Life Sciences 220 (2019) 84–91

Contents lists available at ScienceDirect

Life Sciences

journal homepage: www.elsevier.com/locate/lifescie

MiR-199a-3p mediates the adipogenic differentiation of bone marrow-derived mesenchymal stem cells by regulating KDM6A/WNT signaling

Yi Shuai[a,1], Rui Yang[b,1], Rui Mu[a,1], Yang Yu[c], Liang Rong[a], Lei Jin[a,*]

[a] Department of Stomatology, Jin Ling Hospital (Nanjing General Hospital of Nanjing Military Command), Medical School of Nanjing University, Nanjing, Jiangsu 210002, People's Republic of China
[b] Department of Stomatology, PLA Army General Hospital, Beijing 100000, People's Republic of China
[c] Department of Endodontics, Affiliated Stomatology Hospital of Chongqing Medical University, Chongqing 404100, People's Republic of China

ARTICLE INFO

Keywords:
miR-199a-3p
KDM6A
WNT signaling
Adipogenic differentiation

ABSTRACT

A number of evidences suggest that microRNAs are involved in the adipogenic commitment of mesenchymal stem cells (MSCs). Recent studies have investigated that miR-199a-3p played a pivotal role in adipocyte differentiation. However, the detailed mechanism in this complex biological process remains largely unknown. In current study, we found that the expression of miR-199a-3p was gradually increased during adipogenic differentiation of bone marrow derived mesenchymal stem cells (BMMSCs). Enhanced expression of miR-199a-3p promoted adipogenesis, whereas silence of miR-199a-3p rescued BMMSCs from adipogenic commitment. For further mechanism exploration, KDM6A was confirmed to be the target of miR-199a-3p and the expression of KDM6A was gradually decreased during adipogenic differentiation of BMMSCs. Furthermore, up-regulation of KDM6A markedly abolished the miR-199a-3p overexpression induced adipogenic augmentation, whereas down-regulation of KDM6A suppressed the adipogenic reduction caused by miR-199a-3p silence. In addition, WNT signaling was also verified to be the downstream of miR-199a-3p/KDM6A to regulate adipogenic differentiation of BMMSCs. Taken together, current results indicate that miR-199a-3p regulate adipogenesis of BMMSCs by targeting KDM6A/WNT signaling, which highlights a new insight for a better understanding of molecular mechanism and stem cell based therapy on osteoporotic diseases.

1. Introduction

Bone marrow-derived mesenchymal stem cells (BMMSCs) are a sort of adult multipotent stem cells with self-renewal capability and multi-lineage differentiation capacity, including osteogenesis, adipogenesis and so forth [1]. The imbalance between osteogenic and adipogenic differentiation of BMMSCs has been reported to be crucial predisposing cause in osteoporotic diseases, such as estrogen deficiency induced osteoporosis, age-related osteoporosis and so on [2]. In bone marrow of osteoporotic subjects, BMMSCs shift their cell lineage commitment from osteogenesis to adipogenesis, leading to a massive bone loss and excessive fat accumulation in bone marrow [3,4]. Rescuing cell fate of BMMSCs is an emerging remedy for osteoporosis treatment [5,6]. However, the molecular mechanism of aberrant BMMSCs function remains unknown, which largely affects the development of osteoporosis management. Thus, revealing the detailed mechanisms of adipogenesis of BMMSCs is essential for developing novel strategies to manage osteoporotic diseases.

MicroRNAs (miRNAs) are a kind of small noncoding RNAs with about 22 nucleotides, which exert their biological function via regulating gene expression at post-transcription level [7]. MiRNAs have been reported to be involved in regulation of cellular proliferation, differentiation, apoptosis and so forth [8]. Notably, an increasing number of miRNAs have been revealed to play a pivotal role in regulating osteogenic and adipogenic differentiation of mesenchymal stem cells (MSCs) [4,9–12]. MiR-199a-3p, a tumorigenesis related miRNA [13–15], have been recently found to participate in determination of adipocyte differentiation [16–18]. However, the signalings involved in miR-199a-3p mediated biological process during BMMSCs adipogenic differentiation remains elusive.

H3K27 has been investigated to be methylated by enhancer of zeste homology 2 (EZH2), which controls MSCs multi-lineage commitment,

Abbreviations: miRNAs, microRNAs; BMMSCs, bone marrow derived mesenchymal stem cells; KDM6A, lysine demethylase 6A; PPARγ, peroxisome proliferator-activated receptor γ; aP2, adaptor related protein complex 2; hMSCs, human mesenchymal stem cells; H3K27, histone H3 lysine 27

* Corresponding author.
E-mail address: Ljin@nju.edu.cn (L. Jin).
[1] These authors contributed equally to the study.

https://doi.org/10.1016/j.lfs.2019.01.051
Received 15 September 2018; Received in revised form 28 January 2019; Accepted 29 January 2019
Available online 30 January 2019

such as osteogenesis and adipogenesis [3,19]. It is well known that KDM6A is a kind of histone demethylases, which functions as an epigenetic regulator on H3K27, indicating a relationship between KDM6A and MSCs commitment. KDM6A has been found to participate in many complex biological processes, such as embryos development [20,21], cell proliferation [22,23], apoptosis [24], differentiation [25,26], tumorigenesis [27,28] and so forth. Demethylation of histone H3 lysine 27 (H3K27) mediated by KDM6A led to a transcriptional depression, which was the answer for aforementioned biological process [29]. For regulating stem cell differentiation, KDM6A has been investigated to improve chondrogenic differentiation of periodontal ligament stem cells (PDLSCs) via demethylating SOX9 [25]. Furthermore, previous study has clarified that KDM6A acted as an epigenetic switch to regulate multi-lineage commitment of MSCs, including adipogenic differentiation [29]. WNT signaling is a commonly acceptable downstream pathway of KDM6A modulation. For example, KDM6A has been found to regulate definitive endoderm differentiation from human embryonic stem cells by modulating WNT pathway [26]. In addition, KDM6A has also been investigated to suppress the adipogenic differentiation of BMMSCs by activating WNT signaling [29]. Moreover, bioinformatics analysis of microRNAs target prediction indicated that KDM6A might be a putative miR-199a-3p target with a highly conserved miR-199a-3p binding site (Fig. S1). Thus, the purpose of current study was to reveal the function of miR-199a-3p in the adipogenic differentiation of BMMSCs, and investigate whether miR-199a-3p modulates adipogenic differentiation of BMMSCs by targeting KDM6A/WNT signaling.

2. Materials and methods

2.1. Animals

Twelve 8-week old female C57BL/6 mice (24–26 g) acquired from the Laboratory Animal Research Centre of Nanjing General Hospital of Nanjing Military Command were used to obtain BMMSCs. All the animal experiments were performed in accordance with the guidelines of National Institutes of Health and the Animal Care Committee of Nanjing General Hospital of Nanjing Military Command in this study.

2.2. BMMSCs culture

BMMSCs were isolated from long bone of C57BL/6 mice by flushing bone marrow with α-MEM medium (Gibco, USA). The cell suspension was seeded in 10-cm dishes containing α-MEM medium with addition of 20% FBS, 2 mM L-glutamine (Invitrogen, USA), 100 U/mL penicillin and 100 mg/mL streptomycin (Invitrogen, USA). Cells were incubated in an atmosphere of 37 °C, 5% CO_2 and saturation humidity, and the medium was changed every 3 days. BMMSCs were digested by 0.25% trypsin until 80% confluence. BMMSCs of passage two were used for research.

2.3. Colony formation, osteogenic differentiation and adipogenic differentiation

For colony formation assay, 5×10^2 BMMSCs were seeded in a 5-cm dish containing asal medium. After 10 days, the dish was rinsed with PBS. Cells were fixed using 4% paraformaldehyde and were stained using 1% (w/v) toluidine blue solution Sigma-Aldrich, USA). For osteogenic differentiation assay, BMMSCs were cultured in basal medium before 80% confluence. After confluence, cells were cultured in osteogenic medium (5 mM β-glycerophosphate, 50 μg/mL ascorbic acid and 10 nM dexamethasone (Sigma-Aldrich, USA)) for 28 days. Mineralized nodules were stained using alizarin red solution (Sigma-Aldrich, USA). For adipogenic differentiation assay, BMMSCs were cultured for 14 days in adipogenic differentiation medium supplemented with 0.5 μM dexamethasone, 0.5 mM isobutylmethylxanthine and 60 μM indomethacin (Sigma-Aldrich, USA). The lipid droplets were stained using Oil Red O solution. The colonies, mineralized nodules and lipid droplets were observed under the stereomicroscope (Olympus Optical, Japan).

2.4. BMMSCs transfection

For BMMSCs transfection, cells were seeded into six-well plates and transfection was conducted at about 60% cell confluence. Mimics, inhibitors and negative control (RiboBio, China) of miR-199a-3p were diluted at a working concentration of 50 nM. Mix diluted siPORT™ NeoFX™ Transfection Agent (Ambion, USA) and diluted mimics, inhibitors or negative control of miR-199a-3p respectively, and then incubate the mixture at room temperature for 10 min for further use. BMMSCs suspensions were overlaid onto the transfection complexes and incubate at 37 °C in basal culture for about 24 h. BMMSCs were infected by supplementation of 1×10^8 TU/ml KDM6A overexpression and silence lentivirus (RiboBio, China), 5 μg/ml poly-brene and complete medium (RiboBio, China) at 37 °C in basal culture for about 48 h.

2.5. Target prediction

TargetScan (version 7.2) and miRDB (version 2015) were used to predict targets of miR-199a-3p. Targets with a total context score of ≤ -0.5 in TargetScan, and targets with a target score of ≥ 90 in miRDB were selected for further bioinformatics analysis. Adipogenesis related targets were also selected according to reference review. The aforementioned three parts of candidate targets were overlapped using Venn diagram.

2.6. RNA extraction and qPCR

The isolation of total RNAs was performed using TRIzol reagent (Invitrogen, USA) according to the standard protocol. The cDNA of miRNA was obtained using Mir-X miRNA First-Strand Synthesis Kit (Clontech, USA) from 1000 ng total RNA, and the cDNA of mRNA was obtained using PrimeScript RT Reagent Kit (TaKaRa, Japan) from 1000 ng total RNA. The qPCR was analyzed using the SYBR Premix Ex Taq II kit (TaKaRa, Japan). Thermal cycles for miRNA: Step 1: 95 °C 10 s: Step 2: PCR reaction, GO TO: 39 (40 cycles), 95 °C 5 s, 60 °C 20 s; Step 3: Dissociation Curve 95 °C 60 s, 55 °C 30 s, 95 °C 30 s. Thermal cycles for mRNA: Step 1: 95 °C 30 s ; Step 2: PCR reaction , GO TO: 39 (40 cycles), 95 °C 5 s, 60 °C 30 s; Step 3: Melt Curve. U6 and β-actin were applied as internal control to normalize the expression of miRNAs and mRNAs respectively using $2^{-\triangle\triangle Ct}$ method. The primers were displayed in Table S1.

2.7. Luciferase reporter assay

The KDM6A oligonucleotide sequences were amplified using primers with *Hin*dIII and *Spe*I sites at their extremities to insert the pMIR-Report vector (Ambion, USA). The pMIR-Report, pMIR-KDM6A and pMIR-β-gal plasmids were used as reporter constructs, and were co-transfected into BMMSCs with miR-199a-3p mimics, inhibitors or negative control using Lipofectamine 2000 (Invitrogen, USA). After 48 h, luciferase activity was measured following the manufacturer's protocol, and normalized using β-gal.

2.8. WNT3A and DKK1 treatment

For analyzing effect of WNT3A on adipogenesis, 100 nM WNT3A (Sigma-Aldrich, USA) were supplemented during adipogenic differentiation of BMMSCs. For antagonist WNT3A, 200 nM DKK1 (Sigma-Aldrich, USA) were supplemented during adipogenic differentiation.

2.9. Western blot analysis

Cells were lysed using lysis buffer (Beyotime, China) and ultrasonic

at a low frequency. The harvested protein was quantified using BCA method. The protein was loaded on SDS-PAGE for separation, transferred to a PVDF membrane (Millipore, USA), and blocked with 5% non-fat milk powder solution. After blocking, the PVDF membrane was incubated with the primary antibodies of KDM6A (1:1000, Biolegend, USA), Wnt3a, β-actin and GAPDH (1:1000, Cell signaling, USA) diluted in TBST (0.1% Tween-20) solution overnight (> 8 h) at 4 °C, and then incubated with secondary antibody (1:100,000, Boster, China) at room temperature for 2 h. The blots were then developed using a protein enhancement imaging system. β-actin and GAPDH were applied as an internal control for normalization.

2.10. Statistical analysis

All the data are presented as the mean ± SEM. The data were analyzed using Student's *t*-test for comparison of two groups, and one-way ANOVA for comparison of multiple groups with SPSS software version 13.0 (SPSS, Chicago, IL, USA). $p < 0.05$ was considered as statistical significance.

3. Results

3.1. MiR-199a-3p is up-regulated during adipogenic differentiation of BMMSCs

The BMMSCs expressed positive markers of SCA-1, CD105 and CD73, while showed negative markers of CD11b, CD34 and CD45 (Fig. S2A), suggesting they were mesenchymal derived cells. In addition, colony formation (Fig. S2B), osteogenic differentiation (Fig. S2C) and adipogenic differentiation (Fig. S2D) exhibited the self-renewal capacity and multi-lineage differentiation potential of BMMSCs respectively.

To investigate whether miR-199a-3p is related to adipogenic differentiation, BMMSCs were induced by adipogenic differentiation induction medium. The expression of miR-199a-3p was gradually increased during adipogenic differentiation at day 0, day 7 and day 14 (Fig. 1), which indicates a crucial role of miR-199a-3p in adipogenic differentiation of BMMSCs.

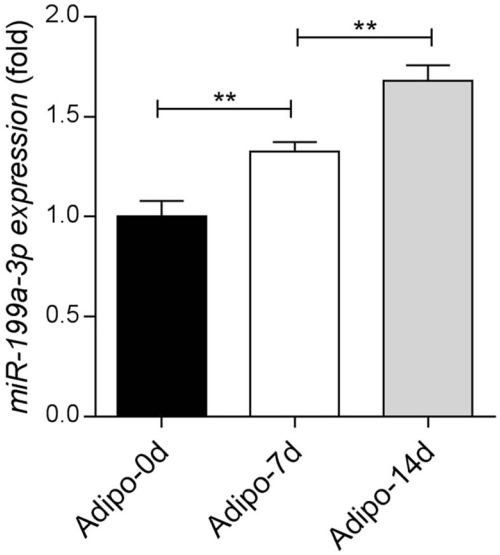

Fig. 1. miR-199a-3p expression during BMMSCs adipogenic differentiation. Normalized expression of miR-199a-3p was increased during adipogenic differentiation at day 0, 7 and 14. $^{**}p < 0.01$.

3.2. MiR-199a-3p positively regulates adipogenic differentiation of BMMSCs

To identify the biological role of miR-199a-3p in adipogenesis of BMMSCs, the gain and loss functional experiments were performed via transfecting mimics and inhibitor of miR-199a-3p into BMMSCs respectively (transfection efficiency see Fig. S3). The expression of miR-199a-3p was increased when BMMSCs were transfected with miR-199a-3p mimics, while was decreased in BMMSCs transfected with miR-199a-3p inhibitor (Fig. 2A). Further analysis of adipogenesis related genes expressions and Oil Red O staining were conducted to clarify the detailed biological function of miR-199a-3p in adipogenesis of BMMSCs. The results showed that *PPARγ*and *aP2* gene expressions were both up-regulated in BMMSCs transfected with miR-199a-3p mimics, while down-regulated in BMMSCs transfected with miR-199a-3p inhibitor (Fig. 2B and C). Moreover, Oil Red O staining also showed lipid droplets formation was positively related with *PPARγ*and *aP2* gene expressions (Fig.2D). Taken together, aforementioned results suggest that down-regulation of miR-199a-3p suppresses the adipogenic differentiation of BMMSCs.

3.3. MiR-199a-3p directly targets KDM6A

In order to explore the down-stream signaling of miR-199a-3p, which mediates the adipogenic differentiation of BMMSCs, candidate target genes were predicted using TargetScan 7.2 and miRDB databases. Notably, *KDM6A* was predicted to be a candidate target gene of miR-199a-3p, which is a newly reported regulator of adipocyte differentiation. The 3′-UTR of *KDM6A* mRNA gives a target binding site for miR-199a-3p sequence (Fig. 3A). Luciferase reporter assay displayed that overexpression of miR-199a-3p largely reduced the luciferase activity of *KDM6A* 3′-UTR, whereas knockdown of miR-199a-3p augmented the luciferase activity of reporter (Fig. 3B). Furthermore, up-regulation or down-regulation of miR-199a-3p scarcely affected *KDM6A* mRNA expression (Fig. 3C), whereas inhibited or promoted KDM6A protein expression respectively (Fig. 3D). Taken together, aforementioned results suggest that miR-199a-3p directly targets to *KDM6A* mRNA 3′-UTR and restrained KDM6A expression at post-transcriptional level.

3.4. MiR-199a-3p regulates adipogenic differentiation of BMMSCs via KDM6A mediated signaling

In order to verify the precise role of KDM6A in adipogenesis, the expression of KDM6A was detected during adipogenic induction of BMMSCs. The data showed that expression of KDM6A protein was down-regulated during adipogenic differentiation at day 0, day 7 and day 14, respectively (Fig. 4A). Furthermore, overexpression of KDM6A reduced *PPARγ*and *aP2* gene expressions, which were activated by miR-199a-3p (Fig. 4B and C). Meanwhile, Oil Red O staining also showed that up-regulation of KDM6A markedly suppressed miR-199a-3p-mediated lipid droplets formation (Fig. 4F). Additionally, knockdown of KDM6A abolished the reductions of adipogenic related gene expression (Fig. 4D and E) and lipid droplets formation (Fig. 4G), which were blocked by down-regulating miR-199a-3p. Taken together, aforementioned results suggest that miR-199a-3p regulates adipogenesis of BMMSCs through targeting KDM6A.

3.5. MiR-199a-3p regulates adipogenesis of BMMSCs via KDM6A/WNT signaling

WNT signaling, especially WNT3A has been reported to be involved in adipocyte differentiation [30,31]. Therefore, we validated the effects of WNT3A and its inhibitor DKK1 on adipogenic differentiation of BMMSCs. The results affirmed that WNT3A depressed adipogenic related gene expressions and lipid droplets formation, whereas the DKK1 eliminated the effect of WNT3A (Fig. 5A-C). To further uncover the

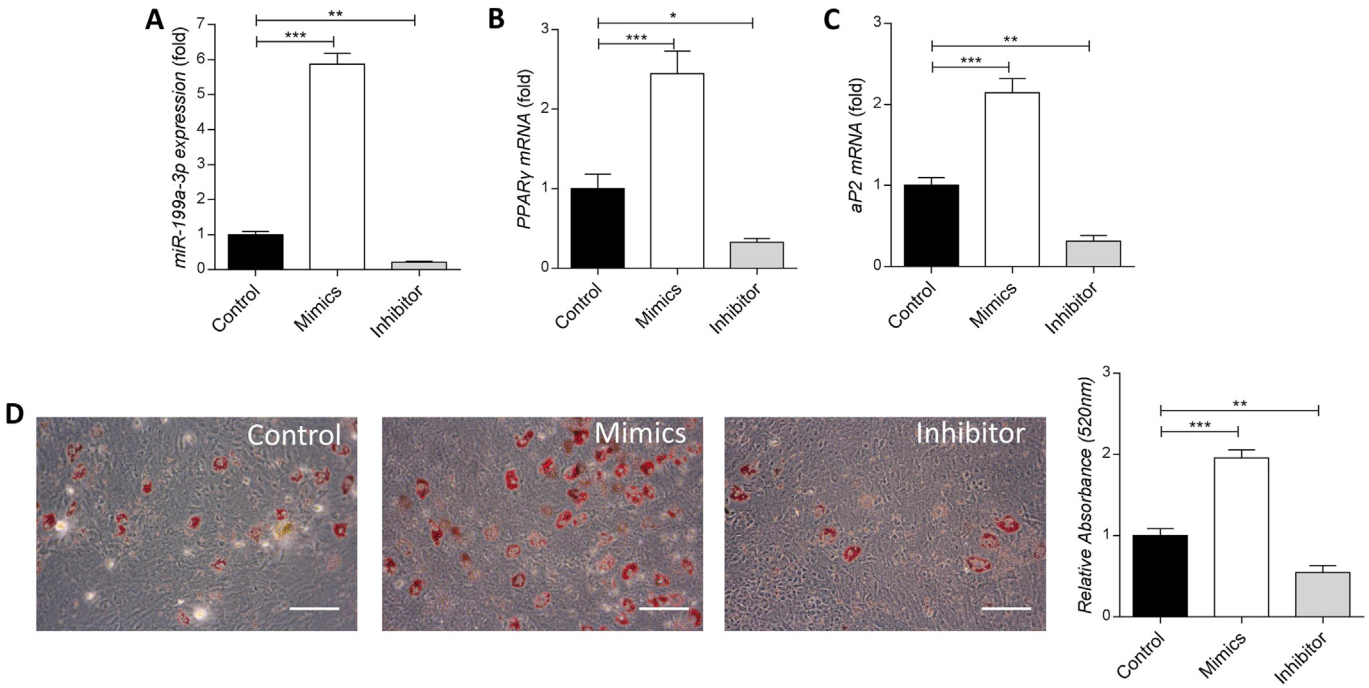

Fig. 2. miR-199a-3p regulates adipogenic differentiation of BMMSCs. (A) Normalized expression of miR-199a-3p was detected after transfecting with miR-199a-3p mimics or inhibitor in BMMSCs. Normalized expression of adipogenic related genes *PPARγ* (B) and *aP2* (C) were detected after transfecting with miR-199a-3p mimics or inhibitor in BMMSCs. Lipid droplets formation and its quantification (D) were determined by Oil Red O staining were detected after transfecting with miR-199a-3p mimics or inhibitor in BMMSCs. Scale bar: 500 μm. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$.

precise mechanism of miR-199a-3p in determining adipogenic differentiation of BMMSCs, we analyzed the modulation of miR-199a-3p on the expression of WNT3A, a downstream target of KDM6A in controlling adipogenesis. The data showed that overexpression of miR-199a-3p markedly suppressed both WNT3A gene and protein expressions, while knockdown of miR-199a-3p improved the expression of WNT3A gene and protein expressions (Fig. 5D and E). Remarkably, silencing of KDM6A largely inhibited the improvement of WNT3A expression, which was activated by down-regulation of miR-199a-3p (Fig. 5F and G). Furthermore, DKK1 abolished the anti-adipogenic effect on BMMSCs, which was induced by miR-199a-3p knockdown (Fig. 5H-J). Taken together, the aforementioned results suggest that miR-199a-3p

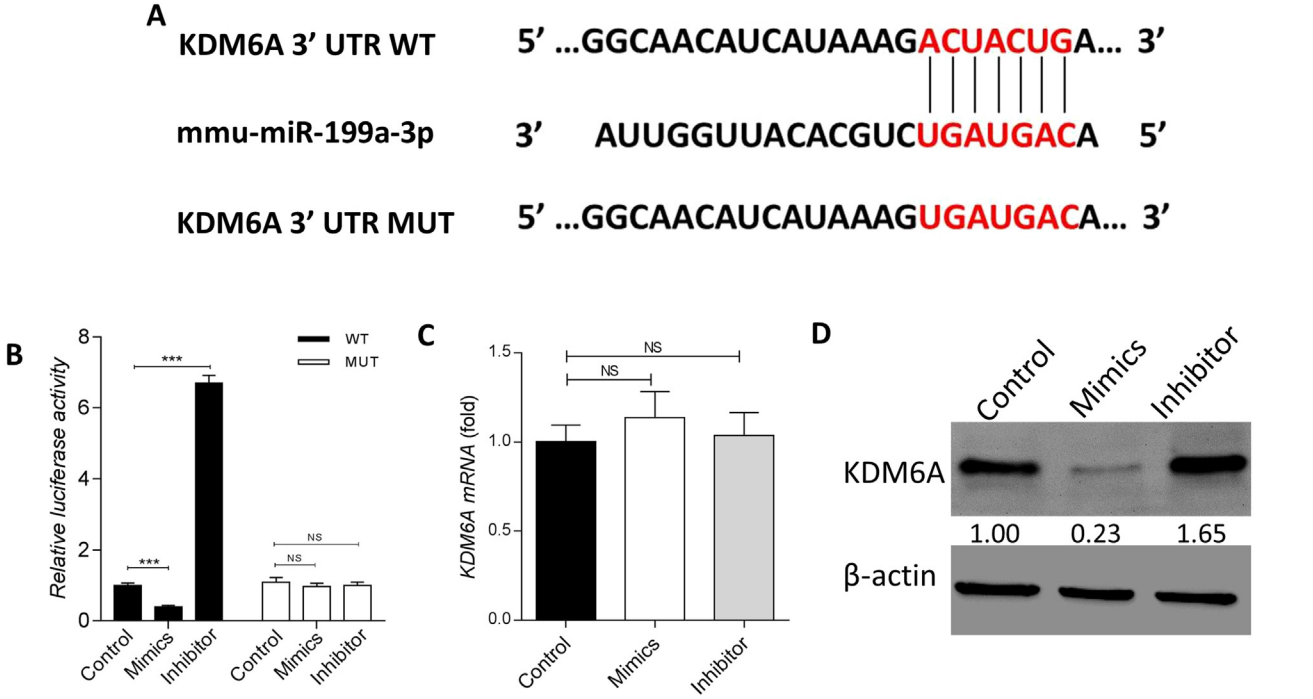

Fig. 3. KDM6A was a target gene of miR-199a-3p. (A) Seed-matched binding site between miR-199a-3p and KDM6A 3′-UTR WT or KDM6A 3′-UTR MUT. (B) Luciferase activity of pMIR-Reporter containing KDM6A 3'-UTR WT or MUT. Normalized expression of KDM6A gene (C) and protein (D) was detected after transfecting with miR-199a-3p mimics or inhibitor in BMMSCs. $***p < 0.001$.

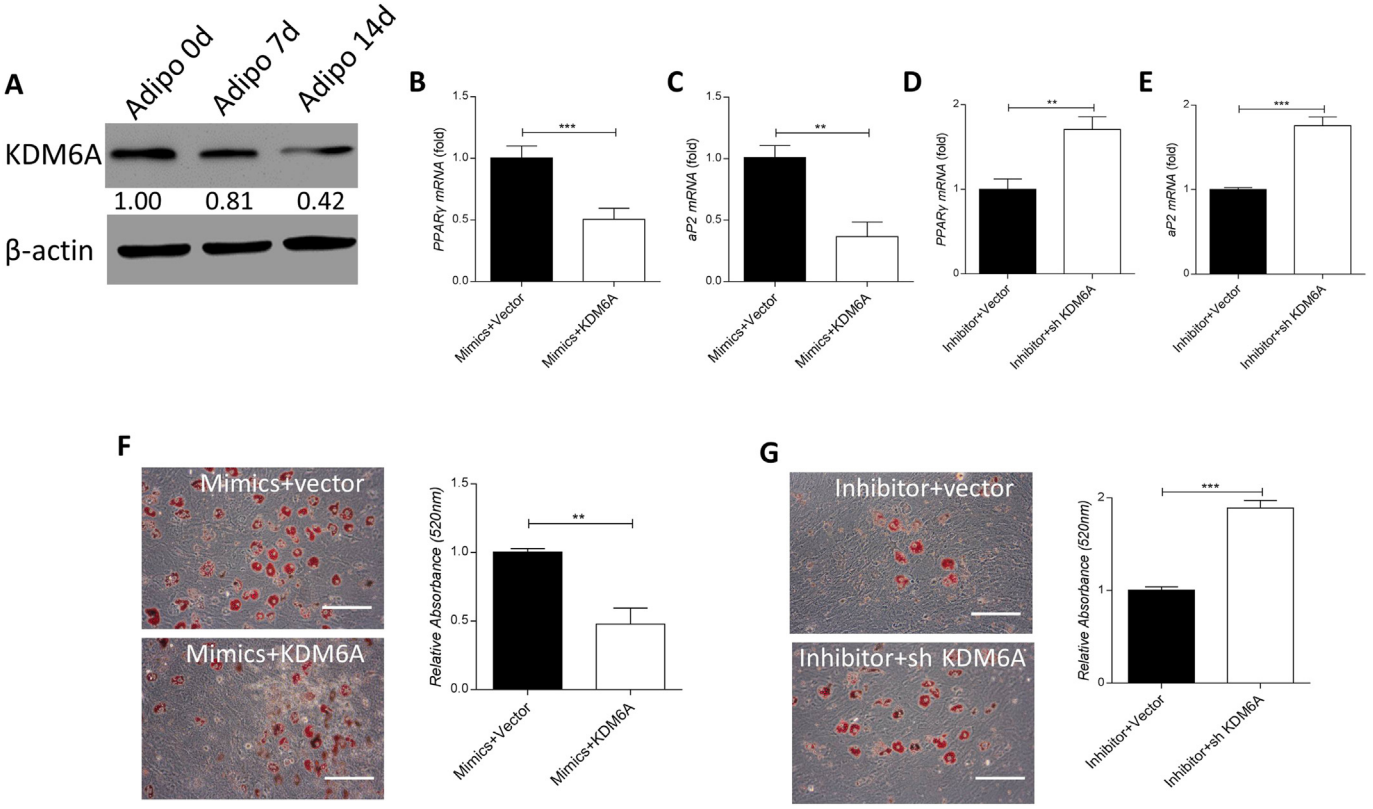

Fig. 4. MiR-199a-3p regulates BMMSCs adipogenesis via targeting KDM6A. KDM6A protein was detected during adipogenic differentiation at day 0, 7 and 14 (A). Normalized expression of *PPARγ* (B) and *aP2* (C) genes, lipid droplets formation and its quantification (F) were detected after co-transfecting miR-199a-3p mimics and KDM6A/vector. Normalized expression of *PPARγ* (D) and *aP2* (E) genes, lipid droplets formation and its quantification (G) were detected after co-transfecting miR-199a-3p inhibitor and shKDM6A/vector. Scale bar: 500 μm. $^{**}p < 0.01$, $^{***}p < 0.001$.

regulates adipogenesis of BMMSCs via KDM6A/WNT3A signaling (Fig. 6).

4. Discussion

Aberrant adipogenic differentiation of BMMSCs impairs the bone mass and quality, and regulation of adipogenesis of BMMSCs is a candidate target of stem cell-based therapy for osteoporotic disease treatment [12,32,33]. However, the detailed molecular mechanisms of BMMSCs multi-lineage commitment remain largely unknown. Recently, an increasing number of evidences indicated that miRNAs participated in regulating adipogenic differentiation of BMMSCs. For example, miR-377-3p inhibited adipogenic differentiation and adipogenic markers of hMSCs by targeting LIFR [34]. Additionally, miR-431 was also reported to be an adipogenic depressor of hMSCs via inhibiting insulin receptor substance 2 [35]. In contrast, miR-128 promoted adipogenesis of human mesenchymal stem cells (hMSCs) by suppressing VEGF pathway [36]. MiR-20a-5p was also investigated as an adipogenic promoter of BMMSCs through regulating Kruppel-like factor 3 [37].

MiR-199a-3p is a commonly recognized as a tumor repressor via not only inhibiting cancer cell proliferation [13,14], migration and invasion [14,15], but also accelerating cell apoptosis [15]. In recent years, miR-199a-3p has been investigated to be involved in adipocyte differentiation determination through modulating mTOR pathway [16], stearoyl-CoA desaturase (SCD) signaling [18]. Additionally, miR-199a-3p was also regulated by free fatty acids and inflammatory factors during fat development [17]. These evidences supported the fact that miR-199a-3p played an important role in adipogenesis. However, the biological function of miR-199a-3p in adipogenesis of BMMSCs remains elusive. Current study displayed that expression of miR-199a-3p was gradually up-regulated during the adipogenic differentiation of BMMSCs, indicating the crucial role of miR-199a-3p in determination of this biological process. Then we revealed the biological function of miR-199a-3p in adipogenic differentiation of BMMSCs. MiR-199a-3p obviously improved adipogenic differentiation of BMMSCs, which was indicated by up-regulation of adipogenic markers PPARγand aP2, as well as augmented lipid droplets formation. However, silence of miR-199a-3p rescued BMMSCs from adipogenic commitment. These results suggested that miR-199a-3p was closely related with adipogenesis of BMMSCs. To determine whether miR-199a-3p functioned as an initiator of adipogenesis or not, we detected adipogenic differentiation of BMMSCs without adipogenic stimuli. The result showed that miR-199a-3p could not improve BMMSCs adipogenesis without adipogenic induction (data not shown), suggesting that miR-199a-3p might be an intermediator of adipogenic process. Further study is essential to uncover which specific stage does miR-199a-3p kick in during adipogenesis of BMMSCs.

According to previous studies, controversial outcome regarding the role of miR-199a-3p in adipogenesis has been documented. It is reported that miR-199a-3p improved adipogenic differentiation in human adipose-derived mesenchymal stem cells [17], which is in accordance with the results of BMMSCs in our system. However, another study reported that although miR-199a-3p promoted adipocyte proliferation, it blunted the lipid accumulation [16]. The difference between the studies indicated that adipogenesis of mesenchymal stem cells and mature adipocyte were quite different. Furthermore, the other study investigated that miR-199a-3p inhibited brown adipocyte differentiation [18]. To our knowledge, yellow adipocytes were the predominant type of adipocytes in bone marrow, which partially stemmed from BMMSCs. Therefore, the difference might credit to the different types of adipocytes.

Although, NLK and KDM6A were screened from various targets for

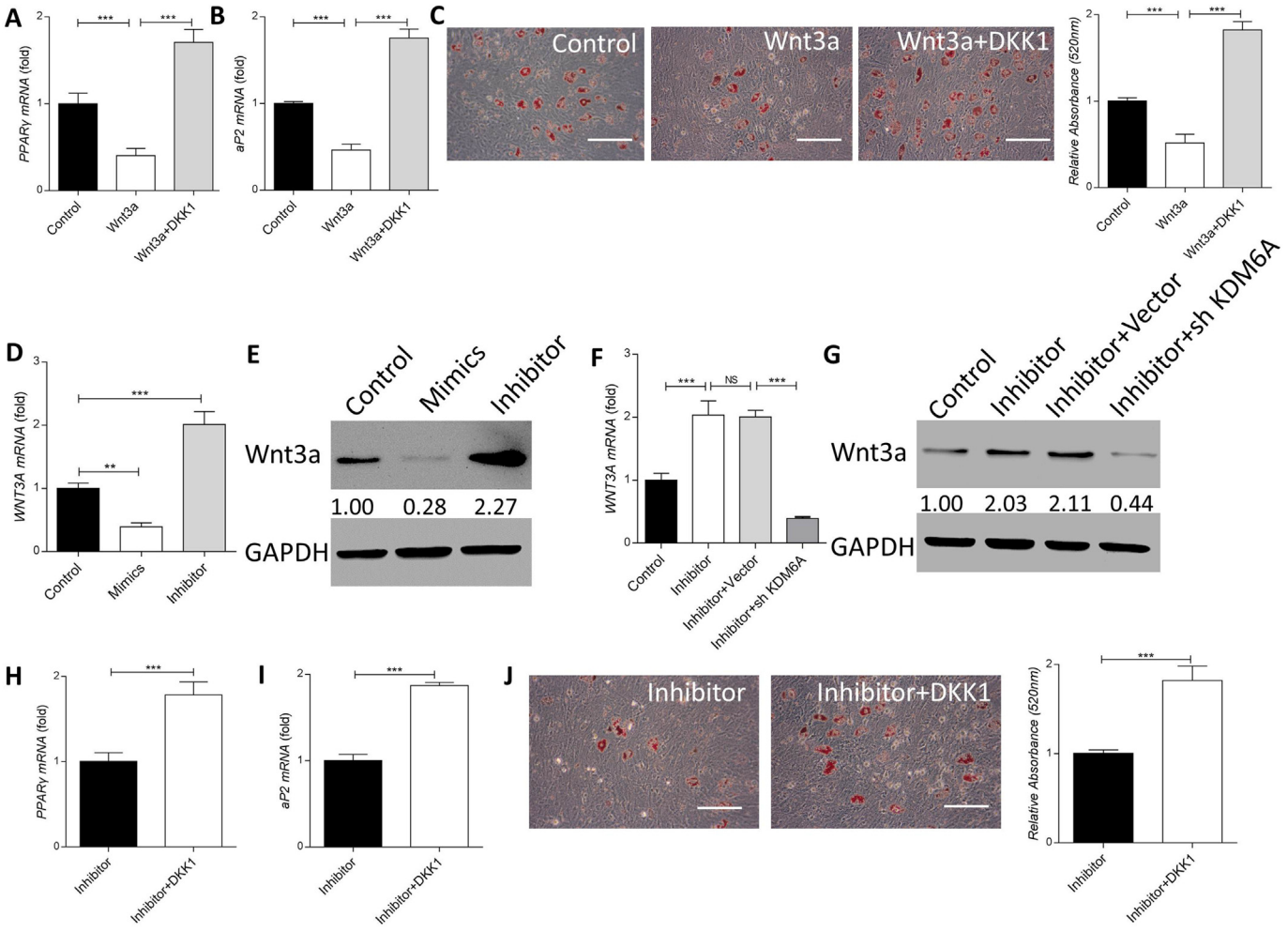

Fig. 5. miR-199a-3p regulates WNT signaling by targeting KDM6A. Normalized expression of *PPARγ* (A) and *aP2* (B) genes, lipid droplets formation and its quantification (C) were detected after treating BMMSCs with Wnt3a and DKK1 to. Normalized expression of *WNT3A* gene (D) and protein (E) was detected after transfecting with miR-199a-3p mimics or inhibitor in BMMSCs. Normalized expression of *WNT3A* gene (F) and protein (G) was detected after co-transfecting with miR-199a-3p inhibitor and shKDM6A/vector in BMMSCs. Normalized expression of *PPARγ* (H) and *aP2* (I), lipid droplets formation and its quantification (J) were detected after transfecting miR-199a-3p inhibitor in BMMSCs with/without addition of DKK1. Scale bar: 500 μm. $^{**}p < 0.01$, $^{***}p < 0.001$.

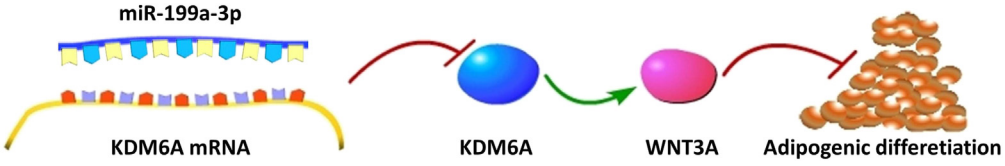

Fig. 6. Schematic diagram of miR-199a-3p/KDM6A/WNT signaling pathway.

miR-199a-3p mediated adipogenic regulation, we only selected KDM6A for further study. Our team has previously investigated that restrain of EZH2 inhibits the adipocytic determination of osteoporotic MSCs, which indicated that the histone demethylases-KDM6A acted an opposite role in adipogenesis. Therefore, we finally concentrated on KDM6A for further analysis. Our results indicated that KDM6A is a target of miR-199a-3p, and the expression of KDM6A was gradually declined during adipogenic differentiation of BMMSCs, and an enforced expression of KDM6A could inhibit adipogenesis of BMMSCs, suggesting that KDM6A is essential for the regulating of miR-199a-3p in adipogenic differentiation of BMMSCs.

It is well-known that the WNT signaling is pivotal for determination of adipogenesis [38–40]. In general, the activated WNT signaling regulates formation and development of adipocytes by an accumulation of β-catenin to translocate into the nucleus, which is evoking or silencing WNT/β-catenin target genes to suppress adipogenic differentiation [38,41,42]. KDM6A, an upstream modulator of WNT signaling, could activate the cytoplasmic WNT3 expression by demethylating H3K27 [29]. In current study, we verified that the activation of the WNT signaling suppress adipogenic differentiation of BMMSCs under the control of KDM6A and miR-199a-3p.

5. Conclusion

Current data supported that miR-199a-3p improved the adipogenic differentiation of BMMSCs by inhibiting KDM6A and sequential downstream inactivation of the WNT signaling pathway, uncovering a novel mechanism underlying adipogenic differentiation and highlighting a potential target for osteoporotic disease therapy.

Conflicts of interest

All the authors declare that they have no competing interests.

Authorship

Lei Jin, Yi Shuai, Rui Yang and Rui Mu conceived and designed the study. Lei Jin supervised the project. Yi Shuai, Rui Yang and Rui Mu conducted the experiments, analyzed the data and wrote the manuscript. Yang Yu and Liang Rong took part in sample collection and provided some technical supports. Lei Jin reviewed and revised the manuscript.

Acknowledgement

This project was funded by the Natural Science Foundation of China (grant nos. 81571817, 81771992, 81800979).

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.lfs.2019.01.051.

References

[1] S. Kokabu, J.W. Lowery, E. Jimi, Cell Fate and Differentiation of Bone Marrow Mesenchymal Stem Cells, Stem Cells Int 2016 (2016) 3753581, https://doi.org/10.1155/2016/3753581.

[2] C. Wang, H. Meng, X. Wang, C. Zhao, J. Peng, Y. Wang, Differentiation of bone marrow mesenchymal stem cells in osteoblasts and adipocytes and its role in treatment of osteoporosis, Med. Sci. Monit. 22 (2016) 226–233 https://www.ncbi.nlm.nih.gov/pubmed/26795027.

[3] H. Jing, L. Liao, Y. An, X. Su, S. Liu, Y. Shuai, X. Zhang, Y. Jin, Suppression of EZH2 prevents the shift of osteoporotic MSC fate to adipocyte and enhances bone formation during osteoporosis, Mol. Ther. 24 (2016) 217–229, https://doi.org/10.1038/mt.2015.152.

[4] L. Liao, X. Yang, X. Su, C. Hu, X. Zhu, N. Yang, X. Chen, S. Shi, S. Shi, Y. Jin, Redundant miR-3077-5p and miR-705 mediate the shift of mesenchymal stem cell lineage commitment to adipocyte in osteoporosis bone marrow, Cell Death Dis. 4 (2013) e600, https://doi.org/10.1038/cddis.2013.130.

[5] S. Liu, D. Liu, C. Chen, K. Hamamura, A. Moshaverinia, R. Yang, Y. Liu, Y. Jin, S. Shi, MSC transplantation improves osteopenia via epigenetic regulation of notch signaling in lupus, Cell Metab. 22 (2015) 606–618, https://doi.org/10.1016/j.cmet.2015.08.018.

[6] Y. Shuai, L. Liao, X. Su, Y. Yu, B. Shao, H. Jing, X. Zhang, Z. Deng, Y. Jin, Melatonin treatment improves mesenchymal stem cells therapy by preserving Stemness during long-term in vitro expansion, Theranostics 6 (2016) 1899–1917, https://doi.org/10.7150/thno.15412.

[7] W. Filipowicz, S.N. Bhattacharyya, N. Sonenberg, Mechanisms of post-transcriptional regulation by microRNAs: are the answers in sight? Nat. Rev. Genet. 9 (2008) 102–114, https://doi.org/10.1038/nrg2290.

[8] D.P. Bartel, MicroRNAs: target recognition and regulatory functions, Cell 136 (2009) 215–233, https://doi.org/10.1016/j.cell.2009.01.002.

[9] A. Asgharzadeh, S. Alizadeh, M.R. Keramati, M. Soleimani, A. Atashi, M. Edalati Fathabad, Z. Kashani Khatib, M. Rafiee, M. Barzegar, H. Razavi, Upregulation of miR-210 promotes differentiation of mesenchymal stem cells (MSCs) into osteoblasts, Bosn. J. Basic Med. Sci. (2018), https://doi.org/10.17305/bjbms.2018.2633.

[10] H. Wang, Z. Xie, T. Hou, Z. Li, K. Huang, J. Gong, W. Zhou, K. Tang, J. Xu, S. Dong, MiR-125b regulates the osteogenic differentiation of human mesenchymal stem cells by targeting BMPR1b, Cell. Physiol. Biochem. 41 (2017) 530–542, https://doi.org/10.1159/000457013.

[11] O. Trohatou, D. Zagoura, N.K. Orfanos, K.I. Pappa, E. Marinos, N.P. Anagnou, M.G. Roubelakis, miR-26a mediates Adipogenesis of amniotic fluid mesenchymal stem/stromal cells via PTEN, cyclin E1, and CDK6, Stem Cells Dev. 26 (2017) 482–494, https://doi.org/10.1089/scd.2016.0203.

[12] B. Li, MicroRNA regulation in osteogenic and Adipogenic differentiation of bone mesenchymal stem cells and its application in bone regeneration, Curr. Stem Cell Res. Ther. 13 (2018) 26–30, https://doi.org/10.2174/1574888X12666170605112727.

[13] P. Phatak, W.M. Burrows, I.E. Chesnick, M.E. Tulapurkar, J.N. Rao, D.J. Turner, A.W. Hamburger, J.Y. Wang, J.M. Donahue, MiR-199a-3p decreases esophageal cancer cell proliferation by targeting p21 activated kinase 4, Oncotarget 9 (2018) 28391–28407, https://doi.org/10.18632/oncotarget.25375.

[14] F. Qu, J. Zheng, W. Gan, H. Lian, H. He, W. Li, T. Yuan, Y. Yang, X. Li, C. Ji, X. Yan, L. Xu, H. Guo, MiR-199a-3p suppresses proliferation and invasion of prostate cancer cells by targeting Smad1, Oncotarget 8 (2017) 52465–52473, https://doi.org/10.18632/oncotarget.17191.

[15] J. Liu, B. Liu, Y. Guo, Z. Chen, W. Sun, W. Gao, H. Wu, Y. Wang, MiR-199a-3p acts as a tumor suppressor in clear cell renal cell carcinoma, Pathol. Res. Pract. 214 (2018) 806–813, https://doi.org/10.1016/j.prp.2018.05.005.

[16] Y. Gao, Y. Cao, X. Cui, X. Wang, Y. Zhou, F. Huang, X. Wang, J. Wen, K. Xie, P. Xu, X. Guo, L. You, C. Ji, miR-199a-3p regulates brown adipocyte differentiation through mTOR signaling pathway, Mol. Cell. Endocrinol. (2018), https://doi.org/10.1016/j.mce.2018.05.005.

[17] N. Gu, L. You, C. Shi, L. Yang, L. Pang, X. Cui, C. Ji, W. Zheng, X. Guo, Expression of miR-199a-3p in human adipocytes is regulated by free fatty acids and adipokines, Mol. Med. Rep. 14 (2016) 1180–1186, https://doi.org/10.3892/mmr.2016.5379.

[18] Z. Tan, J. Du, L. Shen, C. Liu, J. Ma, L. Bai, Y. Jiang, G. Tang, M. Li, X. Li, S. Zhang, L. Zhu, miR-199a-3p affects adipocytes differentiation and fatty acid composition through targeting SCD, Biochem. Biophys. Res. Commun. 492 (2017) 82–88, https://doi.org/10.1016/j.bbrc.2017.08.030.

[19] R.H. Chou, Y.L. Yu, M.C. Hung, The roles of EZH2 in cell lineage commitment, Am. J. Transl. Res. 3 (2011) 243–250 https://www.ncbi.nlm.nih.gov/pubmed/21654879.

[20] G.Y. Bai, S.H. Song, Y.W. Zhang, X. Huang, X.W. Huang, R.Z. Sun, L. Lei, Kdm6a overexpression improves the development of cloned mouse embryos, Zygote 26 (2018) 24–32, https://doi.org/10.1017/S0967199417000673.

[21] T. Porntaveetus, M.F. Abid, T. Theerapanon, C. Srichomthong, A. Ohazama, K. Kawasaki, M. Kawasaki, K. Suphapeetiporn, P.T. Sharpe, V. Shotelersuk, Expanding the Oro-dental and mutational spectra of kabuki syndrome and expression of KMT2D and KDM6A in human tooth germs, Int. J. Biol. Sci. 14 (2018) 381–389, https://doi.org/10.7150/ijbs.23517.

[22] L.D. Ler, S. Ghosh, X. Chai, A.A. Thike, H.L. Heng, E.Y. Siew, S. Dey, L.K. Koh, J.Q. Lim, W.K. Lim, S.S. Myint, J.L. Loh, P. Ong, X.X. Sam, D. Huang, T. Lim, P.H. Tan, S. Nagarajan, C.W. Cheng, H. Ho, L.G. Ng, J. Yuen, P.H. Lin, C.K. Chuang, Y.H. Chang, W.H. Weng, S.G. Rozen, P. Tan, C.L. Creasy, S.T. Pang, M.T. McCabe, S.L. Poon, B.T. Teh, Loss of tumor suppressor KDM6A amplifies PRC2-regulated transcriptional repression in bladder cancer and can be targeted through inhibition of EZH2, Sci. Transl. Med. 9 (2017), https://doi.org/10.1126/scitranslmed.aai8312.

[23] M.L. Nickerson, G.M. Dancik, K.M. Im, M.G. Edwards, S. Turan, J. Brown, C. Ruiz-Rodriguez, C. Owens, J.C. Costello, G. Guo, S.X. Tsang, Y. Li, Q. Zhou, Z. Cai, L.E. Moore, M.S. Lucia, M. Dean, D. Theodorescu, Concurrent alterations in TERT, KDM6A, and the BRCA pathway in bladder cancer, Clin. Cancer Res. 20 (2014) 4935–4948, https://doi.org/10.1158/1078-0432.CCR-14-0330.

[24] S. Cregan, M. Breslin, G. Roche, S. Wennstedt, L. MacDonagh, C. Albadri, Y. Gao, K.J. O'Byrne, S. Cuffe, S.P. Finn, S.G. Gray, Kdm6a and Kdm6b: altered expression in malignant pleural mesothelioma, Int. J. Oncol. 50 (2017) 1044–1052, https://doi.org/10.3892/ijo.2017.3870.

[25] P. Wang, Y. Li, T. Meng, J. Zhang, Y. Wei, Z. Meng, Y. Lin, D. Liu, L. Sui, KDM6A promotes chondrogenic differentiation of periodontal ligament stem cells by demethylation of SOX9, Cell Prolif. 51 (2018) e12413, , https://doi.org/10.1111/cpr.12413.

[26] W. Jiang, J. Wang, Y. Zhang, Histone H3K27me3 demethylases KDM6A and KDM6B modulate definitive endoderm differentiation from human ESCs by regulating WNT signaling pathway, Cell Res. 23 (2013) 122–130, https://doi.org/10.1038/cr.2012.119.

[27] M. Terashima, A. Ishimura, S. Wanna-Udom, T. Suzuki, Epigenetic regulation of epithelial-mesenchymal transition by KDM6A histone demethylase in lung cancer cells, Biochem. Biophys. Res. Commun. 490 (2017) 1407–1413, https://doi.org/10.1016/j.bbrc.2017.07.048.

[28] J. Andricovich, S. Perkail, Y. Kai, N. Casasanta, W. Peng, A. Tzatsos, Loss of KDM6A activates super-enhancers to induce gender-specific squamous-like pancreatic Cancer and confers sensitivity to BET inhibitors, Cancer Cell 33 (512–526) (2018) e518, , https://doi.org/10.1016/j.ccell.2018.02.003.

[29] S. Hemming, D. Cakouros, S. Isenmann, L. Cooper, D. Menicanin, A. Zannettino, S. Gronthos, EZH2 and KDM6A act as an epigenetic switch to regulate mesenchymal stem cell lineage specification, Stem Cells 32 (2014) 802–815, https://doi.org/10.1002/stem.1573.

[30] M. Ukita, T. Yamaguchi, N. Ohata, M. Tamura, Sclerostin enhances adipocyte differentiation in 3T3-L1 cells, J. Cell. Biochem. 117 (2016) 1419–1428, https://doi.org/10.1002/jcb.25432.

[31] M. Wu, Y. Wang, J.Z. Shao, J. Wang, W. Chen, Y.P. Li, Cbfbeta governs osteoblast-adipocyte lineage commitment through enhancing beta-catenin signaling and suppressing adipogenesis gene expression, Proc. Natl. Acad. Sci. U. S. A. 114 (2017) 10119–10124, https://doi.org/10.1073/pnas.1619294114.

[32] Z.W. Teng, Y. Zhu, Q. Na, X.G. Zhang, H. Zhao, G.J. Wei, J. Zhao, X.W. Zhang, G.Z. Wang, J. Liang, Z.J. Zhang, Y.G. Liu, Regulatory effect of miRNA on multi-directional differentiation ability of mesenchymal stem cell in treatment of osteoporosis, J. Biol. Regul. Homeost. Agents 30 (2016) 345–352 https://www.ncbi.nlm.nih.gov/pubmed/27358120.

[33] X. Ye, P. Zhang, S. Xue, Y. Xu, J. Tan, G. Liu, Adipose-derived stem cells alleviate osteoporosis by enhancing osteogenesis and inhibiting adipogenesis in a rabbit model, Cytotherapy 16 (2014) 1643–1655, https://doi.org/10.1016/j.jcyt.2014.07.009.

[34] X. Li, Y. Yang, R. Yan, X. Xu, L. Gao, J. Mei, J. Liu, X. Wang, J. Zhang, P. Wu, W. Li, Z. Zhao, J. Xiong, T. Wang, miR-377-3p regulates adipogenic differentiation of human bone marrow mesenchymal stem cells by regulating LIFR, Mol. Cell. Biochem. (2018), https://doi.org/10.1007/s11010-018-3366-0.

[35] Y. Wang, L. Yang, X. Liu, T. Hong, T. Wang, A. Dong, J. Li, X. Xu, L. Cao, miR-431 inhibits adipogenic differentiation of human bone marrow-derived mesenchymal stem cells via targeting insulin receptor substance 2, Stem Cell Res Ther 9 (2018) 231, , https://doi.org/10.1186/s13287-018-0980-4.

[36] W. Zhang, C. Yao, Z. Wei, Q. Dong, miR-128 promoted adipogenic differentiation

and inhibited osteogenic differentiation of human mesenchymal stem cells by suppression of VEGF pathway, J. Recept. Signal Transduct. Res. 37 (2017) 217–223, https://doi.org/10.1080/10799893.2016.1212375.

[37] E. Zhu, J. Zhang, J. Zhou, H. Yuan, W. Zhao, B. Wang, miR-20a-5p promotes adipogenic differentiation of murine bone marrow stromal cells via targeting Kruppel-like factor 3, J. Mol. Endocrinol. 60 (2018) 225–237, https://doi.org/10.1530/JME-17-0183.

[38] Z. Yuan, Q. Li, S. Luo, Z. Liu, D. Luo, B. Zhang, D. Zhang, P. Rao, J. Xiao, PPARgamma and Wnt signaling in Adipogenic and osteogenic differentiation of mesenchymal stem cells, Curr. Stem Cell Res. Ther. 11 (2016) 216–225 https://www.ncbi.nlm.nih.gov/pubmed/25986621.

[39] H. Ji, K. Ahn, H. Cho, H.E. Kim, Y. Kim, O. Kim, Sanguisorba officinalis L. extracts activate Wnt/beta-catenin pathway, and subsequently control adipo-osteogenic differentiation, Biochem. Biophys. Res. Commun. (2018), https://doi.org/10.1016/j.bbrc.2018.08.196.

[40] J. Zhou, S. Wang, Q. Qi, X. Yang, E. Zhu, H. Yuan, X. Li, Y. Liu, X. Li, B. Wang, Nuclear factor I-C reciprocally regulates adipocyte and osteoblast differentiation via control of canonical Wnt signaling, FASEB J. 31 (2017) 1939–1952, https://doi.org/10.1096/fj.201600975RR.

[41] X. Kong, X. Li, C. Zhang, L. Zhu, C. Liu, Q. Qin, C. Liu, Q. Wang, J. Zhu, X. Wu, H. Wan, W. Chen, N. Lin, Ethyl acetate fraction of Huogu formula inhibits adipogenic differentiation of bone marrow stromal cells via the BMP and Wnt signaling pathways, Int. J. Biol. Sci. 13 (2017) 480–491, https://doi.org/10.7150/ijbs.18430.

[42] S. Bolamperti, M. Signo, A. Spinello, G. Moro, G. Fraschini, F. Guidobono, A. Rubinacci, I. Villa, GH prevents adipogenic differentiation of mesenchymal stromal stem cells derived from human trabecular bone via canonical Wnt signaling, Bone 112 (2018) 136–144, https://doi.org/10.1016/j.bone.2018.04.014.