CiEPCs, CiNSCs	[27, 28, 35, 62]
BRD 7552	Increases acetylation of histone H3 and trimethylation of H3K4 and H3K9	CiBCs	[64]
EPZ5676	DOT1 inhibitor	CiPSCs	[22]
EPZ004777	DOT1L inhibitor	CiPSCs	[20, 22]
SGC0946	DOT1L inhibitor	CiPSCs	[20, 22]
CPI-0610	BET inhibitor		
GS-5829	BET inhibitor		
Histone acetylation modulator			
I-BET151	Inhibitor of epigenetic reader	CiNs	[39, 45, 78]
INCB057643	BET inhibitor		
Metabolic processes			
AM580	RAR agonist	CiPSCs	[20–22]
Bexarotene	RAR agonist	CiAs	[68]
Ch55	RAR agonist	CiPSCs	[21]
Retinoic acid	RAR ligand	CiNSCs, CiNs	[30, 46]
TTNPB	RAR ligand	CiPSCs, CiNs, CiSMCs, CiCCs	[18, 43, 47, 61, 69, 71]

Table 1: Continued.

Name of the compounds	Main mechanism of action	Application in reprogramming or transdifferentiation	References
Bay-K-8644	Ca2+ channel activator	CiEPCs	[35]
ISX9	Neurogenesis inducer	CiNs	[39, 42, 78]
LPA	A ligand activator for EDG-2, EDG-4, and EDG-7	CiBLPCs	[33]
Minocycline hydrochloride	Bind to the bacterial 30S ribosomal subunit and inhibiting protein synthesis	EPSs	[25]
OAC2	Activator of octamer-binding transcription factor 4 (Oct4)	CiCMs	[62]
Parnate (Tranylcypromine)	Monoamine oxidase inhibitor, LSD1 inhibitor	CiPSCs, CiNSCs, CiCMs, CiSMCs	[15, 17, 18, 21, 30, 59, 61, 69, 79]
P7C3	Targets NAMPT enzyme	CiNs	[42]
Rolipram	PDE4 inhibitor		[79]
SMER28	Autophagy modulator	CiNSCs	[30]
(S)-(+)-Dimethindene maleate	Antagonist of muscarinic M2 and histamine H1 receptors	EPSs	[25]
Vitamin C	A strong antioxidant	CiPSCs, CiNSCs, CiBCs	[22, 27, 28, 64]

CiAs: chemical-induced adipocytes; CiBCs: chemical-induced beta cells; CiBLPCs: chemical-induced bipotent liver progenitor cells; CiCCs: chemical-induced cartilaginous cells; CiCMs: chemical-induced cardiomyocytes; CiECs: chemical-induced epithelial colonies; CiEPCs: chemical-induced endodermal progenitor cells; CiLCs: chemical-induced Leydig cells; CiNs: chemical-induced neurons; CiNPCs: chemical-induced neuroprogenitor cells; CiNSCs: chemical-induced neural stem cells; CiPCs: chemical-induced photoreceptor cells; CiPSCs: chemical-induced pluripotent stem cells; CiSMCs: chemical-induced skeletal muscle cells; EPSs: extended pluripotent stem cells.

expression patterns, self-renewal capacity, excitability, and multipotency. Moreover, they can be differentiated into three types of nerve cell lineages *in vitro* and *in vivo*. Based on the same small molecule combination, Pei's team also recently obtained CiNSCs from mouse fibroblasts in hypoxia (5% O_2) [29]. In 2016, Ding's team revealed a new approach to induce mouse fibroblasts into induced neural stem cells (CiNSCs) using a combination of nine small molecules (M9), including LDN193189, A83-01, CHIR99021, bFGF, Hh-Ag 1.5, retinoic acid, RG108, Parnate, and SMER28 [30]. Specific transcription factors Elk1 and Gli2 were upregulated by M9 treatment, which, in turn, upregulated expression of the endogenous master neural gene Sox2 to complete induction.

2.4. Chemical-Induced Bipotent Liver Progenitor Cells (CiBLPCs). In 2017, Ochiya's lab converted mature rat and mouse hepatocytes into bipotent liver progenitor cells with three small molecules (Y-27632, A83-01, and CHIR99021) *in vitro* [31]. Although the reprogramming methods were efficient in rats and mice, they failed in humans. In 2018, Hui's team induced human hepatocytes into bipotent liver progenitor cells using four small molecules A83-01, Y-27632, CHIR99021, and Wnt3a [32]. Later, two research teams obtained human bipotent liver progenitor cells from hepatocytes with different methods such as Y-27632, CHIR99021, A83-01, S1P, and LPA [33] and A83-01, CHIR99021, EGF, and HGF [34], respectively.

2.5. Chemical-Induced Endodermal Progenitor Cells (CiEPCs). In 2016, Pei's team revealed that human gastric epithelial cells could be reprogrammed to endodermal progenitors with a small molecule cocktail (Bay-K-8644, Bix01294, RG108, and SB431542) used to treat tissue-specific mesenchymal feeders [35]. The resulting chemical-induced endodermal progenitors were able to be amplified in culture and differentiated to hepatocytes, pancreatic endocrine cells, and intestinal epithelial cells without generation of teratomas *in vivo*.

Although the mechanism of small molecule induction remains elusive, some clues can be obtained from the current literature. Taken together, to complete reprogramming, the epigenetic barrier has to be overcome and the starting cell identity should be gradually removed, while the target cell identity should be built up. In pure small molecule reprogramming, epigenetic modulators such as DNA methylation writer inhibitors (5-aza-dC, BrdU, DZNep, and RG108), histone methylation writer inhibitors (Bix01294, EPZ004777, EPZ5676, and SGC0946), and histone acetylation eraser inhibitors (VPA) were involved in this process (Table 1 and Figure 2). If fibroblasts are the starting cells, the TGF-β signaling pathway needed to be shut down by chemicals (SB431542, A83-01, and RepSox), which indicates this pathway is essential to keep the identity of fibroblasts. To create target cell identity, the Wnt signaling pathway needed be activated to reverse the induced cells back to an earlier developmental stage during reprogramming with an activator (CHIR99021). Due to cell death caused by oxidative stress and an epigenetically unstable state during the reprogramming process, metabolic regulators ((S)-(+)-Dimethindene maleate, Vitamin C, Parnate, Ch55, SMER28, AM580, and TTNPB)

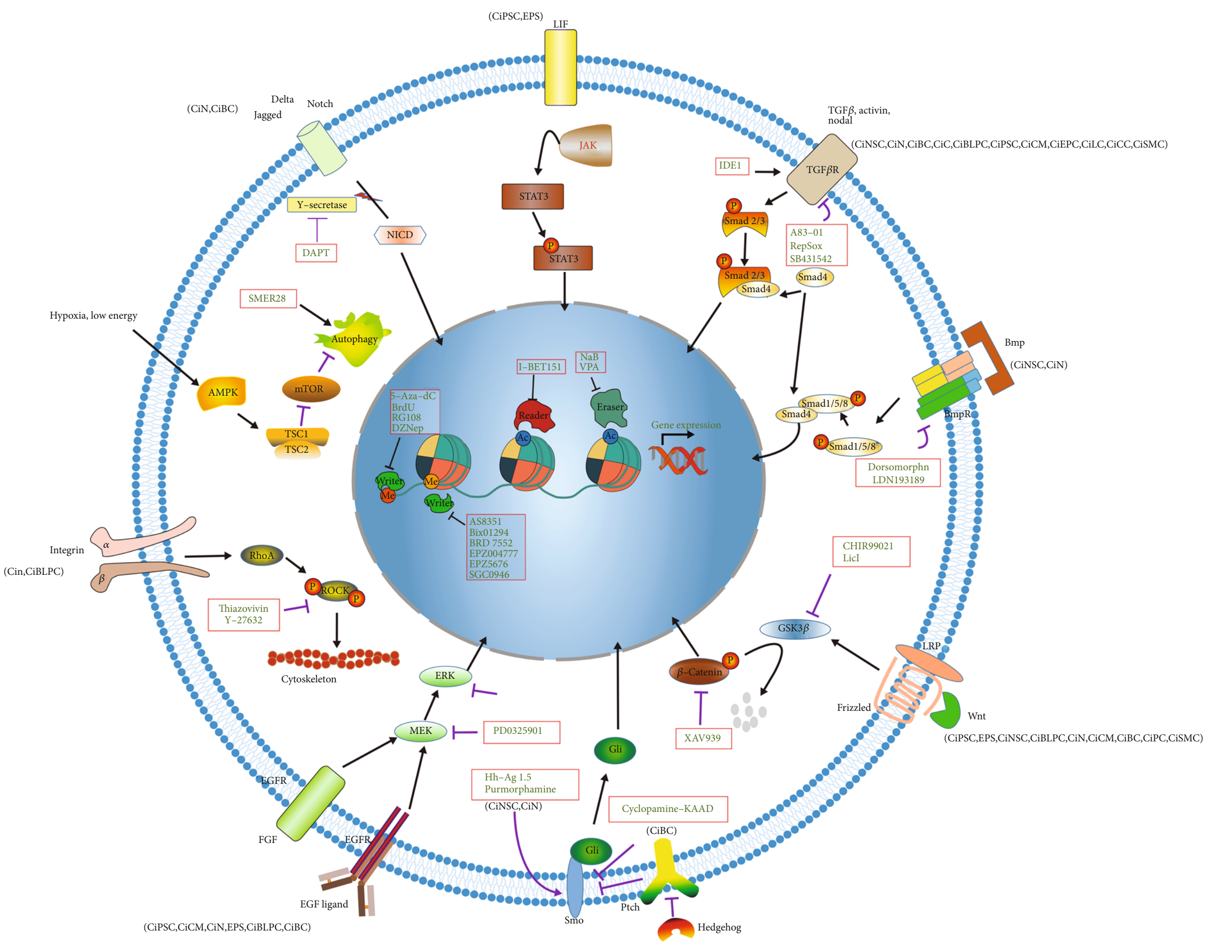

Figure 2: Mechanisms of small molecule induction. Small molecules targeting signaling pathways control target genes and impact cell fate decision. Small molecules also regulate epigenetic modulators modifying chromatin structure and change the epigenome and cell fate. Some other chemical compounds, such as chemicals regulating signaling activity in metabolism or cytoskeleton dynamics, also affect cell fate decision and are shown in Table 1. CiA: chemical-induced adipocyte; CiBC: chemical-induced beta cell; CiBLPC: chemical-induced bipotent liver progenitor cell; CiCC: chemical-induced cartilaginous cell; CiCM: chemical-induced cardiomyocyte; CiEC: chemical-induced epithelial colony; CiEPC: chemical-induced endodermal progenitor cell; CiLC: chemical-induced Leydig cell; CiN: chemical-induced neuron; CiNPC: chemical-induced neuroprogenitor cell; CiNSC: chemical-induced neural stem cell; CiPSC: chemical-induced pluripotent stem cell; CiSMC: chemical-induced skeletal muscle cell; EPS: extended pluripotent stem cell.

have been applied to enhance cell survival during the conversion.

3. Transdifferentiation *In Vitro*

Pluripotent stem cells (ESCs and iPSCs) should be converted into functional target cells before injection for cell therapy because they could generate teratomas *in vivo* [36]. The technology of transdifferentiation (i.e., the transition from one functional cell type to another without a requirement of a pluripotent state) represents a shortcut to achieve sufficiently functional cells for cell therapy [37]. At present, several types of functional cells including neurons, photoreceptor cells, cardiomyocytes, beta cells, adipocytes, skeletal muscle cells, cartilaginous cells, and Leydig cells have been successfully obtained using small molecule-mediated transdifferentiation methods *in vitro*.

3.1. Chemical-Induced Neurons (CiNs). As life expectancy is increasing, the number of people suffering from neurodegenerative disorders such as Alzheimer's and Parkinson's disease is on the rise [38]. Thus, it is urgent to obtain adequate quantities of patient-tailored neural cells for cell therapy and drug screening. Nowadays, scientists have made great progress in small molecule-based direct induction for neurons. In 2015, Deng's team used a combination of four small molecule compounds (Forskolin, ISX9, CHIR99021, and I-BET151) to transdifferentiate mouse fibroblasts into neurons [39]. The authors suggested that I-BET151 (a BET family bromodomain inhibitor) disrupted the fibroblast-specific program, while ISX9 (a neurogenesis inducer) activated neuronal-specific genes. At the same time, Pei's work revealed that human fibroblasts were able to transdifferentiate into neurons by a different chemical cocktail (VPA, CHIR99021, RepSox, Forskolin, SP600125, Gö6983, and Y-27632) [40]. It was also reported that human lung fibroblasts could be

converted into neurons using a similar small molecule combination, including VPA, CHIR99021, DMH1, RepSox, Forskolin, Y-27632, and SP600125 [41].

In 2019, Dai's research group found a rapid and efficient method to convert human fibroblasts into neurons with twelve small molecules (CHIR99021, LDN193189, Dorsomorphin, ISX9, RG108, PD0325901, Purmorphamine, DAPT, Forskolin, ISX9, Y-27632, and P7C3) [42].

In 2015, Chen's team identified a combination of nine small molecules (LDN193189, SB431542, TTNPB, Thiazovivin, CHIR99021, VPA, DAPT, Smoothened agonist, and Purmorphamine) for reprogramming human astrocytes into neurons [43]. These induced neurons could survive for more than 5 months in culture and generated functional synaptic networks *in vitro*, and they were able to survive for over 1 month in mouse brains and merge with local circuits. Later, they also implied that six signaling pathways including SHH, Notch, Wnt, BMP, TGF-β, and JA/STAT played a pivotal role during the transdifferentiation [44]. Similar work was reported by Pei's lab with a different small molecule combination (VPA, Chir99021, RepSox, Forskolin, I-Bet151, and ISX-9) two years later [45].

Furthermore, subtype neurons also have been obtained. In 2018, human and mouse motor neurons were created by a chemical combination containing Kenpaullone, Forskolin, Y-27632, Purmorphamine, and retinoic acid [46]. One year later, Li's team reported that a chemical cocktail (CHIR99021, A83-01, Y-27632, VPA, TTNPB, Forskolin, and NaB) induced human urine-derived cells into neurons, while the majority of induced cells were glutamatergic neurons [47].

3.2. Chemical-Induced Photoreceptor Cells (CiPCs). Vision loss resulting from retinal neuron damage causes retinopathies, including age-related macular degeneration, diabetic retinopathy, and retinitis pigmentosa [48, 49]. As a favorable method, stem cell therapy could substitute for the loss of retinal neurons [50, 51]. Recently, Chavala's team reported five small molecules (VPA, CHIR99021, RepSox, Forskolin, and IWR1) were able to transdifferentiate fibroblasts into photoreceptor-like cells [52]. The authors also confirmed that CiPCs could mend pupil reflex and vision when transplanted into the subretinal space of mice with retinal degeneration. Additionally, they implied that the AXIN2–NF-κB–ASCL1 pathway enhanced retinal lineage commitment and mitochondria were the signaling hub during transdifferentiation.

3.3. Chemical-Induced Cardiomyocytes (CiCMs). It is widely known that the regeneration of the adult mammalian heart after injury is limited [53]. Therefore, heart failure resulting from cardiomyocyte loss is a major cause of mortality around the world [54]. As the most common cell type in the heart, cardiac fibroblasts are considered promising for cardiac reprogramming.

Small molecules are also able to replace transcription factors and provide an alternative means of cardiac reprogramming. It was reported that TGF-β inhibitors (SB431542 or A83-01) could improve the efficiency of cardiomyocyte induction [55–57]. The small molecule Y-27632 also enhanced cardiac reprogramming [58]. Furthermore, Ding's group reported that a small molecule combination (CHIR99021, SB431542, Parnate, and Forskolin) was sufficient to complete the conversion of cardiomyocytes from mouse fibroblasts with Oct4 alone [59]. It was also reported that small molecules (NaB, RA, and ICG-001) were able to improve rat and human cardiac cell generation induced by transcription factors (Gata4, Mef2C, and Tbx5) [60]. In 2015, Xie's team transdifferentiated mouse fibroblasts into cardiomyocytes by passing a cardiac progenitor stage with six small molecules (CHIR99021, RepSox, Forskolin, VPA, Parnate, and TTNPB), while the induced cardiomyocytes were cultured in cardiomyocyte maintenance medium containing CHIR99021, PD0325901, LIF, and insulin [61]. One year later, Ding's lab reported that human functional cardiomyocytes were induced by a combination of nine small molecules (CHIR99021, A83-01, BIX01294, AS8351, SC1, Y-27632, OAC2, SU16F, and JNJ10198409) [62]. Furthermore, the induced human fibroblasts were able to be efficiently converted into cardiomyocyte-like cells in infarcted mouse hearts.

3.4. Chemical-Induced Beta Cells (CiBCs). Diabetes mellitus, which results from pancreatic β cell damage, is an international health epidemic and influences more than 300 million people in the world [63]. Therefore, producing plenty of functional pancreatic β cells for studying diabetes and treating patients is an urgent task. In 2015, we successfully induced human urine cells to insulin-secreting beta cells by passing through three stages with pure small molecules [64]. Firstly, urine cells were induced into an endodermal lineage using a chemical cocktail (IDE 1, LiCl, and VC) for 6 days. The induced cells were then differentiated into pancreatic precursors in two steps. The first step induction medium contained cyclopamine-KAAD, Indolactam V, RA, VC, A83-01, and BRD 7552 for 1 day, while the secondary step induction used chemicals, including cyclopamine-KAAD, Indolactam V, VC, A83-01, and BRD 7552, for 6 days. Insulin-secreting beta cells were obtained in the tertiary induction medium (SB203580, VC, and DAPT) for 9 days. Furthermore, the induced beta cells could reduce glucose levels and enhance survival rates in diabetic mice.

3.5. Chemical-Induced Adipocytes (CiAs). As a promising therapy for obesity and metabolic diseases, brown adipose tissue (BAT) has been intensively studied [65, 66]. The energy balance in the body is balanced with white adipose tissue collecting energy, while BAT expends energy and produces heat [67]. In 2017, Ding's research group converted mouse myoblasts into brown adipocyte-like cells with a retinoid X receptor (RXR) agonist, bexarotene. They implied that Rxrα/γ activation is required for the induction of BAT [68].

3.6. Chemical-Induced Skeletal Muscle Cells (CiSMCs). Muscle-related maladies including muscle wasting and muscular dystrophy have yet-to-be adequately treated using traditional medicine. The cell therapy technique brings a promising

approach to resolve this issue. Recently, it was reported that mouse fibroblasts could be converted to skeletal muscle cells by a combination of six small molecules (VPA, Chir99021, RepSox, Forskolin, Parnate, and TTNPB) [69]. The authors implied that three signaling pathways Wnt, TGF-β, and cAMP were crucial for the transdifferentiation.

3.7. Chemical-Induced Cartilaginous Cells (CiCCs). Cartilage defects cause joint pain and diminish quality of life. Recently, autologous chondrocyte therapy was proposed as a means of cartilage healing [70]. Ouyang's team revealed that mouse embryonic fibroblasts could be converted to functional cartilaginous cells by a chemical cocktail (VPA, CHIR98014, RepSox, TTNPB, and Celecoxib) [71]. These CiCCs could enhance defective healing and restore 63.4% of mechanical function damage *in vivo*.

3.8. Chemical-Induced Leydig Cells (CiLCs). Affecting about 30% of men aged 40–79 years, late-onset hypogonadism (LOH) with a serum testosterone deficiency could result in sexual dysfunction, central adiposity, mood disturbance, osteoporosis, amyotrophy, and other abnormalities [72–74]. Leydig cells produce testosterone, so Leydig cell transplantation could be an ideal tool to heal LOH. Recently, Huang's team reported that functional mouse Leydig cells could be transdifferentiated from fibroblasts using a small molecule combination (Forskolin, 20a-hydroxycholesterol, luteinizing hormone, and SB431542) [75]. Moreover, these CiLCs could survive in the testes and produce testosterone in a circadian rhythm.

As for the mechanism of small molecule transdifferentiation, collectively, in contrast to reprogramming, transdifferentiation is an easier process because it does not need more energy to pull the starting cells to a less differentiated level for cell conversion. Compared to reprogramming, epigenetic modulators, the histone methylation writer inhibitor was replaced with the histone acylation reader inhibiter (I-Bet151) in transdifferentiation, which implies less epigenetic barrier is required to be overcome during transdifferentiation. Furthermore, more metabolic modulators are involved in the confirmation of the new cell identity, such as OAC2 for cardiomyocytes, ISX9 for neurons, and bexarotene for brown adipose tissue.

4. Transdifferentiation *In Vivo*

Although functional cells could be obtained by differentiation from pluripotent stem cells or transdifferentiation from somatic cells, induction efficiency, ultimate maturation of cells, and survival rates after cell transplantation are still the three biggest obstacles to cell therapy [76]. Due to safety and technical difficulties of cell transplantation therapy, *in vivo* reprogramming may become the next generation of regenerative medicine with therapeutic potential [77].

4.1. Neurons. In 2018, Deng's team released their data about *in vivo* transdifferentiation of neurons from mouse astrocytes with a cocktail combination consist of dbcAMP, Forskolin, ISX9, CHIR99021, I-BET151, and Y-27632 [78]. The combination of chemicals was injected into mouse brains at a stable rate for two weeks with an osmotic minipump. The induced cells not only formed endogenous neurons with similar neuron-specific marker expression and electrophysiological properties but also merged with local circuits *in vivo*.

4.2. Cardiomyocytes. In 2018, Xie's team reported that a small molecule combination of CRFVPTM (CHIR99021, RepSox, Forskolin, VPA, Parnate, TTNPB, and Rolipram) mediated transdifferentiation of cardiac fibroblasts into cardiomyocytes in normal adult mice with a low efficiency of 1% [79]. CRFTM were administrated orally and VP were intraperitoneally injected once for 6 weeks. The transdifferentiation only happened in the heart, which suggests the local niche also plays a critical role in small molecule-mediated cardiac induction. Furthermore, the induced cardiomyocytes dramatically repressed the scar formation and promoted cardiac function in mice with a myocardial infarction.

To explore the mechanism of small molecule transdifferentiation *in vivo* and compare transdifferentiation *in vitro* and *in vivo*, additional small molecules were applied to activate the cAMP signaling pathway (dbcAMP for neurons and Rolipram for cardiomyocytes), which suggested targets downstream of the PKA signaling pathway are important to overcome the disturbance from *in vivo* environment during transdifferentiation.

In summary, although the mechanism of full small molecule induction is unknown, some implications can be observed. By examining signaling pathways, it is apparent that certain pathways are preferred for transdifferentiation (Figure 2), such as inhibiting BMP for ectodermal induction, activation of LIF-STAT3 for creating pluripotent stem cells, and inhibition of Notch, SHH, and Rho for the induction of ectodermal or endodermal lineages. On the other hand, some signaling pathways are preferred for induction (e.g., activation of Wnt and inhibition of TGF-β and MAPK/ERK). As for the induction process, it seems that there is an intermediate state by which various target cells could be achieved in certain culture conditions.

5. Perspective

Despite the exciting progress that has been achieved in the field of pure small molecule-induced cells, there are still some key problems such as apoptosis due to oxidative stress, death from an epigenetically unstable state, genomic integrity, genotoxicity, scaling production for large animals' safety and efficacy trials, and producing a safe delivery system as well as induction methods [77]. Moreover, the majority of pure small molecule cocktails for human cells still remain to be determined.

Without cell transplantation, direct *in vivo* reprogramming for local *in situ* conversion of cells is emerging as a new way to produce cells for regenerative medicine. Although *in situ* chemical induction will be a focus for the next decade, how these small molecules could be precisely delivered to the desired tissues or organs to produce fully integrated functional cells is a primary challenge. Biomaterials that can deliver small molecules to targeted organs, for example, nanoparticles containing specific signals for

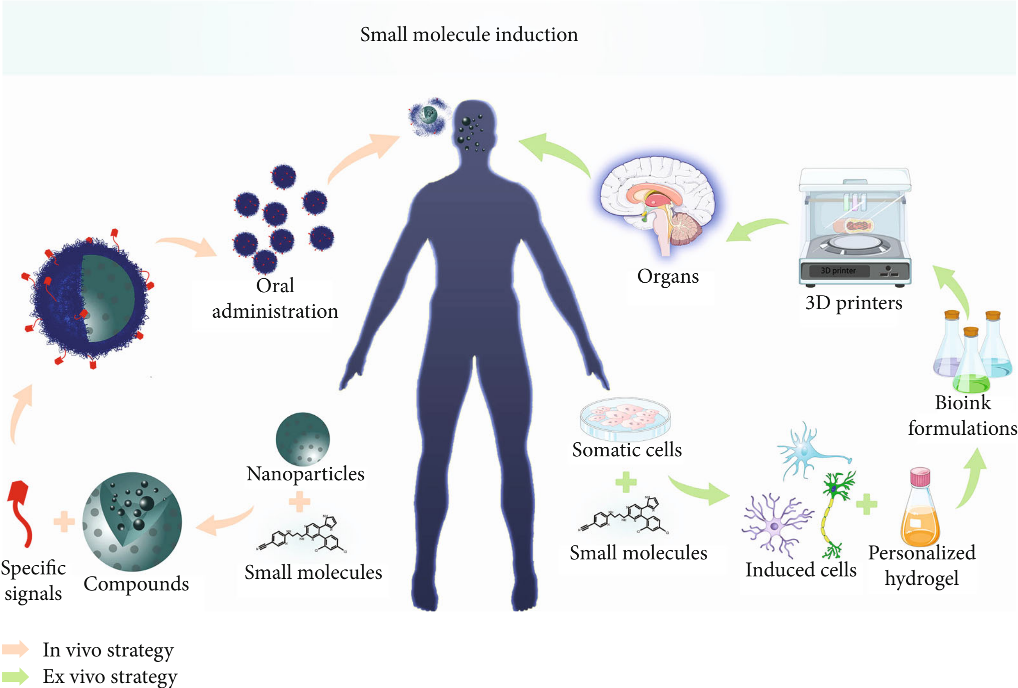

Figure 3: Future trends for small molecule-mediated personalized cell therapy. On the one side, somatic cells will be transdifferentiated to functional cells *in vitro* and then organized to organs by a 3D printer, and the personalized organs will be transplanted into patients finally. On the other side, nanoparticles carrying small molecule cocktails target specific cells for in situ induction *in vivo*.

recognizing specific cell types, can assist *in vivo* reprogramming studies and future clinical applications (Figure 3). On the other hand, small molecule-induced cells could be constructed for organs such as the heart, liver, or brains using 3D printers *in vitro* (Figure 3). In addition, recent scientific tools such as single-cell sequencing [80] and CRISPR-based genome-wide screening [81] will help exploring new chemical cocktails and illustrate the induction mechanisms.

Conflicts of Interest

The authors declare no competing interests.

Authors' Contributions

Z.-D.Y. and W-N.Z. prepared the manuscript. Z.-D.Y. and K-Z.L. drew graphics. Y.-C.H. wrote the manuscript. Z.-P.H. reviewed and edited the manuscript. Z.-P.H drafted the final version of the manuscript. All authors read and approved the final manuscript. Zhao-Di Yuan and Wei-Ning Zhu contributed equally.

Acknowledgments

We thank the members of the Huang Laboratory for the advice and support. We also thank Dr. Da-Zhi Wang and Dr. Douglas Cowan for editing the manuscript. This work is supported by grants from the National Natural Science Foundation of China (81873463), the Guangdong Basic and Applied Basic Research Foundation (2019B151502003), the Guangdong Science and Technology Department (2018A050506026), and the Fundamental Research Funds for the Central Universities (20ykzd06) to Z.-P.H.

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Hindawi
Stem Cells International
Volume 2020, Article ID 8882857, 10 pages
https://doi.org/10.1155/2020/8882857

Research Article

CHD7 Regulates Osteogenic Differentiation of Human Dental Follicle Cells via PTH1R Signaling

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Received 22 July 2020; Revised 16 August 2020; Accepted 28 August 2020; Published 21 September 2020

Academic Editor: Yang Li

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Chromodomain helicase DNA-binding protein 7 (CHD7) is an ATP-dependent chromatin remodeling enzyme, functioning as chromatin reader to conduct epigenetic modification. Its effect on osteogenic differentiation of human dental follicle cells (hDFCs) remains unclear. Here, we show the CHD7 expression increases with osteogenic differentiation. The knockdown of *CHD7* impairs the osteogenic ability of hDFCs, characterized by reduced alkaline phosphatase activity and mineralization, and the decreased expression of osteogenesis-related genes. Conversely, the *CHD7* overexpression enhances the osteogenic differentiation of hDFCs. Mechanically, RNA-seq analyses revealed the downregulated enrichment of PTH (parathyroid hormone)/PTH1R (parathyroid hormone receptor-1) signaling pathway after *CHD7* knockdown. We found the expression of PTH1R positively correlates with CHD7. Importantly, the overexpression of *PTH1R* in *CHD7*-knockdown hDFCs partially rescued the impaired osteogenic differentiation. Our research demonstrates that CHD7 regulates the osteogenic differentiation of hDFCs by regulating the transcription of PTH1R.

1. Introduction

Originated from ectomesenchymal cranial neural crest cells, dental follicle is a loose connective tissue surrounding the cervical margin of unerupted tooth [1]. The dental follicle could give rise to alveolar bone, cementum, periodontal ligament, gingiva, and other periodontal supporting tissues during the process of tooth germ development [2].

hDFCs are abundant in adolescent patients and easy to obtain [3]. The application of hDFCs in clinic also faces little ethical issue [2]. More importantly, hDFCs are capable of the multilineage differentiation into osteoblasts, fibroblasts, adipocytes, and neurons under different induction cues [4]. Therefore, hDFCs are ideal source for periodontal tissue engineering and regenerative medicine [2, 5].

According to the previous researches, a series of transcription factors or signaling pathways participate in the osteogenetic differentiation of hDFCs, e.g., BMP2, DLX3, NOTCH, Hedgehog, and WNT signaling pathway [6]. As for the gene expression, histone modifications and chromatin remodeling are critical regulatory factors [7].

The chromodomain helicase DNA-binding (CHD) protein superfamily is a typical kind of ATP-dependent chromatin remodeling enzymes of eukaryotic organisms [8]. In the chromatin reading state, CHD protein could disrupt the tissue-DNA interaction by translocating the nucleosomes along the DNA strand [9]. With specific function for active or suppressive histone markers, CHD family are critical for the normal gene expression and maintenance of chromatin dynamic structures [10]. Therefore, the CHD superfamily is essential to stem cell maintenance and proliferation, as well as the regulation of cell fate and differentiation [11].

According to the existed researches, one of the major functions of chromodomain is binding to the methylated histone residues, and correspondingly, the CHD superfamily contains the special methy1-binding cages, which could promote interaction with the histone H3 methylated at lysine 4 (H3K4me) [12].

CHD7 is one of the most studied members of the CHD family because of its extensive and important role in organ development [10]. As a kind of ATP-dependent chromatin remodeling enzymes, CHD7 could regulate the position of

nucleosomes and change the accessibility of DNA [13]. Through ChIP, Schnetz et al. found that the recruitment of CHD7 was closely associated with histone modifications, especially H3K4me [14]. Reported previously, CHD7 colocates with H3K4me1 in the enhancer region and with H3K4me3 in the transcription start site [14].

The mutation of CHD7 causes CHARGE syndrome, a developmental disorder that involves multiple organ system defects, including coloboma of the eye, heart defects, atresia of the choanae, retarded growth, genital anomalies, ear malformations, and deafness [15]. The acronym for the six main symptom was defined as CHARGE syndrome by Pagon et al. in 1981 [16].

Previous clinical studies have researched patients with CHARGE syndrome, and most phenotype descriptions were about neural development and neurological disease [17]. Phenotype on bone development was also reported and reviewed in several researches [16]. According to the physical and computed tomography examination, square-shaped face, semicircular canal anomaly, temporal bone abnormality and reduction in bone mineralization can be observed in patients with CHARGE syndrome [18].

We have previously shown that CHD7 plays an important role in osteogenic differentiation of human bone mesenchymal stem cells (hBMSCs) [19]. CHD7 promotes the osteogenic differentiation of hBMSCs by binding to SP7 enhancer and interacting with SMAD1 [19].

In our present work, we focused on the effect of CHD7 on osteogentic differentiation of hDFCs and the downstream signal mechanism. Our results demonstrate that CHD7 promotes osteogenesis of hDFCs via PTH/PTH1R signaling pathway.

2. Methods and Materials

2.1. Human Dental Follicle Immunohistochemistry (IHC) Staining. Human dental follicle was obtained from the unerupted third molar with undeveloped root. Patients aged 12-16 years undergoing the third molar extraction in West China Hospital of Stomatology, Sichuan University, would meet the inclusion criteria. Patients with history of systemic disease, or undergoing maxillofacial surgery, whole body or partial radiotherapy, chemotherapy, periodontitis, oral mucosal disease, or smoking were excluded [20]. All the procedures are approved by the Institutional Review Board and the informed consent of patients.

The human dental follicle was fixed in 4% polyoxymethuylene for 24 hours before sectioning (5 μm). Slides were incubated in sodium citrate antigen retrieval solution at 100°C for 10 min and then incubated with rabbit anti-CHD7 antibody (Sigma, 1: 200) [21].

2.2. Human Dental Follicle Cell Culture. After extraction, the dental follicle was instantly immersed into the phosphate buffer saline (PBS, Gibco) with 1×penicillin-streptomycin (Liquid, Gibco), i.e., PBS with 100 units/mL penicillin and 100 μg/mL streptomycin. The dental follicle was cut into size of 1 mm^3 pieces and digested with PBS with type I collagenase (3 mg/ml, Gibco) and dispase (3 mg/ml, Gibco) for 1 hour in 37°C water bath with agitation [20].

The digested tissue suspension was cultured in 21 cm^2 petri dish in alpha minimum Eagle's medium (α-MEM, HyClone) with 20% fetal bovine serum (FBS, Gibco) and 1×penicillin-streptomycin. Incubated at 37°C with 5% CO_2, the culture medium was changed every 2 days. When cell confluence rate reached 80%, hDFCs were passaged and subcultured in α-MEM with 10% FBS and 1×penicillin-streptomycin. hDFCs at passage 3 were applied for the subsequent research [20].

For the osteogenic induction, hDFCs were cultured with osteogenic medium supplemented with 50 μM ascorbic acid (Sigma), 10 nM dexamethasone (Sigma), and 10 mM β-glycerophosphate (Sigma) [22].

2.3. CHD7 Knockdown. Cells with ~50% confluence were suitable for the siRNA-mediated knockdown. We obtained targeting control and CHD7 siRNA from Shanghai Sangon Biotech Co. (China). According to the manufacturer's protocol, Lipofectamine® RNAiMAX (Invitrogen) and siRNA were, respectively, added into Opti-MEM (Gibco). The transfection system was mixed and incubated at room temperature in dark for 30 minutes. The medium for hDFCs was changed into α-MEM with 10% FBS without antibiotics, then the transfection system with CHD7-siRNA or control-siRNA was added into the corresponding hDFC groups. After 24-hour incubation, the knockdown efficiency was detected via quantitative reverse transcription polymerase chain reaction (qRT-PCR). The sequence of CHD7-siRNA is CCATGA AAGCAATGAGTAA, and of control-siRNA is TACAAC AGCCACAACGTCTAT [19].

2.4. Lentivirus and Adenovirus-Mediated Overexpression. For the lentivirus-mediated overexpression, lentivirus vectors Ubi-MSC-3FLAG-SV40-EGFP-IRES-puromycin expressing CHD7 or blank were purchased from Shanghai Genechem Co. (China). HitransG/A (Shanghai Genechem) was introduced to enhance the infection efficiency [20].

For the adenovirus-mediated overexpression, adenovirus particles pAV [Exp]-CMV > EGFP expressing PTH1R or blank were purchased from Cyagen (US Inc.) [21].

When reached 20-30% confluence, hDFCs were infected with lentivirus vectors or adenovirus particles at MOI = 20. After 72-hour incubation, 70-80% of the hDFC expressed green fluorescence. The overexpression efficiency was detected via qRT-PCR and Western blot after 2.5 μg/mL puromycin (Sigma) selection [20].

2.5. RNA Extraction and qRT-PCR. Total RNA was isolated with TRIzol Reagent (Invitrogen) 3 days and 7 days after the osteogenic induction [20]. RNA was reverse transcribed via a PrimeScript RT reagent Kit (Takara) [23].

Quantitative PCR was performed using SYBR Premix Ex Taq (Takara) and LightCycler 96 (Roche). The housekeeping gene *GAPDH* was used as the baseline to analyze the bone formation-related gene quantitatively [23].

2.6. Total Protein Extraction and Western Blot. The total protein of hDFCs was collected with a protein extraction kit (PE001, Sab-biotech) 7 days after the osteogenic induction

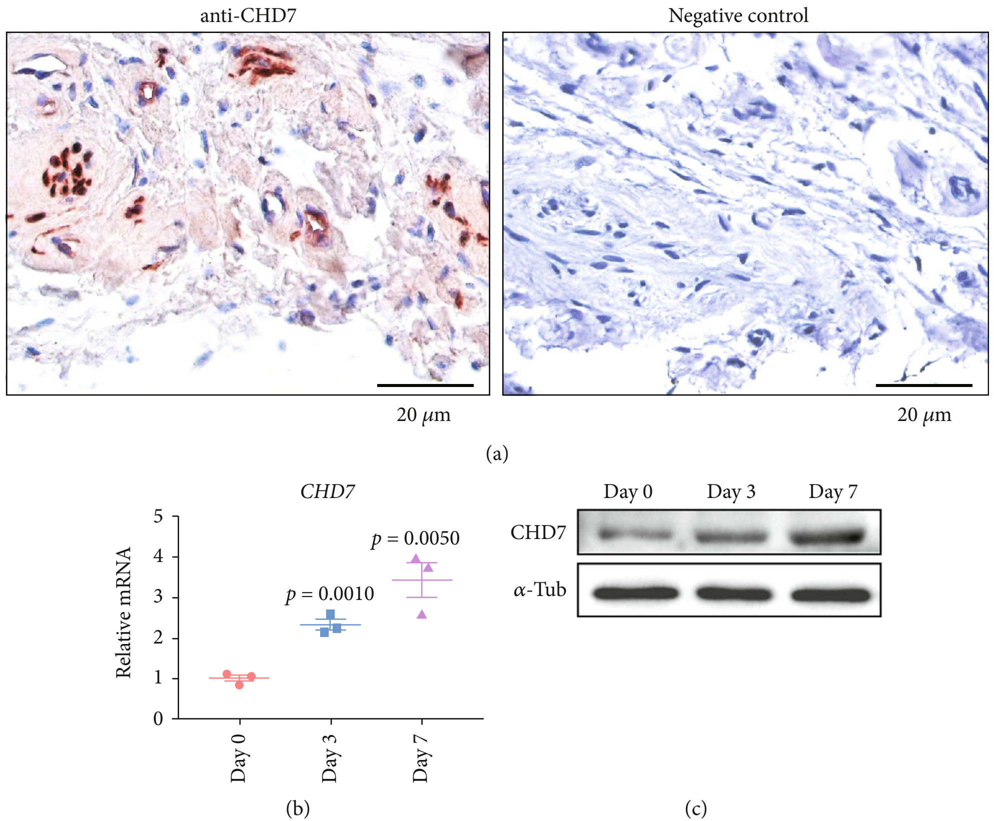

FIGURE 1: The high expression of CHD7 in hDFCs after osteoinduction. (a) Immunohistochemical staining images unraveled that CHD7 is present in human dental follicle. Scale bar, 20 μm. (b), (c) qRT-PCR and Western blot unraveled that the expression of CHD7 increased after 3-day and 7-day osteoinduction.

[4]. The total protein was heated with SDS-PAGE Sample Loading Buffer (Beyotime, China) at 100°C for 5 minutes [24].

After gel electrophoresis separation, protein was transfered to the PVDF membrane (Millipore) via BIO-RAD Powerpac HC. After antigen blocking, the membranes were incubated in rabbit anti-α-tubulin antibody (Sigma, 1: 2500), rabbit anti-CHD7 antibody (Sigma, 1: 1000), and mouse anti-PTH1R antibody (Sigma, 1: 1000) at 4°C overnight. After 1-hour incubation with HRP-labeled Goat Anti-Rabbit IgG or Goat Anti-Mouse IgG (Beyotime, China) at room temperature, the membranes were exposed via ChemiDoc XRS+ (BIO-RAD), to detect the selected protein expression level [20].

2.7. Alkaline Phosphatase (ALP) Staining and Quantitative Analysis of ALP Activity. hDFCs were fixed with 4% paraformaldehyde and then stained with a BCIP/NBT Alkaline Phosphatase Color Development Kit (Beyotime, China) after the 7-day osteogenic induction. After 15-minute light-free incubation at room temperature, the reaction was terminated, and images were obtained with Epson Perfection V370 Photo Scanner [20].

BCA Protein Assay Kit (Beyotime, China) and Alkaline Phosphatase Assay Kit (Beyotime, China) were used for quantitative analysis of ALP activity. The curve of BCA was obtained from the absorbance of BCA protein concentration gradient. By reaction with 0.5 mM p-nitrophenyl phosphate, the corresponding ALP activity was calculated from the standard curve of ALP absorbance [20].

2.8. Alizarin Red S (ARS) Staining and Quantitative Analysis of Mineralization. After the 3-week osteogenic induction in 24-well plates, hDFCs were fixed with 4% paraformaldehyde and then stained with Alizarin Red S solution (Solarbio, China) at room temperature [25].

To quantify the calcium concentration, the calcium nodules were detained by cetylpyridinium chloride for 15 minutes. The quantitative result was measured by absorbance at 562 nm with Multiskan Sky Microplate Spectrophotometer (Thermo Fisher Scientific), in contrast with the standard calcium absorbance curve [21].

2.9. RNA-Sequence. RNA samples of hDFCs, 3 samples in siCTRL group and 3 samples in siCHD7 group, were prepared according to the manufacturer's protocol of a NEBNext Ultra RNA Library Prep Kit for Illumina (USA) [21]. RNA samples were subjected to Illumina HiSeq 2500 (USA). FastQC (v0.11.5) and FASTX toolkit (0.0.13) were used for quality control [21]. On the basis, GO enrichment, KEGG enrichment, heat map, and GSEA analysis were conducted to explore the downstream pathway [21].

2.10. Chromatin Immunoprecipitation (ChIP) Assay. According to the manufacturer's protocol of a ChIP assay kit (Beyotime, China), 2×106 cells were used in each ChIP reaction [19]. By applying 37% formaldehyde solution, protein and DNA of each sample were crosslinked. After

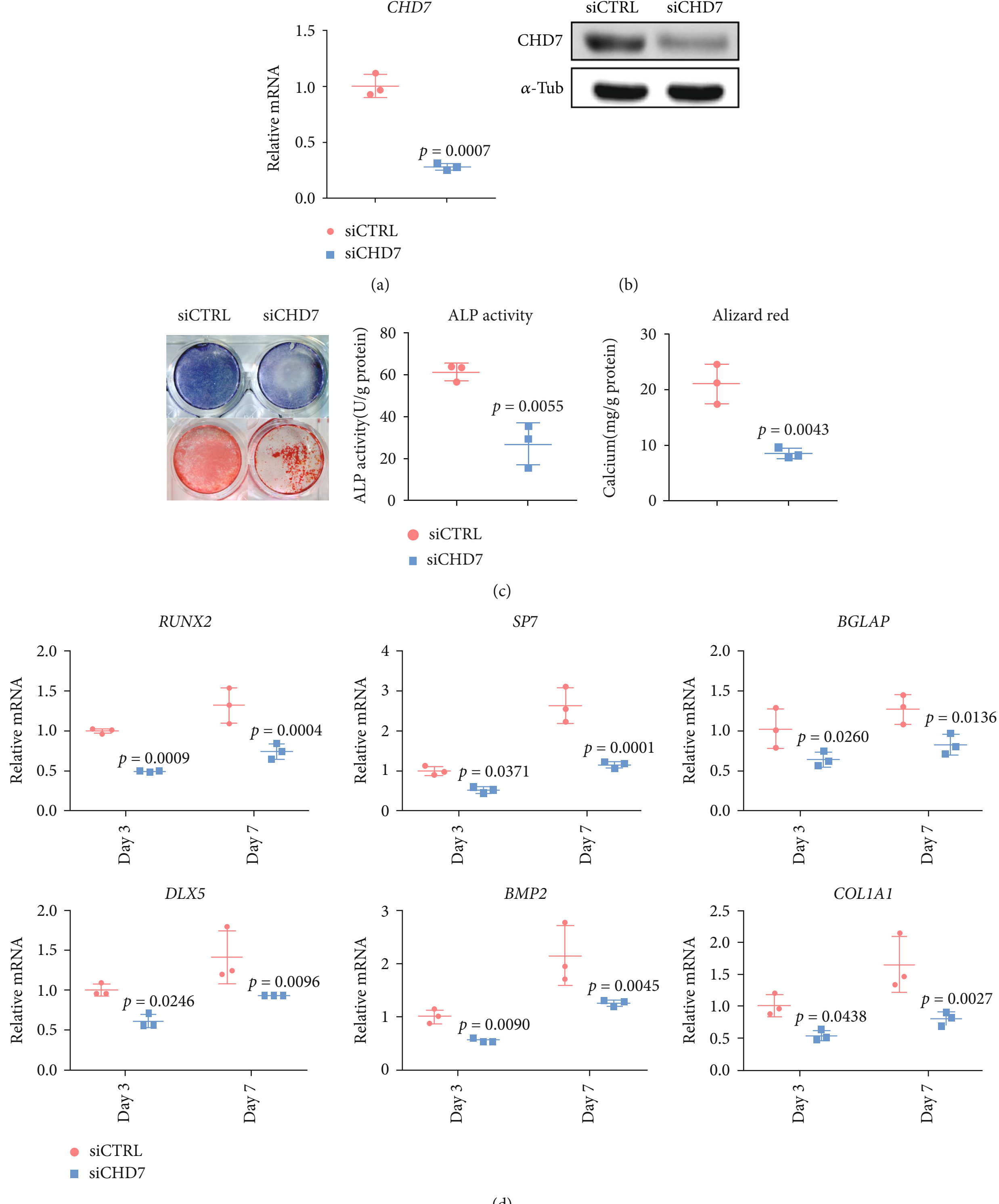

Figure 2: Depletion of *CHD7* decreases osteogenic differentiation of hDFCs. (a), (b) qRT-PCR and Western blot verified the knockdown efficiency of siCHD7. (c) Representative images and quantitative analyses of ALP and ARS staining of hDFCs in the siCHD7 and siCTRL group. (d) qRT-PCR analyses of the expression of *RUNX2*, *SP7*, *BGLAP*, *DLX5*, *BMP2*, and *COL1A1* under osteogenic condition.

cell harvesting, SDS lysis buffer with protease inhibitor cocktail (Roche) was added. After ultrasonication, centrifugation, and precipitation with beads, the precipitated DNA samples were quantified with specific primers using real-time PCR [21].

2.11. Statistical Methods. All data were calculated as the mean ± standard deviation (SD). Statistical difference was calculated via Student's t test for independent sample test or one-way ANOVA for multiple comparison. P value less than 0.05 was considered statistically significant.

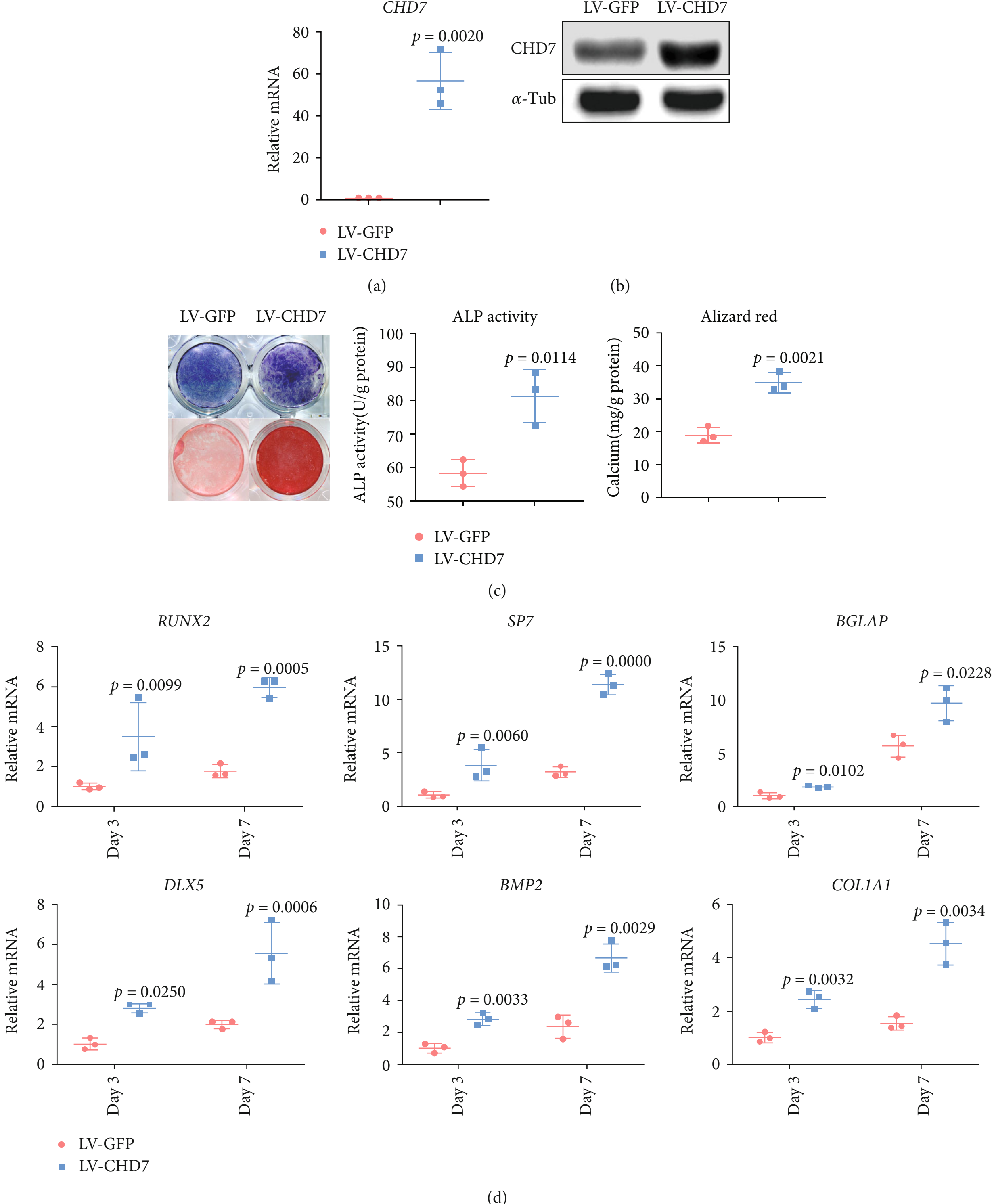

Figure 3: The overexpression of CHD7 promotes osteogenic differentiation of hDFCs. (a), (b) qRT-PCR and Western blot verified the overexpression efficiency of *CHD7*. (c) Representative images and quantitative analyses of ALP and ARS staining of hDFCs in the LV-CHD7 and LV-GFP group. (d) qRT-PCR analyses of the expression of *RUNX2*, *SP7*, *BGLAP*, *DLX5*, *BMP2*, and *COL1A1* under osteogenic condition.

3. Results

3.1. The High Expression of CHD7 in hDFCs after Osteoinduction. We first detected the expression of CHD7 in human dental follicle by IHC staining. Located in nucleus, the high expression of CHD7 can be observed from the slices (Figure 1(a)), which implied that CHD7 might be crucial to the physiological function in dental follicle.

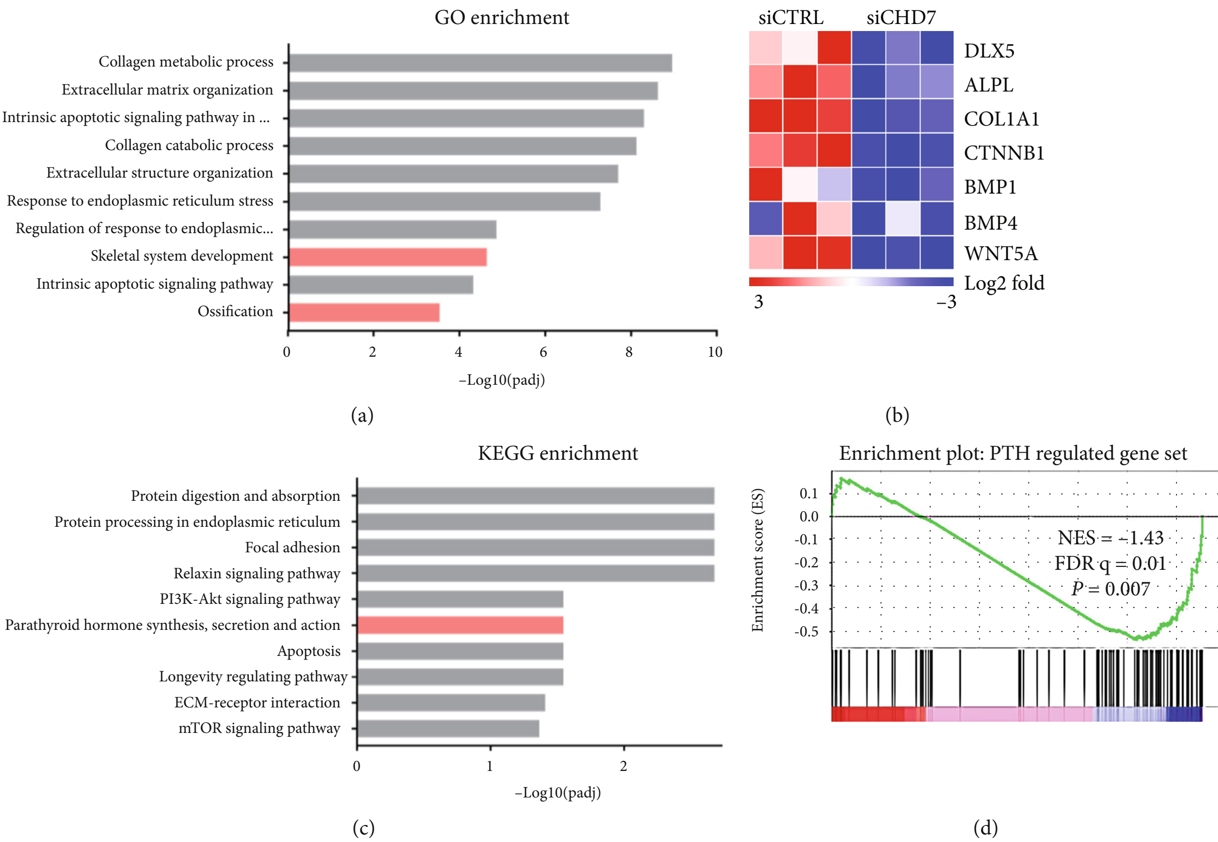

Figure 4: RNA-seq revealed the downregulated enrichment of the PTH-related pathway after CHD7 depletion. (a) GO enrichment unraveled that skeletal system development and ossification were suppressed after *CHD7* depletion. (b) Heatmap of representative osteogenesis associated genes. (c) KEGG enrichment unraveled that the PTH-related pathway was significantly suppressed after *CHD7* depletion. (d) GSEA showed decreased enrichment of PTH-regulated genes in CHD7-deficient hDFCs.

We next conducted the osteogenic induction on hDFCs and analyzed the CHD7 expression changes. As indicated by qRT-PCR, the relative mRNA level of *CHD7* increased after 3-day and 7-day induction (Figure 1(b)). Western blot demonstrated the corresponding trend (Figure 1(c)). These results indicated that CHD7 might be essential to the osteogenic differentiation of hDFCs.

3.2. The Knockdown of CHD7 Decreases Osteogenesis of hDFCs. To elucidate the role of CHD7 in the osteogenic differentiation of hDFCs, we used siRNA to knockdown *CHD7* in hDFCs. qRT-PCR and WB results verified the efficient knockdown of *CHD7* (Figures 2(a) and 2(b)). 7 days after osteoinduction, the ALP staining demonstrated significant reduction in the *CHD7* knockdown group (Figure 2(c)). The quantitative analysis of ALP activity demonstrated the consistent result (Figure 2(c)). The ARS staining and quantitative analysis of the calcium concentration also confirmed the downtrend of osteogenesis after *CHD7* depletion (Figure 2(c)). Moreover, the expression of osteogenesis-related genes *RUNX2*, *SP7*, *BGLAP*, *DLX5*, *BMP2*, and *COL1A1* was significantly downregulated (Figure 2(d)). The results indicated that the knockdown of *CHD7* reduced the osteogenic differentiation of hDFCs.

3.3. The Overexpression of CHD7 Increases Osteogenesis of hDFCs. To further elucidate the role of CHD7 in osteogenesis of hDFCs, we infected lentivirus overexpressing *CHD7*, or *GFP* as control, into hDFCs (Figures 3(a) and 3(b)). The overexpression of *CHD7* significantly promoted the osteogenesis of hDFCs, characterized by increased ALP activity, mineralization (Figure 3(c)), and expression of osteogenesis-related genes (Figure 3(d)).

3.4. The PTH-Related Pathway Is Downregulated after CHD7 Depletion. To elucidate the regulatory mechanism of CHD7, we conducted the RNA-seq analysis. GO enrichment showed that the skeletal system development and ossification were suppressed after *CHD7* depletion (Figure 4(a)). Heatmap was generated with recognized osteogenesis-related genes, and most of them were downregulated after CHD7 depletion (Figure 4(b)).

KEGG enrichment identified that the PTH-related pathway was significantly downregulated (Figure 4(c)). According to the previous researches, the PTH/PTH1R signaling pathway is crucial in bone formation and ossification, which is aligned with our phenotype. In order to confirm the downregulation of PTH1R, we conducted the gene set enrichment analysis (GSEA) with the published gene list of the PTH-

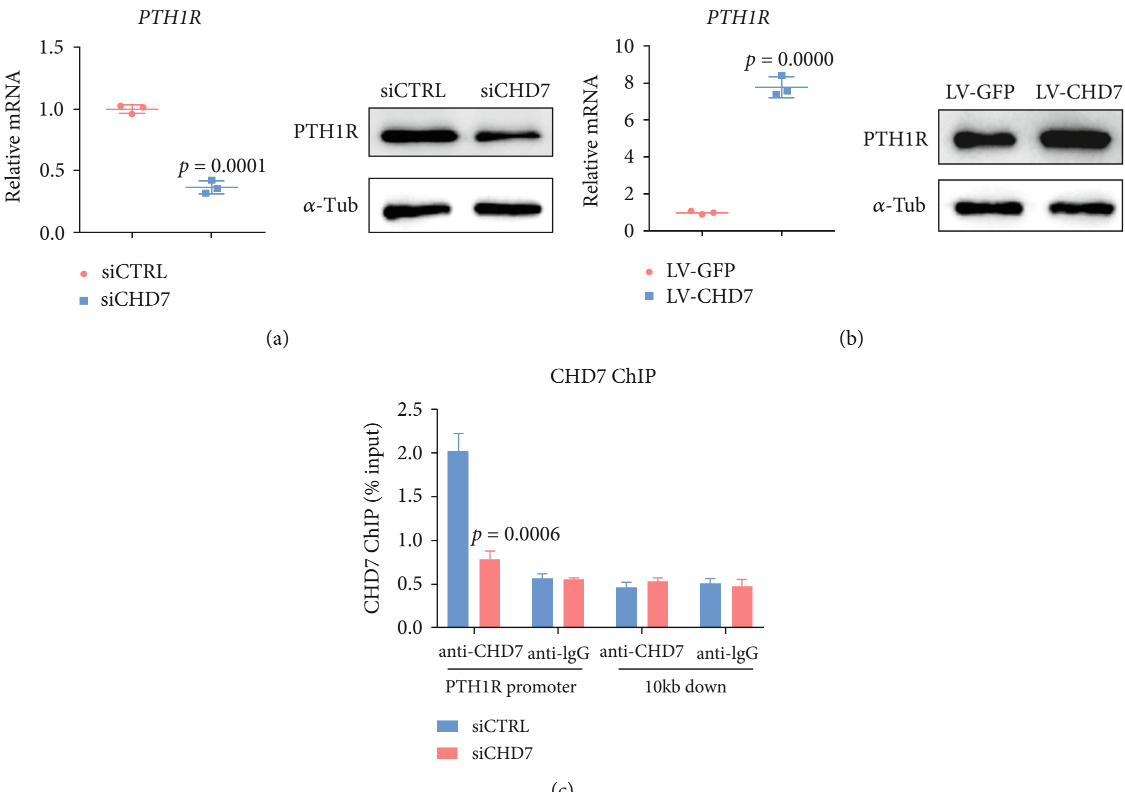

FIGURE 5: CHD7 regulates the expression of PTH1R. (a) qRT-PCR and Western blot of the PTH1R expression after *CHD7* depletion. (b) qRT-PCR and Western blot of the PTH1R expression after the *CHD7* overexpression. (c) Anti-CHD7 ChIP assay. CHD7 can bound to the promoter region of PTH1R, and the ChIP signaling was significantly suppressed after *CHD7* depletion.

related pathway [26]. The normalized enrichment score (NES) was -1.43, indicating that the PTH-related pathway was downregulated ($p = 0.007$) (Figure 4(e)). These results implied that the PTH/PTH1R signaling pathway might be a potential target for CHD7.

3.5. CHD7 Regulates the Expression of PTH1R. To figure out the regulatory role of CHD7 on PTH1R, we first analyzed the gene and protein expression pattern of PTH1R and CHD7. We found that the mRNA and protein level of PTH1R decreased after the *CHD7* knockdown (Figure 5(a)). Correspondingly, the mRNA and protein level of PTH1R increased after the *CHD7* overexpression (Figure 5(b)). To directly clarify the regulation of CHD7 on PTH1R, we conducted an anti-CHD7 ChIP assay. The result showed that CHD7 can bind to the promoter region of PTH1R, and the ChIP signaling was significantly suppressed after the *CHD7* knockdown (Figure 5(c)).

3.6. The Overexpression of PTH1R Partially Rescues the Osteogenesis of CHD7-Defected hDFCs. We next overexpressed *PTH1R* in *CHD7*-knockdown hDFCs (Figures 6(a) and 6(b)). In contrast to *CHD7*-knockdown hDFCs, the overexpression of *PTH1R* significantly promoted the osteogenesis of hDFCs, characterized by increased ALP activity, mineralization (Figure 6(c)), and expression of osteogenesis-related genes (Figure 6(d)). Such results indicated that the overexpression of *PTH1R* could rescue the osteogenic differentiation of *CHD7*-knockdown hDFCs.

4. Discussion

The osteogenic differentiation of hDFCs is a complex process, which involves a variety of intracellular and extracellular signaling pathways [27]. As a chromadomain helicase, the chromatin remodeling function of CHD7 is performed by identifying and binding to specific histone modification sites of nucleosomes, using the energy provided by ATP hydrolysis to make chromatin deagglutination and expose DNA and to increase the accessibility of transcription regulatory elements [28].

CHD7 is not only located in nucleoplasm to regulate the transcription of many genes but also in nucleolus to regulate the production of ribosomal RNA [8, 29]. Once intracellular ribosome production is disturbed, protein synthesis will also be seriously affected. Some rapidly proliferating cells in development like neural crest cells are particularly sensitive to such event [30]. According to previous studies, the neural crest abnormality might be the main cause of the corresponding tissue abnormality of CHARGE syndrome [29, 31]. Dental follicle is also originated from ectomesenchymal cranial neural crest cells [2]. This might be an alternative explanation for the osteogenetic dysfunction of hDFCs after the *CHD7* knockdown, which deserves further study.

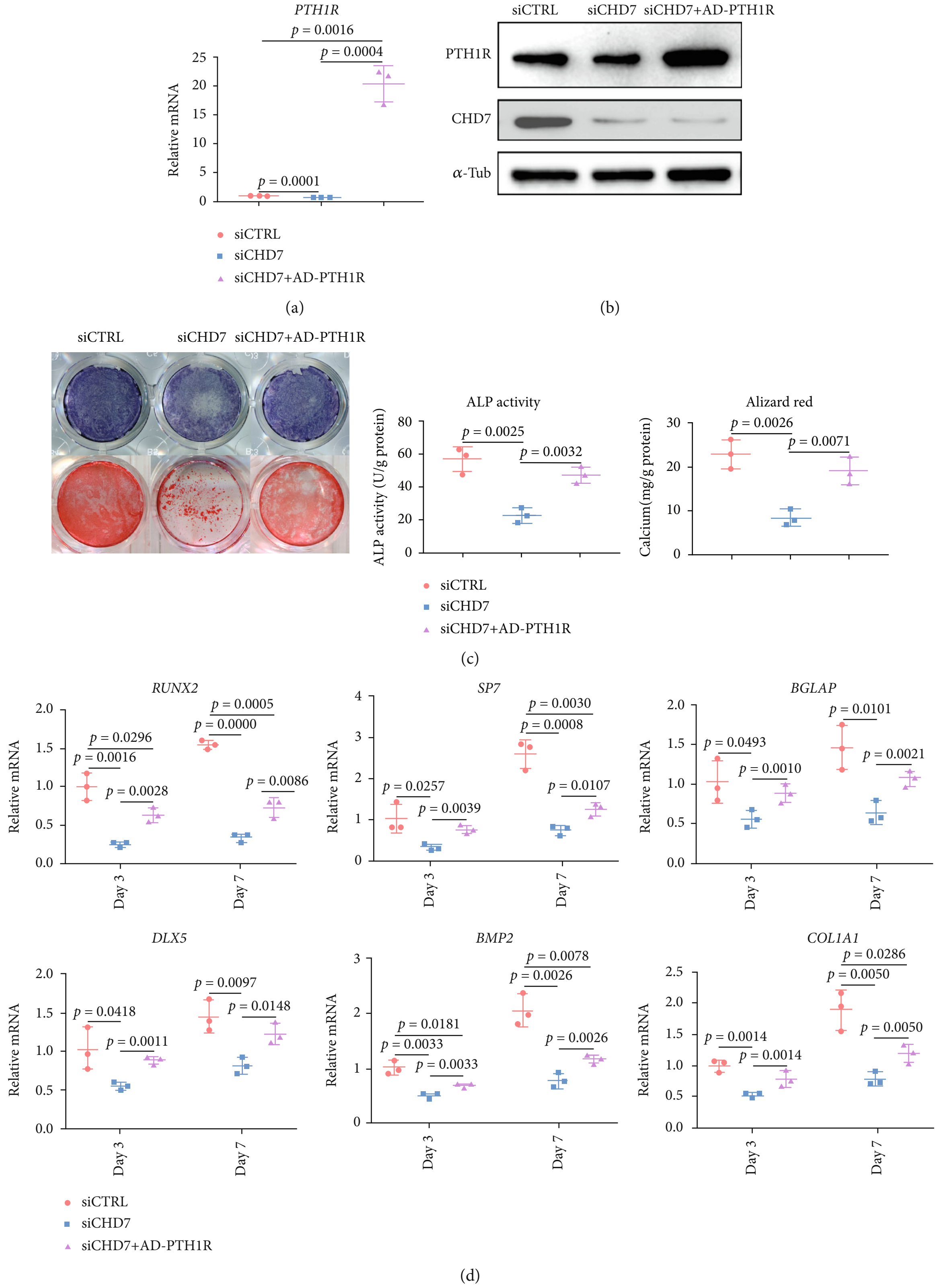

Figure 6: The overexpression of *PTH1R* partially rescues the osteogenic differentiation of CHD7-deficiency hDFCs. (a), (b) qRT-PCR and Western blot verified the overexpression efficiency of *PTH1R*. (c) Representative images and quantitative analyses of ALP and ARS staining of hDFCs in the siCTRL, siCHD7, and rescue group. (d) qRT-PCR analyses of the expression of *RUNX2*, *SP7*, *BGLAP*, *DLX5*, *BMP2*, and *COL1A1* under osteogenic condition.

PTH1R is one of the direct signaling mediators in promoting the osteogenic differentiation of BMSCs [21]. According to our result, CHD7 is vital for the translation of PTH1R during osteogenesis of hDFCs. The *CHD7* knockdown reduces the expression level of PTH1R and impairs the osteogenic function of hDFCs. Subsequently, the *PTH1R* overexpression in the CHD7-knockdown hDFCs partially rescued the impaired osteogenic potential.

The function of the PTH/PTHrP signaling pathway during tooth root formation has been reported by previous researches. The PTH/PTHrP signaling pathway could maintain physiological cell fate of dental follicle mesenchymal progenitor cells to generate functional periodontium and coordinate tooth eruption [24, 32, 33]. During the process of tooth root formation, $PTHrP^+$ dental follicle cells could differentiate into cementoblasts on the basis of acellular cementum with periodontal ligament cells and alveolar bone osteoblasts [34].

According to the qRT-PCR results, although the *PTH1R* overexpression level was considerably high, the rescue of hDFC osteogenic differentiation was still partially, rather than entirely. One possible explanation is that there might be other downstream pathways of CHD7 in hDFCs [6]. On the basis of RNA-seq analysis, exploration in the mechanism, and the previous researches, we inferred that PTH1R is vital and might be the major mechanism of CHD7 during tooth development [34]. Besides, according to RNA-seq analysis, the downregulated gene also includes classic osteogenic regulatory pathway, e.g., *WNT5A*, *NFIC*, and *BMP1*. It could be implied that there can also be other important downstream regulators, which could be our further research targets [4].

Besides CHD7, other member in the CHD family also contribute to tooth root development. Previous research has confirmed high and increasing expression of CHD3 in early and middle stage of tooth root formation, especially in Hertwig's epithelial root sheath [35]. Depletion of CHD3 and cDNA microarray analysis suggested that CHD3 might play a positive role in DNA synthesis in Hertwig's epithelial root sheath cells in tooth development, especially tooth root formation [36].

Several limitations in our research should be noted to provide ideas for the further study. First, for the lack of *CHD7* knockout mice, our research could not conduct corresponding animal experiment. Phenotype in vivo should be studied in the future to reveal the function and mechanism of CHD7 more comprehensively. Moreover, the mechanism of CHD7 regulating the osteogenic differentiation in hDFCs might be associated with the ribosomal RNA production [37]. More studies in molecular genetics and developmental biology could provide more significant evidence.

Data Availability

All Seq data have been deposited into NCBI database with the identifier GSE154822.

Conflicts of Interest

The authors declare that there is no conflict of interest regarding the publication of this paper.

Acknowledgments

This work was supported by the National Natural Science Foundation of China (grant number 81701009).

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Hindawi
Stem Cells International
Volume 2020, Article ID 8852313, 9 pages
https://doi.org/10.1155/2020/8852313

Review Article

Identification of Molecular Signatures in Neural Differentiation and Neurological Diseases Using Digital Color-Coded Molecular Barcoding

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Received 13 May 2020; Revised 26 August 2020; Accepted 28 August 2020; Published 12 September 2020

Academic Editor: De-Meng Chen

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Human pluripotent stem cells (PSCs), including embryonic stem cells and induced pluripotent stem cells, represent powerful tools for disease modeling and for therapeutic applications. PSCs are particularly useful for the study of development and diseases of the nervous system. However, generating *in vitro* models that recapitulate the architecture and the full variety of subtypes of cells that make the complexity of our brain remains a challenge. In order to fully exploit the potential of PSCs, advanced methods that facilitate the identification of molecular signatures in neural differentiation and neurological diseases are highly demanded. Here, we review the literature on the development and application of digital color-coded molecular barcoding as a potential tool for standardizing PSC research and applications in neuroscience. We will also describe relevant examples of the use of this technique for the characterization of the heterogeneous composition of the brain tumor glioblastoma multiforme.

1. Introduction

Pluripotent stem cells (PSCs) can be derived directly from the embryo at the blastocyst stage (embryonic stem cells (ESCs)) or from adult cells by reprogramming (induced PSCs, iPSCs). Regardless of the origin, these cells can be virtually converted into any cell type for basic and translational research. Reprogramming from patients allows generating iPSCs carrying disease mutations, and recent advancement in genome editing techniques has greatly facilitated the generation of mutant PSCs. In particular, the CRISPR/Cas technology is now routinely used for introducing or correcting pathogenic mutations in ESCs and iPSCs. Moreover, development of improved differentiation protocols allows efficient conversion of PSCs into disease-relevant cell types. Such remarkable advancements are mirrored by an expanding toolbox of techniques that aim capturing the transient changes in the transcriptome during differentiation. In this review, we focus on the applications of digital color-coded molecular barcoding for gene expression analysis of PSCs during neural differentiation and in neurological disease models. We also show how this technique can help improving the characterization of glioblastoma multiforme, a brain tumor showing cellular and molecular heterogeneous composition.

1.1. Neural Differentiation of Pluripotent Stem Cells. Human ESCs (hESCs), derived from blastocysts produced by *in vitro* fertilization for clinical purposes, have been described for the first time in 1998 [1]. It became immediately clear that their remarkable replicative capacity and plurilineage developmental potential represented a promise of unlimited supply of specific human cell types. Three years later, the first neural progenitors were obtained by spontaneous differentiation of hESCs *in vitro* [2]. Differentiation was triggered by simply culturing hESCs in suspension in the absence of feeder cells, as floating embryoid bodies (EBs). Plated EBs

developed in characteristic structures that resembled the early neural tube, named neural rosettes. Cells of the neural rosette expressed characteristic neural precursor markers, such as NESTIN, Musashi-1, SOX1, and PAX6 [2–5]. Isolated neural precursors could be expanded in suspension culture as cell aggregates called neurospheres. Further differentiation generated all three central nervous system (CNS) cell types *in vitro*: neurons (mostly glutamatergic), astrocytes, and oligodendrocytes [2]. Notably, hESC-derived neural precursors engrafted into the lateral ventricles of newborn mice migrated, incorporated, and differentiated *in vivo* [2]. As a next step, several groups developed useful protocols for generating individual CNS cell types from ESCs. Human neuron subtypes generated from hESCs, including midbrain dopaminergic neurons [3, 4] and spinal motor neurons [5], became readily available. Since PSCs are pluripotent, the efficiency of conversion into a desired cell type is usually low, as many cells in culture undertake alternative differentiation pathways. A major advancement came from a novel approach leading to highly efficient neural induction. Building up from the notion that vertebrate embryonic cells differentiate by default into nerve cells in the absence of TGFβ signaling [6], the Studer lab obtained rapid and complete neural conversion of hESCs under adherent culture conditions by simultaneous blocking the two canonical branches of the TGFβ signaling (dual-SMAD inhibition) [7]. This method allows directed conversion of PSCs into neural precursors by blocking alternative lineages. Today, dual-SMAD inhibition represents the first neural induction step in most methods used for obtaining CNS cells from human PSCs. Upon neural induction, PSCs undergo a default anterior neural specification pattern, which can be diverted by extrinsic cues to instruct regional patterning along rostro-caudal and dorsoventral axes, producing a wide variety of neuronal subtypes [8].

1.2. Pluripotent Stem Cells for Neurological Disease Modeling. Similar to hESCs, human iPSCs (hiPSCs) can be virtually converted into any cell type (pluripotency) and are endowed with unlimited self-renewal capacity (stemness). iPSCs with pathogenic mutations, either obtained from patients or modified by genome editing, represent a powerful tool for advancing our knowledge on the fundamental mechanisms underlying molecular and functional human nervous tissue homeostasis and disease. HiPSCs have been used to model several diseases of the nervous system, including amyotrophic lateral sclerosis (ALS) [9–11], spinal muscular atrophy (SMA) [12], Alzheimer's disease (AD) [13], Huntington's disease (HD) [14], Parkinson's disease (PD) [15], and Fragile-X syndrome (FXS) [16]. Modeling of neurological disease requires the production of physiological *in vitro* models. A major advancement in this direction has been provided by the development of three-dimensional (3D) models of the nervous system with iPSCs. Brain organoids are 3D structures built by self-organization of differentiating hiPSCs and recapitulate, to some extent, the organization of the human brain and the variety of the cell types contained in it [17]. Brain organoids derived from hiPSCs have been successfully used to model several neurodevelopmental diseases, including microcephaly, Miller-Dieker syndrome, Lissencephaly, Timothy syndrome, and Zika virus infection [18–25]. Recent development of 3D bioprinting technologies provides new opportunities in the field of brain disease modeling with hiPSCs [26].

A major common limiting factor of current brain disease models is the quality of hiPSC-derived cells, which are often not fully representative of their physiological counterparts and include poorly differentiated cells and/or undesired lineages. It is well-documented that cells derived from hiPSCs differentiation often exhibit functional, structural, and metabolic features more similar to foetal or neonatal cells. As an example, differentiation into motor neurons generates mixed populations are often limited to the spinal subtype and represent an immature embryonic stage [27–29]. These have become major and common obstacles to hiPSCs application in modeling and treating late-onset neurological disorders. Moreover, significant variability has been observed in the quality and organization of different brain regions in individual organoids [17, 30]. Thus, improved techniques that allow better characterization of the transient transcriptional landscape of individual cells in hiPSC-derived brain models of human diseases are highly demanded.

1.3. Digital Color-Coded Molecular Barcoding. Among the advanced molecular biology platforms for gene expression analysis and noncoding RNAs (ncRNAs) detection, such as microarrays and high-throughput sequencing, the NanoString Technologies nCounter Analysis System (NanoString Technologies, Seattle, WA98109, USA) is one of the interesting technologies offering high levels of precision and sensitivity, achieving reproducible results and minimizing hands-on time during the experimental setting and data analysis [31]. The NanoString nCounter platform is based on a high-throughput, multiplexed, fluorescence-based digital hybridization technology, suitable for any type of nucleic acid, and therefore, it can be employed for mRNA analysis, genomic mutations, ncRNA expression, and fusion transcripts identification as well as protein levels detection [32, 33].

The detection of molecular signatures, in terms of gene expression profiling and/or ncRNAs expression, represents a potential goal to identify molecular mechanisms in neural differentiation and in neurological diseases, including tumors, developmental, and degenerative disorders. Because most neurological disease diagnosis rely on postmortem confirmation of pathologies and/or on medical imaging during their progression, it is necessary a valid approach to screen a large number of potential markers in a timely and cost-effective manner allowing an early diagnosis. The NanoString technology satisfies these requirements and moreover can be applied to critical samples, such as Formalin-Fixed Paraffin-Embedded (FFPE) and single cells derived from neural cell lines and PSCs.

The automated nCounter NanoString technology utilizes an innovative digital color-coded barcode method detecting and counting hundreds of unique transcripts in a single reaction. This technology is based on hybridization of fluorescent barcodes to specific nucleic acid sequences, in order to measure up to 800 targets for each sample, starting from low amount of material without amplification steps [34]. In

particular, the system uses the reporter probes (short and gene-specific probes), characterized by different combinations of four distinct fluorophores at six contiguous positions; this approach allows to obtain a large diversity of color-based barcodes, each one specific for a gene transcript, that can be mixed together in a single tube reaction for hybridization step and individually identified in the data analysis [35]. A combination of reporter probes and capture probes (biotinylated) makes up a CodeSet that provides a handle for the attachment of molecular targets facilitating downstream digital detection [31, 33]. After hybridization, the excess unbound probes are washed away, and the molecular barcodes, covalently linked to the gene-specific probe sequences in a translucent cartridge, are quantitatively counted using an automated digital scanner (nCounter Digital Analyzer). The raw counts are first normalized for both positive and negative internal controls and for housekeeping genes and then compared within and across samples to obtain the expression of each target [33].

Gene expression analysis using a specific population of cells is an important goal to understand the intracellular molecular mechanisms underlying each cell subtype particularly in the context of neurological diseases, where a specific subset of cells is affected by the different pathologies [36]. In particular, in many neurodegenerative disorders, such as AD, some populations of neurons result vulnerable while others unaffected; for this reason, it is important to evaluate the gene expression profile at a single cell level, e.g., examining human neuronal and glial cells derived from AD iPSCs [37]. The nCounter Single Cell Gene Expression assay allows the gene expression profiling from single cells or from quantities as small as 10 pg of total RNA. In this case, due to the low amounts of mRNA from each single cell, the method requires a preamplification step using specific pairs of multiplexed target enrichment primers (MTE primers) [38].

2. mRNA Signatures

2.1. mRNA Signatures in Neural Differentiation. Stem cell-based neuronal differentiation is frequently used to generate *in vitro* models of neuronal development and disease [39, 40]. Due to the magnitude of research dedicated to understand the gene expression of ESCs and iPSCs, it is important to identify a molecular signature in the different stages of neural differentiation for screening drugs and cell therapies for various diseases. The combination of PSCs for neural progenitor cell (NPC) generation techniques and digital color-coded barcoding for a gene expression profiling has been described (Figure 1). In particular, by using different methods for neuron generation (neuroectoderm and neurosphederm methods) from ESCs and iPSCs, sets of specific neuronal genes from the progenitors (e.g., NEUROG2, NEUROD1, NOTCH1, MYT1, SOX2), mature neurons (MAP2, TUBB3), cortical neurons (e.g., FOXP2, CTIP2, TBR1), and synaptic neurons (e.g., GRIN2B, SYN1, SYP) have been characterized [39]. Moreover, hiPSC-derived forebrain cortical neurons have been well defined by gene expression analysis, showing a robust expression of forebrain cortical transcription factors (FOXG1, SOX1, SOX2, TBR1, TBR2, HES1, HES5) with negligible expression of midbrain and hindbrain transcription factors (EN1, HB9, HOXB6, HOXB13) [41]. Furthermore, astrocyte progenitors from hiPSCs and hESCs transplanted into the ventral horn of the adult rodent spinal cord have been characterized by *in vivo* gene expression analysis; in particular, structural (such as GFAP) and functional (AQP4, CONNEXIN43, MLC1, EAAT1) astrocyte genes have been defined [42].

2.2. mRNA Signatures in in Neurological Disorders. As mentioned, molecular barcoding represents an important approach for molecular signatures identification in neurological diseases, including tumors, disorders of development, and degenerative disorders. The scientific studies based on this technology are providing results to a more complete understanding of neurological disorders and their treatment. Figure 2 summarizes the current scientific evidences concerning nCounter gene expression profile in neurological diseases.

Microglia, the resident immune system macrophages in the brain and cerebrospinal fluid, plays a specific neuroinflammation role in both the normal CNS functions and the neurodevelopmental and/or neurodegenerative diseases progression and resolution; its molecular barcoding signature in induced microglia-like cells represents an important characterization to understand microglia biology in order to target it in the treatment of CNS disease [43, 44]. In particular, the human-induced microglia-like cells (hiMGs) show a very similar expression pattern to foetal primary microglia, characterized by genes highly and/or uniquely expressed in human microglia (P2RY12, C1QA, GAS6, MERTK, GPR34, and PROS1). A similar trend was observed in the microglia microRNA (miRNA) signature as described below [44]. Moreover, Butovsky et al. [43] demonstrated that murine microglia signature is unique in adult microglia cells and that the ESC-derived microglia displays the same gene modulation respect to newborn and primary microglia [43].

2.2.1. mRNA Signatures in Neurological Tumors. About the brain tumors, the glioblastoma multiforme (GBM) represents an important model for gene and miRNA expression evaluation because it is characterized by heterogeneous mixture of cellular and molecular subtypes [45]. Several research data have demonstrated that glioblastoma cells retain many of the features of neural progenitor cells, described as GBM stem-like cells (GSCs), and four molecular subtypes of glioblastoma are identified: proneural, neural, classical, and mesenchymal [45–47]. A GSC molecular barcoding characterization has demonstrated a transcriptional regulation of ESC markers, where NANOG, OCT4, and SALL4 genes show relatively low expression and STAT3 and SOX2 genes display high levels of expression [48]. Moreover, in a xenograft study performed by Garner et al. [45], the adherent GSCs isolated from GBM show a NanoString molecular signature characterized by downregulated (SPP1, ETV1, CCND2) and upregulated (CDH1, NQO1, STAT3, LYN) gene set [45]. Besides, a recent study displays a molecular signature of live quiescent GBM (qGBM) cells and their proliferative counterparts (pGBM) in order to identify GBM molecular subtypes. The digital color-

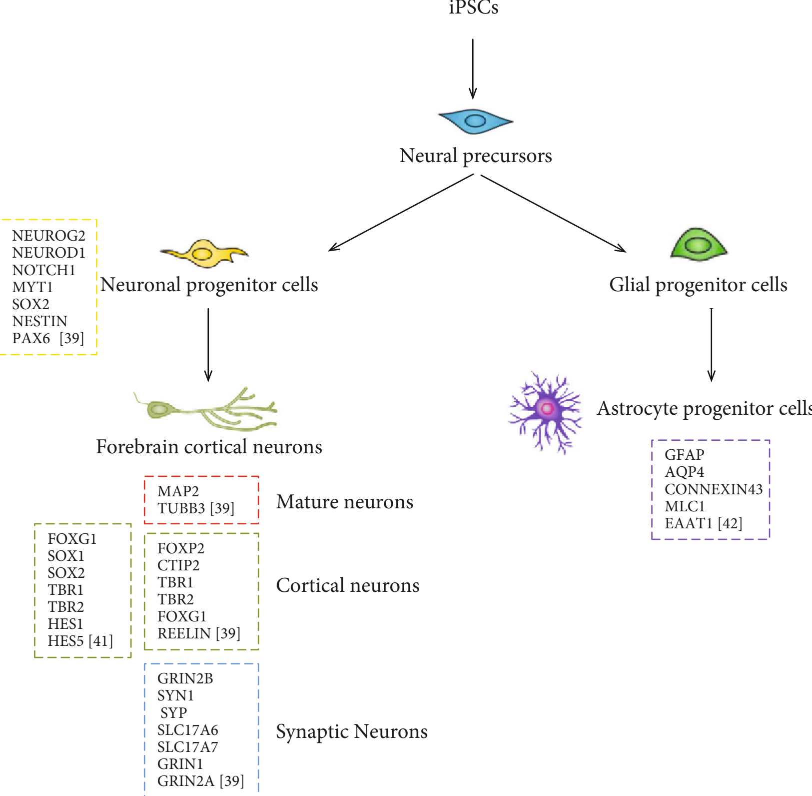

Figure 1: Molecular barcoding gene signatures in human PSC neural differentiation. Schematic representation of the major lineages generated from human PSCs upon neural differentiation. Gene expression signatures identified by digital color-coded barcoding are indicated. References are indicated.

coded barcoding transcriptome analysis reveals a mesenchymal shift as a general feature of qGBM cells: pathway scores for ECM (Extra Cellular Matrix) structure, EMT (Epithelial Mesenchymal Transition), and Cell Adhesion were increased in qGBM relative to pGBM counterparts, indicating that qGBM cells undergo a general shift towards increased mesenchymal features [49].

2.2.2. mRNA Signatures in Neurodevelopmental Disorders. About neurodevelopmental disorders, the molecular barcoding gene expression analyses in stem cells have been conducted on DISC1 gene, implicated in several neurodevelopmental processes (proliferation, synaptic maturation, neurite outgrowth, and neuronal migration), and represented by multiple isoforms [50]. In particular, the effects of DISC1 disruption, limited to exon 2 and exon 8, in NPCs and neurons derived from hiPSCs have been evaluated. In both DISC1 exons disrupted NPCs, a significant decrease of FOXG1 and TBR2 expression has been observed, but only exon 2 disrupted NPCs displayed a modulation of SOX1 and PAX6 genes. The FOXG1 and TBR2 expression decreasing has been confirmed also in DISC1 disrupted neurons; in addition, the exon 2 disrupted neurons showed low levels of mature neuronal genes (VGLUT1, GRIN1, MAP2) and a decreasing expression of the cortical neuronal markers (CTIP2, FEZF2, TBR1), while exon 8 interruption did not significantly alter neuronal layer marker expression. These data suggest that DISC1 exon 2 mutation causes more dramatic deregulation of neurogenesis than DISC1 exon 8 interruption [50]. Moreover, a molecular signature characterized by upregulated (OLFM1, CALB1, FEZF2, NRG1) and downregulated (BRN2, CALB2, EAAT2) genes has been established in DISC1-mutant cerebral organoids by using a custom NanoString panel of 150 genes related to neuronal development, maturity, and cell signaling [51].

Besides, related to neurodevelopmental disorders, a PsychGene NanoString panel has been used to establish a molecular signature in hiPSCs, NPCs, and postmitotic neurons, isolated from bipolar disorder (BD) patients (two parents unaffected and two sons affected). In the NPC gene expression analysis, eighteen genes showed significant expression differences between BD patients relative to unaffected parental controls. Among these genes, NKX2-2, NKX6-1, and IRX3 are known to function in sonic hedgehog-dependent neural patterning to specify the identity of ventral progenitor-derived neurons. Comparing expression levels in BD patient postmitotic neurons to their unaffected parental controls, forty-four genes differentially expressed have been identified. In particular, an increase in expression of the general neural differentiation markers DCX and MAP 2 and a decrease in expression of cortical layers markers (CTIP2, RELN) have been observed in affected BD patients [52].

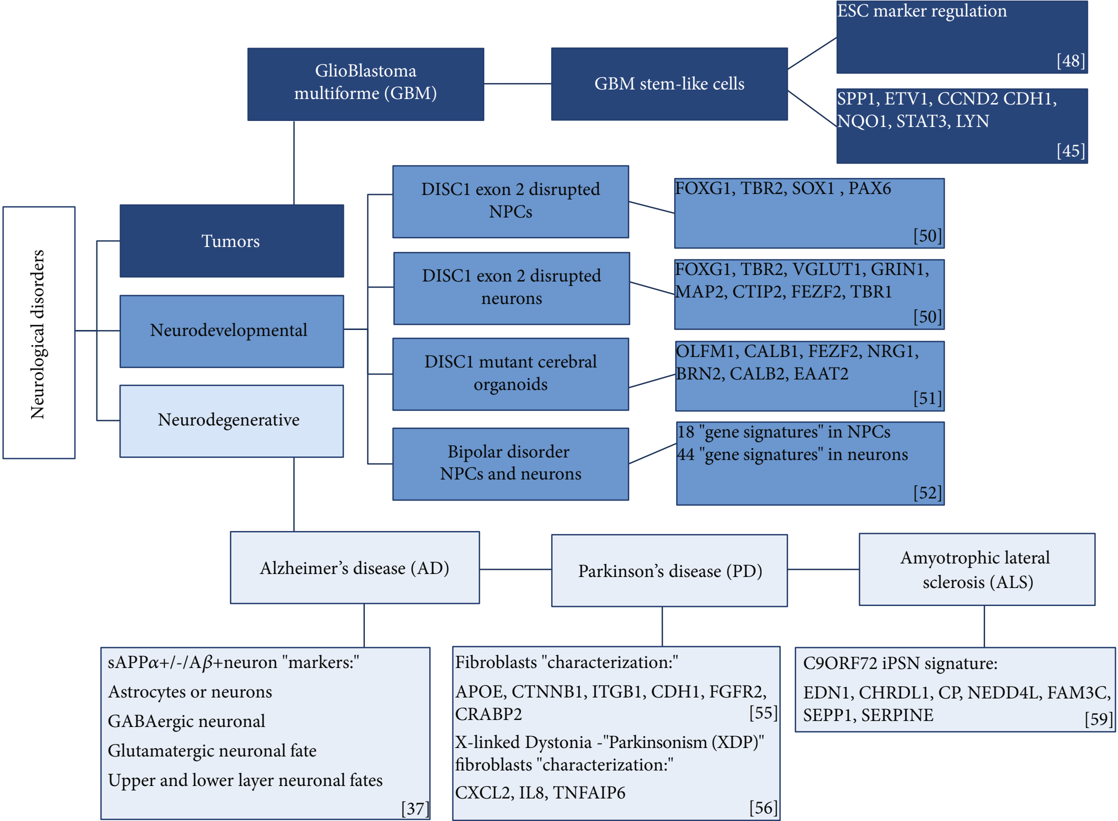

Figure 2: Molecular barcoding gene signatures in diseases of the nervous system. The figure shows gene expression signatures identified by molecular barcoding in several neurological diseases. References are indicated.

2.2.3. mRNA Signatures in Neurodegenerative Disorders. Different research data highlights a gene expression signature in neurodegenerative disorders, such as AD, PD, and ALS, by combining the use of hiPSCs and molecular barcoding approach. Using the NanoString's Single Cell method, Liao et al. [37] established a molecular signature in single living iPSC-derived neurons with different secretion of insoluble extracellular amyloid β (Aβ) and soluble amyloid precursor protein-alpha (sAPPα). In particular, Aβ and sAPPα analytes are crucial to AD pathogenesis, and the transcriptomic characterization of three secretion profiles (sAPPα-/Aβ-; sAPPα+/Aβ-; sAPPα+/-/Aβ+) has identified a specific molecular signature. Individual cells secreting high levels of sAPPα and/or Aβ showed an expression of astrocytes or neurons markers, an elevate expression of GABAergic neuronal markers and glutamatergic neuronal fate markers, as well as upper and lower layer neuronal fates markers [37]. Moreover, a study based on the application of the NanoString's Single Cell and hiPSCs from AD patients harbouring a dominant, fully penetrant mutation in amyloid precursor protein (APP) gene (V717I) highlighted that control and AD iPSCs showed no significant differences in terms of general neuronal or cell fate specific marker expression [53]. Other studies have been conducted on another neurodegenerative disorder, PD, a sporadic, progressive disease linked to a complex genetic architecture and environmental exposures [54] and for these reasons have been attributed to a combination of genetic and nongenetic factors [55]. In order to reduce the effect of genetic variability on the study of this pathology, a characterization of hiPSC lines derived from fibroblasts of the PD affected monozygotic twin, unaffected twin, and a subject with sporadic PD, and healthy subjects have been established; markers for three-germ layer differentiation APOE and CTNNB1 (Endoderm), ITGB1 and CDH1 (Mesoderm), and FGFR2 and CRABP2 (Ectoderm) have been evaluated using the NanoString approach [55]. Moreover, the NanoString fibroblasts characterization in X-linked Dystonia-Parkinsonism (XDP), a progressive neurodegenerative disease causing the loss of medium spiny neurons within the striatum, identified a dysregulation of gene sets. The molecular signature associated to nuclear factor-kappa B (NFκB), and in particular, a strong downregulation of CXCL2, IL8, and TNFAIP6 has been observed in XDP vs. control fibroblasts [56]. Lastly, ALS molecular signature has been performed by digital color-coded barcoding approach as well. The hexanucleotide GGGGCC repeat expansion in the first intron/promoter region (noncoding region) of the C9ORF72 gene is the most common genetic cause of this pathology [57, 58]. Using 50-mer NanoString probes, the levels of the three C9ORF72 RNA variants were determined in samples of patient-derived human brain tissue, ALS fibroblasts, iPSCs, and iPSC-derived neurons (iPSNs). C9ORF72 ALS patient iPSNs showed approximately a 50% reduction in expression of C9ORF72 V1 and V2 variants [59]. Moreover, sixteen aberrantly expressed target genes in C9ORF72 ALS patient tissues have been identified; in particular, seven displayed similar

dysregulation patterns when compared to iPSNs: EDN1, CHRDL1, and CP were upregulated, and NEDD4L, FAM3C, SEPP1, and SERPINE2 were downregulated in C9ORF72 iPSNs versus control [59]. These seven genes represent an important molecular signature for ALS and can be potential candidates for establishing the disease biomarkers to monitor therapeutic approaches.

3. Noncoding RNA Signatures

3.1. microRNA Signatures in Neural Differentiation. Other to mRNAs, also miRNAs have demonstrated their potential as biomarkers for a wide variety of human pathologies. A deregulation of miRNA expression might be involved in neurological dysfunction or neurodegenerative processes [60]. However, while NanoString mRNA signatures by using ESCs and/or iPSCs have been well investigated in cell neural differentiation and in neurological disorders, little information for miRNA expression, and generally for all noncoding RNAs, is known.

A miRNA NanoString profiling over the time course of differentiation (4 days), obtained overexpressing a pair of transcription factors (Neurogenin-1 and Neurogenin-2) in iPSCs (iNGN cells), has been characterized. At day 4 of differentiation, compared to day 0, a signature of eighteen miRNAs downregulated and fifty-five miRNAs upregulated was defined suggesting a rapid change of the miRNA profiles in the course of iNGN differentiation. In particular, at day 0, the uninduced iNGN cells showed miRNA signatures of stem cells; the miR-302/367 cluster dominated their profile confirming its role in regulating self-renewal and preserving pluripotency. At day 4, the miR-124, important for neural differentiation, showed a consistent overexpression along with other neuronal miRNAs (miR-96 and miR-9), establishing neuronal miRNA signatures in iNGN cells [61].

3.2. microRNA and lncRNA Signatures in Neurological Disorders. As well described for mRNA signature, also digital color-coded barcoding technology represents an important approach for ncRNA signature identification in neurological diseases. However, scientific evidences concerning the triplet ncRNA-stem cell-NanoString are still remaining insufficiently characterized, and in particular, no ncRNA signature has been defined for neurodegenerative disorders.

As described for gene signatures, the identification of a unique microglial miRNA NanoString signature in hiMG represents another important goal to understand progression and/or resolution of neurological disease. As a mirror of gene expression data, a hiMG signature represented by nineteen downregulated and one hundred and eleven upregulated miRNAs has been established; this characterization suggests that hiMG most closely resembled foetal primary microglia than the microglia isolated from adult postmortem brain tissue, showing instead an opposite profile [44].

3.2.1. microRNA and lncRNA Signatures in Neurological Tumors. Among neurological tumors, as well as for mRNA signature, the scientific evidences about ncRNA signatures are predominantly in GBM. Importantly, GSC subtype classification was demonstrated by signatures of long noncoding RNAs [47], instead miRNAs have not been shown to predict GBM classification and prognosis by global signature, while being strongly implicated in GBM [62]. In particular, a lncRNA NanoString study revealed a signature in GBM tissue and in GSCs from GBM specimens: fifteen lncRNAs resulted deregulated in GBM tissue, when compared to adjacent tissue, and twenty-seven lnRNAs showed a differentially expression in different subtype of GSCs. The lncRNA HIF1A-AS2 (hypoxia-inducible factor 1 alpha-antisense RNA 2) was significantly enriched in both GBM tissue and in mesenchymal GSCs [47]. Concerning miRNA molecular expression, a NanoString study conducted on two nonmalignant neural stem cells (NSCs) and eight GSC samples showed a signature characterized by four miRNAs downregulated in GSCs: miR-15a, miR-30c, miR-128, and miR-328 [46].

3.2.2. microRNA Signatures in Neurodevelopmental Disease. Related to the ncRNAs NanoString signature in neurodevelopmental disorders, miRNA expression pattern in NSCs was determined considering Autism Spectrum Disorders (ASD) as a model. Generally, the neurodevelopmental disorders are caused by a wide variety of mutations in genes involved in protein translational control, chromatin modification, and cell division and differentiation, such as MBD5 (Methyl-CpG binding domain 5) and SATB2 (Special AT-rich binding protein 2), critical genes in ASD. In particular, an nCounter miRNA expression pattern was evaluated in NSCs in which short hairpin RNA was stably incorporated to suppress MBD5 and SATB2 and in proliferating and differentiating NSCs. A miRNA signature for MBD5 KO and SATB2 KO was established (twenty-one and thirty-one miRNAs, respectively), and interestingly, four miRNAs associated with differentiation or suppression of proliferation (miR-99, miR-9, miR-30b, and miR-92a-3p) were unregulated in MBD5 KO and in differentiating NSCs; while for SATB2 KO, all thirty-one miRNAs (in addition to miR-99, miR-9, miR-30b, and miR-92a-3p, also let-7e, miR-221-3p, and miR-93-5p) showed a significant overlap with the same trend of differentiating rather than proliferating NSCs [63].

4. Conclusions and Future Perspectives

The use of digital color-coded molecular barcoding in PSC-based models has the potential of greatly improving our ability to capture signatures of human development and diseases. This is particularly important for the nervous system. It is indeed crucial to improve our knowledge of the complexity and variety of the cell types that make our brain to understand the pathophysiology of neurological diseases. The molecular color-based barcoding approach offers several key advantages including precision, sensitivity, reproducibility, technical robustness, absence of an amplification step and direct measurement of target molecules, and data analysis easiness [31]. However, this novel technology requires expensive equipment (instruments and experimental kits) distributed by only one company, resulting in a closed platform and may not be cost-effective for low number of samples [64]. As described in this review, digital color-coded

molecular barcoding generates robust results in PSCs, in terms of gene and ncRNA expression; however, to date, molecular information regarding ncRNAs, specifically lncRNAs, are very limited. Definition of specific molecular signatures will allow developing novel therapeutic approaches and design targeted treatments. Given the importance of the nonprotein coding genome in the human nervous system, we expect for the future an increase on the use of molecular barcoding for the characterization of long and short noncoding RNAs expressed in brain cells under physiological or pathological conditions.

Given that many neurological diseases, in particular neurodegeneration disorders, manifest pathologically as proteopathies [65], more emphasis should be placed on direct detection of protein levels in disease models. It would be particularly informative to investigate on genes-proteins association from the same sample, in order to exhaustively study neurological disease pathogenesis, highlighting the importance of combination of genetic and biochemical analyses. For this reason, it would be interesting to take advantage the 3D Biology™ system proposed by NanoString, which allows evaluating RNA, DNA, and proteins in a single assay, allowing a 360 degree view on the profiling of a neurological disease. Another interesting future perspective could be the spatial and simultaneous resolution of RNAs and proteins on a single platform and digital counting of both analytes from a single sample in order to establish a tissue "geography" (Digital Spatial Profiling).

Conflicts of Interest

The authors declare that there is no conflict of interest regarding the publication of this paper.

Acknowledgments

We thank the members of the Rosa lab for helpful discussion. The publication of this article was funded by Sapienza University of Rome to A.R. and Fondazione Istituto Italiano di Tecnologia to D.S. and A.R.

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Hindawi
Stem Cells International
Volume 2020, Article ID 8836258, 17 pages
https://doi.org/10.1155/2020/8836258

Review Article

Epigenetic Regulation in Mesenchymal Stem Cell Aging and Differentiation and Osteoporosis

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Received 17 July 2020; Revised 17 August 2020; Accepted 24 August 2020; Published 10 September 2020

Academic Editor: Quan Yuan

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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 (*PPAR*γ) 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*, $p16^{INK4a}$, 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 vivo* or *in vitro*, 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 *p16*^{INK4a} 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

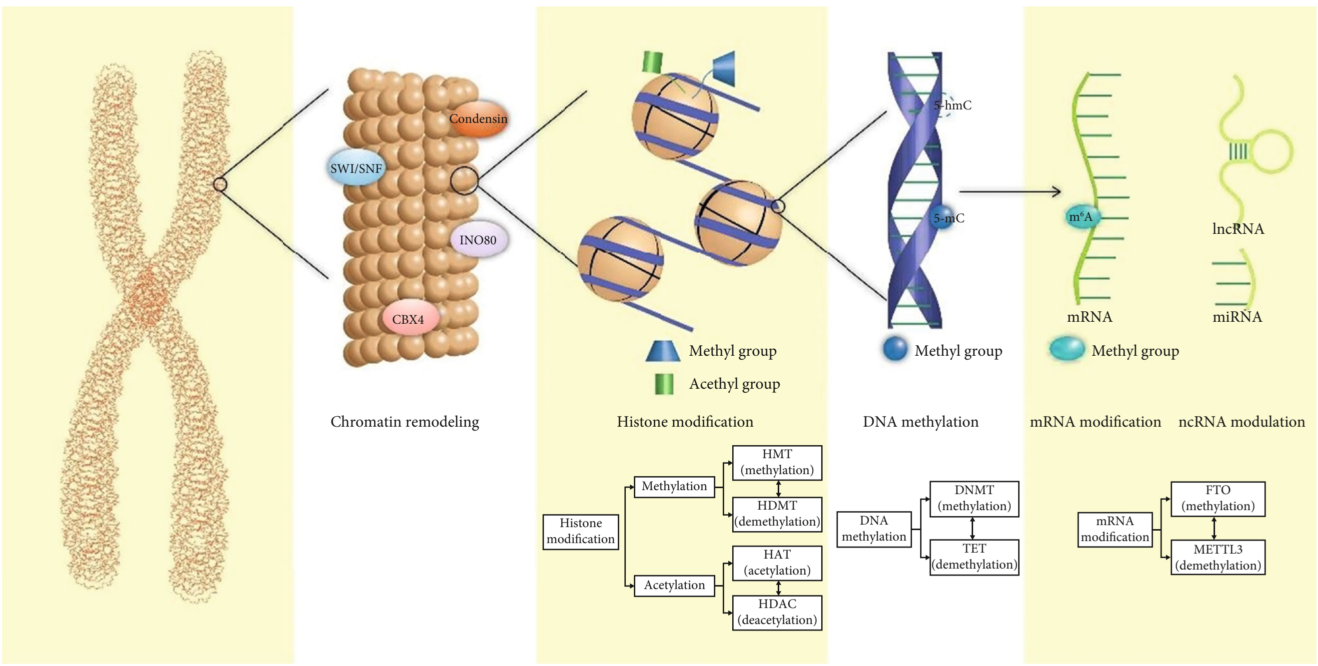

Figure 1: Factors in epigenetic regulation. Epigenetic regulation occurs in several steps of transcription (such as chromatin remodeling, DNA methylation, and histone modification) and posttranscription (such as mRNA processing and ncRNA regulation). Specific regulatory factors participate in each process.

Table 1: DNA methylation in MSC aging and related diseases.

Regulatory factors	Mechanism	*In vivo* or *in vitro*	Consequence	Materials	Ref.
Senescence and aging					
DNMT1↓ DNMT3b↓	Decreases methylation in the promoter region of miRNA targeting *Ezh2* to decrease *Ezh2* expression, thus inhibiting repressive H3K27me3 formation at *p16*'s promoter; directly decreases methylation in CDK inhibitor genes *p16* and *p21*	*In vitro*	Aging	hUCSCs	[28]
DNMT↓	Insufficient to methylate *Tert* promoter; thus upregulated TERT would repair the shortened telomeres with replication	*In vitro*	Antisenescence	hBMSCs	[30]
Skeletal diseases					
DNMT1 changes	Aberrant methylation of *ABCB1* gene leads to a dysregulation of glucocorticoid	*In vitro*	ONFH	hBMSCs	[33]
TET1 and TET2↓	The depletion of TET causes impeded demethylation of *P2rX7* promoter and incapable exosome release, which leads to intracellular accumulated miR-297 targeting Runx2 signaling pathway	Both	Osteoporosis	hBMSCs, mouse BMSCs, mouse model	[34]

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 BMI1, 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 $p16^{\mathrm{INK4A}}$ 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 $p21^{\mathrm{CIP1/WAF1}}$, 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*, *Opn*, 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 (RNAP II) complex to heme oxygenase 1 (*Hmox-1*) gene promoter. More importantly, without the cooperation of SIRT6, expression of *RNAP 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 oculofaciocardiodental (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-2α, 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

Table 2: Histone modification in MSC aging and related diseases.

Epigenetic changes	Factors	Mechanism	*In vivo* or *in vitro*	Consequence	Material	Ref.
Senescence and aging						
Histone acetylation	HDAC↓	Directly upregulates JMJD3 and indirectly downregulates *PcGs* through RB/E2F pathway to inhibit H3K27me3 at $p16^{INK4A}$	*In vitro*	Aging	hADSCs, hUCSCs	[38]
Histone acetylation	HDAC↓	Promotes the transcription of $p21^{CIP1/WAF1}$ 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 RNAP II 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 $p16^{INK4A}$	*In vitro*	Aging	hADSCs, hUSCSs	[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, hUSCSs	[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 *Osx*, *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-2*α* 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]

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- (CBX4-) 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

Table 3: Chromatin remodeling in MSC aging and related diseases.

Regulatory factors	Mechanism	*In vivo* or *in vitro*	Material	Consequence	Ref.
Senescence and aging					
BRG1↓	Inhibits *Nanog* gene expression by facilitating DNMT recruitment and methylation; induces γ-form heterochromatin formation and p53 pathway activation	*In vitro*	hBMSCs	Senescence	[53, 54]
Condensin↓	Fails to alleviate DNA damage by chromatin reorganization	Both	hBMSCs, mouse BMSCs, mouse model	Aging, bone aging	[56]
KAP1↓	Cannot be recruited by insufficient CBX4 at nucleolar rDNA, enhancing the excessive expression of rRNAs and destabilizing nucleolar heterochromatin	Both	hMSCs derived from embryonic cell culture, mouse BMSCs, mouse model	Premature cellular senescence, osteoarthritis	[58]
Skeletal diseases					
INO80↓	Incapable of interacting with Wdr5 that catalyzes H3K4me3 formation, which promotes Wnt pathway activity	Both	hBMSCs, mouse model	Osteoporotic phenotypes	[59]

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 methylation 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 N^6-methyladenosine (m^6A) modification. Osteogenesis induced by methyltransferase like 3 (METTL3) is counterbalanced with lipogenesis promoted by demethyltransferase fat mass and obesity-associated protein (FTO) [61]. During the aging process, expression of *Fto* gene increases and inhibits m^6A formation on *Pparγ* 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) abundance 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

Table 4: ncRNAs in MSC aging and related diseases.

Regulatory factors	Mechanism	*In vivo* or *in vitro*	Consequence	Material	Ref.
Senescence and aging					
miR-10a↓	Insufficient to target *Klf4* 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	Pig ADSCs	[98]
miR-29c-3p↑	Targets *CNOT6* thus inducing senescence via p53/p21 and p16/pRB pathways	*In vitro*	Senescence	hBMSCs	[72, 73]
miR-34a↑	Reduces *CDK2*, *CDK4*, *CDK6*, and *cyclin D* and *E* expression to hinder the SOX2-related self-renewal ability	*In vitro*	Senescence	hADSCs	[70]
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-141-3p↑	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 *Epas1* 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-155-5p↑	Targets *Bag5* that encodes partner protein of PINK1, to inhibit mitophagy and dysfunctional mitochondria elimination	*In vitro*	Aging	hBMSCs	[87]
miR-155-5p↑	Targets the common transcription factor *C/EBP-β* thus repressing antioxidant genes and inducing ROS production	Both	Aging	hBMSCs/mouse BMSCs	[82]
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 *Alpl*, 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 $p16^{INK4A}$ and $p21^{CIP1/WAF1}$	*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]

Table 4: Continued.

Regulatory factors	Mechanism	*In vivo* or *in vitro*	Consequence	Material	Ref.
lncRNA-Bmncr↓	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 *Osx*	*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.

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-

155 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, mi-R141-3p, 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 lncRNAs, although they are regarded as crucial modulators of MSC-mediated ectopic tissue regeneration, only a few studies have reported the influence of lncRNAs on aging. lncRNAs 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 lncRNA-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, lncRNA-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/Pparγ* transcriptional complex to inhibit MSC adipogenic differentiation. When lncRNA-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 lncRNAs 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 *Osx* 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].

lncRNAs often exert their pathogenic effect via miRNAs or other ncRNAs. For example, miR-143, a direct inhibitor of *Osx* gene, is suppressed by lncRNA-MALAT1 in normal MSCs. However, in osteoporosis patients, downregulation of lncRNA-MALAT1 leads to decreased *Osx* gene expression and loss of bone mass [117]. Another team also proved that lncRNA-MALAT1 acts as a sponge of miR-34c to increase the expression of special AT-rich sequence binding protein 2 (SATB2), which is conducive to restoring osteogenesis in osteoporosis conditions [118]. Besides, elevated lncRNA-ORLNC1 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 lncRNA-DEPTOR binds to lncRNA-MEG3 promoter and prevents it from activating BMP4 pathway [120]. It is worth mentioning that in the process of skeletal diseases, alteration of lncRNA expression may work against bone destruction. For example, lncRNA-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, lncRNA-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 lncRNA forecasts the dysfunction of MSCs, and notably, modulative 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 *SIN3A* [24]. Besides, it has been discovered that considerable ncRNAs interplay with complicated molecular networks and pretranscriptional epigenetic marks

in the aging process. For instance, depletion of DNA demethylase TET causes accumulated miR-297; miR-188 inhibits HDAC9-mediated histone deacetylation; and miR-31a-5p can bring about senescence-associated heterochromatin foci formation in aged rat BMSCs [34, 71, 95]. Pursuit in epigenetics about MSCs has never slowed down. It is believable and desirable that more interactive relationships between different epigenetic marks could be established and elaborated. However, according to existing researches and experimental technology, it is difficult and one-sided for us to rank a certain epigenetic mark as a more critical inducing factor for MSC aging. Instead, it is most likely that they function as an interlaced system in coordination and order. Although it has been reported that intervention of a single factor of epigenetic marks could delay or reverse MSC aging to some extent, we believe that other types of accompanied epigenetic regulation may initiate in the biological effect, and even imaginably, proper combinational modulation of two or more epigenetic marks could upgrade the efficacy of therapy for MSC-related diseases.

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 nonaging 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].

Simultaneously, great progress has been made in MSC-dependent epigenetic therapeutics aimed at aging-related skeletal diseases. For histone acetylation, MS-275 subcutaneous injection rescues NF-κB-induced rapid bone loss by interrupting interactions between HDACs and DExH-box helicase Dhx36, which inhibits tissue-nonspecific alkaline phosphatase (TNAP) activity [123]. Pretreatment with KDM5A inhibitor JIB-04 partially rescues bone loss during osteoporosis by increasing the H3K4me3 level on the *Runx2* promoter [45]. And HDAC inhibitor trichostatin prospers osteogenesis of rat adipose tissue-derived stem cells (hADSCs) by histone modifying on *Runx2* promoter [130]; LSD1 inhibitor pargyline rescues osteogenic ability of BMSCs under osteoporosis conditions by modulating H3K4 methylation at the promoter region of *Ocn* and *Runx2* genes [131]. Notably, miRNA inhibitors are also applied in osteoporosis treatment. For example, injection of antagomiR-31a-5p or antagomiR-188 into bone marrow cavity significantly alleviates fat accumulation and remedies bone loss in aged mice [71, 95]. Moreover, atagomiR-132-3p delivered by a bone-targeted $(AspSerSer)_6$-cationic liposome system silences miRNA-132-3p expression in bone tissues, thus effectively preserving bone mass, bone structure, and strength in hindlimb-unloaded mice [132].

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 *Pparγ* genes [137, 138], while collagen-based hydrogel containing agomir-34a elevates bone volume in mouse radiational bone injury area by down-regulating *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. Jumonji 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

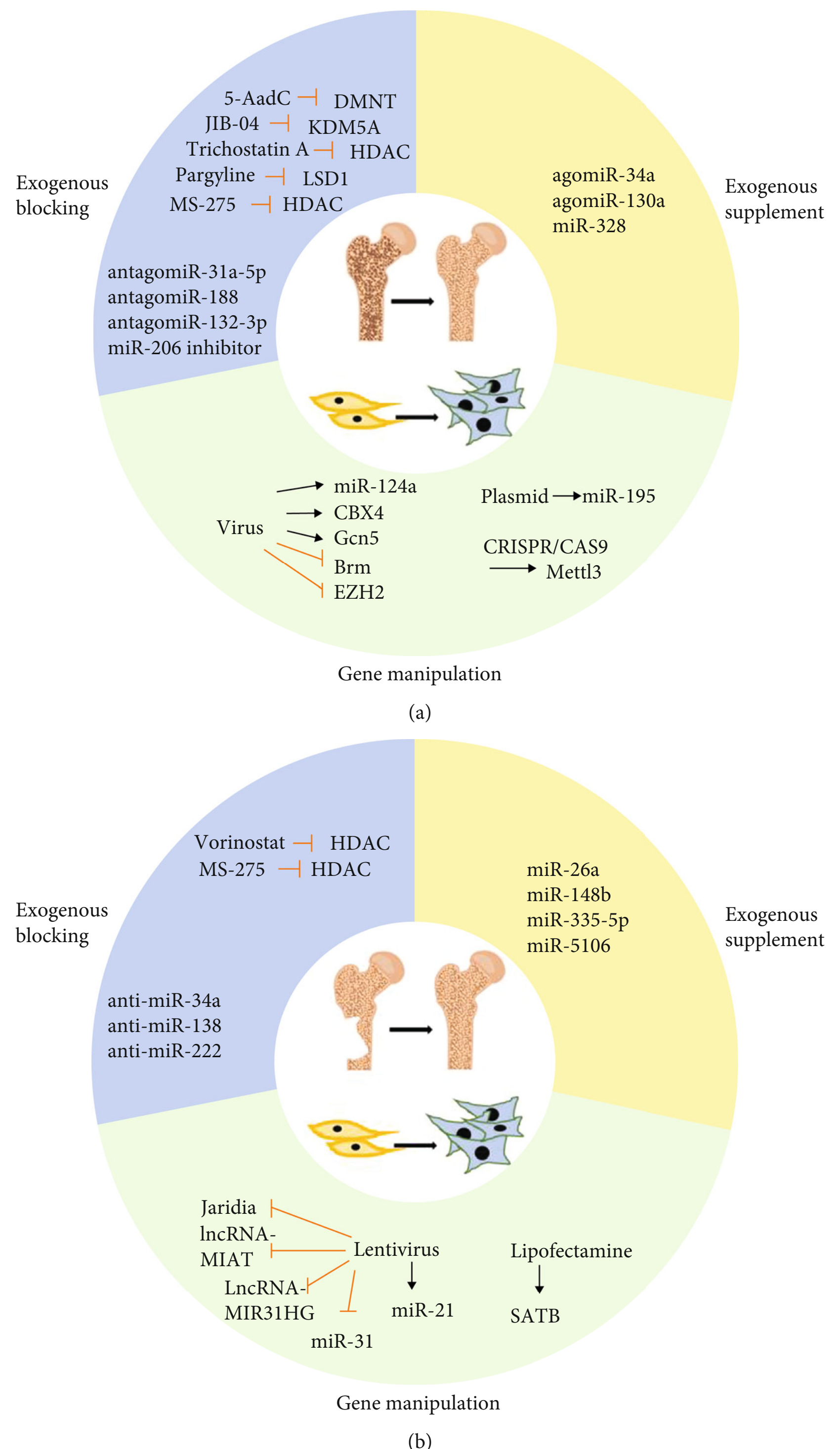

Figure 2: Application of epigenetic regulation in (a) skeletal diseases and (b) bone regeneration. Exogenous blocking or supplement and direct gene manipulation are separately used in both conditions.

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 lncRNA 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.

Acknowledgments

This work was supported by the projects of Ten-Thousand Talents Program (Y.L.), National Natural Science Foundation of China (No. 81871492 (Y.L.) and No. 81670957 (WR.L.)), and Beijing Municipal Natural Science Foundation (No. L182005 (Y.L.)).

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Hindawi
Stem Cells International
Volume 2020, Article ID 8839703, 10 pages
https://doi.org/10.1155/2020/8839703

Review Article

ATP-Dependent Chromatin Remodeling Complex in the Lineage Specification of Mesenchymal Stem Cells

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Received 11 July 2020; Revised 29 August 2020; Accepted 1 September 2020; Published 9 September 2020

Academic Editor: shuibin lin

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Mesenchymal stem cells (MSCs) present in multiple tissues can self-renew and differentiate into multiple lineages including the bone, cartilage, muscle, cardiac tissue, and connective tissue. Key events, including cell proliferation, lineage commitment, and MSC differentiation, are ensured by precise gene expression regulation. ATP-dependent chromatin alteration is one form of epigenetic modifications that can regulate the transcriptional level of specific genes by utilizing the energy from ATP hydrolysis to reorganize chromatin structure. ATP-dependent chromatin remodeling complexes consist of a variety of subunits that together perform multiple functions in self-renewal and lineage specification. This review highlights the important role of ATP-dependent chromatin remodeling complexes and their different subunits in modulating MSC fate determination and discusses the proposed mechanisms by which ATP-dependent chromatin remodelers function.

1. Introduction

Mesenchymal stem cells (MSCs), also known as mesenchymal stromal cells, are multipotent stromal cells that can differentiate into a variety of mesoderm cell types, including osteoblasts, chondrocytes, myocytes, and adipocytes [1–3]. MSCs are a heterogeneous subset of stem cells that can be obtained from different locations of adult tissues including the bone marrow, adipose tissue, and other sources [4–6]. Studies have also indicated that MSCs can differentiate into endoderm and ectoderm lineages, including hepatocytes, epidermal-like cells, neurons, and other cell fates [7–10]. MSCs are a great choice for tissue engineering, regenerative medicine, and clinical therapy. The MSC differentiation process is regulated by different regulatory mechanisms like signaling molecules and epigenetic modifications [11, 12]. All regulatory mechanisms determine the selective transcription of genes with discrete combinations. This selective transcription will define the differentiation process and subsequently determine the specific lineage. Knowledge of how specific lineage differentiation occurs and how epigenetic modifications are involved in this process will accelerate the research and development of cell-based tissue engineering therapy. The current review summarizes our understanding of how ATP-dependent chromatin remodeling complexes regulate multilineage MSC differentiation.

2. ATP-Dependent Chromatin Remodeling Complexes

Specific gene expression programs, which depend largely on the organization of the associated chromatin, define a variety of cellular processes like differentiation, proliferation, and stemness [13]. ATP-dependent chromatin alterations, as one of the major factors that affect chromatin state, can determine a specific gene's transcription level [14–16]. ATP-dependent chromatin alteration is achieved by multisubunit ATP-dependent chromatin remodeling complexes.

These complexes can utilize ATP hydrolysis-derived energy to remodel nucleosome structure, thus modulating transcription factor binding to cognate DNA.

ATP-dependent chromatin remodeling complexes mainly consist of an ATPase and multiple subunits. The ATPase subunit hydrolyzes ATP, while the associated subunits regulate ATPase catalytic activity and genome binding. Therefore, different combinations of ATPase and associated subunits result in various chromatin remodeling complexes with different functions [17, 18]. All ATP-dependent chromatin remodeling complexes include an ATPase subunit from the SNF2 family, which can be divided into four different subfamilies, including SWI/SNF (switch/sucrose nonfermentable), ISWI (imitation SWI), CHD (chromodomain helicase DNA-binding), and INO80 (SWI2/SNF2 related (SWR)) based on sequence similarity between their ATPase domains [16, 19, 20]. The diversity of different isoforms of associated subunits defines a variety of particular properties that are suited to the specific tissue type and assist in recruiting the complexes to specific genomic loci. Complexes with various subunit combinations have been detected in different cell or tissue types during development. For instance, the SWI/SNF complex with BAF60C of the BAF60 subunit functions in gene transcription in the muscles and the heart, while the BAF60A isoform of the complex has a limited role in these tissues [21, 22]. The increasingly identified tissue-specific subunits of ATP-dependent chromatin remodeling complexes indicate the necessity to better understand remodeler subunit composition and how they modulate tissue-specific gene transcription.

The interaction of ATP-dependent chromatin remodeling complexes and histone acetyltransferases (HATs) in gene transcription has been demonstrated. For example, the yeast Spt-Ada-Gcn5-acetyltransferase (SAGA) complex cooperates with the SWI/SNF complex via the cell wall integrity pathway for mandatory nucleosome displacement, which is essential for full gene expression [23]. It has also been found that the yeast and mammalian SWI/SNF complex is involved in the Rb/E2F pathway, which recruits SWI/SNF, histone deacetylases (HDACs), and histone methyltransferases (HMTs) to the E2F promoter that actively represses transcription [24]. In addition, SWI/SNF functions in both transcriptional activation and repression of the pS2 promoter via ligand-specific collaboration with HDAC1, P300, and prohibitin recruitment [25].

ATP-dependent chromatin remodeling complex can also cooperate with DNA methylation in various cellular processes. Several ATP-dependent chromatin remodeling enzymes, including the mammalian SNF2 family and ATPases ATRX and LSH (HELLS), are involved in DNA methylation at the fifth carbon of cytosine (5mC), which is an abundant epigenetic modification in vertebrate genomes [26–28]. The ATRX gene mutation, which resides on the X chromosome, causes a decrease of 30–60% in alpha globin gene expression, and this may result in an unusual form of thalassemia [27]. Patients with ATRX syndrome exhibit both hypermethylation and hypomethylation in highly repetitive elements, including satellite DNA, although the total 5mC level in the genome seems unchanged. However, a dramatic decrease (50%) in 5mC levels was present in LSH-null mice [28]. Fibroblasts from $Lsh^{-/-}$ mouse embryos, which lack DNA methylation from transposons, centromeric repeats, and several gene promoters, can reestablish DNA methylation and silence the misregulated genes with LSH reexpression [29]. The interactions between the complexes and other epigenetic regulation factors in different cell types provide a hint for investigating the potential role of ATP-dependent chromatin remodeling in multilineage MSC differentiation.

3. Role of ATP-Dependent Chromatin Remodeling in MSC Lineage Differentiation

3.1. SWI/SNF. The switch/sucrose nonfermentable (SWI/SNF) complex (also known as BAF) is composed of at least 15 different subunits that invariably include a core ATPase of either Brm (Brahma) or Brg1 (Brahma-related gene 1) that can provide the necessary energy to the complex for nucleosome remodeling activity [16]. BRM and BRG1 share 75% of amino acid sequences and have similar domains, including the ATPase domain, HSA domain (DNA binding), QLQ domain (protein-protein interaction), and bromodomain (acetyl-lysine histone mark recognition) [30]. Several common members including BAF155, BAF45A/B/C/D, BAF47, BAF53A/B, BAF57, BAF60A/B/C, and β-actin are shared by the complexes [31]. The BAF complexes can be divided into BAF250A-containing BAF-A complexes or BAF250B-containing BAF-B complexes depending on the combination of ATPase and associated subunits. Besides, BAF180- (polybromo-), BAF200-, and BRD7-containing complexes connected to the BRG1 ATPase subunit can form a polybromo-associated BAF (PBAF) complex [32].

Targeting of SWI/SNF to genomic sites is partly modulated via interactions of its associated subunits with transcription factors, histone modifications, and noncoding RNAs (ncRNAs), which were recently described. One example is the interaction between long ncRNA (lncRNA) *SChLAP1* and SMARCB1/SNF5. *SChLAP1* is an aberrantly expressed lncRNA identified in prostate cancer tissues [33], while SNF5 is a core subunit of the SWI/SNF complex that is essential for proper assembly and function of the complex [34]. Direct interaction has been found between *SChLAP1* and SNF5 in human prostate cells, while *SChLAP1* overexpression resulted in decreased SWI/SNF occupancy genome-wide [35].

SWI/SNF is critical for stem cell self-renewal and cell differentiation. BRG1, BAF47, and BAF155 depletion can impair the survival of totipotent cells and cause peri-implantation embryonic lethality in mice [36–38]. BRG1, BAF155, and BAF60A expressions are largely correlated with the reprogramming efficiency of induced pluripotent stem cells in the human population [39]. Some components of SWI/SNF have been involved in a transcriptional network that contains core transcription factors like OCT4, SOX2, and NANOG and maintains pluripotency in stem cells, and the Polycomb group (PcG) proteins modify chromatin to arrest differentiation [13, 40]. Deregulation of BRG1 expression induces MSC senescence with suppressed NANOG, and

Table 1: Types of ATP-dependent chromatin remodeling complexes and their subunits in different lineage specification.

Lineage	Types of ATP-dependent chromatin remodeling complexes			
	SWI/SNF	ISWI	CHD	INO80/SWR
Osteogenesis	BRG1, BAF47, BAF200, BAF180, BRD7		CHD1, CHD7, CHD9	INO80
Neurogenesis	BRG1, BAF45A, BAF53A, BAF53B	SNF2H, SNF2L	CHD4, CHD5, CHD7, CHD8	
Adipogenesis	BRG1, BRM, BAF47			
Cardiomyocytes	BRG1, BAF250A, BAF60C		CHD3, CHD4, CHD7	
Hematopoiesis	BAF180, BAF45A	SNF2H, SNF2L	CHD1	P400
Hepatocytes	BRG1, BRM, BAF250A, BAF47, BAF60A			
Chondrogenesis	BRG1			
Muscle cells				ZNHIT1

this is a part of the transcriptional circuitry that manages stem cell functions [41, 42]. BRG1 downregulation leads to an increase in DNA methyltransferase 1 (DNMT1) and Rb recruitment at the NANOG promoter, thus increasing methylation and transcriptionally silencing NANOG. *BRG1* overexpression induces BRG1 occupancy at the NANOG promoter, thereby increasing chromatin compaction and recruiting HDACs [43]. Furthermore, BRG1 knockdown in hematopoietic stem cells and progenitors was shown to result in a compromised capacity of self-renewal both *in vitro* and *in vivo* [44].

Several studies have indicated that SWI/SNF is required for osteogenic induction (Table 1). Brg1 expression was detected in *ex vivo* osteoblast cultures and in skeletal tissues of mouse embryos [45]. This expression depends on the Runx2 expression induced by BMP2. The osteocalcin (OC) promoter region can recruit BRG1 via the transcription factor C/EBPβ; thus, BRG1 can induce OC expression regulated by RNA polymerase II [46]. Brg1 and P300 can also be recruited by Osterix (Osx) to its target gene promoter *in vivo* enhanced by p38 to form a complex that is transcriptionally active [47]. In addition, Brg1 and Baf47 can interact with C/EBPβ-LAP*, which can bind to the Ric-8B promoter. This leads to Ric-8B expression downregulation in differentiating osteoblasts [48]. Several long-term osteogenic signals specifically upregulate the PBAF subunits BAF180, BAF200, and BRD7 in MSCs. The loss of *Baf180*/*Baf200*/*Brd7* largely compromised the osteogenesis and osteolineage gene expression, while *Baf180* loss was found to impair MSC ossification *in vivo* [49]. By comprehensive mapping, SWI/SNF complexes have also been identified in cartilage-expressed transcripts [50]. FGF receptor 3 (FGFR3) expression, which is critical for developing cartilage, can be induced by BMP2. This process is mediated by Sp1, a downstream mediator, and BRG1 can induce FGFR3 expression by selectively remodeling the Sp1 binding site-containing chromatin region that is located at the FGFR3 transcription start site [51].

ATP-dependent chromatin remodeling is also essential in promoter activation during adipogenic differentiation of MSCs. BRG1 and hBRM can cooperate with C/*EBP*α, C/*EBP*β, C/EBPδ, and PP*AR*γ2 to induce uncommitted fibroblasts into adipocytes [52, 53]. In 3T3-L1 preadipocytes and human MSCs, the depletion of BAF47 repressed adipogenic differentiation by interacting with PPARγ2 and C/EBPβ [54]. CARM1 or PRMT5, which are protein arginine methyltransferases, have also been found to mediate BRG1 binding to the PPARγ promoter [55–57]. In MSC cultures with the induction of adipocyte differentiation, BRG1 overexpression promoted the mature phenotypes that were connected with an obvious increase in the expression of the differentiation markers PPARγ and LPL [41]. Moreover, BRM plays an important role in maintaining the balance of MSC lineage selection between adipocytes and osteoblasts. For example, the depletion of BRM in MSCs favored the osteoblast lineage over the adipocyte lineage because BRM deletion in mice exhibited a rescued phenotype in age-related osteoporosis [58]. Furthermore, differentiated adipocytes have been found to exhibit increased miR-143 expression, while the application of antimiR-143 oligonucleotides could suppress differentiation [59]. miRNA378 expression is also relevant to adipocyte differentiation, and miRNA378 overexpression results in triglyceride accumulation and activation of lipogenic genes like PPARγ2 and GLUT4 [60]. This indicated the possibility that SWI/SNF cooperates with miRNAs to participate in adipogenic differentiation.

SWI/SNF is also important for hepatocyte differentiation. During early liver development, BRM or BRG1 can decrease the expression of tryptophan oxygenase, a gene specific to the late stage [61]. During hepatocyte differentiation, the BRM expression is upregulated by degrees while BRG1 is gradually decreased. BRM or BRG1 deficiency causes decreased albumin expression in hepatocytes because BRM and BRG1 can bind to the promoter region of the albumin gene and C/EBPα and RB family proteins [62]. BAF60A can upregulate PPARα target genes while stimulating β-oxidation of fat in hepatocytes [63]. Moreover, BAF47 deletion is accompanied by decreased levels of most genes involved in liver development [64]. On the other hand, the regeneration of the mammalian liver can be substantially improved by deleting Arid1a, a component of the SWI/SNF complexes. The loss of Arid1a leads to chromatin reprogramming that restricts promoter access by transcription factors like E2F4 and C/EBPα, which inhibit cell cycle reentry and enhance differentiation, respectively [65].

BRG1 is essential for regulating gene expression and the differentiation of cardiomyocytes [66]. In a mouse model, *Brg1* deletion in the developing heart results in dysregulated cardiac gene expression and severe cardiac morphogenesis

anomalies. By mediating remodeling of promoter chromatin and BRG1 recruitment, BAF250A regulates the expression of *Mef2c*, *Nkx2-5*, and *Bmp10* during the differentiation of cardiac progenitor cells into beating cardiomyocytes [67]. In addition, BAF250A can interact with nucleosome remodeling and histone deacetylase (NURD), thus occupying the regulatory regions of genes associated with cardiomyocytes [68]. BAF250A is also critical in normal heart function, confirmed by BAF250A deletion in the sinoatrial node that stops Nkx2.5 repression, resulting in sick sinus diseases [69]. Moreover, BAF60C is crucial in reprogramming fibroblasts into cardiovascular precursors by interacting with other cardiac transcription factors, which indicates the important role of BAF60C in cardiac differentiation [70]. BAF60c can function together with Tbx5 and Gata4, the cardiomyocyte-specific transcription factors, to induce cardiomyocyte differentiation when Nodal/BMP signaling is suppressed [71]. Furthermore, BAF45A or BAF180 deficiency in mice results in hematopoietic system defects characterized by a decreased number of hematopoietic stem cells, impaired potential of long-term repopulating, and abnormal development of the hematopoietic lineage [72, 73].

MSCs could also differentiate into astrocytes or neurons when cultured with retinoic acid and neurotrophic factors derived from the brain [74]. Studies have shown that the deficiency of Brg1 and associated proteins resulted in neuronal disorders [75, 76]. During the differentiation of neurons, BAF53A is compromised and replaced by BAF53B, indicating the importance of SWI/SNF activity for proper neuron development [77]. Knockdown of *Baf45a* and/or *Baf53a* mediated by short hairpin RNA leads to decreased proliferation, while *Baf45a* overexpression improves neural progenitor cell mitosis. The SWI/SNF complex specific to neural progenitors regulates Notch and Shh signaling to promote proliferation and maintain the cells in the transition state from progenitors to postmitotic neurons [77]. Meanwhile, the depletion of BAF53B exhibits obvious defects in dendrite development and in memory [78]. Downregulated proliferation in neural progenitor cells with growth retardation in the cerebellum was also observed in mutations in Brg1 and some other SWI/SNF subunits in mice [79].

3.2. ISWI. ISWI complexes contain one of two conserved ATPase SMARCA5 (SNF2H) or SMARCA1 (SNF2L) along with two to four associated subunits [80]. The expression of *Snf2h*, which is critical for early embryonic development, is ubiquitous in various tissue types, while the *Snf2l* expression is restricted to the brain and postnatal reproductive tissues [80, 81]. Therefore, *Snf2h* loss leads to lethality, while mice with a *Snf2l* deficiency can still survive [82]. Furthermore, ISWI is required for nuclear organization and nucleosomal periodicity, and transcription factors depend upon specific remodeling pathways for proper genomic binding [83].

ISWI complexes containing either SNF2H or SNF2L are critical for ectoderm-derived lineage development [82, 84, 85]. In the nervous system, SNF2H is essential for neural progenitor proliferation, which can be partially compensated by SNF2L. Conditional *Snf2h* deletion compromises the proliferation of granule neuron progenitors and Purkinje cells with increased cell death, which leads to defects in postnatal neural maturation [84]. On the contrary, SNF2L was found to decrease the proliferation of neural progenitors to maintain the correct brain size. SNF2L also represses the expression of the transcription factor gene *Foxg1* by binding to its promoter region. Therefore, SNF2L is required to maintain the balance between proliferation and differentiation of neural progenitors during brain development. This can be confirmed by the increased proliferation and self-renewal of neural progenitors in conditional *Snf2l* mutants accompanied by increased FOXG1 expression [82].

ISWI is also essential in mesoderm-derived lineage differentiation. The nucleosome remodeling factor (NURF) complex, which includes SNF2L-containing ISWI, plays an important role in erythropoiesis [81]. On the other hand, SNF2H is essential for hematopoietic progenitor proliferation at an early stage during erythropoiesis [81]. Therefore, the complexes with SNF2H or SNF2L function differently during the early and late stages of hematopoiesis. SNF2L-containing NURF is also required in thymocyte development [86]. Bromodomain PHD finger transcription factor (BPTF), one of the NURF subunits, is critical for CD4 or CD8 single-positive cells to differentiate into mature T cells by regulating DNase I hypersensitivity and cooperating with the transcription factor SRF to mediate the binding of NURF to *Egr1*, a gene specific to thymocyte maturation [86]. *Bptf* mutants were not able to differentiate any ectoderm, endoderm, or mesoderm tissue types, suggesting the important role of BPTF in germ layer formation. In addition, *Bptf* mutants failed to form distal ventral endoderm, and the expression of SMAD-responsive genes depended upon BPTF, suggesting that NURF functions as a transcription cofactor for SMAD [87]. SNF2L is also critical for granulosa cell proliferation and differentiation during folliculogenesis [88, 89]. *Snf2l* mutant mice responded differently under gonadotropin induction, and thus, they yielded significantly fewer eggs and exhibited fewer secondary follicles compared to control WT mice. The study also indicated that *Fgl2* transcription, which can encode a prothrombinase for mouse reproduction to mediate folliculogenesis, is regulated by *Snf2l* [89].

Many genes are heterochromatinized upon differentiation, and thus, regularly spaced nucleosomes are needed for higher order compaction. The ISWI-containing chromatin remodeling complex ACF1 is required for nucleosome assembly. In the meantime, centromeric chromatin is assembled by RSF1, while heterochromatin formation is regulated by NoRC; thus, rDNA repeats can be silenced [90–92]. Deficiency in either *Drosophila* ISWI or BPTF leads to repressed histone H1 levels and a general male X chromosome decondensation [93, 94]. Therefore, ISWI-regulated histone H1 deposition and nucleosome spacing result in higher order chromatin structures and gene repression, which play an important role during the transition between the progenitor cell and the differentiated cell fate [95].

Taken together, ISWI complexes have been shown to have specific roles in cell proliferation, differentiation, or maturation (Table 1). SNF2H-containing ISWI complexes mainly participate in early development and progenitor cell

proliferation, while the complexes containing SNF2L are mostly involved in cell differentiation and maturation.

3.3. CHD. Nine chromodomain helicase DNA-binding (CHD) proteins (CHD1-9), which can either function alone or cooperate with other proteins to form the complexes, constitute a CHD subfamily. Among them, different CHD complexes have distinct roles in early development and cell lineage differentiation.

CHD1 has been shown to be required for maintaining the self-renewal ability and pluripotency of embryonic stem cells [96, 97]. CHD1 was found to interact with RNA polymerases I/II to regulate the transcription of both rRNA and mRNA and maintain proper transcriptional output [98]. CHD1 is also involved in endothelial to hematopoietic transition (EHT), by which hematopoietic stem cells and progenitors derive from endothelial cells in various organs. However, CHD1 is not essential before or after hematopoietic stem cell and progenitor formation, and CHD1 functions to induce the high transcriptional output of hematopoietic progenitors only in a specific time window [99].

In the developing brain, the NuRD complex, which contains CHD4, is required for synapse formation [100]. This complex can compromise a set of developmentally downregulated genes in presynaptic granule neurons to drive synaptogenesis. However, CHD5 is involved in neuronal differentiation to inhibit nonneuronal lineage genes [101–103]. In addition, CHD7 is essential for maintaining the quiescence of neural stem cells in adults by repressing a number of cell cycle activators and inducing Notch signaling [104]. Moreover, CHD7 is critical for neurogenesis during the morphogenesis of the inner ear [105]. In contrast, CHD8 is associated with autism spectrum disorder (ASD). By decreasing half the dose of *Chd8* in neural progenitor cells, the neural developmental genes containing those ASD-related genes were downregulated [106].

CHD complexes also play an important role in heart development. A NuRD complex containing CHD3 or CHD4 is involved in the proliferation of cardiomyocytes by interacting with the transcription factor FOG2 [107]. Once the interaction between FOG2 and NuRD is impaired, it may lead to perinatal lethality because of a thin ventricular myocardium and defects in the atrial and ventricular septum. The FOG2-NuRD interaction maintains cardiomyocyte proliferation by inhibiting *Cdkn1a*, which is a cell cycle inhibitor gene. Therefore, the disruption phenotype in the *FOG2-NuRD* interaction can be rescued through *Cdkn1a* deletion. Furthermore, CHD7 is involved in transcription activity in various heart development processes. *Chd7* mutant mice exhibited CHARGE syndrome in cardiac aspects [108, 109] while CHD7 mutations have been discovered in sporadic cases in congenital human heart defects [110].

In a well-established MSC model with the induction of osteoblast lineage differentiation, CHD1 is essential for osteogenesis by regulating the transcriptional program of osteoblast differentiation, specifically at later stages. Moreover, CHD1 depletion was shown to reduce the induction of lineage-specific genes in adipocyte differentiation, indicating that CHD1 has a more general role in regulating transcriptional programs related to MSC differentiation [111]. CHD7 is also important in osteogenic differentiation since the expression of CHD7 can be induced in MSCs under osteogenic induction medium conditions while CHD7 depletion in MSCs leads to the repression of several osteogenic transcription factors and decreased MSC osteogenesis capability [112]. ChIP analysis showed that CHD9 can bind to skeletal tissue-specific promoters expressed at different stages during osteoprogenitor differentiation. The interactions between CHD9 and the promoter regions involved in the osteogenic process demonstrate the importance of CHD9 in the transcription process in osteoprogenitor cells and its possible role in the MSC maturation direction [113–115]. Another study indicated that nucleolar CHD9 acts as a ribosomal gene transcription regulator, which has also been implicated in cell fate and differentiation of MSCs [116].

Overall, CHD complexes function to regulate transcription or suppression of different genes and induce various lineage differentiations in MSCs (Table 1). This process relies on the cooperation of CHD complexes with histone modifiers and transcription factors specific to different lineages.

3.4. INO80/SWR. The ATPase subunits of INO80/SWR are another subfamily of ATP-dependent chromatin remodeling complexes that exhibit a conserved insertion in the ATPase/-helicase domain. This is required for the interaction between RVB1/RVB2 helicase and these complexes [117]. The INO80 subfamily includes the INO80 complex [118], while SWR is comprised of P400/TIP60 and SRCAP [119]. Histone variant H2A.Z exchange and ATP-dependent nucleosome mobilization are present in INO80-involving chromatin remodeling [120]. However, SWR complexes are mostly required in the process of H2A.Z deposition into nucleosomes that contain H2A [117].

MSCs transfected with siRNAs targeting INO80 resulted in an impaired mineral deposition in osteogenic induction conditions, and the implanted mice with INO80-silencing MSCs also exhibited decreased bone formation. This suggests the essential role of the INO80 complex in MSC osteogenic differentiation and its potential application in tissue engineering in the clinic and osteoporosis treatment [121] (Table 1). INO80 is critical for meiotic recombination during spermatogenesis [122]. A conditional *Ino80* mutation in spermatogonia before meiosis led to reduced synapse formation and double-strand break defects [123, 124]. P400 (EP400), the subunit of the SWR complex, plays an important role during hematopoiesis by regulating the expression of several embryonic globin genes and deregulating HOX gene expression [125, 126]. In bone marrow cells, P400 conditional knockout led to impaired stem and progenitor cell pool of hematopoiesis because of the progression defects in the cell cycle [125]. Moreover, $P18^{Hamlet}$ (ZNHIT1), a SRCAP subunit, is required for muscle differentiation [127]. $P18^{Hamlet}$ is phosphorylated at the promoter region of *Myog*, a muscle-specific transcription factor gene. H2A.Z is then recruited to phosphorylated $P18^{Hamlet}$/SRCAP, forming the chromatin structure necessary for *Myog* transcription.

4. Conclusion

Over the past years, MSCs have become the focus of intense interest. Thus, they have been investigated for their capacities for self-renewal and lineage specification. The application of MSCs has been considered as a solution for the poor ability of adult tissue regeneration and a potential treatment for human diseases. Gene expression programs work at the chromatin level, so the organization of chromatin is essential in both normal and malignant development and tissue regeneration. We propose that the efficiency of differentiation of MSCs into a variety of cell types will be enhanced by modifying the composition of ATP-dependent chromatin remodeling complexes. As mentioned above, ATP-dependent chromatin remodeling complexes catalyze critical functions in self-renewal and multilineage differentiation of MSCs. In addition, the role of ATP-dependent chromatin remodeling in embryonic stem cells in diverse tissue types also raises the possibility that it may have similar functions in MSCs. The diversity of combinations of multiple subunits has specific functions in chromatin remodelers. For example, a specific combination plays an important role in ATP-dependent chromatin remodeling in differentiated cells, while another combination is crucial for some tissue progenitors. Moreover, ATP-dependent chromatin remodelers can regulate specific transcription in various cell types or with different transcriptional programs in the same cell type depending on the collaboration of these chromatin remodelers with histone-modifying complexes that can induce the binding of histone marks to regulatory sites. The insights of the ATP-dependent chromatin remodeling complexes and their roles in MSC fate determination will provide potential strategies for regeneration and cell-based tissue-engineering therapy.

Conflicts of Interest

The authors confirm that this article content has no conflicts of interest.

Acknowledgments

This work was supported by the National Natural Science Foundation of China (NSFC) Grant 81900965 to Wei Du, SCU Postdoctoral Science Foundation (0040304153066 to Wei Du, 0040304153067 to Wen Du), and China Postdoctoral Science Foundation 0040304153062 to Wen Du.

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Hindawi
Stem Cells International
Volume 2020, Article ID 8860185, 7 pages
https://doi.org/10.1155/2020/8860185

Research Article

Histone Demethylase KDM4C Is Required for Ovarian Cancer Stem Cell Maintenance

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Received 8 June 2020; Revised 24 July 2020; Accepted 7 August 2020; Published 29 August 2020

Academic Editor: De-Meng Chen

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Ovarian cancer is a highly deadly disease, which is often diagnosed at a late stage with metastases. However, most ovarian cancers relapse after surgery combined with platinum-based chemotherapy. Cancer stem cells (CSCs) are stem-like cells that possess high tumorigenic capability and display higher resistant capability against current therapies. However, our knowledge of ovarian CSCs and their molecular mechanism remains sparse. In the current study, we found that KDM4C, a histone demethylase, was required for ovarian cancer stem cell (CSC) maintenance. Depletion of KDM4C significantly reduced the CSC population and sphere formation in vitro. Moreover, we found that KDM4C can regulate the expression of stem cell factor OCT-4 via binding to its promoter. These data indicate that KDM4C is relevant for ovarian CSC maintenance and underscore its importance as a potential therapeutic target.

1. Introduction

Ovarian cancer has the highest mortality rate of any gynecological malignancies around the world [1]. Ovarian cancer has an asymptomatic onset, and the majority of cases diagnosed are in the late stages with metastasis. Currently, surgery and chemotherapy are the main treatments for ovarian cancer, whereas a large majority of patients with advanced ovarian cancer relapse due to therapy resistance [2]. To overcome this dilemma in ovarian cancer, it is important to understand the molecular mechanism underlying therapy resistance of ovarian cancer [3]. The recently proposed hypothesis of cancer stem cells (CSCs), also known as tumor-initiating cells, may provide a more effective approach for the treatment of ovarian cancer. CSCs are stem-like cells that possess the tumorigenic capability to self-renew and differentiate into multiple cell types [4]. Moreover, CSCs display higher resistant capability against current therapies and are believed to be responsible for tumor metastasis [5, 6]. Hence, understanding of the molecular regulation mechanism of CSCs might provide new targets for treatment of ovarian cancer.

In recent years, histone modifications, such as histone methylation and acetylation, are emerging as critical mechanisms to regulate gene expression [7]. It plays an important role for coordination and organization of the chromatin structure during a variety of biological processes such as DNA replication, repair, and transcription [7]. Histone methylation status has also been involved in induced pluripotent stem (iPS) cell reprogramming by expression of OCT-4 and Sox2 [8]. In many tumors, changes of histone demethylase expression have been identified as a key characteristic during cancer initiation and progression, suggesting that these genes might be functionally important for cancer development [9]. Indeed, histone demethylases, such as KDM3A and KDM6A, have been shown to be essential for maintenance of CSCs in several types of cancers [10–13].

Deregulation of KDM4C, a H3K9me3 and H3K9me2 demethylase [14], has been identified in several solid tumors, such as esophageal squamous carcinoma, lung cancer, pancreas cancer, and breast cancer [15–18]. In this study, we used the sphere culture approach to enrich the CSC population of ovarian cancer and found that KDM4C is upregulated in the tumospheres. Furthermore, we found that depletion of KDM4C inhibits the migration, invasion, and CSC properties of ovarian cancer cells. Finally, our data revealed that KDM4C can regulate the expression of stem cell factor OCT-4 via binding to its promoter. In summary, we identify a crucial role of KDM4C in the maintenance of CSCs in ovarian cancer.

2. Results

2.1. KDM4C Is Upregulated in the CSC Population in Ovarian Cancer Cell Lines. Cancer stem cells display greater ability to form spheres when placed in low attachment conditions in defined serum-free media (Figure 1(a)). To investigate the epigenetic regulation in ovarian cancer cells, we first performed a qRT-PCR assay to detect the transcription level of 21 histone demethylases in SK-OV-3 ovarian cancer cells grown under adherent or sphere-forming conditions (Figures 1(a) and 1(b)). Our results demonstrated that KDM4C and KDM5C were significantly increased in SK-OV-3 spheres, while the rest of the histone demethylases showed comparable mRNA expression levels (Figure 1(b)). We observed a similar elevation of KDM4C mRNA expression and a significant decrease of KDM3C expression when comparing the adherent and sphere culture of HO-8910 ovarian cancer cells (Figure 1(c)). Together, our data suggested that KDM4C might play a role in ovarian CSC maintenance. In addition, Western Blot results showed that the KDM4C protein level was increased in the SK-OV-3 and the HO-8910 sphere when compared with the monolayer culture (Figure 1(d)). Consistent with previous reports, stem cell factor OCT-4 was also enriched in these CSCs (Figure 1(d)) [19].

2.2. Downregulation of KDM4C Inhibits Cell Migration and Invasion of Ovarian Cancer Cells. Next, we sought to investigate the function of KDM4C in the ovarian CSCs by generation of two stably transfected KDM4C shRNA in SK-OV-3 and HO-8910 cells. Both cell lines transfected with shRNA showed dramatic inhibition of KDM4C mRNA and protein levels compared with the cells transfected with scramble control shRNA (Figures 2(a) and 2(b)). MTT assay results showed that inhibition of KDM4C did not affect the cell proliferation of SK-OV-3 and HO-8910 cells (Figure S1). Transwell assay results showed that KDM4C downregulation significantly reduced the invasiveness of SK-OV-3 and HO-8910 cells (Figures 2(c)–2(e)). In addition, depletion of KDM4C led to inhibition of the migration ability of SK-OV-3 and HO-8910 cells (Figures 2(f)–2(h)).

2.3. Downregulation of KDM4C Inhibits CSC Properties of Ovarian Cancer Cells. Sphere formation and colony formation assays were performed to examine the effect of KDM4C on CSC properties of SK-OV-3 and HO-8910 cells. Our results revealed that the sphere-forming capacity was markedly diminished after depletion of KDM4C in SK-OV-3 and HO-8910 cells (Figures 3(a)–3(c)). In addition, cell colonies were significantly reduced after KDM4C knockdown in SK-OV-3 and HO-8910 cells (Figures 3(d)–3(f)). Furthermore, we evaluated the aldehyde dehydrogenase (ALDH) activity, a known stem cell feature, of ovarian cancer cells after KDM4C knockdown using flow cytometry. The results showed that the percentage of the ALDL^{high} population was reduced in the KDM4C knockdown cells compared with the control (Figure 3(g)). Altogether, these results indicated that KDM4C is required for maintenance of CSC characteristics in ovarian cancer cells.

2.4. KDM4C Regulates OCT-4 Gene Expression via Binding to Its Promoter. KDM4C-mediated H3K9me3 histone di- and trimethylation can regulate gene transcription. To study the role of KDM4C in OCT-4 gene expression, we performed chromatin immunoprecipitation (ChIP) assays to examine whether KDM4C can directly bind to the promoter region of OCT-4 using the SK-OV-3 sphere. Indeed, our results showed that KDM4C bound to the promoter regions of OCT-4 (Figure 4(a)). In addition, our data revealed that depletion of KDM4C led to a dramatic increase in levels of di- and trimethylated H3K9 histones on the OCT-4 promoter (Figures 4(b) and 4(c)), consistent with the decreased OCT-4 expression in these spheres (Figure 4(d)).

3. Discussion

KDM4C, an oncogene that is frequently amplified in esophageal squamous cell carcinomas, is able to demethylate tri- and dimethylated lysine 9 on histone H3 and activates subsequent oncogenic pathways [14, 18]. Furthermore, the important role of KDM4C in CSC maintenance has made this epigenetic factor a promising target for cancer intervention [20–22].

In this study, we used the sphere culture assay to enrich the CSC population in ovarian cancer cells. We screened 21 histone demethylases and identified that KDM4C is upregulated in both SK-OV-3 and HO-8910 cells, suggesting a role of KDM4C in regulating “stemness” of ovarian cancer cells. We found that silencing of KDM4C led to repressed cell migration and invasion. Consistently, a previous study has shown that KDM4C can increase cell migration and invasion via CUL4A in lung cancer [16]. In addition, KDM4C can interact with chromosomes during mitosis to regulate the breast cell proliferation, migration, and invasion [17], suggesting a common oncogenic role of KDM4C in different cancer types.

Our results demonstrated that KDM4C is required for CSC properties. Depletion of KDM4C reduced the sphere-forming, colony-forming ability and the proportion of ALDH^{high} population in ovarian cancer cells. It has been reported that stem cell-like chromatin features in human glioblastoma CSCs are linked to a loss of the H3K9me3 mark [22]. Genetic knockout mouse model results also

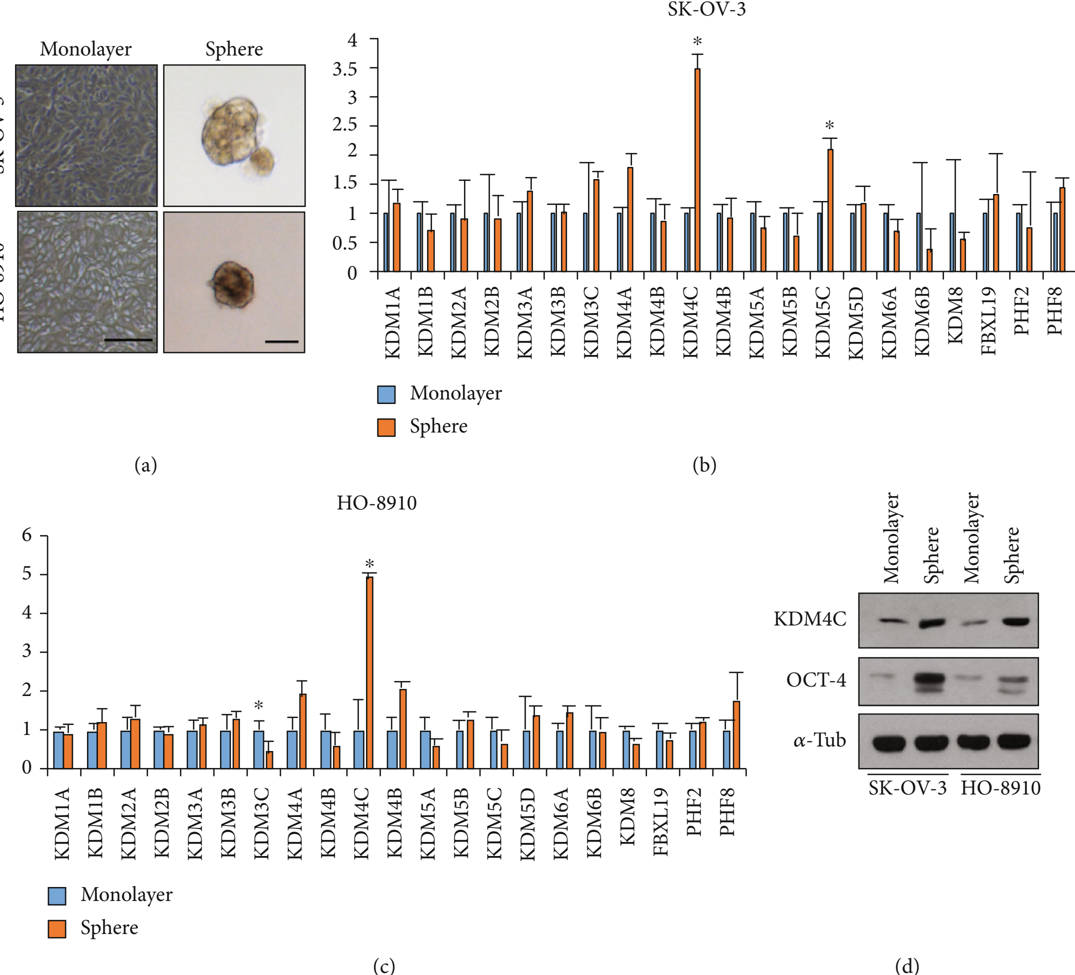

Figure 1: KDM4C is elevated in the tumorsphere of ovarian cancer cell lines. (a) Representative images of the monolayer and spheres of SK-OV-3 and HO-8910 cells. Scale bar, 100 μm. (b) The mRNA expression of histone demethylases in the monolayer and spheres of SK-OV-3 cells was assessed by real-time RT-PCR. (c) The mRNA expression of histone demethylases in the monolayer and spheres of HO-8910 cells was assessed by real-time RT-PCR. (d) Western Blot analysis of the monolayer or spheres of SK-OV-3 and HO-8910 cells for KDM4C and OCT-4 expression. α-Tubulin is used as the control. $^*P < 0.05$.

demonstrated that KDM4 activity is required for hematopoietic stem cell maintenance via accumulation of H3K9me3 on transcription start sites of stem cell-related genes [23]. In addition, our data revealed that KDM4C directly binds to the promoter region of pluripotency factor OCT-4 and regulates its expression. In conclusion, our data provide a novel epigenetic mechanism of CSC regulation in ovarian cancer.

4. Materials and Methods

4.1. Cell Culture. The SK-OV-3 and HO-8910 human ovarian cancer cell line was purchased from China National Infrastructure of Cell Line Resource. For the monolayer culture, cells were maintained in Dulbecco's modified Eagle's medium (DMEM, Invitrogen, China), containing 10% fetal bovine serum (FBS, Gibco, China) and 1% penicillin/streptomycin (Invitrogen, China) at 37°C under an atmosphere of 5% carbon dioxide and 95% air. For the sphere-forming assay, cells were seeded into 6-well ultralow attachment plates (Corning, China) and cultured under 1 : 1 DMEM/F12 medium-containing N2 supplement (100x, Invitrogen, China), B27 supplement (50x, Invitrogen, China), basic fibroblast growth factor (bFGF; 10 ng/ml, PeproTech, China), and human recombinant epidermal growth factor (EGF; 10 ng/ml, PeproTech, China).

4.2. shRNA Transfection. Lentiviral particles containing shRNA against human KDM4C and scrambled lentiviral particles were purchased from GenScript (China). In summary, SK-OV-3 and HO-8910 were cultured up to 70% confluence and were then treated with polybrene (Solarbio, China) and lentiviral particles containing shRNA against KDM4C or scrambled particles. Transfected cells were then selected using puromycin (MCE, China), and knockdown efficiency of KDM4C was assessed by qPCR and Western Blot.

4.3. Detection of Gene Expression by qRT-PCR and Western Blot. Quantitative reverse-transcription polymerase chain

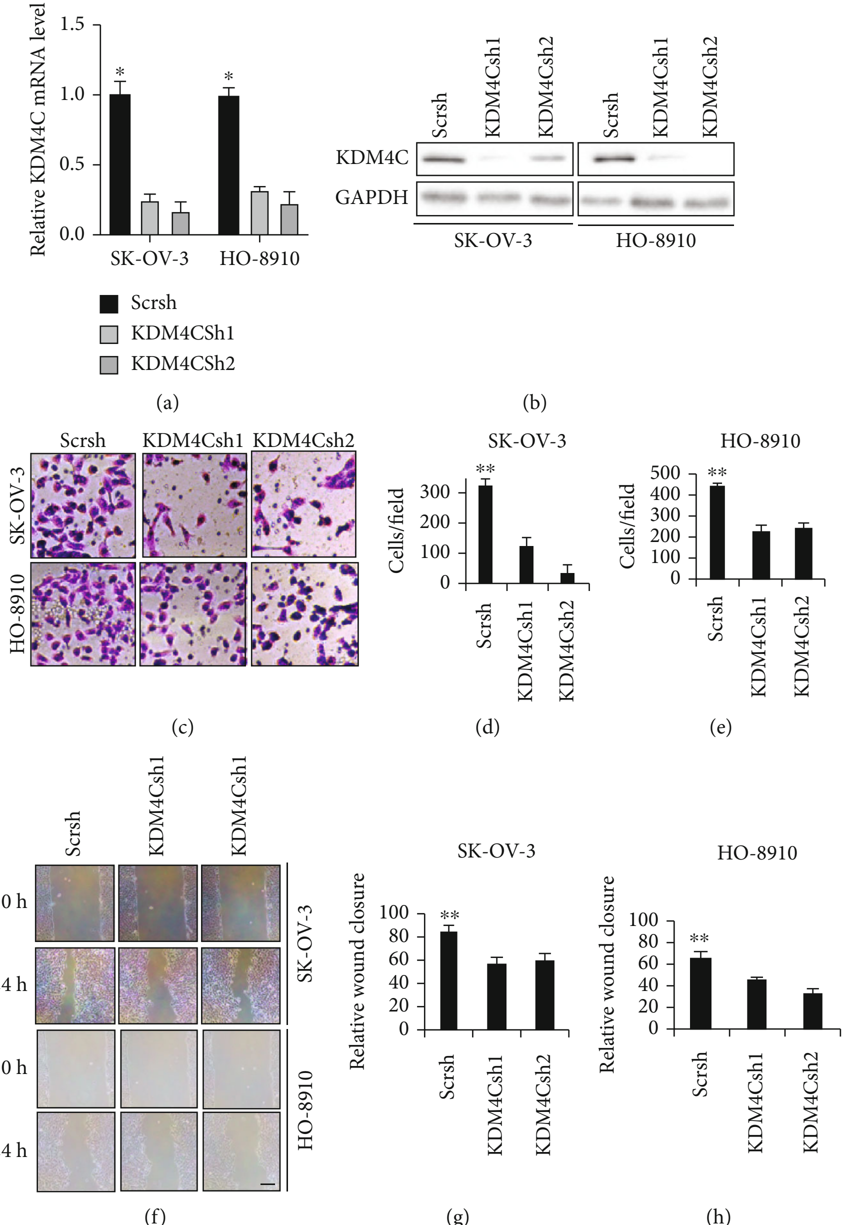

Figure 2: KDM4C is required for the invasion and migration ability of ovarian cancer cells. (a, b) The knockdown of KDM4C by shRNA. KDM4C expression was examined by qRT-PCR and Western Blot analysis. Scramble shell (Scrsh) was used as the control. (c, d) Cell invasive ability of SK-OV-3 and HO-8910 cells determined by transwell assays was decreased after stable knockdown of KDM4C. (e, f) Migratory potential of SK-OV-3 and HO-8910 cells determined by wound-healing assays was decreased after stable knockdown of KDM4C. Scale bar, 100 μm. $^{*}P < 0.05$, $^{**}P < 0.01$.

reaction (qRT-PCR) was used to determine the mRNA expression levels of genes. Briefly, total RNA was extracted from cells by using the TRIzol reagent (Invitrogen, China) according to the manufacturer's instructions. RNA was reverse transcribed to cDNA by using a Reverse Transcription Kit (Takara, China). Real-time PCR analyses were performed with Power SYBR Green (Takara, China), and the primers were synthesized at Invitrogen, China. The primers for each gene were based on a previous report [24]. Results were normalized to the expression of the human β-actin gene.

For the Western Blot analysis, the total protein was lysed in a radioimmunoprecipitation assay buffer (RIPA buffer, Solarbio, China). Samples were prepared under reducing conditions by using SDS-PAGE gels before being blotted and detected using an anti-KDM4C antibody (Abcam, China), OCT-4 (Abcam, China), and α-tubulin (Abcam, China).

4.4. Analysis of ALDH High Cell Subsets. An ALDEFLUOR assay kit (Stem Cell) was used to measure the aldehyde dehydrogenase (ALDH) enzymatic activity following the

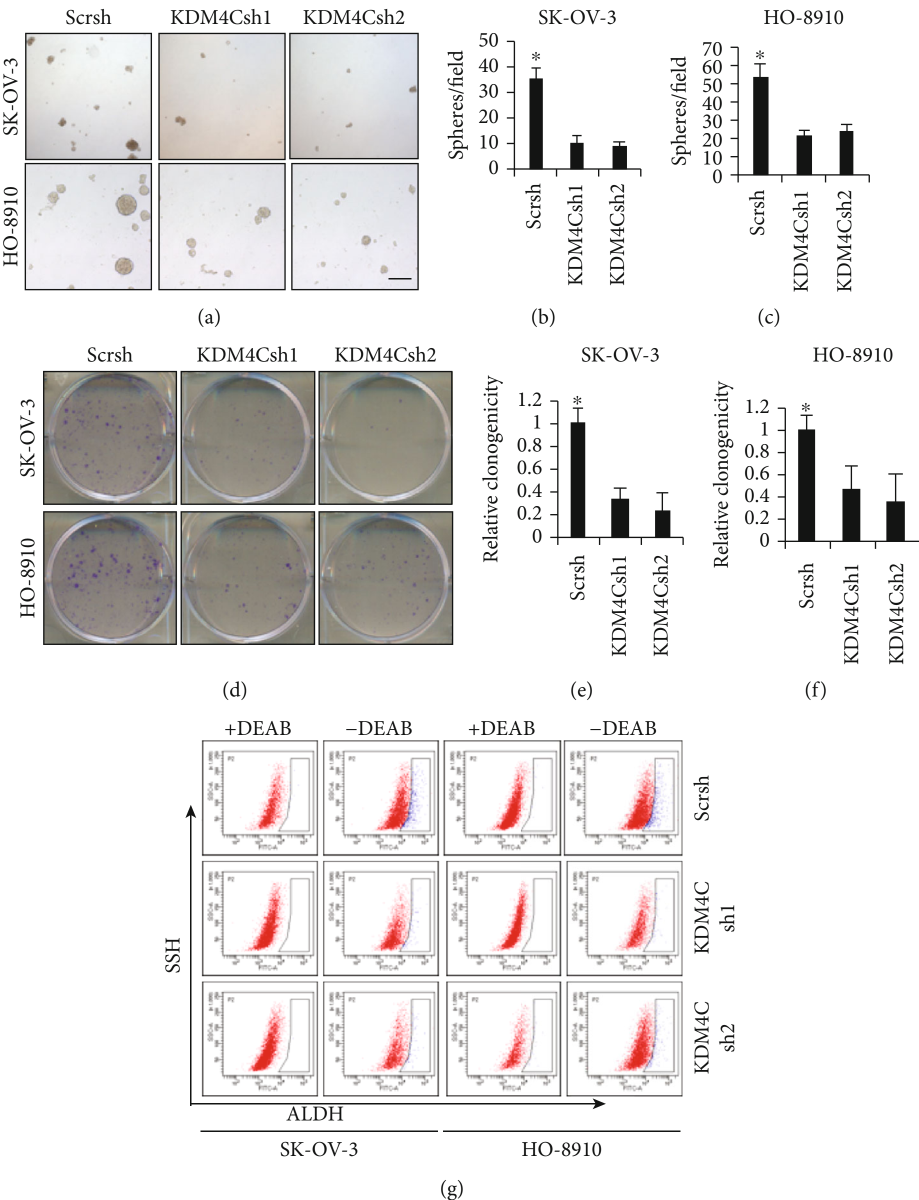

Figure 3: KDM4C is required for stem cell properties of ovarian cancer cells. (a–c) Sphere formation ability of SK-OV-3 and HO-8910 cells was inhibited after KDM4C depletion. Scale bar, 100 μm. (d–f) Colony-forming ability was decreased after knockdown of KDM4C in SK-OV-3 and HO-8910 cells. (g) The percentage of the ALDH^{high} population in SK-OV-3 and HO-8910 cells was reduced after knockdown of KDM4C. $^{*}P < 0.05$.

manufacturer's instructions. In summary, 1,000,000 cells were stained in buffer containing the ALDH substrate with or without DEAB and incubated at 37°C for 30 min. Cells were rinsed in PBS, and the fluorescence intensity was analyzed using a BD FACSCalibur flow cytometer.

4.5. Wound-Healing Assay, Transwell Assay, and MTT Assay. Cell migration capability was evaluated by wound-healing assay. 3×10^5 cells were plated in 6-well plates for 24 h. Monolayer cells were scraped using a sterile 200 μl tip, washed with cold sterile PBS to remove cell debris, and then replenished with fresh culture medium. Representative images were obtained at 0 h and 24 h at 20x magnification using a light microscope. For the transwell assay, 2×10^5 cells were resuspended in serum-free DMEM medium and placed in the transwell chamber (Corning, China), while 600 μl of 1% serum DMEM medium was added to the lower chamber. After incubation for 24 h, cells on the upper surface of the transwell chamber were removed with a cotton swab. The chamber was washed with PBS, fixed in precooled methanol for 5 min, and stained with 0.1% crystal violet solution for 10 min for image acquisition. MTT assays were performed using the manufacturer's guidance (Thermo Fisher, China).

4.6. ChIP Assays. For each ChIP reaction, 2×10^6 spheroid cells were fixed in formaldehyde for 15 min at 37°C.

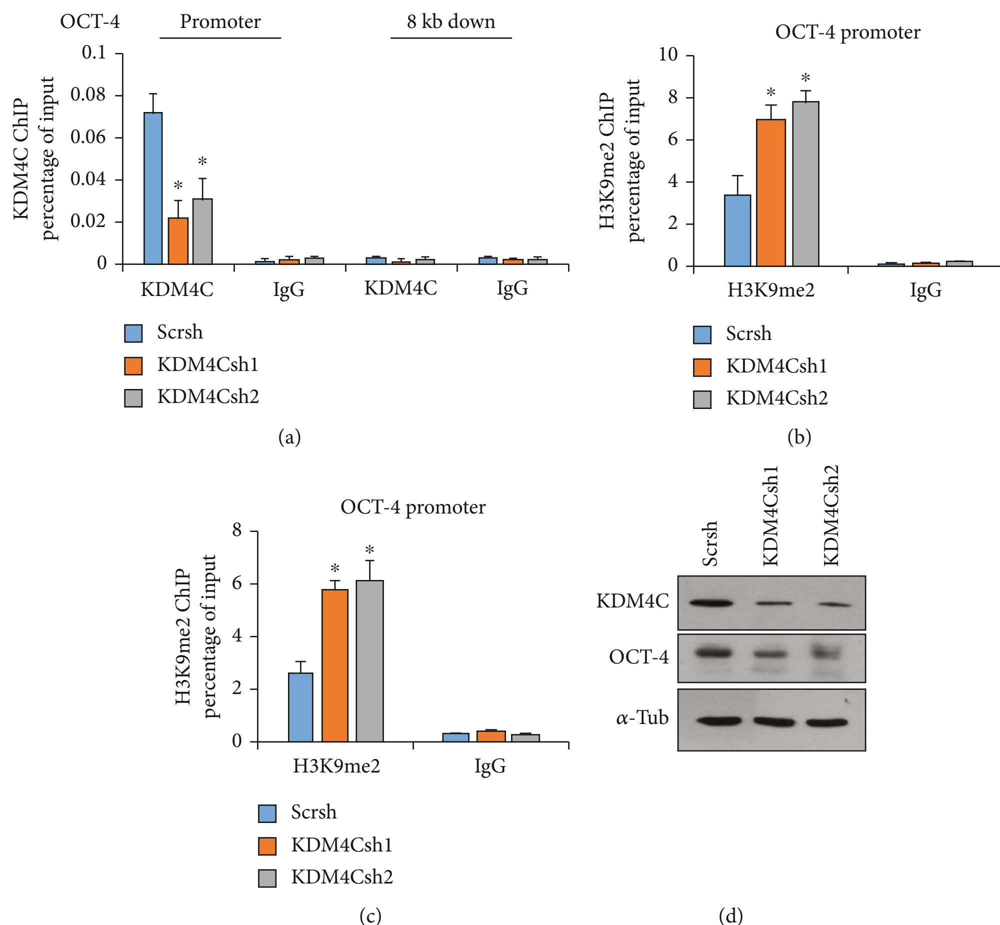

Figure 4: Increased KDM4C occupancy and H3K9 methylation levels at the OCT-4 gene promoter. (a) ChIP analysis of KDM4C occupancy at the OCT-4 promoter after KDM4C inhibition as quantified by real-time PCR. (b) ChIP analysis of H3K9 dimethylation levels at the OCT-4 promoter. (c) ChIP analysis of H3K9 trimethylation levels at the OCT-4 promoter as quantified by real-time PCR. (d) Western Blot analysis of SK-OV-3 spheres for KDM4C and OCT-4 expression. α-Tubulin is used as the control. $^*P < 0.05$.

Crosslinked chromatin was sonicated to obtain 200–500 bp fragments and immunoprecipitated using anti-KDM4C, anti-H3K27me2, or anti-H3K27me3 antibody (Abcam, China). Normal human IgG was used as a negative control.

4.7. Statistical Analysis. Statistical analysis in all the experiments is based on at least three biological replicates, and the error bars are drawn with the standard deviation (SD). The P value is calculated by using Student's t-test.

Data Availability

All data are available upon request.

Conflicts of Interest

No conflicts of interest were disclosed.

Acknowledgments

This work was supported by the National Science Foundation of China (81260385 and 81360383), Clinical Research Project of Shenzhen Municipal Health Commission (SZLY2017017), Doctoral Project of Shenzhen Maternal and Child Health Hospital (FYA2018005), Medical Science and Technology Research Fund Project of Guangdong (A2019363), and Shenzhen Science and Technology Innovation Commission (Grant Numbers ZDSYS20190902092903237, KQJSCX20170728150303243, JCYJ20180305124812444, and JCYJ20170817093928508).

Supplementary Materials

Figure S1: inhibition of KDM4C does not affect proliferation of SK-OV-3 and HO-8910 cells. *(Supplementary Materials)*

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Hindawi
Stem Cells International
Volume 2020, Article ID 8877577, 9 pages
https://doi.org/10.1155/2020/8877577

Research Article

Bmi1 Severs as a Potential Tumor-Initiating Cell Marker and Therapeutic Target in Esophageal Squamous Cell Carcinoma

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Received 21 April 2020; Revised 6 June 2020; Accepted 10 June 2020; Published 20 August 2020

Academic Editor: Quan Yuan

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Esophageal squamous cell carcinoma (ESCC) is a frequent malignant tumor with low 5-year overall survival. Targeting ESCC tumor-initiating cells (TICs) may provide a new research avenue to achieve better therapeutic effects of ESCC. However, the identity and characteristics of ESCC TICs remain poorly understood. Through genetic lineage tracing approach, we found that a group of Moloney murine leukemia virus insertion site 1- (Bmi1-) expressing cell populations present in the invasive front of the esophageal epithelium, providing a continuous flow of tumor cells for ESCC. Subsequently, we found that ablation of $Bmi1^+$ cells from mice with ESCC led to inhibition of tumor growth. In addition, our results demonstrated that PTC-209, an inhibitor of Bmi1, was able to inhibit ESCC progression when combined with cisplatin. In summary, our data suggest that $Bmi1^+$ cells serve as TICs in ESCC.

1. Introduction

Esophageal cancer is one of the most commonly diagnosed cancers, ranking the sixth cancer-related mortality worldwide [1]. It is mainly composed of two histological types: esophageal squamous cell carcinoma (ESCC) and esophageal adenocarcinoma (EAC). ESCC accounts for more than 90% of the whole esophageal cancer cases in China [2]. However, early diagnosis of ESCC is hard to achieve, resulting in a majority of the ESCC patients diagnosed at advanced stages. And the five-year survival rate of ESCC patients remained around 10% owing to high recurrence and distant metastasis [3]. Recently, studies have shown that tumor-initiating cells (TICs) or cancer stem cells are the main cause of tumor recurrence and metastasis [4, 5]. Hence, understanding TICs in ESCC might provide some novel insights in how to improve current treatment of ESCC.

TICs are the cells that are able to self-renew and evolve into the heterogeneous lineages of cancer cells that make up the tumor population [6]. TICs were first identified in leukemia, which have shown that only a limited proportion of transplanted primary tumor cells could cause secondary tumors [7]. Since then, TICs have been successfully isolated from multiple solid tumors by fluorescence-activated cell sorting (FACS) and cell surface markers [8–10]. In ESCC, CD44 is the major marker used in isolation and detection of TICs. For example, $CD44^+/CD24^-$ ESCC cells exhibited higher oncogenous potential in vivo [11]. In addition, selecting cells using both CD44 and ALDH can increase the enrichment effect of TICs by more than 10 times [12]. Other

surface proteins, such as integrin α7, CD90, and Cripto-1, have been reported as potential TIC markers in ESCC [13–15]. However, traditional transplantation assays often disrupt the native microenvironment of TICs and led to alteration of TIC characteristics [16]. To avoid the shortcomings of these assays, lineage tracing of genetically labeled cells has been used to identify TICs in vivo [17–19].

Moloney murine leukemia virus insertion site 1 (Bmi1) is the core component of the polycomb repressive complex 1 (PRC1), which mediates gene silencing through monoubiquitination of histone H2A [20, 21]. Bmi1 functions as a significant stem cell self-renewal factor. It is also involved in multiple tumorigenic processes, including cell migration, invasion, epithelial-mesenchymal transition (EMT), and chemotherapy resistance [22, 23]. The expression of Bmi1 is associated with the progression and invasion of ESCC [24]. Moreover, it is a potential biomarker for the early diagnosis of ESCC [25]. Importantly, the expression of Bmi1 in p75^{NTR}-positive ESCC TICs was higher than that in p75^{NTR}-negative cells [26], which indicates the stemness feature of Bmi1. Study has shown that downregulation of CD44 and Bmi1 in ESCC TICs by administration of nontoxic AUR improved the effect of chemotherapy [27]. Based on these researches, we speculate that Bmi1$^+$ tumor cells might mark CSCs and provide a novel therapeutic molecular target in ESCC.

This article shows that Bmi1$^+$ cells can represent TICs in ESCC and analyzes the related therapeutic value. For this purpose, we used a mature genetic lineage tracing technique, in which the mice with ESCC were induced by 4-nitroquinoline (4NQO). We found that (1) the gene ablation of Bmi1 led to increased apoptosis, decreased proliferation, and weakened stemness of ESCC; (2) the Bmi1$^+$ tumor cells led to the progressive growth of epithelial clones and the Bmi1$^+$ tumor cells were tumor-initiating cells in ESCC; and (3) the cisplatin combined with Bmi1 targeting drug could effectively inhibit tumor growth in ESCC.

2. Materials and Methods

2.1. Animal Assays. All animal use protocols and experiments have been approved by the Institutional Animal Care and Use Committee (IACUC), Sun Yat-sen University. The approval number is SYSU-IACUC-2019-000077. All the animal experiments were carried out in the Laboratory Animal Center, Sun Yat-sen University.

Bmi1CreER, *Rosa^{tdTomato}* and *Rosa^{DTA}* mice were obtained from The Jackson Laboratory. To induce ESCC formation in mice, 4NQO (Sigma, N8141) was prepared with 1,2-propanediol (Sigma, 8223245000) into a 10 mg/mL stock solution and diluted with water for 20 times. The mice aged 8 weeks were fed for 16 weeks and drank normal water for 4 weeks. For lineage tracing, after 16 weeks of 4NQO treatment, in *Bmi1CreER;Rosa^{tdTomato}* or *Bmi1CreER;Rosa^{tdTomato};Rosa^{DTA}* transgenic mice, tamoxifen (0.08 mg/g body weight per day for 3 days; Sigma, T5648-1G) was injected intraperitoneally. Esophageal samples were collected at different time points and frozen sectioned. The expression and distribution of tdTomato fluorescence protein were analyzed under a fluorescence microscope. For drug treatment assay, Bmi1CreER;Rosa^{tdTomato} mice with ESCC were divided into four different treatment groups, including control group, cisplatin (1 mg/g mouse weight every week; Sigma, BP809) treatment group, PTC-209 (1 mg/g mouse weight every week; MCE, HY-15888-5 mg) treatment group, and cisplatin (0.5 mg/g mouse weight every week)+PTC-209 (0.5 mg/g mouse weight every week) treatment group. After 4 weeks of drug treatment, ESCC samples were collected and analyzed.

2.2. Hematoxylin and Eosin Staining. The specimens of ESCC with the adjacent mucosal tissues were fixed in 4% paraformaldehyde (biosharp, BL539A) for up to 24 h and embedded in paraffin. Then, the section with a thickness of 5 μm was cut from the paraffin block and stained with hematoxylin eosin (H&E) kit following the manufacturer's instruction (Solarbio, G1120-100). Briefly, after heating at 65°C, the paraffin sections were dewaxed in xylene and hydrated by serial washing in graded ethanol and distilled water, followed by staining in hematoxylin for 5 minutes, color separation with 1% hydrochloric acid alcohol for 5 seconds, and staining in eosin for 2 minutes. Then, the paraffin sections were dehydrated with graded series of ethanol, removed with xylene, and finally sealed with neutral balsam (Macklin, 822941-100 g).

2.3. Immunohistochemistry (IHC) Staining. Dewaxed paraffin sections were obtained by the same method as mentioned above. Sections were treated with heat-induced epitope recovery with sodium citrate buffer (Bioss, C02-02002), followed with blocking endogenous peroxidase prior to primary antibody incubation. Specific primary antibodies including CD44 (1 : 200; Abcam, ab157107), Tp63 (1 : 200; Abcam, ab53039), and KRT5 (1 : 200; Abcam, ab52635) were used. Horseradish peroxidase (HRP) conjugate was used for DAB staining. Expression levels were based on staining intensity and area of tumor cells.

2.4. Flow Cytometric Sorting. Cancer cells were isolated from the esophagus of tamoxifen-induced *Bmi1CreER;Rosa^{tdTomato}* mice through combining mechanical dissociation with enzymatic degradation by Tumor Dissociation kits (Miltenyi, 130-096-730). To be specific, 0.04-1 g tumor tissue was cut into small pieces of 2-4 mm^3 and was dissociated in a volume of approximately 2.5 mL enzyme mix. Then, isolated cells were filtered through a 75 μm diameter mesh and centrifuged at 300 × g for 7 minutes and supernatant was aspirated completely. Then, cells were washed with 1 × PBS and resuspended with 90 μL 1 × PBS per 10^7 total cells. Then, anti-EpCAM (1 : 500; Abcam, ab221552) was added and reaction solution was incubated for 30 minutes on ice. Cells were washed and resuspended in 500 μL 1 × PBS and incubated with a secondary antibody (1 : 1000; Abcam, ab6717) for 25 minutes on ice. Then, cell sorting was performed on BD FACSAria II. EpCAM$^+$Tomato$^+$ cells were collected. All steps were performed under sterile conditions.

2.5. Tumorsphere Formation Assays. Cells were seeded in 6-well ultralow attachment plates at 3,000 cells/mL in stem cell medium as previously described [18]. Cells were cultured in a humidified 5% CO_2 incubator at 37°C for 7-10 days, during

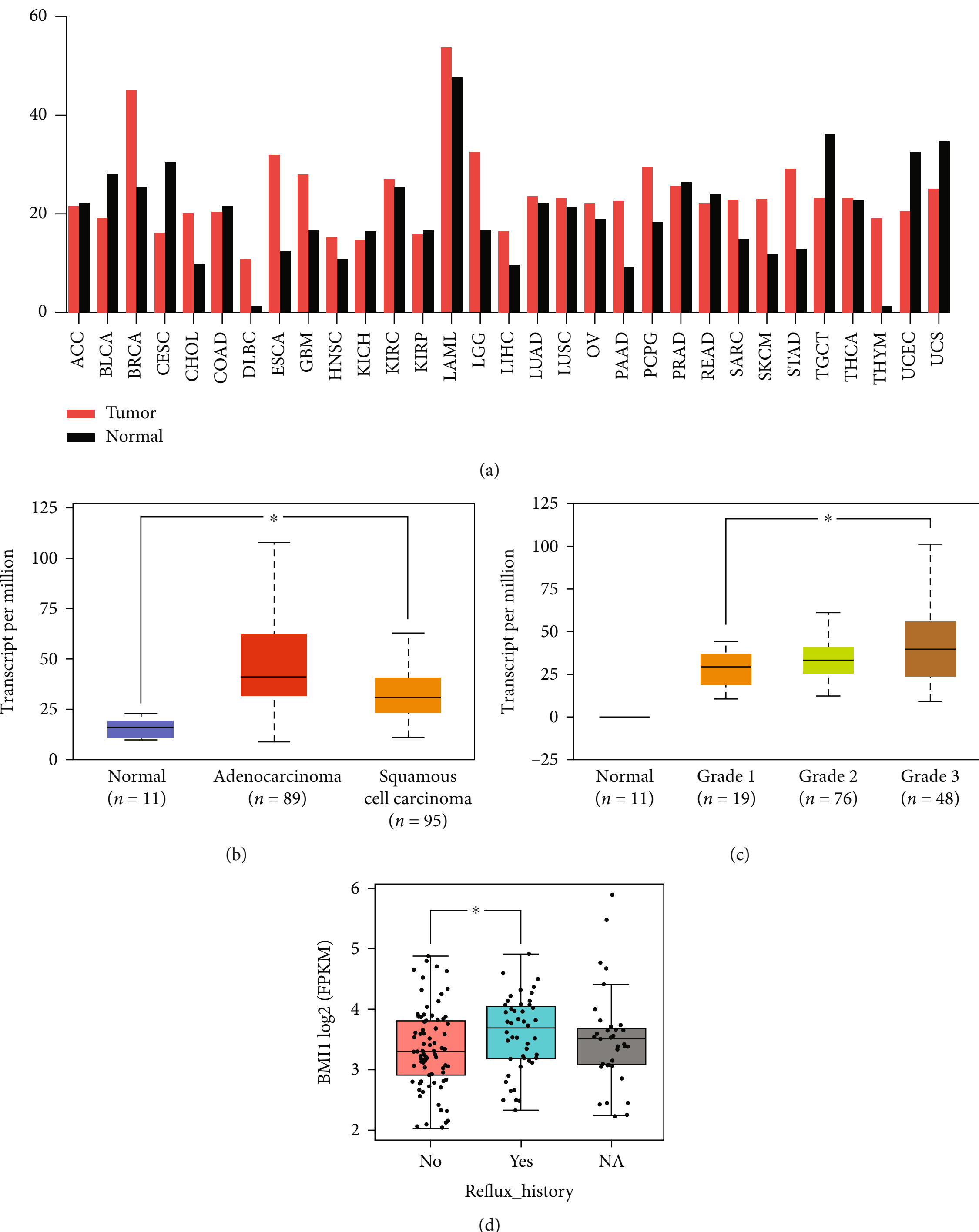

Figure 1: High expression of Bmi1 in esophageal carcinoma. (a) The TCGA database demonstrated that Bmi1 gene expression was significantly upregulated in esophageal carcinoma tissues. (b) Bmi1 expression levels in ESCA based on tumor histology were compared with normal tissues in the TCGA database ($n = 195$). (c) Bmi1 expression levels in different grades of esophageal carcinoma from TCGA database. (d) Gastroesophageal reflux is more likely to occur in patients with high expression of Bmi1. No = patients without gastroesophageal reflux; Yes = patients with gastroesophageal reflux; NA = data missing. $^*p < 0.05$.

which serum-free media were changed every other day until the spheres formed. Then, tumorspheres were collected, washed with $1 \times$ PBS, and incubated with Trypsin-EDTA for two minutes at 37°C. Then, the number of tumorspheres was counted. Three dishes were used for each group and all experiments were repeated three times.

2.6. TUNEL Assays. TUNEL assays are carried out using a commercial kit (KeyGEN, KGA703). According to the instructions provided by the manufacturer, freezing sections were fixed in 4% paraformaldehyde fix solution at room temperature (15-25°C) for 20-30 minutes and rinsed in $1 \times$ PBS three times for 15 minutes. Then, sections were treated with

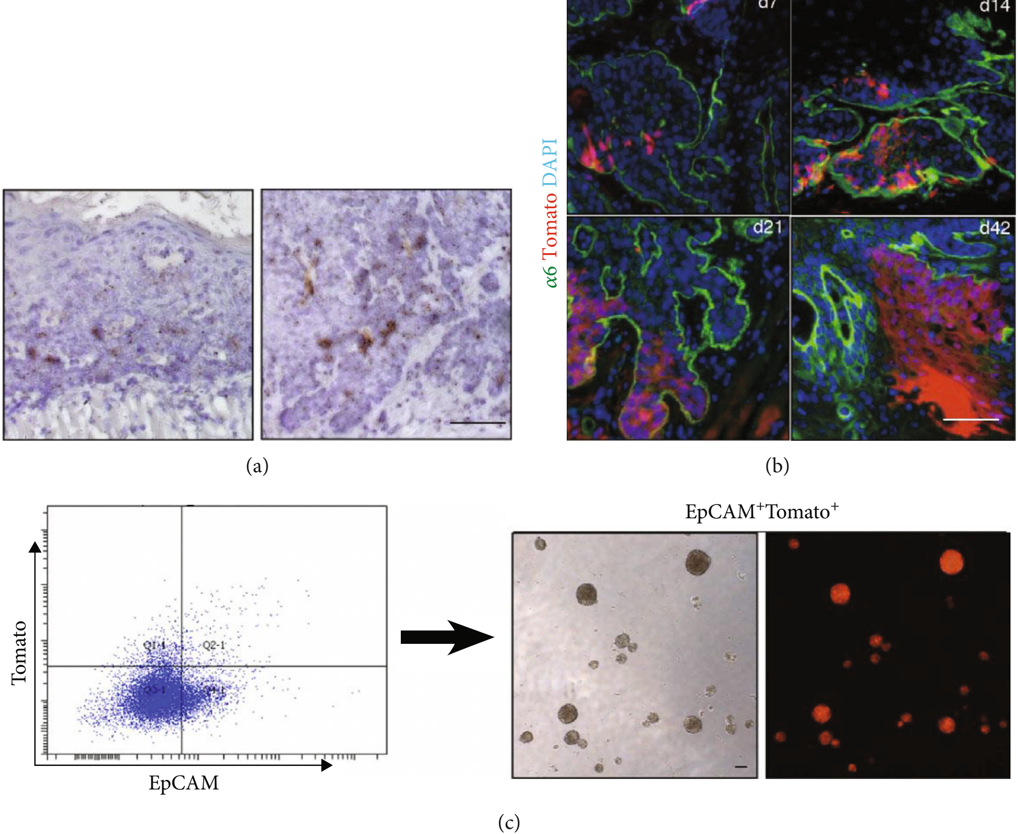

Figure 2: Bmi1$^+$ cells and their progeny cells can self-renew in vivo and in vitro. (a) In situ hybridization of Bmi1 in ESCC samples induced by 4NQO for 15 weeks (left) and 22 weeks (right). The brown area is the expression signal of the Bmi1 gene. (b) Bmi1$^+$ cells are labeled in *Bmi1CreER;Rosa*^{tdTomato} mice following a single dose of tamoxifen and traced for 7, 14, 21, and 42 days. Red represents Tomato-positive Bmi1-expressing cells and their differentiated progeny cells. Green labels integrin $\alpha 6$, a marker of the epithelial basement membrane. Nuclei are labeled in blue. (c) Three days after tamoxifen injection, EpCAM$^+$Tomato$^+$ double-positive cells sorted by flow cytometry were cultured into tumorspheres in vitro. Scale bars are 100 μm.

100 μL proteinase K for 30 minutes at 37°C and rinsed in 1 × PBS three times for 15 minutes again. After being immersed in 3% H_2O_2 sealing liquid for 10 minutes and rinsed, the slides were added with 100 μL DNase I reaction liquid containing 2000-3000 U, 40-60 μL DNase I (50 U/μL), and 60-40 μL DNase I buffer and rinsed. Later, TdT enzyme reaction solution, streptavidin-HRP working fluid, and DAB working fluid were added 50 μL after each time the slides were rinsed in 1 × PBS three times for 15 minutes and drained with blotting paper. Dyed in hematoxylin stain for 30 seconds to 5minutes and washed with distilled water, the slides were put in methanol hydrochloride solution for differentiation for 5 seconds and washed with distilled water again, followed by 70%, 85%, 95%, and absolute ethyl alcohol each for 5-minute rinse and xylene twice for ten minutes. After being dried, the samples were added with neutral balsam (Macklin, 822941-100 g), covered with glass slides, photographed, and observed under an optical microscope.

2.7. In Situ Hybridization. Dewaxed paraffin sections were obtained by the same method as H&E. Incubation with 0.3% H_2O_2 at room temperature for 30 minutes was done to remove endogenous peroxidase activity. After washing with ddH_2O, proteinase K was dripped onto paraffin sections and incubated at 37°C for 25 minutes. Then, the probe-free hybridization solution was added to pre-treat the slice (this step can be omitted). After washing with ddH_2O, the probe-containing hybridization solution was added and incubated at 46°C overnight. Sections were soaked three times with 46°C ddH_2O for 15 minutes each. Peroxidase-labeled sheep anti-digoxin antibody was added and incubated at room temperature for 2 hours. Observation under an optical microscope after color development with DAB was done.

2.8. Statistical Analysis. The two-tailed unpaired Student *t*-test was used to measure statistical significance. Data were presented as mean ± SD. All the data statistical analysis is carried out through GraphPad Prism 8.0. For an alpha probability of 0.05, the sample size needed to detect statistical significance difference is at least 6 animals in each group.

3. Results

3.1. Expression Levels and Clinical Value of Bmi1 in Esophageal Carcinoma. According to the data of The Cancer

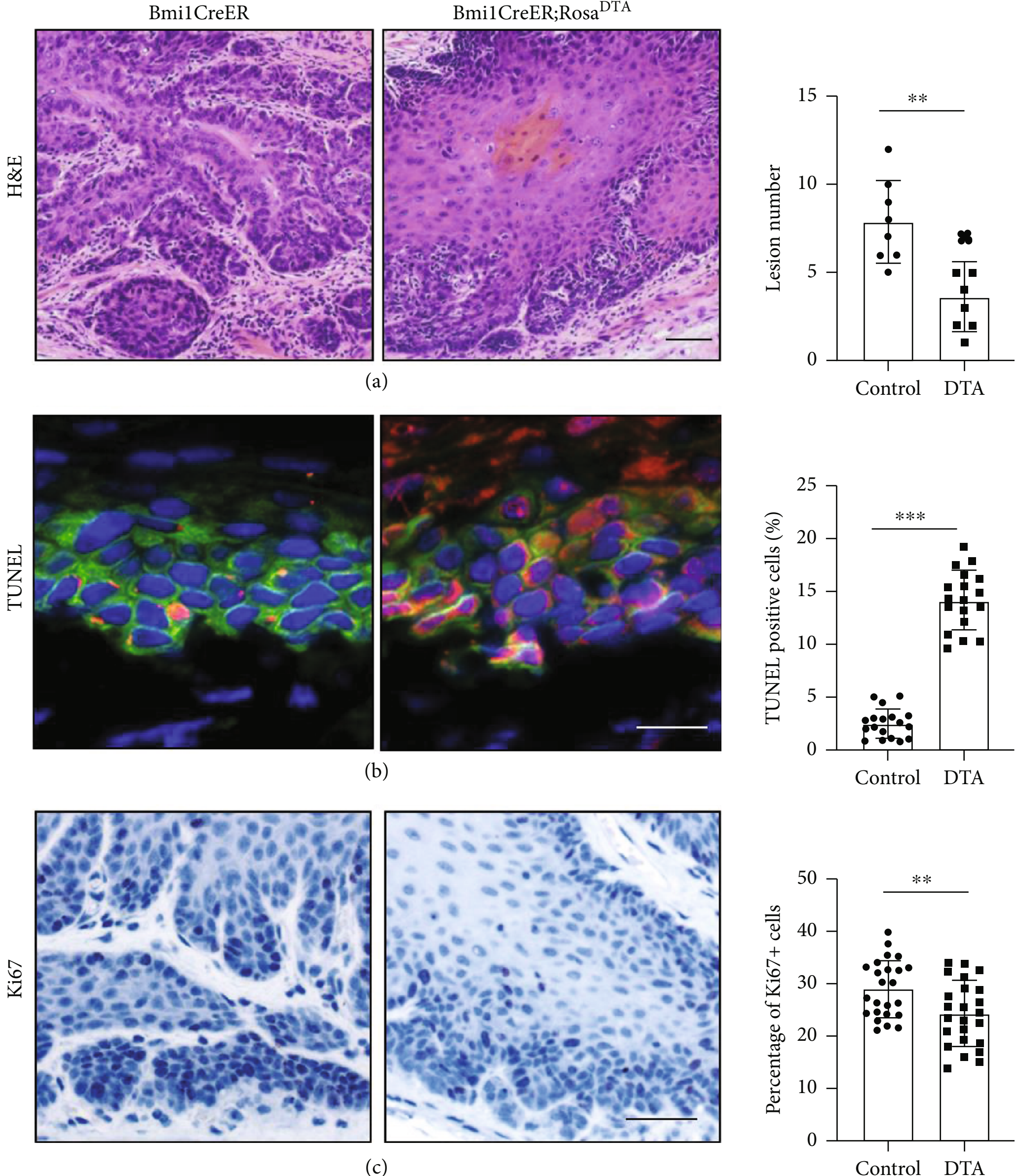

Figure 3: Depletion of Bmi1$^+$ cells led to decreased proliferation and increased apoptotic phenotype in ESCC. (a) *Bmi1CreER;Rosa^{tdTomato};Rosa^{DTA}* mice were compared with the control in the malignant degree of ESCC. (b) Apoptosis of the *Bmi1CreER;Rosa^{tdTomato};Rosa^{DTA}* group was higher than that of the control group under the TUNEL test. Red indicates apoptotic cells. (c) Ki67 signal was reduced in ESCC without Bmi1$^+$ cells, and Ki67 signal was represented by black. $^*p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$. Scale bars are 100 μm.

Genome Atlas (TCGA) database, the expression level of Bmi1 mRNA in esophageal carcinoma (ESCA) was upregulated in the esophageal carcinoma tissues compared with normal tissues (Figure 1(a)). And both ESCC and EAC tissues' Bmi1expression levels were significantly higher than normal tissues (Figure 1(b)). In addition, the expression of Bmi1 was correlated with the tumor grade of ESCA; although there were marginal differences between grades 1 and 2 and grades 2 and 3, there was still significant difference between grades 1 and 3 (Figure 1(c)). Furthermore, the clinical phenotype of Bmi1 expression pattern demonstrated that patients with gastroesophageal reflux tended to express higher Bmi1 leve1 (Figure 1(d)). And gastroesophageal reflux was considered to be one of the precancerous diseases of esophageal cancer [28]. Overall, these data indicated the potential clinical significance of Bmi1 in esophageal cancer patients.

3.2. Bmi1$^+$ Cells Are the TICs in Mouse ESCC. To investigate whether Bmi1 is expressed in the mouse ESCC, we performed in situ hybridization assay. Our results indicate that Bmi1 is expressed in the subsets of ESCC cells in the areas near the basement membrane 15 weeks and 22 weeks after 4NQO treatment (Figure 2(a)), suggesting a possible role of Bmi1-expressing cells. To verify whether Bmi1-expressing

cells have a role in ESCC, we bred *Bmi1CreER;Rosa^{tdTomato}* transgenic mice and carried out lineage tracing assay. Our data showed that a small number of Tomato$^+$ cells were presented near the basement membrane 7 days after tamoxifen injection. In the samples taken at 14 days, 21 days, and 42 days, the number of Tomato$^+$ cells increased with time and distributed in both the epithelial basal layer and the epithelial interstitial layer. Especially on the 42nd day, Tomato$^+$ cells were widely distributed in the esophageal epithelium (Figure 2(b)). This shows that in ESCC, the offspring cells differentiated from Bmi1$^+$ cells located in the basement membrane gradually develop into tumor parenchyma cells of ESCC over time. To further probe the stemness of Bmi1$^+$ cells, we harvested ESCC mice three days after injection of tamoxifen and isolated EpCAM$^+$Tomato$^+$ double-positive cells for sphere formation assay. We found that ESCC Bmi1$^+$ cells could form spheres in vitro (Figure 2(c)). Together, these data showed that the Bmi1$^+$ cells served as TICs in mouse ESCC.

3.3. Bmi1$^+$ Cells Are Critical for ESCC Progression. In order to explore the role of Bmi1$^+$ cells in the progression of ESCC, we also treated *Bmi1CreER;Rosa^{tdTomato}; Rosa^{DTA}* mice with 4NQO. At 16 weeks of 4NQO treatment, Bmi1$^+$ cells underwent apoptosis caused by diphtheria toxin after tamoxifen injection, resulting in almost no Bmi1$^+$ cells in the tumor. After the removal of Bmi1$^+$ cells, we can see that the lesion number was reduced (Figure 3(a)). Our results also showed that after the removal of Bmi1$^+$ cells, the proportion of apoptosis of ESCC cells increased and the ability of proliferation was decreased (Figures 3(b) and 3(c)). These results indicate that Bmi1$^+$ cells are important for the growth of ESCC.

3.4. The Expression of ESCC TIC Markers Was Inhibited after Bmi1$^+$ Cell Ablation. Further, we examined the expression of known ESCC TIC markers, CD44, tp63, and KRT5, in ESCC tissue after Bmi1$^+$ cell removal. Our data showed that Bmi1$^+$ cells in ESCC were almost eliminated after tamoxifen administration (Figure 4(a)). In the absence of Bmi1$^+$ cells, we found that the expression of three tumor stem cell markers (CD44, Tp63, and Krt5) decreased in ESCC (Figures 4(b)–4(d)). It shows that after the scarcity of Bmi1$^+$ cells, the stemness characteristics of ESCC are largely inhibited, suggesting that Bmi1 is important for the stemness of ESCC.

3.5. Cisplatin Combined with PTC-209 in the Treatment of ESCC. Our previous studies have proved that Bmi1 is an important tumor stem cell marker of ESCC, so the targeted therapy of Bmi1$^+$ cells may provide a possibility for the clinical treatment of ESCC. Based on the above data, we carried out the animal drug experiment of cisplatin combined with PTC-209 (a small molecular inhibitor of Bmi1). The experimental results show that although cisplatin and PTC-209 alone have a certain therapeutic effect, the combination of cisplatin and PTC-209 has the least lesion number and the best therapeutic effect (Figures 5(a) and 5(b)).

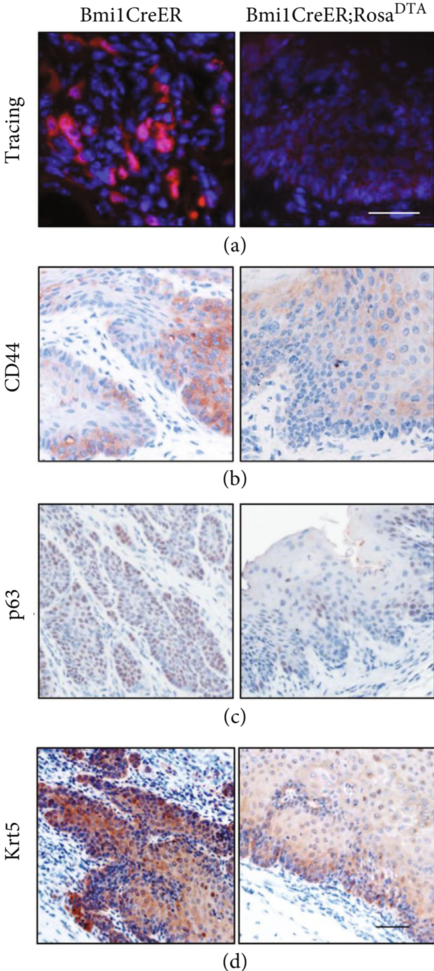

Figure 4: The stemness phenotype of ESCC without Bmi1$^+$ cells was inhibited. (a) Samples of control and *Bmi1CreER; Rosa^{tdTomato};Rosa^{DTA}* mice were collected on the 21st day after a single intraperitoneal injection of tamoxifen. Bmi1$^+$ cells of *Bmi1CreER;Rosa^{tdTomato};Rosa^{DTA}* mice were effectively removed. Red labels Bmi1$^+$ cells and their progenies. (b–d) In ESCC samples, all three types of TIC markers, CD44 (b), tp63 (c), and Krt5 (d), were reduced after Bmi1$^+$ cells were removed. Brown represents the signals of interest. Scale bars are 100 μm.

4. Discussion

Bmi1 plays the important part in maintaining the dynamic balance of mitochondrial function and redox and can function like stem cells and progenitor cells to regulate cellular metabolism [29]. Notably, despite the key role of Bmi1 in the self-renewal of various somatic cancer stem cells that has been reported [30], there is no research focusing on Bmi1 in ESCC. In our study, we first confirmed the high expression of Bmi1 in esophageal carcinoma and patients with high expression prone to malignant transformation. After that, we generated *Bmi1CreER;Rosa^{tdTomato}* transgenic mice and explored the role of Bmi1$^+$ cells as a source of carcinogenesis in ESCC. We utilized genetic lineage tracing analysis [31] and identified a subpopulation of Bmi1$^+$ tumor cells that give rise to progressively growing epithelial clones.

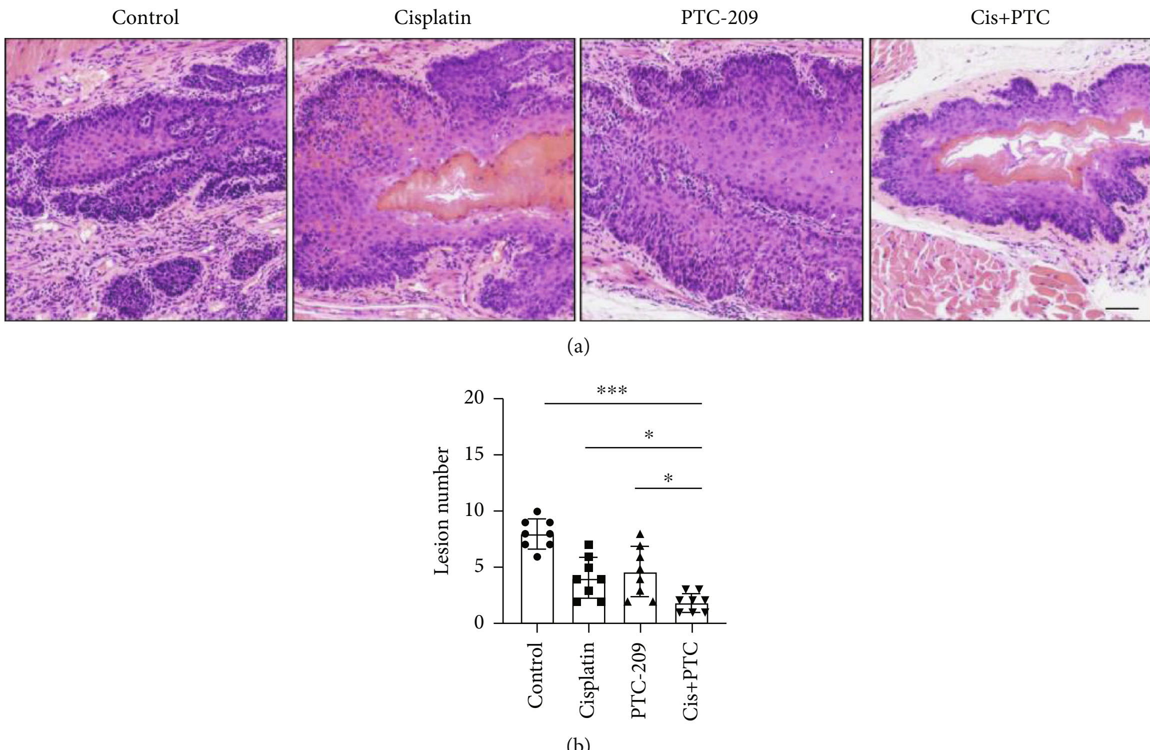

FIGURE 5: Treatment effect of cisplatin combined with PTC-209 on ESCC. (a, b) From left to right, the histopathology (a) and histogram (b) of ESCC in the control group, cisplatin-treated group, PTC-209-treated group, and the cisplatin^{+}PTC-209-treated group. The processing time of the four groups was four weeks. Cisplatin combined with PTC-209 has the lowest malignancy. $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$. Scale bars are 100 μm.

In addition, we demonstrated that genetic ablation of Bmi1 resulted in increased apoptosis and decreased proliferation. We also compared different treatment strategies of ESCC, and we found that the therapeutic effect of targeting both the tumor bulk and TICs was better than monotherapy [32–34].

The esophagus has a keratinized squamous epithelium consisting of four to five cell layers with rapid turnover characteristics, which is maintained by proliferative basal cells. These basal cells can renew, differentiate, and migrate to the lumen, producing the upper basal layer of terminally differentiated cells. In previous studies, clonal analysis using CreER transgenic mice suggests that Sox2- and K15-labeled progenitor and/or stem cell populations have a higher potential for self-renewal than that of committed progenitors [35, 36]. In the present study, Bmi1 actively expressed in the ESCC and Bmi1^{+} cells were enriched in ESCC with stem cell properties. Importantly, Bmi1^{+} cells differentiated into a large chunk of the tumor of the *Bmi1CreER;Rosa^{tdTomato}* mouse over time, consistent with previous studies [37, 38]. Moreover, Bmi1^{+} cell depletion by using Bmi1CreER;Rosa$^{td\text{-}Tomato}$;Rosa^{DTA} mouse further proves that Bmi1^{+} cells function as TICs in ESCC.

In terms of the treatment of esophageal cancer, the effect of monotherapy is not all satisfactory. Cisplatin is capable of killing proliferative cells [39] but has limited effect on relatively resting cells such as TICs, resulting in poor efficacy. Our study showed that Bmi1^{+} cells are endowed with malignant phenotype. We treated mouse with ESCC using monotherapy or combined therapy and analyzed the treated tumors. We found that targeting both the tumor bulk and Bmi1^{+} cells achieved the most efficacious tumor control. Consistently, a recent study on glioblastomas shows that combined Bmi1 targeting and another molecular targeting drug proved more effective than either agent alone both in culture and in vivo [32]. In addition, Bmi1 inhibitor PTC-209 has been proved to inhibit tumor growth by targeting CSC self-renewal in head and neck squamous cell carcinoma [18]. Our results demonstrate the significant efficacy of combined inhibition of Bmi1 and cisplatin, but we did not detect cures: the efficacy against each subtype was preferential, but not absolute. Future studies will determine how to achieve the desired results.

5. Conclusions

To sum up, our data provide specific experimental evidence that Bmi1^{+} cells are the cell origin of ESCC. Monotherapy alone against ESCC tumor growth near the limits of detection and combinational therapy of Bmi1 inhibitor and cisplatin were the most effective in reducing tumor burdens. Although the molecular basis of Bmi1-derived carcinogenesis and the clinical significance of Bmi1-derived ESCC have yet to be further studied, exploring the carcinogenic mechanism of multiple malignant tumors from the perspective of cell origin will provide us with new and promising therapeutic strategies.

Data Availability

The data used to support the findings of this study are available from the corresponding author upon request.

Conflicts of Interest

The authors indicate no potential conflicts.

Authors' Contributions

Xiaochen Wang and Kang Li contributed equally to this work.

Acknowledgments

This paper was supported by grants from the Natural Science Foundation of Guangdong Province (no. 2018A030313610) and the National Natural Science Foundation of China (no. 81872409).

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Hindawi
Stem Cells International
Volume 2020, Article ID 8897138, 14 pages
https://doi.org/10.1155/2020/8897138

Research Article

Ascorbic Acid, Inflammatory Cytokines (IL-1β/TNF-α/IFN-γ), or Their Combination's Effect on Stemness, Proliferation, and Differentiation of Gingival Mesenchymal Stem/Progenitor Cells

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Received 1 May 2020; Revised 16 July 2020; Accepted 30 July 2020; Published 17 August 2020

Academic Editor: De-Meng Chen

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Objective. Ascorbic acid (AA) and controlled inflammatory stimuli are postulated to possess the ability to independently exert positive effects on a variety of proliferative, pluripotency, and differentiation attributes of gingival mesenchymal stem/progenitor cells (G-MSCs). The current study's objective was to explore and compare for the first time the impact of the major inflammatory cytokines (IL-1β/TNF-α/IFN-γ), AA, or their combination on multipotency/pluripotency, proliferative, and differentiation characteristics of G-MSCs. *Design*. Human G-MSCs ($n = 5$) were isolated and cultured in basic medium (control group), in basic medium with major inflammatory cytokines; 1 ng/ml IL-1β, 10 ng/ml TNF-α, and 100 ng/ml IFN-γ (inflammatory group), in basic medium with 250 μmol/l AA (AA group) and in inflammatory medium supplemented by AA (inflammatory/AA group). All media were renewed three times per week. In stimulated G-MSCs intracellular β-catenin at 1 hour, pluripotency gene expression at 1, 3, and 5 days, as well as colony-forming units (CFUs) ability and cellular proliferation over 14 days were examined. Following a five-days stimulation in the designated groups, multilineage differentiation was assessed via qualitative and quantitative histochemistry as well as mRNA expression. *Results*. β-Catenin significantly decreased intracellularly in all experimental groups ($p = 0.002$, Friedman). AA group exhibited significantly higher cellular counts on days 3, 6, 7, and 13 ($p < 0.05$) and the highest CFUs at 14 days [median-CFUs (Q25/Q75); 40 (15/50), $p = 0.043$]. Significantly higher Nanog expression was noted in AA group [median gene-copies/PGK1 (Q25/Q75); 0.0006 (0.0002/0.0007), $p < 0.01$, Wilcoxon-signed-rank]. Significant multilineage differentiation abilities, especially into osteogenic and chondrogenic directions, were further evident in the AA group. *Conclusions*. AA stimulation enhances G-MSCs' stemness, proliferation, and differentiation properties, effects which are associated with a Wnt/β-catenin signaling pathway activation. Apart from initially boosting cellular metabolism as well as Sox2 and Oct4A pluripotency marker expression, inflammation appeared to attenuate these AA-induced positive effects. Current results reveal that for AA to exert its beneficial effects on G-MSCs' cellular attributes, it requires to act in an inflammation-free microenvironment.

1. Introduction

Periodontitis is an inflammatory disorder of tooth-investing and tooth-supporting tissues, branded by a gradual damage of alveolar bone, periodontal attachment, and eventually gingiva, associated with bacterial dysbiosis. The commencement of this multifaceted disease process commonly entails challenging of the periodontal immune-inflammatory system through virulent microbial biofilms. Subsequently, an inflammatory reaction is mounted, with the release of inflammatory cytokines, most prominently tumor necrosis factor alpha (TNF-α), interleukin (IL) 1 beta (IL-1β), IL-4, IL-6, and interferon gamma (IFN-γ) [1]. Duration and intensity of the resultant host reaction govern the personalized course and outcome of the inflammatory process, as well as affect the outcome of any subsequent periodontal reparative/regenerative approach.

Ascorbic acid (AA) is one of the pivotal biomolecule, with decisive effects on wound healing and collagen biosynthesis [2]. Investigations on adult [3, 4], embryonic [5], and induced pluripotent [6] stem/progenitor cells outlined the beneficial effects of AA on increasing cellular proliferation, impeding apoptosis, and triggering pluripotency markers' expression. Furthermore, in additional to its host modulatory effects in periodontal disease [2], it was suggested that AA supplementation could positively affect the outcome of periodontal reparative/regenerative therapies [7, 8].

Gingival mesenchymal stem/progenitor cells (G-MSCs) possess notable periodontal reparative and regenerative potentials [9, 10], with inflammation-resistance properties [11] and immunomodulation abilities in their local microenvironment [12]. The latest investigations outlined an individual G-MSCs-TLRs' expression profile [13] and defined two TLR-generated immunomodulatory phenotypes in G-MSCs challenged by TLR-agonists [14]. G-MSCs obtained from inflamed gingival tissues demonstrated a differentiation/regenerative aptitude comparable to G-MSCs from uninflamed tissues [15]. Furthermore, recent studies outlined positive short-term stimulatory effects of controlled inflammatory microenvironments on the G-MSCs' pluripotency, proliferation, and differentiation attributes [16–18]. Short-term inflammatory stimuli in isolation or combined with retinol supplementation boosted the stemness, proliferative, and differentiation capabilities of G-MSCs [19]. Recently, AA was shown to possess the ability to similarly enhance G-MSCs' proliferative aptitude and pluripotency marker expression [20]. The current study's aim is to investigate for the first time the AA and controlled inflammatory impacts, isolated or combined, on pluripotency, proliferation, Wnt/β-catenin pathway activation, and differentiation of G-MSCs.

2. Materials and Methods

2.1. Isolation/Characterization of G-MSCs. Human G-MSCs were obtained from healthy gingival collars ($n = 5$) at the Christian-Albrechts University of Kiel. The patient was taken as the experimental unit, and cells were not pooled. The present study was approved by the Ethics Committee of Christian-Albrechts University of Kiel (IRB D513/17). Cells' isolation, culturing in basic medium and immuno-magnetic cell sorting, employing anti-STRO-1 antibody (BioLegend, San Diego, CA, USA) and anti-IgM MicroBeads (Miltenyi-Biotec, Bergisch Gladbach, Germany), was done as formerly described [19, 21]. Colony-forming units (CFUs) capability and the expression of the surface markers CD14, 34, 45, 73, 90, and 105 on second passage G-MSCs, using FACS Calibur E6370 and FACS Comp 5.1.1 software (Becton Dickinson), were further conducted as formerly described [16, 17, 21].

2.2. Multilineage Differentiation

(i) Osteogenic differentiation: 2×10^4 second passage G-MSCs/well were cultivated in six-well plates in osteogenic inductive medium (PromoCell, Heidelberg, Germany) as well as basic medium (controls) for 21 days. Alizarin red (Sigma-Aldrich) staining of both cultures was done to assess calcified nodules formation and quantified

(ii) Adipogenic differentiation: 3×10^5 second passage G-MSCs/well were cultured in six-well culture plates in adipogenic inductive medium (PromoCell) as well as basic medium (controls) for 21 days. Oil-Red-O staining (Sigma-Aldrich) was used to examine the presence of intracellular lipid droplets and quantified

(iii) Chondrogenic differentiation: 3D micromasses of second passage 3×10^4 G-MSCs/tube were cultured in chondrogenic induction medium (PromoCell) and in basic medium (controls) (both in Eppendorf tubes, Eppendorf, Hamburg, Germany) for 35 days. Alcian blue and nuclear-fast-red counterstaining (Sigma-Aldrich) evaluated glycosaminoglycans' formation and quantified (all quantification methods are described below).

2.3. Experimental Groups. Second passage G-MSCs were cultured in basic medium (control group), in basic medium, with 1 ng/ml IL-1β, 10 ng/ml TNF-α, and 100 ng/ml IFN-γ (Pepro Tech Inc, Rocky Hill, NJ, USA) [13, 14, 16, 19, 22, 23] (inflammatory group), in basic medium and 250 μg/ml AA [20] (AA group), or in inflammatory medium with 250 μg/ml AA (inflammatory/AA group). Culture media were exchanged three times per week.

2.4. Intracellular Wnt/β-Catenin Evaluation by ELISA. Intracellular total β-catenin was measured (ELISA Kit, Invitrogen, CA, USA). 8×10^4 G-MSCs per well were cultivated on a six-well plate and stimulated for 1 hour, according to the groups defined above, followed by PBS washing and 350 μl lysis buffer addition. 50 μl standard or sample was mixed with 100 μl β-catenin (total) detection antibody and incubated for one hour at room temperature. After addition of 100 μl of Ant-Rabbit IgG HRP Working Solution, shaking the plates for 30 minutes, 100 μl of Stabilized Chromogen was supplemented for 30 minutes in the dark, followed by 100 μl Stop Solution, and OD documented at 450 nm (MultiskanGO Microplate Spectrophotometer, Thermo Fisher, Langenselbold, Germany). Intracellular total β-catenin was determined using standard curves.

2.5. CFUs and Cellular Proliferation. 1×10^4 second passage G-MSCs per well of each experimental group were cultivated in 24-well culture plates, and their cellular counts were determined every day for 14 days.

MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl-tetrazoliumbromide) test was conducted at 24 and 72 hours (MTT Cell Proliferation Kit-I, Roche Diagnostics GmbH, Mannheim, Germany) [24] to test the cells metabolic activity. No phenol-red serum-free medium (RPMI 1640, PAN-Biotech, Aidenbach, Germany) and 0.5 mg/ml MTT-labelling reagent was added to the G-MSCs cultures and left for 4 hours, followed by 1 ml of the Solubilization solution (37°C, 5% CO_2, overnight). The spectrophotometrical absorbance was recorded at 570 nm wavelength (MultiskanGO Microplate Spectrophotometer, Thermo Fisher). Metabolic activity

was calculated using standard curves. The assays were conducted in duplicate and averaged.

Second passage 1.63/cm^2 G-MSCs were cultivated in 10-cm-diameter dishes for the CFUs assay. On the 14th day, cell cultures were fixed using 100% methanol (ice-cold, for 10 min) and stained with 0.1% crystal violet for 10 min. CFUs were evaluated by two independent examiners, using phase-contrast inverted microscopy. Aggregations of ≥50 cells were counted as a colony.

2.6. G-MSCs' mRNA Expression. To test for the pluripotency gene expressions (Nanog, octamer-binding-transcription-factor 4A (Oct4A) as well as sex-determining-region-Y-box 2 (Sox2)), mRNA extraction was done in the four experimental groups outlined above at 24, 72, and 120 hours, using the RNeasy kit (Qiagen, Hilden, Germany). Complementary DNA (cDNA) was synthesized from RNA (1 μg/μl) via reverse transcription (QuantiTect reverse transcription kit, Qiagen) in a volume of 20 μl reaction mixture (4 pmol of each primer, 10 μl of the LightCycler Probes Master mixture (Roche) and 5 μl specimen cDNA). Real-time polymerase chain reaction (rt-PCR; LightCycler 96 Real-Time PCR System, Roche Molecular Biochemicals, Indianapolis, Indiana, USA) was conducted. Nineteen possible reference genes were preexamined to decide on the most suitable reference gene in the G-MSCs, which would not be regulated by the experiment (NormFinder). Except for PGK1, all were regulated. Hence, PGK1 (housekeeping gene) was determined to be employed as a reference gene (Table 1). The relative quantification of the examined genes was conducted employing the $2^{-\Delta\Delta Ct}$ method and assays done in triplicate and averaged. Gene expressions of each of the tested genes were normalized to PGK1.

2.7. Multilineage Potential of Stimulated G-MSCs. G-MSCs were prestimulated for five days in the experimental groups designated above, followed by osteogenic (21 days), adipogenic (21 days), or chondrogenic (35 days) differentiation. mRNA expression of alkaline phosphatase (ALP) and Runt-related transcription factor 2 (Runx2), as well as the qualitative and quantitative Alizarin red staining, was conducted to assess the G-MSCs' osteogenic aptitude. For the quantification of the Alizarin red staining, 200 μl 10% acetic acid was supplemented into the osteogenically induced G-MSCs cultures for 30 minutes on a shaker, followed by detachment of the cellular monolayer, and transferred into a 1.5 ml tubes (Eppendorf) and vortexing for 30 seconds. Following 10 minutes 85°C heating and ice-cooling, the mixture was centrifuged at 20,000 rpm (15 minutes). 200 μl supernatant was transported to a new 1.5-ml tube and the pH neutralized with 10% ammonium hydroxide. Spectrophotometrical absorbance of 50 μl of the sample and Alizarin red standards were recorded at OD405 (Thermo Fisher), and relative Alizarin red quantities were determined [25].

mRNA expression for lipoprotein lipase (LPL) and proliferator-activated receptor gamma (PPAR-γ) and quantitative and qualitative Oil-Red-O evaluation were assessed as evidence for the adipogenic differentiation aptitude. For Oil-Red-O quantification, isopropanol (1 ml/well) was added, and the cultures were incubated for 15 minutes on a shaker, and 100 μl of the resultant mixture's spectrophotometrical absorbance was measured at OD540 (Thermo Fisher), and finally, the relative Oil-Red-O quantities were determined [26].

To assess chondrogenic differentiation, Aggrecan (ACAN) mRNA expression and Alcian blue/nuclear-fast-red staining were evaluated. For quantification of Alcian blue and nuclear-fast-red staining, automated digital image quantification of the chondrogenic differentiation was performed as previously described [27]. All primers used in the real-time PCR were supplied by Roche (Table 1).

Table 1: Real-time PCR primers (Roche).

Gene	Gene symbol	Accession ID	Assay ID
RUNX-2	RUNX2 H.sapiens	ENST00000359524	113380
ACAN	ACAN H.sapiens	ENST00000439576	138057
ALP	ALP H.sapiens	ENST00000374840	103448
LPL	LPL H.sapiens	ENST00000311322	113230
Nanog	Nanog H.sapiens	ENST00000229307	148147
Oct4A	Oct4 H.sapiens	ENST00000259915	113034
PGK-1	PGK1 H.sapiens	ENST00000373316	102083
PPARγ	PPARγ H.sapiens	ENST00000287820	110607
Sox2	Sox2 H.sapiens	ENST00000325404	111867

Abbreviations: ACAN: Aggrecan; ALP: alkaline phosphatase; LPL: lipoprotein lipase; Oct4A: octamer-binding-transcription-factor 4A; PGK-1: phosphoglycerate kinase-1; PPARγ: proliferator-activated receptor gamma; RUNX-2: Runt-related transcription factor 2; Sox2: sex-determining region Y-box 2.

2.8. Statistical Analysis. The normality of the data was evaluated, using the Shapiro-Wilk-test. Differences in intracellular total β-catenin, CFUs, MTT, mRNA expression, and quantitative adipogenic and osteogenic differentiation between the experimental groups were analyzed employing the Friedman-test. Differences in MTT and mRNA expression at different time points and pairwise comparisons were conducted using the Wilcoxon-signed-rank-test (SPSS 11.5, IBM, Chicago, IL, USA). The significance level was set at $p = 0.05$.

3. Results

3.1. Characterization of G-MSCs. Following adhesion, cells grew out of the gingival tissues, demonstrating fibroblast-like morphology (Figure 1(a)). Characteristic CFUs were demonstrated by the G-MSCs (Figure 1(b)), which were negative for CD14, CD34, weakly positive for CD45, and highly positive for CD73, CD90, and CD105 surface markers expression (Figure 1(c)). Osteogenically induced G-MSCs formed significantly higher Alizarin red-labelled calcified deposits, as opposed to unstimulated controls (Figures 1(d)–1(f)). The G-MSCs' adipogenic differentiation formed significantly higher Oil-Red-O positively stained lipid inclusions, as opposed to unstimulated controls (Figures 1(g)–1(i)). Chondrogenically induced G-MSCs formed significantly higher Alcian blue-positive glycosaminoglycans deposits, as opposed

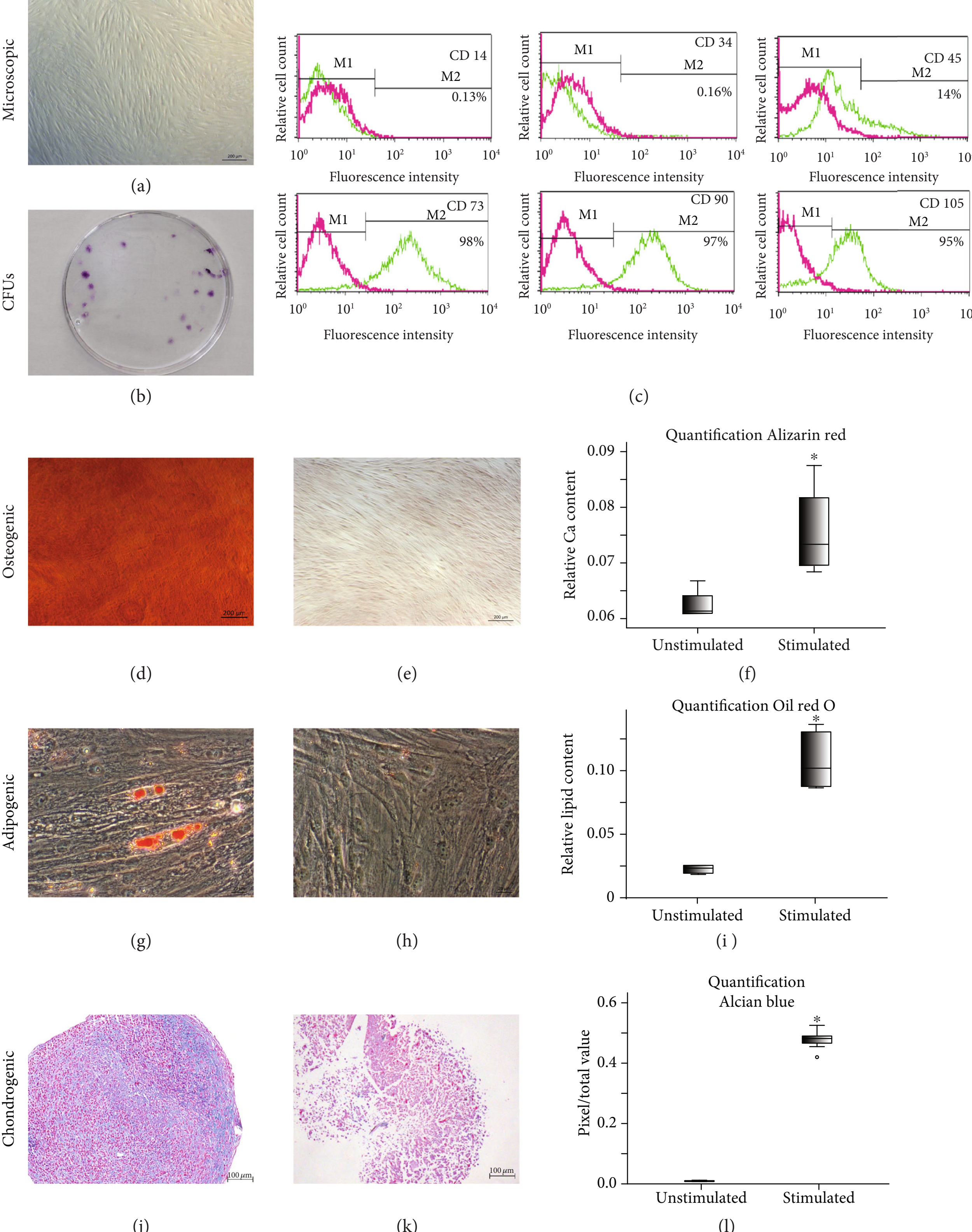


Figure 1: Phase contrast inverted microscopic picture of gingival cells growing out from a gingival connective tissue specimen (a). G-MSCs' colony-forming units (CFUs) (b). G-MSCs surface markers' expression flowcytometrically (c). Osteogenic induction of stimulated G-MSCs (Alizarin red staining; d) and respective unstimulated controls (e) with quantification (f). Adipogenic induction of stimulated G-MSCs (Oil-Red-O stained; g) and respective unstimulated controls (h) with quantification (i). Chondrogenic induction of stimulated G-MSCs (Alcian blue/acid-fast red staining; j) and respective unstimulated controls (k), with quantification (l). Significant differences denoted with asterisks ($n = 5$, $^*p < 0.05$, Wilcoxon-signed-rank-test).

to unstimulated controls (Figures 1(j)–1(l); $p < 0.05$, Wilcoxon-signed-rank-test).

3.2. The Intracellular Wnt/β-Catenin Pathway. G-MSCs' intracellular Wnt/β-catenin pathway was activated by AA and inflammation (Figure 2(a)), significantly reducing the total intracellular β-catenin, as compared to the control group. β-Catenin levels intracellularly were the greatest (median% expression, Q25/Q75) in control group (12.8%, 11.9/16.3), trailed by AA group (11.8%, 10.4/14.6), inflammatory/AA group (11.8%, 10.1/13.4), and finally the inflammatory group (11.7%, 11.0/14.6; $p = 0.002$, Friedman).

3.3. CFUs and Cellular Proliferation. Over 14 days, the greatest cellular counts were evident in AA group, trailed by inflammatory/AA group, inflammatory group, and finally control group (Table 2, Friedman). The highest cellular metabolic activity was demonstrated between 24 hours and 72 hours in the control (6109, 4457/7290), trailed by the inflammatory group (5558, 4444/6815), the inflammatory/AA group (5116, 4055/6434), and finally the AA group (4083, 3466/4845; $p = 0.004$). At 14 days, CFUs were the greatest (Median CFUs, Q25/Q75) in AA group (40, 15/50), trailed by control group (12, 5/32), and minute CFUs in inflammatory challenged groups ($p = 0.002$, Friedman, Figures 2(b) and 2(c)).

3.4. M-RNA Expression of Pluripotency Markers. Regarding pluripotency gene expressions (Figure 3), significantly, higher expressions for Sox2 were noted overtime (median gene copies/PGK-1, Q25/Q75) from (0.00006, 0.00000/0.00019) to (0.00041, 0.00000/0.00014; $p = 0.04$, Friedman). Significantly, higher expression of Nanog was noted in AA group (0.0006, 0.0002/0.0007), trailed by the inflammatory group (0.0004, 0.0001/0.008), the control group (0.0002, 0.0000/0.0005), and inflammatory/AA group (0.00006, 0.0000/0.0002; $p < 0.001$, Friedman). Overall, Oct4A and Sox2 were the highest expressed in the inflammatory/AA group ((0.0002, 0.0001/0.0006) and (0.0002, 0.0000/0.0004), respectively).

3.5. Stimulated G-MCSs' Multilineage Differentiation Potential. G-MSCs in the different experimental groups exhibited a remarkable multilineage differentiation aptitude (Figure 4). No significant differences were observed for RUNX2, ALP, LPL, PPAR-γ, or ACAN expression between the different groups in their respective inductive media. Histochemical staining of cellular cultures in the experimental groups exhibited calcified nodules after osteogenic induction, Oil-Red-O-positive lipid inclusions after adipogenic induction, and glycosaminoglycan deposition, following chondrogenic induction. For osteogenic and chondrogenic stimulation, significantly, the highest Alizarin red and Alcian blue with the lowest nuclear-fast-red staining was, respectively, evident in the AA group as compared to the other examined groups ($p < 0.05$, Wilcoxon-signed-rank-test).

4. Discussion

Reparative/regenerative approaches in periodontology are based principally on recapitulating the chief periodontal developmental events, encompassing stem/progenitor cells' proliferation, migration, homing, differentiation, and finally maturation [28]. Clinically, these healing stages primarily take place in an initially inflamed periodontal microenvironment, with inflammatory cytokines orchestrating the course of the inflammatory periodontal disease progression [29], possible healing/regeneration [30], as well as periodontal stem/progenitor cells' attributes [18, 19].

Apart from its important roles in periodontal wound healing, tissue regeneration [31], and collagen synthesis of bone, teeth, and gingiva, AA demonstrates potent cellular protective antioxidative properties in response to periodontitis-induced oxidative inflammatory reactions [32, 33]. It further possesses immunomodulatory abilities, which could markedly downregulate IL-1α, IL-1β, IL-6, tumor necrosis factor beta (TNF-β), and nitric oxide production in periodontal lesions [34, 35]. AA could further induce a pluripotent stage [36] and enhance the reprogramming efficiency [6] in embryonic stem cells.

Similar to previous investigations [21, 37–40], the tested G-MSCs showed all predefined stem/progenitor cells' hallmarks encompassing CFUs ability, surface markers' expression, and a remarkable multilineage differentiation capacity [41]. Recently, multiple investigations have revealed that local microenvironmental inflammatory stimuli could uplift G-MSCs' reparative and regenerative potentials [16–19, 42, 43]. The currently investigated G-MSCs were stimulated via periodontal proinflammatory cytokines, namely IL-1β, TNF-α, and IFN-γ [16], by AA or their combination. Subsequently, the G-MSCs' pluripotency, proliferation, and differentiation potentials were assessed. Challenging the G-MSCs' through AA and inflammatory stimuli appeared to activate their Wnt/β-catenin pathway, culminating in a significant reduction of their β-catenin levels intracellularly, possibly affecting their stemness [44], proliferative [45], and differentiation capacity [46].

Comparable to oral wound healing [47], periodontal wound healing undergoes five phases, namely a hemostatic phase, an inflammatory phase, a cellular recruitment and proliferation phase, and finally a tissue remodeling one. Hereby, G-MSCs rely primarily on their stemness in performing theses periodontal tissue reparative and regenerative actions, primarily proliferation and multilineage differentiation potential. The current study examined the effect of the inflammatory cytokines mixture together and in combination with AA on the pluripotency markers' expression, namely Nanog, Oct4A, and Sox2 at 24, 72, and 120 hours. In line with earlier studies, in the present one Nanog, Oct4A, and Sox2 expressions were amplified in the AA- [3, 20, 48] and inflammation-challenged [19, 49] G-MSCs. The AA-induced increase in the pluripotency markers Oct4A and Nanog could be ascribed primarily to the capability of AA, similar to Retinol [19], to activate the ten-eleven-translocation (TET) demethylases, eliminating methylation of the DNA and thereby inciting intracellular epigenetic reprogramming actions, encompassing pluripotency amplification [19, 50, 51] in G-MSCs. Yet, the current study further demonstrated their synergistic effect, with the highest upregulation of Oct4A and Sox2 expression being notable on the combination of AA with the inflammatory cocktail. It appears

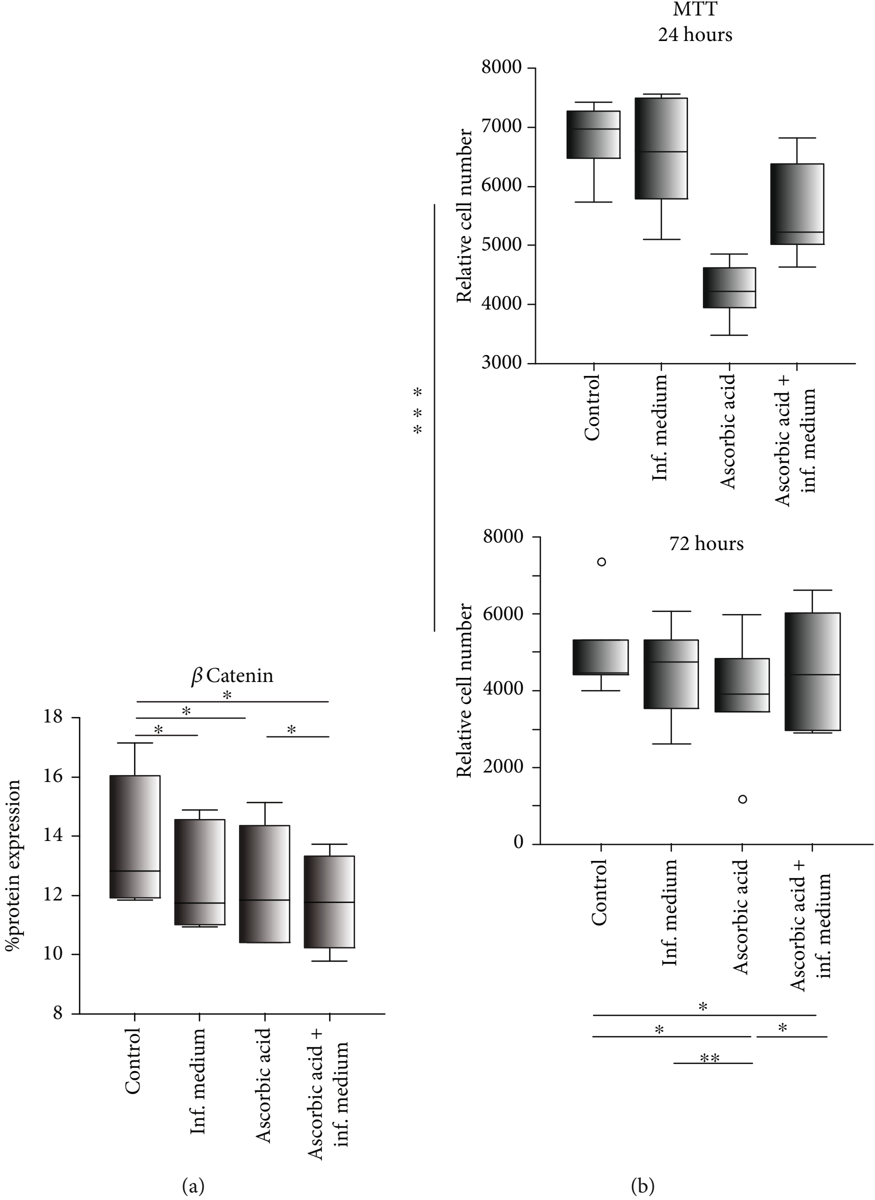
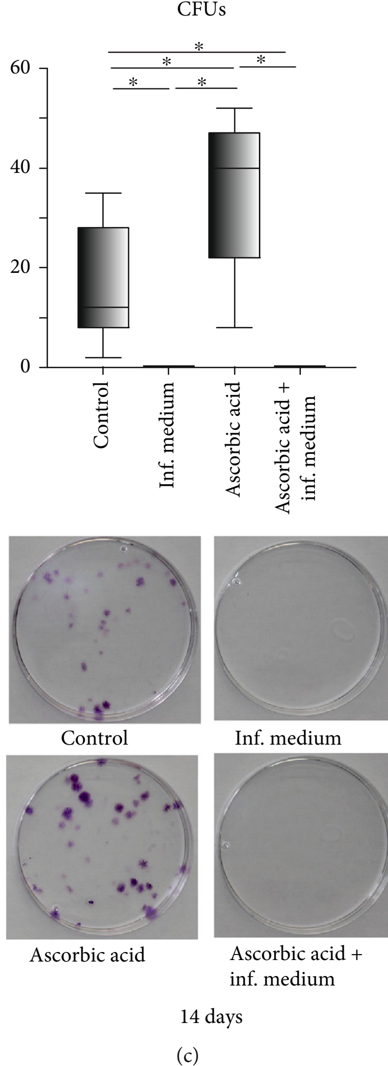


Figure 2: Continued.



(c)

Figure 2: Wnt/β-catenin pathway activation, MTT, and CFUs following ascorbic acid and inflammatory stimulation of G-MSCs: ELISA examination of the Wnt/β-catenin signaling pathway for total intracellular β-catenin following G-MSCs' challenging by ascorbic acid and inflammation (a; box and whisker plots with medians/quartiles). Relative G-MSCs metabolic activity following ascorbic acid and inflammatory stimulation for 24 h and 72 h (b; box and whisker plots with medians/quartiles). CFUs-assay/CFUs' numbers following G-MSCs' stimulation via ascorbic acid and inflammation (c; box and whisker plots with medians/quartiles). Significant differences denoted with asterisks ($n = 5$, $^{*}p < 0.05$; $^{**}p < 0.01$; Wilcoxon-signed-rank-test). Abbreviations: CFUs: colony-forming units; β-catenin: total β-catenin.

that a combination of AA and inflammation could augment their action on the expression of nuclear markers of pluripotency in the G-MSCs, thereby upregulating their stemness.

Cellular proliferation represents a primary requirement of any periodontal regenerative/reparative approach to obtain cellular counts able of executing the ensuing phases of migration/homing and functional tissue differentiation. A fundamental hallmarks of stem/progenitor cells remains to be their ability for colonogenic self-renewal, demonstrated through their CFUs producing potential. Inflammatory stimulation, in line with previous investigations [17], appeared to enhance G-MSCs' metabolic activity at 72 hours. Surprisingly, AA stimulation initially appeared to decrease cellular metabolic activity in the early phase and even diminish the augmenting effects of the inflammatory stimuli in the inflammatory/AA group. The observed attenuation of G-MSCs' metabolic activity, while inducing proliferation could be attributed to the earlier reported property of AA to suppress cellular growth arrest encoding genes, namely growth arrest/-DNA-damage-inducible 45α (Gadd45a) as well as apoptosis-inducing genes, namely caspase-1 [35]. It further can be ascribed to the potential of AA to increase ERK1/2 phosphorylation with a concomitant attenuation of the mitogen-activated kinase pathway [52]. In the current study, AA exhibited over 14 days a proliferative-induction potential, which was previously reported in longer-term cultures [48, 53], and significantly higher CFUs formation. This property could be primarily attributed to an increase in the AA-mediated upregulation of the proliferation-related-Fos-transcriptional-factor [52]. In contrast, the tested inflammatory microenvironment drove the G-MSCs to self-senescence on lengthier stimulation [17]. Short-term inflammatory stimuli

Table 2: Cellular counts in the control-, inflammation-, ascorbic acid- and inflammation/ascorbic acid-stimulated G-MSCs over 14 days (median and Q25/Q75 quartiles; Friedman test).

		Control	Inflammation	Ascorbic acid	Inflammation/ascorbic acid	p value
Day						
	Median	1.83E + 04	1.33E + 04	1.83E + 04	1.58E + 04	
1	Q25	1.25E + 04	1.33E + 04	1.50E + 04	1.42E + 04	0.33
	Q75	1.92E + 04	1.67E + 04	2.58E + 04	2.00E + 04	
	Median	3.33E + 04	2.08E + 04	3.75E + 04	2.92E + 04	
2	Q25	2.50E + 04	1.58E + 04	2.75E + 04	2.67E + 04	0.06
	Q75	3.92E + 04	2.25E + 04	4.17E + 04	3.50E + 04	
	Median	4.42E + 04	3.50E + 04	5.25E + 04	3.92E + 04	
3	Q25	3.67E + 04	3.17E + 04	5.25E + 04	3.75E + 04	0.04
	Q75	6.50E + 04	3.67E + 04	5.83E + 04	4.42E + 04	
	Median	4.50E + 04	4.67E + 04	6.17E + 04	5.25E + 04	
4	Q25	3.58E + 04	4.58E + 04	5.58E + 04	4.08E + 04	0.45
	Q75	4.58E + 04	6.17E + 04	6.83E + 04	5.92E + 04	
	Median	5.42E + 04	5.58E + 04	7.17E + 04	5.92E + 04	
5	Q25	5.08E + 04	4.83E + 04	5.67E + 04	5.75E + 04	0.34
	Q75	5.50E + 04	5.83E + 04	7.75E + 04	7.75E + 04	
	Median	5.50E + 04	6.25E + 04	9.58E + 04	6.75E + 04	
6	Q25	4.33E + 04	5.67E + 04	8.25E + 04	4.92E + 04	0.03
	Q75	5.58E + 04	7.33E + 04	1.03E + 05	6.75E + 04	
	Median	6.42E + 04	5.67E + 04	9.50E + 04	6.00E + 04	
7	Q25	4.67E + 04	5.17E + 04	7.67E + 04	5.25E + 04	0.04
	Q75	9.00E + 04	5.83E + 04	9.92E + 04	7.00E + 04	
	Median	5.33E + 04	8.58E + 04	6.58E + 04	1.00E + 05	
8	Q25	3.83E + 04	7.75E + 04	5.75E + 04	6.25E + 04	0.52
	Q75	7.17E + 04	8.58E + 04	1.13E + 05	1.00E + 05	
	Median	7.33E + 04	6.58E + 04	8.92E + 04	6.08E + 04	
9	Q25	5.33E + 04	5.83E + 04	7.17E + 04	5.08E + 04	0.72
	Q75	7.50E + 04	7.83E + 04	9.42E + 04	7.00E + 04	
	Median	8.17E + 04	5.42E + 04	7.17E + 04	7.00E + 04	
10	Q25	6.25E + 04	4.83E + 04	7.08E + 04	5.83E + 04	0.41
	Q75	8.25E + 04	9.92E + 04	8.83E + 04	8.83E + 04	
	Median	7.17E + 04	5.75E + 04	8.25E + 04	7.83E + 04	
11	Q25	5.67E + 04	4.50E + 04	7.50E + 04	4.58E + 04	0.73
	Q75	8.67E + 04	8.92E + 04	8.75E + 04	7.83E + 04	
	Median	6.58E + 04	6.08E + 04	7.54E + 04	7.17E + 04	
12	Q25	5.50E + 04	3.92E + 04	4.67E + 04	4.00E + 04	0.55
	Q75	7.50E + 04	8.08E + 04	1.07E + 05	7.42E + 04	
	Median	5.58E + 04	6.67E + 04	1.13E + 05	6.75E + 04	
13	Q25	4.50E + 04	5.67E + 04	8.83E + 04	6.08E + 04	0.06
	Q75	5.67E + 04	1.11E + 05	1.26E + 05	1.06E + 05	
	Median	7.08E + 04	6.92E + 04	9.58E + 04	9.42E + 04	
14	Q25	5.17E + 04	5.83E + 04	7.83E + 04	6.42E + 04	0.22
	Q75	7.58E + 04	8.50E + 04	1.00E + 05	1.00E + 05	

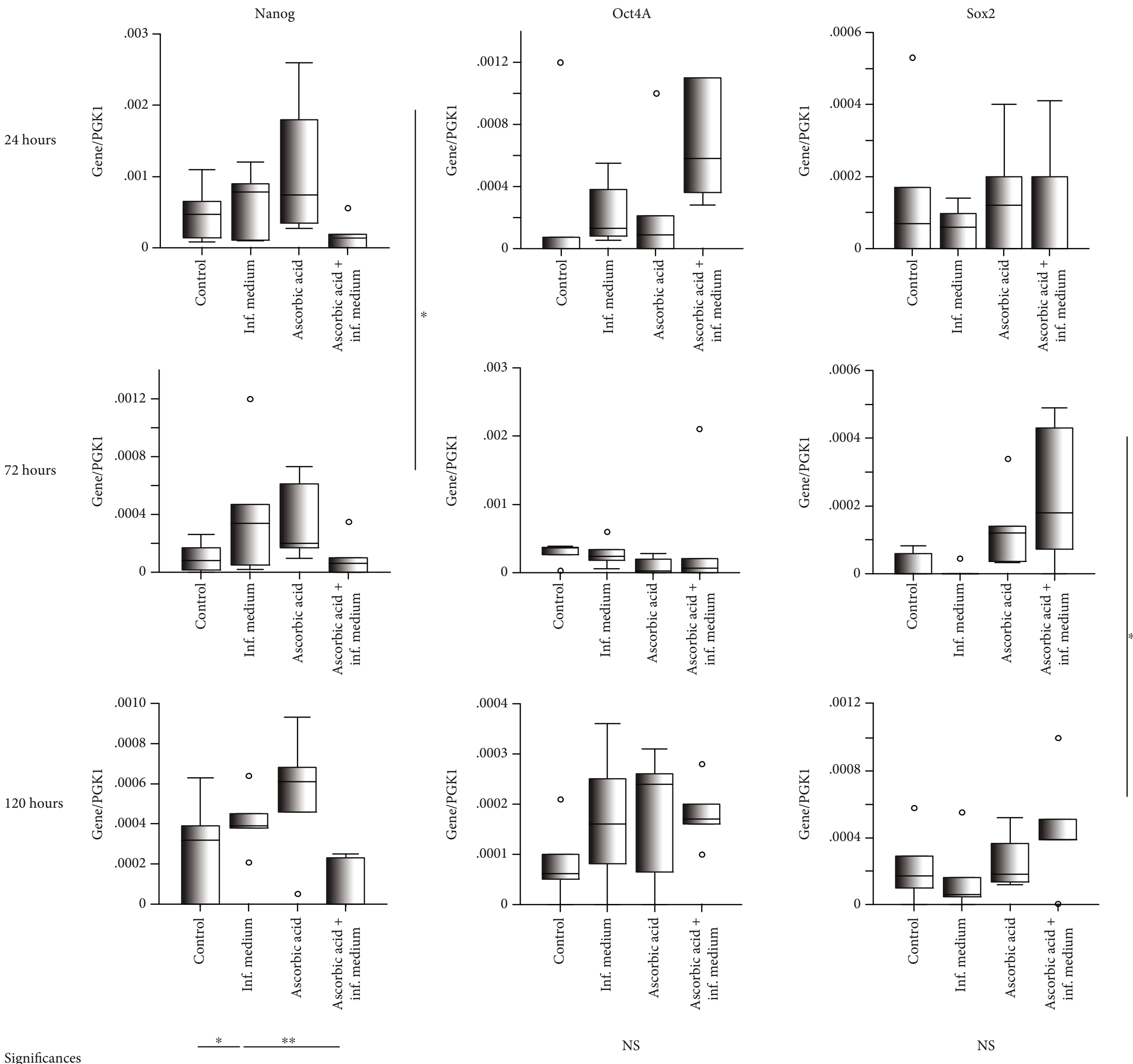
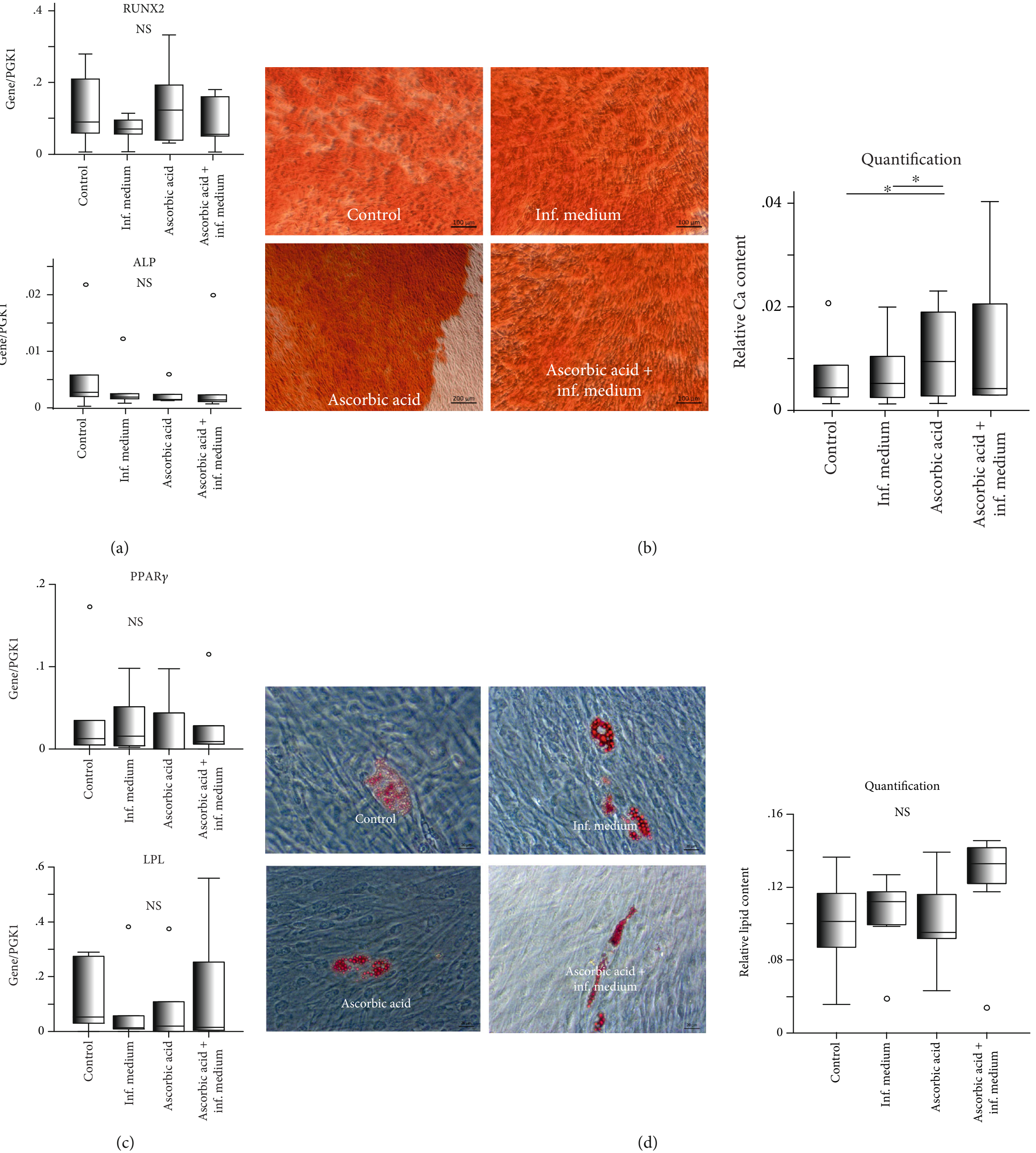


Figure 3: mRNA pluripotency genes expression (Nanog, Oct4A, Sox2) in G-MSCs challenged by ascorbic acid and inflammation at 24, 72, and 120 hours (box and whisker plots with medians/quartiles). Significant differences denoted with asterisks ($n = 5$, $^{*}p < 0.05$, $^{**}p < 0.01$; Wilcoxon-signed-rank-test). Abbreviations: Sox2: sex-determining region Y-box 2; Oct4A: octamer-binding-transcription-factor 4A; NS: nonsignificant.

could induce cellular proliferation through Wnt/β-catenin activation and a subsequent subdual of the noncanonical Wnt/Ca^{2+} pathways [18, 45], which was evident by the reduced total intracellular β-catenin observed in the challenged groups. Yet on excessive stimulation, TNF-α could induce self-senescence of the stem/progenitor cells, especially in the presence of IFN-γ and cultivation at lower cellular densities, as in the CFU experiment. Through altering the IFN-γ-activated, nonapoptotic form of TNF-receptor-superfamily-member-6 (Fas) signaling into a caspase 3- and caspase 8-associated proapoptotic cascade, the G-MSCs' apoptotic pathway could have been activated [54]. AA and short-term controlled inflammation appear to have opposed biphasic impacts on the short-term as well as the long-term G-MSCs' proliferation.

AA is generally characterized by its ability to modulate cell growth, metabolism, and morphogenesis during osteogenesis [55, 56], as well as to induce extracellular matrix production [3]. AA stimuli boosted the G-MSCs' multilineage differentiation potentials, particularly osteogenic as well as chondrogenic differentiation capacity. In this context, the observed Wnt/β-catenin pathway activation, with a substantial reduction in the total β-catenin intracellularly plays an important role. An intracellular β-catenin accumulation, with a subsequent translocation to the nucleus and resultant stimulation of the lymphocyte enhancer-binding factor-1 (Lef-1), silences the RUNX2/Osterix-associated axis of osteogenesis [46]. Hence, the currently noted intracellular total β-catenin downregulation, aside from a proliferatory enhancement effect, would heighten the G-MSCs' differentiation capacity.



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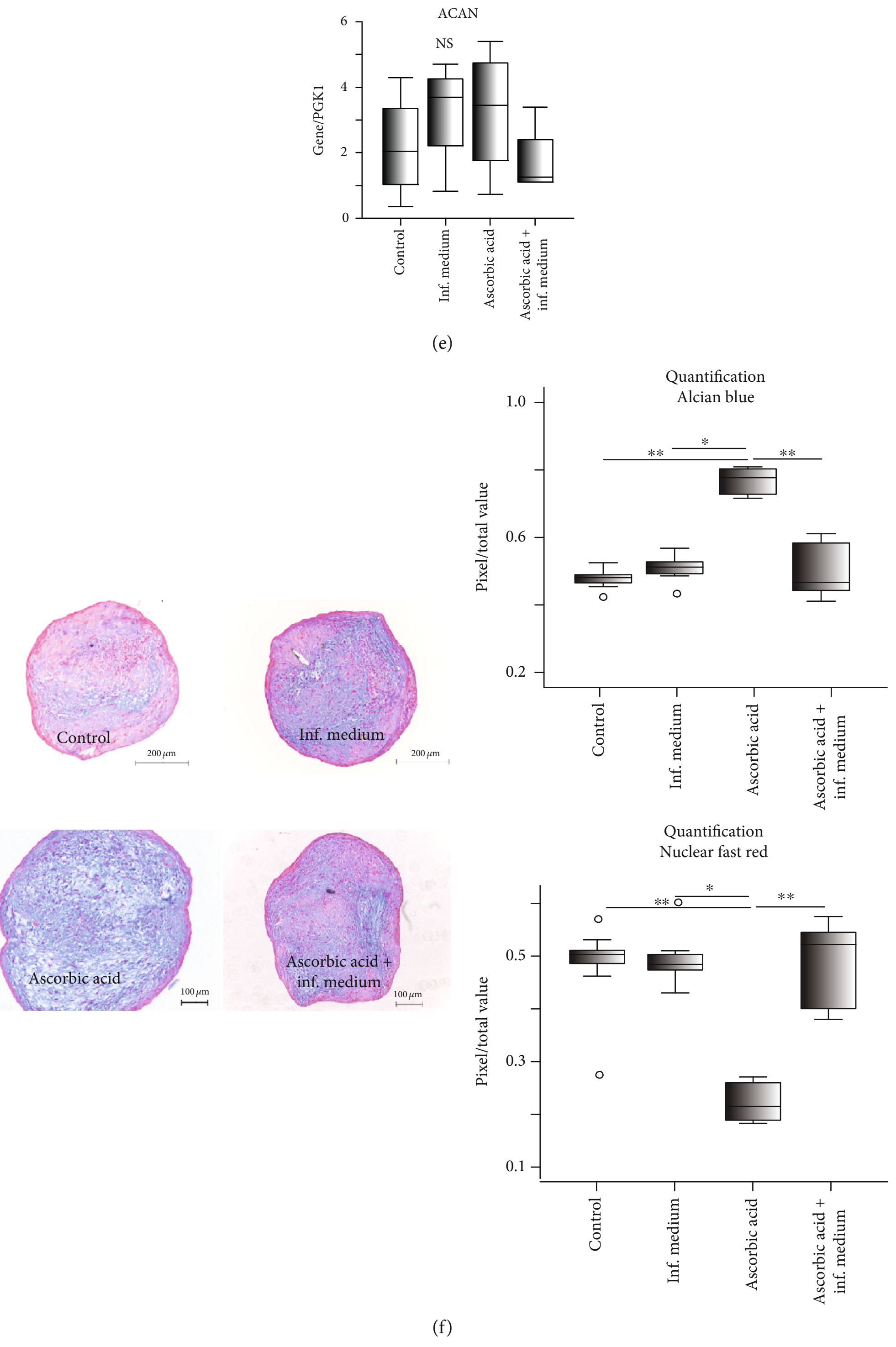

Figure 4: G-MSCs multilineage differentiation following stimulation by ascorbic acid and inflammation: gene expressions of ALP and RUNX2 following a 21-day osteogenic stimulation (a; box and whisker plots with medians/quartiles). Ca^{2+} quantification and Alizarin-Red staining following a 21-day osteogenic induction of ascorbic acid and inflammation stimulated G-MSCs (b; box and whisker plots with medians/quartiles). LPL and PPARγ gene expression after 21 days of adipogenic stimulation of ascorbic acid and inflammation challenged G-MSCs (c; box and whisker plots with medians/quartiles). Oil-Red-O staining and lipid amount quantification of ascorbic acid and inflammation stimulated G-MSCs after 21 days of adipogenic stimulation (d; box and whisker plots with medians/quartiles). ACAN gene expression following a 35-day chondrogenic induction of ascorbic acid and inflammation stimulated G-MSCs (e; box and whisker plots with medians/quartiles). Alcian blue/nuclear-fast-red staining and their quantification in ascorbic acid and inflammation stimulated G-MSCs following a 35-day chondrogenic induction (f). Significant differences denoted with asterisks ($n = 5$, $^{*}p < 0.05$, $^{**}p < 0.01$; Wilcoxon-signed-rank-test). Abbreviations: ACAN: Aggrecan; ALP: alkaline phosphatase; LPL: lipoprotein lipase; NS: nonsignificant.

Notably, the greatest multilineage differentiation capability was evident in the AA group, emphasizing the importance of an inflammation-free environment for a successful multilineage differentiation of G-MSCs boosted by AA.

Combined, current results point at enhanced G-MSCs' characteristics in the presence of AA, which, apart from an initially observed synergistic effect on cellular metabolism as well as Sox2 and Oct4A pluripotency markers expression, were attenuated in the presence of an inflammatory microenvironment. The observed effects appeared to be associated with a Wnt/β-catenin pathway activation. Similar to previous investigations, precise short-termed microenvironmental inflammatory stimuli could enhance early cellular attributes and pluripotency, while an AA-induced boosting of cellular proliferation and differentiation would require an inflammation-free microenvironment. The present results denote that an early short-termed controlled G-MSCs' inflammatory stimulation, followed by a AA stimulation in an inflammation-free microenvironment could provide an interesting scheme for enhancing their cellular attributes in regenerative approaches.

Data Availability

All data used to support the findings of this study are included within the article.

Conflicts of Interest

All authors declare no conflict of interest related to this work.

Acknowledgments

The authors are thankful to Dr. Toelle, Mrs. Paymard-Stolz, Mrs. Marx, Mrs. Marquardt, and Dr. Kruse for their excellent scientific support. This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

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Hindawi
Stem Cells International
Volume 2020, Article ID 1047896, 9 pages
https://doi.org/10.1155/2020/1047896

Review Article

Epigenetic Clock: DNA Methylation in Aging

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Received 27 March 2020; Revised 11 June 2020; Accepted 20 June 2020; Published 8 July 2020

Academic Editor: Yang Li

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Aging, which is accompanied by decreased organ function and increased disease incidence, limits human lifespan and has attracted investigators for thousands of years. In recent decades, with the rapid development of biology, scientists have shown that epigenetic modifications, especially DNA methylation, are key regulators involved in this process. Regular fluctuations in global DNA methylation levels have been shown to accurately estimate biological age and disease prognosis. In this review, we discuss recent findings regarding the relationship between variations in DNA methylation level patterns and aging. In addition, we introduce the known mechanisms by which DNA methylation regulators affect aging and related diseases. As more studies uncover the mechanisms by which DNA methylation regulates aging, antiaging interventions and treatments for related diseases may be developed that enable human life extension.

1. Introduction

Aging is an inevitable biological progress in which the functions of multiple organs gradually decline with time, leading to an increased susceptibility to diseases and environmental stressors [1, 2]. In humans, age has become the primary risk factor for various diseases, such as cardiovascular diseases, cancer, neurodegeneration diseases, and diabetes [3]. Scientists have made unremitting efforts to elucidate the causal mechanisms involved in the phenotypic alterations associated with age. Since many genes appear to show altered expression during the aging process, researchers have focused on studying the long-term effects of environment stress on gene expression regulation. Importantly, epigenetic modifications are proposed to play a crucial role in the progression of aging.

Epigenetic changes are known as genetic variations triggered by the environment and include alterations in histone modifications, DNA methylation, noncoding RNAs, transcription factor binding, and nucleosome positioning [4, 5]. DNA methylation is a biological process by which methyl groups are added to DNA molecules. Histone modifications include lysine methylation, arginine methylation, lysine acetylation, and serine phosphorylation. These modifications alter the extent to which DNA is wrapped around histones and the availability of genes within the DNA to be activated. Recently, numerous studies have emphasized the unique role of DNA methylation in aging [6, 7]. In addition to its effects on the aging process, a function shared by other epigenomic regulators, DNA methylation can also predict aging status. In this review, we will discuss the relationship between DNA methylation and aging as well as summarizing the primary mechanisms by which DNA methylation regulates the aging process.

2. DNA Methylation

As is shown in Figure 1, DNA methylation modification involves two different processes: the addition and removal of a methyl group at the fifth position of cytosine or the sixth position of adenine in DNA [8]. DNA 5-methylcytosine (5mC) is the most prevalent DNA methylation modification in eukaryotic genomes and primarily occurs on cytosines that precede a guanine nucleotide (CpG sites) [9]. The presence of 5mC is generally believed to prevent transcription factors from binding to a promoter region and thus suppress gene

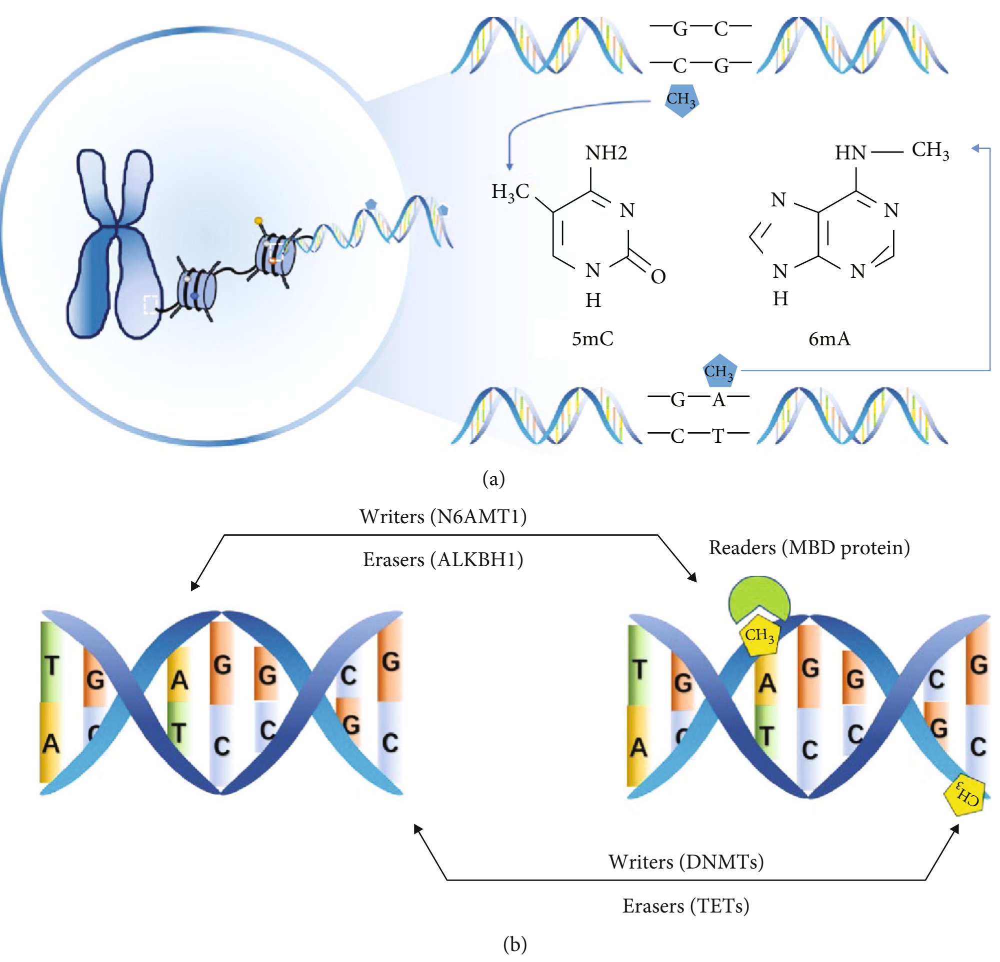

Figure 1: Modulation of DNA methylation in mammals. (a) DNA 5mC methylated site is the fifth position of cytosine, while 6mA modification occurs at the sixth position of adenine in DNA. (b) Methylation marks are established by writers, such as DNMTs and N6AMT1. These modifications are identified by readers, like methyl-CpG-binding domain (MBD) proteins. Erasers, among which TETs and ALKBH1 are representatives, can make all the marks invalid by oxidizing or removing methyl groups.

expression. DNA 6-adenine methylation (6mA) is a recently discovered epigenetic modification in the human genome that has been demonstrated to affect mammalian development [10, 11].

The two types of DNA methylation modifications, 5mC and 6mA, involve different enzymatic systems in mammals (see Table 1). 5mC methylation marks are established by DNA methyltransferases (DNMTs), including DNMT1, DNMT3A, and DNMT3B. DNMT1 is primarily responsible for the maintenance of methylation status across mitosis, while DNMT3A and DNMT3B work in a de novo methylation process. 5mC methylation can be oxidized and changes into 5-hydroxymethylcytosine (5hmC) by ten-eleven translocation (TET) enzymes, which contain TET1, TET2, and TET3 [12]. Moreover, recent studies have revealed the potential role of DNMTs in active DNA demethylation [13]. To date, only a few enzymes involved in 6mA methylation, such as N6AMT1 and ALKBH1, have been identified in mammals [10, 14]. However, a recent study challenged currently used 6mA detection techniques and suggested that more powerful evidence is needed to support the presence of 6mA [15]. DNA methylation modifications typically occur in promoter

Table 1: Enzymes involved in mammalian DNA methylation.

Type of DNA methylation	Role of enzymes	Family	Members
5mC	Writer	DNMT	DNMT1 DNMT3A DNMT3B
	Eraser	TET	TET1 TET2 TET3
	Reader	MBD	MeCP2 MBD1 MBD2
6mA	Writer	HemK Mettl	N6AMT1 Mettl4
	Eraser	ALKBH	ALKBH1 ALKBH4

regions and then recruit classical readers of DNA methylation, methyl-CpG-binding proteins (MBDs). The variation in the expression of DNA methylation-associated enzymes has been observed in some age-related condition, which will be described in greater detail later.

Although continual changes in methylation status are supposed to be adaptive to aging, sometimes this process fails and becomes maladaptive. Ineffective adaptation accelerates the biological aging process and conversely impacts functional and phenotypic aging. Thus, fluctuations in DNA methylation levels in the human genome can serve as an "epigenetic clock" that regularly changes with age [16–18]. Studies have shown that DNA methylation may function as an accurate biomarker to estimate "biological age," which predicts age-related changes [19]. Furthermore, scientists have begun to understand the potential role of genome-wide patterns of DNA methylation in age-related diseases. To understand the causal connection between DNA methylation and aging, rigorous studies on the underlying biological mechanisms of DNA methylation-induced aging are currently in progress. The mechanisms of aging are complicated and involve multiple factors. Compared with other hallmarks of aging, phenotypic changes resulting from DNA methylation are thought to be associated with a loss of proteostasis, mitochondrial dysfunction, stem cell exhaustion, and immunosenescence [20–22]. Thus, understanding the molecular nature of aging is a key to allow for the identification of targets for interventions to prevent age-related multimorbidity and disability.

3. DNA Methylation in Aging

3.1. DNA Methylation and Biological Age. It is generally acknowledged that chronological age is not an accurate indicator for the aging process, making it difficult to identify and validate effective measures to promote longevity and healthy aging. Thus, the concept of biological age has been proposed to accurately predict the aging status of an organ or person [23]. Enormous progress has been made in recent years in the development of several potential biological age estimators, of which DNA methylation level is the most promising [19]. Previous studies have clearly proven that genome-wide DNA methylation levels are associated with chronological age throughout the entire human lifespan [24, 25]. Some age-related DNA methylation changes occur in specific regions of the genome and are directional, indicating the existence of differentially methylated regions associated with aging [26]. Therefore, DNA methylation-based biomarkers enable accurate age estimation, which has also been proven by many investigations involving tissues, individuals, and populations [27, 28].

DNA methylation clocks, built from epigenetic DNA methylation marks, rely on the combined use of a mathematical algorithm and sets of CpGs to estimate the biological age of cells, organs, or individuals. In general, age-associated variations in DNA methylation levels comprise locus-specific hypermethylation and global hypomethylation [29]. Since the site-specific CpG methylation fraction can be different in cells from different DNA sources, it has been used to reflect both chronological and biological age.

To date, several human DNA methylation biological clocks have been built that are based on sets of CpGs across tissues and age spectra [30–32]. Analyzing the whole blood samples of 656 individuals, Hannum et al. measured more than 450,000 CpG markers and used the resulting data to generate a quantitative model of aging [30]. In this model, 15% of the identified CpG markers demonstrated a remarkable association between percent DNA methylation and age. Similarly, Horvath built a multitissue predictor of age using 82 published DNA methylation datasets [31]. Interestingly, Horvath also showed that the estimated DNA methylation age of embryonic stem cells was nearly zero and increased with cell passaging, indicating that DNA methylation age is consistent with biological age at the cellular level [31].

Furthermore, the results of a growing body of epidemiological studies indicate remarkably high correlations between DNA methylation-based age and various aging-associated conditions [8]. Detailed assessments of DNA methylation clocks promote a better understanding of the biological foundation of aging and inform us about age-related disease risks. This approach is a typical example of interactions between epigenetics and aging, and its development will be informative and enable subsequent functional studies in humans. Most likely, the most exciting feature of epigenetic clocks is the reversibility of the DNA methylation process, which makes it possible to develop antiaging interventions [33, 34].

3.2. DNA Methylation and Age-Related Diseases. Longevity can indeed be regarded as a manifestation of healthy aging. In contrast, accelerated aging is always accompanied by the onset of chronic diseases, particularly degenerative diseases, which ultimately result in disability or premature death. Some researchers also consider age-related degenerative diseases as one of the factors that accelerate the aging process [35]. Emerging evidence indicates that DNA methylation is a crucial factor in age-related diseases.

Cardiovascular disease (CVD), one of the most common age-related diseases, accounted for 31% of global deaths in 2015 [36]. Systematic studies have associated CVD with DNA methylation [37]. In a 10-year follow-up study of 832 participants, increases in biological age, estimated by DNA methylation biomarkers, were accompanied by a 4% rising risk of CVD for each year [38]. CVD risk factors, such as smoking, also induce dysregulated DNA methylation [39]. In contrast, DNA methylation can directly regulate cardiovascular function by modifying the promoters of specific genes and reducing their expression. For example, reduced methylation of the angiotensin I-converting enzyme-encoding gene promoter affects the expression of this gene expression and eventually leads to hypertension [40]. Interestingly, a new role for DNA methylation in the metabolic reprogramming of ischemic cardiomyopathy has recently been discovered, a mechanism that is believed to contribute to the reprogramming of cardiac tissue during ischemia [41].

The incidence of most cancers exponentially increases with age. Aberrant DNA methylation patterns have been

observed in variety of tumor cells, including colon, ovarian, and breast cancer cells [42–44], indicating that they may serve as biomarkers in early diagnosis and treatment of cancer [45]. Two DNA methylation modifications appear to be primarily associated with cancer, including the hypomethylation of open sea regions and hypermethylation of promoter CpG islands, with other constituents only playing supplementary roles in the promoter or open sea region methylation process [46, 47]. Current DNA methylation-based biomarker studies focus on the influence of promoter hypermethylation in tumor suppressor genes (such as *RYR2*), which may alter cancer signaling transduction and promote the formation and development of cancer [48–50]. These findings are useful in the development of demethylation drugs aimed at specific tumor treatments.

DNA alterations that occur with age have also been investigated in neurodegenerative diseases. A similar aberrant DNA methylation pattern is shared in patients with Parkinson's disease, Alzheimer's disease, and Down syndrome [51]. During the course of these neurodegenerative diseases, disrupted CpG methylation has been reported to be similar in a set of genes involved in many cellular pathways [51]. Of the identified genes, DNA methylation of the ankyrin 1 gene shows specificity in different brain regions and neurodegenerative diseases [52]. 5hmC has recently been shown to be a potential epigenetic marker in cognitive deterioration [53]. However, additional evidence is needed to demonstrate causality between DNA methylation variability and neurodegenerative disease pathology.

In accordance with these findings, it should be emphasized that DNA methylation has been identified as an early detection maker of age-associated diseases and may also serve as a novel therapeutic target. In vivo studies with animal disease models will be necessary to provide causal evidence of the association between age-related diseases and the aging process.

4. Mechanisms of Aging Induced by DNA Methylation

During aging, predefined genes constantly undergo epigenetic modifications and exhibit altered expression in response to internal and external environmental stress. Changes in DNA methylation may occur hundreds of times over the lifespan of an individual in the form of a fully adaptive response. However, in some cases, this methylation acts as a switch for the acceleration of pathological aging, resulting in negative consequences [54]. Thus, global fluctuations in DNA methylation are not only a consequence but also a cause of aging. Understanding the biological mechanisms underlying the observed associations may reveal novel targets for reversing aging-related phenotypes and ultimately prolonging lifespan.

4.1. Loss of Proteostasis. Proteostasis maintenance depends on chaperones and two proteolytic systems, the lysosome-autophagy and ubiquitin-proteasome systems [55]. An increase in the disruption of protein homeostasis is one of the primary features of aging. In most organisms, a gradual loss of proteostasis has been observed during aging, and it is reported that long-lived species tend to have more stable proteomes [56]. Additionally, the accumulation of unfolded, misfolded, or aggregated proteins is the leading cause of some neurodegenerative diseases [57].

Evidence has emerged showing that decreased autophagic activity is involved in DNA methylation. DNA methylation inhibits autophagy processes in two ways, one of which is the direct modification and silencing of autophagy-related genes by DNMTs. The promoter regions of *Atg5* and *LC3* are hypermethylated in aged mice, which suppresses gene expression and disrupts the completion of autophagosomes [58]. Whole-body overexpression of *Atg5* results in antiaging phenotypes, extending the median lifespan of mice by 17.2% [59]. Furthermore, researchers have recently shown that DNA methylation inhibitors can rescue phenotypic changes associated with aging by reactivating autophagy-related genes [60, 61].

Another mechanism associated with the dysfunctional autophagy caused by DNA methylation is the modification of genes such as *miR-129-5P* and *FoxO3a*, encoding autophagy-related signaling molecules. In disc degeneration, hypermethylation in the promoter region of *miR-129-5P* reduces gene expression, which then blocks autophagy through downregulation of *Beclin-1* [62]. *FoxO3a* is a protective transcriptional regulator that maintains cell homeostasis from environmental stress by increasing autophagy [63]. DNA hypermethylation of the *FoxO3a* promoter indirectly inhibits autophagy, contributing to aging-related endothelial dysfunction [64]. Abnormal methylation in some tumor-related genes, such as *TCF21*, *CELF2*, and *NOR1*, is also involved in autophagy regulation, which contributes to some premature aging disorders [65–67].

While DNA methylation has gained recognition for its involvement in protein degradation, information regarding its effect on protein synthesis in senescent cells is just beginning to emerge. The expression of ribosomal RNA (rRNA) determines translational rate and protein synthesis, which decreases during physiological aging. It has been reported that abnormal methylation of ribosomal DNA (rDNA) promoters occurs with age [68]. Recent studies have confirmed that increased CpG methylation in rDNA promoter regions inhibits transcription and thus significantly reduces the expression of 18S, 5.8S, and 28S rRNA [69]. Furthermore, DNMTs have been shown to affect the synthesis of proteins associated with long-term memory, providing an explanation for memory impairments that occur with age [20].

Speculation has increased that experimental perturbation of proteostasis precipitates pathological change and accelerates aging. Therefore, further studies are needed to determine if genetic manipulations can successfully maintain proteostasis in aged mammals.

4.2. Mitochondrial Dysfunction. Mitochondria are considered to be the powerhouses of cells and are important for energy production through respiration and cellular metabolism regulation [70]. The accumulation of damage to mitochondria can reduce energy metabolism and increase the production of reactive oxidative species (ROS), leading to aging-associated changes. The expression of mitochondrial

methyltransferases has been shown to be age-dependent, indicating that mitochondria are involved in the development of proaging features [71].

Increasing methylation of *Elovl2* has recently been reported to be a crucial driver of aging by inducing a stress response in the endoplasmic reticulum and promoting mitochondrial dysfunction [6]. In some degenerative diseases, mitochondrial DNA and genes encoding the enzymes responsible for mitochondrial biogenesis, such as *Mfn2* in diabetes, are hypermethylated and compromise the electron transport chain [72]. Increasing methylation of the D-loop region and mitochondrial NADH dehydrogenase 6 causes insulin resistance in obese individuals, which can easily develop during aging [73]. The upregulation of 5mC and DNMTs in neuronal mitochondria has been postulated to be an important feature of neurodegeneration. In addition, DNMT inhibitors can improve the protection against oxidative damage in senescent cells [74]. There is also a positive correlation between DNA methylation and base mismatch in the dysfunctional mitochondria of vascular diseases [75]. Moreover, the DNMT inhibitor 5-azacytidine has recently been shown to reverse the aged phenotype of mesenchymal stem cells (MSCs) by reducing ROS and nitric oxide levels, the accumulation of which results in low viability and mitochondrial dysfunction [21].

These findings raise the possibility that rejuvenation of mitochondrial dysfunction may be a potential approach for prolonging life. Thus, further investigation is warranted with respect to determining whether directly targeting DNA methylation can ameliorate mitochondrial damage and delay aging progression using animal models.

4.3. Stem Cell Exhaustion. Adult stem cells are of great importance in maintaining tissue homeostasis and regeneration over a lifetime. Stem cell exhaustion can be described as a qualitative and quantitative decline of stem cells. This process has been observed in many senescent tissues and organs and is regarded as one of the driving forces of aging. Importantly, epigenetic regulators have been shown to control stem cell fate [76, 77]. The role of epigenetic dysregulation in stem cell exhaustion has recently become the subject of intense research, and DNA methylation is thought to be an age-dependent upstream regulatory factor affecting cell-specific gene expression as stem cells become more specialized.

A great deal of evidence has revealed that changes of DNA methylation level regulate genes involved in self-renewal in aging hematopoietic stem cells (HSCs). Adelman et al. demonstrated that epigenetic reprogramming of human HSCs, including redistribution of DNA methylation, occurs with age [78]. Supporting this finding, the inactivation of DNMT3a enhances cell self-renewal at the cost of differentiation potential in vivo [79]. Ablation of both DNMT3a and DNMT3b results in a more severe effect on cell differentiation capacity, which is one of the characteristics of natural HSC aging [80]. A deficiency of DNA demethylases, known as TET, yields similar results, which reduces genomic levels of 5hmC and contributes to lineage skewing towards myelopoiesis in HSCs [81]. Further evidence has revealed a direct connection between TET and HSC function in leukemogenesis [82].

Significant differences in DNA methylation levels are observed at specific CpG sites, especially in differentiation-related genes, throughout both the aging processes and long-term cultures of MSCs [83]. The DNMT inhibitor RG108 has been reported to modulate the transcription of prosenescence genes and alleviate oxidative stress-mediated damage in human bone marrow-derived MSCs (BM-MSCs) [74, 84]. DNMT3a and DNMT3b methylate the promoters of stem cell functional genes during the chondrogenic differentiation of BM-MSCs, and the reversion process can be regulated by the demethylating agent 5-azacitidine [85]. Similar results have been obtained in human hair follicle MSCs in which the upregulation of DNMTs suppresses downstream genes associated with stem cell properties, which is essential for maintaining self-renewal capacity and reversing cell senescence [86]. In addition, the DNA 6mA demethylase ALKBH1 has also been shown to affect the ability of MSCs to differentiate, providing new evidence of the potential regulatory role of DNA 6mA in mediating the aging process [87].

Considering the gradual decline of regenerative ability in muscle tissue with age, muscle stem cell (MuSC) senescence is of great concern. There are many studies on the relationship between age-related alterations of DNA methylation and transcriptional variability in senescent MuSCs. Changes in DNMT and TET expression have been observed in MuSCs resulting from proliferation to differentiation and between quiescence and proliferation [88, 89]. Furthermore, the regeneration of skeletal muscle after injury is markedly inhibited in *Dnmt3a*-knockout mice [90]. Bigot et al. observed that age-related DNA methylation in MuSCs primarily acts on the sprouty1 pathway and that through its suppression, the self-renewal capacity of senescent MuSCs is destroyed [91].

4.4. Immunosenescence. The deterioration of the immune system in aging, known as immunosenescence, is characterized by immune lineage skewing and higher levels of inflammatory markers. This process is one of the causes of inflammaging, a sterile, low-grade, and chronic proinflammatory condition of older organisms [22]. Tserel et al. observed different DNA methylation levels and skewing in human CD8+ T cells isolated from different age groups [92]. Age-related DNA methylation involves variations in both the levels of immune-related factors and the proportions of immune cell types.

A recent study observed age-sensitive hypermethylation in the promotor region of *Klf14* in several human tissues, which affects the differentiation of CD4+ T cells via suppression of *FOXP3* [93]. Garg et al. demonstrated that regulatory T cells from aged mice have an intensified inhibitory impact on effector T cells due to the hypomethylation of the *FoxP3* enhancer, which consequently increases immune suppression with age [94]. Similarly, the methylation content of *GSTM1* is involved in type 1 T helper cell differentiation [95]. Alterations in *Tet2* expression have been observed in myeloid malignancies, and TET2 has been shown to regulate myeloid and erythroid lineage differentiation [96]. The deficiency of DNMT1 in mice has been suggested to cause

immune senescence and is involved in the development of early autoimmunity [97].

Abnormal DNA methylation induced by aging also accompanies disturbances in inflammatory cytokines. Shinozaki et al. measured DNA methylation levels in brain and blood samples and observed a significant negative association between aging and the DNA methylation of inflammatory factor genes (such as TNF-alpha and IL-6) [98]. Methylation levels in the mouse *Klf14* promoter region increase with age and obesity and appear to be a regulatory factor of chronic inflammation in adipose tissue [99]. Furthermore, elevated 5hmC levels have been shown to promote the appearance of Iba1-positive inflammatory microglia in a study investigating age-related cerebrovascular alterations [100]. DNA methylation has also been suggested to have a role in the development of inflammation in several age-associated chronic diseases, such as cancer, osteoarthritis, and neurodegenerative diseases [101–103].

5. Prospects and Conclusion

The underlying mechanisms of aging have perplexed scientists for decades. Detailed evaluations of global DNA methylation changes have provided insights into the process of aging, with DNA methylation serving as a biomarker of biological age and a driving force of aging. Current findings should stimulate further discussion and experimentation based on epigenetic regulation in cell-, tissue-, and disease-specific aging models. Future studies focusing on the mechanisms by which specific parameters, such as stress, affect methylation patterns will uncover additional details of the aging process. Identification of the target genes modified by DNA methylation-related regulatory elements in aging individuals is highly informative to figure out the hormone-like effectors and signal pathways that mediate these alterations as well as related diseases. The interaction among epigenetic regulators during aging should also be highly valued. Further studies should focus on the cross-talk among these epigenetic regulators, such as DNA methylation, RNA methylation, histone methylation, and noncoding RNAs, which will aid in providing a full picture of epigenetics and aging. The results of such studies may pave the way for antiaging interventions as well as treatments for related diseases, enabling human life extension.

Conflicts of Interest

The authors declare no conflicts of interest.

Acknowledgments

This work was partly supported by grants from the Research Funding for Talents Developing, West China Hospital of Stomatology Sichuan University (No. RCDWJS2020-23), and the China Postdoctoral Science Foundation Grant (No. 2019TQ0218).

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Hindawi
Stem Cells International
Volume 2020, Article ID 8863539, 12 pages
https://doi.org/10.1155/2020/8863539

Review Article

Ribosomes: An Exciting Avenue in Stem Cell Research

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Received 9 April 2020; Revised 12 June 2020; Accepted 16 June 2020; Published 6 July 2020

Academic Editor: shuibin lin

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Stem cell research has focused on genomic studies. However, recent evidence has indicated the involvement of epigenetic regulation in determining the fate of stem cells. Ribosomes play a crucial role in epigenetic regulation, and thus, we focused on the role of ribosomes in stem cells. Majority of living organisms possess ribosomes that are involved in the translation of mRNA into proteins and promote cellular proliferation and differentiation. Ribosomes are stable molecular machines that play a role with changes in the levels of RNA during translation. Recent research suggests that specific ribosomes actively regulate gene expression in multiple cell types, such as stem cells. Stem cells have the potential for self-renewal and differentiation into multiple lineages and, thus, require high efficiency of translation. Ribosomes induce cellular transdifferentiation and reprogramming, and disrupted ribosome synthesis affects translation efficiency, thereby hindering stem cell function leading to cell death and differentiation. Stem cell function is regulated by ribosome-mediated control of stem cell-specific gene expression. In this review, we have presented a detailed discourse on the characteristics of ribosomes in stem cells. Understanding ribosome biology in stem cells will provide insights into the regulation of stem cell function and cellular reprogramming.

1. Introduction

Ribosomes are subcellular cytoplasmic biomolecules composed of rRNA and dozens of proteins. Ribosome sedimentation coefficients in eukaryotic cells and prokaryotic cells are 80S and 70S, respectively. Ribosomes primarily participate in translation, but recent research shows their involvement in multiple biological processes, such as cellular proliferation, differentiation, homeostasis, and development of cancer (these are known as "heterogeneous ribosomes") [1, 2]. The ribosome filter hypothesis posits that, besides constituting the translation machinery, ribosomes influence the selective expression of mRNAs, thereby differentially regulating cellular function [3]. The efficiency of ribosome biosynthesis depends on specific environments, thereby differentially regulating the function of various cells, such as stem cells. Self-renewal is an attribute of stem cells that requires high translation efficiency [4–8]. Inhibiting translation of genes using transcriptional repressors leads to reduced stemness [4]. Hematopoietic stem cells also require significant ribosomal activity [9]. Cells can internalize ribosomes via trypsin-activated endocytosis to generate cell clusters similar to embryonic bodies expressing pluripotency markers [10]. It has been reported that ribosomes regulate stem cell differentiation and embryonic growth [11]; however, the mechanisms involved in this process remain to be understood. This review summarizes characteristics of "stem ribosomes".

1.1. Ribosome-Mediated mRNA Translation. mRNA translation primarily involves 3 steps: initiation, elongation, and termination [12]. And the mRNAs have dynamic interactions of the small and large subunits of the ribosome, aided by multiple auxiliary factors during the process of translation [13]. Ribosomes read the codons (genetic code) in the mRNA; each codon corresponds to the addition of an amino acid [14]. Initiation is an important rate-limiting step in translation [15]. During this step, initiation factors facilitate the recruitment of the 40S subunit to the mRNA $5'$ end, scanning of the $5'$ untranslated region (UTR), start codon recognition and 80S subunit joining to form an elongation-competent ribosome [16–18]. mRNAs possess regulatory elements that regulate the frequency of translation initiation, choice of the open reading frame (ORF), global and local

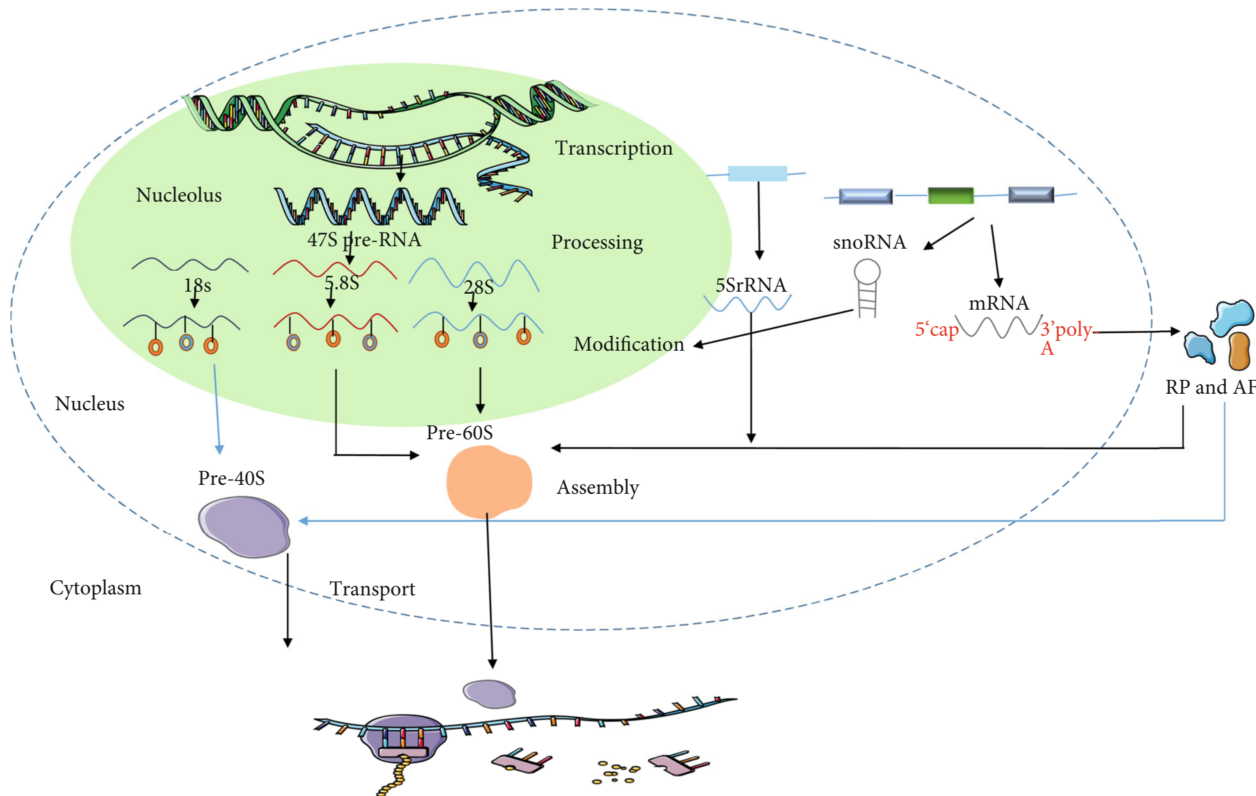

Figure 1: Eukaryotic ribosome synthesis. Eukaryotic ribosome synthesis is a complex process that comprises 5 steps, including transcription, processing, modification, assembly, and transport. (1) Transcription: RNA polymerase I transcribes rDNA into 47S preRNA. RNA polymerase III transcribes 5S rRNA. snoRNAs are transcribed by RNA polymerase II or III from non-protein-encoding regions or mRNA introns. RNA polymerase II transcribes the mRNAs for ribosome proteins (RPs) and assembly factors (AFs). (2) Processing: 47S pre-rRNA is processed to 18S, 28S, and 5.8S rRNAs. (3) Modification: there are two primary kinds of modifications on rRNA that are mediated by snoRNAs: 2′-O-methylation (2′-O-Me) and pseudouridines (Ψ). (4) Assembly: RPs and AFs are translated in the cytoplasm and shuttled to the nueclus for ribosome assembly. The pre-60S subunits comprise 28S, 5.8S, and 5S rRNA, and the pre-40S subunit includes an additional 18S rRNA. (5) Transport: the subunits are transported to the cytoplasm via the nuclear pore to be assembled as needed during translation.

rates of elongation, and protein folding [19]. Structured or excessively short 5′ UTRs [20, 21] and upstream open reading frames (uORFs) [20, 22] negatively influence translation efficiency, while internal ribosome entry sites (IRESs) [23, 24], other regions of direct ribosomal recruitment [25, 26], and codon bias at the sites of initiation sites [27, 28] enhance initiation in response to ribosome shortage. The efficiency of elongation depends on codon usage, secondary structures in the mRNA, and ribosome density. Finally, translation terminates when the ribosome encounters a termination codon [19]. Thus, the cis-elements in mRNAs can be used in combinations to regulate the activity of ribosomes, thereby resulting in selective gene expression. This gives rise to ribosome heterogeneity that includes subsets of ribosomes with differential selectivity for mRNA subpools [2].

1.2. Assembly of Ribosomes. Ribosome synthesis is an energy-intensive process that requires complex machinery comprising numerous proteins and RNAs (Figure 1) [29]. Ribosomes are assembled from large and small subunits: large and small subunits predominantly function in peptide bond transfer and mRNA decoding, respectively [30]. There are four main components of ribosome synthesis: ribosome proteins (RPs), assembly factors (AFs), ribosomal RNAs (rRNAs), and small nucleolar RNAs (snoRNAs) [1]. Ribosome precursors are synthesized in nucleoli whose internal structure comprises three characteristic regions: fiber center (FC), dense fiber component (DFC), and particle component. rRNAs are transcribed between FC and DFC. rRNAs and their binding proteins reside in the DFC. rRNAs are also cleaved, processed, and modified in the DFC. The ribosome precursor is assembled in the particle component [31]. In eukaryotic nucleoli, RNA polymerase I transcribes rDNA into 47S preRNA that is spliced to form 5.8S, 28S, and 18S rRNA [32, 33]. In the eukaryotic nucleus, RNA polymerase III transcribes 5S rRNA that participates in the formation of the 60S subunit with 28S and 5.8S rRNA. The 40S subunit is composed of 18S rRNA and 33 RPs, while the 60S subunit comprises 5S, 5.8S, and 28S rRNA and 47 RPs.

rRNAs can be modified or processed by snoRNAs [34] that are transcribed by RNA polymerase II or III or arise from pre-mRNA introns. snoRNAs are found in the nucleus and provide a direct role in the post transcription of rRNA and mRNA [35]. snoRNAs interact with proteins to form

small nuclear ribonucleoproteins (snoRNPs) that direct rRNA processing and modification [36].

There are ~80 RPs [37], majority of which are cotranscribed with rRNA [38]. mRNAs for RPs are translated in the cytoplasm following which they are transported back to the nucleus to form the precursor of ribosomal subunits. To enable efficient protein translation, ribosome assembly also requires specific AFs [39]. Eukaryotes possess more than 500 AFs [40]. AFs are associated with rRNA at specific stages including rRNA processing and modification, thereby facilitating the binding of RP and influencing ribosome biogenesis [41]. AFs mainly consist of multiple enzymes and proteins with known protein or RNA-binding domains. Specific AFs such as FBL and BYSL are overexpressed in stem cells and maintain pluripotency by promoting ribosome biogenesis [42–44].

Differentiation of embryonic stem (ES) cells can be caused by a decrease in ribosomal abundance. Inhibition of protein synthesis influences numerous proteins with short half-lives. The expression of key proteins with short half-lives depends on multiple factors [45]. In human ES cells, the expression of the short-lived Nanog protein is erratic. The proteolysis of Nanog is mediated by the ubiquitin–proteasomal pathway [46]. Mouse embryonic stem cell (mESCs) can be treated with the transcription inhibitor 4EGI-1 to result in the rapid reduction of the protein levels of Nanog, Esrrb, and Tfcp2l1 and a steady time-dependent reduction in their mRNA levels [47]. Ribosome biogenesis is composed of five main steps involving transcription, processing, modification, assembly, and transport of ribosome precursors. Careful regulation of the multiple steps in ribosome biogenesis enables efficient translation and is critical for maintaining pluripotency.

1.3. Ribosome-Induced Cellular Transdifferentiation. Transdifferentiation involves the reprogramming of somatic cells into those of a different lineage without going through the intermediate proliferative pluripotent stem cell stage; it is a new method to generate functional cells [48–50]. *Mycobacterium leprae* transdifferentiates Schwann cells into pluripotent cells by downregulating differentiation markers (SOX10, Mpz, and p75) and upregulating genes associated with mesodermal development (Sox2, CD44, and CD43) [51]. *Helicobacter pylori* infection in intestinal epithelial cells promotes the expression of CDX1 [52]. CDX1 induces the expression of pluripotency factors KLF5 and SALL4, thereby transdifferentiating gastric epithelial cells into intestinal epithelial-like cell [52, 53]. Proteins from *Wolbachia pipientis*, especially W20, accelerate mammalian cell reprogramming [54]. Lactic acid bacteria (LAB) convert human dermal fibroblasts (HDFs) into pluripotent cells [55]. LAB-differentiated cell clusters have the potential to form three germ layer cells along with increasing the expression of the marker for pluripotency, Nanog [55]. Thus, bacteria promote host cell reprogramming, but the mechanisms involved remain to be investigated. To understand LAB-induced transdifferentiation of HDFs, LAB lysates were used to treat trypsinized HDFs; the protein fraction of size > 100 kDa obtained from ultrafiltered lysates was found to induce cell cluster formation [10]. Owing to the size of the fraction, the "transdifferentiation factor" was speculated to be the ribosome. Purified ribosomes obtained by ultracentrifugation promoted HDF transdifferentiation. These ribosome-induced cell clusters can enhance the expression of pluripotency factors and give rise to endodermal, mesodermal, and ectodermal cells, but they could not form teratomas and chimeras [10]. Ribosome-induced cell clusters need to be induced with trypsin [56]. Since the diameter of a ribosome is ~20 nm [57], it can undergo nucleocytoplasmic shuttling and internalized by other cells via endocytosis by endosomal vesicles that are ~10 μm in size [58]. The characteristics of this ribosome that promote transdifferentiation and express stem cell markers remain to be understood fully.

1.4. rRNA Transcription Efficiency Determines the Fate of Stem Cells. The nucleus of ES cells quickly adapts to increases in cellular proliferation that requires rapid transcription of rRNAs [59, 60]. To promote the initiation of transcription, RNA polymerase I specifically binds to the promoter region of rDNA via transcription factors, such as upstream binding factor (UBF) and promoter selectivity factor (SL1/TIF-IB) [61]. The efficiency of rRNA transcription determines the speed of ribosome biosynthesis and assembly. Stem cells heavily transcribe rRNAs, but their levels decrease as cells differentiate [32]. The expression of c-Myc, an important stem cell marker, decreases during differentiation [62]. A reduction in the levels of RNA polymerase-associated factors downregulates rRNA synthesis [63], thereby inducing cell differentiation [64]. Downregulation of rRNA correlates with an increase in the levels of linage-specific factors that are responsible for differentiation into specific cell types (e.g., MyoD and myogenin during myogenesis, Runx2 during osteogenesis, and C/EBP-β, C/EBP-δ, and C/EBP-α during adipogenesis); these factors hinder rRNA transcription by interacting with UBF or rDNA promoters [65]. An ex vivo experiment demonstrated that actinomycin D-mediated inhibition of rRNA transcription induces the differentiation of mouse hematopoietic stem cells (HSCs). Thus, it is generally accepted that a decrease in rRNA transcription correlates with cellular differentiation.

In eukaryotes, 75% of rRNAs are transcribed by RNA polymerase I [1]. This enzyme complex comprises Udd, TAF1B, and a TAF1C-like factor in *Drosophila*. Increased transcription by RNA polymerase I inhibits cell differentiation, while inhibition of RNA polymerase I-mediated transcription limits ribosome biogenesis and promotes cellular differentiation [66]. FBL methylates a glutamine residue in histone H2A and stimulates RNA polymerase I binding on rDNA gene promoters [67].

A recent study has shown that 17 pluripotency-associated factors bind rDNA loci in mESCs [32]. Moreover, silencing of rDNA genes and downregulated ribosome biogenesis are associated with stem cell ageing in murine HSCs [68]. In general, stem cells have higher rRNA transcription efficiency than the daughter cells and rRNA synthesis is downregulated by phenotype-specific transcription factors during differentiation. rDNA transcription is quantitatively regulated in stem cells and the rate of rDNA transcription influences cell fate.

Beyond rDNA transcription: many factors at all steps of the process appear to play stem cell-specific roles.

1.5. rRNA Processing and Stem Cells. rRNA processing is an evolutionarily conserved phenomenon that is essential for ribosome assembly. Ribosome assembly and pre-rRNA processing are closely linked, and the primary 47S transcript is cleaved to the 20S and 32S intermediates that are processed to the mature 18S and 5.8/28S rRNAs (components of the 40S and 60S ribosomal subunits, respectively). Stem cell AFs promote rRNA processing to improve the efficiency of ribosome synthesis. Small subunit processome (SSUP) is a pre-18S processing complex composed of snoRNA U3 and 54 proteins encoded by six genes (Krr1, Ddx47, Ddx52, Nol6, Pdcd11, and Rrp7a) in mESCs [4]. These SSUP genes are overexpressed in stem cells but downregulated during embryoid body formation. Depleting cells of the SSUP reduces Nanog expression, while knocking out SSUP genes hinder cellular reprogramming. Krr1, a conserved yeast homolog of SSUP [45], promotes the cleavage of 18S rRNA at sites A0, A1, and A2 to generate the 40S subunit [69]. SSUP stimulates pluripotency by enhancing translation. ES cells exhibit an upregulation in the subunits of SSUP, thereby enhancing the rate of translation and regulating pluripotency.

Lrrc34 (leucine-rich repeat-containing 34) is another gene that is robustly expressed in mESCs and is downregulated during differentiation [70]. Lrrc34 is a nucleolar protein that interacts with nucleophosmin and nucleolin regulate pluripotency-related genes, such as OCT4, and is important in rRNA processing and ribosome formation [71]. Urb2, another nucleolar protein, plays a role in 27S pre-RNA processing and 60S subunit biogenesis [72]. Moreover, mutations in Urb2 impair HSC development by disrupting the biogenesis of ribosomal subunits and rRNAs in zebrafish [11, 72].

Nucleostemin is overexpressed in proliferating cells, such as central nervous system stem cells, ES cells, and cancer cell lines, and downregulated during differentiation. It contains an N-terminal basic domain that is involved in nucleolar localization and two GTP-binding motifs that regulate its transport between the nucleolus and nucleoplasm [73, 74]. Nucleostemin regulates cell proliferation via p53 signaling and is involved in ribosomal biogenesis, especially pre-RNA processing. It is a large protein complex (>700 kDa) comprising five ribosomal subunits (RPS6, RPS8, RPS24, RPL13, and RPL14), three nucleolar proteins (DDX21, Pes1, and EBP2), and a translation initiation factor (eIF2B1) [75]. DDX21 is a DExD/H box protein that uses energy from ATP hydrolysis to unwind RNA or disrupt RNA-protein complexes that could alter RNA [76]. It stabilizes 28S rRNA, promotes the conversion of the 20S pre-RNA into 18S RNA in *Xenopus*, and processes of 18S and 28S rRNAs in humans [77]. Pes1 is also involved in processing the 12, 36, and 32S pre-rRNAs in mammals, thereby promoting the biogenesis of the 60S ribosomal subunit [78]. EBP2 interacts with ribosomal proteins L36, L34, and L8; L36 is important for processing 27SA2, 27SA3, and 27SBL pre-rRNAs [79]. Furthermore, nucleostemin and 60S subunits can be found in the same fraction following sucrose gradient centrifugation, indicating the involvement of nucleostemin in ribosome synthesis [75]. In summary, the interactions between DDX21, Pes1, EBP2, and nucleostemin enhance pre-RNA processing to promote 60S ribosomal subunit synthesis and improve the efficiency of translation.

Bystin-like (BYSL) is detected in abundance in rapidly proliferating embryo and cancer cells and is evolutionarily conserved across eukaryotes, especially the C-terminus that regulates its nuclear localization [80–83]. Knocking out BYSL inhibits the synthesis of 18S rRNA and enables the accumulation of 20S rRNA precursors without affecting 28S rRNA. Moreover, there is a decrease in the cytoplasmic content of the 40S subunit, suggesting the role of Bysl in the export of the 40S subunit [33]. Bysl is also a key regulator of c-Myc and is overexpressed in stem and cancer cells [84, 85]. Enp1 is the yeast ortholog of Bysl that is predominantly localized to the nucleolus. Similar to Krr1, Enp1 functions in 18S rRNA processing and cleavage of the 35S pre-RNA at sites A0, A1, and A2 [86]. Enp1 has been observed to coimmunoprecipitate with a cohort of proteins, including Nop1 (the yeast ortholog of FBL) [87]. Enp1 and Nop1 interact with snoRNAs U3 and U14 and stimulate rRNA processing.

1.6. Specific rRNA Modifications in Stem Cells. rRNA modifications change according to different stimuli, diseases, and development, and this results in ribosome heterogeneity, thereby differentially regulating gene expression [34]. Eukaryotic rRNAs possess 91 pseudouridines (Ψ), 105 sugars containing $2'$-O-methylation ($2'$-O-Me), and 10 methylated bases [88]. Modifications are primarily found in the functional regions of the ribosome and are induced by snoRNPs wherein snoRNAs complementary to specific rRNA sequences determine the methylation site [89, 90]. snoRNAs can be divided into C/D or H/ACA box-containing snoRNAs [91]. C/D box snoRNAs predominantly undergo $2'$-O-Me modification, while H/ACA box snoRNAs undergo substitution with Ψ [92]. rRNA modification alters the secondary and tertiary structure of ribosomes that is important for ribosome biogenesis and function [93]. Differential modification of particular rRNA sites results in ribosome heterogeneity.

Fragile X mental retardation protein (FMRP) is an RNA-binding protein that is important for neuronal development and differentiation. In animal and human stem cells, FMRP maintains pluripotency, regulates cell fate, and determines the speed of generating neuronal lineage-committed cells [94–97]. FMRP has been shown to function predominantly in the cytoplasm; however, recent evidence has demonstrated its role in the nucleus [98, 99]. In the nuclei of human embryonic stem cells, FMRP directly interacts with C/D box snoRNAs and results in the $2'$-O-Me modification of rRNA, thereby causing ribosome heterogeneity by affecting rRNA folding and ribosomal assembly [100, 101]. In the cytoplasm, FMRP identifies $2'$-O-Me-modified ribosomes to enable specific translation of its target mRNAs [101, 102]. FMRP promotes the expression of genes involved in stem cell intracellular pathways, such as mTOR, PI3K, ERK, and Gsk3β [103–106].

Fibrillarin is a protein that is involved in proliferation [107], cancer [108], and stem cell differentiation [43].

Fibrillarin is enriched in the DFC region of the nucleolus and contains an N-terminal domain rich in glycine and arginine residues (namely the GAR domain), a central RNA-binding domain comprising an RNP-2-like consensus sequence, and a highly conserved C-terminal helical domain that may act as methyltransferases [109, 110]. The human GAR domain enables fibrillarin-interacting pre-RNAs to process nascent 47S pre-rRNAs and demarcate the DFC region. As a part of C/D box snoRNPs, FBL catalyzes the 2′-O-Me of rRNAs to regulate ribosome biogenesis and translation [111, 112]. Thus, fibrillarin functions in pre-rRNA processing and modification, thereby regulating ribosomal biogenesis. It can also enhance the activity of RNA polymerase I. Nop1, the yeast homolog of fibrillarin, also processes pre-RNAs, especially 18S rRNA. Fibrillarin has been reported to be overexpressed in mouse embryonic stem cells and maintains pluripotency state even in the absence of LIF [43]. During stem cell differentiation and neurogenesis, fibrillarin is downregulated and may affect the 2′-O-Me modification of rRNAs to regulate ribosome biogenesis with modified translational specificity such that IRES-containing mRNAs (e.g., *cMYC*, *FGF1*, and *VEGFA*) are preferentially translated instead of 5′-capped transcripts [43, 107, 108].

1.7. RP Heterogeneity in Stem Cells. Differences in RP composition and isoform lead to ribosome heterogeneity [113] that enables the recognition of sequence-specific elements or structures in mRNAs and selective expression [2, 114, 115]. Various RPs express to different extents in different tissues of the developing mouse embryo [116].

Quantitative mass spectrometry was used to measure the RP abundance and identify heterogeneous compositions of translationally active ribosomes in mESCs [2]. Ribosomes containing RPS25 or RPL10A translate specific transcript subpools, including mRNAs encoding key components in metabolism, the cell cycle process, and development, while the depletion of RPL10A does not affect the overall polysome profiles but reduces translation efficiencies of mRNAs associated with metabolism [2]. The heterogeneous RPs identified by SRM are located on the surface of the ribosome in important functional regions including the mRNA exit tunnel and the L1 stalk and thus directly interacts with mRNAs [117, 118]. RPL10A directly interacts with the IRES and engages the 80S ribosome independent of some or all initiation factors to achieve translational regulation of mRNAs, highlighting the importance of cis-regulatory elements in selective mRNA translation [2, 119, 120].

Diamond-Blackfan anemia is a special hematological disease. Patients present with a decrease in the population of erythroid precursors and progenitors in the bone marrow that is caused by heterozygous loss-of-function mutations in one of 18 different RP genes (e.g., RPL11, RPS19), thereby resulting in RP haploinsufficiency [121, 122]. Knockdown of RPL11 or RPS19 reduces IRES-mediated translation, especially of Bag1 that protects GATA1 from caspase-3-mediated cleavage during terminal erythroid differentiation [123–125]. RP mutations reduce the key lineage-determining hematopoietic transcription factor GATA1 mRNA in Diamond-Blackfan anemia [125].

Mutations in RPL21 are linked to stem cell-specific defects, such as loss of body hair [126]. RPL38 mutant embryos show no change in global protein synthesis but selectively affect the translation of a subset of Homeobox mRNAs [116].

Collectively, these findings suggest that RPs are regulated to confer a new layer of specificity in the control of gene expression, mammalian development, and stem cell biology.

1.8. AFs Interact with RPs to Regulate Stem Cell Function. RP synthesis is closely linked to other biological processes [127]. RPs are translated in the cytoplasm by preexisting ribosomes following which they enter the nucleoli and bind to rRNA to form ribosomes. RPs may play selective roles in eukaryotic ribosomes during cellular homeostasis and development [114]. Some AFs directly interact with and stabilize RPs, while others associate with DNA to stimulate transcription. UBA52 encodes a fusion protein of ubiquitin and RPL40 that is important for embryonic development. The RPL40 cleaved from UBA52 is important in protein biogenesis and forms a ribosomal complex with ubiquitin cleaved from UBA52. Efficient protein synthesis requires the cleavage of RPL40 from the fusion protein [128].

Bmi1 is a member of the polycomb group of proteins that bind to the promoter of target genes and induce epigenetic modifications in the chromatin to regulate cancer and stem cell biology [129–131]. Bmi1 affects the proliferation and differentiation of HSCs as well as other stem cells, such as mesenchymal stem cells and neural stem cells [132, 133]. In K562 cells, Bmi1 binds to the promoter of ribosomal genes, such as *RPL5*, *RPL1*, *RPL23*, *RPS14*, and *RPS19*; thus, a loss in this interaction downregulates ribosomal proteins and results in impaired ribosome biogenesis, thereby reducing global translation efficiency [134, 135]. Bmi1 promotes the transcription of RPs by recruiting active histone marks including H3K9ac and H3K4me3.

Runx1, another transcription factor, binds to the promoters of RP-encoding genes and rDNA repeats to regulate the transcription of rDNA and ribosomal biogenesis in HSPCs [65, 136]. Runx1 forms the core ribosomal promoter element with RUNX1, GATA2, and FLI1 that affects ribosomal biogenesis in conjunction with cooperative hematopoietic transcription factors [137, 138]. Runx1 is regulated by the global regulator of ribosome biogenesis, Myc [139]. Moreover, Bmi1 directly interacts with RUNX1 to recruit polycomb repressor complex 1 to regulate ribosome biogenesis and assembly [140].

The phosphatidylinositol-3-kinase (PI3K)/Akt and mammalian target of rapamycin (mTOR) signaling pathways (PI3K/Akt/mTOR) are pivotal for cell growth and survival [141–144]. Cells can be reprogrammed by activating IGF1/AKT/mTOR signaling and increasing the translation of RPs in cells depleted of MeCP2 (Figure 2) [145]. mTOR phosphorylates downstream effectors, including S6 kinase (s6K) [146] and eukaryotic initiation factor 4E (eIF4E) binding protein 1 (4E-BP1) [147, 148]. eIF4E inhibits translation, while mTOR-phosphorylated eIF4E relieves translational suppression to promote cap-dependent translation [148]. The phosphorylation of S6K promotes the biogenesis of

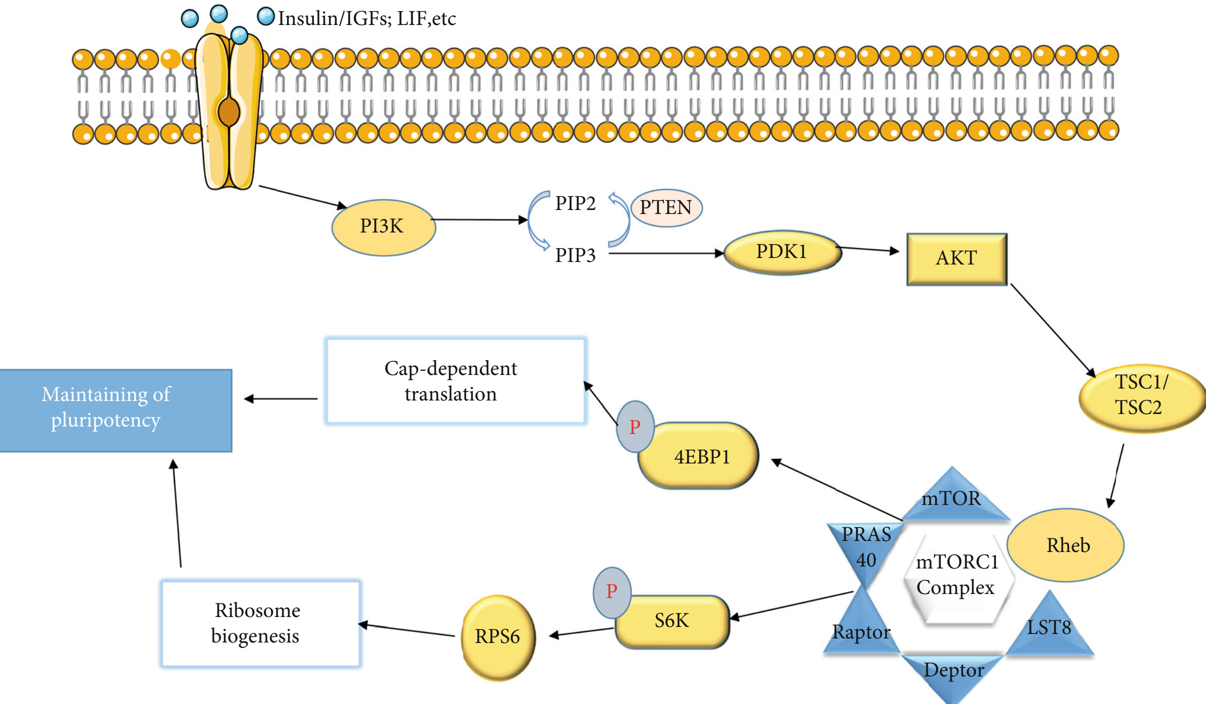

Figure 2: PI3K/AKT/mTORC1 signaling involved in translation and pluripotency. PI3K is activated by cytokines, such as LIF, Wnt, and growth factor receptors, to induce cell proliferation and regulate mTOR signaling to maintain pluripotency. This primarily involves the mTORC1 complex that phosphorylates S6K and 4EB-P1. Phosphorylated 4EB-P1 relieves suppressed translation by stimulating cap-dependent translation; phosphorylated S6K increases the levels of ribosomal protein S6 that is important in the biogenesis of 40S subunits. This helps maintain stem cell pluripotency by enhancing ribosome biogenesis to promote the translation of specific stem cell genes.

RpS6 (component of the 40S subunit) and translation [149, 150]. mTOR is important for the development of ES cells and can be regulated by PI3K signaling involved in ES cell pluripotency [151–153].

1.9. Ribosome Assembly and Transport in Stem Cells. PDCD2 is a conserved protein in eukaryotes that is present in mouse ES cells and other rapidly proliferating cells, such as cancer cells, and detected in abundance (if at all) in differentiated or slow-growing cells [154–157]. Zfrp8, the homolog of PDCD2 in *Drosophila*, functions in the maintenance of HSCs [158]. PDCD2 is a member of TYPP domain-containing proteins (TSR4, YwqG, PDCD2L, and PDCD2), among which TSR4 regulates rRNA processing and ribosome maturation [159]. Zfrp8/PDCD2 directly interacts with the 40S ribosomal subunit via RpS2, thereby regulating the cytoplasmic levels of RpS2 and stability of the 40S subunit [160]. The 40S subunit consists of more than 30 RPs that bind to numerous non-RPs to regulate translation, subunit assembly, and nucleocytoplasmic transport [161, 162]. Thus, Zfrp8/PDCD2 plays a key role in translation; however, it is not essential during general translation [160]. Zfrp8/PDCD2 can recruit different RNA-binding proteins, such as FMRP/Fmr1 and NUFIP1/Nufip (nuclear FMRP-interacting protein), form mRNA-RNP complexes that bind specifically to the 40S subunit, and spatiotemporally regulate target gene expression [163–165]. Zfrp8/PDCD2 also regulates the translation of protein-coding genes by promoting nuclear export of the mRNAs [160]. Thus, Zfrp8/PDCD2 is important in ribosome assembly and regulates the transport of specific mRNAs to maintain properly functioning stem cells.

2. Conclusions

Ribosomes are tools that are important for translation in different kinds of cells. However, recent research has shown that it exists in heterogeneous forms to differentially regulate gene expression [2]. Ribosome biogenesis is a very complex process. Although the basic steps of ribosome synthesis are conserved [166], there are various factors that can regulate the different processes [167] to modulate the translation efficiency of specific genes. Most of these factors are highly expressed in stem cells; knockout or mutation affects stem cell function and leads to cell death. Ribosome heterogeneity is when ribosomes have different composition, such as rRNAs, RPs, and AFs, and allows the selective translation of mRNAs to generate the appropriate types and amounts of proteins needed to regulate cellular function to the environment. Specific features in mRNAs, such as cis-elements, are recognized by specialized ribosomes, thereby enabling selective translation [2]. What is more, mRNA recognition and translation by the ribosome are based on combinatorial sets of RNA–RP interactions; thus, ribosome heterogeneity and its role in translational control may be mainly determined by RP composition and modification [115]. We know that there is a long way to go to decipher the heterogeneity ribosome, and recently, research discovered that

stem cells and differentiated cells express different subsets of tRNAs [168, 169].

Stem cells differentiate into lineage-committed cells that proliferate to form specific tissues, organs, and systems in our body, thereby highlighting their importance as ideal sources for repair of damaged cells and tissues. Owing to the limited abundance of stem cells, Yamanaka and colleagues expressed four specific genes (*OCT4*, *KLF4*, *SOX2*, and *CMYC*; OSKM) to reprogram differentiated cells into induced pluripotent stem cells [170]. However, since reprogramming is an inefficient process, there is ongoing research on the identification of factors that accelerate reprogramming [171, 172]. Numerous studies have shown that the presence of long noncoding RNAs (lncRNAs) promote the maintenance of stem cell function [173]. LncRNAs, such as Peblr20 and SNHG14, significantly improve reprogramming efficiency [174, 175]. This review focuses on the diversity of ribosomes associated with the translational control of stem gene expression and identification of specific recognition elements in the mRNAs associated with stemness. AFs in stem cells improve the efficiency of ribosome biogenesis and promote the translation of stem cell-related genes. Thus, using these AFs with the factors involved in reprogramming (lncRNAs, proteins, etc.) will promote ribosome synthesis and improve reprogramming efficiency. However, further research is required on the mechanisms by which ribosomes specifically regulate the expression of selective stem cell-related genes. Addressing this will pave way for a new direction in stem cell research that will help stimulate stem cell reprogramming and promote the clinical application of stem cells.

Data Availability

No data were used to support this study.

Conflicts of Interest

The authors declare that they have no conflict of interest.

Acknowledgments

We would like to thank Editage (http://www.editage.cn) for English language editing. Publication of this review was supported by the National Natural Science Foundation of China (grant numbers 81372835 and 81670143) and National Key Research and Development Program of China (grant number 2018YFA0106902).

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Hindawi
Stem Cells International
Volume 2020, Article ID 8849218, 12 pages
https://doi.org/10.1155/2020/8849218

Research Article

The m⁶A Methylation-Regulated AFF4 Promotes Self-Renewal of Bladder Cancer Stem Cells

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Received 25 April 2020; Revised 9 June 2020; Accepted 11 June 2020; Published 2 July 2020

Academic Editor: Quan Yuan

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The dynamic N^6-methyladenosine (m^6A) modification of mRNA plays a role in regulating gene expression and determining cell fate. However, the functions of m^6A mRNA modification in bladder cancer stem cells (BCSCs) have not been described. Here, we show that global RNA m^6A abundance and the expression of m^6A-forming enzyme METTL3 are higher in BCSCs than those in non-CSCs of bladder cancer (BCa) cells. The depletion of the *METTL3* inhibited the self-renewal of BCSCs, as evidenced by decreased ALDH activity and sphere-forming ability. Mechanistically, METTL3 regulates the m^6A modification and thereby the expression of AF4/FMR2 family member 4 (AFF4), knockdown of which phenocopies the *METTL3* ablation and diminishes the tumor-initiating capability of BCSCs *in vivo*. AFF4 binds to the promoter regions and sustains the transcription of *SOX2* and *MYC* which have critical biological functions in BCSCs. Collectively, our results demonstrate the critical roles of m^6A modification in self-renewal and tumorigenicity of BCSCs through a novel signaling axis of METTL3-AFF4-SOX2/MYC.

1. Introduction

Cancer stem cells (CSCs, also known as tumor-initiating cells), a relatively rare population of cancer cells, have characteristics of self-renewal capability, tumorigenic capacity, and pluripotency, which contribute to the driving force of tumorigenesis and metastasis. These stemness properties make CSCs resistant to conventional chemotherapies and cause subsequent recurrence, leading to clinical treatment failure [1]. Effective therapeutics and strategies targeting CSCs are desperately needed, whereas our knowledge of the CSCs is still incomplete so far.

Bladder carcinoma (BCa) is one of the most common malignancies and is characterized by rapid progression and high risk of recurrence [2, 3]. To better understand and eventually eliminate the bladder cancer stem cells (BCSCs), we and other groups have successfully identified several different BCSCs and determined their roles in BCa progression *in vivo* [4–7]. Moreover, we have found low-dose decitabine (a DNA methyltransferase inhibitor) could diminish the stemness of BCSCs without causing severe cytotoxicity [8], suggesting an important role of epigenetic regulation in BCSCs.

Besides the DNA methylation, recently we and others have found that aberrant N^6-methyladenosine (m^6A) methylation was also implicated in BCa progression [9–11]. RNA m^6A is the most prevalent chemical mark observed in approximately 25% of eukaryotic mRNAs [12–14]. In mammalian cells, this dynamic modification is catalyzed by a methyltransferase complex consisting of several "writers," which include methyltransferase-like 3 (METTL3), METTL14, Wilms tumor 1-associated protein (WTAP), VIRMA (KIAA1429), and RBM15 [15–19], and removed by two "erasers": fat mass and obesity-associated protein (FTO) [20] and alkylation repair homolog protein 5 (ALKBH5) [21]. Aberrant m^6A modification plays crucial roles in the progression of different types of cancer [22], especially as

the modulator of CSCs of breast cancer [23], glioblastoma [24, 25], and leukemia [26, 27]. However, its function and mechanism in regulating CSCs seem to be context-dependent and have not been described in BCSCs so far.

In our previous study, we found that m^6A abundance of both *MYC* and *AFF4* mRNAs was regulated by aberrantly expressed METTL3 in BCa cells [11]. As a core component of the super elongation complex (SEC), AFF4 is involved in the regulation of transcription elongation of many genes encoding the pluripotency factors [28, 29]. For instance, AFF4 could upregulate *SOX2* transcription to promote the tumor-initiation capacity of head and neck squamous cell carcinoma (HNSCC) [30], and *MYC* is another known target of AFF4 [11, 31]. Inspired by these results, we hypothesized that m^6A plays a role in promoting the stemness of BCa cells by regulating AFF expression.

Here, we provide unequivocal evidences that the expression of METTL3 and RNA m^6A level is significantly higher in the CSCs relative to the non-CSCs of BCa; METTL3 promote the self-renewal capability of BCSCs by regulating the mRNA m^6A level and therefore the expression of AFF4, which in turn bind to the promoter regions of *SOX2* and *MYC* to activate their transcription. Our findings reveal the role and mechanism of RNA m^6A in regulating the stemness of BCSCs and will inspire future studies regarding their applications in clinical treatment.

2. Materials and Methods

2.1. Cell Culture, Flow Cytometry, and Sphere Formation Assays. BCa cell lines 5637 (ATCC NO. HTB-9) and UM-UC-3 (ATCC NO. CRL-1749) were purchased from the Chinese Academy of Cell Resource Center (Shanghai, China) and maintained as previously described [32]. Cell lines were routinely tested for mycoplasma and not cultured for longer than 20 passages. Specific siRNAs were transfected into cells by Lipofectamine™ RNAiMAX Transfection Reagent (13778-075) according to the manufacturer's instructions.

For flow cytometry analysis, BCa cells were stained using the ALDEFLUOR assay kit (StemCell Technologies) according to the manufacturer's instructions. Acquisition and sorting were then performed using the BECKMAN Moflo XDP (Beckton Dickson, Mountain View, CA). Gates for fluorescence fractionations were established using unstained and isotype controls.

For sphere formation assays, FACS-sorted cells were cultured in 24-well ultralow attachment plates (Corning Inc., Corning, NY, USA) at a density of 1,000 cells per well. Cells were cultured in serum-free DMEM/F12 supplemented with growth factors EGF, β-FGF, and IGF-1 at a concentration of 20 ng/ml (PeproTech, Rocky Hill, NJ, USA). Spheres with a diameter of over 20 μm were counted 7 days after plating.

2.2. Detect Gene Expression. For mRNA level examination, total RNA of BCa cells was extracted using Trizol reagent (Invitrogen). Complementary DNA (cDNA) synthesis was performed with the PrimeScript™ RT reagent Kit with gDNA Eraser (Takara, RR047A) using 1 μg RNA per sample. qPCR reactions were performed using TB Green® Premix Ex Taq™ (Takara RR820A) to determine mRNA transcript levels. Primers for qRT-PCR are listed in Supplementary Table S1, siRNAs are used to knockdown METTL3, and AFF4 expression is listed in Supplementary Table S2.

For Western blotting, BCa cells were lysed with RIPA buffer as a standard protocol. The cell lysate was then mixed with loading buffer and incubated at 100°C for 5 min and subjected to conventional Western analysis. Antibodies are listed in Supplementary Table S3. The relative levels of proteins were quantified using densitometry with the Gel-Pro Analyzer (Media Cybernetics, Rockville, MD, USA). The target bands were densitometrically quantified and indicated under each band.

2.3. m^6A Quantification. RNA m6A levels were evaluated by the m^6A RNA quantification kit (Epigentek, P-9005) according to the manufacturer's protocol. Briefly, 200 ng total RNA of each sample was bound to the strip well of a 96-well plate, followed by m^6A antibody capture and washing. After incubated with the substrate for 5 min before the reaction was stopped, the absorbance of each well was read on a microplate reader (Multiskan FC, Thermo Scientific) at 450 nm.

2.4. CHIP Assay. Chromatin immunoprecipitation (ChIP) assay was performed using a Simple ChIP Assay Kit (Cell Signaling Technology, Danvers, MA) according to the manufacturer's instruction. The precipitated DNA samples were purified and measured by qPCR. Results were shown as the percentage of input controls. Primers and antibodies used for CHIP assay are listed in the supplementary Table S1 and Table S2, respectively.

2.5. Limiting Dilution Transplantation Assay. Stable AFF4 knockdown 5637 cells and control cells were serially diluted (1×10^5-2.7×10^6), resuspended in 50 μl of Matrigel (Corning, 354230), and injected subcutaneously into BALB/cA-Slac-nu nude mice (Shanghai Laboratory Animals Center, SLAC). Subsequent tumors were monitored weekly until mice presented signs of distress, and the mice were sacrificed. All animal procedures were performed under a protocol approved by the Laboratory Animal Center of Anhui Medical University.

Paraffin sections of samples from xenografts were antigen retrieved, blocked, and processed as described before [33]. The intensity of immunostaining was measured by Image-Pro Plus 6.0 image analysis software (Media Cybernetics). The intensity of each image was calculated by normalizing the average integrated optical density (IOD) with the total selected area of interest (AOI).

2.6. Statistics. All experiments were performed at least three times, unless otherwise noted. Data are presented as the means ± standard deviation (S.D.) or standard error (S.E.). All of the statistical analyses were performed using Excel (Microsoft, Redmond, WA) or Prism (GraphPad Software Inc., La Jolla, CA). The two-tailed Student's t-test was used, and a p value of <0.05 was considered significant. For limiting dilution assay, a statistical test was performed as described previously [34].

3. Results

3.1. RNA m^6A Levels Are Elevated in BCSCs. To estimate the potential role of RNA m^6A modification in regulating the stemness of BCa, we examined the global RNA m^6A levels of CSCs and non-CSCs of BCa. Aldehyde dehydrogenase 1 family, member A1 (ALDH1A1) was used as a marker to isolate BCSCs [35] from two established cancer cell lines, 5637 and UM-UC-3, by flow cytometry (Figure 1(a)), and RNA m^6A methylation abundance was evaluated by the m^6A RNA quantification kit. The results showed that ratios of m^6A RNA/total RNA in ALDH1-positive ($ALDH1^+$) cells isolated from both 5637 and UM-UC-3 were significantly higher than those in ALDH-negative ($ALDH1^-$) proportion (Figure 1(b)). We then checked the expression patterns of known m^6A writers (i.e., *METTL3*, *METTL14*, and *WTAP*) and erasers (i.e., *FTO* and *ALKBH5*) by quantitative RT-PCR to determine which subunit may account for m^6A dysregulation of CSCs and found that the expression of *METTL3* rather than other regulators was significantly elevated in $ALDH1^+$ BCa cells (Figure 1(c)). The protein level of METTL3 was further validated to be higher in $ALDH1^+$ BCa cells by Western blot (WB) (Figure 1(d)). All these data indicate that METTL3 is upregulated in BCSCs and may be implicated in self-renewal.

3.2. Targeting METTL3 Expression Impairs BCSC Self-Renewal. To determine whether METTL3 is important to BCSC self-renewal, we used two distinct siRNAs (si-METTL3-1 and si-METTL3-2) to ablate METTL3 expression in 5637 and UM-UC-3 cells. Compared with a nontargeting control siRNA (si-GFP), both specific siRNAs significantly reduced METTL3 mRNA and protein levels (Figures 2(a) and 2(b)). Two characteristics to identify populations of BCSCs are the ability to generate clusters of daughter cells when they are cultured on ultralow adherence plates (sphere assay) and high ALDH activity which can be quantified by flow cytometry using a fluorogenic substrate [7].

36 hours after siRNA transfection, cells with high ALDH activity were examined and sorted by flow cytometry, and the same amount of cells with high ALDH activity from different transfection groups was further transferred to ultralow adherence plates with stem cell medium. One week later, the number of formed spheres was counted. Both the percentages of cells with high ALDH activity (Figures 2(c) and 2(d)) and sphere formation frequency (Figures 2(e) and 2(f)) were significantly decreased upon *METTL3* knockdown. With the above evidences, we concluded that METTL3 is required for the BCSC self-renewal *in vitro*.

3.3. AFF4 Is Regulated by METTL3 in BCSCs. In the previous study, we performed transcriptome sequencing and m^6A sequencing followed by a series validation in 5637 cells, which proved the m^6A modification and expression of *AFF4* mRNA were directly regulated by METTL3 [11]. To identify if AFF4 was also the target of METLL3 in BCSCs, we then checked both mRNA and protein levels of AFF4 in $ALDH1^+$ and $ALDH1^-$ cells from 5637 and UM-UC-3, respectively. Not surprisingly, a significantly higher level of AFF4 expression was observed in the $ALDH1^+$ proportion compared to the corresponding $ALDH1^-$ counterpart in BCa cells (Figures 3(a) and 3(b)). Moreover, gene-specific m^6A-qPCR using primers to amplify either the m^6A peak region (indicated by our m^6A-sequencing results) or a control (non-peak) region showed a markedly increased m^6A abundance of *AFF4* mRNA in $ALDH1^+$ BCa cells (Figures 3(c) and 3(d)), which suggest the difference of AFF4 expression between CSCs and non-CSCs is regulated by METTL3-mediated m^6A modification primarily. To validate if *AFF4* acted downstream of METTL3 in BCSCs, we further analyzed the effect of AFF4 deficiency on the BCa self-renewal using a similar strategy to METTL3 knockdown. With effective ablation of AFF4 expression by siRNAs in both 5637 and UM-UC-3 cells (Figures 4(a) and 4(b)), both ALDH activity (Figures 4(c) and 4(d)) and sphere formation frequency (Figures 4(e) and 4(f)) showed a significant decrease upon *AFF4* knockdown, which mimic the phenotype resulting from METTL3 knockdown and indicate the regulatory relationship between AFF4 and METTL3 in BCSC self-renewal.

3.4. AFF4 Directly Regulates MYC and SOX2 Gene Expression in BCa Cells. As an essential component of SEC, AFF4 can bind to DNA directly and regulate the transcription elongation of many genes. MYC and SOX2, well-known pluripotency factors of CSCs, have been reported to be regulated by AFF4 in BCa [11] and HNSCC [30], respectively. To investigate whether MYC and SOX2 are effectors of AFF4 in regulating the self-renewal capability of BCSCs, we performed CHIP assay in 5637 and UM-UC-3 cells and found AFF4 directly bound to *MYC* and *SOX2* promoter regions, which were barely detectable after *AFF4* knockdown (Figure 5(a)). Besides, we also confirmed the expression of MYC and SOX2 in response to *AFF4* knockdown by qRT-PCR and Western blot. The results showed knockdown of *AFF4* drastically reduced the expression of these two genes at both mRNA level and protein level (Figures 5(b) and 5(c)).

3.5. AFF4 Promotes BCSC Self-Renewal In Vivo and Is a Negative Prognostic Factor for BCa Patients. To further evaluate the effect of *AFF4* depletion on the self-renewal capacity of BCSCs *in vivo*, we conducted limiting dilution transplantation assay, a method widely used to assess cancer stem cell content. *AFF4* expression was stably ablated by short hairpin RNA (sh-AFF4) in 5637 cells, which were then injected subcutaneously into immune-deficient mice, and tumor growth was measured over time. Consistent with the *in vitro* results, AFF4-deficient cells exhibited a significantly lower tumor-propagating potential than the control cells (sh-GFP) comprising the tumor bulk (Figures 6(a) and 6(b)). Following ALDH1 staining and FACS analysis showed a clear reduction of ALDH-positive ratio (Figure 6(c)), along with AFF4, SOX2, and MYC expression in xenografts generated from sh-AFF4 5637 cells relative to the control tumors (Figure 6(d)).

The cancer stemness properties of BCSCs contribute to the chemoresistance, metastasis, and recurrence, which are often related to poor clinical outcome. We queried The

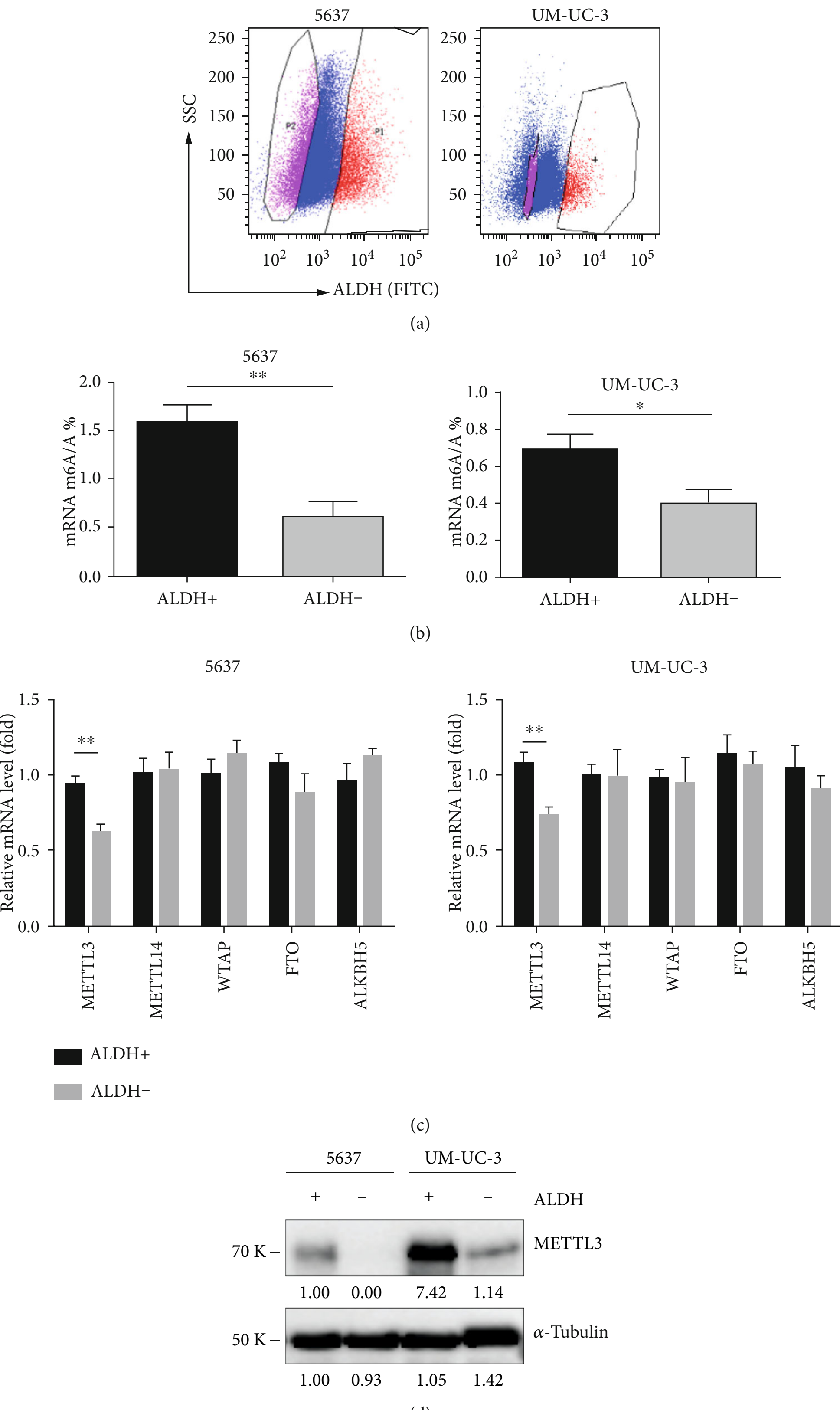


Figure 1: Differential m^{6}A levels between CSCs and non-CSCs of BCa. (a) Representative gating scheme for FACS sorting of ALDH1-stained 5637 and UM-UC-3 cells. (b) Quantification of m^{6}A levels in ALDH1-positive and ALDH1-negative BCa cells ($^*p < 0.05$, $^{**}p < 0.01$, Student t-test). (c) mRNA levels of RNA m^{6}A writers and erasers in ALDH1-positive and ALDH1-negative BCa cells ($^{**}p < 0.01$, Student t-test). (d) Protein levels of RNA m^{6}A methyltransferase METTL3 in ALDH1-positive and ALDH1-negative BCa cells.

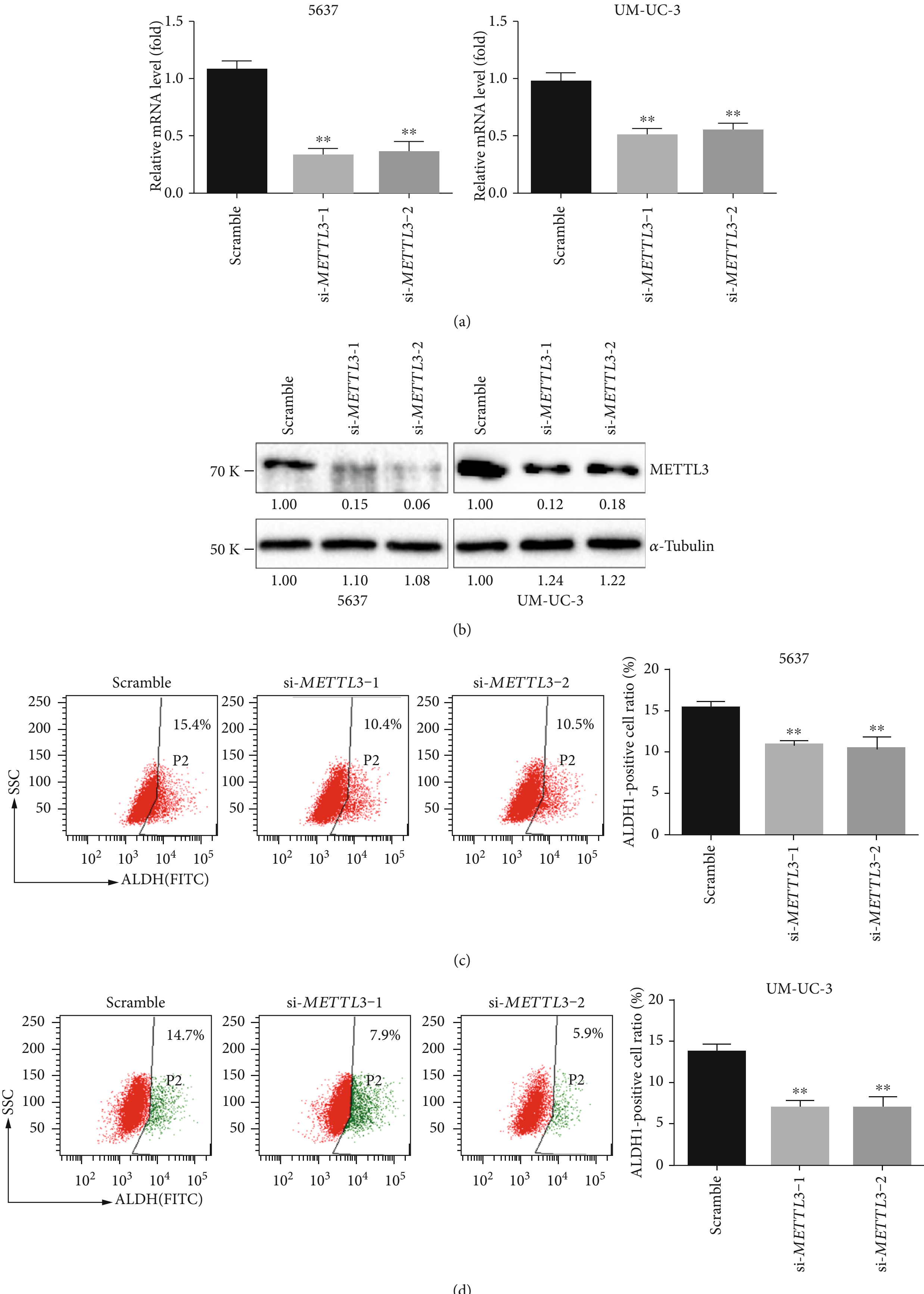


Figure 2: Continued.

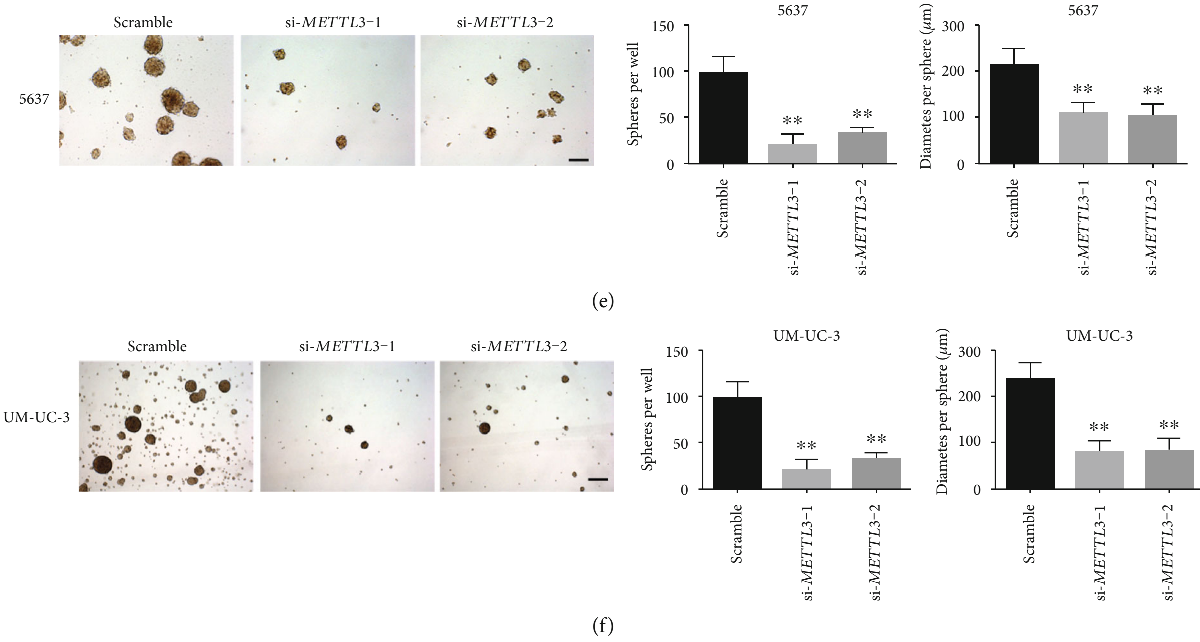


Figure 2: METTL3 is required to sustain self-renewal of BCa cells. The knockdown effect of specific siRNAs (si-METTL3-1 and si-METTL3-2) in 5637 and UM-UC-3 cells was verified at both the mRNA ((a) by qRT-PCR) and protein levels ((b) by Western blot). Ratio of cells with high ALDH activity (c, d); number and size of spheres formed in stem cell medium (e, f) of the BCa cells transfected with indicated siRNAs are plotted, and representative images are presented. $^{**}p < 0.01$ compared to the scramble group, by Student t-test. Scale bar, 250 μm.

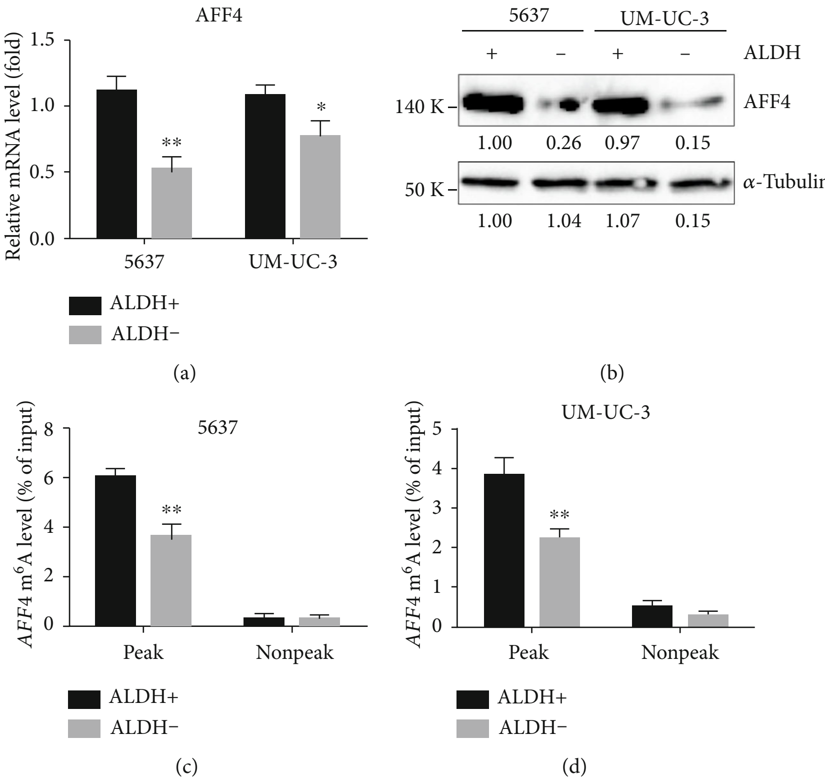


Figure 3: Differential expression level and m^{6}A levels of *AFF4* between CSCs and non-CSCs of BCa. mRNA levels (a) and protein levels (b) of AFF4 in ALDH1-positive and ALDH1-negative BCa cells. m^{6}A modification in specific regions of *AFF4* transcripts in ALDH1-positive and ALDH1-negative 5637 (c) and UM-UC-3 (d) cells was tested by gene-specific m^{6}A-qPCR assay. $^{*}p < 0.05$, $^{**}p < 0.01$ compared to the scramble group, by Student t-test.

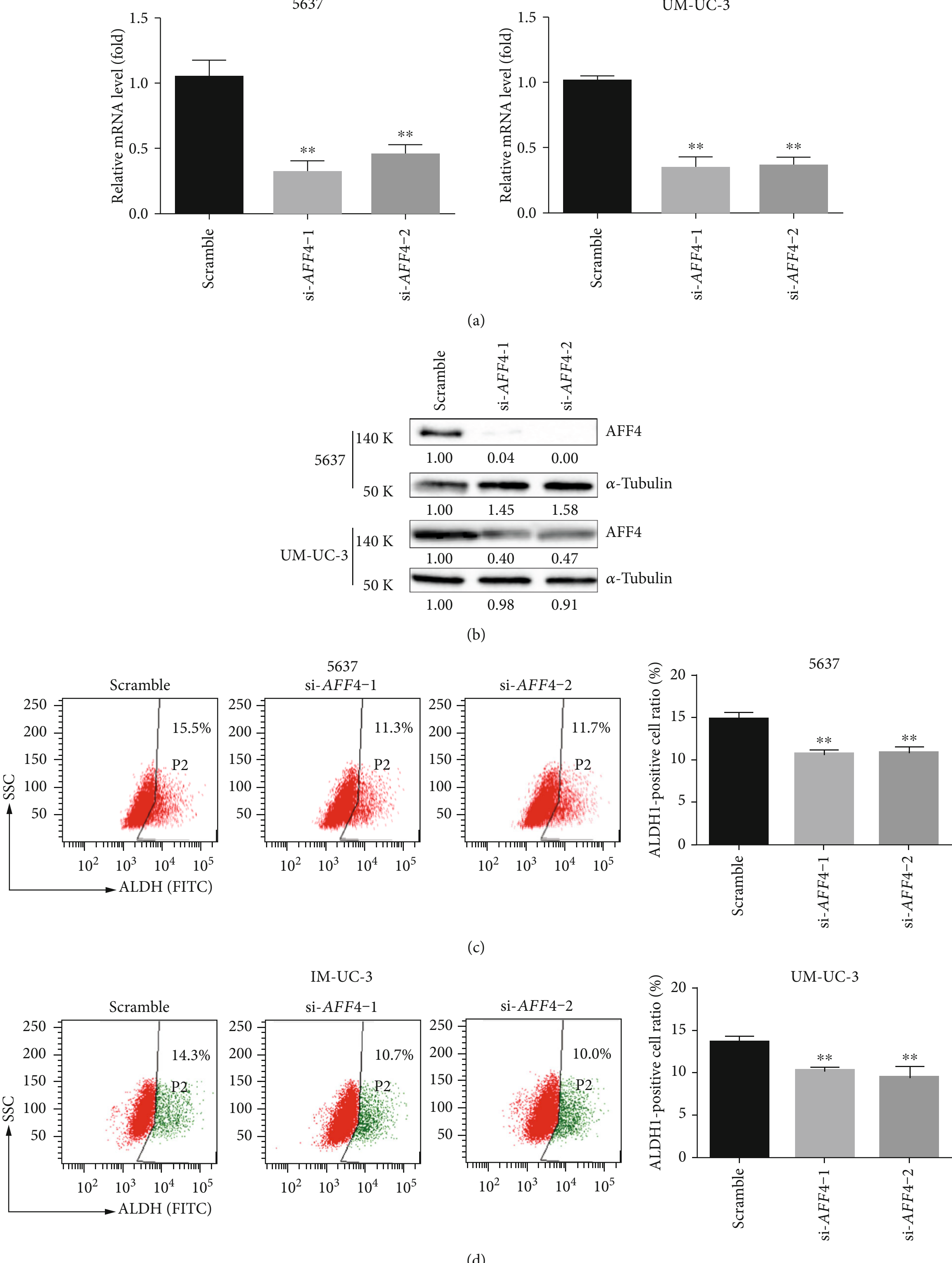


Figure 4: Continued.

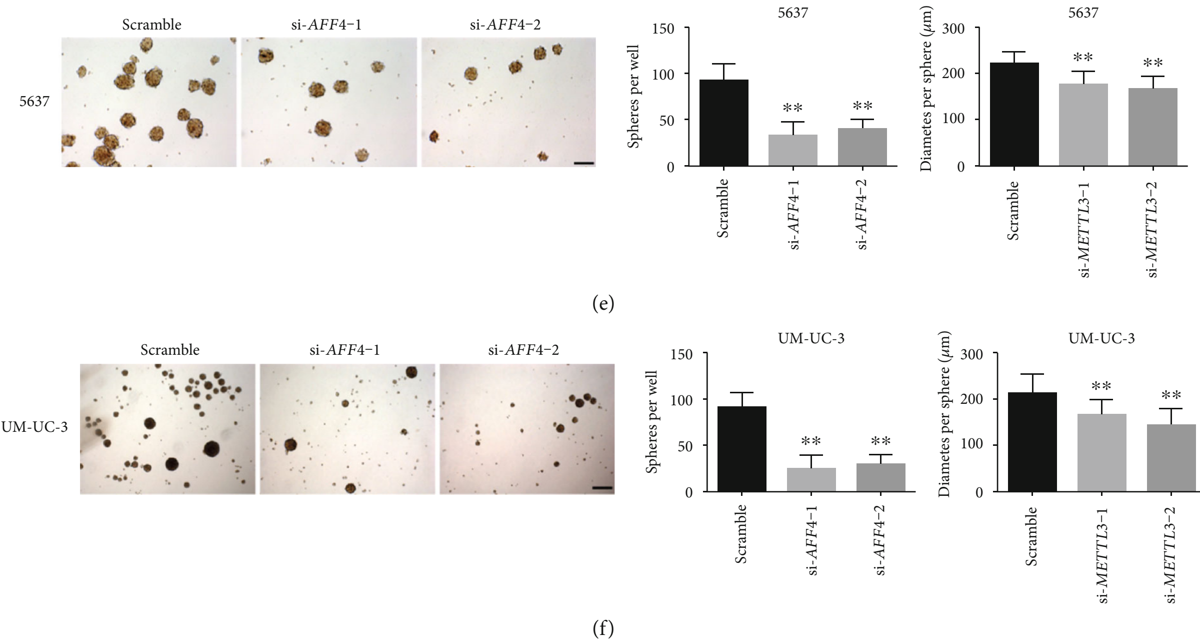


Figure 4: *AFF4* mimic the phenotype of *METTL3* in regulating the stemness of BCSCs. The knockdown effect of specific siRNAs (si-*AFF4*-1 and si-*AFF4*-2) in 5637 and UM-UC-3 cells was verified at both the mRNA ((a) by qRT-PCR) and protein levels ((b) by Western blot). Ratio of cells with high ALDH activity (c, d); number and size of spheres formed in stem cell medium (e, f) of the BCa cells transfected with indicated siRNAs are plotted, and representative images are presented. $^{*}p < 0.05$, $^{**}p < 0.01$ compared to the scramble group, by Student t-test. Scale bar, 250 μm.

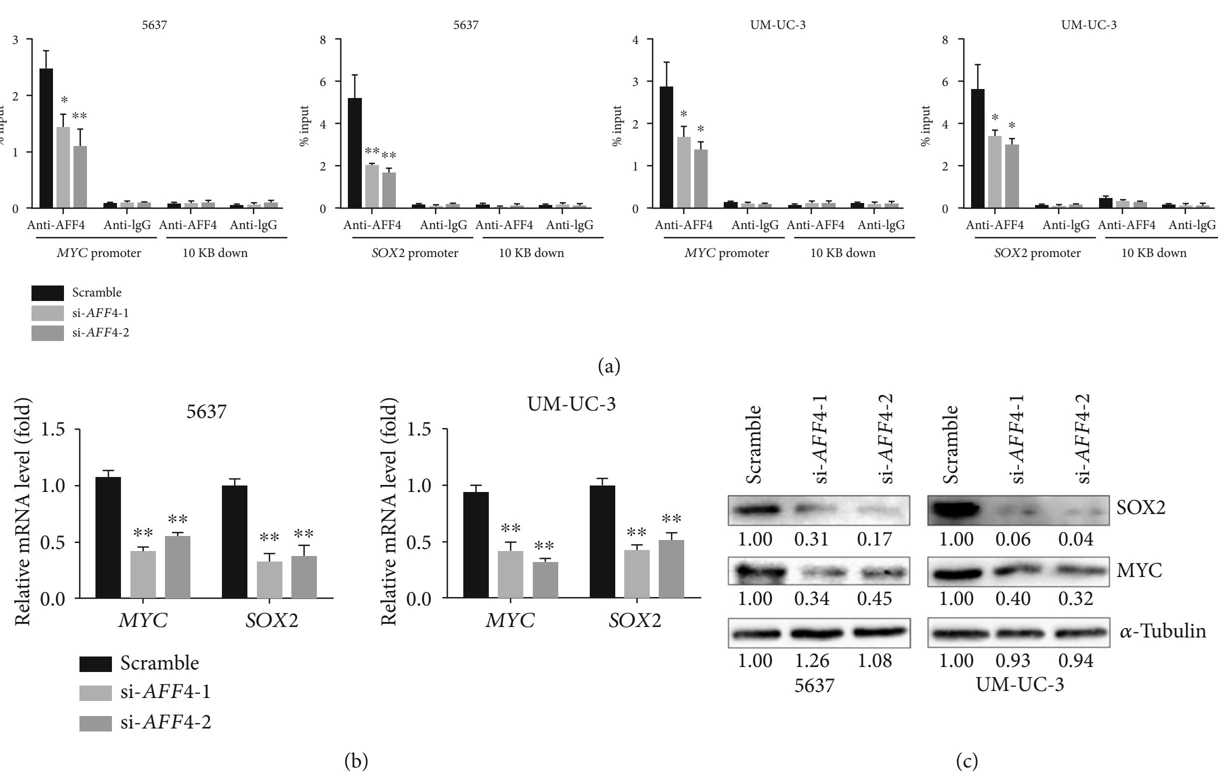
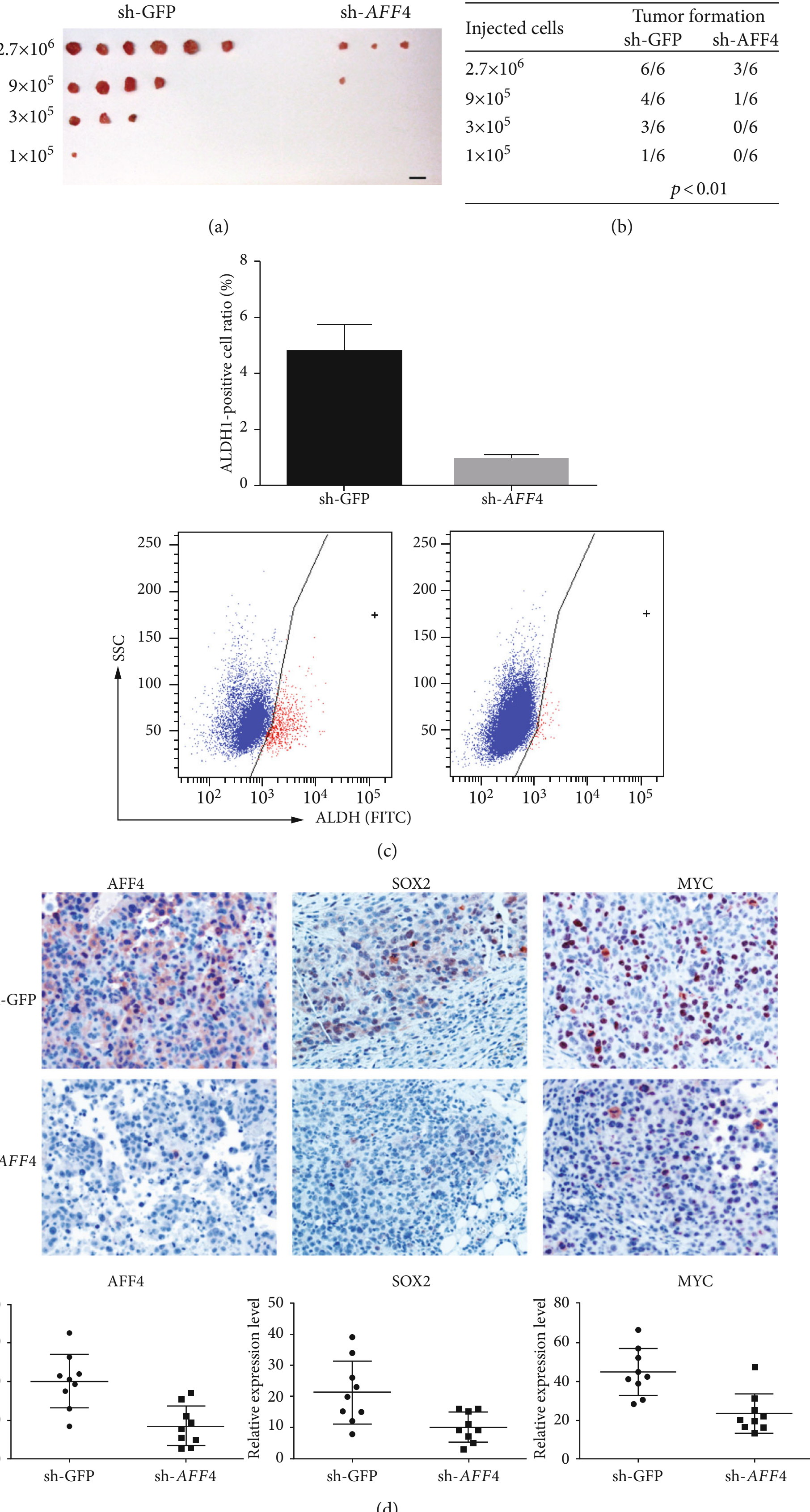


Figure 5: AFF4 regulates SOX2 and MYC expression in BCa cells. (a) ChIP assay showed the recruitment of AFF4 at *MYC* and *SOX2* promoter regions in 5637 and UM-UC-3 cells transfected with indicated siRNAs at 48 h posttransfection. Expression of MYC and SOX2 in 5637 and UM-UC-3 cells transfected with indicated siRNAs (si-*AFF4*-1 and si-*AFF4*-2) was verified at both the mRNA ((b) by qRT-PCR) and protein levels ((c) by Western blot). $^{*}p < 0.05$, $^{**}p < 0.01$ relative to the scramble group by Student t-test.



Injected cells	Tumor formation sh-GFP	sh-AFF4
2.7×10^6	6/6	3/6
9×10^5	4/6	1/6
3×10^5	3/6	0/6
1×10^5	1/6	0/6
	$p < 0.01$	

Figure 6: Continued.

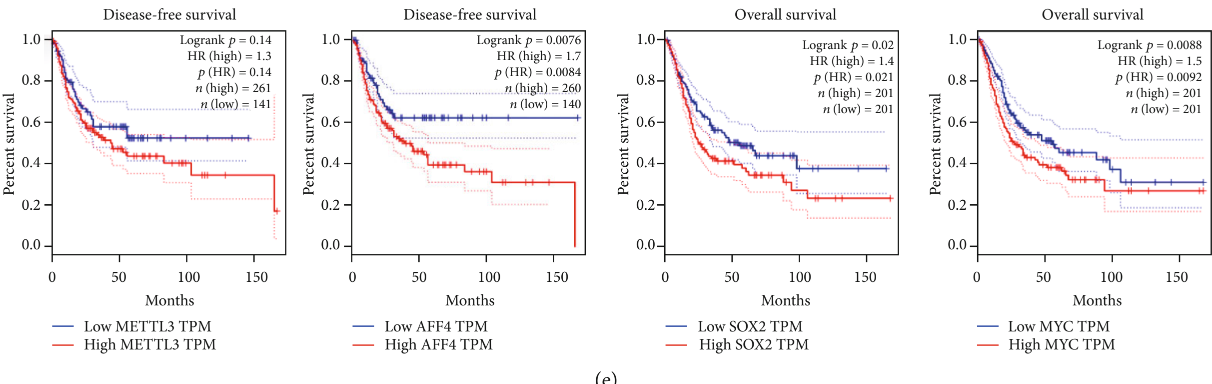


Figure 6: AFF4 is essential for BCa tumor propagating *in vivo*. Graph (a) and quantification (b) of the percentage of tumor-free mice 30 days after subcutaneous injection of different dilutions of AFF4 knockdown 5637 cells or control cells into immunodeficient mice ($n = 6$ for each dilution). (c) Ratio of ALDH-positive cells from the xenografts. $^{**}p < 0.01$ by Student t-test. (d) Quantitative measurement and representative images of AFF4, SOX2, and MYC expression in xenografts generated by AFF4 stable knockdown BCa cells and control cells. ($^{**}p < 0.01$ by Student t-test). Scale bar, 50 μm. (e) Correlation between METTL3, AFF4, SOX2, and MYC mRNA expression and survival of BCa patients in TCGA dataset. Disease-free or overall patient survival in groups of high and low expression was analyzed by the Kaplan-Meier survival curve and compared by the log-rank test.

Cancer Genome Atlas (TCGA) database and analyzed the survival curve of BCa with the help of the GEPIA online tool [36]. A worse disease-free survival was found in the METTL3 high expression group than that in the METTL3 low expression group, and the p value shows a certain trend toward significance (Figure 6(e), $p = 0.11$), while higher expression of *AFF4* was clearly a significant indicator of poor prognosis of BCa (Figure 6(e), $p = 0.008$). Besides, the higher expression of SOX2 (Figure 6(e), $p = 0.02$) and MYC (Figure 6(e), $p = 0.009$) was also significantly associated with worse overall survival. Taken together, aberrant expression of AFF4 is associated with BCSCs within the tumor bulk which may lead to poor prognosis.

4. Discussion

We have shown in our previous study that METTL3 plays a critical role in the pathogenesis of BCa, by positively regulating the expression of IKBKB, RELA, AFF4, and MYC through m^{6}A-based posttranscriptional regulation [11]. Here, we demonstrate that mRNA m^{6}A modification is critical for maintaining BCSC self-renewal and tumor development. The knockdown of *METTL3* expression reduced the self-renewal of BCSCs. Emerging data have suggested that the global abundance of m^{6}A and expression levels of its regulators, including writers, erasers, and readers, are often dysregulated in various types of cancers and are critical for cancer initiation, progression, metastasis, and drug resistance and cancer relapse [22]. Intriguingly, reasons of m^{6}A dysregulation in CSCs are different among various types of cancer, considering the roles of FTO, ALKBH5, and METTL3 in glioblastoma stem cells [24, 25] and of METTL14 and FTO in leukemia stem cells [26, 27]. In BCa, our data shows METTL3 is the only regulator that is aberrantly expressed and critical for BCa pathogenesis and BCSC maintenance. This study uncovered a critical role of mRNA m^{6}A modification in regulating BCSCs self-renewal and tumorigenesis. Nevertheless, the reason for aberrant *METTL3* expression in BCa is still unknown and awaits further investigation.

AFF4 is a core component and required for SEC stability and activity, by acting as a scaffold to assemble the SEC [37, 38]. Evidences showing that AFF4 might play a role in regulating pluripotency include its involvement in the osteogenic differentiation of human mesenchymal stem cells [28] and odontogenic differentiation of human dental pulp cells [29]. AFF4 is also required for the tumor-initiating capacity of stem-like cells in HNSCC [30]. In our previous study, AFF4 was indicated by our transcriptome and m^{6}A sequencing data to be a direct target of METTL3 in BCa cells; we then demonstrated that *AFF4* mRNA is regulated by METTL3 in a m^{6}A-dependent manner [11]. In the current study, we reveal that both the m^{6}A abundance and the expression level of *AFF4* mRNA are elevated in BCSCs, which is consistent with the expression pattern of METTL3. Moreover, ALDH activity and sphere-forming ability *in vitro* as well as tumor-initiating capacity *in vivo* were all abrogated upon *AFF4* knockdown. Besides, there was a clear correlation between AFF4 expression and BCa invasion potential [11], which is another commonly used indicator of tumorigenicity. Taken together, our data suggest AFF4 is a *bona fide* target of METTL3 in regulating the self-renewal capacity of BCSCs.

Our previous work proved Sox2 as a marker for stem-like tumor cells of BCa *in vivo* [7]. Besides, there are evidences indicated that downregulation of c-Myc suppressed CSC differentiation in BCa, and overexpression of c-Myc increased the levels of stem cell markers including SOX2 [39]. Therefore, SOX2 and MYC both are master regulators of self-renewal and differentiation of CSCs and are essential for BCa initiation and progression. *SOX2* mRNA was reported to contain m^{6}A modification in embryonic stem cells [40] and glioblastoma stem cells [24], and m^{6}A modification of *MYC* mRNA was found in the CSCs of acute myeloid leukemia. Indeed, we have also confirmed MELLT3 could regulate

MYC expression by promoting the m^{6}A modification of its mRNA in BCa cells [11]. It is likely that METTL3 promote the expression of SOX2 and MYC through m^{6}A-based post-transcriptional regulation as well as AFF4-mediated regulation at the transcriptional level, which reinforces the signal activating the tumor-initiating and self-renewal capabilities of BCa cells. Meanwhile, methyltransferase METTL3 has a global effect on many RNAs; just like AFF4/SEC, MYC and SOX2 exert a broad effect on the expression of various pluripotency-related genes by binding to multiple sites of DNA. Therefore, the role of METTL3 regulating BCSCs might not merely rely on AFF4. Other potential target genes involved in BCa initiation and self-renewal need to be investigated.

In summary, we found m^{6}A modification of *AFF4* RNA was upregulated by METTL3 and their expression was elevated in BCSCs, which in turn promotes the expression of SOX2 and MYC to enhance tumorigenesis and tumor-initiating capacity of BCa. Our findings indicate AFF4 may serve as a biomarker and a potential target of therapies for patients with BCa.

Data Availability

All data is available upon request by contacting the corresponding authors: Yang Li, Ph.D. Department of Genetics, School of Life Science, Anhui Medical University, Hefei, Anhui 230031, China; Tel.: 86 551-65160327; E-mail: liyang@ahmu.edu.cn; and Yingyin Zhang, Department of Genetics, School of Life Science, Anhui Medical University, Hefei, Anhui 230031, China; Tel.: 86 551-65169646; E-mail: liyang@ahmu.edu.cn.

Conflicts of Interest

All the authors have declared no conflict of interest.

Authors' Contributions

Qian Gao and Jin Zheng contributed equally to this work.

Acknowledgments

The authors thank the Center for Scientific Research of Anhui Medical University and Facilities of School of Life Science for valuable help in our experiment. This work was supported by the National Natural Science Foundation of China (81872313 and 81672776 to YL, 81802391 to QG, and 31501838 to XH Z), the Anhui University Natural Science Research Project (KJ2019A0236 to YY Z), and the Anhui Provincial Natural Science Foundation (1808085QH266 to QG).

Supplementary Materials

Supplementary Table 1: information of primers. Supplementary Table 2: information of siRNAs. Supplementary Table 3: information of antibodies. Supplementary Figure 1: backbone of the plasmids used for shRNA construct. *(Supplementary Materials)*

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