Stem Cells International

Base Modifications: Regulation of Stem Cell Functions and Diseases

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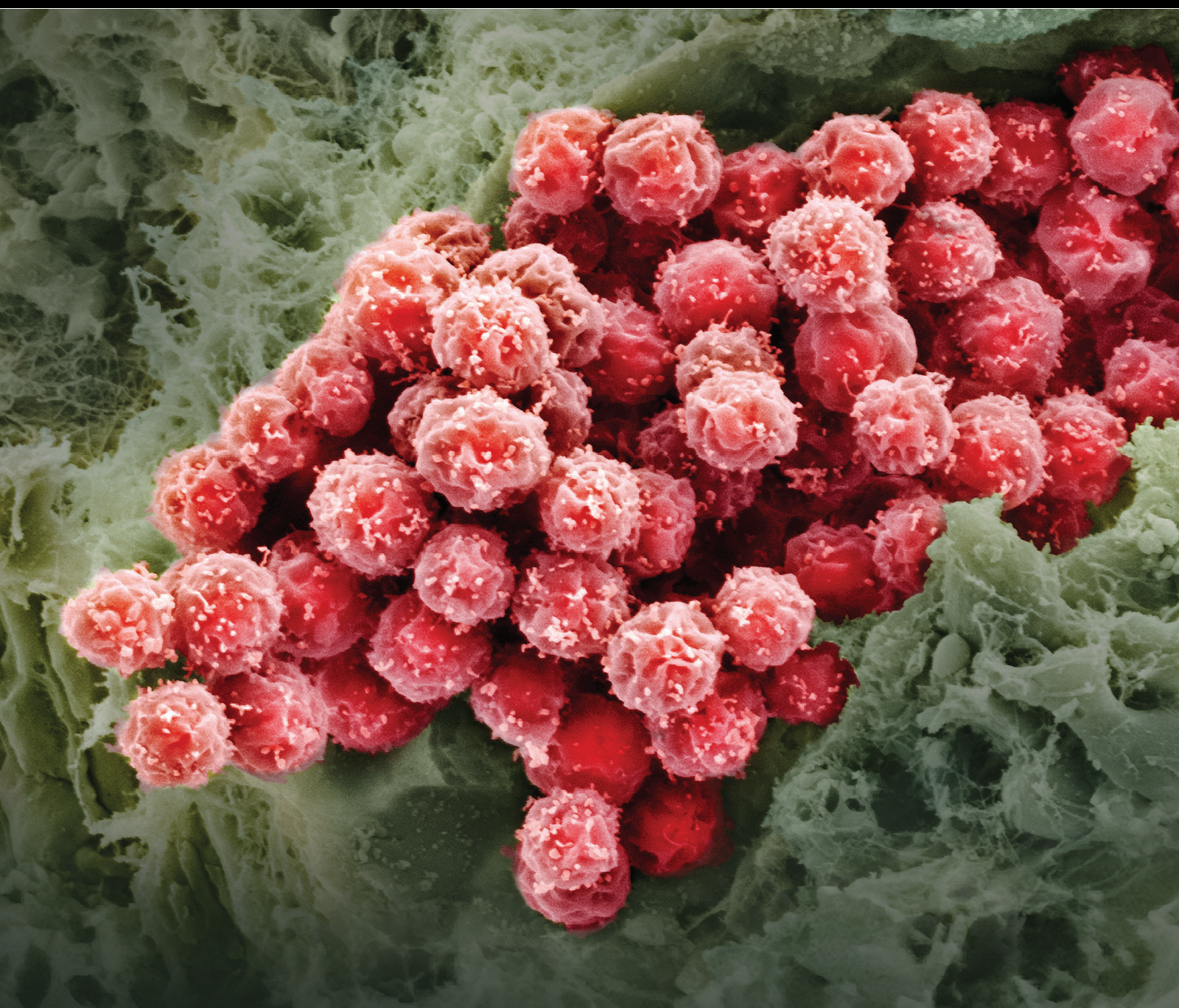

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Editorial Board

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Editorial

Base Modifications: Regulation of Stem Cell Functions and Diseases

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Vast emerging lines of evidences support importance of the crosstalk between base modifications and stem cell functions such as lineage commitment, specification, self-renewal, quiescence, proliferation, and differentiation. The main forms of base modifications in DNA and RNA (coding and noncoding RNA) are N6-methyladenosine (m6A) and 5-methylcytosine (5mC) and its oxidative derivatives 5-hydroxymethylcytosine (5-hmC), 5-formylcytosine (5-fC), and 5-carboxylcytosine (5-caC). The delicate spatiotemporal alteration of base modifications constitutes an integral part of controlling mechanisms of self-renewal, ensure quiescence, proliferation, and differentiation, which is critical for ensuring successful development and maintaining postnatal life.

Over the past decades, significant progress has been made in appreciation of the link between aberrant base modifications in stem cells and diseases, such as cancers and neurodegenerative disorders. These achievements keep inspiring scientists to further uncover the epigenetic mechanisms for stem cell development and to dissect pathogenesis of the multiple diseases conferred by developmental dysfunctions of the stem cells. The special issue "Base Modifications: Regulation of Stem Cell Functions and Diseases" mainly focuses on the original research and review articles that address the recent advances in base modification-mediated regulation of stem cell functions and their link with the diseases.

In this special issue, H. Tao et al. investigated the expression pattern of ten-eleven translocation proteins (TETs) which catalyze the oxidation of 5-mC to 5-hmC and further to 5-fC and 5-caC and the levels as well as the distribution of the 5-hmC, 5-fC, and 5-caC in postnatal neurons and adult neuronal stem cells (aNSCs). The authors demonstrated that the three distinct forms of the modified bases and their writer TETs are highly enriched in multiple brain regions and in the aNSCs and display temporal and spatial patterns during the postnatal neuron development, contributing to dynamic epigenetic regulation of the related gene expression.

In a separate review article, H. Zhou et al. highlighted the recent advances in the critical roles of TETs, the 5-mC and 5-hmC markers, noncoding RNAs, and histone modifications in proliferation and differentiation of the NSCs and tumorigenesis. Furthermore, the article addressed the potential link between the aberrant 5-hmC modification and the neurological disorders such as Alzheimer's disease (AD), Huntington's disease (HD), Rett syndrome, and major depressive disorders (MDD). Finally, the perspective is given regarding the future approaches to comprehensively understand the epigenetic roles that the 5-hmC marker and its writer TETs play in the neurological disorders as well as to discover the potential therapeutic targets for these disorders.

N6-Methyladenosine in both DNA and RNA has also attracted significant attention due to its essential regulation roles in the expression of genes involved in stem cell fate, CNS development and functions, and some diseases including cancers. A. Shah et al. reported that DDX3, a member of the family of DEAD box RNA helicases, functions as a regulator for ALKBH5 which can catalyze demethylation of the m6A in mRNAs. A series of experiments revealed that the regulation of DDX3's function is dependent upon the interaction between the ATP domain of DDX3 and the DSBH domain of ALKBH5 which has been acknowledged as a main m6A RNA demethylase. In addition to the regulation of the mRNA demethylation, DDX3 could regulate the methylation

status of microRNAs through the interaction with AGO2 as well. This discovery adds one more clue to understand the regulation of ALKBH5 by other components as well as the crosstalk between N6-methyladenosine modification and the microRNA pathway.

A review article by P. Ji et al. systematically highlighted the research progress in N6-methyladenosine as an epitranscriptomic and epigenetic player implicated in determination of stem cell fate. Firstly, the article summarized the characteristics of the N6-methyladenosine marker complexes including the writers, the erasers, the readers, and other regulation components at the biochemical, molecular, and phenotypical levels. Then, the potential clinical applications of the components in the complexes as the therapeutic targets were discussed. Furthermore, the article summarized the dynamic regulation and fine-tuned coordination of the levels and the landscapes of the N6-methyladenosine marker in accordance with stages of the growth, development, and reproduction as naturally programmed during the lifespan. More specifically, the article addressed the association between aberrant m6A modification in stem cells and initiation/development of the diseases such as cancers. Finally, the future research directions are discussed.

Expanded further from the base modification, N. Xie et al. emphasized the advances in the CRISPR/Cas9-based novel epigenetic modulation techniques. Starting from the epigenome editing, the first part of the article discussed the rewriting of the epigenetic markers at the given loci in the genome to uncover the effects caused by the targeted rewriting. This epigenome editing strategy could also confer the potential application to screening and annotation of the epigenetic elements such as enhancers. Furthermore, the article summarized the research progress in temporal and spatial control of epieffectors induced by light and chemicals in combination with the CRISPR/Cas9 system to determine cell phenotypes during development, aging, and disease pathogenesis. Besides, the interesting and the valuable progress in the CRISPR/Cas9-based characterization of the chromatin structures and the interactions were addressed. Finally, the article addresses the current drawbacks and the future directions including the potential clinical applicability.

Other than studies on base modification study, M. Mellado-López et al. reported two important functions of Plasma Rich in Growth Factors (PRGF) in adipose-derived stem cells (ASCs). Firstly, the PRGF could favor survival and proliferation of human and canine ASCs and delay human ASC senescence and autophagocytosis in comparison with serum-containing cultures. Additionally, PRGF could efficiently enhance the differentiation of the canine- and human-derived ASC into osteocytes, adipocytes, or chondrocytes. The PRGF-induced AKT phosphorylation to avoid the lethal concentrations of hydrogen peroxide was proposed as the mechanism for prevention of ASC death. It is well known that ASCs have become a promising therapeutic alternative for tissue repair in various clinical applications. Given that the conventional serum-based ASC culture methods confer the restrictive cell survival, differential tissue integration, and undirected cell differentiation after transplantation in a hostile microenvironment, this discovery could be a breakthrough for ASC culture and future SC-based regenerative medicine.

The authors of this special issue hold strong wishes that the research and the review articles published here, technically and scientifically, could provide important information more conveniently for the readers with strong interest in the closely related research field.

Conflicts of Interest

The editors declare that they have no conflicts of interest regarding the publication of this special issue.

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Changwon Park
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Review Article

N6-Methyladenosine in RNA and DNA: An Epitranscriptomic and Epigenetic Player Implicated in Determination of Stem Cell Fate

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Vast emerging evidences are linking the base modifications and determination of stem cell fate such as proliferation and differentiation. Among the base modification markers extensively studied, 5-methylcytosine (5-mC) and its oxidative derivatives (5-hydroxymethylcytosine (5-hmC), 5-formylcytosine (5-fC), and 5-carboxylcytosine (5-caC)) dynamically occur in DNA and RNA and have been acknowledged as important epigenetic markers involved in regulation of cellular biological processes. N6-Methyladenosine modification in DNA (m6dA), mRNA (m6A), tRNA, and other noncoding RNAs has been defined as another important epigenetic and epitranscriptomic marker in eukaryotes in recent years. The mRNA m6A modification has been characterized biochemically, molecularly, and phenotypically, including elucidation of its methyltransferase complexes (m6A writer), demethylases (m6A eraser), and direct interaction proteins (readers), while limited information on the DNA m6dA is available. The levels and the landscapes of m6A in the epitranscriptomes and epigenomes are precisely and dynamically regulated by the fine-tuned coordination of the writers and erasers in accordance with stages of the growth, development, and reproduction as naturally programmed during the lifespan. Additionally, progress has been made in appreciation of the link between aberrant m6A modification in stem cells and diseases, like cancers and neurodegenerative disorders. These achievements are inspiring scientists to further uncover the epigenetic mechanisms for stem cell development and to dissect pathogenesis of the multiple diseases conferred by development aberration of the stem cells. This review article will highlight the research advances in the role of m6A methylation modifications of DNA and RNA in the regulation of stem cell and genesis of the closely related disorders. Additionally, this article will also address the research directions in the future.

1. Introduction

Epigenetics is defined as the gene expression alterations heritable to next generations caused by nongenetic but heritable cellular memory other than DNA sequence variations [1]. The epigenetic memories including dynamic base modifications (DNA methylation/demethylation), histone modifications, chromatin architecture, and noncoding RNAs maintain all the biological processes in the programmed tracks. It is true that a microevent in base modifications could lead to strong "earthquake" in metabolic pathways and the consequent alteration of organism phenotypes. Therefore, any aberrant alterations could lead to development of abnormality and initiation of diseases such as neurological disorders and cancers as reviewed in [2–8].

DNA base modifications such as methylation of 5-cytosine (5-mC) [9–14] and 5-hydroxymethylcytosine (5-hmC) [15–21] have been acknowledged as the best characterized epigenetic markers in mammalian brains [22–25] and ES cells [26–28], essentially regulating chromatin structure and consequently gene expression with the potential mechanisms. In the present review article, we highlight advances in another base modification N6-methyladenine which exists in both DNA (m6dA) and RNA (m6A) and is

not new in terms of its discovery history, but its biological functions are being gradually unveiled only in recent years in regulation of the development and stem cell fate. Meanwhile, the future research directions in N6-methyladenine are addressed as well.

1.1. RNA m6A Modification. Fine-tuning functions and metabolic regulation require posttranscriptional modifications of RNA transcripts. Among more than 100 of the chemical modifications in RNA from almost all the known living organisms [29–31], N6-methyladenosine (m6A) has been recognized as the most abundant in quantity and prominent in its power and range of the regulation functions in eukaryotic mRNA, leading to the significant efforts paid particularly in recent years with invention and application of high-throughput sequencing as well as advances in modern molecular and genetic technologies.

RNA m6A is catalyzed by a multicomponent methyltransferase complex (the "writer"), preferentially bound by binding proteins (the "readers"), and could be removed by specific demethylases (the "erasers") (Figures 1 and 2, Table 1). Recent studies on mRNA m6A modification have linked the m6A-dependent control of mRNA homeostasis to posttranscriptional regulation of gene expression involved in a wide spectrum of metabolic pathways, consequently.

1.2. DNA N6-Adenosine Modification (m6dA). N6-Methyladenine modification is not only a RNA marker (m6A) but also a genomic DNA marker (m6dA). The initial discovery of m6dA was from prokaryotes [32] particularly in bacteria, but later on it was detected in lower eukaryotes as well [33–43]. In higher eukaryotes, alteration of m6dA levels from the most abundant during embryogenesis to the significant decrease in adult tissues suggests its importance for development and a potential link with regeneration maintenance. Like 5-hmC loss as a hallmark for cancer cells, a significant decrease in m6dA levels has also been reported in a variety of cancer cells (unpublished data).

2. Distribution of m6A and m6dA in Epitranscriptomes and Epigenomes

2.1. m6A Distribution in Epitranscriptomes. The sequencing data from mRNAs of several organisms indicated that m6A-methylated mRNA accounts for only ~25% of the total cellular mRNA, suggesting the high selectivity and specificity of m6A sites in the target mRNAs although related mechanisms remain to be elusive.

The m6A distribution was nonrandom and asymmetric in a way that majority of m6A sites were highly enriched within 5′UTR, 3′UTR, stop codon, and long introns relative to the coding region (Table 2) [44, 45]. In addition, the m6A landscape is dynamically altered in accordance with the development stages and physiological conditions, but highly conserved among the mammal species at the corresponding conditions, indicating the regulation of the development and the significant functional relevance [44]. However, some studies argue that m6A functions as an even faster approach to posttranscriptionally enhance gene expression [46]. Additionally, m6A is believed to have a special function during developmental transitions by leading the m6A-marked transcripts to degradation [46].

2.2. m6dA Distribution in Epigenomes

2.2.1. m6dA Distribution in the Genomes of Eukaryotes. The genome-wide distribution of m6dA in genome has been identified and characterized by using multiple strategies ([42, 47], Yao et al., unpublished data [42, 48–51]). However, so far none of these methods alone could offer the accurate detection of m6dA distribution in the genomes, implicating the indispensable multiple strategy-based cross-validation for the high efficiency and sensitivity.

m6dA-IP-seq, SMRT-seq, and single-molecule long-read-seq have contributed significantly to identification of genomic loci of m6dA in genomes of *C. elegans* [39], *Drosophila* [40], *Chlamydomonas* [36], and fungi [52] as summarized in Table 2. Unlike the distribution of the m6A sites in the epitranscriptomes, the distribution of m6dA greatly varies from genome to genome. Using single-molecule long-read-seq, m6dA levels and genomic distribution were compared in 16 diverse fungal genomes. It turns out that the ratios of m6dA to all adenine bases (A) reach up to 2.8%; dramatically higher levels than all other eukaryotes so far have been identified [52]. 80–99.6% of the m6dA sites among the diverse genomes were located at the AT motif symmetrically and significantly enriched in the heavily methylated m6dA clusters near the downstream TSSs of the actively expressed gene promoters [52]. More interestingly, m6dA distribution was inversely correlated with abundance of 5-mC.

While in *C. elegans* m6dA showed no region preference across the genome, it mainly distributed in transposon elements as well as in CNS in fly genome ([40, 42], and unpublished data). Particularly, our group found a large percentage of 6mA on intragenic regions with particular enrichment in introns and untranslated regions (UTRs) in *Drosophila* neuron cells BG3C2 (Yao et al., unpublished data). By contrast, m6dA is preferentially enriched at transcription start sites (TSSs), in promoter, genic, and intergenic regions [53], and in the nucleosome-linker DNA with an A-T sequence motif in *Chlamydomonas* [36, 53].

SMRT-seq based m6dA mapping in the genome of *Tetrahymena* indicated that m6dA is enriched at the 5′ end of the gene body and AT motif of the linker DNA regions flanked by nucleosomes particularly H2A.Z- (a variant of the H2A) containing nucleosomes [54]. In addition, m6dA is specifically associated with Pol II-transcribed genes, altogether suggesting that m6dA serves as an indispensable component of the chromatin landscape, playing a part in chromatin remodeling and gene expression at the transcription level.

In the mouse brain, m6dA was substantially biased in its genomic distribution, depending on the gain or loss of m6dA in accordance with stress or normal physiological conditions. The gain of m6dA upon stress is highly enriched in the intergenic regions of the prefrontal cortex (PFC), while it intragenically associated with introns and is excluded from most coding exons [42]. The SMRT-ChIP-seq-based assays have

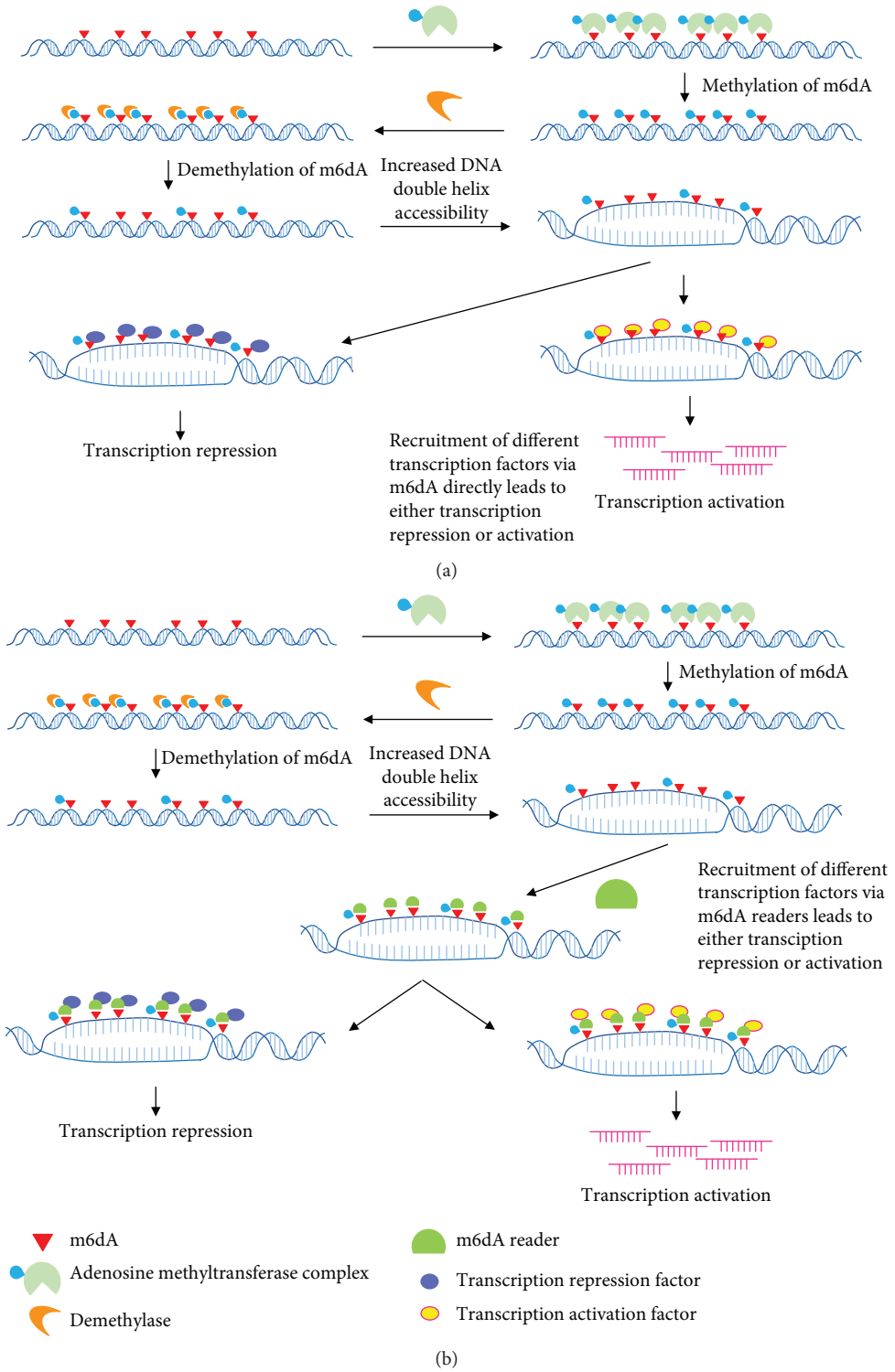

Figure 1: Dynamic regulation of genomic DNA N6-methyladenosine (m6dA) levels by unknown/known components and the potential functions of m6dA in the regulation of gene expression. The coordination between m6dA writer(s) and m6dA erasers maintains the m6dA levels in accordance with physiological conditions and the development and growth stages. (a) The hypothesis for m6dA-mediated regulation of gene expression is that by decreasing the binding energies of base pairs, m6dA could destabilize the DNA duplexes, facilitating m6dA-enriched regions of DNA, unwinding, or making the DNA structure more open for transcription initiation. The m6dA readers (to be identified) are highly affinitive to and bind to the m6dA sites, then the readers may recruit their interaction factors involved in transcription initiation, repression, and so on. (b) Alternatively, it is possible though that these transcription factors might serve as reader(s) of m6dA, directly functioning as regulators of gene expression.

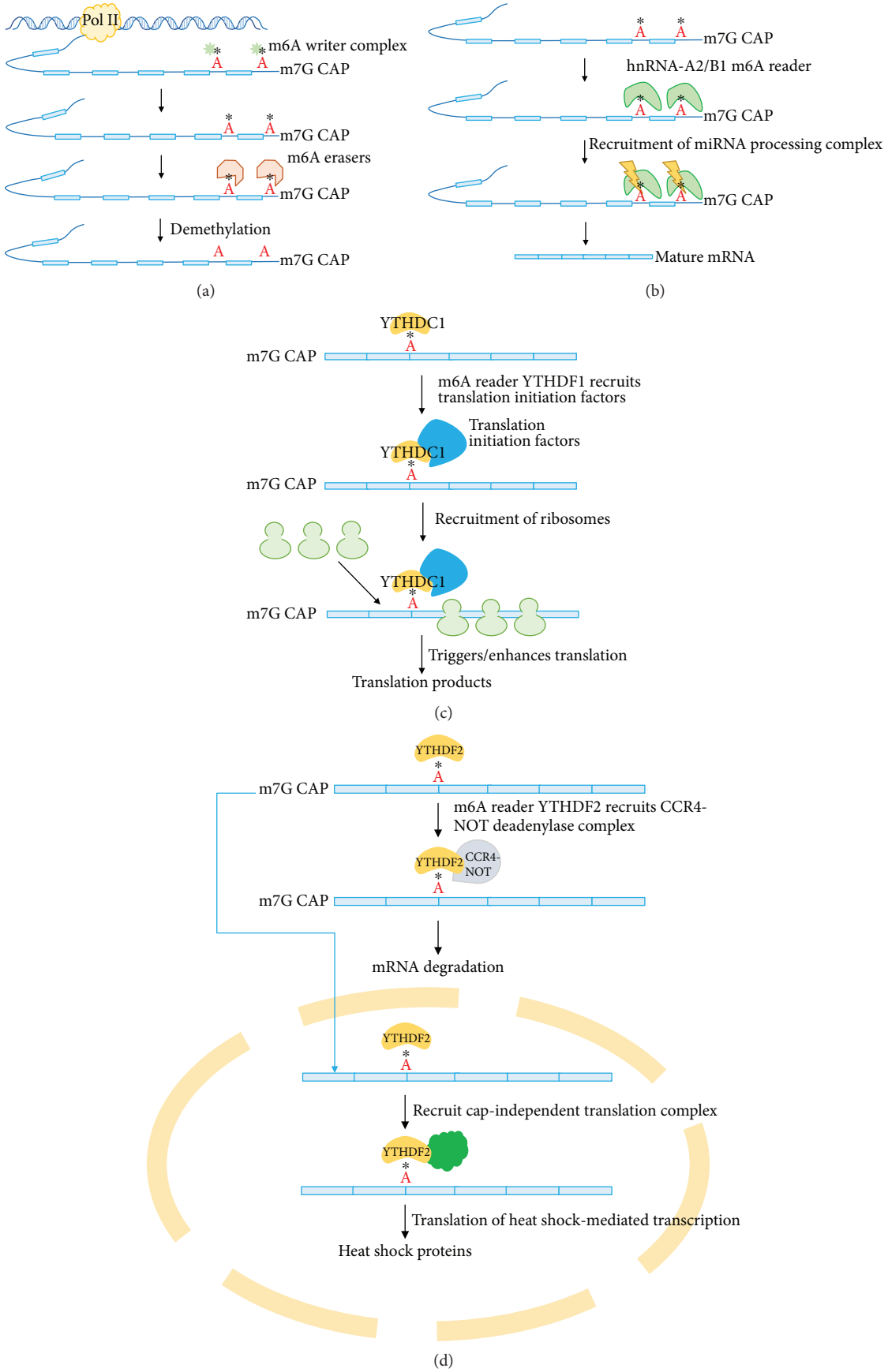

Figure 2: Continued.

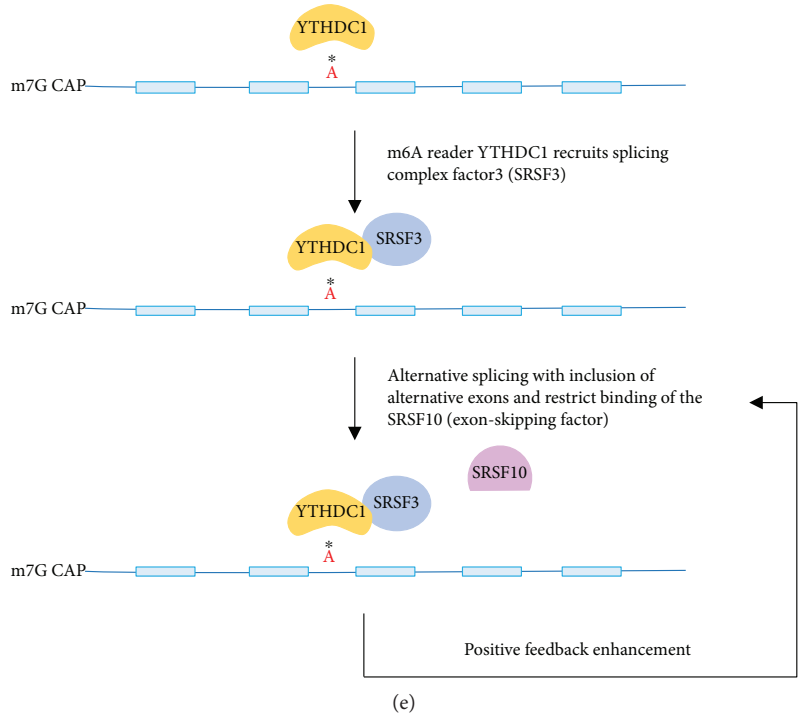

Figure 2: Dynamic regulation of RNA m6A levels by the m6A processing machinery and the known functions of m6A in regulation of RNA metabolism. (a) The coordination between m6A writers and m6A erasers maintains the m6A levels in accordance with the physiological conditions and the development and growth stages. (b) m6A reader hnRNP-A2/B1 mediated microRNA processing. The hypothesis for m6dA-mediated regulation of gene expression is that m6dA readers (to be identified) are highly affinitive to and bind to the m6dA sites, then the readers may recruit their interaction factors involved in transcription initiation, repression, and so on. (c) Via binding to m6A sites to recruit the translation initiation factors, m6A reader YTHDF1 triggers initiation of translation and releases the RNA transcripts to the ribosomes. (d) By recruiting the CCR4-NOT deadenylase complex after binding to m6A sites, the reader YTHDF2 could enhance mRNA decay. On the other hand, facing heat shock, YTHDF2 could transport to the nuclei to trigger the cap-independent translation to translate the heat shock-related RNA transcripts into heat shock proteins. (e) YTHDC1 binds to the m6A sites on the pri-mRNA transcripts and recruit splicing complex factor 3 (SRSF3) to trigger alternative splicing with inclusion of alternative exons. Meanwhile, by recruiting SRSF3, YTHDC1 could restrict binding of SRSF10, further enhancing alternative splicing.

identified the significant enrichment of m6dA in deposition regions of H2A.X (a H2A variant) and at intergenic but not at gene-rich regions as well as at transposon LINE-1 in mouse and mouse ES cells [54]. The motif diversity and non-random distribution of m6dA in distant genomes suggest the potentially biological functions unique to a specific organism.

3. Methyltransferases (Writers) for m6A Methylation in RNA and DNA

3.1. Writers of RNA m6A in Eukaryotes. A multiple component complex consisting of heterodimer of METTL3–METTL14 linked with WTAP and KIAA1429 has been characterized as main writers to methylate base adenosine in the conserved region ACU [55–60]. The different components in the complex have been specified for their individual roles and work together concordantly to carry out their functions more efficiently from recognition and precise localization of the m6A methylation sites to methylate the adenosine sites to m6A. Knockout or knockdown of either Mettl3 or Mettl14 led to depletion or dramatic decrease in the m6A levels in RNA, suggesting their function as methylase for RNA m6A methylation [58, 59, 61].

Although METTL3 has been acknowledged as the main methyltransferase, more and more components in the methyltransferase complex are being identified, such as ZFP217, RMB15, and RMB15B binding to the specific target sites of the RNA to execute specific functions. WTAP is believed to be responsible for recruiting the METTL3–METTL14 complex to nuclear speckles [57, 58] where RNA adenosine methylation occurs. METTL14, the partner of METTL3, though no methyltransferase activity was detected, could facilitate RNA methylation site recognition [62]. Additionally, RNA-binding motif protein 15 (RMB15) and its paralogue RMB15B recruits the METTL3-WTAP complex to m6A consensus sites for methylation [63].

3.2. Writers of DNA m6dA in Eukaryotes. Any base modification can be dynamically regulated in accordance with stages of growth, development, and reproduction, including generation by writers and removal by erasers. While methyltransferases and demethylases for m6A RNA modification have been identified and well characterized, so far only limited information is available for the methylation and demethylation of DNA m6dA modification. The main methyltransferases for RNA m6A such as the complex consisting

Table 1: Machinery of adenosine methylation in DNA and RNA.

Machinery	Component	Roles in the complex	Localization	Organism	Biological function
Writers for m6dA	DAMT-1	Methylation of DNA adenosine	Nuclei	*C. elegans*	
Erasers for m6dA	DMAD	Demethylation of m6dA	Nuclei	*Drosophila*	
	NMAD-1	Demethylation of m6dA	Nuclei	*C. elegans*	
	ALKBH1	Demethylation of m6dA and tRNA	Nuclei/cytoplasm	Mammals	
	FTO	Demethylation of m6dA and RNA m6A	Nuclei/cytoplasm	Mammals	
Readers for m6dA	Unknown	Unknown			
Writers for m6A	METTL3	m6A methyltransferase	Nuclei		
	METTL14	Core component of the m6A methyltransferase in human	Nuclei		
	MT-A70	Complex	Nuclei		
	WTAP	Regulatory component of the complex	Nuclei		
	KIAA1429	Regulatory component of the complex	Nuclei		
Erasers for m6A	ALKBH5	m6A demethylase	Cytoplasm	Mammals	mRNA export and RNA metabolism
	FTO	m6A demethylase	Cytoplasm	Mammals	Mammal fertility convert m6A to h-m6A, f-m6A
Readers for m6A	YTH family				
	(i) YTHDF1	m6A reader	Cytoplasm	Mammals	Trigger/enhance translation of mRNA bearing m6A
	(ii) YTHDF2	m6A reader	Cytoplasm		mRNA decay Mouse female fertility cap-independent Translation in nuclear
	(iii) YTHDF3	m6A reader	Cytoplasm	Mammals	Concordance of YTHDF1 and YTHDF2
	(iv) YTHDC1	m6A reader	Nuclei	Mammals	Facilitate inclusion of alternative exons
	(v) YTHDC2	m6A reader	Cytoplasm	Mammals	Fertility of mouse
	hnRNP family				
	(i) hnRNP-A2/B1	m6A reader	Cytoplasm	Mammals	Alternative splicing processing of miRNA
	(ii) hnRNP-C	m6A reader	Cytoplasm	Mammals	Alternative splicing processing of miRNA
	(iii) hnRNP-G	m6A reader	Cytoplasm	Mammals	
	(iv) hnRNP-H1	m6A reader	Cytoplasm	Mammals	
	(v) hnRNP-H2	m6A reader	Cytoplasm	Mammals	
	KH/RM/RBD family				
	(i) FMR1	m6A reader	Cytoplasm/nuclei mammals		FMR1/YTHDF1 share overlapping mRNA targets
	(ii) FXR1	m6A reader	Cytoplasm/nuclei		
	(iii) FXR2	m6A reader	Cytoplasm/nuclei		
	(iv) KHSRP	m6A reader	Cytoplasm/nuclei		

Table 1: Continued.

Machinery	Component	Roles in the complex	Localization	Organism	Biological function
Repellers for RNA m6A	(i) G3BP1	Repel to m6A	Cytoplasm/nuclei	mammals	Positively affect stability of their target mRNAs
	(ii) G3BP2	Repel to m6A	Cytoplasm/nuclei		
	(iii) CAPRINI	Repel to m6A	Cytoplasm/nuclei		
	(iv) USP10	Repel to m6A	Cytoplasm/nuclei		
	(v) METTL16	Repel to m6A	Cytoplasm/nuclei		

Table 2: Distribution of the methylated adenosine in DNA and RNA.

Species	DNA m6dA distribution	Functions
D. melanogaster	Transposons, intergenic regions, nucleosomal biased, preferential for repeat sequences	Promotion of transposon expression Repression of many genes involved in CNS functions Promote GSC differentiation
C. reinhardtii	TSS of more than 14,000 genes actively Linker DNA biased Intergenic regions	Mainly promote gene transcription
C. elegans	No preference in the genome Nucleosomal biased	Mainly promote gene transcription
T. thermophilus	5′ end of the gene body AT motif of the linker DNA regions flanked by nucleosomes particularly H2A.Z-containing nucleosomes associated with Pol II-transcribed genes	Enhance transcription of the genes bearing m6dA sites
Danio rerio	Preferential for repeat sequences	
Xenio laevis	Depleted at TSSs	
Mus musculus	Depleted at TSSs, enriched on LINE-1 in ESC Varies in accordance with physiological conditions	Epigenetic silencing of LINE-1 and surrounding Enhancers and genes Involved in ESC self-renewal and differentiation
Homo sapiens	Depleted at TSSs, enriched on LINE-1	Similar to mouse
Species	RNA m6A	Biological functions
Mammals	5′UTR and 3′UTR Stop codon Low abundance in coding regions long introns	Regulation of gene expression RNA metabolism including mRNA, rRNA, tRNA, miRNA, snoRNA, and circRNA Determination of cell fate

of METTL3/METTL14 [62, 64–66] have only weak activity in DNA methylation in humans [58]. In other well-characterized DNA methyltransferases for 5-cytosine methylation such as DNMT family members, so far no evidences show their activity to transfer the methyl group to the 6dA base to generate m6dA. Likewise, for N6-methyladenosine transferases for the formation of RNA m6A, such as the IME4 inducer in *Saccharomyces cerevisiae* [67, 68], the MT-A70 domain in humans [69], and DAMT-1 in *C. elegance* [39], so far there are no direct biochemical evidences to show whether they really function as genomic DNA methyltransferases as well. Collectively, it seems that the majority of methyltransferases for N6-adenosine methylation in RNA have either weak or no activity at all for genomic DNA adenine methylation, indicating that although it is completely the same base methylation event, few crosstalks occur between the event in DNA and that in RNA.

4. Readers of RNA m6A and DNA m6dA

4.1. Readers/Effectors of RNA m6A. Transformation from the epitranscriptomic information engraved in RNA m6A to functional signals is carried out by a special class of proteins defined as m6A readers or effectors. The readers/effectors are highly affinitive to the m6A sites due to alteration of the secondary or tertiary structure in specific domain(s) of the target RNAs where the m6A sites were disposed. Since none of the known m6A readers were confirmed to be directly involved in miRNA biogenesis, mRNA maturation, splicing factors, or mRNA half-life, functions of the m6A marker are most probably executed by the m6A reader-mediated downstream events (Figure 2). By binding to the m6A surrounding domain(s), the m6A readers/effectors could alter the RNA-protein conformation to pave the way for recruitment of the second protein component either by direct interaction with readers or by binding to the new site(s) created during the protein-RNA conformation remodeling. The recruitment of the second protein may determine the fate of the target RNA as the recruited proteins were referred to be involved in mRNA metabolism. So far, a category of m6A reader protein components has been identified and these components are classified as several families, including the YTH domain [70–82], hnRNP family including hnRNP-A2/B1, hnRNP-C, hnRNP-G, hnRNP-F, hnRNP-H1, and hnRNP-H2 [83–87], KH domain, zf-CCHC domain, RBD, RRM, and zinc knuckle domain protein families [88–92], as summarized in Table 1.

4.2. RNA m6A Repellers. In addition to the m6A readers, the m6A repel proteins (or m6A repeller) were identified as well in a recent study [87]. The m6A repellers preferentially interact with an unmodified RNA sequence but repelled by m6A,

such as G3BP1 and G3BP2 known as stress granule proteins [93, 94], USP10 and CAPRINI (interaction partners of G3BP1 and G3BP2), and METTL16, an adenosine methyltransferase for small nuclear RNA. Compared to the m6A readers, these repellers were more diverse and cell type-dependent [87]. It has been confirmed both in vivo and in vitro that the RNA m6A repellers positively affect the stability of the target mRNAs by binding to their mRNA targets [87].

5. Functions of RNA m6A and m6dA Erasers

5.1. RNA m6A Erasers. Adenosine methyltransferases and demethylases (erasers) concordantly work together to regulate dynamic levels of m6A and the landscapes during the stages of generation, development, and reproduction. The functional study on m6A has been lagged behind until the discovery of its erasures in recent years [85, 95, 96]. So far, several demethylases have been identified and characterized for both DNA m6dA and RNA m6A.

5.1.1. FTO. So far, only two members have been identified to exhibit the comparable demethylase activity, including FTO and ALKBH5 [77, 95–97]. FTO, belonging to the AlkB family of non-heme Fe (II)/a-ketoglutarate- (a-KG-) dependent dioxygenases, was the first demethylase identified to demethylate m6A in RNA [96]. FTO is mainly expressed in the brain and adipose tissue [95, 98, 99]. More specifically, like TET proteins that convert 5-mC to 5-hmC, 5-fC, and 5-caC, FTO could oxidize m6A to its intermediate form N6-hydroxymethyladenosine (h-m6A) and N6-formyladenosine (f-m6A). However, these intermediates' functions remain to be elusive whether they are just intermediates with short lifespans to be finally converted to regular adenosine or they serve as special modification markers to further affect RNA-protein interactions [100].

5.1.2. ALKBH5. Four of the nine *E. coli* AlkB family homologs in mammalian (ALKBH1-9) cells have been characterized as diverse demethylases functioning as removal of the methyl group from ribonucleobases, including ALKBH1, ALKBH5, ALKBH8, and ALKBH9, respectively [85, 95, 101]. Next to FTO, ALKB5 was the second demethylase identified to erase the methyl group of m6A in eukaryotic RNA, regulating mRNA export and RNA metabolism as well as fertility in mammals phenotypically. In contrast to FTO with preferential expression in the brain and adipose tissues [98, 99], ALKBH5 is highly expressed in testes [95], suggesting that the tissue-preferential expression of demethylase is responsible for local demethylation activity. Disorders of the ALKB family level in mammals induce many types of diseases, suggesting the essential roles of the dynamic m6A levels in the life process.

More recently, DDX3, a member of DEAD box RNA helicases, was found to interact with ALKBH5 through its ATP domain and DSBH domain of ALKBH5 to modulate mRNA demethylation activity. Moreover, DDX3 regulated the m6A methylation status of microRNAs. This result suggests that the potential partners for demethylases such as DDX3 could regulate the demethylase activity more efficiently and precisely [102].

5.2. DNA m6dA Erasers. As for demethylation, although 5-mC could be demethylated by ten eleven translocation protein (TET) family members in eukaryotic genomic DNA particularly in mammals [103], it seems that majority of these members are not functional for m6A demethylation in RNA. Likewise, the identified majority of enzymes for demethylation of RNA 6mA such as ALKBH5, one member of the AlkB family of dioxygenases [95], showed very weak or even no activity at all for m6dA in DNA. However, FTO has been identified to catalyze demethylation of m6dA in synthetic DNA [96] and an even stronger activity than in RNA m6A demethylation under in vitro conditions, but still lacks evidence if it works for genomic DNA in vivo.

5.2.1. DMAD. The homologue of the mammalian ten eleven translocation protein family (TET) [104] is the first demethylase for erasure of m6dA in DNA identified in *Drosophila* ([40], Yao et al., unpublished data). DMAD belongs to the TET protein superfamily, which functions in demethylation of 5-mC in mammals, but so far no report is available for mammalian TETs that could catalyze the demethylation of 5-mC to 5-hmC. A histone H3K4me2 demethylase SPR-5, a potential m6dA demethylase in *C. elegans*, could function as a putative m6dA demethylase as the SPR-5 deficiency mutant elevates the level of m6dA transgenerationally [39], but further biochemical evidences are required to support the conclusion.

5.2.2. ALKBH1. The second demethylase has been characterized in mammalian ES cells to catalyze the demethylation of the DNA m6dA [47, 105, 106]. So far, it is not clear if this demethylase functions as demethylation of m6A in RNA.

5.2.3. FTO. Under in vitro conditions, the first identified demethylase FTO for RNA m6A also shows an even stronger activity for demethylation of synthetic m6dA in DNA strands than that in RNA strands [96], suggesting the potentially strong demethylase of m6dA in genomic DNA. Later on, Huang et al. [107] further confirmed an inverse correlation between FTO expression and the m6dA levels in genomic DNA, suggesting that FTO functioned as a DNA m6dA eraser although in vitro biochemistry evidence is still not available.

6. Regulation Functions of RNA m6A

Even though significant efforts have been made in the study on RNA m6A modification, precise regulation mechanisms remain largely unknown. However, emerging evidences suggest that RNA m6A modification is indispensably involved in a wide range of spectrum of biological functions at both molecular and phenotypical levels. At molecular levels, m6A regulates RNA metabolism, including mRNA [56, 61, 74, 80, 83, 84, 95, 108–116], rRNA, tRNA, miRNA [83, 115], and circRNA [116].

6.1. For mRNA. m6A modification regulates mRNA stability [56, 61, 74, 108, 117], clearance [75], alternative splicing [80, 109–111], transportation and localization [95], translation efficiency [112, 113], and mRNA-protein interactions [84, 114].

6.2. Reciprocal Regulation of miRNA Maturation and m6A Methylation. hnRNP protein family members such as hRNPA2/B1 and hnRNP-C serve as m6A readers. hnRNP-A2/B1 shows high affinity to m6A that was methylated by METTL3 and located in pri-miRNAs. After binding to m6A, hRN-A2/B1 recruits the microprocessor complex to the miRNA precursors, enhancing processing of the precursors into mature miRNAs [83, 115]. Reciprocally, by base priming with their specific target mRNA sequences, miRNAs regulate the m6A modification level via repression of the binding of METTL3 to mRNAs that contain miRNA-targeting sites evidenced by the fact that 6mA sites are enriched at the miRNA-binding sites of target mRNAs in mouse pluripotency cells and differentiated cells [118].

6.3. Regulation of Long Noncoding RNA (lncRNA) by m6A Methylation. The functional secret behind the significantly high abundance of m6A in the eukaryote lncRNAs relative to other RNA molecules [41, 107, 119] has not yet been unveiled until recently, and the inverse correlation between m6A methylation levels in lnc-XIST and its silencing function was discovered [120]. As a long noncoding RNA X-inactive specific transcript, XIST functions as a gene silencer on the X chromosome at the transcriptional level. One of the m6A readers, YTHDC1, preferentially binds to m6A markers on XIST and is indispensable for XIST-conferred transcriptional silencing in human cells [121–123].

6.4. Regulation of m6A on circRNAs. Circular RNAs (circRNAs) belong to a new type of ncRNAs bearing the covalently closed-loop structures and universally expressed in lower and higher eukaryotes [124]. While their functions remain largely elusive, emerging data suggest that circRNAs could regulate gene expression [125, 126] and are pathologically involved in the progression of some diseases, such as cancer [127] and neurological disorders [128]. A recent study showed that endogenous circRNAs may generate proteins, expanding a novel mode of cap-independent translation [129]. Recently, Zhou et al. have identified widespread m6A modifications in circRNA by genome-wide mapping of m6A sites [116, 130]. It turns out that m6As in circRNAs share the same writer and reader protein complexes with those in mRNAs, while significant distinctions exist between many m6A sites in circRNA and those in mRNAs. One of those distinctions is in the way m6A circRNAs are generated from unmethylated exons in mRNAs, and circRNAs derived from m6A-methylated exons tend to be unstable mediated by YTHDF2, suggesting that m6A modification directed the regulation of circRNAs.

6.5. For tRNA Methylation. tRNA serves as a key component of protein synthesis machinery. Among the heavy modifications in tRNA, presence of m6A has been confirmed, and the dynamic regulation of m6A in tRNA critically impacts its functions as well. Mammalian ALKBH1, in addition to its function as demethylator of the DNA m6dA, has been also tested to be a tRNA demethylase for demethylation of N1-methyladenosine (m1A). Enhanced expression of ALKBH1 leads to attenuated translation initiation due to demethylation of the target tRNAs, therefore giving rise to a decrease in the usage of tRNAs for protein synthesis. The dynamic regulation of the tRNA m6A is in a glucose availability-dependent manner, altogether suggesting that dynamic m6A in tRNA regulates gene expression posttranscriptionally [131].

6.6. For DNA Damage Response. More recently, it was reported that RNA m6A modification could regulate UV-induced DNA damage response by rapidly recruiting Pol K, a DNA polymerase implicated in DNA damage repair, to the damage sites for quick repair to confer cell survival [130].

6.7. Phenotypical Correlation with m6A Alterations. Phenotypically, m6A is involved in the regulation of sex determination [132, 133], male infertility [95, 134], circadian clock [135], neurological disorders [132, 133, 136], and other diseases, such as cancer [113, 137–141].

7. Potential Functions of DNA m6dA Modification

The development of the restriction-modification (R-M) system conferred by the abundance of m6dA in prokaryotes such as *E. coli* [38] has been unanimously acknowledged. The potential function of m6dA, although progress has been made such as that bacterial DNA m6dA could lead to differentiation of mammalian tumor cells [142], remains largely to be elusive. Dynamic alteration of m6dA in genomic DNA was associated with brain functions ([42, 136], Yao et al., unpublished data), embryogenesis [131], reproduction [40, 131], and ES cell development [47] in a range of organisms, suggesting the fundamentally biological functions of m6dA in eukaryotes besides affecting protein-DNA interaction in eukaryotes [143–145], rather than the R-M system as in prokaryotes.

7.1. DNA m6dA-Mediated Chromatin Remodeling. The functions of m6dA are thought to be via the m6dA-mediated regulation at chromatin structural and transcriptional levels. It has been shown that m6dAs are distributed in the linker DNA regions of H2A variant-containing well-positioned nucleosomes, such as the H2A.X deposition region in mouse ES cells [47] and H2A.Z in *Tetrahymena* [54]. This discovery suggests the function of m6dA in chromatin remodeling. In addition, some m6dA sites have high affinity with Pol II-transcribed genes, enhancing the transcription of these genes [54].

7.2. DNA m6dA-Mediated Dual Functions of Gene Expression. m6dA reader proteins have not yet been identified and characterized so far. Similar to MeCP2, by binding to m6dA-distributed regions, m6dA readers may recruit partners to remodel the chromatin structure. However, in

contrast to 5-mC-mediated transcription silencing, m6dA confers both transcriptional activation and repression depending on the organisms and the tissues or developmental stages even on the same organism [36, 39, 40, 52, 54].

Studies showed m6dA-conferred transcription repression like the 5-mC regulation manner in many organisms ([36, 40, 42, 146]). In *Drosophila*, 6mA levels are inversely correlated with the transposon expression in the ovary [40]. In mouse, the significantly increased level of m6dA following environmental stress is negatively associated with expression of a group of neuronal genes and LINE transposons [42]. Through genome-wide 6mA and transcriptome profiling, we found that 6mA may serve as a repressive epigenetic mark on a group of genes involved in neurodevelopment and neuronal functions in *Drosophila*.

In mouse ESCs, m6dA deposition is strongly biased on the evolutional age of L1 transposons. m6dA is significantly enriched at young relative to old L1 elements, positively correlating with epigenetic silencing of such L1 transposons together with their surrounding enhancers and genes in mammalian genome [47].

In contrast to the m6dA-associated repression of gene expression, m6dA accumulation activates the expression of genes in some organisms or in some specific tissues or developmental stages in *Chlamydomonas* [36] as well as early embryogenesis of zebrafish [147], fungi [52], and adult mouse brain [148]. Alternatively, compared to the nonmethylated adenine base in DNA, m6dA can decrease the binding energies of base pairs [149] and therefore destabilize DNA duplexes, facilitating m6dA-enriched regions of DNA, unwinding, or making the DNA structure more open for transcription initiation and the downstream processing [150].

8. Regulation of Stem Cell Fates by RNA m6A Modification

Dynamic changes of m6A sites or levels alter the m6A landscape in epitranscriptomes of stem cells. This could lead to the enhanced or impeded expression of the key genes responsible for proliferation, differentiation, or specification during the embryogenesis and normal development of tissues/organs/individual organisms. Consequently, the fates of the stem cells are determined. Although the exact functions of RNA m6A in stem cell regulation remain to be elusive, emerging evidences have suggested the indispensable roles of mRNA m6A in ES cells, including iPS cells, ES cells, bone marrow ES cells, blood stem cells, and neuronal stem cells [61, 111, 136, 151–154] as summarized below.

8.1. m6A-Mediated Regulation of Somatic Cell Reprogramming. Significant demethylation of 5-mC mainly in the promoter regions of the genes encoding some pluripotency factors such as Oct4, Nanog, Sox2, and Klf4 serves as the prerequisite during somatic cell reprogramming toward induced pluripotent stem cells (iPSCs). The demethylation is mainly catalyzed by TET, consequently leading to overexpression of the defined reprogramming factors [155]. In contrast to the inverse correlation of the DNA 5-mC methylation levels and the reprogramming efficiency during somatic cell reprogramming [155], paradoxically, the elevated mRNA methylation level of m6A enhances the efficiency [156]. This was confirmed by the fact that overexpression of METTL3 and the four Yamanaka factors (*Oct4*, *Sox2*, *Klf4*, and *c-Myc*) in mouse embryonic fibroblasts (MEFs) led to elevation of m6A abundance and dramatically promoted the number of iPSC colonies. Accordingly, downregulation of methyltransferase *METTL3* expression leading to a decreased m6A level repressed the expression of Yamanaka factors and consequently inhibited the reprogramming efficiency, altogether suggesting the essential roles of the finer-tuned regulation by combining the modifications of cytosine and adenosine at both DNA and RNA simultaneously when the cells face to the significant turning point of the life processes.

8.2. Regulation of Normal Hematopoietic Stem and Progenitor Cells (HSPCs) by RNA m6A Modification. Recent studies are gradually unveiling the link between RNA m6A modification and regulation of normal hematopoietic and leukemia cells as well as vertebrate embryogenesis [59, 157]. METTL3 depletion in normal human hematopoietic stem/progenitor cells (HSPCs) and leukemia cells leads to a decline in RNA m6A levels, to promotion of differentiation, and to reduction of proliferation in HSPCs and myeloid leukemia cells. Conversely, overexpression of METTL3 could reverse the phenotype conferred by METTL3 depletion [59]. Comparing with healthy HSPCs or other types of tumor cells, the expression of *METTL3* at both transcriptional and translational levels was dramatically enhanced in acute myeloid leukemia (AML) cells. Furthermore, mRNA m6A modification promotes translation of *c-MYC*, *BCL2*, and *PTEN* mRNAs in human AML cell lines. METTL3 deficiency induces the differentiation and apoptosis of human myeloid leukemia cell lines, partially being ascribed to the increased levels of phosphorylated AKT. More interestingly, METTL3 depletion delays leukemia progression in in vivo mice, altogether suggesting the potential of METTL3 as a therapeutic target for AML [59].

During zebrafish embryogenesis, dynamic mRNA m6A modification levels coordinately regulate the fate of the earliest HSPCs in endothelial-to-hematopoietic transition (EHT). Similar to human HSPCs, *mettl3*-deficient embryos, a significant decrease in m6A abundance strongly represses HSPC generation mechanistically due to the delayed YTHDF2-mediated mRNA decay of the arterial endothelial genes *notch1a* and *rhoca* [157].

8.3. Adult Neural Stem Cell Differentiation Regulation by m6A at the RNA Level. The RNA m6A modification levels were altered dynamically from the remarkable enrichment during early embryogenesis to a rapid drop and then maintenance of the low dose thereafter. However, the overall level of m6A remains substantially higher in heads and ovaries compared to other organs/tissues [132], suggesting the potential functions of mRNA m6A modification in the nerve and reproduction system. The mutant flies with methyltransferase deficiency reduce their lifespans and accompanied by multiple behavior defects mainly exhibited in flying and locomotion [132, 133]. This result suggests the aberrant regulation

of neurological regulation associated with m6A loss. Accordingly, m6A overaccumulation in *Fto*-KO mice show postnatal neurodevelopment defect and repression of both proliferation and differentiation in adult neural stem cells [136]. Consequently, this leads to a reduced brain size and poor learning and memory. Altogether, it suggests that RNA m6A modification levels must be tightly regulated to optimal levels in accordance with the physiological conditions during embryogenesis and at the normal development stages.

8.4. Regulation of ES Cell Pluripotency and Differentiation by RNA m6A Modification. During embryogenesis and ES cell development, expression levels between the pluripotency factors and the differentiation factors are precisely and dynamically regulated by RNA m6A methylation. RNA m6A conferred regulation, among other epigenetic modifications, to determine the fate of ESC towards self-renewal or differentiation [154]. In mESC, the Mettl3 knockdown-caused deficiency of RNA m6A methylation leads to loss of self-renewal capability. The mechanism is the m6A methylation loss-mediated degradation of the transcripts coding for developmental regulators among a large number of others. By contrast, a conflict report is available for mESCs with Mettl3 KO in a way that RNA m6A modification loss enhances self-renewal and inhibits differentiation efficiency [151]. More studies demonstrated that chromatin-associated zinc finger protein 217 (ZFP217) could coordinate distinct epigenetic and epitranscriptomic networks to play essential roles in maintaining the pluripotency of ESC and somatic cell reprogramming by two mechanisms. The one is that ZFP217 directly regulates transcription of key pluripotency and reprogramming genes. The other is that ZFP217 sequestrates METLL3 by interacting with it to repress m6A RNA deposition in a subset of RNAs including the pluripotency and reprogramming factors such as Nanog, Sox2, Klf4, and c-Myc for their stability [55].

8.5. Regulation of Cancer Stem Cells by RNA m6A Modification. Cancer stem cells (CSCs) are a driving force for tumor initiation and metastasis. Exposure of breast cancer cells to hypoxia promotes demethylation of m6A in NANOG and KLF4 mRNA, leading to an increased expression of these pluripotency factors. Further study confirmed that the demethylation of m6A in these mRNAs is caused by induced expression of ZNF217 and mediated by ALKBH5; exposure to hypoxia also induces ZNF217-dependent inhibition of m6A methylation. All these inductions and enhanced demethylation are in an HIF-1α-dependent manner [158].

RNA m6A modification regulates generation, growth, self-renewal, and metastasis/tumorigenesis of human glioblastoma stem cells (GSC). Knockdown of METTL3, a key component of the RNA methyltransferase complex, significantly enhances GSC growth and self-renewal, caused by a dramatic decrease in m6A methylation. Further study shows the alteration of mRNA m6A distribution and the consequent mRNA expression of the genes under conditions of METTL3 or METTL14 knockdown. Inversely, overexpression of METTL3 or FTO deficiency inhibits GSC growth and self-renewal. Interestingly, FTO deficiency represses tumor progression and increases the lifespan of GSC-grafted mice substantially, suggesting FTO as a potential therapeutic target for glioblastoma [140].

9. Regulation of Stem Cells by DNA m6dA Methylation Modification

Although DNA m6dA methylation was discovered almost at the same time as RNA m6A methylation was, the progress in understanding the biological functions largely lags behind that in RNA m6A methylation. To date, while progress has been made in understanding stem cell regulation by RNA m6A modification, stem cell regulation from DNA m6dA modification remains to be a super mystery.

9.1. Insect Germline Stem Cell (GSC) Regulation. The dynamic status of DNA m6dA methylation plays essential roles during *Drosophila* embryogenesis [40]. In accordance with life processes starting from fertilization, embryogenesis, to later development, expression levels of methyltransferase (not yet identified) and DMAD, the first identified demethylase of m6dA, must be tightly regulated to maintain the appropriate levels of m6dA in the genome. Overexpression or KO/KD of DMAD leads to prenatal or postnatal lethality. It seems that m6dA could maintain the self-renewal state, while removal of m6dA by its eraser DMAD promotes GSC differentiation.

9.2. ESC Regulation. ALKBH1, the second identified demethylase for m6dA, functions as dioxygenase specifically removing the methyl group from histone H2A. ESC with ALKBH1 deficiency enhances pluripotency but represses differentiation particularly for neural differentiation. Further study suggests that by interaction with the core transcriptional pluripotency factors, ALKBH1 plays important roles in regulation of ESC self-renewal and differentiation [105]. More evidence came from where m6dA preferentially deposited on young L1 transposons over old L1 on X chromosomes and confers L1 silencing in ESC [47].

9.3. Regulation of Human Bone Marrow-Derived MSCs by m6dA. In bone marrow-derived stem cells (MSC), m6dA elevation due to ALKBH1 deficiency significantly represses differentiation of MSCs, leading to the aberrant bone phenotype [159]. Molecularly, by interacting with the promoter regions of core factors indispensable for osteoblastic differentiation including Atf4, Runx2, and Osterix, ALKBH1 removes m6dA on the promoter regions of these genes. Thus, the repression mechanism could be dissected as the increased m6dA levels at the promoter regions of these core factors in accordance with ALKBH1 deficiency, hampering the expression of these differentiation-conferring factors.

10. Concluding Remarks

In the recent two decades, significant achievements have been made in an epigenetic study particularly 5-mC and its intermediates such as 5-hmC, 5-fC, 5-caC, and more recently 6mA and m6dA. Introduction of bacterial m6dA-bearing DNA to mammalian tumor cell lines led to the differentiation

of tumor cells [142], shedding light onto m6A modification-mediated tumor therapy. Since then, m6A study was significantly enhanced in identification of more writers, erasers, and particularly readers of m6dA modification in genomes as well as their partners for network coordination-based regulations. So far, significant achievements have been made in understanding the generation, dynamic alteration, machinery, distribution, and biological functions molecularly and phenotypically in the recent few years. However, a large number of unknown mysteries behind the RNA m6A and the DNA m6dA remain to be elusive. Since RNA m6A and DNA m6dA belong to different layers of modifications, we separately discuss about them.

So far, information on m6dA writers, erasers, and m6dA readers remains largely unknown. To better understand the functions of m6dA, it is of significance to dissect the exact mechanisms of m6dA-mediated regulations on a wider range of species. (1) Additional components of the machinery for m6dA methylation (writers)/demethylation (erasers), readers, and associated effectors need to be identified. (2) Once these machinery components are identified, their functions should be targeted at molecular, physiological, and phenotypic levels. (3) It is of importance to understand the molecular and cellular mechanisms of the deposition of m6dA in the genomes particularly in stem cell genomes. (4) 5-mC can be oxidized by TET to generate 5-hmC, 5-fC, and 5-caC intermediates, and likewise, RNA m6A could be converted by FTO to generate 6-hmA and 6-fdA as its intermediates. Thus, it is necessary to determine if m6dA could be converted into 6-hmdA, 6-fmdA, and 6-cadA as well either by TET or erasers such as FTO and ALKBH1. If it is true, their functions will be an interesting target as intermediate products for the final removal of methyl groups or as epigenetic markers for any known biological functions. It is well known that 5-hmC functions as an intermediate during demethylation of 5-mC in eukaryotes. Additionally, 5-hmC also serves as an important epigenetic marker involved in a wide range of spectra of biological pathways such as reprogramming, proliferation/differentiation, and tumorigenesis. Do h-m6A and 6-fmdA function as epigenetic markers like 5-hmC? (5) Evidences suggest the inverse correlation between m6dA levels and the complexity of eukaryotic genomes. Relative to the dominant abundance and the significant epigenetic regulation roles of 5-mC in vertebrate genomes, it is paradoxical so far how the 102- to 103-fold lower levels of the m6dA marker still play important roles in proliferation and differentiation of mammalian ESCs. [160] suggest the temporal or spatial distribution of m6dA to serve as a complementary and alternative DNA marker instead of being relatively constitutive from the generation/disposition to the functions. Since the extremely low dosages of m6dA in higher eukaryote mammals do not seem to come from relics during the evolution from prokaryotes to eukaryotes, it remains to be an interesting and essential issue for understanding the different layers of epigenetic regulations. (6) Loss of DNA 5-hmC has become a hall marker for cancer cells. Likewise, compared to the adjacent normal tissue, significant m6dA loss in human primary tumors has been detected (unpublished data). Thus, it is of great importance to compare the levels of 6mA in a variety of tumors to confirm if loss of 6mA modification could be a novel hallmark of cancers for epigenetic diagnostics of cancers or other diseases. (7) Furthermore, some compounds that could induce DNA damages have been acknowledged to play essential roles in cancer therapies. Overexpression of ALKBH family members in some cancers, such as bladder, prostate, and pancreatic cancers, inhibits cancer DNA damage, leading to cancer cell proliferation and chemotherapy resistance [161, 162]. Thus, efforts were worthy of being paid to study whether or not ALKBH family members such as ALKBH1 could serve as therapeutic target(s) for clinical cancer therapy.

For RNA m6A modification, although significant progress has been made in recent years, significant challenges remain. (1) More components in the processing machinery complexes for m6A methylation or demethylation may exist and wait to be identified. Discovery of more components could help us understand the regulation of the dynamic alteration of m6A levels. ZFP217 is the first modifier that could coordinate the distinct epigenetic and epitranscriptomic networks to maintain the pluripotency of ESCs and somatic cell reprogramming. It will be of great significance to further identify and characterize more coordinators/modifiers that could directly regulate transcription of key regulation genes. Simultaneously, the potential coordinators/modifiers could interact with m6A RNA methylation/demethylation machinery complexes. Consequently, these modifiers/coordinators could regulate the transcription and m6A RNA disposition in a subset of RNAs including the factors indispensable for pluripotency, differentiation, and reprogramming, and other key metabolic pathways. (2) More precise techniques are required to analyze the exact distribution of m6A in epitranscriptomics from different organisms. Since the exact molecular mechanisms of the selection of mRNA targets and the m6A sites in the targets remain largely unknown, efforts should be made for determination. (3) Further identification of m6A readers and repellers and characterization of their functions will help us understand the m6A-based epitranscriptomic regulation of the wide spectrum of biological processes. (4) Given that the known m6A RNA demethylases ALKBH1 and FTO could catalyze demethylation of m6A in both RNA (mRNA and tRNA) and DNA, it is of importance to investigate if other major components in the m6A methylase complex machinery such as METLL3–METTL4 could function as m6dA writers for DNA modification as well. (5) It has been identified that there are 6-hmA and 6-fmA during demethylation of m6A in the RNA, but their functions remain to be elusive. (6) It is also of importance to test if mammalian TET family members could catalyze the demethylation of m6A and m6dA in both DNA and RNA modifications, although these members did not show the demethylation activity of m6A RNA in our lab. (7) METTL3 depletion delays leukemia progression in in vivo mice, shedding light on the potential of METTL3 as a therapeutic target for human AML [59]. Thus, further exploring therapeutic targets involved in m6A machinery complexes might be very promising for some stubborn diseases such as cancers and

neurological diseases. These extensive studies may unveil more exact mechanisms and the regulation roles in multiple biological processes.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

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Review Article

Novel Epigenetic Techniques Provided by the CRISPR/Cas9 System

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Epigenetics classically refers to the inheritable changes of hereditary information without perturbing DNA sequences. Understanding mechanisms of how epigenetic factors contribute to inheritable phenotype changes and cell identity will pave the way for us to understand diverse biological processes. In recent years, the emergence of CRISPR/Cas9 technology has provided us with new routes to the epigenetic field. In this review, novel epigenetic techniques utilizing the CRISPR/Cas9 system are the main contents to be discussed, including epigenome editing, temporal and spatial control of epigenetic effectors, noncoding RNA manipulation, chromatin in vivo imaging, and epigenetic element screening.

1. Introduction

Epigenetics classically refers to the inheritable changes of hereditary information without perturbing DNA sequences. DNA methylation, demethylation, hydroxyl-methylation, histone modification, chromatin remodeling, gene imprinting, and noncoding RNA are the central mechanisms involved. They play important roles in diverse biological processes including gene regulation, iPSC reprogramming and maintenance, genomic imprinting, X-chromosome inactivation, aging, neurodegeneration, autoimmune modulation, and tumorigenesis [1–4].

Adapted from a natural immune defense system in bacteria, the clustered regularly interspaced short palindromic repeat- (CRISPR-) associated protein 9 (Cas9) system, abbreviated as the CRISPR/Cas9 system, is a site-specific genome editing tool that could be implemented to target and mutate specific genomic regions in eukaryotic cells, especially in mammalian cells [5]. The rationale is described below: the in vivo CRISPR/Cas9 system comprises two core components, a Cas9 nuclease and a guide RNA sequence. The guide RNA is programmable. By changing the sequences of guide RNA, researchers could target Cas9 nuclease to almost any locus in the genome precisely. After being delivered into the cell of interest, guide RNA will direct Cas9 nuclease to the target via complementary matching with the corresponding genomic DNA sequence. Flanking by a NGG protospacer adjacent motif (PAM) is a prerequisite for a piece of DNA sequence to be a qualified target. Once guide RNA finds its target, the Cas9 nuclease will transit from the binding state to the cutting state with the help of PAM, subsequently generating a double-stranded break (DSB). Depending on the presence of a repair template, the DSB will be rejoined either by the nonhomologous end joining (NHEJ) or the homology-directed repair (HDR) mechanism. The former is more error-prone, while the latter is more precise [6–11]. Briefly, by designing specific guide RNA sequence and inducing appropriate downstream repair mechanism, researchers can utilize this method to achieve genome modifications flexibly.

Notably, since the emergence of CRISPR/Cas9 technology, diverse applications have been explored beyond genome editing. Here, we will focus on the new toolkit that CRISPR/Cas9 has provided to us for epigenetic research.

1.1. Epigenome Editing. Epigenome editing refers to the targeted rewriting of epigenetic markers [1, 12]. On the one hand, it could be used to selectively modify epigenetic marks at a given locus to explore mechanisms of how targeted epigenetic alterations would affect transcription regulation and cause subsequent phenotype changes. For example, it has been reported that inducing histone methylation or acetylation at the *Fosb* locus in the mice brain reward region, nucleus accumbens, could affect relevant transcription network and thus control behavioral responses evoked by drug and stress [13–15]. On the other hand, epigenome editing has the potential for epigenetic treatment, especially for the disorders with abnormal gene imprinting or epigenetic marks. Targeted epigenetic silencing or reactivation of the mutant allele could be a potential therapeutic approach for diseases such as Rett syndrome and Huntington's disease [12, 16–19]. DNA-binding protein domains, such as zinc finger (ZFN) or transcription activator-like effector nuclease (TALEN), fused with transactivators have been demonstrated to be feasible methods for epigenome editing; however, the protein synthesis step is costly and labor-consuming, which makes it difficult for such methods to be widely used, for the binding specificity of zinc finger and TALEN is determined by the amino acid sequences within their repeat domains. Changing genomic targets means changing amino acid sequences [20–23]. On the contrary, the CRISPR/Cas9 method described above overcomes this shortcoming due to its cost-effectiveness and easy-manipulating. Instead of redesigning amino acid sequences and synthesizing new DNA-binding proteins, what we need to do in the CRISPR/Cas9 system is redesign the programmable guide RNA sequences and synthesize a new expression cassette. Thanks to the advent of the novel CRISPR/Cas9 technology, the field of epigenome editing begins to thrive.

To achieve CRISPR/Cas9-mediated epigenome editing, the main strategy is fusing the Cas9 protein with a transcription repressor or activator domain, which was known as an epigenetic effector (epieffector) [21]. To be specific, the adaption is inactivating the Cas9 nuclease first and further fusing it with an epieffector domain. The deactivated (dCas9) has no nuclease activity but functions as a DNA-binding domain. Accumulating evidence has proved that this dCas9-epieffector fusion complex is an efficient epigenome editing tool.

For example, when the fused epigenetic effector domain was Krüppel-associated box (KRAB), using the dCas9-KRAB complex to target and induce locus-specific deposition of H3K9me3 at the *HS2* enhancer region, researchers successfully silenced multiple globin genes in K562 cells. Off-target perturbation in global gene expression seemed not to be an issue in this study [24].

When the fused domain was LSD1, using the dCas9-LSD1 complex to target *Tbx3*, a gene implicated in the maintenance of pluripotency, researchers observed down-regulation of *Tbx3*, loss of H3K27Ac at the enhancer, and impaired pluripotency in embryonic stem cells. In addition, this study compared specificity for enhancer targeting between dCas9-LSD1 and dCas9-KRAB complexes. Interestingly, it was concluded that dCas9-LSD1 was enhancer-specific; however, the specificity of the dCas9-KRAB complex was questionable, for it may actually be silencing the promoter or changing the chromatin structure instead of targeting the enhancer to achieve *Tbx3* downregulation. The difference in the experimental methods, such as origins of Cas9 nuclease, cell types of interest, and genes to be manipulated, might account for such contradictory findings [25].

When the fused domain was p300 core, which was an acetyltransferase, using this dCas9-p300 core complex, researchers activated the *Myod* gene at a regulatory region distal to the promoter, upregulated the *Oct4* gene from a regulatory region proximal to the promoter, and induced transcription in three-fourths of downstream hemoglobin genes by targeting several DNase hypersensitive sites within the β-globin locus control region. These findings updated the conventional view that controlling gene expression with engineered transcription factor at sites other than promoters was limited [26].

The fusion domain could also be the transactivation domain VP64 or VPR, the DNA methyltransferase 3A (DNMT3A), or the DNA demethylase TET. The dCas9-VPR complex is an escalated version of dCas9-VP64, where VP64 was further fused by two other transcription factors p65 and Rta to increase the transactivation efficiency. Both of them have well been demonstrated to be reliable gene activation tools. The dCas9-VP64 complex could directly activate the silenced *Oct4* gene in both human and mouse cells [27]. By targeting the *Ngn2* and *Neurod1* genes, the dCas9-VPR complex was shown to be capable to aid induced pluripotent cell (iPSC) neuronal differentiation [28, 29]. The dCas9-DNMT3A complex was proved to be able to induce methylation at targeted CpG sites within multiple gene promoters. The highest methylation rate was estimated to be 50% [30]. The dCas9-TET complex has been shown to be able to rescue epigenetically silenced gene via inducing demethylation at the targeted region in a B2MtdTomato K-562 cell line. Transient expression of dCas9-TET and guide RNA together leads to a long-term reactivation effect highly specifically [31].

Efforts have been made to improve epigenome editing efficiency. The first strategy is incorporating SunTag into the dCas9-epieffector complex. SunTag is a repeating peptide array that can simultaneously bind with multiple copies of a certain protein. Several studies have highlighted the utility of the dCas9-SunTag system in improving transactivation robustness. For example, via recruiting multiple transactivation domains, the dCas9-SunTag complex has been proved to be able to augment a transactivation effect significantly compared to the conventional dCas9-single activator domain complex [32]. In another example, demethylation efficiency of the dCas9-SunTag-TET1 complex could reach as high as 90% in 4 out of 7 loci tested, resulting in 1.7- to 50-fold upregulation of targeted genes both in vitro and in vivo [33]. Moreover, SunTag could also be fused with the dCas9-DNMT3A complex to augment CpG methylation at targeted loci. Using the dCas9-SunTag-DNMT3A complex, researchers methylated a region of 4.5 kb residing the *Hoxa5*

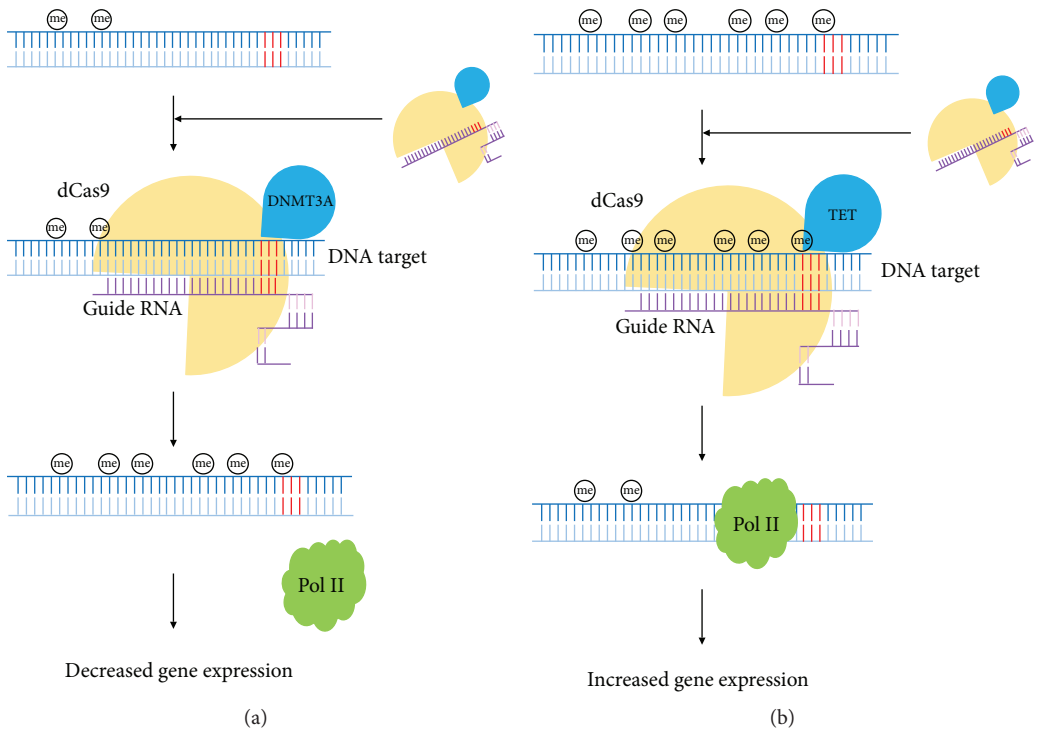

Figure 1: DNA methylation and demethylation mediated by Cas9. DNA sequences are represented by the blue lines. PAM sequences are highlighted by red color. Guide RNA sequences are shown in purple color. DNA polymerase II (PoI II) is represented by the green irregular shape. The circled "me" represents methylation at a specific CpG site. Basically, dCas9 functions as a DNA-binding protein. DNMT3A or TET is the epieffector. The dCas9-epieffector complex is guided to the DNA target by guide RNA via Watson-Crick base pairing to execute (a) DNA methylation or (b) demethylation, thus inducing decreased or increased gene expression.

gene with fairly low off-target to on-target ratio [34]. The second strategy is the CRISPR-SAM system developed by Feng Zhang Lab. SAM represents synergistic activation mediator. In this system, the tetraloop and stem loop2 are two RNA loops protruding outside the Cas9-guide RNA complex. RNA aptamers are added onto the two loops to recruit more activation domains [35]. Similar to the dCas9-SunTag-epieffector strategy, the CRISPR-SAM system could also be used to recruit epieffector domains to improve the efficiency of epigenome editing. Another strategy is to optimize the system components, which may be of more uncertainty. For instance, in primary mouse T cells, researchers did not stabilize *Foxp3* expression significantly using the dCas9-TET1 complex, although demethylation at the targeted enhancer region was induced successfully, while switching to the dCas9-p300 complex sustained *Foxp3* expression even under inflammatory condition by inducing acetylation at the targeted promoter region [36].

Briefly, the dCas9-epieffector complex could achieve methylation and demethylation at DNA level, rewriting histone marks by inducing methylation or acetylation at nucleosome level, and be optimized to improve the editing efficiency (Figures 1 and 2, Table 1).

1.2. Temporal and Spatial Control of Epieffectors. In a broad sense, epigenetics refers to the temporal and spatial control of gene expression, of which the total effect would determine cell phenotypes during development, aging, and disease pathogenesis [2, 37]. Thus, controlling gene expression with temporal and spatial precision is of great value. By inducing or repressing the expression of interested epigenetic regulators in iPSC models, differentiated cell lineages, and animal models, researchers may observe stage- and lineage-specific functions [14, 37, 38]. Generally, there are two inducing strategies, light and chemicals. Their combination with the CRISPR/Cas9 system is briefly summarized here.

Studies prior to the emergence of CRISPR have proved the utility of optogenetics in temporal control of gene expression. Basically, there need to be two components, a DNA-binding protein fusing to a light-sensitive cryptochrome protein, and a chromatin modifier fusing to a cryptochrome protein interaction partner, vice versa. [39, 40] When recruiting, the chromatin modifier to the targeted DNA sequence was controlled by blue light illumination, which would trigger conformation changes and rapid association between the cryptochrome protein and its interactor [1, 41, 42]. In the first example, the optically inducible dimerizing protein pair CRY2-CIB1 was fused to DNMT3A and telomere repeat binding factor-1 (TRF-1), respectively. The CRY2-DNMT3A fusion construct was the epigenetic rewriter, while the CIB1-TRF1 fusion construct was the target locator. Being shed with blue light, the CRY2-DNMT3A fusion construct would be targeted to the subtelomeric region to bind with the CIB1-TRF1 fusion construct and to execute locus-specific cytosine methylation. This system provided a method for understanding the role of subtelomeric methylation in telomere length

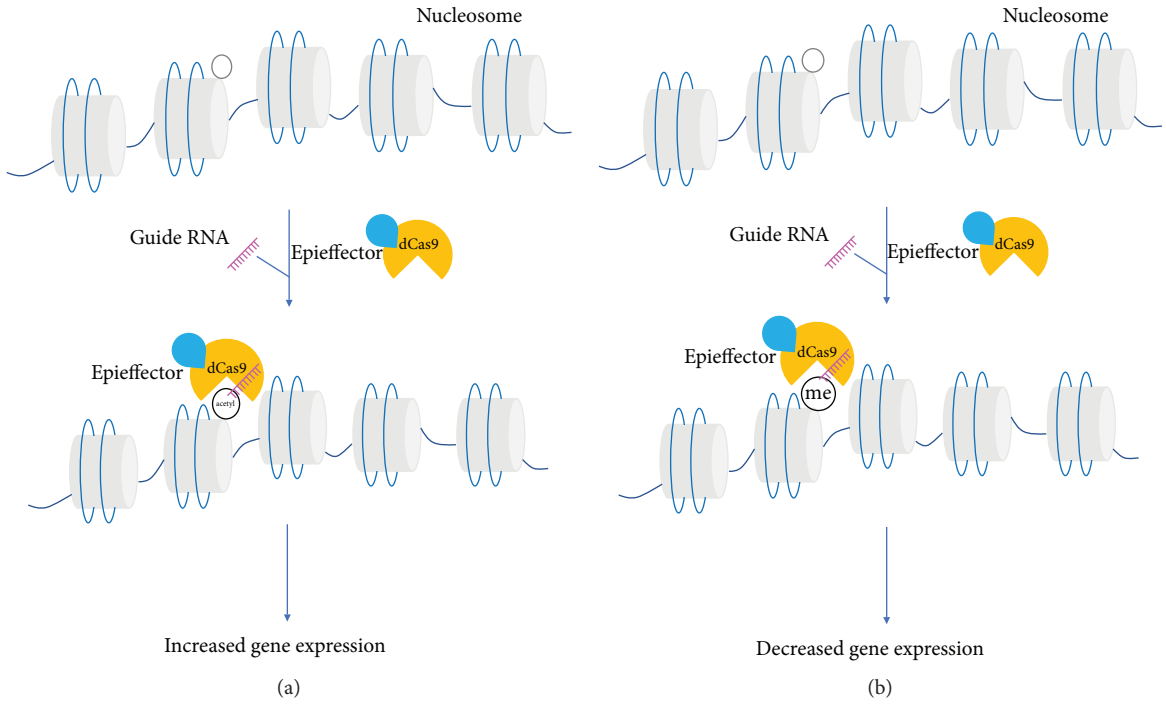

Figure 2: Histone acetylation and methylation mediated by Cas9. The empty circle attached to the nucleosome represents a specific amino acid of the histone side chain. The circled "acetyl" and "me" represents acetylation and methylation of amino acids, respectively. The dCas9-epieffector complex could be guided to a selected DNA target to achieve (a) acetylation or (b) methylation at histone level to regulate gene expression.

Table 1: Validated representative dCas9-epieffector complex.

Type	Mechanism	Function	Reference
dCas9-KRAB	Histone methylation	Gene downregulation	[21, 27]
dCas9-DNMT3A	DNA methylation		
dCas9-LSD1	Histone acetylation	Gene upregulation	[22–26, 28]
dCas9-p300	Histone acetylation		
dCas9-TET	DNA demethylation		
dCas9-VP64 or VPR	Recruitment of active transcription factors		
dCas9-SunTag-epieffector	Recruitment of multiple epieffector domains by the repeating peptide array SunTag	Effect augmentation	[29–32]
dCas9-SAM-epieffector	Recruitment of multiple epieffector domains by the protruding guide RNA loops		

maintenance [43]. The DNA-binding domain used in this experiment is TRF-1; it is reasonable to speculate that substitution of dCas9 with TRF-1 may achieve the same goal. Similarly, another study achieved site-specific epigenome editing at the *Ascl1* gene promoter using a CIB1-TALE fusion construct as the locus-specific binding complex. When DNMT3A catalytic domain was the rewriter, the *Ascl1* promoter was hypermethylated, resulting in decreased gene expression. When TET1 catalytic domain was the rewriter, the *Ascl1* promoter was hypomethylated, resulting in increased gene expression. By controlling the gene expression temporally, researchers were able to affect the differentiation preference of neural stem cells [44]. Moreover, the LITE system developed by Feng Zhang Lab was also a typical example of the optogenetic two hybrid systems. In this system, programmable TALE-DNA-binding domain was fused with CRY2 protein, while transcription activator domain VP64 or histone effector domain was fused with CIB1. Upon light stimulation, CRY2 would interact with CIB1, bringing effector domains to the genomic loci targeted by TALE to achieve transactivation or histone modification. With a suitable delivery method, this system could be applied both in vivo and in vitro [42].

By the same principle, the dCas9 DNA-binding domain paired with optical-inducible proteins could also be utilized to recruit the epieffector domain to the targeted DNA site in an inducible and reversible manner described above.

In a light-activated CRISPR/Cas9 effector (LACE) system, CRY2 and CIB1 were fused to transactivation domain VP64 and catalytically deactivated Cas9, respectively.

Cotransfection of these fusion protein pairs with guide RNA resulted in detectable levels of gene activation in the presence of blue light. Interestingly, when the N-terminal fragment of CIB1 was fused to both N- and C-terminus of dCas9, gene activation level in response to blue light was significantly increased, which is consistent with previous observation that simultaneous recruitment of VP64 domains to the target site had a synergistic effect on gene activation [45].

Another similar strategy from a different angle is to split Cas9 into fragments and further tether them with optical inducible protein pairs. Upon light stimulation, Cas9 fragments would be brought together via light-induced dimerization to reconstruct nuclease activity. Interestingly, in a photoactivatable CRISPR/Cas9 system, when split Cas9 fragments were fused with the CRY2-CIB1 pair, no light-induced Cas9 activity was induced. Given the steric block effect may be at play, a different pair of proteins with much smaller molecular weight called magnets was used. The nuclease activity was successfully reconstructed upon light stimulation when Cas9 fragments were tethered with positive and negative magnets, respectively [46]. In addition to the CRY2-CIB1protein pair and magnets, the split Cas9 could also be bound with other ligand-binding protein pairs, which would be brought together by different chemicals, to achieve multiplexed genome or epigenome regulation simultaneously and rapidly [47, 48].

As to the chemical-controlled strategy, the optimized inducible gene knockdown or knockout (OptiKO) system was a good example. It was a strategy harnessing the CRISPR/Cas9 technology and tetracycline-inducible expression cassette in human iPSC. The OptiKO system has two main components. Cas9 was constitutively expressed while guide RNA (gRNA) was tetracycline-inducible. Clonal lines incorporating both the CAG-Cas9 cassette and the TET-ON/CAG-gRNA cassette was first isolated. In the following experiments, the addition of tetracycline to the isolated clonal cells at different time points could achieve targeted region knockdown or knockout flexibly. In particular, inducible knockdown of the *DPY30* gene, an epigenetic modifier, during the iPSC differentiation process revealed that *DPY30* not only was indispensable for germ layer specification but also played dynamic roles in iPSC differentiation and maturation of specific lineages, which exemplified the utility of OptiKO in an epigenetic study [49].

Although more and more evidence is being reported regarding the feasibility of optical or chemical controllable epigenome editing, the detailed mechanisms remain to be elusive. Whether the local chromosomal or nucleosome context affects the binding affinity of the DNA-binding protein remains to be determined. What are the exact effects of locus-specific epigenome editing on DNA epigenetic profiling, histone modifications, and chromosomal interactions? What are the transcription factors participating in transcription activation or repression mediated by epigenome editing? Do we have the confidence to ensure every shot with unfailing accuracy? Such questions need to be answered to achieve reliable epigenome editing consequence and bona fide conclusions.

Elusive as the mechanism is, studies discussed above indeed enlightened us with new possible routes to perturb epigenome precisely. It is conceivable that through temporal and spatial control of genes playing indispensable roles in epigenetic dynamic changes, we may acquire new insights into the underlying mechanisms (Figure 3).

1.3. Noncoding RNA Manipulation. Noncoding RNA plays important roles in gene imprinting and chromatin remodeling; thus, it is an indispensable topic in the epigenetic research field [50, 51]. Accumulating evidence suggests that the CRISPR/Cas9 system is also a potential tool for studying noncoding RNAs [52, 53]. Firstly, CRISPR/Cas9 has been shown to be potential for manipulating noncoding RNA expression, including microRNA, long noncoding RNA, and miRNA families and clusters. For example, integrated Drosha and Dicer sites are essential for miRNA biogenesis. In a recent study, these two sites were used as the target cut sites of Cas9 nuclease in a cell model. The double-stranded breaks generated by Cas9 were further repaired by NHEJ mechanism. In this way, the integrity of the Drosha and Dicer sites was disturbed, which resulted in the downregulation of the corresponding miRNA and upregulation of genes regulated by them [54]. Another interesting example is using epigenome editing to aid a functional study of an imprinted gene, in which CRISPR/Cas9 was used to knock out a maternally expressed lncRNA gene, *Rian*, in mouse models. With a simultaneous delivery of multiple guide RNAs spanning the targeted region, the knockout rate could reach 33%. The *Rian*−/− mouse model facilitated functional studies of the gene knockout impact on nearby relevant gene expression in different tissues [55]. In addition, with multiple guide RNAs being delivered at the same time, CRISPR/Cas9 could be a scalable and multiplex system for miRNA family mutagenesis. The mutant rate could be as high as 90% [56]. Moreover, the CRISPR/Cas9 deletion-based method could be used to screen lncRNA involved in transcription regulation. In a recent research, researchers first generated a NF-kappa B reporter cell line constitutively expressing Cas9 nuclease. The activity of NF-Kappa cells was reflected by GFP signal via FACS. In the following steps, guide RNAs were delivered, along with Cas9, to achieve targeted lncRNA deletion. Changes in the GFP signal intensities and the levels of relevant proteins regulating or regulated by NF-kappa were further measured to determine whether the deleted lncRNA had a role in the interfered pathway. In this way, two lncRNAs, lincRNA-Cox2 and lincRNA-AK170409, were identified as potential negative regulators of NF-kappa expression in macrophage immune functions [57]. Conversely, noncoding RNA within cells could also be used to modulate Cas9 activity. A miRNA responsive CRISPR/Cas9 system was developed recently. Sequences complementary to a specific type of miRNA were incorporated into the 5′-UTR of the gene encoding Cas9, rendering the CRISPR/Cas9 system only switch-on in cells without such miRNA expression [58].

Most recently, Feng Zhang Lab developed a RNA editing tool, named as the RNA Editing for Programmable A to I Replacement (REPAIR) system. It was adapted from the type IV CRISPR/Cas13 system, where Cas13b was deactivated for

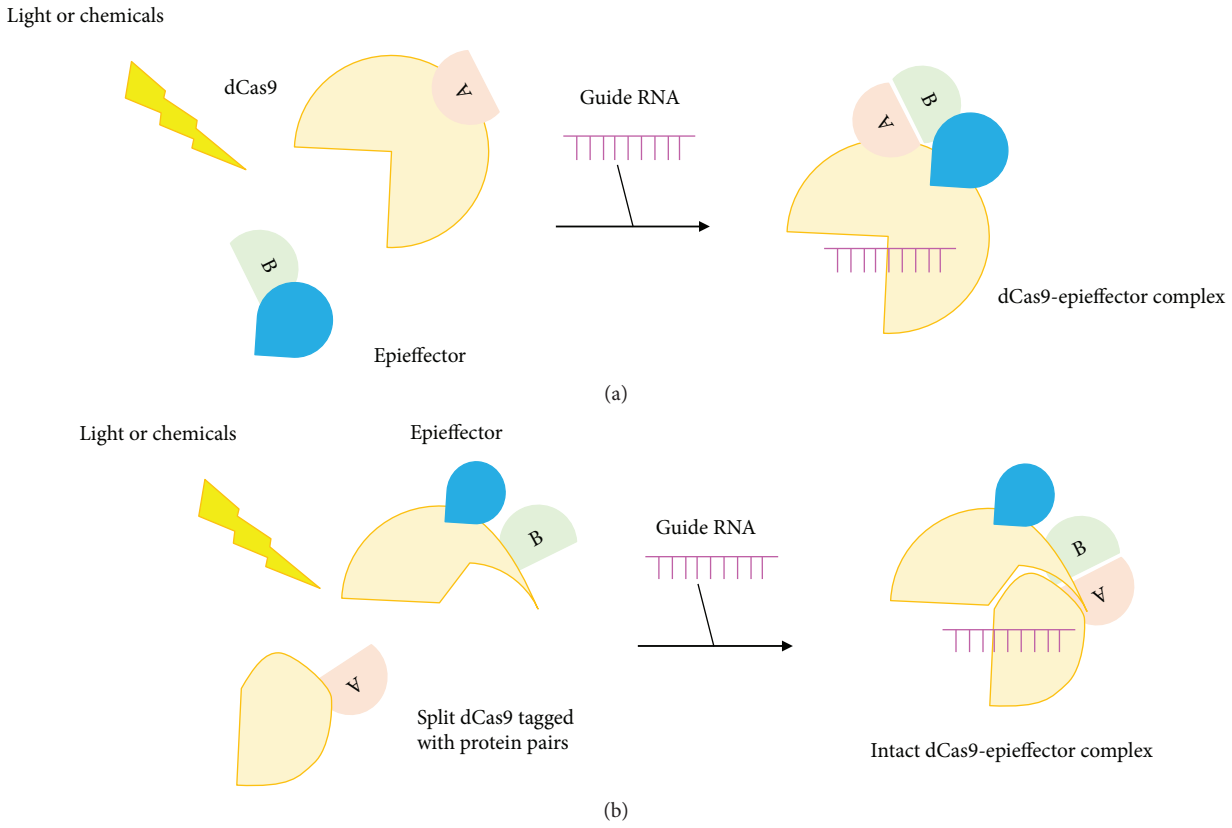

Figure 3: Schematic of temporal and spatial control of epigenome editing. The semicircles labeled with (A) and (B) represent a protein pair. The magenta comb-like lines represent guide RNA sequences. (A) is bound to dCas9, which would be directed by the guide RNA to the DNA target. (B) is bound to the epieffector. (a) Upon stimulation of light or chemicals, (A and B) would pair with each other thus bringing the dCas9 and epieffector together to achieve site-specific epigenome editing at a given time point. (b) Another strategy is splitting the dCas9 into two parts, each of which is bound by protein (A) or (B). Upon light or chemical stimulation, (A) and (B) would gather together to reconstruct an intact dCas9-epieffector complex to achieve site-specific epigenome editing at a given time point.

its RNA cleavage capacity while the RNA-binding ability was reserved. Deactivated Cas13b was further fused with an adenosine deaminase enzyme called ADAR. After being directed to the targeted RNA transcript, the Cas13b-ADAR complex would deaminize adenosine to inosine, which is an analog to guanine in diverse biological processes, resulting in A to I conversion. Although this CRISPR/Cas13-based RNA editing tool seemed to have no overwhelming advantage over the CRISPR/Cas9-based DNA editing method in terms of specificity due to a substantial number of off-target events across the transcriptome, it still presented some promising merits. For example, no PAM sequence constraints within the RNA target meant more flexible target binding capacity of Cas13b compared to the classical Cas9. Direct conversion of RNA base without reliance on endogenous repair mechanism also rendered it applicable in mitotic cells such as neurons. Additionally, the transient nature of RNA editing may provide a basis for the temporal control of targeted RNA transcription and downstream translation [59].

Altogether, it seems that Cas9 instead of dCas9 is playing important roles in noncoding RNA manipulation, as more and more Cas proteins are being characterized, such as cpf1 [60, 61]. It may be likely that the design idea for CRISPR/Cas9 could also be expanded to other Cas protein types. We should keep learning from and trying to borrow wisdom from the world of microbes.

1.4. Characterization of Chromatin Structures and Interactions. DNA wraps around histones to form nucleosomes. Nucleosomes continue to assemble in a spiral pattern. Through several rounds of such repeated twisting, DNA and protein are finally packaged into the superhelix structure, chromatin. Chromatin has two statuses, the tightly packed heterochromatin and the loosely packed euchromatin [1, 62]. The former is inaccessible to transcription or translation, while the latter is transcriptionally or translationally active. Switch between these two conformations and chromatin-chromatin interactions play an important role in gene regulation [63]. The novel methods investigating chromatin structures and interactions presented by the emergence of the CRISPR/Cas9 technology are briefly summarized here.

Live cell imaging is a valuable method for studying structure changes and interactions of chromatin in epigenetic regulation. Efforts have been made to adapt the CRISPR/Cas9 system for the in vivo imaging experiments. Generally, the design rationale is tagging the guide RNA loops or the dCas9 protein with fluorescent dyes to form a dCas9-gRNA-

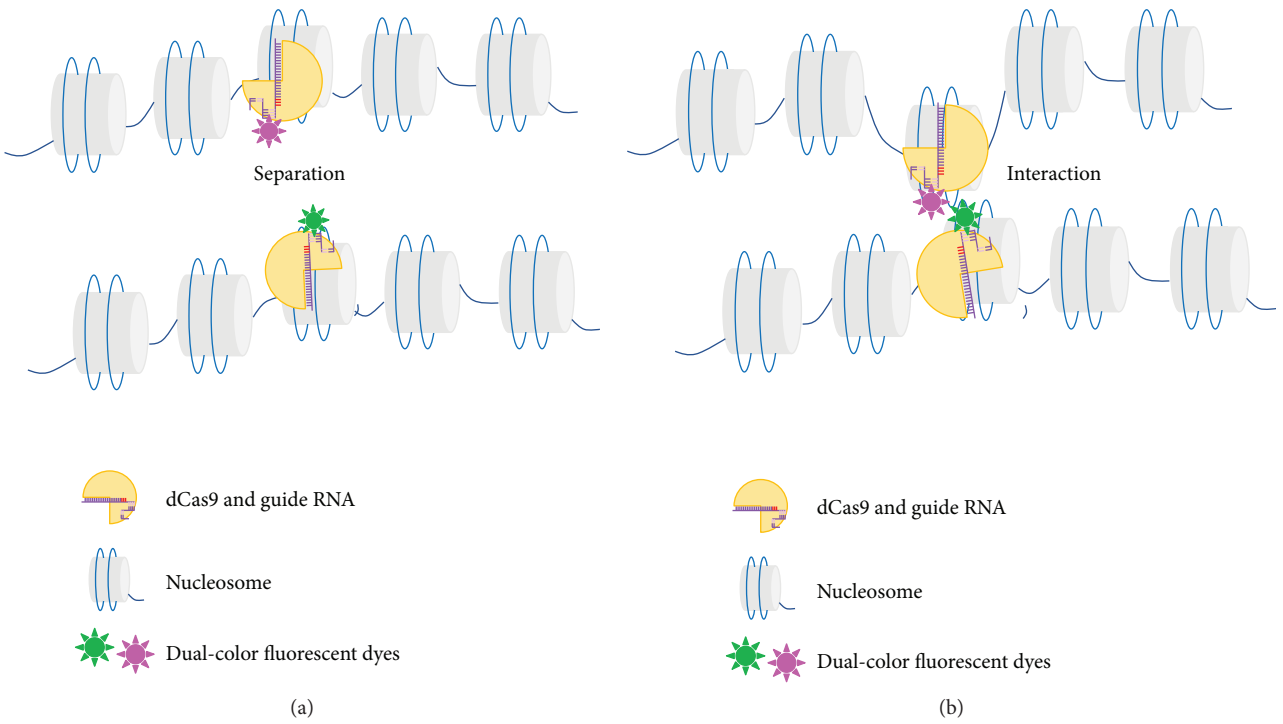

Figure 4: In vivo imaging of chromatin interaction mediated by Cas9. Guide RNAs direct dCas9 and fluorescent proteins to bind with selected DNA targets, forming the dCas9-gRNA-fluorescent dye complex. At chromosomal level, spatial position changes between chromosomal regions could be reflected by the (a) seperation or (b) interaction of the dual-color fluorescent signals.

fluorescent dye complex. In a recent study, a dCas9 expression cassette was first incorporated into cells of interest. Guide RNA scaffold was redesigned, with two new stem loops protruded. One of the loops was tethered by protein MS2; the other was bound by protein PP7, which were able to recruit EGFP-conjugated MCP and mCherry-conjugated PCP, respectively. Clonal cells expressing dCas9, EGFP, and mCherry were selected as the tested platform. The delivery of guide RNAs into these cells would bring dCas9 and fluorescent proteins together at targeted loci. Colocalization and separation of the two-color fluorescent signals indicated possible interactions between the targeted loci on chromosomal level. Using this dual-color system, researchers achieved real-time labeling of major satellites, minor satellites, and two single loci on chromosomes [64]. In another study, fluorescent proteins were tagged with dCas9 to achieve dual-color imaging in live human RPE cells. Researchers first isolated a cell line expressing dCas9-EGFP and dCas9-mCherry stably. Next guide RNAs were delivered to lead dCas9-fluorescent dye complex to the target, giving out observable fluorescent signals [65] (Figure 4). Intriguingly, based on similar design principles, an earlier study has explored the possibility of CRISPR/Cas9-mediated multicolor imaging in live human cells and successfully utilized this method to measure the distance between loci on the same or different chromosomes [66]. Moreover, in living cells, the CRISPR/Cas9 system has also been optimized for visualization of repetitive and nonrepetitive genomic sequences, to monitor telomere length dynamically and to track MUC4 locus variations in the mitosis process. Stably and inducible expression of dCas9-EGFP in the cell line being tested was a prerequisite. Delivering guide RNA into these cells at different stages would give out observable fluorescent signals, to some extent reflecting the length and conformational structure of the targeted genomic loci. Repetitive sequences need only one type of guide RNA, while nonrepetitive sequences need a bunch of guide RNAs spanning the target region to achieve enrichment of fluorescent signal at the targeted loci [67].

In addition to live cell imaging, CRISPR/Cas9 could also be used to characterize locus-specific regulatory composition. In a recent study, the deactivated cas9 nuclease was biotinylated to capture telomeric factors and components of enhancers regulating human β-globin gene expression. Chromatin interactions were isolated at high resolution, unbiased analysis of which suggested spatial features that may be involved in transcription regulation [68]. Another example is the engineered DNA-binding molecule-mediated chromatin immunoprecipitation (enChIP) technique, which could be comprehended as an adapted version of the conventional ChIP-seq method. dCas9 and guide RNA here were used to locate an interested genomic region. Next, cell lysis was crosslinked and sonicated to yield chromatin fragments, the dCas9-gRNA tagged of which was pulled down for further RNA-seq or mass spectrometry analysis [69, 70].

Chromatin structures and interactions are gaining increasing academic attention in recent years. It has been widely accepted that the dynamic chromatin changes play pivotal roles in the temporal-spatial regulation of genes.

The in vivo imaging technique and several other attempts described above indicated that CRISPR/Cas9 is a promising research tool for this topic.

1.5. Epigenetic Element Screening. The accumulation of work has demonstrated the capacity of CRISPR/Cas9 for screening both coding and noncoding DNA sequences on a genome-wide scale [71]. Although the amount of evidence focusing on epigenetic screening approach is far less, efforts have been made to prove that CRISPR/Cas9-mediated epigenome editing is a potential method for enhancer screening and annotation [37]. For example, a recent study developed a CRISPR/Cas9-based epigenetic regulatory element screening system, abbreviated as CERES, for regulatory element annotation in the native chromosomal background. Firstly, cells constitutively expressing dCas9-KRAB repressor or dCas9-p300 activator were, respectively, transduced with gRNA libraries targeting regions containing putative regulatory elements surrounding the gene of interest for loss-of-function or gain-of-function screens. Next, given the interested gene was a transmembrane protein, FACS was used to sort cells for increased and decreased gene expression. Finally, guide RNA enrichment analysis was used to identify functional enhancers [72]. Albeit publications on this topic are being few at present, it is still believable that the CRISPR/Cas9-mediated epigenome editing has the potential to pave the way for studying putative regulatory elements in their native chromosomal background in a high-throughput manner.

1.6. Potential Clinical Applications. By transcriptionally activating or deactivating a specific gene that is normally silent or active, epigenome editing exhibited therapeutic potentials. It is easy to imagine that diseases with aberrant epigenetic marks as the underlying pathophysiologic mechanisms would be excellent targets to test epigenome editing-based therapeutics. The disease spectrum may include cancers, neurodegenerative diseases, neuropsychiatric diseases, neurodevelopmental diseases, and imprinting disorders. For example, using a dCas9-SAM-VPR complex, researchers reactivated a heavily methylated tumor suppressor gene, *Maspin*, in lung cancer cells both transcriptionally and translationally, which limited cancer cell growth and apoptosis [73]. Another interesting example is that the delivery of ZFN-VP64 complex into mouse models of Alzheimer's disease could rescue the memory deficit phenotype by upregulating a major synaptic protein PSD95 [74]. Although the DNA-binding domain used in this study was ZFN, it still validated the possibility of dCas9-mediated epigenome editing for treating neurodegenerative disorders to some extent. As to the neurodevelopmental diseases, epigenome editing theoretically is a potential therapeutic strategy worth a try, especially for diseases caused by aberrant imprinting patterns without underlying genetic mutations, such as the Angelman syndrome, where hypomethylated imprinting center of the maternal chromosome led to epigenetic silencing of the *Ube3a* gene on both alleles [75]. With a suitable dCas9-epieffector complex, the imprinting center may be remethylated to correct the abnormal imprinting pattern and thus reactivate the *Ube3a* gene epigenetically. Other representative candidates may include the Rett syndrome, Huntington's disease, and Friedreich ataxia, where dCas9-epieffector complex may be harnessed to restore or repress the responsible gene to cure the disease [12, 76, 77].

In addition, epigenome editing has the potential to facilitate stem cell therapy. In recent years, iPSCs have been a major source for cell replacement therapy due to its accessibility and pluripotency [78–82]. iPSCs experience active and delicate epigenome reset to become differentiated cells, and vice versa [83]. By changing the epigenome landscape at a desired time point or cell stage, epigenome editing may be utilized to improve the reprogramming efficiency or to confine the differentiation direction to yield more pure target cell populations for cell replacement therapy [84, 85]. Moreover, epigenome editing provides a new route for the transdifferentiation process. For example, using the CRISPR-SAM system to target and activate pdx1, a gene essential to pancreatic development, researchers successfully transdifferentiate liver cells into insulin-producing pancreatic cells to treat diabetes mellitus in mouse models [86]. Last but not least, epigenome editing holds a great promise for the in vivo reprogramming process. In vivo overexpression of the Yamanaka factors have proven to be able to fully or partially help somatic cells to regain pluripotency in situ. These rejuvenated cells would subsequently differentiate again to replace the lost cell types [87–89]. Since the reprogramming process essentially is the reset of epigenome, it is fair to envision that epigenome editing designed to reset the soma epigenome to a naïve state may be a more controllable and precise method for in vivo reprogramming.

Taken together, these studies demonstrated the therapeutic potential of CRISPR/Cas9-mediated epigenome editing in various diseases and stem cell therapy.

2. Concluding Remarks and Future Perspectives

In conclusion, all of the studies described above indicated that CRISPR/Cas9-mediated epigenome editing holds a great promise for epigenetic studies and therapeutics. However, there are still some limitations to be scrutinized. First of all, in terms of basic science studies, although most studies claimed high specificity in their experiments, however, the high specificity usually is the result of repeated optimization. A precise model that could predict deleterious off-target effects during the experiment design stage is still lacking. In addition, although transactivation or repression effects on multiple genes were well documented in publications, mechanisms underlying the phenomenon were not clear. Epigenetic mark profiling on epigenome scale was not sufficient. Local CHIP-seq data usually only focused on the characterization of one or few histone marks. Theoretically, we hope that epigenome editing could achieve targeted gene regulation by changing epigenetic marks specifically and freely according to our wills. To achieve this goal, high specificity and clarified mechanisms are the prerequisite. Therefore, more thorough off-target event assessments and more studies focusing on mechanisms underlying epigenome editing are needed.

Moreover, in terms of clinical applications, several issues need to be addressed prior to successful clinical translation. Firstly, the endurance of gene activation or a repression effect mediated by CRISPR/Cas9 remains to be undetermined. It has been thought that epigenome-editing-induced gene activation or repression is short-term [90, 91]. On the contrary, there was also evidence showing that a gene silencing effect mediated by the hit-and-run epigenome editing strategy could also be long-term and inheritable [31]. A short-term effect is more suitable for antagonizing acute pathogenic factor exposure and the transdifferentiation process. However, for treating chronic diseases, a long-term effect is expected. Additionally, a safer and efficient delivery method should be developed [92]. Adeno-associated virus (AAV) vectors have been the prevailing delivery method for some time. By tagging a synthetic surface peptide, splitting the Cas9 protein or using its smaller orthologues, and choosing a suitable administration route, researchers significantly improved the packaging capacity and delivery efficiency of AAV vectors [93–96]. However, for clinical applications, more optimization is required. The immunogenicity of AAV vectors, dCas9 proteins, and guide RNAs should be determined precisely. The off-target effects of AAV vectors or dCas9-epieffector complex should be minimized as much as possible to ensure clinical safety [97].

Conflicts of Interest

The authors have declared that there is no conflict of interest.

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Review Article

Epigenetic Regulations in Neural Stem Cells and Neurological Diseases

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Among the regulatory mechanisms of the renewal and differentiation of neural stem cells, recent evidences support that epigenetic modifications such as DNA methylation, histone modification, and noncoding RNAs play critical roles in the regulation on the proliferation and differentiation of neural stem cells. In this review, we discussed recent advances of DNA modifications on the regulative mechanisms of neural stem cells. Among these epigenetic modifications, DNA 5-hydroxymethylcytosine (5hmC) modification is emerging as an important modulator on the proliferation and differentiation of neural stem cells. At the same time, Ten-eleven translocation (Tet) methylcytosine dioxygenases, the rate-limiting enzyme for the 5-hydroxymethylation reaction from 5-methylcytosine to 5-hydroxymethylcytosine, play a critical role in the tumorigenesis and the proliferation and differentiation of stem cells. The functions of 5hmC and TET proteins on neural stem cells and their roles in neurological diseases are discussed.

1. Introduction

Human beings are developed from a fertilized egg into a complete individual; during the whole process, a series of precise regulations on the development are included, such as gene expression and gene silence [1], transcriptional regulation [2], posttranscriptional regulation [3], hormone regulation [4], chromosome behavior regulation [5], and apoptosis [6]. For these different regulative pathways, their target cells are embryonic stem cells (ESCs). ESCs are totipotent stem cells that had a capability to proliferate and differentiate into appropriate lineages to form specialized cells and organs and play a central role in the developmental process [7]. Due to the powerful plasticity and potential of ESCs as a high potential cell replacement therapy for many diseases, stem cells are considered to have an appreciable translational prospect in the field of regenerative medicine [8]. Except for ESCs at the embryonic stage of the development, adult stem cells (ASCs) exist in different tissues at the adult stage of the development [9]. ASCs are often in a resting state in individuals and exhibit different potentials of regeneration and differentiation under pathological conditions or special incentives. Reynolds and Weiss first found that the neurons isolated from the striatum of the adult mouse brain could proliferate and differentiate in vitro with epidermal growth factors [9], indicating the existence of neural stem cells (NSCs) in the mature nervous system. They also demonstrated that NSC has the ability to self-renew and differentiate into other types of cells like neurons, astrocytes, and oligodendrocytes under many conditions such as growth factors, neurotransmitters, hormones, injury, or environmental factors [9]. However, the renewal and differentiation ability of NSC is limited; in the process of aging or pathological conditions, neuronal cell loss is much more than newly generated neurons and glial cells from NSCs, resulting in different neurological disorders including Alzheimer's disease [10], Parkinson's disease [11], Huntington's disease [12], neuroendocrine tumors [13], and ataxia [14]. Therefore, the regulation on the renewal and differentiation of NSCs or NSC transplantation therapy are considered an important

therapeutic strategy for the treatment of these neurodegenerative diseases.

Among the regulatory mechanisms of the renewal and differentiation of NSCs, epigenetic modification plays a critical role in monitoring the phase transition during individual development, maintaining the directional differentiation of stem cells, regulating the proliferation of specific cells, and controlling the process of differentiation [15, 16]. For example, in the process of umbilical cord mesenchymal stem cells (UMSCs) being differentiated to neural stem-like cells (uNSCLs), E1A-like inhibitor of differentiation 3 (EID3), an important member of EID gene family that has the main function of p300/CBP inhibitors (a transcriptional coactivator) in response to cell transformation, growth arrest, or cell apoptosis, directly interacts with DNMT3A, a DNA methyltransferase (DNMT) for DNA methylation, suggesting that DNA methylation may be involved the regulation of transdifferentiating from UMSCs to uNSCLs as a key mechanism in epigenetic regulation of stem cell reprogramming [17]. So far, epigenetic modification is a hot topic in recent years. Except for DNA methylation, histone modification, micro-RNA, chromatin remodeling, and other epigenetic modification are found to play important roles in the regulation of stem cells [18]. In this article, we will review the recent advances of different epigenetic modifications on NSCs, but mainly focus on the role of 5hmC as a new player in the regulation of the renewal and differentiation of ESCs or NSCs.

2. Recent Advances on Epigenetic Regulation on Stem Cells

It is strongly believed that the basis of cell differentiation in ontogeny is based on the regulation of intracellular factors, while environmental factors also play a role as a main cause [19]. Epigenetic modifications including methylation, acetylation, ubiquitination, and phosphorylation on DNA, RNA, or proteins mediate the interaction between the environment and the organism [20]. Interestingly, recent evidences demonstrate that epigenetic modification changes can be inherited to the next generation [21]. Here, we present a brief overview of current advances on epigenetic modifications and NSCs.

2.1. DNA Methylation. The increasing evidences demonstrate that DNA methylation is involved in the proliferation and differentiation of stem cells [22]. DNA methylation prevents transcriptional factors from binding to promoters, such as Oct4 and Nanog, thereby limiting gene expression [23]. The process of DNA methylation is catalyzed by DNA methyltransferase, mainly DNMT1, DNMT3A, and DNMT3B. DNMT3 enzyme is a de novo methyltransferase [24] and DNMT1 is mainly involved in the maintaining of DNA methylation in dividing somatic cells [25]. The deletion of DNMT3A in hematopoietic stem cells impaired the differentiation of transplanted hematopoietic stem cells and increased the level of hematopoietic stem cells in the bone marrow [22]. In skeletal muscle stem cells, the DNA methylation of CpG dinucleotide in the promoter or enhancer region reduces gene expression of Pax7 and MyoD [26]. Similarly, Uhrf1 (ubiquitin-like PHD ring finger-1; also known as Np95) mainly interacts with DNMT1 to maintain DNA methylation in NSCs; the deletion of Uhrf1 in NSCs leads to increase the global DNA methylation and delayed neurodegeneration [27]. Recent evidences showed that Methyl CpG binding domain protein 1 (MBD1) is expressed in neural stem cells (aNSCs) of dentate gyrus of the adult hippocampus and maintains the integrity and stemness of NSC by inhibiting differentiation [28]. MBD1 and Methyl CpG binding protein 2 (Mecp2) belong to the methyl-CpG-binding protein family and play a key role to link DNA methylation and transcriptional regulation on differentiation genes [29]. MBD1 deficiency leads to the accumulation of undifferentiated NSCs and impaired transition into the neuronal lineage [28]. DNA methylation is closely related to stem cell-related diseases. A recent study found that there are a large number of gene mutations of DNMT3A in acute myeloid leukemia which is a malignant tumor characterized by clonal stem cell proliferation and aberrant block in differentiation [30]. Fetal alcohol syndrome showed that alcohol exposure to cultured NSCs altered normal DNA methylation programming of key neural stem cell genes and retarded NSC migration and differentiation [31], supporting the role of aberrant patterns of DNA methylation in fetal neural development after embryonic alcohol exposure.

2.2. Histone Modification. Histone modification refers to the process of histone methylation, acetylation, phosphorylation, polyadenylation, ubiquitination, and ADP glycosylation under the action of related enzymes. Histone-mediated epigenetic gene silencing is to remove acetyl groups from histone tails catalyzed by histone deacetylase (HDAC) enzymes and enhance the binding of histones to DNA and the aggregation of chromosomes, preventing transcription factors into the regulatory region [32]. HDAC1 is highly expressed in the oligodendrocyte differentiation period of the corpus callosum; HDAC inhibitors blocked oligodendrocyte differentiation and cause demyelination in the corpus callosum of postnatal rats [33]. The recent study indicated that the Arf-p53 axis also might be involved in the regulation of histone acetylation on the proliferation and senescence of the neurospheres [34].

Histone demethylation is also an important histone modification. It has two families, LSD1 (Lysine-specific demethylase 1) and JmjC (a domain), to regulate the proliferation and differentiation of stem cells. Inhibiting the activity of LSD1 or knockdown of LSD1 expression leads to the decreased proliferation of neural stem cells [35]. In addition, LSD1 plays a crucial role in maintaining the silencing of several developmental genes in human embryonic stem cells by regulating the balance between H3K4 (lysine 4 on histone H3 protein) and H3K27 methylation in its regulatory region [36]. Thus, histone modifications play a role in inducing NSC differentiation into neurons and glial lineages, but the mechanisms are still not clear.

2.3. Noncoding RNA. Noncoding RNAs (ncRNAs) are a class of RNA molecules that have no ability to translate into proteins but function as regulatory factors at transcriptional or

posttranscriptional levels, including ribosomal RNAs (rRNAs), microRNAs (miRNAs), piwi-interacting RNAs (piRNAs), long noncoding RNAs (lncRNAs), and others [37]. These ncRNAs have shown to play distinct but also conserved roles in regulation of differentiation of NSCs [38–40]. Among different ncRNAs, current evidences demonstrate that miRNAs play critical roles in the regulation of differentiation of NSCs. miRNAs are a group of small RNA molecules of 20–24 nucleotides widely found in eukaryotes. They bind to target mRNAs to regulate their gene expression by promoting the degradation of target mRNAs. Similarly, microRNA is also involved in the regulation of NSC differentiation and proliferation dynamic homeostasis; for example, high levels of miR-184, which are inhibited by methyl-CpG binding protein 1, promote stem cell proliferation but inhibit adult neural stem/progenitor cell (aNSCs) differentiation [41]. MiR-145 directly regulates Nurr1 (a nuclear receptor) expression level, and overexpression of miR-145 inhibits the differentiation effect of BMP2; knockdown of miR-145 promoted the upregulation of Nurr1, resulting in the differentiation of NSCs into dopaminergic neurons [42]. MicroRNA can regulate many factors such as CT4, SOX2, and KLF4 in embryonic stem cells that are the direct targets of miR-145. The deletion of miR-145 increases the expression of OCT4, SOX2, and KLF4 and further inhibits the differentiation of NSC [43]. Recent studies showed that aging process begin when hypothalamic stem cells that coexpress Sox2 and Bmi1 are ablated accompanying with substantial loss of hypothalamic cells; the injection of exosomal miRNA in the cerebrospinal fluid, greatly prevented the cell aging process [44]. Therefore, more and more evidences showed that ncRNAs like miRNA are involved in the regulation of differentiation of NSCs.

3. Ten-Eleven Translocation (Tet) Proteins-5hmC Modification-Related Enzymes

Tet family proteins are a group of α-ketoglutarate (α-KG) and Fe2+ -dependent monooxygenase to catalyze the conversion of 5mC to 5hmC, concluding Tet1, Tet2, and Tet2 [45]. In 2009, Tahiliani et al. found that Tet1 catalyzes the reaction of 5mC to 5hmC [46]; thereafter, Tet2 and Tet3 have been found to have similar catalytic activity [47]. Although the main functions of the three enzymes are to oxidize 5mC to 5hmC, the distribution of the enzymes is different. The expression of Tet1 protein in embryonic stem cells and nervous system is high [48–50]; Tet2 is widely distributed and relatively high in hematopoietic system; Tet3 is mainly expressed in colon, muscle tissues, and less in brain tissues [51]. The three Tet enzymes contain a structurally similar carboxyl terminal catalytic region, which catalyzes the synthesis of 5hmC activity [45]. The catalytic domain of Tet proteins has 3 metal ion (Fe2+) and α-KG binding site to enhance its catalytic activity [46]. Tet1 and Tet3 have an amino terminal CXXC zinc finger protein domain, whereas Tet2 lacks this structure and needs to be assisted by IDAX protein with similar functions [52]. The CXXC domain protein of Tet2 is encoded by a distinct gene IDAX. The IDAX CXXC domain binds DNA sequences containing unmethylated CpG dinucleotides, localizes to promoters and CpG islands in genomic DNA, and interacts directly with the catalytic domain of Tet2 [52]. IDAX (also known as CXXC4), a reported inhibitor of Wnt signaling, regulates Tet2 protein expression [53]. Unexpectedly, IDAX expression results in caspase activation and Tet2 protein downregulation in a manner that depends on DNA binding through the IDAX CXXC domain, suggesting that IDAX recruits Tet2 to DNA before degradation [52]. Notably, the IDAX-related protein CXXC5 resembles IDAX in inhibiting Wnt signaling [54]. Therefore, the distribution and structure of Tet enzymes determine the distribution of 5hmC modifications in brain and their different roles in different diseases. Tet1 knockout mice showed impaired hippocampal neurogenesis resulting in learning and memory deficiency [55]. Tet2 functional disruption or knockout influences hematopoietic cell homeostasis and hematopoietic differentiation and promotes the development of myeloid malignancies [56]. Although either Tet1 or tet2 knockout mice are viable and fertile, Tet3 knockout mice are perinatally lethal [51]. These demonstrate the different roles of Tet proteins in the different tissues and in the devolvement of different organs. The functions of Tet proteins and its related phenotypes in rodent animals and diseases in human are summarized in Table 1.

5-Hydroxymethylcytosine (5hmC), the oxidative product of 5mC, was found in mammals with surprisingly high abundance in 2009 [46, 57]. Recent studies showed that 5mC is not the final chemical steps for gene silencing; Tet protein-associated DNA demethylation can transform 5-methyl cytosine (5mC) into 5-hydroxymethycytosine (5hmC), 5-formylcytosine (5fC), and 5-carbosycytosine (5caC), but 5fC and 5caC is much less than 5-hydroxymethylcytosine [58, 59]. Interestingly, for individual tissues, the levels of 5hmC, 5fC, and 5caC were not significantly related; for example, although 5hmC is more abundant in mouse brains than in ESCs, the levels of 5fC and 5caC are less abundant [45]. 5fC and 5caC can be further removed by the base excision repair (BER) pathway and thymine-DNA glycosylase [60]. This pattern suggests that the different steps of demethylation cycle are different in different tissues [61]. In addition, Tet protein overexpression or depletion can increase or decrease the content of 5hmC, 5fC, and 5caC in the genome [61]. The discovery of Tet proteins speeds the exploration of the functions of 5hmC [46, 57]. Because of its important functions, 5hmC in DNA has been considered as the sixth base. More evidences show that demethylation by 5hmC regulates the proliferation of NSCs and neurogenesis [27]. Therefore, we further discuss the regulation of 5hmC on NSCs and related neurological diseases.

4. Tet Proteins and DNA 5hmC Modifications Are Involved in the Regulation on the Proliferation and Differentiation of NSCs

The direction of cell differentiation is determined by the specific expression of tissue-specific genes, while DNA 5mC is involved in the regulation of gene expression and

Table 1: Tet proteins and their functions.

Genes	Distribution	Structure	Functions of Tet enzymes Knockout phenotypes in rodents	Related diseases in humans
Tet1	Mainly in ESCs and nervous system [48].	Contains CXXC, Cys-rich, and DSBH domains	(1) Abnormal hippocampal neurogenesis, with learning and memory fading [55]. (2) Antidepressive phenotypes [94] (3) Skews differentiation towards extraembryonic lineages in the teratoma [99].	(1) Acute leukemia [100]. (2) Gastric cancer ([101, 102], Deng, [103]). (3) Breast cancer [104].
Tet2	Widely distributed and high in hematopoietic system [48].	Contains Cys-rich and DSBH domains without CXXC domain	(1) Hematopoietic cell homeostasis and hematopoietic differentiation impairment, myeloid malignancies [56]. (2) Retinal neurons developmental failure in zebrafish [105].	(1) Polycythemia vera [106, 107]. (2) Primary myelofibrosis [107]. (3) Myelodysplastic syndrome [106]. (4) Myeloproliferative neoplasm [108]. (5) Melanoma [109].
Tet3	Mainly in colon and muscle tissues, less in brain tissue [51].	Contains CXXC, Cys-rich, and DSBH domains	(1) Developmental failure [72] and embryonic sublethality [110]. (2) Impaired differentiation and increased apoptosis [72]. (3) Fear extinction impairments in mice [111]. (4) Abnormal morphogenesis of retinal neurons in zebrafish [105]. (5) Abnormal neural differentiation and skewed toward cardiac mesodermal fate in mouse ESC [112].	NA
	Tet1/2 DKO		Embryonic stage death and little normal growth [113]	NA
	Tet1/3 DKO		(1) Dendritic arborization inhibition in mice [114] (2) Holoprosencephaly [115].	NA
	Tet1/2/3 TKO		Developmental disorders [116]	NA

CXXC: Cys-X-X-Cys domain; DSBH: double-stranded beta helix; DKO: double knockout; ESCs: embryonic stem cells; TKO: triple knockout; NA: not available.

differentiation of cells in a specific direction [62–64]. Previous studies have shown that about 1.4% of CpG islands undergo a significant remethylation process during the differentiation of embryonic stem cells into NSCs and NPCs [65]. The increasing line of evidences indicate that 5mC directly inhibits transcription factors to bind to DNA [23] or recruits MeCP2 and MBD to form a complex and further prevent gene transcriptions that relate to the differentiation of NSCs [66]. Therefore, DNA methylation plays an important role in neural cell differentiation. Apparently, as an important demethylation mechanism, DNA 5hmC modification and Tet enzymes can be involved in the regulations of NSCs in theory.

Recently, 5hmC has been found in the mammalian genome and has been shown to be about 10 times more abundant in neurons than in some peripheral nervous tissues [67]. This suggests that 5hmC may be a stable epigenetic marker involved in cell specific mechanisms to achieve its function in the brain. More and more evidences demonstrated that Tet enzymes and Tet-mediated 5hmC modifications are involved in the proliferation and differentiation of ESCs and NSCs [29, 58, 68, 69]. Hahn et al. found that the increase of 5hmC modification in gene bodies is associated with genes important for neuronal functions during neuronal differentiation in mouse brain regions; however, gene activation for neuronal differentiation is not related to substantial DNA demethylation [69]. At the same time, overexpression of Tet2 and Tet3 also promotes the progression of neuronal differentiation [69]. Similarly, in Sirt6-knockout ESCs, the expression of Oct4, Sox2, and Nanog (the downstream of Sirt6) is inhibited and the upregulation of Tet enzymes and the significant increase of DNA 5hmC are found, resulting in ESC skewed development towards neuroectoderm [68]. This suggests that Sirt6-regulated ESC differentiation is in a Tet enzyme and 5hmC-dependent manner [68], supporting Hahn et al.'s results. A recent study further demonstrates that 5hmC dynamics is correlated with the differentiation of aNSCs; however, Tet2 primarily contributes to 5hmC acquisition during the differentiation of aNSCs [58]. Therefore, these evidences support the critical role of 5hmC modifications in the differentiation of NSCs.

Tet proteins, as the important enzymes for the conversion of 5mC to 5hmC, also showed their functions on the proliferation/differentiation of NSCs. Tet1 depletion impairs hippocampal neurogenesis accompanied with poor learning and memory in mice; at the same time, Tet1 deficiency results in reduced neural progenitor pool in adult subgranular zone [55]. These results provided in vivo evidences that

Tet1 deficiency in the central nervous system decrease the proliferation of adult NSCs in the hippocampal dentate gyrus. Moran-Crusio et al. showed that the depletion of Tet2 stimulates aNSCs proliferation but impairs the differentiation of aNSCs [56]. Tet2 interacted with the neuronal transcription activator Foxo3a, a member of the helix-turn-helix-like family proteins [70], and coregulated key genes involved in aNSC differentiation [58]. Moreover, Tet3 plays critical roles in neural progenitor cell maintenance [71] but is not required for NSC fate [72]. However, how Tet proteins interact with cofactors to regulate target genes responsible for the proliferation and differentiation of NSCs remains unclear. The possible regulative mechanisms are proposed in Figure 1.

5. Abnormal 5hmC and Neurological Diseases

The growing evidences demonstrate that 5hmC has high abundance in the brain and play a critical role to in the maintenance of normal neurodevelopment and functions of central nervous system. Thus, accumulating evidences showed that abnormal 5hmC modifications are involved the pathophysiology of different neurological diseases.

5.1. Alzheimer's Disease (AD). Alzheimer's disease (AD) is one of the most common age-related neurodegenerative disorders in the central nervous system, characterized by progressive cognitive decline and loss of neuronal cells [73]. The pathogenesis of AD has yet to be defined, but there are evidences to support its genetic abnormalities, such as the mutations in β-amyloid precursor gene and presenilin1/2. Previous study has shown that AD is associated with DNA methylation [74]. It has been found that levels of 5mC and DNMT in neurons are reduced in patients with AD [74]. At the same time, 5hmC level was reported to decrease in the hippocampal tissue of patients with AD [75]. However, a study has shown that brain 5mC and 5hmC levels increased in patients with AD [76]. The reasons for this inconsistency need to be further investigated. In APP-presenilin1 double transgenic mice, 5hmC abundance in different brain regions showed differential response to the pathogenesis [77]. Further gene ontology analyses indicated that differential hydroxymethylation region- (DhMR-) associated genes are highly enriched in multiple signaling pathways involving neuronal development/differentiation [77], suggesting that DNA 5hmC modification is an epigenetic modifier on neurogenesis or NSC differentiation in aging or AD [78]. Interestingly, Tet1 is found to decrease in the hippocampus of patients with AD [79]. Tet1 knockout mice show impaired hippocampal neurogenesis as well as learning and memory defects [55, 80]. Therefore, Tet1 functions as a critical enzyme to regulate 5hmC modifications on those genes related to the proliferation and differentiation of NSCs and further promotes neurogenesis in adult brains.

5.2. Huntington's Disease (HD). HD is an autosomal dominant disorder characterized by chorea, dystonia, slow and unexpected decline in cognitive function, and mental disorders [81]. At present, Huntington gene exon CAG repeats are considered as the major cause that leads to abnormal accumulation of the first amino acid polyglutamine in huntingtin proteins. Despite extensive research, the pathogenesis of neurodegeneration in HD is still unknown. ADORA2A gene encodes an adenylate A2A receptor, a G protein-coupled receptor that is highly expressed in the normal basal ganglia and is severely reduced in HD [82]. Recent studies have shown that HD results in an increase of 5mC expression and a decrease of 5hmC expression at the $5'$-UTR end of the ADORA2A gene compared with the same age group [83]. Except for the decreased of ADORA2A gene 5hmC modification, a significant decrease of global 5hmC modification is found in HD mice with 128 CAG repeats, indicating the involvement of 5hmC in the pathogenesis of HD and a novel epigenetic marker in HD [82]. Further 5hmC profiling analysis indicates that most genes with differentially hydroxymethylated regions are highly related to the pathological changes in HD, suggesting that gene 5hmC modifications are involved in the regulation of neurogenesis, neuronal function, and survival in HD brain [82]. Because previous studies have shown the abnormal neurogenesis in HD [84], aberrant epigenetic regulation on relevant genes may impair the neurogenesis in brains with HD. Recent study demonstrated that targeting histone modification to downregulate the key genes for the pathology of HD causes beneficial effects in a Drosophila model of HD [85]. Therefore, the modulation of 5hmC signature in HD may be an effective strategy to ameliorate the symptoms of HD.

5.3. Rett Syndrome. Rett syndrome is considered as an inherited disease characterized by progressive mental decline, autistic behavior, ataxia, and anxiety in the early life of those who suffer from the disease. The etiology and genetic pattern of this disease remain unknown. The primary cause of Rett syndrome is caused by methyl CpG binding protein 2 (MeCP2) gene mutations that result in loss of function of MeCP2 [86]. Because brains have the highest expression of MeCP2, MeCP2 functional deficiency causes neurological diseases such as Rett syndrome [87]. Recent study showed that MeCP2 was identified as the major 5hmc binding protein in the brain to facilitate gene expression by organizing the chromatin [88]. Previous study showed a reverse correlation between MeCP2 and 5hmC level, suggesting that MeCP2 binds to 5mC blocking the conversion of 5mC to 5hmC [67]. MeCP2 mutations such as R133C (an MeCP2 residue mutated in Rett syndrome) preferentially abolish its binding ability to 5hmC and account for the role of 5hmC in the pathophysiology of Rett syndrome, supporting that 5hmC and MeCP2 constitute an epigenetic regulation complex to control cell differentiation or chromatin structure [88]. Recent studies have shown that MeCP2 is required for brain development and neuronal differentiation by inhibiting the ID1/Her2 (the zebrafish ortholog of mammalian Hes5) axis in zebrafish because genetic depletion of MeCP2 inhibited neuronal differentiation but its overexpression promoted neuronal differentiation [89]. However, it is still unclear whether the blocking of MeCP2 binding to 5hmC is responsible for neuronal differentiation in Rett syndrome, as awaits more investigations.

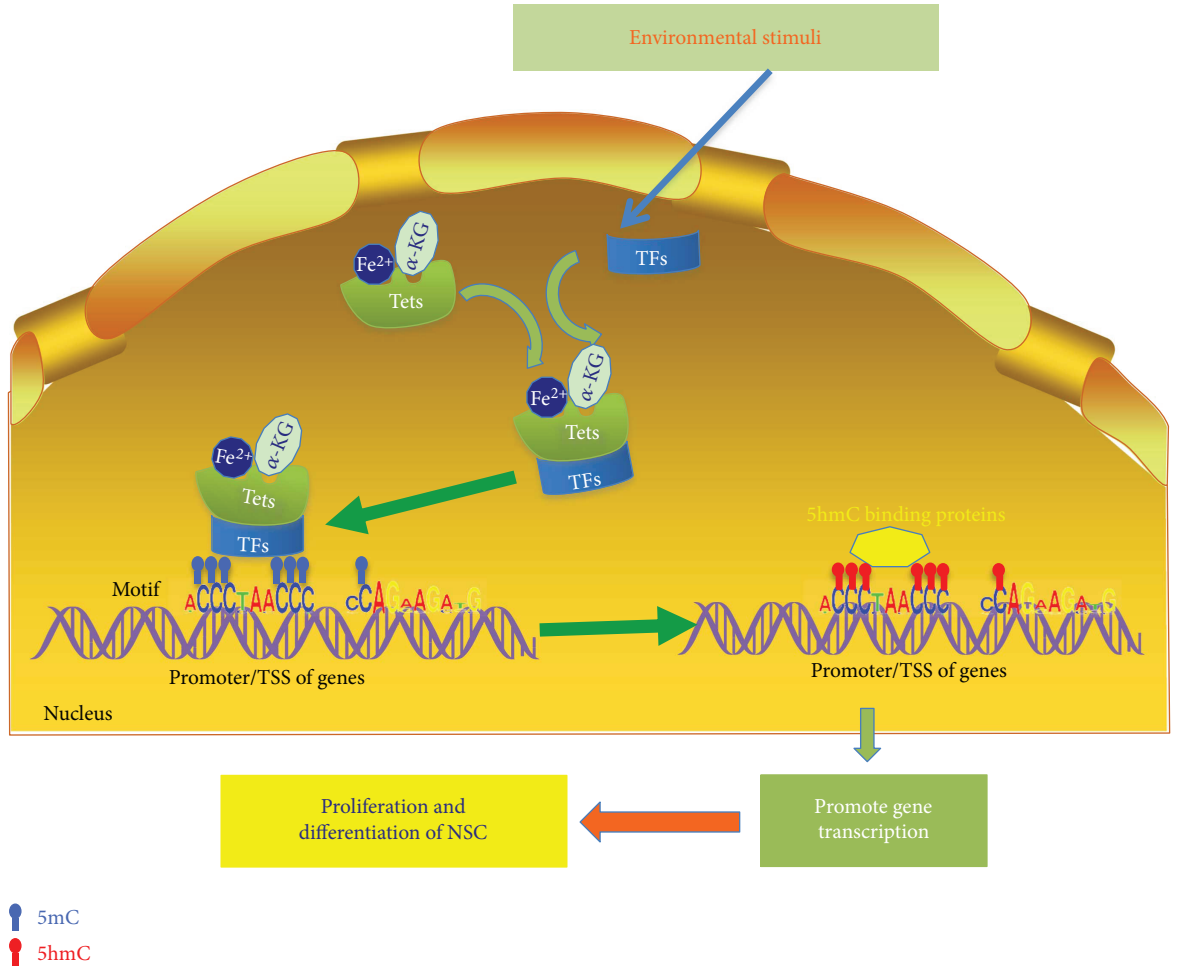

Figure 1: Tet proteins and 5-hmC mediated regulation of NSC proliferation and differentiation. Under the conditions of environmental stimuli, some transcriptional factors (TFs) such as FOXO3a enhance the affinity to Tet proteins along with cofactors of Tet enzymes including α-KG and Fe^{2+} to form a functional complex. By binding to DNA motifs of the targeting genes, the TFs guide the Tet enzymes to catalyze the conversion of 5mC to 5hmC. Generation of 5hmC facilitates the recruitment of the 5hmC binding proteins or other factors to enhance the transcription of targeting genes, thereby regulating the proliferation and differentiation of NSCs.

5.4. Major Depressive Disorders (MDD). The high morbidity and suicide of depression has become a major health concern in the world [90]. However, the pathogenesis of MDD remains unclear. So far, genetic and environmental factors are considered to interact and participate in the MDD, in which environmental factors mainly affect gene transcription and expression through epigenetic modification. DNA methylation is considered a major epigenetic modification from environmental stress [91]. 5hmC functions as a new DNA demethylation mechanism, however, its role in depressive disorders is unclear. Epigenetic 5hmC modification, to some extent, provides a possible mechanism for explaining environmental factors that affect gene expression. Recent reports showed that patients with MDD had decreased gray matter volume and white matter integrity in the hippocampus [92]. In addition, Bansal et al. found structural changes in the cerebral cortex of patients with MDD, indicating that thickening of the cerebral cortex is a compensatory nerve growth response [93]. Recent studies have also provided evidence that Tet1 knockout showed antidepressive phenotypes by affecting neurogenesis in the hippocampus [94]. Therefore, Tet proteins-mediated 5hmC modifications on depression-related genes are involved the regulation of neurogenesis in the mechanisms of MDD.

6. Conclusions

Epigenetic modification is likely to be the collective response to changes in environmental factors as a means of cells or organisms to mitigate the adverse effects [95]. The dynamic changes of methylation (5mC) and demethylation (5hmC) in DNA could affect its structure as well as the functions of genes and further lead to different kinds of diseases. Recent advances on 5hmC modification have demonstrated that Tet proteins and Tet-mediated 5hmC play important roles in the proliferation and differentiation of NSCs. However, it is unclear how Tet protein, Tet-interacting factors, and DNA 5hmC in target genes interplay and regulate the devolvement of NSCs. These need more investigations in the future. Recently, DNA N6-Methyldeoxyadenosine (6 mA) is

emerging as a new DNA modification and plays an important role in the regulation of the proliferation and differentiation of NSCs [96–98]. The interaction or crosstalking of DNA 5hmC modification and 6 mA modification will be an interesting topic. Considering the critical role of neuronal stem cells in the neurological diseases, targeting epigenetic regulation, especially on DNA 5hmC modification, is a promising strategy for the treatment of these neurological diseases.

Disclosure

Hang Zhou and Bin Wang are co-first authors.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

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Research Article

The Dynamic DNA Demethylation during Postnatal Neuronal Development and Neural Stem Cell Differentiation

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Background. DNA demethylation, the conversion of 5-methylcytosine (5mC) to 5-hydroxymethylcytosine (5hmC), 5-formylcytosine (5fC), and 5-carboxylcytosine (5caC), plays important roles in diverse biological processes and multiple diseases by regulating gene expression. *Methods.* In this study, utilizing DNA dot blot, immunofluorescence staining, and qRT-PCR, we studied the expression pattern of Tets, the enzymes governing DNA demethylation, and the levels of 5hmC, 5fC, and 5caC during the postnatal neuronal development of mice. *Results.* It was found that 5hmC, 5fC, and 5caC were highly enriched in multiple brain regions and aNSCs and displayed temporal and spatial patterns during postnatal neuronal development and the differentiation of aNSCs. Consistently, the expression of *Tets* also exhibited temporal and spatial patterns. *Conclusion.* DNA demethylation displayed dynamic features during postnatal neuronal development and the differentiation of aNSCs of mice, which could contribute to appropriate gene expression.

1. Introduction

In mammalians, DNA methylation at the fifth carbon of cytosine (5-methylcytosine, 5mC), the most intensively studied DNA modification, plays pivotal roles in multiple biological processes including chromatin structure, gene imprinting, X chromosome inactivation, and genomic stability; embryonic and postnatal development; ageing; and diseases by regulating gene expression [1–4]. It has been uncovered that 5mC can be oxidized to 5-hydroxymethylcytosine (5hmC), an active DNA demethylation process in mammalians, by ten-eleven translocation (Tet) dioxygenases (Tet1, 2, and 3) [5, 6]. Besides, 5hmC can be further oxidized to 5-formylcytosine (5fC) and 5-carboxylcytosine (5caC) by *Tets* [5, 7]. Both 5fC and 5caC can be targeted by thymine DNA glycosylase (TDG) and subsequently processed through the base excision repair (BER) DNA repair pathway, and they generated an unmodified cytosine [8, 9]. Mounting evidences indicate that 5hmC-mediated epigenetic modification plays critical roles in neuronal development and function, and the aberrant DNA demethylation is associated with neurological disorders including Rett syndrome, autism, Alzheimer's disease, Huntington's disease, and FXTAS [7, 10–14].

Previous studies have found that 5hmC widely exists in mammalian tissues, displays a cell-/tissue-specific distribution pattern, and is abundant in neural tissues [7, 14–18]. Further, 5hmC also exhibits dynamic features during embryonic and postnatal neuronal development [7, 14, 18, 19]. However, the distribution pattern of 5fC and 5caC during postnatal neuronal development and adult neural stem cell (aNSC) differentiation remains largely unknown. Meanwhile, the expression of *Tets* during postnatal neuronal development is also unclear. In the present study, we studied the distribution patterns of 5hmC, 5fC, and 5caC in the postnatal mouse brain, cultured adult neural stem cells (aNSCs) with immunostaining and dot blot, and compared their global levels. We found that 5hmC, 5fC, and 5caC were all detectable in neuronal cells in multiple brain regions. During the

postnatal neuronal development, the global levels of 5hmC increased in all these three brain regions, whereas 5fC did not show a significant change. 5caC significantly decreased in the cortex while the alteration is slight in the hippocampus and cerebellum. QRT-PCR results showed that the mRNA levels of *Tets* were decreased in the cortex during postnatal neuronal development, and *Tet2* had the highest expression level in the cortex and hippocampus. Further, 5hmC, 5fC, and 5caC were all detected in aNSCs and the global levels also changed during aNSC differentiation. Our results indicated the existence of 5hmC, 5fC, and 5caC in the brain and aNSCs, and *Tets* exhibited dynamic expression patterns during the postnatal neuronal development.

2. Materials and Methods

2.1. Animals and Tissue Preparation. C57BL/6 male mice and pregnant mice were purchased from the Shanghai Experimental Animal Center (Shanghai, China). The generation of *Tet1* and *Tet2* mice was described as previously [20, 21]. The animals were housed in the animal center of Zhejiang University on a 12 : 12 light/dark cycle with free access to food and water. All experimental procedures were performed according to protocols approved by the Animal Care and Use Committee of Zhejiang University. In this study, the day of birth was considered as postnatal day 1 (P1), 14 days after the birth as P14, and 8 weeks as adult.

Mice were deeply anesthetized with chloral hydrate (50 mg/kg, i.p.) and transcardially perfused with cold phosphate-buffered saline (PBS) followed by perfusion of 4% paraformaldehyde (PFA). The brains were gently removed and postfixed in 4% PFA overnight at 4°C. On the second day, the brain samples were transferred into 30% sucrose solution for dehydration at 4°C until they settled down in the solution. The brain samples were embedded in OCT (Thermo Fisher Scientific) and sectioned in the coronal plane (20 μm) with a cryostat (Leka). Sections were collected into the cryoprotectant solution and stored at −20°C until further processing.

2.2. The Isolation, Proliferation, and Differentiation Assays of NSCs. After being deeply anesthetized with chloral hydrate (50 mg/kg, i.p.), adult male mice were sacrificed and brains were removed and put into cold PBS. ANSCs were isolated from the forebrain as described previously [22] and cultured with DMEM/F-12 medium (DM-25, Omega Scientific) containing 20 ng/ml basic fibroblast growth factor (FGF-2, PeproTech), 20 ng/ml epidermal growth factor (EGF, PeproTech), 2% B27 supplement (Gibco), 1% penicillin-streptomycin (Gibco), and 2 mM L-glutamine (Gibco) in a 5% CO_2 incubator at 37°C.

For the proliferation assay of aNSCs, aNSCs were plated onto coverslips (BD Biosciences) with fresh medium. 5-Bromo-2′-deoxyuridine (BrdU, Sigma-Aldrich) was added into the culture medium at the final concentration of 5 μM for 8 h. ANSCs were then washed with PBS and fixed with 4% PFA for 30 min at room temperature. For the differentiation assay, aNSCs were treated with 5 μM forskolin (Sigma-Aldrich) and 1 μM retinoic acid (Sigma-Aldrich) for 2 days and then fixed with 4% PFA for 30 min at room temperature.

2.3. 5hmC, 5fC, and 5caC Immunofluorescence Staining and Imaging. Brain sections or cell samples were washed with PBS for 30 min and treated with 1 M HCl for 30 min at 37°C followed by RNase A treatment. After being washed with PBS for 15 min, samples were blocked with PBS containing 3% normal goat serum (Vector Laboratories) and 0.1% Triton X-100 (Sigma-Aldrich) for 1 h at room temperature and then incubated with primary antibodies. The primary antibodies used for immunofluorescence were as follows: the polyclonal rabbit antibodies anti-5-hydroxymethylcytosine (Active Motif), anti-5-formylcytosine (Active Motif), and anti-5-carboxylcytosine (Active Motif); the mouse monoclonal antibody anti-NeuN (Millipore); the rabbit polyclonal antibody anti-Sox2 (Millipore); the mouse monoclonal antibody anti-Nestin (BD Biosciences); the mouse monoclonal antibody anti-β III-tubulin (Promega); the rat monoclonal antibody anti-BrdU (Abcam); and the rabbit polyclonal antibody anti-glial fibrillary acidic protein (Dako). On the second day, samples were washed with PBS for 30 min and then incubated with secondary antibodies Alexa Fluor 488 goat anti-rabbit IgG, Alexa Fluor 568 goat anti-mouse IgG, and Alexa Fluor 568 goat anti-rat IgG (Invitrogen). DNA was counterstained with 4′-6-diamidino-2-phenylindole (DAPI, Sigma-Aldrich). After final washes, sections were mounted onto glass slides and coverslipped with mounting medium (Vector Laboratories). All immunostaining experiments were repeated with the sections from at least three animals of each age. Fluorescence images were viewed and captured with a Zeiss confocal microscope.

2.4. Genomic DNA Isolation and Dot Blot. Genomic DNA was extracted as described previously [7]. Briefly, the dissected brain samples or cells were homogenized in lysis buffer (5 mM EDTA, 0.2% SDS, 200 mM NaCl in 100 mM Tris-HCl, pH 8.5) supplemented with proteinase K (Sigma-Aldrich) and were digested at 56°C overnight. On the second day, the samples were treated with RNase A for at least 12 h at 37°C. An equal volume of phenol : chloroform : isoamyl alcohol (25 : 24 : 1, Sigma-Aldrich) was added, mixed completely, and centrifuged at 14,000 rpm for 10 min. An equal volume of isopropanol was added to the supernatant to precipitate DNA, which was dissolved with 10 mM Tris-HCl (pH 8.0). The concentration of DNA was quantified with NanoDrop (Thermo Fisher Scientific).

Dot blot was performed as described previously [7, 23]. Briefly, DNA samples were denatured and spotted onto the membrane (Amersham Biosciences) on a Bio-Dot apparatus (Bio-Rad). After being heated in a hybridization oven for 30 min at 80°C, the sample membrane was blocked with 5% fat-free milk in Tris-buffered saline (TBS) for 1 h before incubation with the primary antibody at 4°C overnight. The following primary antibodies were used for dot blot: the polyclonal rabbit antibodies anti-5-hydroxymethylcytosine (Active Motif), anti-5-formylcytosine (Active Motif), and anti-5-carboxylcytosine (Active Motif). On the second day, the sample membranes were washed with TBS for 30 min

and incubated with anti-rabbit horseradish-peroxidase-conjugated secondary antibody for 30 min at room temperature. After washing with TBS for 30 min, the chemiluminescence signals were visualized with the Tanon detection system (Tanon). The signal intensities of 5hmC, 5fC, and 5caC were quantified with the Adobe Photoshop software.

2.5. RNA Isolation and Real-Time PCR. Total RNA was extracted by the TRIzol reagent (Invitrogen), and 1 μg total RNA was used for reverse transcription according to the manufacturer's protocol (Life Technologies). The relative gene expression levels were measured by SYBR qPCR master mix (Life Technologies) with Applied Biosystems Viia 7. The following PCR primers were used: *Tet1*-F 5′-AGGGCCAAA ATGAAGCAGAA-3′ and *Tet1*-R 5′-GAGGCTGATGAAA AGCTCTTAGTGT-3′, *Tet2*-F 5′-GGCAAATGTGAAGGA TGCAA-3′ and *Tet2*-R 5′-CCAGCTCCTAGATGGGTAT AATAAGG-3′, and *Tet3*-F 5′-CGCCTCACGGGAGACA AT-3′ and *Tet3*-R 5′-AGTGGCCAGATCCTGAAAGCT-3′. *18S* was used for internal control: forward 5′-CGGCTA CCACATCCAAGGAA-3′ and reverse 5′-CCTGTATTGTT ATTTTTCGTCACTACCT-3′. The PCR program starts with the denaturation step of 10 min at 95°C, followed by 40 cycles of 15 sec at 95°C and 1 min at 60°C. The relative expression levels were then calculated using the $2^{-\Delta\Delta CT}$ method.

2.6. Statistical Analysis. All statistical analysis was performed with the GraphPad Prism software. Data were presented as mean ± SEM. For comparisons between two groups, a two-tailed unpaired Student *t*-test was used. For multiple group comparisons, a one-way ANOVA followed by Tukey post hoc test was used. $p < 0.05$ was considered statistically significant.

3. Results

3.1. The Immunofluorescence Staining of 5hmC, 5fC, and 5caC in Brain Sections. To detect 5hmC, 5fC, and 5caC in the brain, we first performed immunofluorescence using 5hmC-, 5fC-, or 5caC-specific antibodies together with a neuronal cell marker NeuN antibody. It was found that nearly all neuronal cells ($NeuN^+$) were also positive for 5hmC labeling in the cortex (Figure 1(a)), hippocampus (Figure 1(d)), and cerebellum (Figure 1(g)). Moreover, 5fC and 5caC were also detected in neuronal cells in those brain regions (Figures 1(b), 1(c), 1(e), 1(f), 1(h), and 1(i)). Taken together, these results indicated that active DNA demethylation (5hmC, 5fC, and 5caC) occurred in the genome of neuronal cells in different brain regions.

3.2. The Quantification of the Global Levels of 5hmC, 5fC, and 5caC in Different Brain Regions. Considering that 5hmC, 5fC, and 5caC were detectable in multiple brain regions, we next performed DNA dot blot to quantify the global levels of 5hmC, 5fC, and 5caC in those brain regions during the postnatal development (postnatal day 1 (P1), day 14 (P14), and adult (8 w)). Dot blot results showed that the 5hmC level significantly increased from P1 to P14 in the cortex and hippocampus (Figures 2(a)

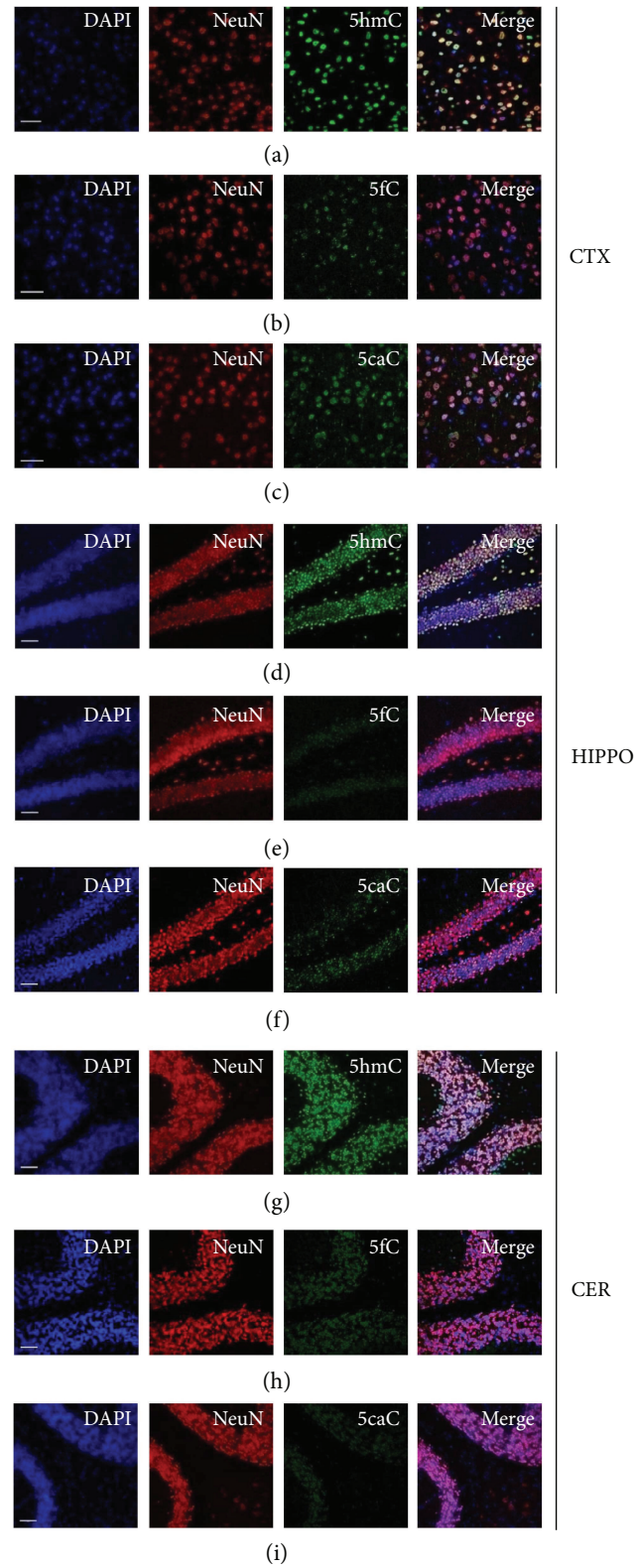

Figure 1: The representative immunofluorescence images of 5hmC, 5fC, and 5caC in the cortex, hippocampus, and cerebellum. Immunofluorescence of 5hmC (a), 5fC (b), and 5caC (c) in the 8 w cortex. Immunofluorescence of 5hmC (d), 5fC (e), and 5caC (f) in the 8 w hippocampus. Immunofluorescence of 5hmC (g), 5fC (h), and 5caC (i) in the 8 w cerebellum. Scale bar, 200 μm.

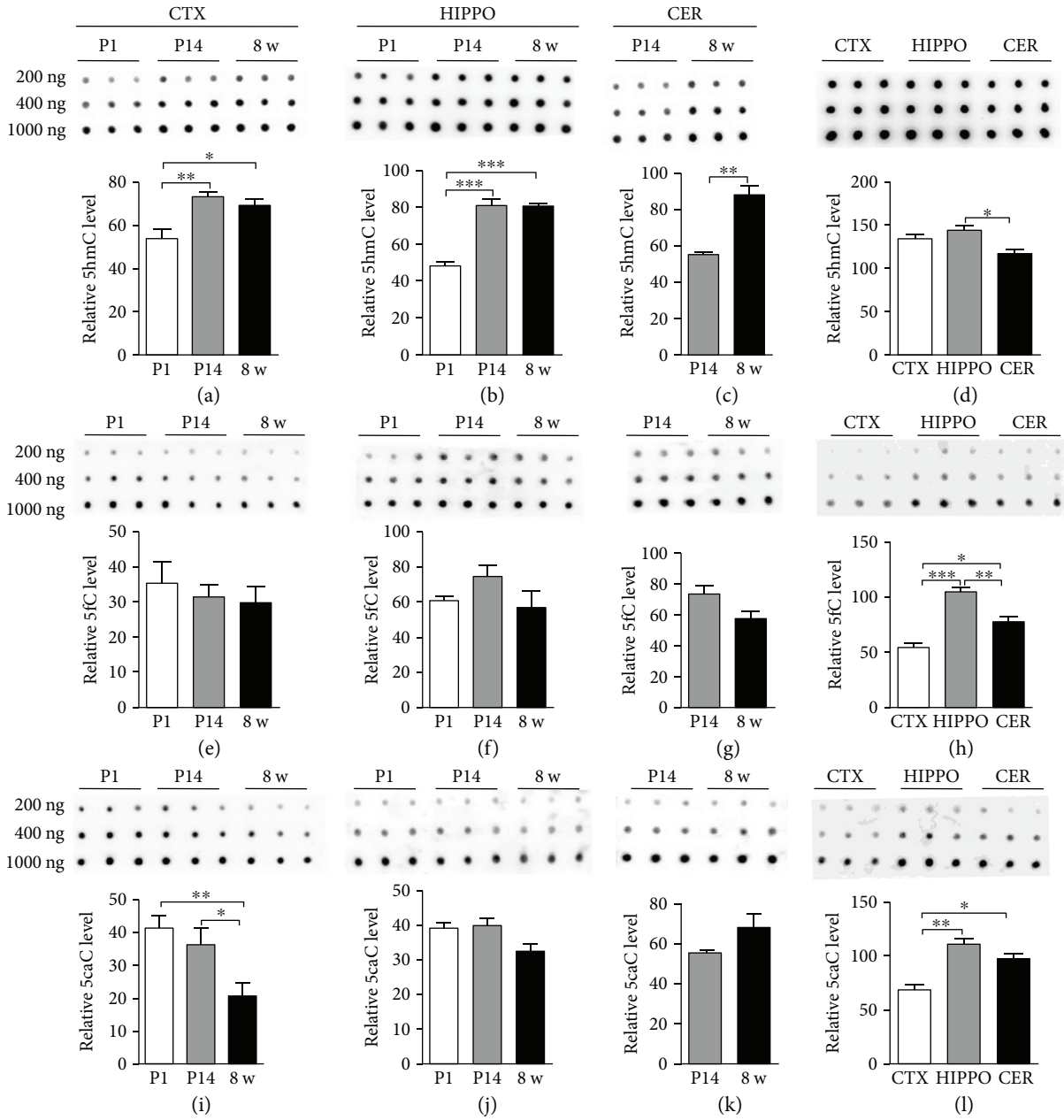

Figure 2: 5hmC, 5fC, and 5caC in different brain regions using DNA dot blot. The relative levels of 5hmC, 5fC, and 5caC in the P1, P14, and 8 w cortex ((a), (e), (i)), hippocampus ((b), (f), (j)), and cerebellum ((c), (g), (k)). Comparison of 5hmC (d), 5fC (h), and 5caC (l) levels between different brain regions at 8 w. Data are represented as mean ± SEM ($n = 3$). Statistically significant differences were indicated: $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$.

and 2(b)). Of note, from P14 to adult, the 5hmC level maintained stably in the cortex and hippocampus, but significantly increased in the cerebellum (Figures 2(a)–2(c)). The 5fC level did not display a significant change in those three brain regions during the postnatal neuronal development (Figures 2(e)–2(g)). Further, the 5caC level in the cortex significantly decreased in adults compared to P1 and P14 (Figure 2(i)), but it did not alter significantly in the hippocampus and cerebellum (Figures 2(j) and 2(k)).

We further compared the 5hmC, 5fC, and 5caC levels in different brain regions at adult age. The 5hmC level was similar between the cortex and hippocampus, but higher than that of the cerebellum (Figure 2(d)). 5fC and 5caC were significantly enriched in the hippocampus compared to the cortex and cerebellum (Figures 2(h) and 2(l)). Taken together, these results suggest that DNA demethylation exhibited a temporal and spatial feature during the postnatal neuronal development.

3.3. The Expression of Tets in Different Brain Regions by qRT-PCR. In mammalian brain, DNA demethylation was catalyzed by Tet family proteins. We next studied the expression

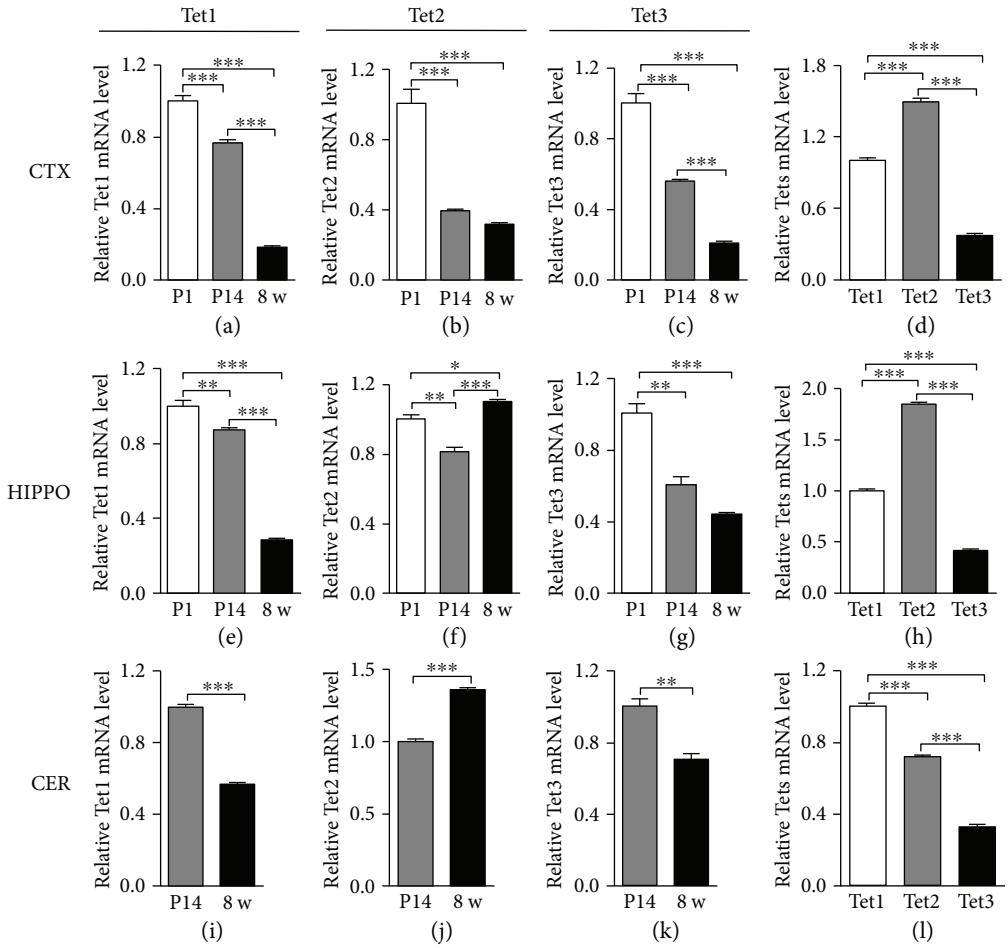

Figure 3: The mRNA levels of Tets in different brain regions. The relative mRNA levels of *Tet1*, *Tet2*, and *Tet3* in the cortex from P1 to 8 w ((a)–(c)). The relative mRNA levels of *Tet1*, *Tet2*, and *Tet3* in the hippocampus from P1 to 8 w ((e)–(g)). The relative mRNA levels of *Tet1*, *Tet2*, and *Tet3* in the cerebellum from P1 to 8 w ((i)–(k)). Comparison of *Tet1*, *Tet2*, and *Tet3* mRNA levels in different brain regions at 8 w ((d), (h), (l)). Data are represented as mean ± SEM ($n = 3$). Statistically significant differences are indicated: $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$.

of *Tets* by qRT-PCR in those three brain regions from P1 to adult. It was found that the mRNA levels of *Tet1*, *Tet2*, and *Tet3* decreased from P1 to adult in the cortex (Figures 3(a)–3(c)). In the hippocampus and cerebellum, the mRNA levels of *Tet1* and *Tet3* decreased during postnatal neuronal development while *Tet2* increased (Figures 3(e)–3(g) and 3(i)–3(k)). We then compared the expressions of *Tet1*, *Tet2*, and *Tet3* in different brain regions at adult age. It was found that *Tet2* had the highest expression level in the cortex and hippocampus compared to *Tet1* and *Tet3* (Figures 3(d) and 3(h)); however, the expression of *Tet1* was the highest in the cerebellum (Figure 3(l)). These results suggested that the expression of *Tets* also displays dynamic features during the postnatal neuronal development.

To reveal which Tet plays dominant roles in DNA demethylation during the postnatal neuronal development, *Tet1* and *Tet2* knockout (KO) mice were adopted and genomic DNA was extracted from the cortex, hippocampus, and cerebellum regions, respectively. Dot blot results showed that *Tet1* and *Tet2* KO could significantly decrease 5hmC levels in those three brain regions of mice. *Tet2* depletion displayed more dramatic effects on 5hmC levels compared to *Tet1* depletion (Figures 4(a)–4(c)). For 5fC and 5caC, *Tet1* and *Tet2* depletion showed a distinct effect in different regions. The 5fC level was decreased in the cerebellum induced by the depletion of *Tet1* and *Tet2* while no significant change was observed in the cortex and hippocampus (Figures 4(d)–4(f)). The 5caC level decreased in the cortex while there was no observable change in the hippocampus and cerebellum (Figures 4(g)–4(i)). These results suggested that Tet1 and *Tet2* both catalyzed DNA demethylation, and *Tet2* showed a more dominant effect.

3.4. DNA Demethylation in Cultured Adult Neural Stem Cells. To further characterize DNA demethylation in neuronal development, we isolated neural stem cells (aNSCs) from the brain of adult mice. More than 97% of the cultured aNSCs were positive for NSC markers Nestin and Sox2 (Supplemental Figures 1(a)–(d)), suggesting the homogeneity of aNSCs. The cultured aNSCs could incorporate BrdU during proliferation (Supplemental Figures 1(e)–(g)) and generate neurons and astrocytes upon differentiation

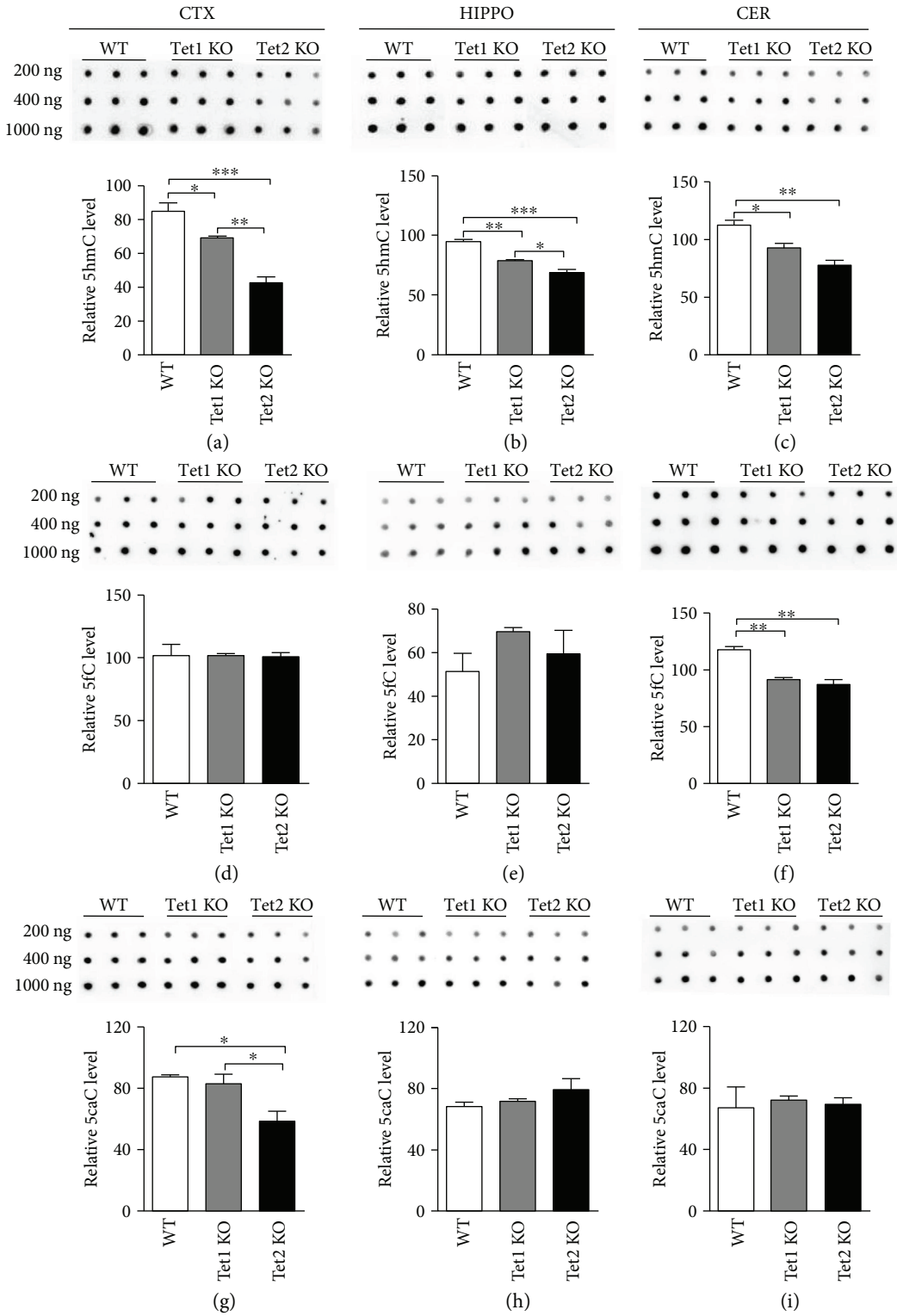

Figure 4: 5hmC, 5fC, and 5caC in *Tet1* and *Tet2* knockout brain using DNA dot blot. The levels of 5hmC, 5fC, and 5caC in the cortex ((a), (d), (g)), hippocampus ((b), (e), (h)), and cerebellum ((c), (f), (i)) of adult *Tet1* and *Tet2* KO mice. Data are represented as mean ± SEM ($n = 3$). Statistically significant differences are indicated: $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$.

(Supplemental Figures 1(h)–(k)), suggesting their capabilities of self-renewal and multipotency.

Next, we performed immunofluorescence staining and found that 5hmC, 5fC, and 5caC were well colocalized with aNSC marker Sox2 at the proliferating condition (Figures 5(a)–5(c)). 5hmC, 5fC, and 5caC could be detected in the nuclei of neuronal cells (Tuj1^{+}) generated during aNSC differentiation (Figures 5(d)–5(f)). Dot blot results showed

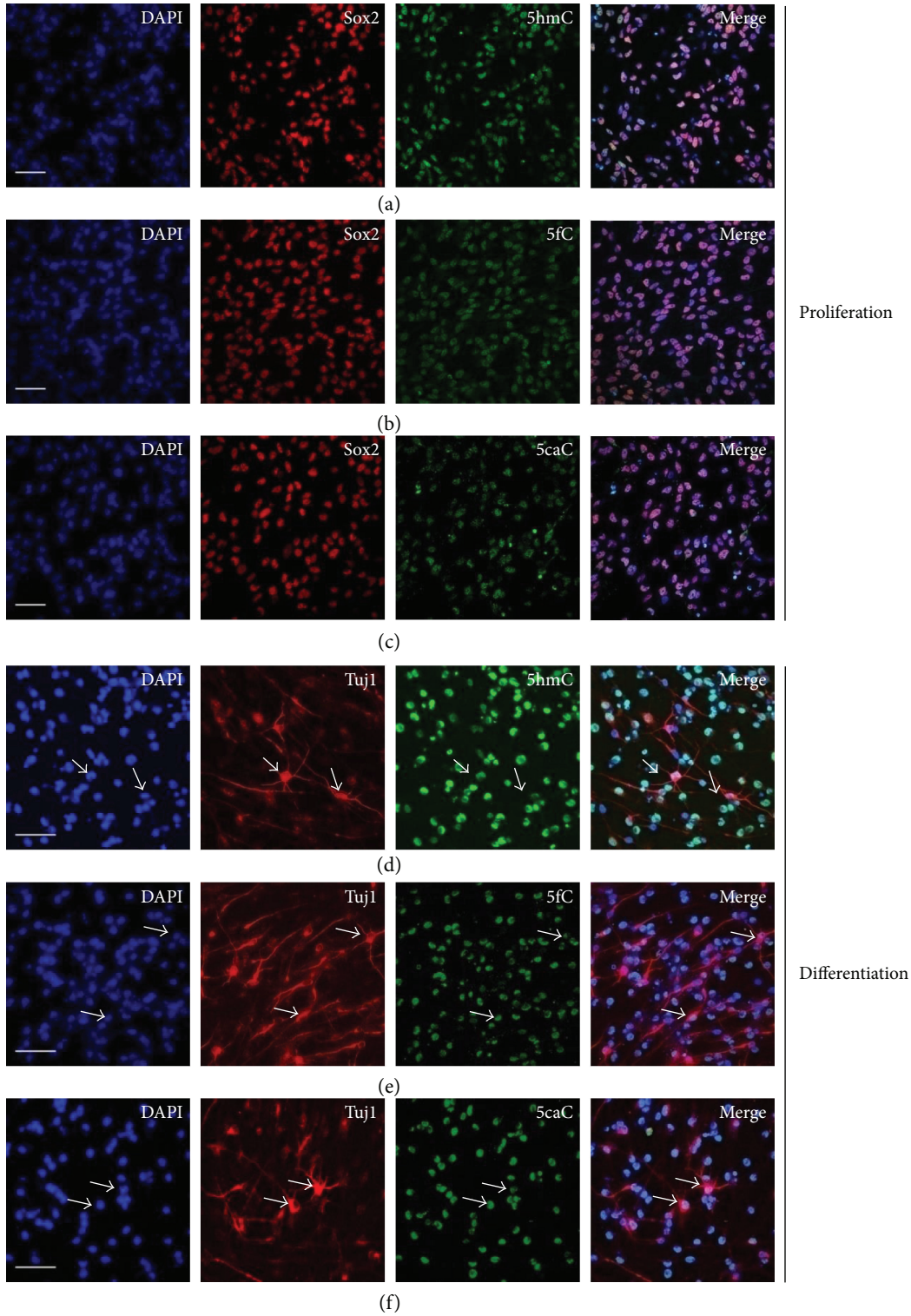

Figure 5: The representative immunofluorescence images of 5hmC, 5fC, and 5caC in cultured aNSCs. Immunofluorescence of 5hmC (a), 5fC (b), and 5caC (c) in cultured aNSCs. Neuronal cells generated from aNSCs were positive for 5hmC (d), 5fC (e), and 5caC (f). Scale bar, 200 μm.

that 5hmC, 5fC, and 5caC were all detectable in the genomic DNA of aNSCs (Figure 6(a)). 5hmC significantly increased while 5fC and 5caC decreased from proliferation to differentiation of aNSCs (Figures 6(b)–6(d)). Together, these results indicated that DNA demethylation exhibited dynamic features during the differentiation of aNSCs.

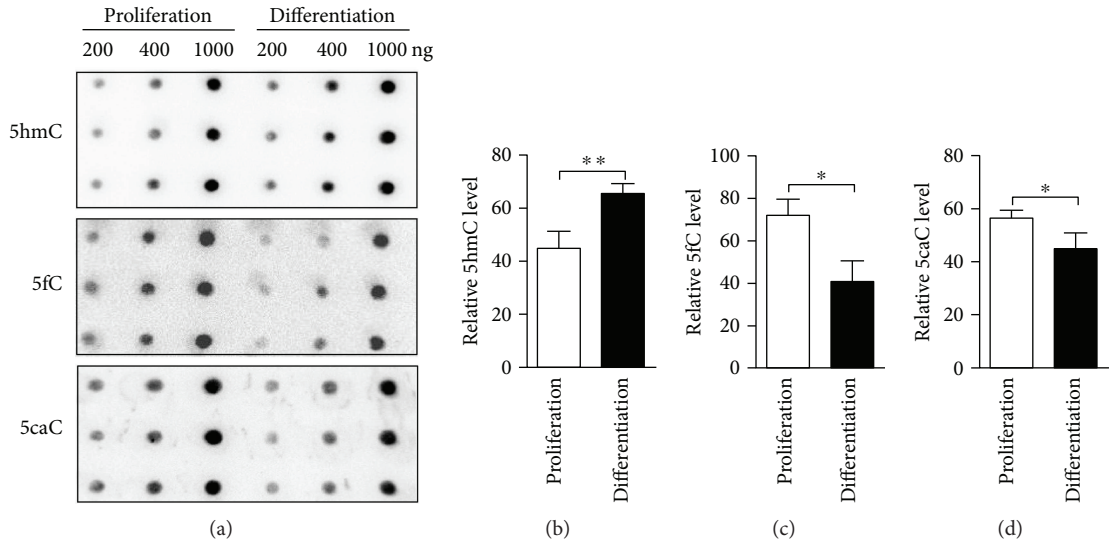

Figure 6: DNA demethylation in cultured aNSCs. DNA dot blots of 5hmC, 5fC, and 5caC in proliferating and differentiated aNSCs (a). The quantification of dot blots of 5hmC, 5fC, and 5caC, respectively ((c)–(d)). Statistically significant differences are indicated: $^{*}p < 0.05$ and $^{**}p < 0.01$.

4. Discussion

In the present study, we detected and compared DNA demethylation in multiple brain regions and aNSCs. Our results indicated that 5hmC-, 5fC-, and 5caC-mediated DNA demethylation actively occurred not only in the brain during postnatal development but also in aNSCs. 5hmC was acquired in all three brain regions during neuronal maturation, while 5fC and 5caC displayed slight changes in the hippocampus and cerebellum but a significant decrease in the cortex. The mRNA levels of *Tet1*, *Tet2*, and *Tet3* were decreased overall in all three brain regions during postnatal neuronal development, and *Tet2* had the highest expression level in the cortex and hippocampus. We also found that 5hmC, 5fC, and 5caC existed in the genome of aNSCs, and the global level of 5hmC was increased while 5fC and 5caC were successively decreased in aNSCs. Our study showed the features of the temporospatial-specific alteration of 5hmC, 5fC, and 5caC in mammalian brain and aNSCs.

Previous studies had indicated that DNA demethylation played essential roles in neuronal development, learning and cognitive ability, and neurological disorders [7, 11, 19, 24–27]. 5hmC was acquired during embryonic and postnatal brain development and highly enriched in neuronal cells [7, 15, 18, 28–30]. During neuronal development, 5hmC not only displayed cell- and tissue-specific features but also displayed dynamic features in the genome [7, 18, 19]. 5hmC, 5fC, and 5caC were shown to be enriched in distinct genomic regions including promoters and enhancers [7, 31–33] and to display dynamic alteration during lineage specification [34, 35]. Thus, it is rational to speculate that the dynamic and specific distribution of active DNA demethylation could contribute to the proper gene expression during neuronal development.

Although 5fC and 5caC had been shown as stable epigenetic markers [36], their functions were still on the way to be explored. Genome-wide profiling studies revealed 5fC enriched in CpG islands (CGIs) of promoters, exons, and enhancers and involved in the regulation of gene expression during development [32, 37]. Recently, it was found that the level of 5caC increased in human breast cancer and during the differentiation of neural stem cells [34, 38]. Our present results indicated that 5fC and 5caC also exhibited dynamic features during the postnatal neuronal development. Together with these studies, 5fC and 5caC, like 5hmC, might also contribute to the regulation of cell- and region-specific gene expression.

In line with the temporospatial feature of DNA demethylation, the expression of Tets exhibited tissue-/cell-specific patterns and dynamic features during neuronal development [7, 19]. These findings suggested that despite having a similar structure, Tet1, 2, and 3 might have differential binding or catalytic activities towards the substrates, that is, 5hmC, 5fC, and 5caC [39]. The preference towards the differential substrates and distinct genomic loci could lead to the differential function. In the adult mouse brain, *Tet1* and *Tet2* deletion both regulated adult neurogenesis but displayed some differential effects [19, 20]. *Tet2* deletion preferentially induced 5hmC loss and hypermethylation at enhancers and gene bodies, but *Tet1* depletion predominantly reduced 5hmC levels at transcription start sites and promoter regions [19, 40, 41]. All these results suggested the distinct roles of *Tets* in different brain regions during development.

Abbreviations

aNSC: Adult neural stem cell
Tet: Ten-eleven translocation
5mC: 5-Methylcytosine
5hmC: 5-Hydroxymethylcytosine
5fC: 5-Formylcytosine
5caC: 5-Carboxylcytosine

CTX: Cortex
HIPPO: Hippocampus
CER: Cerebellum.

Conflicts of Interest

The authors declare that they have no competing interests.

Authors' Contributions

Xuekun Li conceived and designed the project. Huikang Tao, Pei Xie, Yuhang Cao, Liping Li, Junchen Chen, Liqi Shu, Guangfeng Tian, and Yingliang Zhuang performed experiments. Xuekun Li wrote the manuscript. All authors commented on the manuscript.

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Supplementary Materials

Supplemental Figure 1: cultured NSCs express NSC markers and display the capabilities of self-renewal and multipotency. Cultured NSCs expressed the neural progenitor markers Sox2 and Nestin ((a)–(d)). Proliferative NSCs stained with BrdU ((e)–(g)). Cultured NSCs could differentiate into $GFAP^+$ astrocytes and $Tuj1^+$ neurons ((h)–(k)). Scale bar, 200 μm. *(Supplementary Materials)*

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Review Article

Focus on Mesenchymal Stem Cell-Derived Exosomes: Opportunities and Challenges in Cell-Free Therapy

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Mesenchymal stem cells have been at the forefront of regenerative medicine for many years. Exosomes, which are nanovesicles involved in intercellular communication and the transportation of genetic material transportation that can be released by mesenchymal stem cells, have been recently reported to play a role in cell-free therapy of many diseases, including myocardial infarction, drug addiction, and status epilepticus. They are also thought to help ameliorate inflammation-induced preterm brain injury, liver injury, and various types of cancer. This review highlights recent advances in the exploration of mesenchymal stem cell-derived exosomes in therapeutic applications. The natural contents, drug delivery potency, modification methods, and drug loading methods of exosomes are also discussed.

1. Introduction

Mesenchymal stem cells (MSCs) originate from the mesoderm of many tissues, including bone marrow, liver, spleen, peripheral blood, adipose, placenta, and umbilical cord blood, and have the capacity to self-renew and the ability to generate differentiated cells. Over the last decade, MSCs have emerged as a popular research topic because of their potential role in regenerative medicine, immunoregulation, neuroprotection, and antitumor effects originally attributed to direct cell replacement. However, experimental data indicates that most MSCs are largely cleared, while a small proportion will integrate into injured tissue after intravenous injection [1]. Furthermore, the "cell replacement theory" does not account for the sufficient durations in a variety of disease models [2, 3]. Recently, several mechanisms have been put forward regarding the therapeutic potential of MSCs, including (1) paracrine factors involving proteins/peptides and hormones and (2) the transfer of mitochondria or exosomes/microvesicles packaging multitudinous molecules [4].

Exosomes are a family of nanoparticles with a diameter in the range of 40–100 nm that are generated inside multivesicular endosomes or multivesicular bodies (MVBs) and are secreted when these compartments fuse with plasma membrane [5]. Exosomes are enriched in endosome-derived components as well as many bioactive molecules such as proteins, lipids, mRNAs, microRNAs (miRNAs), long noncoding RNAs (lncRNAs), transfer RNA (tRNA), genomic DNA, cDNA, and mitochondrial DNA (mtDNA) [6–12]. It has also been reported that exosomes may be released from multiple cell types, including reticulocytes [13], immunocytes, tumor cells, and MSCs [14]. This suggests that the secretion of exosomes is a general cellular function that plays an important role in the intercellular transfer of information.

In this review, we focus on the mechanisms of exosomes/microvesicles, covering the current knowledge on biological characteristics and their potential cell-free therapeutic applications for MSC-derived exosomes.

2. Characterization and Isolation of Exosomes

Exosomes were first discovered by Harding's group as "a garbage can" in maturing sheep reticulocytes [13]. Originally, they were thought to have a typical "cup-shaped" or "saucer-like" morphology when analyzed by electron morphology

Table 1: Characterization of various extracellular vesicles.

	Exosomes	MVs	Apoptotic bodies
Size	30–100 nm	50–2000 nm	500–4000 nm
Surface markers (used most)	CD63	ARF6	TSP
	CD9	VCAMP3	C3b

[15, 16]. Zabeo's group revealed a wide diversity in exosome morphology when purified from homogeneous cell types (the human mast cell line HMC-1). They classified exosome morphology into nine categories: (1) single vesicle; (2) double vesicle; (3) triple vesicle or more; (4) small double vesicle; (5) oval vesicle; (6) small tubule; (7) large tubule; (8) incomplete vesicle; and (9) pleomorphic vesicle [17]. This categorization suggested that different morphologies of exosomes may be accompanied by various and specific functions. Exosomes also contain surface proteins unique to the endosomal pathway, which are generally used to characterize exosomes and distinguish them from microvesicles (MVs), apoptotic bodies, and other vesicles (Table 1), such as tetraspanins (CD63, CD81, and CD9), heat shock proteins (Hsc70), lysosomal proteins (Lamp2b), the tumor-sensitive gene 101 (Tsg101), and fusion proteins (CD9, flotillin, and annexin) [12, 18]. Exosomes are released in almost all types of extracellular fluids, including blood, urine, amniotic fluid, ascites, hydrothorax, saliva, breast milk, seminal fluid, and cerebrospinal fluid. Exosomal content greatly depends on cellular origin. For example, exosomes derived from B lymphocytes that bring functional MHCI, MHCII, and T cell costimulatory molecules can stimulate T cell proliferation [19]. Furthermore, cancer cell-derived exosomes contain gelatinolytic enzymes and other cell adhesion-related molecules to help tumor progression and metastasis [20]. Importantly, these cancer cell-derived exosomes are actively incorporated by MSCs in vitro and in vivo, in that the transfer of exosomal proteins and miRNAs acquire the physical and functional characteristics of tumor-supporting fibroblasts [21, 22]. For more details on the molecular cargos and extracellular signal transmission pathway of exosomes, the reader may refer to ExoCarta (http://www.exocarta.org) or EVpedia (http://evpedia.info), as well as the American Society for Exosomes and Microvesicles (http://www.asemv.org), for an in-depth exploration.

Ultracentrifugation and a commercial kit rooted in polymer-based precipitation are the most well-established purification protocols [16]. Other conventional validated isolation methods described in the literature include ultrafiltration, chromatography, and affinity capture [23]. New protocols have been established in order to facilitate the large-scale and high-purity manufacture of exosomes. Microfluidic techniques [24] are based on electrochemical, electromechanical, viscoelastic [25], optical, nonoptical, and other principles, yet the isolation is a mixed population of small nanoparticles without further demonstration of their intracellular origin. Thus, we use the term exosomes in this review to refer to extracellular vesicles characterized by exosome-specific surface markers, regardless of the primitive appellations in the published data.

3. Cargos and Functions of MSC-Derived Exosomes

The abundance of cargos identified from MSC-derived exosomes attracts broad attention because of their therapeutic potential in cardiovascular disease, tissue (kidney, liver, skin, and cornea) repair, immune disease, tumor inhibition, and neurological disease (Figure 1). They function largely via the constant transfer of miRNAs and proteins, resulting in the alteration of a variety of activities in target cells via different pathways.

3.1. Protein. Over 900 species of proteins have been collected from MSC-derived exosomes according to ExoCarta. With the exception of some common proteins involved in cell metabolism and the cytoskeleton, many proteins have been found in different tissue sources of MSC-derived exosomes. Proteomic studies by Kang's group identified 103 proteins from neural stem cell-derived exosomes. For example, the presence of polymyositis/scleroderma autoantigen 2 (PM/Scl2), a highly specific nuclear autoantigen, indicates that exosomes may be involved in triggering autoimmunity. They also found an imparity between exosomes larger than the baseline (50 nm) and those of smaller morphology [26]. These findings may explain the phenomenon recently observed by Caponnetto et al. regarding size-dependent cellular uptake of exosomes by target cells [27]. Intriguingly, all enzymes involved in the ATP synthesis of glycolysis (glyceraldehyde 3-phosphate dehydrogenase (GAPDH), phosphoglycerate kinase (PGK), phosphoglucomutase (PGM), enolase (ENO), and pyruvate kinase m2 isoform (PKm2)), as well as the rate-limiting glycolytic enzyme phosphorylated PFKFB3 that upregulates phosphofructose kinase, were identified in MSC-derived exosomes. Furthermore, oxidative stress was reduced via peroxiredoxins and glutathione S-transferases in MSC-derived exosomes [28], which suggests that replenishing glycolytic enzymes to increase ATP production, as well as additional proteins to reduce oxidative stress through exosomal transportation, may help reduce cell death in myocardial ischemia/reperfusion injury. Comparable levels of VEGF, extracellular matrix metalloproteinase inducer (EMMPRIN), and MMP-9 have also been reported in MSC-derived exosomes. These three proteins play a vital role in stimulating angiogenesis [29], which could be fundamental for tissue repair. Recent experimental evidence summarized by Burrello suggests that transcriptional factors, such as Nanog, octamer-binding transcription factor 4 (Oct-4), HoxB4, and Rex-1, play an important role in the immune system [30]. For example, HoxB4 has been shown to affect DC maturation and T-cell proliferation, differentiation, and activation through WNT signaling. Interestingly, membrane proteins and exosome-specific surface markers, such as CD81, CD63, and CD9, may affect the immune response by regulating cell adhesion, motility, activation, and signal transduction [31]. Several studies have also shown that exosomes derived from MSCs harbor cytokines and growth factors, such as TGFβ1, interleukin-6 (IL-6), IL-10, and hepatocyte growth factor (HGF), which have been proven to contribute to immunoregulation [30].

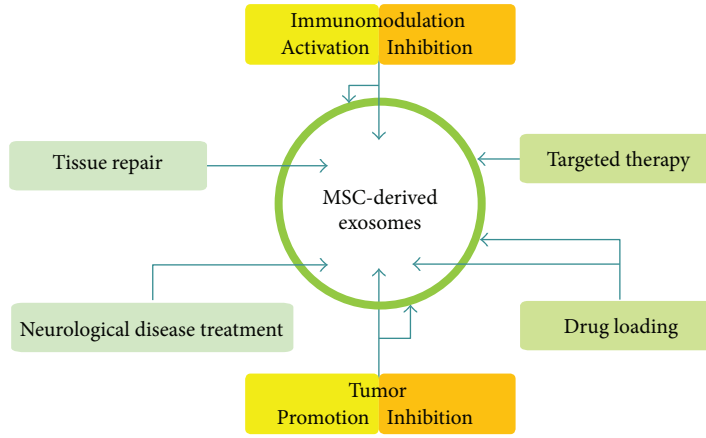

Figure 1: The main functions of MSC-derived exosomes. The external bilayer (green circle) is the membrane and the internal bilayer (white circle) is packed with various bioactive compounds.

3.2. miRNAs. miRNAs consist of a class of small noncoding RNAs that regulate gene expression posttranscriptionally by targeting mRNAs to induce suppression of protein expression or cleavage [32]. Many miRNAs have been found in MSC-derived exosomes and are reportedly involved in both physiological and pathological processes such as organism development, epigenetic regulation, immunoregulation, tumorigenesis, and tumor progression. Notably, exosomes with membrane structure act as preservers and deliverers of miRNAs, transferring functional miRNAs into recipient cells. It has been reported that exosomal miR-23b, miR-451, miR-223, miR-24, miR-125b, miR-31, miR-214, and miR-122 [33, 34] may inhibit tumor growth and stimulate apoptosis through different pathways. For instance, miR-23b promotes dormancy in metastatic breast cancer cells via the suppression of the target gene MARCKS, which encodes a protein that promotes cell cycling and motility [34]. MiR-16, shuttled by MSC-derived exosomes, has also been found to suppress angiogenesis by downregulating VEGF expression in breast cancer cells [35]. Recently, let-7f, miR-145, miR-199a, and miR-221, which are released from umbilical MSC-derived exosomes, have been found to largely contribute to the suppression of hepatitis C virus (HCV) RNA replication [36]. Di Trapani's group evaluated the immunomodulatory effects exerted by MSC-derived exosomes on unfractionated peripheral blood mononuclear cells and purified T, B, and NK cells. They observed that exosomes had higher levels of miRNAs compared to MSCs and could also induce inflammatory priming via increasing levels of miR-155 and miR-146, which are two miRNAs involved in the activation and inhibition of inflammatory reactions [37]. Similar immunosuppressive functions have also been reported in animal experiments by Cui et al. [38]. Exosomes from MSCs effectively increased the level of miR-21 in the brain of AD mice. Additionally, replenishment of miR-21 restored the cognitive deficits in APP/PS1 mice and prevented pathologic features by regulating inflammatory responses and restoring synaptic dysfunction [38]. Recent studies have also shown that aging is substantially controlled by hypothalamic stem cells, partially through the release of exosomal miRNAs [39]. However, contradictions regarding these outcomes remain. A quantitative analysis of exosomal miRNA abundance and stoichiometry by Chevillet's group quantified both the number of exosomes and the number of miRNA molecules in replicate samples isolated from diverse sources. Regardless of the source, the study indicated that, on average, over 100 exosomes would need to be examined to observe one copy of a given abundant miRNA, suggesting that most individual exosomes do not carry biologically significant numbers of miRNAs and are thus unlikely to be functional individually as vehicles for miRNA-based communication [40].

3.3. Others. In 2006, MSC-derived exosomes that could modulate the phenotype of target cells, supporting self-renewal of hematopoietic progenitors and multipotency by transfer of growth factors and mRNA, were first reported. For instance, exosomal SOX2 was found to initiate innate responses against microbial infection through neutrophil activation [41]. Although MSC-derived exosomes have the same morphology as exosomes from other cells and carry typical markers, they are quite different in regard to compartmentalization and protein and RNA composition. For example, studies have indicated that not all MSC-derived exosomes are equivalent [42]. Baglio's group [6] characterized the small RNAome of exosomes released by early passage adipose-MSCs (ASC) and bone marrow-MSCs (BMSCs). They found a large discrepancy in the proportion of miRNAs in total small RNA content between cells (19–49%) and exosomes (2–5%), suggesting that the miRNAs in exosomes do not merely reflect the cellular content. Further studies regarding the overrepresentation of small RNA content-tRNAs revealed a similar outcome. The most abundant tRNA in ASC exosomes, tRNA GCC (Gly), represented only a small fraction (5%) of the total cellular tRNA. Importantly, the authors also determined that the striking differences in tRNA species seemed to be associated with the differentiation status of MSCs. Recent research has shown that the stability of exosome composition is susceptible to localized environmental conditions. For example, hypoxia and inflammatory signals, such as lipopolysaccharides, may be strong interference factors [43].

4. Exosomes as Drug Delivery Vehicles

Optimal features of drug delivery vehicles may be applied to improve carrier qualities, including cellular tropism, efficient therapeutic cargos, appropriate physicochemical properties, and sufficient immune tolerance. Among the many drug platforms, liposomes have been the preferred pharmaceutical vehicles for drug delivery. A wide range of liposome products have been approved for the treatment of diseases, including fungal infections, pain management, hepatitis A, influenza, and various types of cancer [44, 45]. In contrast to liposomes, exosomes are optimal for drug delivery because of their natural properties and plasticity with minor modifications. Here, we compare exosomes and liposomes and suggest that exosomes may be a promising star for drug delivery.

4.1. Exosomes versus Liposomes. Exosomes and liposomes are both coated with a phospholipid membrane. The membrane structure of exosomes is inlayed with multiple natural biomolecules, such as surface proteins and MHCs, while liposomes may be modified with targeting ligands or inert polymeric molecules such as oligosaccharides, glycoproteins, polysaccharides, and synthetic polymers [45]. The size of liposomes is in the range of 30 nm to several microns [46]. Smaller liposomes (as small as exosomes) display a prolonged circulation time compared to larger ones, but the capacity for optimal drug reservation and release profiles is partly lost. For more details regarding circulation time and biodistribution, readers can refer to other sources [46]. Regarding cellular interactions and uptake, liposomes can be equipped with targeting ligands, which can bind to receptors or other molecules that are specific or overexpressed by target cells for interactions and the intracellular delivery of drugs [46]. However, the drug delivery of liposomes is not efficient, since many modifications have been designed to minimize clearance and poisonousness. In general, liposomes accumulate in the macrophages of the liver and spleen after intravenous injection. Few liposomes are interspersed in other tissues, which may be due to the lack of immunocompatibility. On the other hand, exosomes are born with many features of an ideal drug delivery vehicle. For example, they exhibit lower toxicity compared to liposomes. In addition, they are well tolerated by the immune system, even across the blood-brain barrier, avoiding phagocytosis or degradation by macrophages [47]. Exosomes exhibit an innate targeting tendency. For instance, MSC-derived exosomes home preferentially to inflamed tissues and tumor tissues [48]. Furthermore, abundant bioactive materials within exosomes or on the surface provide primitive treatment potential, and there are abundant modification methods for membrane targeting and drug loading. Alvarez-Erviti et al. engineered dendritic cells to express Lamp2b, an exosomal membrane protein, fused to the neuron-specific RVG peptide and loaded these modified exosomes with siRNAs by electroporation. These intravenously injected exosomes showed a strong knockdown of BACE1 (mRNA (60%) and protein (62%)), a therapeutic target of Alzheimer's disease, in wild-type mice [49].

4.2. Exosomal Modification and Cargo Loading. To amplify the therapeutic effects, many studies try to modify and load various treatment factors into exosomes via various methods. To date, these methods can be classified into two categories: (1) loading after isolation and (2) loading exosomes during biogenesis.

The first approach has been applied to load chemotherapeutic agents, siRNAs, and miRNAs. To reduce immunogenicity and toxicity of doxorubicin, Tian's group facilitated exosomal tumor targeting by engineering mouse immature dendritic cells (imDCs) to express a well-characterized exosomal membrane protein (Lamp2b) fused to a breast cancer-specific iRGD peptide (CRGDKGPDC). Chemotherapeutic agents were loaded via electroporation. The results showed an encapsulation efficiency of up to 20% and exosomal-delivered doxorubicin specific to breast cancer cells in vitro, leading to strong antiproliferative activity without overt toxicity after intravenous injection of BALB/c nude mice [50]. For nucleic acid, electroporation method has also been the first-rank used reported in several studies [49, 51]. Although these studies provided positive delivery outcomes, debates remain. Some studies indicate that siRNA encapsulation is an illusion caused by nonspecific aggregate formation, independent of the exosomes. In addition, no significant encapsulation of siRNA could be measured when aggregate formation was blocked [52]. Therefore, it is necessary to establish multiple protocols for loading exosomes with nucleic acid.

The second approach is based on transfection methods to package active proteins, nucleic acid, and other active molecules into exosomes, where cells are transfected with an engineered effector-expressing vector. Liu's group used this method to load cells with opioid receptor Mu (MOR) siRNA in order to treat drug addiction via downregulating the expression of MOR, the primary target for opioid analgesics used clinically, including morphine, fentanyl, and methadone. This novel study provided a new strategy for the treatment of drug addiction [53]. Similarly, synthesized RNA oligonucleotides were transferred to MSCs in order to produce miR-143-rich exosomes, inhibiting the migratory potential of osteosarcoma cells [54]. Akt was transfected into umbilical cord-derived MSCs by using an adenovirus transfection system that improved cardiac function in animals treated with modified exosomes [55]. In addition, Pascucci reported that MSCs can acquire strong antitumor properties after incubation with paclitaxel (PTX), including the uptake of high drug doses followed by release into exosomes, inhibiting tumor growth activity. This method provides the possibility of using MSCs for the development of drugs with a higher cell-target specificity [56]. Sterzenbach reported the usage of the evolutionarily conserved late-domain (L-domain) pathway as a mechanism for loading exogenous proteins into exosomes [57]. They labeled an intracellular target protein with a WW tag, which was recognized by the L-domain motifs on Ndfip1, resulting in the loading of the target protein into exosomes.

For better tissue-targeting and an enhanced exosomal therapeutic effect, surface modification of exosomes was recently attempted by many groups using gene transfection

Table 2: Studies focusing on MSC-derived exosomes, their loading methods, therapeutic cargos, and biological function.

Origin	Disease models	Biological function	Contributing factors	Artificial modification & loading methods	Articles
Mouse bone marrow-derived MSCs (BM-MSCs)	Breast cancer	Suppress angiogenesis and tumor progression	miR-16	/	Lee et al. [35]
Human adult liver stem cells (HLSCs)	Hepatoma	Transfer genetic information that interferes with the deregulated survival and proliferation	miR451, miR223, miR31, and so on	/	Fonsato et al. [33]
MSCs	Glioma	Reduce glioma xenograft growth in rat brain	miR-146b	Transfection and electroporation	Katakowski et al. [65]
Mouse BM-MSCs	Pancreatic adenocarcinoma	Inhibit in vitro tumor growth	Paclitaxel	Incubation	Pascucci et al. [56]
Human umbilical cord Wharton's jelly mesenchymal stem cells (hWJMSCs)	Bladder tumor	Downregulated phosphorylation of Akt protein kinase and upregulated cleaved caspase 3 during the process of antiproliferation and proapoptosis	Akt and caspase 3	/	Wu et al. [66]
MSCs	Liver injury	Induces an anti-inflammatory effect	Cationized pullulan	Incubation	Tamura et al. [59]
Mouse embryonic fibroblast (MEF) glioblastoma cells	/	Engineer exosomes containing Cre recombinase that have been used to identify functional delivery of exosomes across the blood-brain barrier to recipient neurons in the brain	Labeling of a target protein, Cre recombinase, with a WW tag	Transfection	Sterzenbach et al. [57]
BM-MSCs	Colonic carcinoma gastric carcinoma	Promote VEGF and CXCR4 expression in tumor cells through ERK1/2 and p38 MAPK pathways	/	/	Zhu et al. [67]
Mouse immature dendritic cells (imDCs)	Breast cancer	Target tumor therapy	Doxorubicin	Electroporation transfection	Tian et al. [50]
Human umbilical cord-derived MSCs (hucMSCs)	Acute myocardial infarction	Accelerate endothelium cell proliferation, migration, and vessel formation	Platelet-derived growth factor D (PDGF-D)	Transfection	Ma et al. [55]
BM-MSCs	Status epilepticus (SE)	Minimize the adverse effects of SE in the hippocampus and prevent SE-induced cognitive and memory impairments	/	/	Long et al. [68]
BM-MSCs	Ameliorate inflammation-induced preterm brain injury	Ameliorate inflammation-induced neuronal cellular degeneration, reduce microgliosis, and prevent reactive astrogliosis	/	/	Drommelschmidt et al. [69]
hucMSCs	PBMCs	Immunosuppressive function	/	/	Monguio-Tortajada et al. [70]
Cardiomyocyte progenitor cell (CMPC) MSCs	Human microvascular endothelial cells (HMECs) and human umbilical vein endothelial cells (HUVECs)	Proangiogenic effects	Extracellular matrix metalloproteinase inducer (EMMPRIN)	/	Vrijsen et al. [29]
MSCs	Breast cancer	Deliver antagomiRs to chemosensitize the BCCs and to prevent dormancy	Anti-miR-222/-223	Transfection	Bliss et al. [71]

Table 2: Continued.

Origin	Disease models	Biological function	Contributing factors	Artificial modification & loading methods	Articles
BM-MSCs	PBMCs	Incorporated by monocytes, lowering the effect on lymphocyte populations	/	/	Di Trapani et al. [37]
BM-MSCs	Hepatocellular carcinoma, ovarian cancer, Kaposi's sarcoma	Induce in vitro cell cycle arrest and apoptosis or necrosis of different tumor cell lines and in vivo inhibit growth of established tumors	/	/	Bruno et al. [72]
BM-MSCs	Breast cancer	Induce dormant phenotypes through the suppression of a target gene, MARCKS, which encodes a protein that promotes cell cycling and motility	miR-23b	/	Ono et al. [34]
hucMSCs	Hepatitis C virus (HCV)	Suppress HCV RNA replication	let-7f, miR-145, miR-199a, and miR-221	/	Qian et al. [36]
BM-MSCs	Osteosarcoma	Suppress the migration of the 143B osteosarcoma cell line	miR-143	Transfection	Shimbo et al. [54]

Table 3: Clinical trials of exosome–based therapies.

Study title	Disease	Intervention	Phase	NCT
Effect of plasma-derived exosomes on intractable cutaneous wound healing: prospective trial	Ulcer	Autologous exosomes Rich plasma	Early phase 1	NCT02565264
Study investigating the ability of plant exosomes to deliver curcumin to normal and colon cancer tissue	Colon cancer	Curcumin delivery by exosomes	Phase 1	NCT01294072
Effect of microvesicles and exosomes therapy on β-cell mass in type I diabetes mellitus (T1DM)	Type I diabetes mellitus	Umbilical cord blood-derived MSC microvesicles	Phase 2 Phase 3	NCT02138331
Preliminary clinical trial investigating the ability of plant exosomes to abrogate oral mucositis induced by combined chemotherapy and radiation in head and neck cancer patients	Head and neck cancer Oral mucositis	Dietary Supplement: grape extract Drug: lortab, fentanyl patch, mouthwash	Phase 1	NCT01668849
Pilot study of metformin in head and neck cancer and its effect on proinflammatory cytokines and exosomes implicated in acute and chronic toxicity	Head and neck cancer	Radiation: external beam radiation therapy Drug: metformin hydrochloride Other: placebo	Early phase 1	NCT03109873
Phase II trial of a vaccination with tumor antigen-loaded dendritic cell-derived exosomes on patients with unresectable non-small-cell lung cancer responding to induction chemotherapy	Non-small-cell lung cancer	Tumor antigen-loaded dendritic cell-derived exosomes	Phase 2	NCT01159288
Clinical trial of tumor cell-derived microparticles packaging chemotherapeutic drugs to treat malignant pleural effusion	Malignant pleural effusion	Biological: tumor-derived microparticles Drug: cisplatin	Phase 2	NCT02657460

techniques. The conventional method for surface protein loading was the expression of a genetic fusion between the targeted peptide and a protein that natively localized on the exosomal surface, such as Lamp2 [50]. Similarly, Ohno and colleagues engineered donor cells to express the transmembrane domain of platelet-derived growth factor receptor fused to the GE11 peptide, which efficiently delivered let-7a miRNA to epidermal growth factor receptor- (EGFR-) expressing breast cancer cells [58]. Furthermore, Tamura modified the exosomal surface by a simple mixing of original exosomes and cationized pullulan through an electrostatic interaction of both substances, thus targeting injured liver tissue and enhancing the therapeutic effects [59].

The fate of nucleic acid cargos in target cells remains controversial. For example, Kanada et al. suggests that exosomes cannot deliver functional nucleic acids to target cells by detecting differential fates of transfection-loaded biomolecules (plasmid DNA (pDNA), mRNA, and siRNA) delivered to target cells [60].

4.3. MSCs as an Ideal Source of Exosomes for Drug Delivery. Despite the fact that the properties of natural MSC-derived exosomes are disputed and distinctive of different origins, the use of MSC-derived exosomes has been confirmed for the cell type-specific targeting of drug delivery as a better alternative because of several features. First, exosomes do not elicit acute immune rejection, and there is no risk for tumor formation [61]. Second, MSCs are efficient mass producers of exosomes, which can be manufactured large scale in culture [62], providing support for individualized therapy. Third, the safety of exosomes has been confirmed in vivo by different animal models [63, 64]. To achieve cell-specific targeted drug delivery, several studies have tested donor cells, loading methods, and therapeutic cargos of MSC-derived exosomes. Bone marrow stem cells are typically used as the donor cells, and miRNAs are typically used for therapeutic cargos (Table 2). A phase II-III study has also been processed by a group in Egypt, who hypothesized that intravenous infusion of cell-free umbilical cord blood-derived MSC microvesicles may reduce the inflammatory state, thus improving the β-cell mass and glycemic control in patients with type 1 diabetes (T1DM). However, these outcomes remain controversial, particularly in reference to dose responses. The data reported in several studies is highly dependent on the drug-loading of exosomes, not the quantity of exosomes.

5. Exosomes for Cell-Free Therapy

Currently, the use of exosomes as early diagnostic tools for various types of cancer is underway. In addition, the use of exosomes as diagnostics for prostate cancer is undergoing FDA-approved tests. While complexities surrounding the therapeutic potential of exosomes continue to unravel, several clinical trials (Table 3, data from http://clinicaltrials.gov) have been completed or are underway in order to evaluate this therapeutic potential. In these trials, largely modified exosomes were used rather than native exosomes.

6. Conclusion and Perspective

The therapeutic potential of exosomes presents exciting new avenues for intervention in many diseases. The ability instinct to transport genetic messages and to protect the cargos to natural preferential recipient cells has drawn a rapid rise in attention. Therefore, specialized journals and websites have been established to disseminate this continuously unraveling information. While various clinical trials are underway to evaluate the safety and effectiveness of exosomes as therapeutic targets, many issues still remain. Questions regarding how clinical-grade exosomes can be produced in quantity and how various loading and isolation strategies impact the potency of exosome-based drug delivery remain unanswered. Therefore, there is an urgent need to closely examine the following aspects of exosomes: (1) natural therapeutic potential; (2) biogenesis mechanism; and (3) circulation kinetics and biodistribution. There is still a long road ahead, from promising phenomenological observations to clinical applications.

Conflicts of Interest

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

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Research Article

The DEAD-Box RNA Helicase DDX3 Interacts with m^{6}A RNA Demethylase ALKBH5

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DDX3 is a member of the family of DEAD-box RNA helicases. DDX3 is a multifaceted helicase and plays essential roles in key biological processes such as cell cycle, stress response, apoptosis, and RNA metabolism. In this study, we found that DDX3 interacted with ALKBH5, an m^{6}A RNA demethylase. The ATP domain of DDX3 and DSBH domain of ALKBH5 were indispensable to their interaction with each other. Furthermore, DDX3 could modulate the demethylation of mRNAs. We also showed that DDX3 regulated the methylation status of microRNAs and there was an interaction between DDX3 and AGO2. The dynamics of m^{6}A RNA modification is still a field demanding further investigation, and here, we add a link by showing that RNA demethylation can be regulated by proteins such as DDX3.

1. Introduction

DEAD- (Asp-Glu-Ala-Asp-) box (DDX) proteins are the largest family of RNA helicases [1]. This family plays pleiotropic functions in cells by an interaction with other proteins or different forms of RNA, to maintain the integrity of the secondary and tertiary structure of RNAs and facilitate multiple RNA processing procedures [1–3]. These helicases contain a highly conserved catalytic core domain that mediates the ATPase and helicase activities as well as the less conserved N and C-termini, which are thought to confer functional specificity and subcellular localization of individual DDX helicases [4–6]. DDX3 is multifunctional and is ubiquitously expressed in a wide range of tissues [7, 8]. The protein shuttles between the nucleus and cytoplasm and can localize to P-bodies under stress conditions [9–12]. Over the last years, DDX3 has been reported to play important roles in key biological processes like cell cycle progression, apoptosis, cancer, stress response, hypoxia, and response to radiation [8, 13–15]. DDX3 participates in many steps of RNA metabolism including RNA transcription, RNA splicing, mRNA transportation, and translation initiation [10, 16–19]. Multiple lines of evidence suggest that specific cofactors can modify the functionality of DDX3, such as DDX3 forms functional complex with the transcription factor SP1 (specificity protein 1) and enhances the expression activity of its cognate promoters [16].

The 6-methyladenosine (m^{6}A) RNA methylation is the most prevalent and enriched modification of both coding and noncoding RNAs [20–26]. m^{6}A accounts for about 50% of total methylated ribonucleotides and is present in 0.1%–0.4% of all adenosines in total cellular RNAs [20, 23, 24]. The presence of m^{6}A affects nuclear retention, pre-mRNA splicing, stability of mRNAs, and stability of small RNAs [22, 27–29]. The knockdown of ALKBH5 enhances mRNA export to the cytoplasm [29], whereas the depletion of METTL3 inhibits mRNA export [30]. The demethylase FTO (fat mass and obesity associated) modulates alternative splicing by removing m^{6}A around splicing sites and by inhibiting the binding of serine- and arginine-rich splicing factor

2 (SRSF2) [31]. The presence of m^6A at the 5′ UTR (untranslated region) improves cap-independent translation [32], and eIF3 (eukaryotic initiation factor 3) is proposed to interact with m^6A mark and facilitates ribosome loading [33].

m^6A in mRNAs affects cell differentiation and the expression of numerous genes including transcription factors [34]. For instance, m^6A affects the differentiation of preadipocytes during adipogenesis [31, 35]. Exposure of the breast cancer stem cell (BCSC) to hypoxia induces m^6A demethylation of a key pluripotency factor, NANOG, by ALKBH5 [36]. Demethylation of NANOG increases transcript stability and promotes BCSC proliferation [36]. The depletion of methyltransferases *Mettl3* (methyltransferase-like 3) and *Mettl14* (methyltransferase-like 14) leads to low levels of m^6A and reduced self-renewal of mouse ES cells [37].

The m^6A modification is posttranscriptionally installed by a multicomponent methyltransferase complex with at least three core proteins, namely, METTL3, METTL14, and Wilms' tumor 1-associating protein (WTAP) [38, 39]. In recent years, the discoveries of the two RNA m^6A demethylases, FTO and alkylated DNA repair protein AlkB homolog 5 (ALKBH5), demonstrate that the m^6A RNA modification can be erased [29, 40].

In this study, we have showed that DDX3 and ALKBH5 interacted with each other and DDX3 could modulate the m^6A demethylation of RNAs. Since the dynamics of m^6A RNA modification was largely elusive, here, we demonstrated that RNA demethylation could be subjected to regulation.

2. Materials and Methods

2.1. Cell Culture and Transfection. HEK293T and HeLa cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 1% antibiotics and 10% fetal bovine serum (FBS). Cells were cultured at 37°C in a humidified 5% (vol/vol) CO_2 incubator. All plasmid or siRNA transfections were carried out using Lipofectamine 2000 (Invitrogen) according to the manufacturer's protocol.

2.2. Plasmid Construction. All plasmids were constructed with recombinant method and/or restriction enzyme digestion and ligation. The full-length and all deletion constructs for DDX3 were cloned into p3xFLAG-Myc-CMV vector (Sigma-Aldrich). The full length and all deletion constructs for methyltransferases and demethylases were cloned into pKH3-HA vector. All constructs were confirmed by sequencing. Primer sequences used along with other oligo information were included in Supplementary Table S1 available online at https://doi.org/10.1155/2017/8596135.

2.3. Immunoprecipitation. The cells were washed twice with ice-cold PBS and cross linked with UV 12000 J/cm^2 for 2 min in PBS. The cells were incubated on ice in modified RIPA lysis buffer (150 mM NaCl, 50 mM Tris pH 8.0, 1% Nonidet P-40, 0.5% deoxycholate, and a protease inhibitor mixture (Roche Applied Science, Mannheim, Germany)) for 20 min and rotated at 4°C for 10 min. Then, lysate was sonicated typically for 5 min at 30% amplitude, 3 sec pulses followed by 6 sec rest period. The lysates were clarified by centrifugation for 20 min at 14,000 rpm at 4°C. Fifty (50 μl) of Dynabeads Protein G magnetic particles (Invitrogen) were resuspended, 500 μl of lysis buffer. Then, 1.5 μg of respective antibodies was added to the beads and incubated on a rotating wheel at room temperature for 30 min. Beads were precipitated by magnet and finally resuspended again in cleared cell lysates and incubated for 2 h at 4°C. In the last step, the beads were resuspended in 50 μl of lysis buffer and subjected to Western blots.

2.4. Western Blotting. The proteins either from cell lyses or isolated from the IP beads were subjected to SDS-PAGE and then transferred on to a polyvinylidene difluoride (PVDF) membrane. Following blocking the membrane was probed using the corresponding primary antibodies overnight at 4°C. HA-Tag (C29F4) rabbit monoclonal, monoclonal anti-FLAG M2 (Sigma-Aldrich), mouse DDX3 (C-4) mAb, rabbit polyclonal anti-ALKBH5 (Sigma-Aldrich), and mouse anti-GAPDH antibody (Signalway) were used. After washing, the membrane was incubated with secondary horseradish peroxidase-conjugated goat anti-rabbit IgG (1 : 5000; ZB-2301) or anti-mouse IgG antibodies for 2 h at room temperature. Enhanced chemiluminescence substrates (EMD Millipore) were then applied, and signals were detected using a chemiluminescence imaging system (ChemiDoc™ MP Imaging System; Bio-Rad Laboratories Inc.).

2.5. RNA Extraction and Real-Time Quantitative PCR (qRT-PCR). Total RNA from cells was isolated using TRIzol reagent (Invitrogen) according to the manufacturer's protocol. The RNA concentration was determined by spectrophotometer. cDNA for qRT-PCR was synthesized from total RNA by the GoScript Reverse Transcription System (Promega) according to the supplied protocol using random hexamer primer and oligo dT. qPCR was performed with the GO Taq qPCR Master Mix (Promega) on the PikoReal Real-time PCR System (Thermo Scientific) according to the standard procedures.

2.6. MicroRNA and Poly(A) mRNA Isolation. MicroRNA and poly(A) mRNA from cells were extracted using the mirVana™ miRNA Isolation Kit and PolyAT tract mRNA Isolation System (Promega), respectively, according to the manufacturer's protocol.

2.7. Protein Complex Modeling. The complex modelling was performed by docking $ALKBH5_{190–293}$ to $DDX3_{211–402}$ on a Rosetta web server (http://rosie.rosettacommons.org/docking2). The parameters used for docking are set to default [41–43]. Docking results are open to public. The docking job of $ALKBH5_{190–293}$ to $DDX3_{211–402}$ was assigned an ID (33161) or can be accessed with the following link http://rosie.rosettacommons.org/docking2/viewjob/33161. Model with the best score was analyzed with PyMOL software (The PyMOL Molecular Graphics System, Version 1.8 Schrödinger, LLC). Structures used for modelling are

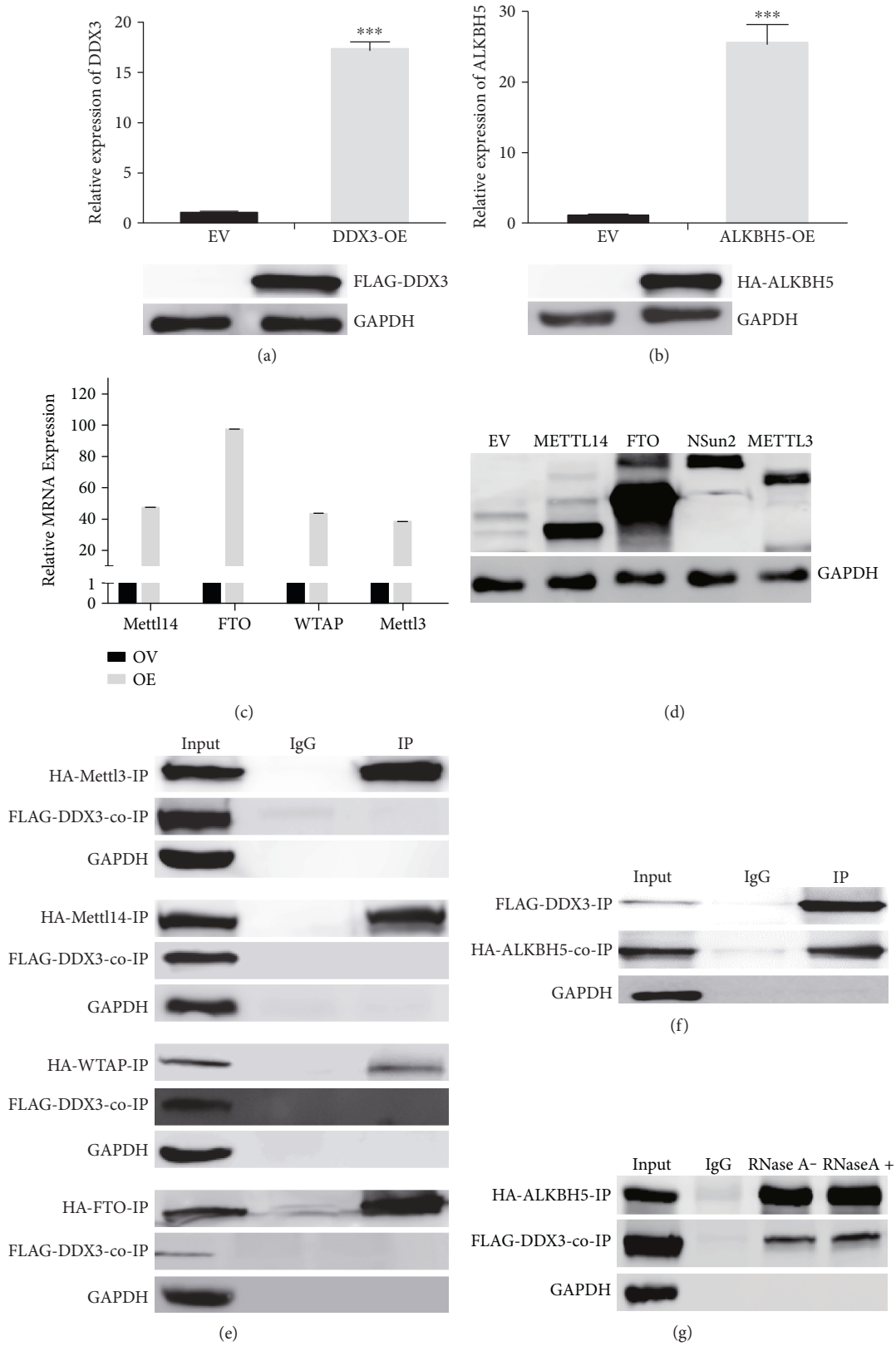

Figure 1: Continued.

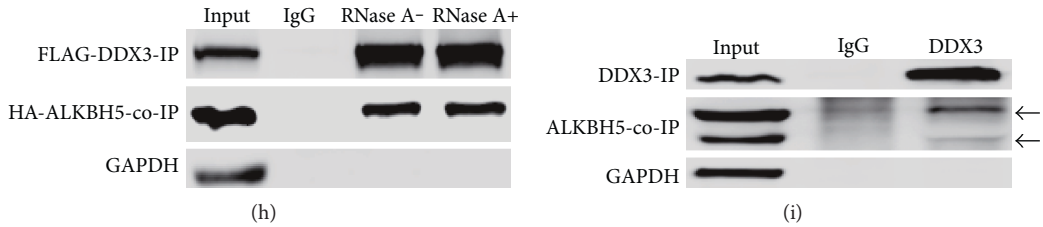

Figure 1: Identification of interaction between DDX3 and ALKBH5. (a) FLAG-DDX3 overexpression in HEK293T cells, relative DDX3 mRNA (upper panel), and protein levels (lower panel) are shown. (b) HA-ALKBH5 overexpression in HEK293T cells, ALKBH5 mRNA (upper panel), and protein levels (lower panel) are shown. (c) Overexpression of methyltransferases and demethylases in HEK293T cells, mRNA levels examined by qPCR. (d) Overexpression of the corresponding protein (indicated with arrowhead) examined by Western blots. (a–d) EV, empty vector; OE, overexpression. (e) DDX3 showed no interaction with the METTL3, METTL14, WTAP, or FTO, examined with IP and co-IP. (f) The interaction of ALKBH5 with DDX3 examined with FLAG-DDX3 IP and HA-ALKBH5 co-IP in HeLa cells. (g) The interaction of ALKBH5 with DDX3 in the presence or absence of RNase A, examined with HA-ALKBH5 IP and FLAG-DDX3 co-IP in HEK293T cells. (h) The interaction of ALKBH5 with DDX3 in the presence or absence of RNase A, examined with FLAG-DDX3 IP and HA-ALKBH5 co-IP in HEK293T cells. (i) Endogenous DDX3 interacted with endogenous ALKBH5, examined with IP and co-IP; two known isoforms of ALKBH5 are indicated with arrowheads. IP and co-IP were performed in triplicates, and representative results are shown. $^{***}P < 0.001$; P values were determined with two-tailed Student's t-test; error bars represent standard deviation (SD).

obtained from protein data bank, accession numbers are 2I4I for $DDX3_{211-402}$ [44] and 4061 for $ALKBH5_{190-293}$ [45].

2.8. Analysis of m^6A Level Using Dot-Blot Assay. The m^6A dot-blot was performed on a Bio-Dot Apparatus (Bio-Rad Laboratories Inc.). In brief, the RNA samples were denatured and spotted to nitrocellulose membrane under vacuum. After UV cross-linking, the membranes were baked at 80°C for 1 hr, and methylene blue staining was used to examine equal RNA loading. For detecting m^6A levels, rabbit anti-m^6A antibody (Millipore Sigma) was diluted with 1 : 500 in 0.1% TBST and 5% nonfat dry milk and incubated with the membranes overnight (4°C). Following extensive washing with 0.1% TBST, the blot was incubated with horseradish peroxidase- (HRP-) conjugated anti-rabbit IgG secondary antibody for 2 h at 25°C. The membrane was washed again with 0.1% TBST and visualized by ECL Western Blotting Detection Kit (Thermo Scientific). Dots were quantified with imageJ.

2.9. Cell Proliferation Assay. Cell viability was measured with the MTT Cell Proliferation and Cytotoxicity Detection Kit (Keygentec, Nanjing, China) according to the manufacturer's recommendations. Cells in 96-well plates were plated at a density of 2×10^3 per well. Cells were then transfected with siRNAs or scrambled control. MTT reagents were added at indicated time points. Four hours later, the supernatant was removed, and DMSO was added to dissolve the blue precipitates. The number of live cells was determined by the OD value, which was measured by a plate reader (MultiSkan GO, Thermo Scientific).

3. Results

3.1. DDX3 Interacted with ALKBH5. To unveil the role of DDX3 in RNA methylation, we started to determine whether DDX3 physically interacted with m^6A methyltransferases and demethylases. We constructed expression plasmids of METTL3, METTL14, WTAP, FTO, and ALKBH5. We cotransfected HEK293T cells with individual expression plasmids together with DDX3 expression plasmid (Figures 1(a), 1(b), 1(c), and 1(d)). The successful overexpression was confirmed by real-time qPCR and Western blot (Figures 1(a), 1(b), 1(c), and 1(d)). We then performed immunoprecipitation (IP) to examine their interaction with DDX3 (Figures 1(e), 1(f), 1(g), 1(h), and 1(i)). It was found that IP of METTL3, METTL14, WTAP, or FTO could not co-IP DDX3 (Figure 1(e)). In contrast, IP of ALKBH5 could co-IP DDX3 (Figures 1(f) and 1(g)). Furthermore, IP of DDX3 could co-IP ALKBH5 (Figure 1(f)). These data demonstrated that ALKBH5 was the only protein among the five methyltransferases and demethylases that interacted with DDX3.

The interaction between DDX3 and ALKBH5 was not RNA dependent, as the interaction was essentially unchanged with or without RNase A treatment (Figures 1(f) and 1(g)). We further examined whether they interacted with each other endogenously by performing IP with antibodies against DDX3 and indeed ALKBH5 could be co-IPed (Figure 1(h)).

3.2. ATP Domain of DDX3 Interacted with DSBH Domain of ALKBH5. We further asked which domain of DDX3 interacted with ALKBH5. For this purpose, 5 partially deleted constructs of DDX3 were constructed (Figure 2(a)). Each of these constructs was then used to perform IP, and full length ALKBH5 was then examined for the co-IP in HEK293T cells (Figures 2(a), 2(b), 2(c), and 2(d)). Among these constructs, deletion of either N-terminal domain, Linker domain, Helicase domain, or the C-terminal domain still showed interaction with the full length ALKBH5 (Figure 2(b)). However, upon deletion of the ATP-binding domain (DDX3-ΔATP), the binding with ALKBH5 was abolished (Figure 2(c)). IP of full length ALKBH5 also did not co-IPed DDX3-ΔATP (Figure 2(c)). Interestingly, the ATP-binding domain of DDX3 itself could interact with the full length ALKBH5 (Figure 2(d)). These results showed that ATP–binding

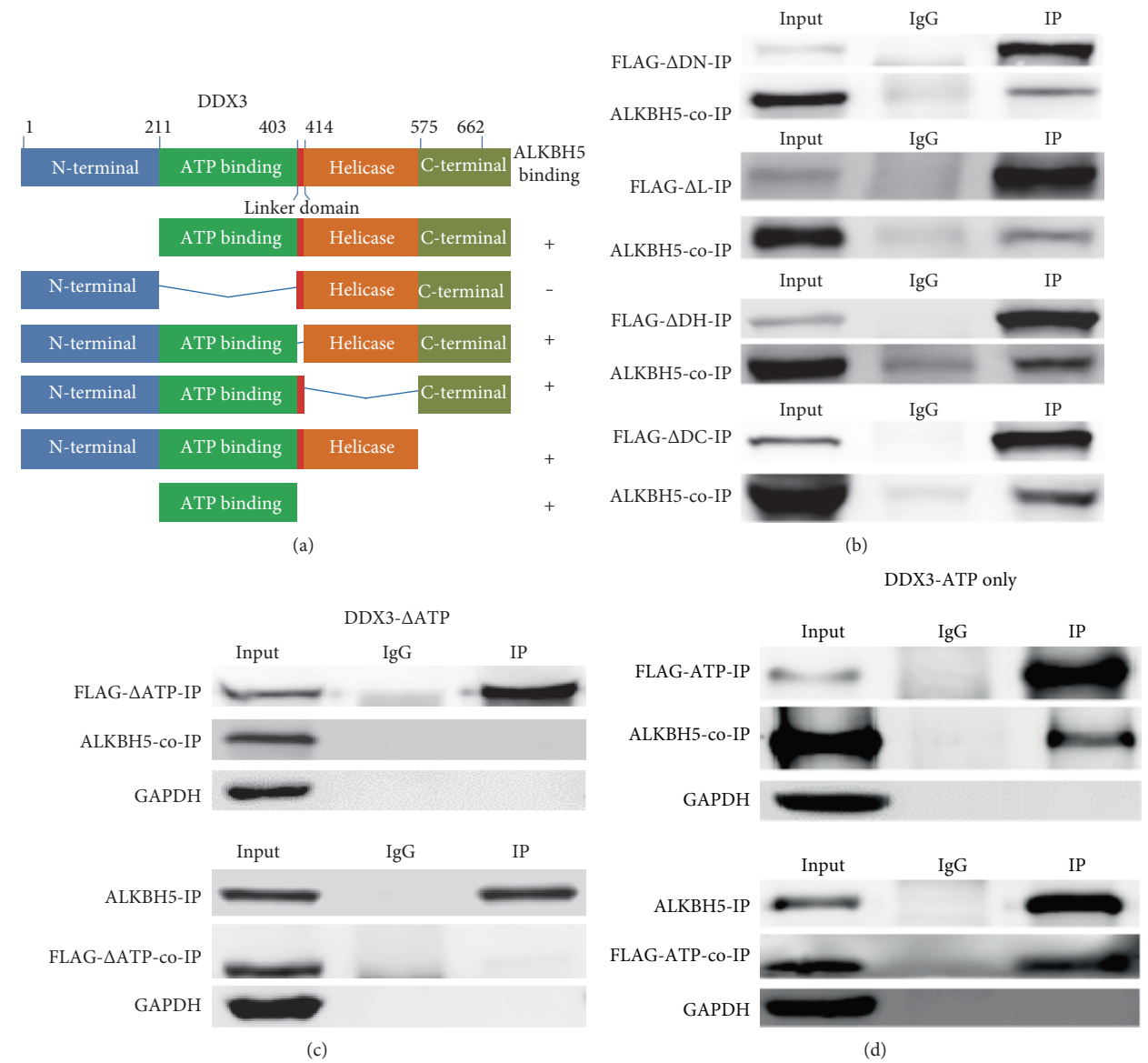

Figure 2: Determination of the binding domain of DDX3 with ALKBH5. (a) Schematic diagram of full-length DDX3 and the corresponding partial deletion constructs; summary of the results of interaction with ALKBH5 was also shown. (b) Deletion of N-terminal domain, Linker domain, Helicase domain, or the C-terminal domain of DDX3 showed interaction with the full-length ALKBH5, as examined with IP and co-IP. (c) The interaction was abolished when ATP domain of DDX3 was deleted, as examined with IP and co-IP. (d) ATP domain of DDX3 alone interacted with full-length ALKBH5, examined with IP and co-IP. All IP and co-IP were performed in triplicates, and representative results were shown.

domain but not the other domains of DDX3 was indispensable to interact with ALKBH5.

3.3. DSBH Domain of ALKBH5 Interacted with ATP Domain of DDX3. Next, we mapped the domain in ALKBH5 responsible for its interaction with DDX3. For this purpose, 4 partially deleted constructs of ALKHB5 were constructed (Figure 3(a)). Then, each construct was examined for interaction with the full-length DDX3 using co-IP analyses. Among these constructs, deletion of the N-terminal domain, D-domain, or C-terminal domain had no effect on the binding efficiency with DDX3 (Figure 3(b)). However, when the DSBH domain of ALKBH5 was deleted, the interaction with full-length DDX3 was abolished (Figure 3(c)). Furthermore, the DSBH domain of ALKBH5 alone could interact with the full-length DDX3 (Figure 3(d)). These results showed that DSBH domain of ALKBH5 is necessary and sufficient for the interaction with DDX3. Additionally, ATP-binding domain of DDX3 and DSBH domain of ALKBH5 could interact with each other (Figures 3(e) and 3(f)).

A model illustrating interactions between $DDX3_{211–402}$ and $ALKBH5_{190–293}$ was predicted by Rosetta docking server (Figure 3(g)). The results imply a direct interaction between DDX3 and ALKBH5. Residues potentially essential for DDX3-ALKBH5 interactions locate in loop region of these two proteins. The model suggests hydrogen bond occurring

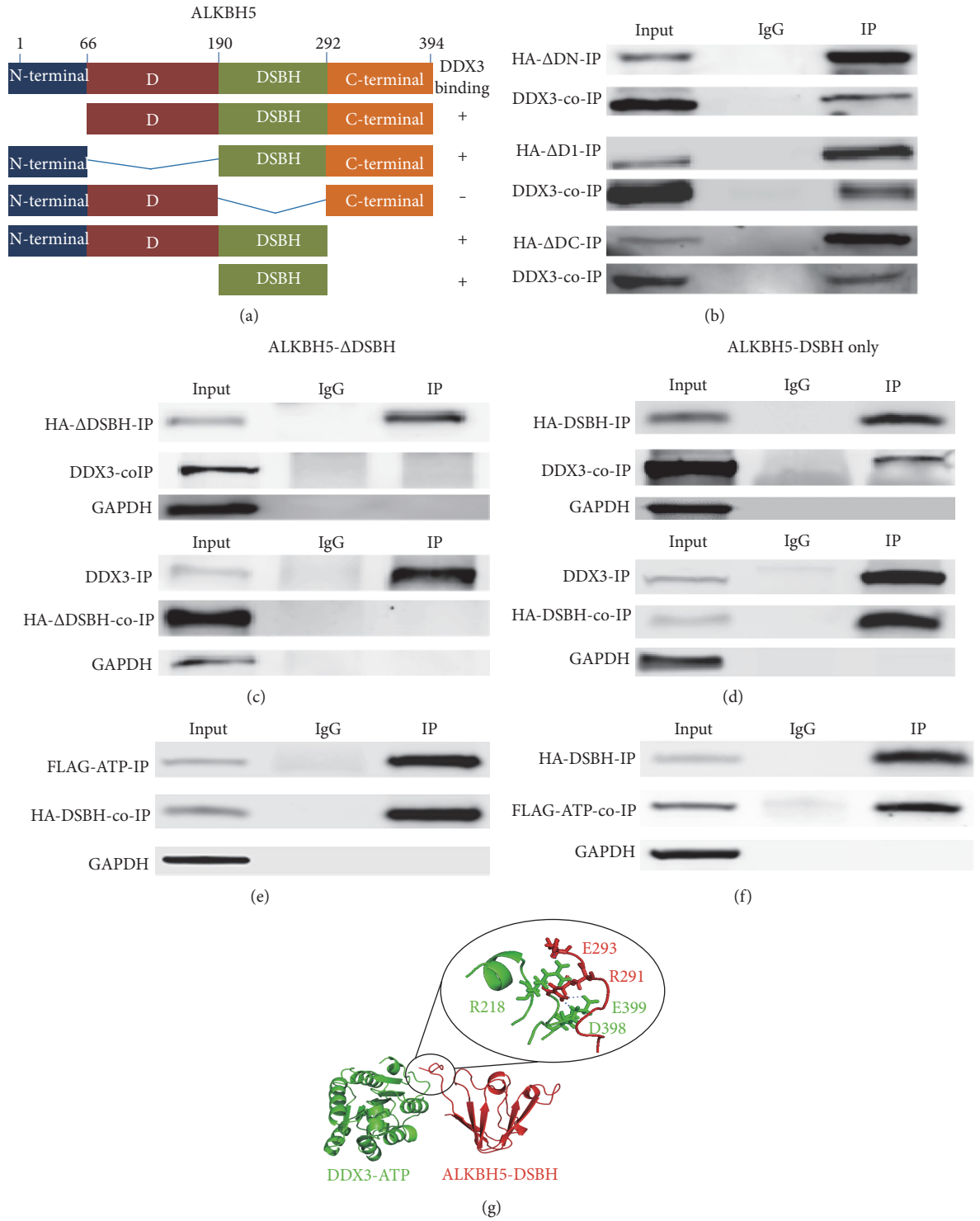

Figure 3: Determination of the binding domain of ALKBH5 with DDX3. (a) Schematic diagram of full-length ALKBH5 and the corresponding partial deletion constructs; summary of the results of interaction with DDX3 was shown. (b) Deletion of the N-terminal domain, D-domain, or C-terminal domain of ALKBH5 showed interaction with full-length DDX3, examined with IP and co-IP. (c) Deletion of the DSBH domain of ALKBH5 abolished its interaction with DDX3 when examined with IP and co-IP. (d) DSBH domain of ALKBH5 alone interacted with full-length DDX3, examined with IP and co-IP. (e) ATP domain of DDX3 interacted with DSBH domain of ALKBH5, examined with IP and co-IP. (f) DSBH domain of ALKBH5 interacted with ATP domain of DDX3, examined with IP and co-IP. (b–f) All IP and co-IP were performed in triplicates, and representative results are shown. (g) The predicted interaction between ATP domain of DDX3 ($DDX3_{211–402}$) and DSBH domain of ALKBH5 ($ALKBH5_{190–293}$). $DDX3_{211–402}$ is labeled in green, and $ALKBH5_{190–293}$ is labeled in red. The circle indicates the interacting region of these two domains, and a magnified view displays residues involved in formation of intermolecular hydrogen bonds. The hydrogen bonds are presented in blue dash lines.

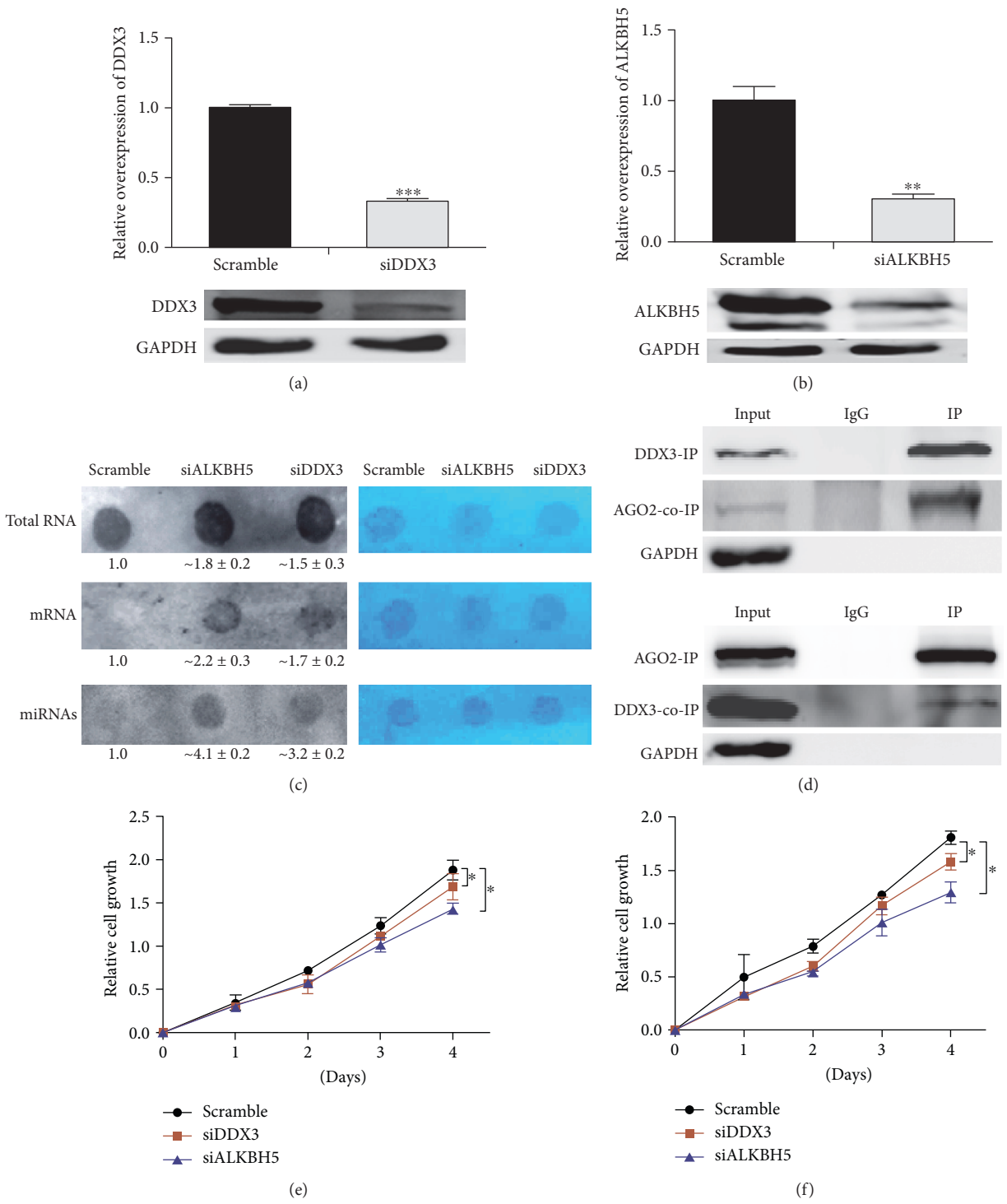

FIGURE 4: DDX3 modulated m^{6}A RNA demethylation. (a) DDX3 knockdown in HEK293T cells, relative DDX3 mRNA (upper panel, qPCR), and protein levels (lower panel, Western blots) are shown. (b) ALKBH5 knockdown in HEK293T cells, ALKBH5 mRNA (upper panel, qPCR), and protein levels (lower panel, Western blots) are shown. (c) Left panel: dot-blot analyses of m^{6}A levels of isolated total RNA, mRNA, and miRNA from ALKBH5 knockdown, DDX3 knockdown, and control (NC, siRNA with scrambled sequences) cells. Right panel: methylene blue staining showing equal RNA loading. (d) IP of endogenous DDX3 could co-IP endogenous AGO2, and IP of endogenous AGO2 could co-IP endogenous DDX3 in HEK293T cells. (e) Quantification of cell proliferation (MTT assay) after knockdown of DDX3 or ALKBH5 in HEK293T cells. (f) Quantification of cell proliferation (MTT assay) after knockdown of DDX3 or ALKBH5 in HeLa cells. Dot blots, IP, and co-IP were performed in triplicates, and representative results were shown. Scramble siRNA with scrambled sequences. $^*P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$. P values were determined with two-tailed Student's t-test. Error bars represent standard deviation (SD).

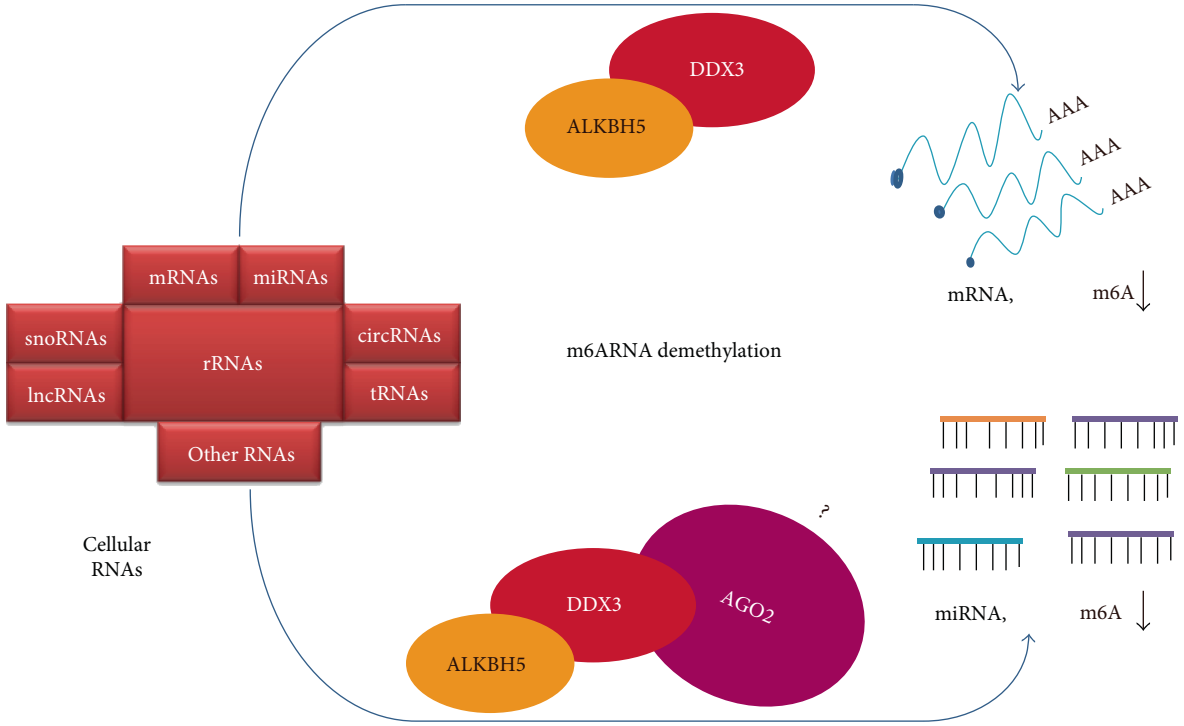

Figure 5: Working model for the role of ALKBH5 and DDX3 in m^{6}A RNA demethylation. DDX3 interacts with ALKBH5 to regulate mRNA demethylation and additionally, by interacting with AGO2, may modulate microRNA demethylation. Whether there is direct interaction between ALKBH5 and AGO2 requires further investigations.

between residue Arg218 of DDX3 and Glu293 of ALKBH5 and between Arg291 of ALKBH5 and Asp398 and Glu399 of DDX3, respectively.

3.4. DDX3 Modulated m^{6}A RNA Demethylation. We carried out siRNA-mediated knockdown of DDX3 and ALKBH5, respectively, in HEK293T cells. The mRNA and protein levels of either DDX3 or ALKBH5 were significantly downregulated (Figures 4(a) and 4(b)). We then analyzed m^{6}A modification in total RNA, mRNA, and miRNA. After knockdown of ALKBH5 or DDX3, increased m^{6}A modification signals were observed (Figure 4(c)). Thus, DDX3 positively modulated the demethylation effect. It has been reported previously that DDX3 and AGO2 colocalize with each other [46] which prompted us to determine whether DDX3 could physically interact with AGO2. Indeed, in co-IP experiments, AGO2 protein was shown to interact with DDX3 (Figure 4(d)). Together, these lines of evidence suggested a possible role of DDX3 in miRNA demethylation through interacting with AGO2.

3.5. Knockdown of DDX3 and ALKBH5 Decreased Cell Proliferation. m^{6}A RNA methylation is known to affect stem cell renewal and differentiation [34, 35]. To investigate the effect of DDX3 and ALKBH5 on cell growth, DDX3 and ALKBH5 were downregulated in HEK293T and HeLa cells with siRNAs. MTT assay showed that a significant decrease in the growth curve of both cell lines (Figures 4(e) and 4(f)). These results indicated a potential role of DDX3 and ALKBH5 in regulating cell growth, highly possibly through the modulating of m^{6}A levels and in proliferating cells.

4. Discussion

Our results demonstrate that DDX3 interacts with RNA demethylase ALKBH5 and AGO2. These interactions and the results of DDX3 effects on the m^{6}A levels suggest a tantalizing working model that DDX3 serves as a "mediator" for the modulation of demethylation of either mRNAs or microRNAs by ALKBH5 (Figure 5).

The m^{6}A is a conserved posttranscriptional modification of both coding and noncoding RNAs, which has essential roles in multiple cellular processes [20, 21, 47–49]. Recent researches have demonstrated cellular and physiological roles of m^{6}A [27, 28, 50]. It is well accepted that m^{6}A methylation plays critical roles in mRNA splicing and translation [27, 28]. Methylation of microRNAs has also been shown to have functional consequences [25, 26]. Various cellular conditions exhibit changes in m^{6}A RNA methylation, which is associated with changes in the expression of methyltransferases and demethylases [22, 32, 34, 50]. Yet, the molecular regulatory mechanisms of either the addition or the removal of m^{6}A modification require further investigations. Our results indicate that DDX3 may mediate or at least modulate the demethylation activities of ALKBH5. It seems that DDX3 is rather specific for ALKBH5 among the methyltransferases and demethylases tested (Figure 2). It is tempting to propose that other methyltransferases or demethylases may also have the corresponding specific modulators.

DDX3 plays diverse cellular functions by interacting with different proteins through its different domains. In the present study, we describe a new role for DDX3 in RNA demethylation by physical interaction with the m^{6}A RNA demethylase ALKBH5 and AGO2. The ATP domain (AA 212–403) of DDX3 and the DSBH domain (AA 191–292) of ALKBH5 are responsible for their interaction (Figures 2 and 3). The conserved core segment of DDX3 (AA 227–534) is responsible for interaction with PABP1 [19]. DDX3 C-terminal region AA 260–517 fragment is required for its association with CRM1 [9]. DDX3 specifically represses cap-dependent translation by binding to eIF4E through its N-terminal 100 amino acid fragment [18]. Thus, DDX3 is involved in many biological processes via its different domains to interact with distinct proteins.

m^{6}A RNA methylation is known to affect cell renewal and differentiation [31, 35]. In the present study, we have shown that knockdown of DDX3 or ALKBH5 decreases cell proliferation in both HEK293T and HeLa cell lines (Figures 4(e) and 4(f)). Interestingly, previous study has shown that BCSCs under hypoxic conditions have higher levels of ALBKH5 expression in a HIF-1α- and HIF-2α-dependent way, which eventually leads to enrichment of BCSCs in the hypoxic tumors [36]. On the other hand, previous studies have also shown that depletion of methyltransferase *Mettl13* or *Mettl14* reduced self-renewal of mouse ESCs [37].

In this study, we have identified DDX3 as a partner of ALKBH5 and AGO2 to regulate the demethylation of mRNAs and miRNAs. Further studies would help to elucidate how these interactions contribute to regulated dynamics of m^{6}A epitranscriptome and the functional relevance and physiological roles of DDX3 in the context of m^{6}A modification.

5. Conclusion

In this study, we found that DDX3 interacted with ALKBH5, an RNA m^{6}A demethylase. We found ATP domain of DDX3 and DSBH domain of ALKBH5 to be indispensable to their interaction with each other. Furthermore, DDX3 could modulate the demethylation of mRNAs. We also found the interaction between DDX3 and AGO2, and DDX3 could modulate the demethylation of miRNAs. The dynamics of m^{6}A RNA modification was largely elusive, and here, we showed that RNA demethylation could be regulated by proteins such as DDX3.

Conflicts of Interest

The authors declare that there are no conflicts of interest.

Authors' Contributions

Abdullah Shah, Farooq Rashid, and Hassaan Mehboob Awan contributed equally to this work.

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Research Article

Plasma Rich in Growth Factors Induces Cell Proliferation, Migration, Differentiation, and Cell Survival of Adipose-Derived Stem Cells

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Adipose-derived stem cells (ASCs) are a promising therapeutic alternative for tissue repair in various clinical applications. However, restrictive cell survival, differential tissue integration, and undirected cell differentiation after transplantation in a hostile microenvironment are complications that require refinement. Plasma rich in growth factors (PRGF) from platelet-rich plasma favors human and canine ASC survival, proliferation, and delaying human ASC senescence and autophagocytosis in comparison with serum-containing cultures. In addition, canine and human-derived ASCs efficiently differentiate into osteocytes, adipocytes, or chondrocytes in the presence of PRGF. PRGF treatment induces phosphorylation of AKT preventing ASC death induced by lethal concentrations of hydrogen peroxide. Indeed, AKT inhibition abolished the PRGF apoptosis prevention in ASC exposed to 100 μM of hydrogen peroxide. Here, we show that canine ASCs respond to PRGF stimulus similarly to the human cells regarding cell survival and differentiation postulating the use of dogs as a suitable translational model. Overall, PRGF would be employed as a serum substitute for mesenchymal stem cell amplification to improve cell differentiation and as a preconditioning agent to prevent oxidative cell death.

1. Introduction

Adipose-derived stem cell (ASC) transplantation has already demonstrated effectiveness and continues to be an important avenue of research and development due to their extraordinary therapeutic aptitude. ASC transplantation has shown remarkable restorative ability reflected in the significant recovery of function in patients with a range of diseases such as Parkinson's, Alzheimer's, spinal cord injury, heart disease, and rheumatoid arthritis [1]. Nevertheless, new approaches for ASC isolation and amplification have been developed [2–4], to generate sufficient amount of stem cells to optimize clinical applications [5]. However, the paucity of information regarding ASC survival after transplantation lends itself to further investigation of ASC quantity and quality before transplantation. For instance, ASC activation with defined stimuli prior transplantation may enhance ASC repair capabilities and improve success rates for regenerative treatments. Recent studies have focused on increasing the yield, efficiency, and therapeutic capability of ASC by treating them with growth factors like PDGF or bFGF [6–8] or as a xeno-free alternative for mesenchymal stem cell expansion [5, 9]. A major source of endogenous growth factors is the plasma rich in growth factors (PRGF) from platelet-rich plasma [10]. PRGF has been extensively used in many species to reduce healing time and to improve tissue regeneration [7, 11]. PRGF beneficial effects are modulated by the degranulation of alpha granules in platelets [12, 13] which contain

several important growth factors that stimulate cell growth, proliferation, and differentiation [14, 15]. PRGF stimulates undifferentiated stem cells to proliferate and differentiate and has been used for tissue regeneration [8, 16–18] purposes. Undifferentiated stem cells migrate to the concentration of platelet-releasing growth factors triggering proliferation of the cells at the site [19]. Moreover, platelet-derived growth factors enhance sternness of ASC [20] being proposed as a gold standard fetal bovine serum replacing method for human cell propagation for clinical applications [5, 21, 22]. Additionally, PRGF has also shown synergistic properties on mesenchymal stem-induced differentiation which accelerate bone [23] or cartilage repair [24].

Minimizing aging of ASC cultures represents a significant challenge for tissue engineering especially for autologous cell-based approaches in geriatric patients [25]. New approaches have been considered to avoid ASC senescence for prolonged ex vivo expansion or induced differentiation avoiding, for instance, hypertrophic phenotypes on chondrocytes-induced differentiation process [26], by forcing the expression of hTERT, and telomerase activity induction, to extend the lifespan of the mesenchymal cells on culture. Previous reported data shows that hTERT-transduced mesenchymal stem cells have prolonged replicative capacity in vitro and keep the adipo-, chondro-, and osteogenic differentiation potential in vitro and osteogenic potential in vivo [27, 28]. Importantly, although there was no evidence for tumor formation or cell transformation, nongenetic manipulations will be more suitable for further potential clinical applications.

The serine/threonine kinase AKT represents an initial signaling node within all cells of higher eukaryotes contributing significantly to the regulation of survival, growth, proliferation, angiogenesis, and metabolism of many cell types [29]. AKT activation by PRGF enhances survival and regenerative function in ASC [30]. Furthermore, conditioning of ASC by inducing AKT activity increases cell survival and proangiogenic capacities [31]. The induction of cell survival signals, such as AKT, would also confer a resistance to hostile environments such as those associated with an inflammatory response, which induce cell death by oxidative stress [32].

2. Material and Methods

2.1. ASC Isolation and Culture. Adipose tissue was collected from dogs and humans, both of which were suffering from osteoarthritis, and was performed in an operating room by veterinarians and physicians, respectively. A 10 g biopsy of subcutaneous fat from dog patients [8] or from the suprapatellar fat pad from human patients [33] was collected, $n = 4$ (dogs) and $n = 4$ (humans). All procedures were performed under sterile conditions, and the adipose tissue was placed into sterile conical tubes containing sterile saline. The experimental procedures for dogs did not require evaluation by the animal Ethics Committee because the procedure only included a cession of part of the amplified ASCs needed for cell transplantation, and for this purpose, the canine owners voluntarily signed an informed consent for the use of surplus adipose tissue utilized for the derivation of ASCs and further research purposes. The human samples were anonymized, and this experimental procedure has been evaluated and accepted by the Regional Ethics Committee for Clinical Research with Medicines and Health Products following the Code of Practice 2014/01. As exclusion criteria, no samples were collected from patients with a history of cancer or infectious diseases at the time of the surgery (viral or bacterial). All human patients voluntarily signed an informed consent document for the use of surplus adipose tissue and donation of peripheral blood (20 ml) collected sodium citrate containing tubes for PRGF isolation prepared following the standardized method described in Anitua et al. [14], pooled to minimize differences between individuals and stored at −20°C.

Adipose tissue was transferred from the surgery room in an enclosed package at 4°C in sterile solution and arrived at the laboratory within 24 h after extraction. Each sample was washed multiple times in PBS plus antibiotics to clean the tissue and remove residual blood. Adipose tissue was then placed into sterile Petri dishes (10 g adipose tissue per 100 mm Petri dish), in a solution containing PBS, 100 units/ml penicillin and 100 μg/ml streptomycin (Gibco 15140), collagenase type I-A (0.07%, Sigma-Aldrich C9891 CA, USA), and dispase I (0.2 mM, Sigma-Aldrich). The adipose tissue was manually cut into small pieces using sterile surgical scissors in a laminar flow hood and transferred to a cell flask for overnight digestion in a shaker at 37°C, 20% O_2, and 5% CO_2. On the following day, the digested adipose tissue was collected and washed multiple times with PBS plus antibiotics by serial centrifugation. The cell pellet was then resuspended in growth medium (DMEM containing 2 mM L-glutamine, 30% L-glucose, 100 units/ml penicillin, and 100 μg/ml streptomycin), plus 10% fetal bovine serum (FBS) for canine samples or 10% human serum (HS) for human samples. On the following day, the medium was removed and replaced with fresh medium and attached cells were allowed to grow until nearly confluent and then subjected to cell proliferation, survival studies, and cell differentiation assays.

2.2. Cell Proliferation Assay. At passages 3-4, canine and human ASCs, 10^4 cells were seeded in 24-well plates in the presence of 10% of FBS or HS, respectively, for 24 hours. Then, the medium was replaced for 0, 1, 2.5, 5, or 10% of FBS or HS or PRGF-containing medium and cells were maintained on culture for 24 hours incubation. For cell quantification, each well was trypsinized and counted in a Neubauer® chamber. Three independent experiments were performed in triplicates. Data was expressed as mean ± SD.

2.3. Cell Migration and Invasion Analysis by In Vitro Scratch Assay. Cell migration for wound healing of the induced in vitro scratch was performed in the IncuCyte® S3 live-cell analysis [34, 35]. 15×10^5 ASCs were seeded in 96-well plates for a monolayer cell culture in the presence of 10% of HS. 24 hours after seeding, the scratch was performed at the time in all wells by using the WoundMaker tool. Then, the medium was replaced by 0% HS or PRGF, 2.5%, or 10% of HS or PRGF in triplicates. Cell growth and migration were monitored under phase-contrast microscopy connected to

a time-lapse recording system at the scratched area, every hour during 16 h. A picture from four different fields at each experimental condition was recorded for time-lapse reconstruction and to quantify the cell density of repopulated scratched area. Image analysis was automatically performed by the associated software by following the reported mathematical analysis in [35]. The results are shown as the mean ± SD of the % of relative wound density at every experimental condition.

2.4. Senescence-Associated Beta-Galactosidase (SAβGal) Activity. Cells were seeded at 2000 cells/cm^2 in six-well plates in the presence of 10% of HS. 24 hours after seeding, the medium was replaced for 2.5% PRGF, 2.5% HS, 10% HS, or 0% of PRGF or HS. 48 hours after incubation, cells were fixed in 4% paraformaldehyde for 10 minutes and assayed for SAβ-Gal activity as described by Debacq-Chainiaux et al. [36]. SAβGal-positive cells were counted with a minimum of 200 cells overall for each condition. Three independent experiments were performed. Data was expressed as the mean ± SD of the percentage ratio of βGal-positive cells within the total assayed cell culture.

2.5. Transmission Electron Microscopy and Autophagosome Quantification. Cells were seeded at 2000 cells/cm^2 in Lab-Tek chamber slides (Nalge Nunc International, Naperville, IL) in the presence of 10% of HS. 24 hours after seeding, the medium was replaced for 2.5% PRGF, 2.5% HS, 10% HS, or 0% of PRGF or HS. 48 hours after incubation, cells were fixed in 3% glutaraldehyde for 1 hour at 37°C. Cells were post fixed in 2% OsO_4 for 1 hour at room temperature and stained in 1% uranyl acetate in the dark for 2 h at 4°C. Finally, cells were rinsed in distilled water, dehydrated in ethanol, and infiltrated overnight in Durcupan resin (Fluka, Sigma-Aldrich, St. Louis, USA). Following polymerization, embedded cultures were detached from the chamber slide and glued to araldite blocks. Serial semithin sections (1.5 μm) were cut with an Ultra cut UC-6 (Leica, Heidelberg, Germany) and mounted onto slides and stained with 1% toluidine blue. Selected semithin sections were glued with Super Glue-3, Loctite (Henkel, Düsseldorf, Germany) to araldite blocks and detached from the glass slide by repeated freezing (in liquid nitrogen) and thawing. Ultrathin sections (0.06–0.08 μm) were prepared with the Ultra cut and stained with lead citrate. Finally, photomicrographs were obtained under a transmission electron microscope FEI Tecnai G2 Spirit (FEI Europe, Eindhoven, Netherlands) using a digital camera Morada (Olympus Soft Image Solutions GmbH, Münster, Germany). Autophagosomes were morphologically identified (*) and quantified from at least 10 different pictures, at equal magnification, for each experimental condition and normalized to the total cell area by using the ImageJ software in pixels (px^2). Three independent experiments were performed, and data was expressed as mean ± SD of autophagosomes/total cell area (px^2).

2.6. ASC-Directed Differentiation. Confluent canine and human ASCs at passage 4 were subjected to directed differentiation towards adipocytes, osteocytes, or chondrocytes [37]. All three differentiation processes were performed in parallel with 2.5% thawed PRGF plus heparin (40 U) or heparin alone. All directed differentiation media were obtained from Lonza Group Ltd.

2.6.1. Adipogenesis. ASCs were seeded at a cell density of 10,000 cells/cm^2, and when ASCs were >90% confluent, the growth medium was changed to differentiation medium containing insulin, dexamethasone, IBMX (3-isobutyl-methyl-xantine), and indomethacin (adipose-derived stem cell Basal Medium; Lonza Group Ltd.). The cells were then incubated for 10–12 days. Adipogenic differentiation was evaluated by Oil Red O staining of the lipid vacuoles in formalin-fixed cultures.

2.6.2. Osteogenesis. ASCs were seeded at a cell density of 10,000 cells/cm^2 in collagen I-coated plates (Sigma-Aldrich; 10 mM) in medium containing 0.1 μM dexamethasone, 50 μM Asc2P, and 10 mM μ-glycerophosphate (osteogenic basal medium; Lonza Group Ltd.) with 10% human serum. ASC cultures were maintained in this medium for 4 weeks (with medium changes every 3 days). For detection of extracellular calcium deposits, Alizarin Red staining was used in formalin-fixed cultures. Immunodetection, by immunofluorescence, was performed to detect actin filaments by phalloidin and osteocytes by Connexin 43.

2.6.3. Chondrogenesis. Differentiation of chondrocytes was performed using a micromass. Starting with a high concentration of ASC in a minimal volume (1×10^5 cells/100 μl) in the presence of TGF-β 1 and 3 (10 ng/ml), Asc 2P (50 μM), and insulin (6.25 μg/ml) (Chondro BulletKit; Lonza Group Ltd.), these ASCs were cultured for four weeks in this medium, with medium changes every 3 days. Alcian blue was used to detect the presence of enrichment of sulfated proteoglycans in the extracellular matrix. Before staining, the micromass cultures were fixed in formalin, embedded in paraffin, and cut into 5 μm sections.

2.7. Immunocytochemistry. ASC monolayer was fixed with 4% PFA at room temperature for 15 minutes, permeabilized with 0.1% Triton X-100, and subsequently blocked with 10% FBS. Micromass sections from chondrocyte-induced differentiation were previously dewaxed. Incubation with one of the following primary antibodies was performed overnight (1 : 200) at 4°C: Connexin 43 (Abcam; UK); Sox9 (Chemicon, USA). After removing primary antibodies and washing thoroughly, one of the following secondary antibodies (1 : 400) was added and incubated for 1 h at room temperature; Oregon green 488 goat anti-mouse IgG or Alexa Fluor 647 mouse anti-rabbit (Thermo Fisher Scientific, USA). Phalloidin conjugated with FITC (1 : 1000; Invitrogen, USA) was incubated for 45 minutes before visualization. All cells were counterstained by incubation with 4,6-diamidino-2-phenylindole dihydrochloride (DAPI) from Molecular Probes (Invitrogen, USA) for 3 min at room temperature followed by washing steps. Samples were mounted using FluorSave Reagent (Calbiochem, USA). Confocal Microscopy (Leica, Germany) was employed to visualize the signals; at least six different fields per condition and assay were analysed.

2.8. ASC Treatments and Survival Studies in the Presence of Hydrogen Peroxide. The H_2O_2 (Sigma-Aldrich, USA) and AKT inhibitors (Calbiochem, USA; AKT inhibitor VIII 124018; 10 μM) were freshly prepared from 1 M and 10 mM stock solutions, respectively. PRGF and H_2O_2 combined treatments were incubated at the same time. AKT inhibitor was preincubated for 30 minutes prior to secondary treatments.

The cell viability was determined by the CellTiter 96® AQueous Non-Radioactive Cell Proliferation Assay (Promega Co., Madison, WI, USA) following the manufacturer's instructions. Briefly, 10^5 ASCs at passages 2–5 were seeded onto 96-well plates and allowed to grow for 24 h in growth medium. After removing the growth medium, the cells were treated with PRGF (0 or 2.5%, plus 40 U of heparin), 2.5% of HS, H_2O_2 (100 μM), and/or AKT inhibitor (Calbiochem, USA; AKT Inhibitor VIII 124018; 10 μM) for 24 h in the absence of serum. Every condition was assayed in quadruplicate in three different experiments. The viability of cells at each assayed condition was expressed as the percentage ratio of the mean ± SD.

2.9. Annexin V Detection by FACS Analysis. ASCs were trypsinized, and 10^5 cells per condition were diluted into 100 μl of PBS and incubated with 1 : 50 dilution of annexin V-FITC-conjugated antibody (Invitrogen, USA) in the dark for 45 min at room temperature and then washed three times with PBS and resuspended in 0.3 ml of cold PBS for flow cytometry analysis (FC500, Beckman Cultek, USA). The mean ± SD of the annexin V-positive population (in percentage) of all tested samples were represented.

2.10. Western Blotting Analysis. ASCs were collected, and proteins were extracted by using Lysis Buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.02% NaN_3, 0.1 SDS, 1% NP40, 1 mM EDTA, 2 μg/ml leupeptin, 2 μg/ml aprotinine, 1 mM PMSF, and 1x Protease Inhibitor Cocktail (Roche Diagnostics, USA)). Equal amounts of protein extracts (20 μg) were loaded onto a 10% SDS-polyacrylamide gel and resolved by standard SDS-PAGE. Proteins were electrophoretic transferred onto PVDF membranes. Membranes were blocked with 5% skim milk in PBST for 60 min and incubated overnight with one of the following specific primary antibodies (1 : 1000): PARP (Abcam, UK); P-AKT, AKT, or Cyclin D (Cell Signaling, USA). β-Actin (1 : 5000) (Sigma-Aldrich, USA) was used as a loading control. Subsequently, membranes were incubated with rabbit anti-mouse or rabbit anti-goat horseradish peroxidase-conjugated secondary antibody (1 : 5000) (Sigma-Aldrich, USA). Blots were visualized by the ECL detection system (Amersham, UK).

2.11. Statistical Analysis. Statistical comparisons by pairs were assessed by Student's *t*-test. All *P* values were derived from a two-tailed statistical test using the SPSS 11.5 software. A value of $P < 0.05$ was considered statistically significant.

3. Results

3.1. PRGF Induces Proliferation and Migration of ASCs. Human ASCs in the presence of growing concentrations of HS or PRGF (1, 2.5, 5, or 10%), for 24 h, exhibited significant increased proliferation in comparison with absent of growth factors (0%; Figure 1(a), left graph; $^*P > 0.05$). 10% of PRGF induced the highest proliferation rates and was significantly different to the HS proliferative activity (Figure 1(a), left graph; $^\$P > 0.05$). Representative phase-contrast images of human ASCs in the presence of 10% HS or PRGF are shown in Figure 1(a) (right). Similarly, in a cell invasion scratch assay, 10% of PRGF induced the highest cell migration activity, significantly different in comparison with ASC in the presence of HS (Figure 1(b), left graph). Representative photograms from time-lapse analysis, 16 hours after PRGF or HS treatments, evidenced both the increase of cell density and the accelerated wound invasion induced by 10% PRGF (Figure 1(b), right panels). Canine ASCs showed comparable respond to human ASCs. 10% of canine PRGF induced higher proliferation rates in comparison with FBS containing canine ASC cultures (Supplementary Figure 1 available online at https://doi.org/10.1155/2017/5946527).

3.2. PRGF Reduces Senescence and Autophagocytosis of In Vitro Expanded ASC. Ex vivo amplification of ASCs has shown to be limited to a certain number of passages constituting a limitation for generation of sufficient high cell quantities on clinical usage. Indeed, cellular alterations occurring during in vitro aging have been suggested to be similar to differences observed on ASCs from aged and young donors [38]. There is increasing evidence that cellular senescence is a cause of stem cell aging and malignancy [39]. Herein, we report that 2.5% of PRGF reduces senescence of human ASCs upon starvation, in the absence of growth factors, but not the equivalent concentration of HS, in comparison with the total absent of growth factors (Figure 2(a)). Similarly, the significant accumulation of autophagosomes, a hallmark of cell aging [40], induced by the lack of growth factors, was significantly prevented with 2.5% of PRGF and not with the same concentration of HS (Figure 2(b); graph). Transmission electron microscopy representative images show an extensive accumulation of autophagosome bodies (∗) and vacuoles ($) in the complete absence of growth factors and in less extend, but also significant, in the ASCs in the presence of 2.5% of HS (Figure 2(b); upper panels). A dose response of PRGF on reducing senescence and autophagocytosis was assayed also for 5 and 10% of PRGF, and no significant differences were found in comparison with 2.5% PRGF (data not shown).

3.3. PRGF Accelerates ASC-Directed In Vitro Differentiation of Adipocytes, Osteocytes, and Chondrocytes. Human ASCs in the presence of PRGF differentiated more rapidly toward adipocytes, osteocytes, and chondrocytes (Figures 3(a), 3(b) and 3(c)). Human ASCs were induced to differentiate into the three mesodermal lineages, in the presence or absence of 2.5% PRGF in the defined differentiated mediums for each lineage. Directed adipogenesis revealed a more rapid accumulation of fat-containing cells in the presence of PRGF, as visualized and quantified by the Oil Red O staining (Figure 3(a)). Osteocyte differentiation in the presence of PRGF was even more robust and accelerated. PRGF

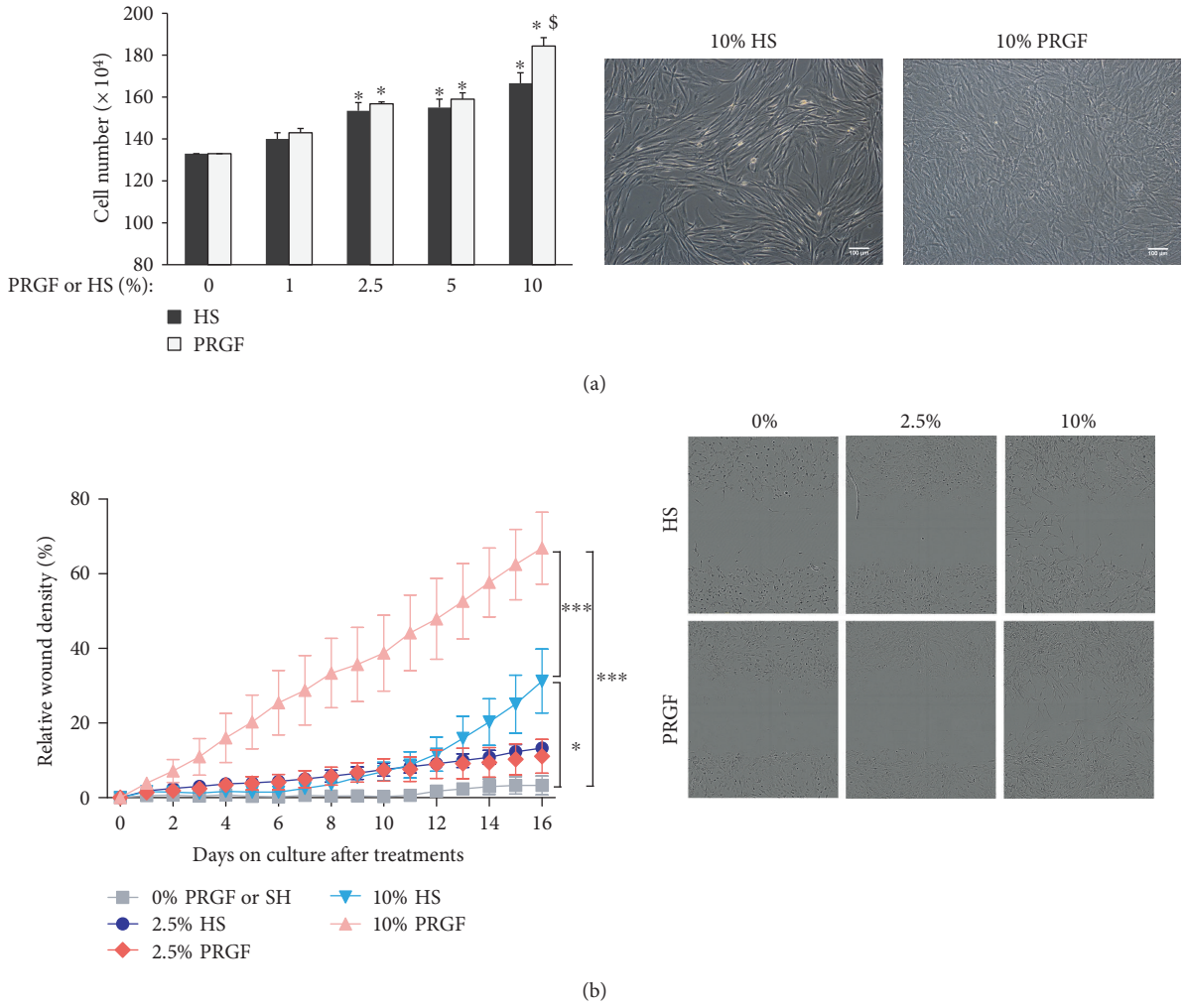

Figure 1: PRGF induces proliferation and migration of human ASCs. (a) Left: Human ASCs were cultured with HS or PRGF at growing concentration (1, 2.5, 5, or 10%) or in the absent of growth factors (0%) for 24 hours and subjected to cell viability test analysis. 10% PRGF induced a significant difference on the cell numbers in comparison with 10% of HS; $^{*}P > 0.05$ versus 0% and $^{\$}P > 0.05$ versus 10% HS; Right: representative phase-contrast images of ASCs 24 hours after incubation with 10% HS or 10% PRGF; scale bar: 100 μm. (b) Cell migration and invasion assay was performed in 96-well plates by the IncuCyte S3 live-cell analysis. Left: cell density quantification at the wound area showed a faster and significant increase of cell density induced by 10% of PRGF since 2 hours after stimulation. 10% HS significantly induced the cell migration and invasion in comparison with 0% since 10 hours of incubation. $^{***}P > 0.001$; $^{*}P > 0.05$. Right: representative phase-contrast images of human ASCs in the presence of different concentrations of HS or PRGF 16 hours after incubation.

treatment leads to increased expression of Cx43, a known marker of mature osteocytes, at four weeks of the differentiation process and a higher number of calcium containing osteocyte cell clusters as demonstrated by Alizarin Red staining quantification (Figure 3(b)). ASCs differentiated toward chondrocytes showed higher Sox9 expression in the presence of PRGF (Figure 3(c), right panels). In addition, cell number and density increased in the presence of PRGF, with an enrichment of collagen in the micromass cultures for chondrocyte induction as visualized and quantified by Alcian blue staining (Figure 3(c), left panels). Canine PRGF also improved the yield on the directed differentiation process into adipocytes, osteocytes, and chondrocytes as shown in the representative images of Supplementary Figure 2.

3.4. PRGF Exhibits Improved Tolerance to Hydrogen Peroxide Cytotoxicity in Human ASCs by AKT Induction. Acute bouts of oxidative stress stimulate cell proliferation in ASCs [41]; however, high doses, or extended exposure to low doses of reactive oxygen species, dramatically reduce ASC viability [42]. Treatment with 100 μM H_2O_2 for 24 h was significantly toxic to human ASCs in the absence of growth factors (Figure 4(a)). Consistent with the perceived cytoprotective effect of PRGF, quantification of cell viability revealed that in the presence of 2.5% PRGF, but not 2.5% HS, PRGF significantly prevented the cell death that was significantly compromised by the addition of 100 μM H_2O_2 (Figure 4(a)).

Pharmacological inhibition of AKT by the preincubation for 30 minutes with 10 μM of the Inhibitor VIII 124018 (Calbiochem) blocked the protective effect of 2.5%

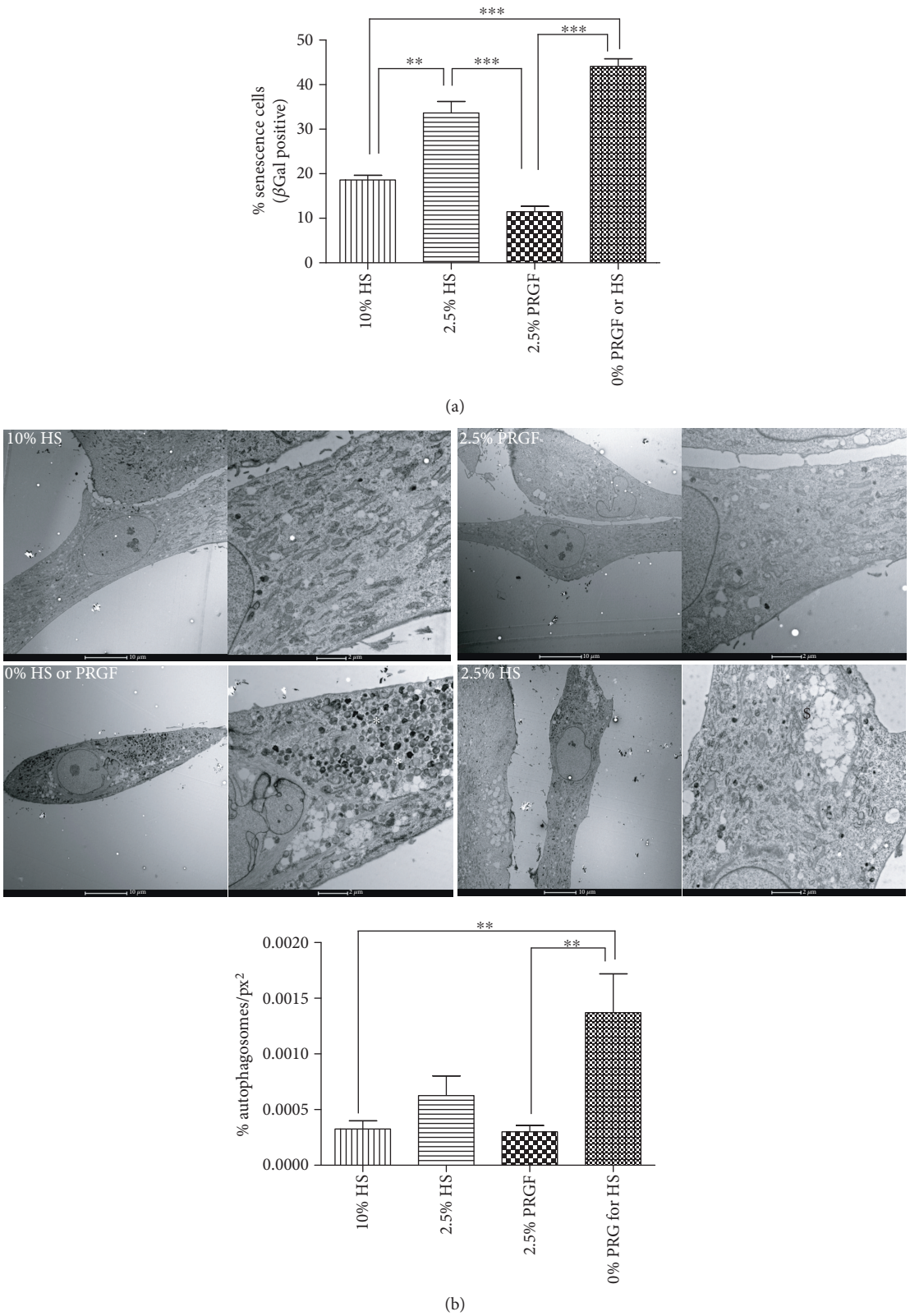

Figure 2: PRGF prevents in vitro ASC aging. (a) Senescence cell quantification was performed by quantification of SAβGal-positive cells in bright field microscope at 20x magnification. Four different fields, containing a minimum of 200 cells, were analysed in three independent experiments. (b) Autophagosome quantification was performed from higher magnification TEM pictures from at least 200 different cells. The total number of autophagosomes was normalized to the total area analyzed with ImageJ software. Three independent experiments were analysed and represented as the mean ± SD of autophagosomes/total cell area (px^2) $^{**}P > 0.01$; $^{***}P > 0.0001$.

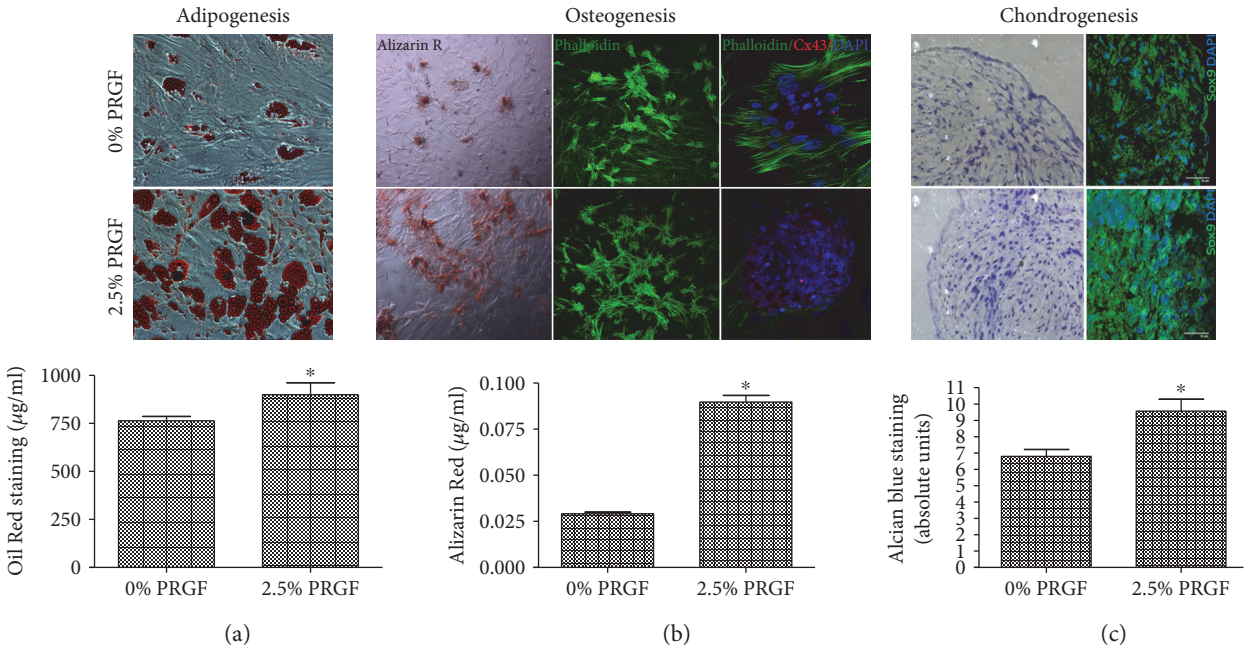

Figure 3: PRGF accelerates adipocyte, osteocyte, and chondrocyte in vitro differentiation of human ASCs. Human ASCs were induced to differentiate toward the three mesodermal lineages in the presence or absence of 2.5% PRGF; (a) adipogenesis: the presence of PRGF induced higher intracellular lipid content by Oil Red O staining; (b) osteogenesis: both calcium deposits visualized by Alizarin Red staining and the phalloidin (green: which indicates actin cytoskeleton growth in osteocytes) and Cx43 expression (red: a known marker of mature osteocytes) were improved in the presence of PRGF; (c) chondrogenesis: Alcian blue staining (marker of the proteoglycan aggrecan deposits) and immunostaining of Sox9 (green: marker of chondrogenesis and chondrocyte differentiation) were higher in the presence of PRGF. Scale bar: 50 μm. $^*P < 0.05$ versus 0% PRGF.

of PRGF on ASCs exposed to a cytotoxic H_2O_2 dose (Figure 4(b)). Furthermore, the percentage of the population of apoptotic cells, quantified by the expression of annexin V, was determined in the presence or absence of PRGF and when treated with H_2O_2 alone or with AKT inhibitor. The results demonstrated that PRGF induced cell survival via activation of AKT since the inhibition of AKT leads to a significant population of annexin V-positive cells, including those in the presence of PRGF (Figure 4(c)).

ASCs require of trophic factors since in the absence of PRGF or HS, there was an increase of basal activation of apoptosis as evidenced by the cleavage of PARP, a protein associated with the induction of apoptosis signaling [43] (Figure 4(d)). Moreover, the presence of PRGF blocked the induced PARP cleavage when ASCs were treated with 100 μM H_2O_2. In the presence of PRGF, phosphorylated AKT (active form) and cyclin D (downstream effector of AKT) are expressed when PARP has not been cleaved.

4. Discussion

ASCs and PRGF have been shown as promising therapeutic alternatives for tissue repair in mesoderm-related tissues, like, for instance, in the repair of the damaged cartilage in osteoarthritis. ASCs are easily expanded in culture and are capable of differentiating by their multipotent nature in various mature cell lineages allowing to reproduce in vitro processes such as osteogenesis, chondrogenesis, or adipogenesis [44]. However, still there is much uncertain information about the ex vivo amplification, behaviour of the transplanted cells in terms of cell survival, tissue integration, or cell differentiation in a hostile microenvironment. Nowadays, PRGF offers an autologous source of known regenerative properties being able to synergistically improve the benefits of ASC treatments. PRGF favors ASC proliferation [7, 8] and is able to stimulate undifferentiated stem cell differentiation for tissue regeneration [17, 45]. Undifferentiated stem cells migrate to the concentration of PRGF growth factor gradients, and the growth factors trigger proliferation of these cells once they are at the site of administration [46]. The combinatory use of PRGF and ASCs has already shown improved benefits in a number of tissue repair process, including osteoarthritis or by accelerating ossification of fractures [47–49].

Cell transplantation efficiency is directly depending on cell survival, cell aging, and cell fate specification. Rejuvenation of aged progenitor cells has been largely reported by exposure to a young or regenerative environment by reducing the cell senescence [50]. Recent studies suggest that blood from young donors could reverse age-related diseases based on experiments of blood transference from young mice which could rejuvenate aged tissues in older animals [50–52]. After analyzing several circulating factors, Loffredo et al. [52] suggested that GDF11 could be responsible for these effects. Recently, Bueno et al. [53] have shown that GDF11 is at least ten times more concentrated in platelet lysate than in serum or plasma, indicating that GDF11 is stored in platelets contributing most probably to the

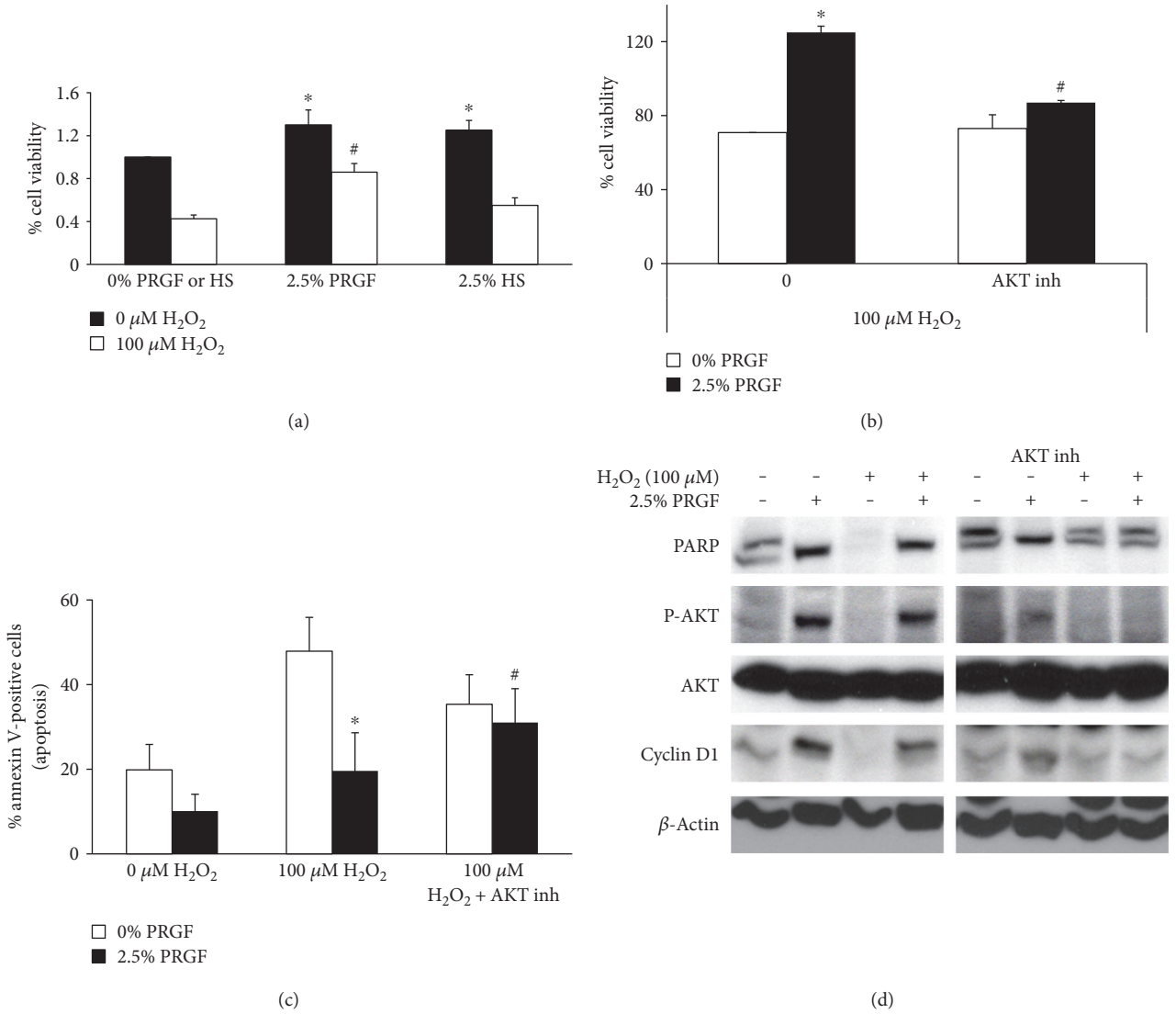

Figure 4: AKT mediates PRGF survival and prevention of H_2O_2 cytotoxicity. (a) Human ASCs were treated with 100 μM H_2O_2 for 24 h in the presence or absence (0%) of 2.5% PRGF or 2.5% HS, and the cell viability was analyzed by MTT assay. The significant reduction on cell viability induced by hydrogen peroxide was prevented by 2.5% of PRGF. $^*P < 0.05$ versus 0 μM H_2O_2 and $^\#P < 0.05$ versus 100 μM H_2O_2. (b) Preincubation with 10 μM of the AKT inhibitor (Calbiochem, VIII 124018) abolished the cell protective effect of PRGF of the cytotoxic effects of 100 μM H_2O_2. $^*P < 0.05$ versus 0% PRGF. (c) FACS analysis of annexin V showed the percentage of apoptotic cells according to each treatment. $^\#P < 0.05$ versus 2.5% PRGF. (d) Western blot analysis of total protein lysates of the human ASCs treated with 2.5% PRGF (+) or 0% PRGF (−) in the presence of 100 μM, preincubated or not for 30 min with 10 μM AKT inhibitor for 24 h. Activation or cleavage of the apoptotic protein PARP is confirmed when two bands visible. β-Actin was employed as a loading control. Representative blots of three different experiments are shown; $^*P < 0.05$.

differential effect of PGRF on cell proliferation, cell differentiation, or cell aging [54]. We have observed a significant dependency on the AKT signaling activation with survival responses in ASCs in the absence of growth factors and by oxidative damage. PRGF by inducing phosphorylation of AKT significantly improves cell survival of ASCs when are exposed to proapoptotic concentrations of hydrogen peroxide. The hydrogen peroxide is employed to reproduce the oxidative stress found at the injury and transplantation area. The inflammatory-related oxidative stress in fact compromises the cell function and survival of the entire joint, including the cartilage in the osteoarthritic patients [55]. In this context, PRGF treatment would prevent cell death associated to oxidative stress by inducing prosurvival signals in the joint by activation of AKT [56].

The efficiency of ASCs for osteoarthritis has been already probed with significant functional improvements. Dogs provide a suitable translational model for further clinical research on osteoarthritis, with close histopathological and functional features [57]. We recently showed in a randomized study performed in canine patients, with moderate to severe osteoarthritis, that intra-articular transplantation of ASC provided a significant joint functional improvement, reducing dog's pain and improving physical function up to

six months [58, 59]. In fact, ASCs from canine samples were also tested in vitro, in response to PRGF, and we found a similar response to cell proliferation, survival, and differentiation found in human ASCs (Supplementary Figure). Based on in vitro experimentation, significant improvements would be expected for activated ASCS by PRGF stimulation; however, further in vivo analysis is needed to disclose it. Thus, ASCs in culture pretreated with PRGF before transplantation may confer improved survival, proliferation, and antiapoptotic capabilities and render the cells a powerful source for cell therapy and tissue regeneration. Canine and human ASCs showed a comparable response to PRGF of cell proliferation, cell differentiation, and AKT induction. Thereby, dogs provide a suitable translational model for further clinical research on ASC-based treatments.

5. Conclusion

PRGF treatment is a potent stimulator of ASCs and can be used as an autologous preconditioning agent to enhance the therapeutic potential of ASC transplantation.

Conflicts of Interest

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

Authors' Contributions

Richard J. Griffeth, Montserrat García, Ramón Cugat, Victoria Moreno-Manzano, Maravillas Mellado-López, and Jose Meseguer-Ripolles collected and/or assembled, analysed, and interpreted the data. Montserrat García, Ramón Cugat, and Victoria Moreno-Manzano gave their financial support. Maravillas Mellado-López and Richard J. Griffeth contributed equally to this work.

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