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miR-20a-5p promotes adipogenic differentiation of murine bone marrow stromal cells via targeting Kruppel-like factor 3

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Abstract

miR-20a-5p has recently been identified to induce adipogenesis of established adipogenic cell lines in our previous study. However, its role and molecular mechanisms in the regulation of adipocyte lineage commitment of bone marrow-derived stromal cells (BMSCs) still need to be explored. In this report, we demonstrated the expression of miR-20a-5p was promoted gradually during adipogenic differentiation in BMSCs. We also confirmed that miR-20a-5p has a positive function in the adipogenic differentiation of BMSCs by gain-of-function study with overexpression lentivirus or synthetic mimics of miR-20a-5p, and loss-of-function study with sponge lentivirus or synthetic inhibitor of miR-20a-5p. Dual luciferase reporter assay, GFP repression assay and Western blotting suggested Kruppel-like factor 3 (*Klf3*) was a direct target of miR-20a-5p. Furthermore, siRNA-mediated silencing of *Klf3* recapitulated the potentiation of adipogenesis induced by miR-20a-5p overexpression, whereas enhanced expression of *Klf3* attenuated the effect of miR-20a-5p. As *Klf3* was reported to play an inhibitory role in adipogenesis at the initial stage of differentiation, the findings we present here indicate that miR-20a-5p promotes adipocyte differentiation from BMSCs by targeting and negatively regulating *Klf3* in the early phase during the procedure of adipogenesis.

Key Words

- ▶ microRNA
- ▶ adipogenesis
- ▶ MSCs
- ▶ *Klf3*

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Introduction

Bone marrow-derived stromal cells (BMSCs) in culture can differentiate into several distinct cell lineages, including adipocytes, osteoblasts or chondrocytes (Pittenger *et al.* 1999, Caplan & Brude 2001). There are two major phases during the procedure of adipogenesis *in vitro*: the first phase was named as determination or commitment, which involves the conversion of the stem cell to pre-adipocyte; the second phase was termed as terminal differentiation, which results in the maturation of adipocyte from the pre-adipocyte (Rosen & MacDougald 2006). Adipocyte

differentiation is a well-orchestrated and multistep process that requires the sequential activation of signaling pathways and expression of numerous transcription factors. In general, CCAAT/enhancer-binding protein (C/EBP) β and C/EBP δ are activated very early during adipogenesis, which transcriptionally activates the expression of low levels of C/EBP α and peroxisome proliferator-activated receptor γ (PPAR γ), and both of the latter factors can then induce each other's expression in a positive feedback loop (Yeh *et al.* 1995, Lefterova *et al.*

2008). Finally, many downstream target genes are induced specially for promoting and maintaining the differentiated state of the adipocyte such as adipocyte protein 2 (aP2) (Tang & Lane 2012).

Adipogenesis is also regulated by lots of other transcription factors, for instance, the Kruppel-like factor (KLF) family (Sarjeant & Stephens 2012). KLFs are a large family of zinc-finger transcription factors, and it is derived from the drosophila protein 'Kruppel', which shares homology of proteins to the DNA-binding domains (Zuo *et al.* 1991). KLFs are critical in many physiological and pathological processes including cell proliferation, differentiation, inflammation and apoptosis. Recently, many KLF members have been identified to be involved in both adipogenesis and lipogenesis (Wu & Wang 2013). *Klf3*, as a member of the KLF family, could directly bind to the endogenous *C/ebpa* promoter and repress its activity, and overexpression of *Klf3* resulted in decreased adipogenesis from 3T3-L1 cells (Sue *et al.* 2008).

microRNAs (miRNAs) are ~22 nucleotides non-coding RNAs that function as guide molecules in RNA silencing by base pairing with its target mRNAs (Ha & Kim 2014). miRNAs are involved in nearly all developmental and pathological processes in animals, and recent emerging evidences suggest that miRNAs have the capacity to regulate adipogenesis by suppressing various target genes associated with adipogenic differentiation. For instance, miR-17-92 cluster, miR-204, miR-30e, miR-223 and miR-148a-3p promote adipocyte differentiation by negatively regulating retinoblastoma family (pRb) gene *Rb2/p130*, runt-related transcription factor 2 (*Runx2*), low-density lipoprotein receptor-related protein 6 (*Lrp6*), fibroblast growth factor receptor 2 (*Fgfr2*) and lysine-specific demethylase 6b (*Kdm6b*) respectively (Wang *et al.* 2008, 2013, Huang *et al.* 2010, Guan *et al.* 2015, Tian *et al.* 2017). miRNA let-7, miR-448, miR-24, miR-302a and miR-215 impair adipocyte differentiation by suppressing high-mobility group AT-hook 2 (*Hmga2*), *Klf5*, fatty acid-binding protein 4 (*Fabp4*, also termed *aP2*), PPAR γ , fibronectin type III domain-containing 3B (*Fndc3b*) and catenin, beta-interacting protein 1 (*Ctnnbip1*) respectively (Sun *et al.* 2009, Kinoshita *et al.* 2010, Kang *et al.* 2013, Jeong *et al.* 2014, Peng *et al.* 2016).

miR-20a-5p is a member of miR-17-92 family, we have previously demonstrated that miR-20a-5p was induced in primary cultured bone marrow stromal cells and established adipogenic cell lines after adipogenic treatment (Zhou *et al.* 2015). We also found supplementing miR-20a-5p could enhance adipogenesis in mouse adipogenic cell

lines such as 3T3-L1 preadipocytes, ST2 stromal cells and C3H10T1/2 mesenchymal cells. However, the effects of miR-20a-5p on adipogenic differentiation in BMSCs were not investigated, and the exact mechanisms by which miR-20a-5p determines the adipogenic fates of BMSCs is not yet been clarified.

In current study, we found the expression of miR-20a-5p was kept in a high level during adipogenic differentiation. Furthermore, we show that miR-20a-5p plays a positive role in the regulation of adipogenic differentiation in BMSCs by targeting *Klf3*. Our findings shed new light on the mechanisms of miR-20a-5p in adipogenesis.

Materials and methods

Ethics statement, mice

Four-week-old male C57BL/6J mice were purchased from HFK bioscience company (Beijing, China). All animal experiments and procedures were approved by the Animal Ethical and Experimental Committee of the Tianjin Medical University Metabolic Diseases Hospital.

Cells culture

BMSCs were isolated from femurs and tibias of 4-week-old C57BL/6J mice and cultured in α -MEM supplemented with 10% fetal bovine serum (FBS) in 10 cm dish as previously described (Zhou *et al.* 2017). When cells reached about 90% confluences, they were cultured in adipocyte-inducing medium (AIM, α -MEM containing 10% FBS, 0.5 mM dexamethasone, 0.25 mM methylisobutylxanthine, 5 mg/mL of insulin, and 50 mM indomethacin) for 72 h, followed by treatment for an additional 48 h with 5 mg/mL insulin alone. Human embryonic kidney (HEK)-293T cells were cultured in DMEM (Hyclone, ThermoFisher) with 10% FBS. All cells were maintained in a humidified incubator containing 5% CO₂ at 37°C.

Reagents

For Western blot assay, anti- β -actin was purchased from Sigma Chemical, anti-C/EBP α , anti-PPAR γ and anti-aP2 were purchased from Cell Signaling Technology; anti-KLF3 was purchased from Abcam and anti-KLF5 and anti-KLF7 were purchased from Protein Tech Group Ptglab (Wuhan, China). Lipofectamine RNAiMAX was purchased from Invitrogen.

Quantitative RT-PCR

Total RNA was extracted from cells using a total RNA isolation kit (Omega BioTek, Norcross, GA, USA). 0.5 µg total RNA of each sample was reverse-transcribed (RT) to cDNA with Reverse Transcription Kit (Thermo Fisher). Quantitative PCR (qPCR) analyses for the mRNA expression of *C/ebpα*, *Pparγ*, *aP2* and *Klf3* were performed by using SGExcel Fast SYBR Mixture kits (Sangon Biotech, Shanghai, China) on the Light Cycler 96 Real-Time PCR System (Roche Diagnostics), the mRNA level of NONO

was used as an internal control (Arsenijevic *et al.* 2012), and the detail parameters was followed as previously described (Zhu *et al.* 2016). For the expression analysis of miR-20a-5p, 0.5 µg RNA were reverse-transcribed to cDNA using the specific RT primer. Subsequently, miR-20a-5p was PCR amplified on a real-time PCR system using specific forward primers and a universal reverse primer, its expression level was normalized to U6, the detail parameters was followed as previously described (Zhou *et al.* 2015). All relative expression was calculated using

Table 1 Sequences of oligonucleotides used in this article.

Name	Sequence
Primer for stem-loop qRT-PCR studies to miRNA	
miR-20a-5p-RT	5'-GTC GTA TCC AGT GCA GGG TCC GAG GTA TTC GCA CTG GAT ACG ACC TAC CTG CAC TAT A-3'
U6-RT	5'-GTC GTA TCC AGT GCA GGG TCC GAG GTG CAC TGG ATA CGA CAA AAT ATG G-3'
miR-20a-5p-specific F	5'-ATG CTA AAG TGC TTA TAG T-3'
U6-specific F	5'-TGC GGG TGC TCG CTT CGG C-3'
Universal R	5'-CCA GTG CAG GGT CCG AGG T-3'
Primer for qRT-PCR analyses to mRNA	
<i>C/ebpα</i> -F	5'-CTG ATT CTT GCC AAA CTG AG-3'
<i>C/ebpα</i> -R	5'-GAG GAA GCT AAG ACC CAC TAC-3'
<i>PPARγ</i> -F	5'-CTT GAC AGG AAA GAC AAC GG-3'
<i>PPARγ</i> -R	5'-GCT TCT ACG GAT CGA AAC TG-3'
<i>aP2</i> -F	5'-AAA TCA CCG CAG ACG ACA GG-3'
<i>aP2</i> -R	5'-GGC TCA TGC CCT TTC ATA AAC-3'
<i>Klf3</i> -F	5'-GAA CCA TCC TTC CGT CAT CG-3'
<i>Klf3</i> -R	5'-TCA TCA GAC CGA GCG AAC TT-3'
<i>Nono</i> -F	5'-TGC TCC TGT GCC ACC TGG T-3'
<i>Nono</i> -R	5'-CCG GAG CTG GAC GGT TGA AT-3'
Ectopic expression vector construction	
<i>Klf3</i> -F	5'-CGG GAT CCG CCA GGA TGC TCA TGT TTG ATC CAG TCC-3'
<i>Klf3</i> -R	5'-GCT CTA GAT CAG ACT AGC ATG TGG CGT TTC C-3'
Report vector construction	
<i>Klf3</i> -3'UTR-F	5'-CGA GCT CGT CTT GAA GGA GTT GCT AAC G-3'
<i>Klf3</i> -3'UTR-R	5'-CCC AAG CTT AAT GCA ATA AGC CTG CTA TG-3'
<i>Klf3</i> -3'UTR-MUT-F	5'-CAA ATT CAA CCT CAT ATT TTT CCT GTT ATG TAC CTA GTG TTA GAG GG-3'
<i>Klf3</i> -3'UTR-MUT-R	5'-AAA AAT ATG AGG TTG AAT TTG TAA AAC ACA TTG TAG CCC TCT T-3'
Lentivirus vector construction	
Pre-miR-20a-5p-F	5'-AGA ATT CTG TCG ATG TAG AAT CTG CCT G-3'
Pre-miR-20a-5p-R	5'-AGG ATC CGA CAG TTT GAT TGG GCG ACA G-3'
miR-20a-5p-sponge-F	5'-TGC ACT ATA AGC ACT TTA GCT ACC TGC ACT ATA AGC ACT TTA TTT TTT ACG CGT GAA TTC ACT GGC CGT CGT T-3'
miR-20a-5p-sponge-R	5'-GGT AGC TAA AGT GCT TAT AGT GCA GGT AGC TAA AGT GCT TAT AGT GCA GGT AGC TAA AGT GCT TAT AGT GCA GGT AGC GGA TCC TCT AGA GTC GAC CT-3'
siRNA sequences	
si <i>Klf3</i> -1	5'-CCCUGCAAUUACAUAAGAA-3'
si <i>Klf3</i> -2	5'-CCAGUACCUGAAUUGAAU-3'
miRNA mimics, inhibitor and NC for transfection	
miR-20a-5p mimics-sence	5'-UAA AGU GCU UAU AGU GCA GGU AG-3'
miR-20a-5p mimics-anti-sence	5'-ACC UGC ACU AUA AGC ACU UUA UU-3'
miR-20a-5p inhibitor	5'-CUA CCU GCA CUA UAA GCA CUU UA-3'
mimics NC-sence	5'-UUC UCC GAA CGU GUC ACG UTT-3'
mimics NC-anti-sence	5'-ACG UGA CAC GUU CGG AGA ATT-3'
inhibitor NC	5'-CAG UAC UUU UGU GUA GUA CAA-3'

F, forward; NC, negative control; R, reverse.

the comparative threshold cycle method. The primer sequences used in the study were listed in [Table 1](#).

Construction of plasmids, lentivirus packaging and transduction

For lentivirus-mediated overexpression of miR-20a-5p, genomic sequence spanning the mouse miR-20a-5p-coding region flanked by approximately 100 base pairs from 5' or 3' on either end was cloned into lentiviral vector pCDH-CMV-MCS-EF1-copGFP (CD511B-1, System Bioscience, Mountain View, CA, USA) between the *EcoR* I site and the *BamH* I site. For control, the lentivirus packaged with the empty vector.

miR-20a-5p sponge vector was constructed as follows: the fragment containing 6 copies of miR-20a-5p-binding sites was amplified by PCR from pUC19 vector with two long specific primers, and then, this fragment was cloned into the pLVX-shRNA2 vector (Clontech Laboratories) at the *BamH* I site and the *EcoR* I site. The lentivirus packaged with the empty pLVX-shRNA2 vector served as a control. Virus was produced and target cells were infected according to the user's manual.

The construction of report vectors was based on the pMIR-REPORT and pMIR-GFP-REPORT for miR-20a-5p targets. The 3'-UTR mRNA sequences of *Klf3* containing the miR-20a-5p-binding site were amplified by PCR, and PCR products were cloned into vectors at *Spe* I and *Hind* III sites. Moreover, the mutant report vectors was constructed by In-Fusion cloning technology using Mut Express II Fast Mutagenesis Kit V2 (Vazyme, Nanjing, China) according to the user's manual.

The coding sequence of *Klf3* was amplified by PCR and cloned into pCDNA3.1 (+) vector at *BamH* I and *Xba* I sites.

The sequences of primers used are shown in [Table 1](#).

Western blotting

Treated cells were lysed by RIPA Lysis Buffer (Beyotime, Shanghai, China), and the immunoblotting was performed as previously described ([Zhu *et al.* 2014](#)). Briefly, the protein was separated in 12% SDS denatured polyacrylamide gel and then transferred onto a polyvinylidene difluoride membrane. The membranes were blocked with 5% skim milk at room temperature for 1 h and were incubated with appropriate antibodies at 4°C overnight respectively. Membranes were washed and incubated with horseradish

peroxidase (HRP)-conjugated secondary antibodies (1:3000, TransGen Biotech, Beijing, China) according to the manufacturer's instructions. Finally, the protein of interest was visualized using Immobilon Western Chemiluminescent HRP Substrate (Millipore).

Oil red O staining

Treated adipocyte-inducing BMSCs were gently washed twice with cold 1×PBS, and then fixed in 4% paraformaldehyde for 10 min. Next, the cells were washed twice with deionized water, and then stained with 60% saturated oil red O for 5 min. For oil red O quantification, isopropanol was added to each well, and then the plate was rocked on a shaker for 15 min. Light absorbance by the extracted dye was measured at 520 nm.

Cell transfection

Transfection was performed with Lipofectamine RNAiMAX Reagent according to the manufacturer's protocol. Briefly, cells were seeded in plates the day before transfection to ensure a suitable cell confluent on the day of transfection. For transfection of the oligonucleotides, 50 nM miR-20a-5p mimics, mimics negative control RNA (miR-NC), inhibitor and inhibitor NC (GenePharma, Shanghai, China) were used. For plasmids, transfection was performed with Lipofectamine 2000 Reagent, 80 ng of each firefly luciferase reporter vector, 20 ng of Renilla luciferase control vector and 200 ng GFP report vectors were used.

Luciferase assay and GFP repression experiments

HEK-293T cells were seeded in a 96-well plate, and 5000 cells were placed in each well the day before transfection. The cells were transfected with each firefly luciferase reporter vector, Renilla luciferase control vector pRL-TK (Promega), and either miR-20a-5p mimics or miR-NC for 48 h. The luciferase assay was performed as described ([Zhu *et al.* 2014](#)).

For GFP repression experiments, HEK-293 cells were seeded in a 12-well plate at 1×10^5 cells per well the day before transfection, and then were co-transfected with either miR-20a-5p mimics or miR-NC, and various GFP reporter vectors for 48 h. The cells were analyzed by fluorescence microscope and flow cytometer as described previously ([Zhu *et al.* 2014](#)).

Statistical analysis

The results are expressed as mean $\pm$ standard deviation (s.d.). A two-tailed Student's *t* test was applied to analyze the differences between the two groups, and one-way ANOVA was used followed by Tukey's test to analyze the differences between multiple groups. Statistical analysis was performed with SPSS software (version 17). Statistical differences were declared significant at $P < 0.05$ level. Statistically significant data are indicated by asterisks ($P < 0.05$ (*), $P < 0.01$ (**), and $P < 0.001$ (***)).

Results

miR-20a-5p was upregulated during adipocyte differentiation in BMSCs

We have found that miR-20a-5p was increased in BMSCs at 72 h after adipogenic treatment and demonstrated that supplementing miR-20a-5p activity favored adipogenic differentiation from preadipocyte line 3T3-L1, stromal cell line ST2 and mesenchymal cell line C3H10T1/2 (Zhou *et al.* 2015). In this study, to further define the function and mechanism of miR-20a-5p in adipogenic differentiation, we switched to the primary cultured BMSCs cell system. We monitored the expression of miR-20a-5p during adipocyte differentiation in BMSCs. Total RNA was collected at various stages throughout BMSCs differentiation and analyzed by RT-qPCR. As expected, the relative expression of adipogenic differentiation marker genes *C/ebp α* , *Ppar γ* and *aP2* was induced in BMSCs after adipogenic treatment (Fig. 1A, B and C). The expression of miR-20a-5p was upregulated gradually for the first 3 days, and its expression remained elevated at following 2 days compared to day 0 (Fig. 1D). Based on these results, we speculated that miR-20a-5p might play a regulatory role in BMSCs adipogenic differentiation.

Overexpression of miR-20a-5p enhanced adipocyte formation in BMSCs

To certify the role of miR-20a-5p during adipocyte differentiation from BMSCs, we made lentiviral constructs for miR-20a-5p manipulation *in vitro*. We constructed a plasmid-encoding pre-miR-20a-5p and used the empty vector as NC. Lentiviruses were packaged and named as LV-miR and LV-miR-ctrl respectively, and then the BMSCs were infected with LV-miR-20a-5p or LV-miR-ctrl for 2 days. The increased expression of miR-20a-5p was confirmed by RT-qPCR (Fig. 2A). Following adipogenic

Figure 1

miR-20a-5p was upregulated during adipocyte differentiation in BMSCs. Total RNA was extracted from BMSCs at the indicated time points after induction of adipogenic differentiation. (A, B and C) The mRNA expression levels of *C/ebp α* , *Ppar γ* and *aP2* were measured by RT-qPCR and normalized to *Nono* levels. (D) The expression levels of miR-20a-5p was tested by RT-qPCR and normalized to U6 level. The levels of the measurements at day 0 were set as 1. All data are representative of three independent experiments. The values are represented as means $\pm$ s.d. ($n = 3$). * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, compared with the day 0 group (ANOVA followed by Tukey's test was applied).

treatment, the miR-20a-5p expressing virus led to a significant increase of differentiated adipocyte numbers (28% increases in oil red O staining compared to LV-miR-ctrl group) (Fig. 2B and C). Accordingly, the protein levels of PPAR γ , C/EBP α and aP2 were significantly increased 3 days after adipogenic treatment (Fig. 2D). Furthermore, BMSCs were transfected with miR-20a-5p mimics to supplement the activity of miR-20a-5p, and the validity of miR-20a-5p ectopic expression was confirmed by RT-qPCR (Fig. 2E). We further demonstrated that miR-20a-5p mimics also significantly promoted adipogenic differentiation from BMSCs (26% increases in oil red O staining compared to miR-NC group) (Fig. 2F and G). Consistent with this, enhanced expression of PPAR γ , C/EBP α and aP2 was confirmed by Western blotting analysis (Fig. 2H). These results reveal that overexpression of miR-20a-5p can promote the adipogenic differentiation from BMSCs.

Inhibition of miR-20a-5p suppressed adipocyte formation in BMSCs

To further verify the promotion of adipogenesis by miR-20a-5p, loss-of-function analysis was performed using miR-20a-5p sponge lentivirus (LV-sponge). Dramatically, inhibition of miR-20a-5p attenuated the adipocyte

Figure 2

Overexpression of miR-20a-5p enhanced adipocyte formation from BMSCs. (A) Relative expression of miR-20a-5p was measured by RT-qPCR in BMSCs infected with pre-miR-20a-5p-encoding lentivirus (LV-miR-20a-5p) or its NC lentivirus (LV-miR-ctrl) for 2 days. (B, C and D) BMSCs were infected with lentivirus for 2 days, and then cultured in adipocyte-inducing medium (AIM). After 5 days, the adipocyte-inducing BMSCs were stained with Oil Red O, and representative images were shown (B). Oil Red O extracted with isopropanol was measured at OD520 (C). After 3 days, the adipocyte-inducing BMSCs were harvested, and the protein levels of PPAR γ , C/EBP α and aP2 were tested by Western blot analysis (D). (E) Relative expression of miR-20a-5p was measured by RT-qPCR in BMSCs transfected with miR-20a-5p mimics or its negative control (NC) for 2 days. (F, G and H) BMSCs were transfected with miR-20a-5p mimics or NC for 2 days, and then cultured in AIM. After 5 days, the adipocyte-inducing BMSCs were labeled with Oil Red O, and representative images were shown (F). Oil Red O extracted with isopropanol was measured at OD520 (G). The protein levels of PPAR γ , C/EBP α and aP2 were analyzed by Western blotting 3 days after adipogenic treatment (H). All data above represent three separate experiments. All photographs were taken using a light microscope at 200 $\times$ magnification, and the scale bars are presented at 100 μ m. The value in D and H under each lane indicates the relative expression level of the putative target gene, which is represented by the intensity ratio between PPAR γ , C/EBP α or aP2 and β -actin bands in each lane; β -actin was used as the internal control. The values are mean $\pm$ s.d. ($n=3$), * $P<0.05$, ** $P<0.01$, *** $P<0.001$, presented relative to NC group (Student's t test was applied in A and E, and ANOVA followed by Tukey's test was applied in C and G).

formation from BMSCs, and oil red O quantification revealed a 27% decrease after LV-sponge infection compared to control LV-sponge infection (LV-sponge-ctrl) (Fig. 3A and B). Immunoblotting further confirmed decreased protein expression levels of PPAR γ , C/EBP α and aP2 (Fig. 3C). Next, the effect of miR-20a-5p antisense oligonucleotide inhibitor was examined on adipogenesis from BMSCs. As shown in Fig. 3D, the expression level of miR-20a-5p was significantly reduced by this inhibitor. Consistently, repressed expression of miR-20a-5p through its inhibitor could reduce the adipogenic differentiation from BMSCs (30% decrease in oil red O staining compared to inhibitor NC group) (Fig. 3E and F). Furthermore, miR-20a-5p inhibitor also decreased the protein levels of PPAR γ , C/EBP α and aP2 (Fig. 3G). These results suggested that inhibition of miR-20a-5p can restrain the adipogenic differentiation from BMSCs.

Klf3 was a direct target gene of miR-20a-5p

For further assessing the function of miR-20a-5p, it is important to determine which host mRNAs are regulated. We used TargetScanMouse (version 7.1 http://www.targetscan.org/mmu_71/) to predict the candidate target genes of miR-20a-5p. *Kdm6b* and *Tgfb β 2* have been identified as direct targets of miR-20a-5p in our previous study (Zhou *et al.* 2015). In this report, we also found that there was a putative miR-20a-5p-binding site within the 3'-UTR of *Klf3* (Fig. 4A). To determine whether *Klf3* represent as a direct target of miR-20a-5p, we made the luciferase report constructs that contain the putative wild-type or mutant miR-20a-5p-binding sites within 3'UTR. We observed marked reduction in luciferase activity ($P < 0.01$) when co-transfecting the recombinant luciferase reporter constructs and miR-20a-5p mimics, compared to the cells co-transfected with the recombinant luciferase reporter constructs and NC; by contrast, mutation of the putative binding site rendered the construct loses the responsiveness to miR-20a-5p mimics (Fig. 4B). To confirm this, GFP repression assay was performed. GFP fluorescence was significantly reduced in HEK-293T cells transfected with GFP reporter constructs containing binding sites and miR-20a-5p mimics (Fig. 4C, D and E). Furthermore, overexpression of miR-20a-5p in BMSCs resulted in the downregulation of the mRNA and protein level of *Klf3*, and in contrast, miR-20a-5p inhibitor increased the mRNA and protein level of *Klf3* (Fig. 4F and G). Taken together, these data suggest that *Klf3* is a potential target of miR-20a-5p.

Klf3 was potentially involved in miR-20a-5p-promoted adipocyte formation

To investigate whether *Klf3* is involved in miR-20a-5p-promoted adipogenic differentiation, we first tested if the silencing expression of *Klf3* may have the similar pro-adipogenic effect as miR-20a-5p overexpression. BMSCs were transfected respectively with two *Klf3* siRNAs or NC RNA (si-ctrl) for 48 h. As shown in Fig. 5A, the mRNA level of *Klf3* could be reduced by its siRNAs. Subsequently, *Klf3* siRNAs obviously displayed stimulatory effect on the adipocyte formation from BMSCs (Fig. 5B and C). Silencing of *Klf3* also significantly promoted the mRNA expression of *Ppar γ* , *C/ebp α* and *aP2* (Fig. 5D), which phenocopied the effect of miR-20a-5p overexpression. Furthermore, we investigated whether *Klf3* could counteract the pro-adipogenic function of miR-20a-5p. The expression construct pCDNA3.1-*Klf3* (*Klf3*), which encoded the entire coding sequence of *Klf3*, was made. We demonstrated the overexpression of *Klf3* in BMSCs with RT-qPCR and immunoblotting (Fig. 5E). As shown in Fig. 5F and G, overexpression of *Klf3* abrogated miR-20a-5p-promoted adipogenesis. Consistently, the mRNA levels of adipogenic marker genes were decreased in the cells co-transfected with miR-20a-5p mimics and pCDNA3.1-*Klf3*, as compared to the cells co-transfected with miR-20a-5p mimics and vector (Fig. 5H). These results suggested that *Klf3* is potentially involved in miR-20a-5p-promoted adipocyte formation.

Discussion

Adipogenic differentiation from BMSCs is regulated by multiple regulatory factors and signaling pathways. Upon induction of adipogenic factors, C/EBP β and δ activate C/EBP α and PPAR γ to coordinate the expression of adipogenic genes characteristic of terminally differentiated adipocytes like aP2 (Kang & Hata 2015). Moreover, emerging evidences suggest that miRNAs also regulate fate decisions of BMSCs to influence adipocyte differentiation (Scheideler 2016). In our previous report, we have shown that miR-20a-5p was upregulated in BMSCs and adipogenic cell lines 3T3-L1 and C3H10T1/2 3 days after adipogenic treatment (Zhou *et al.* 2015). In the current study, we further demonstrated the expression of miR-20a-5p was promoted gradually at the first 3 days during adipogenic differentiation from BMSCs, and its expression remained high level at the next 2 days (Fig. 1).

miR-20a-5p belongs to the miR-17-92 cluster that is highly expressed in various cancers (Hayashita *et al.* 2005,

Figure 3

Inhibition of miR-20a-5p suppressed adipocyte formation from BMSCs. (A, B and C) BMSCs were infected with miR-20a-5p sponge lentivirus (LV-sponge) or control LV-sponge (LV-ctrl) for 2 days, and then cultured in AIM. After 5 days, the adipocyte-inducing BMSCs were labeled with Oil Red O, and representative images are shown (A). Oil Red O extracted with isopropanol was measured at OD520 (B). After 3 days, the adipocyte-inducing BMSCs were harvested, and the protein levels of PPAR γ , C/EBP α and aP2 were analyzed by immunoblotting (C). (D) Relative expression of miR-20a-5p was measured by RT-qPCR in BMSCs transfected with miR-20a-5p inhibitor or its negative control (Inhibitor NC) for 2 days. (E, F and G) BMSCs were transfected with miR-20a-5p inhibitor or inhibitor NC for 2 days, and then cultured in AIM. After 5 days, the adipocyte-inducing BMSCs were labeled with Oil Red O, and representative images were shown (E). Oil Red O extracted with isopropanol was measured at OD520 (F). After 3 days, Immunoblotting for PPAR γ , C/EBP α and aP2 was performed (G). All data are from three separate experiments. All images were taken using a light microscope at 200 $\times$ magnification, and the scale bars are presented at 100 μ m. The value in C and G under each lane indicates the relative expression level of the putative target gene, which is represented by the intensity ratio between PPAR γ , C/EBP α or aP2 and β -actin bands in each lane; β -actin was used as the internal control. The values are mean $\pm$ s.d. ($n=3$), * $P<0.05$, ** $P<0.01$, presented relative to NC group (ANOVA followed by Tukey's test was applied).

Figure 4

Klf3 was a candidate target gene of miR-20a-5p. (A) Sequence alignment of miR-20a-5p and its putative target site in the 3'UTR of *Klf3* mRNA. Mutation was made in the complementary sites for the seed region of miR-20a-5p as indicated. (B) Luciferase activity analysis. HEK-293T cells were transiently co-transfected with Renilla luciferase control vector pRL-TK, firefly luciferase reporter constructs containing either wild-type or mutant 3'UTR of *Klf3*, and either miR-20a-5p NC or mimics for 48 h. Firefly luciferase activities were normalized to the activity of Renilla luciferase. (C and D) GFP repression assay. HEK-293T cells were transfected with the wild-type or mutant GFP construct, and either miR-20a-5p mimics or NC. GFP fluorescence was monitored by fluorescence microscope (C) and FCM analysis (D). (E) The statistical analysis of GFP repression rate for experiments in D is shown. (F) BMSCs were transfected with miR-20a-5p mimics, mimics NC, miR-20a-5p inhibitor or inhibitor NC. After 72 h, the mRNA level of *Klf3* was analyzed by RT-qPCR. (G) Western blot analysis for *Klf3* in transfected cells in F is shown. The value under each lane indicates the relative expression level of the putative target gene, which is represented by the intensity ratio between KLF3 and β -actin bands in each lane, β -actin was used as the internal control. All data above are representative of at least three independent experiments. All image magnification is 100 $\times$, and the scale bars are presented at 50 μ m. The values are mean $\pm$ s.d. ($n=3$), * $P < 0.05$, ** $P < 0.01$, presented vs control group (Student's t test was applied in F, and ANOVA followed by Tukey's test was applied in B and E).

Figure 5

Klf3 was potentially involved in miR-20a-5p-promoted adipocyte formation. (A) RT-qPCR was used to monitor the expression of *Klf3* 48 h after transfection with *Klf3* siRNAs (si*Klf3*) or NC RNA (si-Ctrl), *Nono* served as internal control. (B, C and D) BMSCs were transfected with si-Ctrl or si*Klf3* for 2 days, and then cultured in AIM. After 5 days, the adipocyte-inducing BMSCs were stained with Oil Red O, and representative images are shown (B). Oil Red O extracted with isopropanol was measured at OD520 (C). RT-qPCR was performed to test the mRNA expression of adipocyte specific genes *Clebpα*, *Pparγ* and *aP2* (D). (E) RT-qPCR and Western blotting were used to test the expression of *Klf3* 2 days after transfection with indicated expression construct, the value under each lane of Western blotting indicates the relative expression level of the putative target gene, which is represented by the intensity ratio between *Klf3* and β -actin bands in each lane. *NONO* and β -actin served as internal controls respectively. (F, G and H) BMSCs were transfected with indicated RNA duplex for 1 day, and transfected with indicated expression vector for another day. Next, the cells were cultured in AIM. After 5 days, the BMSCs were stained with Oil Red O, and representative images are shown (F). Oil Red O extracted with isopropanol was measured at OD520 (G). The mRNA levels of *Clebpα*, *Pparγ* and *aP2* were measured with RT-qPCR (H). All data are from three independent experiments. All image magnification is 200 $\times$, and the scale bars are presented at 100 μ m. The values are mean $\pm$ s.d. ($n=3$), * $P<0.05$, ** $P<0.01$, *** $P<0.001$, presented vs control group (ANOVA followed by Tukey's test was applied).

Takakura et al. 2008, Mu et al. 2009, Tsuchida et al. 2011). In recent years, several members of miR-17-92 cluster have been identified to be involved in the regulation of adipogenesis. miR-17-92 cluster accelerates adipocyte differentiation by negatively regulating tumor-suppressor *Rb2/p130* in 3T3-L1 cells (Wang et al. 2008). miR-17-5p and miR-106a promote adipogenesis in human adipose-derived MSCs by targeting BMP2 (Li et al. 2013). We previously reported that miR-20a-5p increased adipogenesis in murine cell lines 3T3-L1, ST2 and C3H10T1/2 cells, which is dependent upon its inhibitory effects on KDM6B and TGF- β signaling (Zhou et al. 2015). In the present study, we switched to the primary cultured BMSCs cell system to further clarify the function and mechanism of miR-20a-5p in adipogenic differentiation by gain- or loss-of-function studies. We found ectopic expression of miR-20a-5p, either by overexpression lentivirus or synthetic mimics, favored adipocyte differentiation; by contrast, inhibition of endogenous miR-20a-5p, either by sponge lentivirus or synthetic inhibitor, reduced adipocyte differentiation (Figs 2 and 3). These findings that miR-20a-5p affects adipogenesis of BMSCs coincided with our previous report (Zhou et al. 2015), and it suggests that miR-20a-5p have a pivotal role in adipogenic differentiation of BMSCs. We also found adipocyte differentiation could not be triggered by overexpression of miR-20a-5p without hormonal cocktail induction (data not shown), and we have previously shown that miR-20a-5p had no effect when the cells have differentiated after 3 days of adipogenic treatment (Zhou et al. 2015). All these results suggested that miR-20a-5p is a necessary adjunct to hormonal signals at the initial stage of adipogenic differentiation.

Next, it is important to identify target genes of miR-20a-5p for exploring the molecular mechanism underlying its function. Although *Kdm6b* and *Tgfb2* have been validated to involve in the regulation of adipogenesis by miR-20a-5p in our previous study (Zhou et al. 2015), there is no evidence to indicate that they play a regulatory role in initiate differentiation. In present study, we focused on whether there were target genes of miR-20a-5p at the early stage of adipocyte differentiation. Recently, numerous evidences suggest several KLF family members have important roles as immediate early regulators in adipogenesis. Oishi and coworkers reported that KLF5 expression is induced by C/EBP β and δ and can directly bind to the endogenous PPAR γ promoter to modestly stimulate the activity of PPAR γ promoter (Oishi et al. 2005). KLF4 modulates adipocyte formation by directly binding to the C/EBP β promoter and together with Krox20 cooperatively transactivates C/EBP β (Birsoy et al. 2008). KLF2 directly

binds to PPAR γ 2 promoter and inhibits the promoter activity to exert its suppressive role during adipogenesis (Banerjee et al. 2003). Overexpressing KLF7 decreases the differentiation of the human preadipocyte and inhibits glucose-induced insulin secretion in pancreatic β -cell line (Kawamura et al. 2006). Sue and coworkers reported that (I) the mRNA level of *Klf3* was the highest at day 0 in 3T3-L1 preadipocytes and gradually diminished upon adipocyte differentiation; (II) overexpression of *Klf3* could inhibit adipogenic differentiation of 3T3-L1 cells *in vitro*; (III) *Klf3* can directly bind to the endogenous C/EBP α promoter and repress its activity (Sue et al. 2008). Considering that both C/EBP α and PPAR γ can positively regulate the expression of one another in the early stage of adipogenic commitment (Mota de Sa et al. 2017), we conclude that *Klf3* may suppress the activity of *C/ebp α* to reduce the expression of *Ppar γ* , and then, the expression of *C/ebp α* and *Ppar γ* will be decreased to a lower level in a negative feedback loop. Therefore, *Klf3* was selected basing on its established inhibitory role in adipogenesis at the initial stage of differentiation, and we tried to identify whether it was a direct target of miR-20a-5p.

With the assistance of prediction programs, *Klf3* was predicted to be a putative target because there was a targeting site for miR-20a-5p in its 3'UTR (Fig. 4A). Subsequent luciferase assay and GFP repression experiments provided evidence that miR-20a-5p directly targeted the 3'UTR of *Klf3* mRNA (Fig. 4B, C, D and E). We also showed that miR-20a-5p regulated *Klf3* expression at the transcriptional level (Fig. 4F and G); in addition, the manipulate of miR-20a-5p mimics or inhibitor did not affect the protein level of two other KLF family members KLF5 and KLF7 (Supplementary Fig. 1, see section on supplementary data given at the end of this article), which indicates that *Klf3* may be the only direct target of miR-20a-5p in KLF family members, and there may not be any compensatory changes of other KLF family members upon *Klf3* upregulation or downregulation. Next, we found silencing of *Klf3* expression had similar stimulatory effect on adipogenic differentiation compared with ectopic expression of miR-20a-5p (Fig. 5B, C and D), and overexpression of *Klf3* could counteract the effect of increasing adipogenesis by miR-20a-5p (Fig. 5F, G and H); moreover, simultaneous overexpression of *Klf3*, *Kdm6b* and *Tgfb2* could be more effectively to neutralize the role of miR-20a-5p in promoting adipogenesis (Supplementary Fig. 2). Based on our experimental data, we propose the following model: after hormonal cocktail induction, the expression of miR-20a-5p is upregulated, resulting in a decrease of *Klf3*; then, the inhibition of C/EBP α is

abrogated by the downregulation of KLF3; finally, PPAR γ is activated to stimulate the expression of downstream adipogenic genes characteristic of mature adipocytes.

In summary, a series of present findings provided evidence that miR-20a-5p has a positive effect on adipogenic differentiation from BMSCs. We for the first time demonstrated miR-20a-5p promoted adipogenesis from BMSCs by directly targeting *Klf3*. Our findings expand our knowledge on the mechanism underlying the function of miR-20a-5p in adipogenesis and indicate the potential therapeutic application of miR-20a-5p in metabolic disorders caused by adipogenic dysregulation such as obesity.

Supplementary data

This is linked to the online version of the paper at <https://doi.org/10.1530/JME-17-0183>.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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Author contribution statement

Endong Zhu analyzed and interpreted the data and drafted the manuscript. Endong Zhu, Juanjuan Zhang, Jie Zhou, Hairui Yuan and Wei Zhao analyzed the data; Baoli Wang critically revised the manuscript and responsible for important intellectual content. All authors approved the final version for publication.

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