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Studies on microRNA regulation of multidirectional differentiation of dental pulp stem cells: a narrative review

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Abstract. – OBJECTIVE: Dental pulp stem cells (DPSCs) are adult stem cells of neural crest origin, are readily available, have good self-renewal and multidirectional differentiation properties, can differentiate into a variety of cells, are abundant, less harmful to donate, have no ethical issues, low immunogenicity, and therefore, are widely used as seed cells in the field of tissue engineering and regenerative medicine. MicroRNA (miRNA) is a single-stranded non-coding small RNA consisting of about 22 nucleotides, which plays an important regulatory role in various aspects of cellular activities, such as proliferation, differentiation, and apoptosis. In this paper, we review the regulatory role of small RNA in the differentiation of DPSCs and its mechanism in the past 5 years. This paper aims to reveal the important role of miRNAs in differentiation in DPSCs.

MATERIALS AND METHODS: MicroRNAs (miRNAs), differentiation, and DPSCs were extensively searched in three databases from 2014 to 2021. These databases include PubMed, Cochrane Library, Embase.

RESULTS: Our study reviews the microRNAs (miR-145, miR-143-3p, miR-140-5p, miR-488, miR-218, miR-125a-3p, miR-27a-5p, miR-223, miR-21, miR-143, miR-215, miR-219a-1-3p, miR-31, miR-496, miR-218, miR-24-3p, miR-146a-5p, miR-196a, miR-188-3p, miR-424, miR-378a, miR-135, miR-124) in the differentiation of DPSCs.

CONCLUSIONS: A large body of evidence supports the involvement of miRNAs in differentiation associated with mesenchymal stem cells (MSCs), although the mechanisms involved are not yet clear. Most of the current studies are from *in vitro* studies, but the ultimate goal is to apply these studies to the clinic, and studies involving *in vivo* models are needed.

Key Words:
MicroRNA, DPSCs, Osteogenic differentiation.

Introduction

Non-coding RNAs are transcriptional products that do not encode proteins and are considered "junk sequences" generated during evolution and were not given much attention. DPSCs are MSCs derived from dental pulp tissue. They can be isolated and cultured in living pulp tissue. They have robust proliferation, self-renewal, and differentiation into various cells and are easy to obtain. It has abundant sources, minor damage to the donor, and no ethical issues. It is widely used in tissue engineering and regenerative medicine[1]. The regulatory mechanism of DPSCs directed differentiation has been a hot topic of research in this field. However, there are still many difficulties, which have also greatly limited the clinical application of DPSCs. In recent years, it has been discovered that non-coding RNA has a significant regulatory effect on stem cells and has attracted widespread attention. MiRNA is a kind of non-coding RNA. This article reviews the latest research progress of miRNA regulation on dental pulp stem cells.

Biological Properties and Multidirectional Differentiation Potential of DPSCs

Human dental pulp-derived stem cells (hDPSCs) are mesenchymal stem cells and, like other stem cells, have a plastic adherent and fibroblast-like morphology. They have been shown to express the MSC-specific markers CD44, CD90, CD105, CD73, and STRO-1, but not the hematopoietic markers CD14 and CD19, meeting the minimum criteria for defining multipotent MSCs proposed by the International Society for Cellular Therapy in 2006[2]. However, DPSCs are a highly heterogeneous population with different clonal and expression markers and differences in proliferation and differentiation capacity[3]. Therefore, the purification of DPSCs is essential for successful clinical applications. Specific cell surface markers help to isolate particular subpopulations of DPSCs that can subsequently be differentiated into specific cell types for clinical use.

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Since 2000, when DPSCs were first isolated from the pulp of human interrupted third molars and cultured *in vitro*, some studies[4,5] have demonstrated the self-renewal capacity, the multilineage differentiation potential of DPSCs. DPSCs have now been shown to differentiate into various cells, including osteoblasts, dentin-forming cells, adipocytes, endothelial cells, neurons, myocytes, and chondrocytes[6,7] (Figure1). Furthermore, surprisingly, DPSCs retain their properties even after two years of cryopreservation[8]. Therefore, the differentiation and clinical potential of DPSCs in regenerative medicine are receiving increasing attention.

Biogenesis of MiRNAs

Only 1%-2% of RNA molecules can be translated into proteins, called coding RNAs, and most are non-coding RNAs (ncRNAs)[9]. As an introductory class of ncRNAs, miRNAs are composed of 19-25 nucleotides in length. The first two miRNAs identified were lin-4 and let-7, and both were found in nematodes[10,11].

The biogenesis of miRNA involves several key steps: in the nucleus, transcription by RNA polymerase II (polII) to form primary mi RNA (pri-mi RNA), followed by cleavage by a microprocessor consisting of the RNA-binding protein DGCR8 and the RNase III enzyme Drosha to form precursor miRNA containing short hairpins (pre-miRNA). RNA-GTP and exportin 5 transport the pre-miRNA into the cytoplasm, and finally, the mature miRNA is formed by Dicer treatment[12-18]. The mature miRNA is incorporated into the RNA-induced silencing complex (RISC), where it can be linked to the 3' untranslated region (3'U') of the target mRNA by the RAN-GTP exportin 5[19]. The RISC is paired with bases in the 3' untranslated region (3'UTR) of the target gene, resulting in inhibition of mRNA translation if the pairing is incomplete (common in mammals) and degradation of mRNA if the pairing is complete[20,21] (Figure 2).

Regulation of the Multidirectional Differentiation Ability of DPSCs by MiRNA

Regulation of MiRNAs During the Differentiation of DPSCs Into Adult Dentin-Like Cells Regulatory Role and Its Mechanism

The most distinctive feature of DPSCs is their ability to form dentin-pulp-like complexes[22,23]. The differentiation of DPSCs into adult dentin-like cells is a complex process regulated by multiple signal transduction pathways. Gong et al[24] used microarray analysis to screen and compare changes in the miRNA profile of hDPCs during odontogenic differentiation and found that 22 miRNAs were differentially expressed, 12 of which were upregulated and 10 downregulated. According to the literature, miRNAs play an active role in differentiating adult dentin cells in DPSCs[25,26].

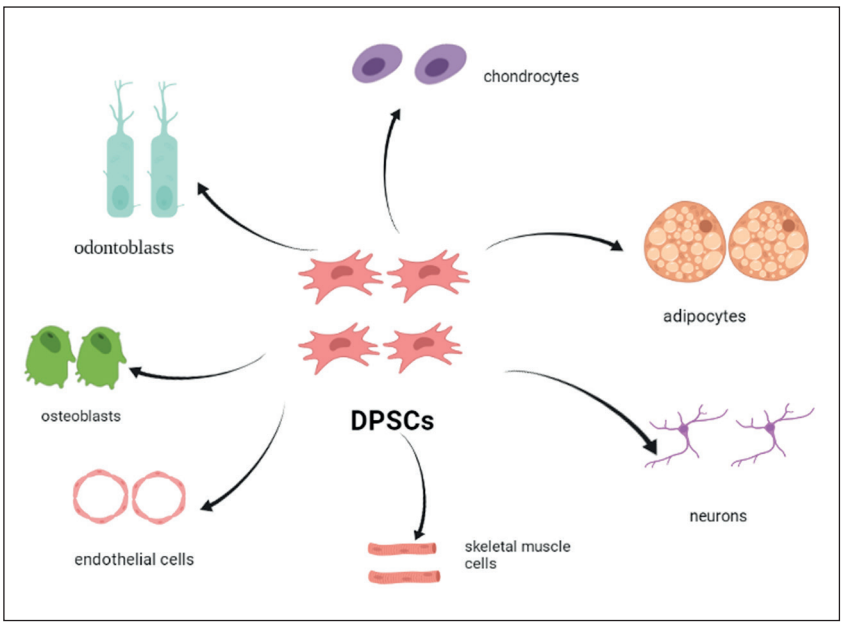

Figure 1. Multi-directional differentiation potential of DPSCs.

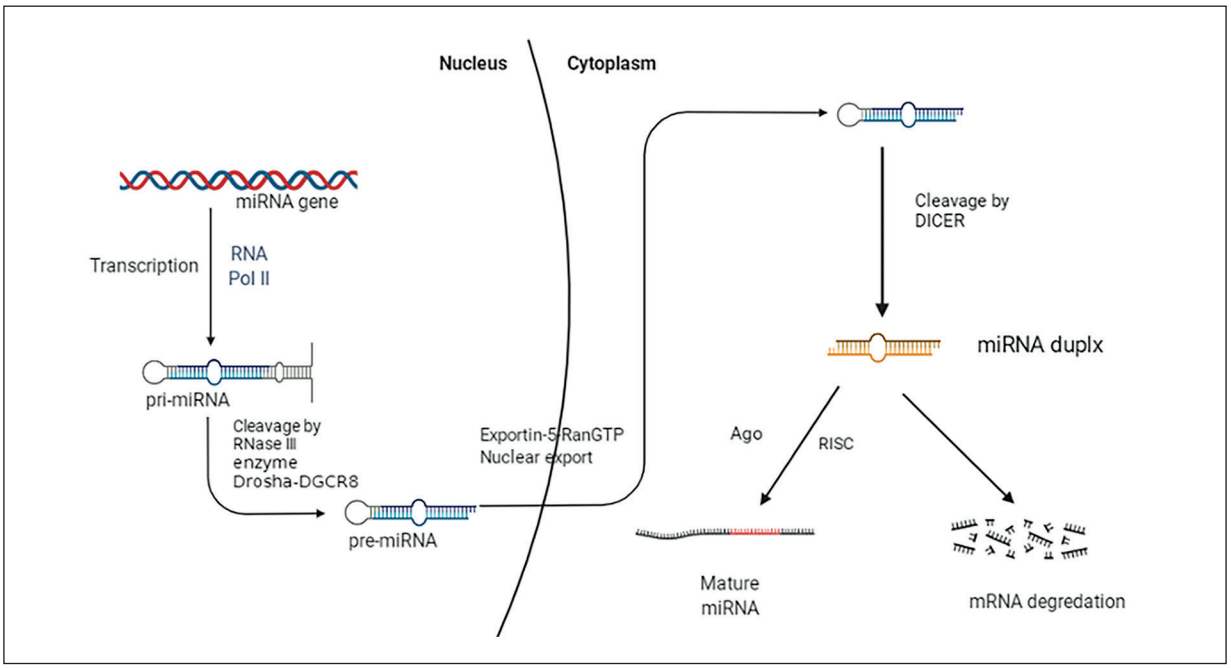

Figure 2. Mechanism of miRNA biogenesis. MiRNAs are transcribed by RNA polymerases II (Pol II) in the form of a first precursor called primary miRNA (pri-miRNA). The pri-miRNA is converted into the precursor miRNA (pre-miRNA) via the cutting activity of the Drosha enzyme, a nuclear endoribonuclease III. Pre-miRNAs are exported into the cytoplasm as a result of the action of Ran-GTP and Exportin-5, a nuclear export factor. In the cytoplasm, Dicer clivates the pre-miRNA into a double stranded miRNA (miRNA duplex) of about 18-22 nt. The mature miRNA strand is incorporated into the RNA- induced silencing complex (RISC), which guides the miRNAs to the 3 UTR of its target.

In 2013, Liu et al[27] showed through loss-of-function and gain-of-function experiments that could promote the differentiation of mouse DPSCs odontoblasts and increase the expression of dentin sialophosphoprotein (DSPP) and dentin matrix protein 1 (DMP1) by downregulating miR-145 and miR-143. MiR-145 can bind to the 3'-UTRs of Krüppel-like factor 4 (KLF4) and osterix (OSX) genes and inhibit the expression of these two genes. The reduction of miR-143 can partially downregulate the expression of miR-145, thereby releasing the expression of target genes KLF4 and OSX. Both KLF4 and OSX upregulated the odontoblast marker genes DSPP and DMP-1, thereby inducing the differentiation of odontoblasts. Yang et al[28] showed that downregulation of miR-143-3p led to upregulation of nuclear factor-κB (RANK), activating the osteoprotegerin (OPG)/nuclear factor-κB ligand (RANKL) signaling pathway and promoting hDPSCs to form odontoblasts. Simultaneous downregulation of miR-143-3p inhibited the cycle progression of hDPSCs and induced apoptosis through activation of the OPG/RANKL/RANK pathway. Lu et al[29] also found that low expression of miR-140-5p promoted the differentiation of odontoblasts of DPSCs, while the overexpression of miR-140-5p inhibited the differentiation of odontoblasts. However, the expression of miR-140-5p does not affect the activity and proliferation of DPSCs, which is contrary to the results of Sun et al[30], and different detection conditions may cause this difference. In addition, the target gene Wnt1 of miR-140-5p was determined by Luciferase reporter gene assay. Combined with the results of this study, it is believed that miR-140-5p regulates the differentiation of odontoblasts of DPSCs through the Wnt1/β-Catenin signaling pathway. Sun et al[30] found that miR-140-5p may be closely related to the differentiation of DPSCs odontoblasts. Later, Chang et al[31] found that miR-218 also negatively regulated dentin formation in DPSCs. However, Wang et al[32] found that miR-125a-3p overexpression increased the ability of DPSCs to differentiate into odontoblasts, whereas miR-125a-3p knockdown decreased the ability of DPSCs to differentiate into odontoblasts. They further demonstrated that miR-125a-3p could target Fyn and regulate NRP1, thereby further regulating the odontoblastic differentiation of DPSCs. Hu

et al[33] similarly found that miR-27a-5p and miR-223 promoted the odontoblastic differentiation of DPSCs by upregulating the protein expression of DSPP, DMP-1. Furthermore, Luciferase reporter gene assay and siRNA experiments confirmed that Smad3 is one of the miR-223 target genes involved in these processes[34]. The researchers observed that under inflammatory conditions, upregulation of miR-21 may synergistically promote odontoblastic differentiation of DPSCs with signal transducer and activator of transcription 3 (STAT3). This may provide a new therapeutic strategy for regulating the differentiation of DPSCs into dentin cells[35].

Role of MiRNA Regulation in the Osteogenic Differentiation of DPSCs and Its Mechanism

DPSCs are considered to be a promising source for tissue engineering, particularly for osteogenic tissues[36]. Increasing their osteogenic capacity is crucial for their potential application in tissue engineering. Therefore, it is important to identify new therapeutic targets and elucidate the mechanisms regulating the osteogenic differentiation of DPSCs. There is growing evidence that miRNAs influence the osteogenic differentiation of DPSCs by regulating various aspects of their osteogenic differentiation process. A scholar identified eight differentially expressed DPSCs miRNAs (DEmiRNAs), five of which were downregulated (miR-1273g-3p, miR-146a-5p, miR-4508, miR-4516 and miR-6087) and three were upregulated (miR-146b-5p, miR-337-3p, miR-382 -3p) in bone differentiation[37]. In addition, Iaculli et al[38] found that miR-133 and miR-135 play a key role in the differentiation of DPSCs and can be used as markers of osteogenic differentiation.

Zhang et al[39] in 2018 found that the overexpression of miR-143 inhibited osteogenic differentiation of DPSCs. In addition, bioinformatics analysis and Luciferase reporter analysis showed that tumor necrosis factor alpha (TNF-α) was a target of miR-143 in DPSCs and that miR-143 regulated TNF-α expression through post-transcriptional binding to its 3'UTR. Functional analysis revealed that miR-143 inhibited TNF-α-induced osteogenic differentiation of DPSCs, suggesting that miR-143 inhibited osteogenic differentiation of DPSCs by downregulating TNF-α. Furthermore, they found that inhibition of miR-143 promoted osteogenic differentiation of DPSCs through activation of the NF-κB signaling pathway. Yao et al[40] found increased expression of let-7b-5p, miR-98-5p, miR-215, miR-219a-1-3p and miR-295-5p in rat DPSCs in late *in vitro* culture. In late passaged DPSCs, increased miR-215 and miR-219a-1-3p downregulated Heat Shock Protein B8 (HspB8) expression and reduced osteogenic differentiation. The increased miR-295-5p expression also reduced osteogenic differentiation, but not by downregulating heat shock protein B8. The hsa-miR31 inhibitor was transfected into DPSCs by Xie et al[41]. Alkaline phosphatase (ALP) activity assays, and alizarin red staining was performed at 14- and 21-days post-transfection. By ALP analysis and ARS, it was found that downregulation of hsa-miR-31 promoted osteogenic differentiation of DPSCs. Ji et al[42] found that miR-496 inhibitors downregulated miR-496 expression and promoted the expression of DPSCs by upregulating the expression of β-catenin. In addition, circRNA124534 promoted osteogenic differentiation of human DPSCs by regulating the miR-496/β-catenin pathway. Gay et al[43] (2014) isolated periodontal ligament stem cells (PDLSCs), DPSCs, and gingival stem cells (GSCs) from human third molars and performed osteogenic induction. Research of miRNA expression profiles upon completion of induction showed that hsa-miR-218 targeted RUNX2, and inhibition of hsa-miR-218 promoted mineralization induction in DPSCs and promoted runt-related protein 2 (RUNX2) expression.

However, Wu et al[44] demonstrated that transforming growth factor beta (TGF-β) receptors 1(TGFBR1) is a downstream target of miR-24-3p in the regulation of osteogenesis in DPSCs. Furthermore, LEF1-AS1 and miR-24-3p synergistically regulated osteogenic differentiation in DPSCs. Qiu et al[45] found that the expression levels of RUNX2, OSX, ALP, and DSPP were upregulated in the differentiation profile after overexpression of miR-146a-5p in STRO-1+ DPSCs, while the opposite was true for inhibitor transfection. However, osteocalcin (OCN) expression was not affected by miR-146a-5p, which may be related to the regulation of other post-transcriptional mechanisms. Furthermore, the downregulation of miR-146a-5p promoted the proliferation of STRO-1$^+$DPSCs. Gardin et al[46] found that the expression levels of RUNX2, OSX, ALP, and DSPP were upregulated in the differentiation profile after overexpression of miR-146a-5p in STRO-1$^+$DPSCs, while the opposite was true for inhibitor transfection. However, OCN expression was not affected by miR-146a-5p, which may be

related to the regulation of other post-transcriptional mechanisms. Furthermore, the downregulation of miR-146a-5p promoted the proliferation of STRO-1^{+}DPSCs[47].

The Role of MiRNA Regulation in the Differentiation of DPSCs Into the Vascular Endothelium and its Mechanisms

For pulp regeneration, vascular regeneration is essential. The formation of a functional, truly vascularized pulp is our goal. In recent years, miRNAs have been found to affect the vascular regeneration of dental pulp stem cells. MiRNAs indirectly regulate the differentiation of DPSCs into vascular endothelial cells through the regulation of these cellular factors, such as hypoxia-inducible factor-1 (HIF-1) and vascular endothelial-derived growth factor (VEGF).

Liu et al[48] identified VEGF and kinase insert domain-containing receptor (KDR) as targets of miR-424 during endothelial differentiation of human dental pulp cells (HDPCs). Inhibition of miR-424 function promotes endothelial differentiation of HDPCs, while miR-424 overexpression inhibits its angiogenic potential. Inhibition of miR-424 promoted the secretion of angiogenic factors and upregulated the expression of these receptors, thereby enhancing differentiation efficiency. Zhou et al[49] showed that miR-378a was enriched in periodontitis-compromised dental pulp stem cells (P-EVs) and confirmed that it promotes angiogenesis in P-EVs. Sufu was identified as a downstream target gene of miR-378a, and P-EV-mediated miR-378a delivery significantly affected the expression of Sufu, Gli1, and VEGF in endothelial cells. Some scholars have verified at the molecular level that miR-411 can act directly on HIF-1α mRNA through a dual-luciferase gene assay. The miR-411 mimic reduced the expression level of hypoxia-inducible factor-1α (HIF-1α) in cells, and the miR-411 inhibitor increased the expression level of HIF-1α in cells, suggesting that miR-411 can target and negatively regulate HIF-1α[50]. It was also reported that DPSCs modified with HIF-1α were successfully induced to endothelial differentiation *in vitro*[51]. In conclusion, it can be speculated that miR-411 inhibitor can promote endothelial differentiation of DPSCs by regulating HIF-1α expression, which needs to be confirmed by *in vitro* and *in vivo* experimental data.

The Role of MiRNA Regulation in the Differentiation of DPSCs Into Skeletal Muscle and its Mechanisms

Many scholars[52] have demonstrated that DPSCs can differentiate into myogenic cells, but tapping into new factors, especially biologic factors, that can help induce differentiation of DPSCs into myogenic cells will undoubtedly accelerate the research process of treating muscle regeneration disorders through DPSCs. In recent years, it has been demonstrated that microRNAs can promote myogenic differentiation of DPSCs.

Some scholars[53] transfected anti-miR-135 and anti-miR-143 into DPSCs separately. At day 24, anti-miR-135 transfection alone resulted in about 10% of DPSCs entering cardiomyocytes, and anti-miR-143 transfection alone resulted in about 16% of DPSCs entering cardiomyocytes. Transfection of either anti-miR-135 or anti-miR-143 alone would only result in a small proportion of cells differentiating into cardiomyocytes. However, when both anti-miR genes were transfected, about half of the cells exhibited distinct cardiomyocyte characteristics and eventually fused to form myotubes. Furthermore, transfection with the anti-miRNAs resulted in significantly higher levels of the myogenic markers myocyte enhancer factor 2C (MEF2C), myogenic differentiation (MyoD), myogenin (MyoG), and myosin heavy chain (MyHC), and higher protein levels were obtained with co-transfection compared to transfection alone. These findings suggest that miRNAs play a decisive role in the induction of myogenic differentiation of DPSCs and provide new insights into the use of DPSCs in the treatment of muscle regeneration disorders[53].

The role of MiRNA Regulation in the Neural Differentiation of DPSCs and its Mechanism

DPSCs are considered a promising source of stem cells for neural regeneration due to their stem cell properties and ease of access, and they can differentiate into neural cell-like cells under specific induction conditions[54]. The literature on promoting neural stem cell differentiation by microRNAs has been reported recently, but reports are scarce. The binding of miR-124 to epidermal growth factor (EGF)/basic fibroblast growth factor (bFGF) has been reported to upregulate the expression of peripherin, β-tubulin III, and microtubule-associated protein 2 (MAP2) in DPSCs, suggesting that it promotes

the expression of neuronal markers, but the exact mechanism of regulation has not been elucidated[55] (Table I).

DPSCs have many advantages, such as being non-invasively obtained, highly proliferative, and remaining viable during transport. Dental pulp stem cells can be highly expressive of stem cell markers and retain the ability to differentiate into a wide range of cell lineages. However, the differentiation mechanism of dental pulp stem cells is not known. There is growing evidence that different epigenetic factors, including miRNAs, regulate the differentiation of DPSCs.

MiRNAs directly or indirectly contribute to the differentiation of DPSCs to various cell types and functions through different signaling pathways *in vitro*. The miRNAs provide new ideas and approaches for using DPSCs as seed cells for tissue regeneration, which has significant value and potential applications in tissue engineering and regenerative medicine, but its application to regenerate tissues or organs *in vivo* has rarely been reported. Among the problems that may face, its application in the clinic today is the following: (1) although many miRNAs have been found to change during multiple differentiation of MSCs, the focus must be on miRNAs with significant up-or down-regulation and meaningful targets; (2) the discovery of miRNAs that are efficiently targeted for regulation at different temporal stages of differentiation of DPSCs; (3) as differentiation is complex, essential differentiation genes may be targeted by more than one miRNA; it is noteworthy whether regulation of a single miRNA can achieve the goal of mediating differentiation; (4) a single miRNA can bind to multiple targets and affect multiple differentiation or physiological processes in DPSCs, while multiple miRNAs can also target the same gene; (5) most of the above findings are from *in vitro* studies, but studies involving *in vivo* models are needed to achieve the ultimate goal of translating these findings into clinical applications for human use; (6) how to overcome immune rejection of allogeneic transplants.

Conclusions

A large body of evidence supports the involvement of miRNAs in MSCs-related differentiation, although the mechanisms involved are not yet precise. Further studies are needed to discover new miRNAs and understand their complex mo-

Table I. Regulation of microRNA in the differentiation of dental pulp stem cells.

DPSCs	miRNA	Target gene	Effect	References
Odontogenic differentiation	miR-145/143	KLF4 OSX	(-)	27
	miR-143-3p	RANK	(-)	28
	miR-140-5p		(-)	29,30
	miR-218		(-)	31
	miR-125a-3p	Fyn	(+)	32
	miR-27a-5p		(+)	33
	miR-223	Smad3	(+)	34
	miR-21	TNF-α	(+)	35
Osteogenic differentiation	miR-143	TNF-α	(-)	39
	miR-215	HspB8	(-)	40
	miR-219a-1-3p		(-)	40
	hsa-miR-31		(-)	41
	miR-496	β-catenin	(-)	42
	miR-218	RUNX2	(-)	43
	miR-24-3p	TGFBR1	(+)	44
	miR-146a-5p		(+)	45
	miR-196a		(+)	46
	miR-188-3p		(+)	47
Vascular endothelial differentiation	miR-424	VEGF, KDR	(-)	48
	miR-378a		(+)	49
Skeletal muscle differentiation	miR-135/143	Sufu	(-)	53
Neuronal differentiation	miR-124		(+)	55

lecular activities at gene targets. Understanding the mechanisms by which specific miRNAs regulate targeted differentiation will be necessary for the future treatment of various related diseases.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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Authors' Contribution

Lili Cao wrote the paper. Feng Tian, Jiawei Wang and Yujuan Zhang provided constructive advice and edited the paper. ChunFang Wang revised the manuscript.

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