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miR-17-5p and miR-106a are involved in the balance between osteogenic and adipogenic differentiation of adipose-derived mesenchymal stem cells

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Abstract Mesenchymal stem cells (MSCs) can differentiate into several distinct cell types, including osteoblasts and adipocytes. The balance between osteogenic and adipogenic differentiation is disrupted in several osteogenic-related disorders, such as osteoporosis. So far, little is known about the molecular mechanisms that drive final lineage commitment of MSCs. In this study, we revealed that miR-17-5p and miR-106a have dual functions in the modulation of human adipose-derived mesenchymal stem cells (hADSCs) commitment by gain- and loss-of-function assays. They could promote adipogenesis and inhibit osteogenesis. Luciferase reporter assay, western blot and ELISA suggested BMP2 was a direct target of miR-17-5p and miR-106a. Downregulation of endogeneous BMP2 by RNA interference suppressed osteogenesis and increased adipogenesis, similar to the effect of miR-17-5p and miR-106a upregulation. Moreover, the inhibitory effects of miR-17-5p on osteogenic and adipogenic differentiation of hADSCs could be reversed by BMP2 RNA interference. In conclusion, miR-17-5p and miR-106a regulate osteogenic and adipogenic lineage commitment of hADSCs by directly targeting BMP2, and subsequently decreased osteogenic TAZ, MSX2 and Runx2, and increased adipogenic C/EBPα and PPARγ.

Abbreviations: ELISA, enzyme-linked immunosorbent assay; MSCs, mesenchymal stem cells; hADSCs, human adipose-derived mesenchymal stem cells; BMP2, bone morphogenetic protein 2; TAZ, transcriptional co-activator with PDZ-binding motif; MSX2, msh homeobox 2; Runx2, runt-related transcription factor 2; OSX, Osterix; ALP, alkaline phosphatase; OPN, osteopontin; OCN, osteocalcin; C/EBPα, CCAAT/enhancer binding proteins alpha; PPARγ, peroxisome proliferator-activated receptor gamma; LPL, lipoprotein lipase; AP2 (FABP4), fatty acid binding protein 4, adipocytes; ID1, helix-loop-helix proteins1.

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Introduction

Human mesenchymal stem cells (hMSCs) have multilineage differentiation potential. These cells can differentiate to form a variety of tissues such as bone, cartilage, adipose and endothelium (Cao et al., 2005; Phinney and Prockop, 2007; Pittenger et al., 1999; Reyes et al., 2001; Fink et al., 2011). The balance between osteogenic and adipogenic differentiation of human mesenchymal stem cells is disrupted in various human diseases. For example, decreased bone formation accompanied by an increase in bone marrow adipogenesis occurs with aging, immobility or osteoporosis (Kajkenova et al., 1997; Nuttall and Gimble, 2000), whereas increased bone formation or calcification is observed in progressive osseous hyperplasia (Bostrom et al., 1993; Kaplan and Shore, 2000; Parhami and Demer, 1997). Therefore, elucidation of the molecular mechanisms regulating adipogenic and osteogenic differentiation of MSCs is of extreme importance for finding new treatments of these diseases.

Adipogenesis is a highly regulated process in which a coordinated cascade of transcription factors leads to the formation of mature adipocytes (Rosen et al., 2000; Sethi and Vidal-Puig, 2007). This cascade begins with the transient expression of C/EBPβ and C/EBPδ which activate C/EBPα and PPARγ. C/EBPα and PPARγ together coordinate the expression of adipogenic genes underlying the phenotype of terminally differentiated adipocytes. Osteogenesis is also a highly coordinated process and is initiated by the transcription factors *Runx2* and *Osterix* (*OSX*), which lead to the terminal osteoblast phenotype characterized by calcification of the extracellular matrix. The genes involved in this mineralization process include *alkaline phosphatase* (*ALP*) and *osteopontin* (*OPN*) in early phase, and *osteocalcin* (*OCN*) in late differentiation phase (Gallea et al., 2001; Jaiswal et al., 1997; Nakashima and de Crombrugghe, 2003).

Mounting evidence showed that multiple pathways are involved in regulating osteogenesis and adipogenesis, such as TGFβ/BMPs/Smads, Wnt/β-catenin, Notch, JAK/STAT, MAPK, PI3K/Akt and Hedgehog pathways (Bellido et al., 1997; Chiba, 2006; Fritzius and Moelling, 2008; Gallea et al., 2001; Ross et al., 2000; Suh et al., 2006; ten Dijke et al., 2003). But what factors are involved and how they orchestrate to regulate the specification of cell fate remain elusive. Therefore, investigating the mechanisms that fine-tune the balance between osteogenic and adipogenic differentiation of MSCs is of high importance. Recent study revealed gene repression to be most prevalent prior to commitment in osteogenesis and adipogenesis, and computational analysis suggested that gene repression before commitment is mediated by miRNAs (Scheideler et al., 2008).

Recently, emerging evidence suggests that miRNAs are involved in regulating differentiation and cell fate decisions (Ivey and Srivastava, 2010). miR-196a, -29b, -2861, -3960 and -335-5p were reported to enhance the osteogenic differentiation (Hu et al., 2011; Kim et al., 2009; Li et al., 2009; Zhang et al., 2011a), miR-26a, -133, -135, -141 and -200a could impede osteogenic differentiation (Itoh et al., 2009; Li et al., 2008; Luzi et al., 2008), and miR-143, -24, -31, -30c and -642a-3p were involved in regulating adipogenesis (Esau et al., 2004; Sun et al., 2009; Yang et al., 2011; Zaragosi et al., 2011). But so far, only a few key miRNAs controlling the balance between osteogenesis and adipogenesis had been identified, such as miR-22 and miR-637 (Huang et al., 2012; Zhang et al., 2011b).

In this study, we found that miR-17-5p and miR-106a regulate lineage specification of hADSCs between osteogenesis and adipogenesis by directly targeting BMP2. These miRNAs, as post-transcriptional regulators of osteogenic and adipogenic differentiation, may serve as novel therapeutic agents for osteogenesis- or adipogenesis-related disorders.

Materials and methods

Isolation and expansion of MSCs from adult human adipose tissue

Human adipose tissue was obtained from donors undergoing liposuction according to procedures approved by the Ethics Committee at the Chinese Academy of Medical Sciences and Peking Union Medical College. The isolation and culture procedure of Cao et al. (2005) was adopted.

Osteogenic differentiation

The culture-expanded cells of 3rd passage at 80% confluence were induced in the following osteogenic medium for 8 days: high-glucose Dulbecco's modified Eagle's medium (H-DMEM) supplemented with 10% FCS, 10 mM β-glycerophosphate, 10 nM dexamethasone, and 0.2 mM ascorbic acid. The expression of the osteogenic phenotype was evaluated after induction by the simultaneous monitoring expression of genes involved in osteogenic differentiation (qRT-PCR) and mineralization (ALP and alizarin red staining). Eight days after osteogenic induction, ~70% of hADSCs showed an osteoblast-like phenotype.

Adipogenic differentiation

The culture-expanded cells of 3rd passage at 100% confluence were induced in the following adipogenic medium for 8 days: H-DMEM supplemented with 10% FCS, 1 μM dexamethasone, 0.5 mM isobutylmethylxanthine, and 1 mM ascorbic acid. Adipogenesis was determined by oil red O staining. All reagents used in osteogenic and adipogenic differentiation were from Sigma Aldrich.

ALP, oil red O, and alizarin red staining

For detection of calcification during osteogenic differentiation, the induced cells in 24-well plates were washed twice with PBS and fixed with 95% ethanol (500 μl/well) for 20 min. The fixed cells were stained with 1 ml of alizarin red solution (Sigma) in each well and incubated in 37 °C for 30 min. The procedure of ALP staining was performed according to the manufacturer's instructions of ALP staining Kit (Institute of Hematology & Blood Diseases Hospital, Chinese Academy of Medical Sciences). For oil red O staining, cells were washed twice with PBS and fixed with 10% formalin for 10 min at room temperature. After fixation, cells were stained with filtered oil red O solution (stock solution: 3 mg/ml in isopropanol; working solution: 60% oil red O stock solution and 40% distilled water) for 1 h at room temperature. After staining, cells were washed with water

to remove unbound dye, visualized by light microscopy, and photographed. In order to quantify the degree of adipogenic differentiation, after visualized by light microscopy, the dye in cells was extracted with isopropanol and OD value was measured at 510 nm wavelength.

MiRNA profiling analysis

miRNA profiling was performed by high-throughput quantitative qRT-PCR using the TaqMan® Array Human MicroRNA Cards V3.0 (Applied Biosystems) following the manufacturer's instructions. Ten nanograms of total RNA was used for each reaction. PCRs were repeated at least twice, and the mean relative expression level was calculated. The U6 was used for data normalization (Applied Biosystems). Experiments were performed in triplicate.

MiRNA mimic, miRNA inhibitor, and siRNA transfection

Human ADSCs were transfected with liprofectamine2000 (Invitrogen) according to the manufacturer' procedures before differentiation. All oligonucleotides were obtained from GenePharma (Shanghai, China). Transfection efficiency was assessed by percentage of fluorescent-positive cells under fluorescence microscope 24 h after transfection. Cells were induced and harvested at various time points for protein and mRNA assays.

RNA-reverse transcription and qRT-PCR

Total RNA was extracted using Trizol total RNA isolation reagent (Invitrogen) and purified with the Column DNA Erasol kit (TianDz, Beijing, China) according to the manufacturer's instructions. qRT-PCR was carried out to check mRNA levels with SYBR Green I (TaKaRa). The expression level of genes was normalized to *GAPDH*. All experiments were performed in triplicate. Primers used for amplification are listed in supplemental Table 1. Reverse transcription of miRNAs was performed with miScript Reverse Transcription Kit (Qiagen, Shanghai, China). Expression of mature miRNAs was determined using miRNA-specific quantitative qRT-PCR (TaKaRa). The expression levels were normalized to U6, an internal control, and measured by comparative *C*t ($\Delta\Delta C$t) method.

DNA constructs, reporter plasmids, and luciferase assays

Putative miR-17 family-recognition element from the *BMP2* gene was cloned in the 3′-UTR of the firefly luciferase reporter vector according to the manufacturer's guidelines. The oligonucleotide sequences were designed to carry the Xba1 and Not1 sites at their ends to facilitate ligation into corresponding sites of pRL-TK-Report (Promega). The oligonucleotides used in these studies are showed in supplemental Table 2. For luciferase activity assay, 5.0×10^4 cells in 24-well plates were cotransfected with 0.5 μg of the indicated pRL-TK firefly luciferase construct and 40 ng of a PGL3 renilla luciferase normalization control, together with 200 nM miR-17-5p and miR-106a mimic or their inhibitor, or its corresponding control. Luciferase assays were performed 48 h after transfection using the Dual Luciferase Reporter Assay System (Promega). Firefly luciferase activity was normalized to renilla luciferase activity for each transfected well. All experiments were done thrice in triplicate.

Western blot, ELISA and antibodies

Cells were harvested in RIPA lysis buffer (Beyotime, Shanghai, China). Whole cell protein extracts were qualified by BCA assay. Proteins from total cell lysates were separated onto NuPAGE 12% polyacrylamide gels (Invitrogen), transferred to nitrocellulose membranes (Bio-Rad), blocked in 5% non-fat milk in TBST, and hybridized with antibodies for BMP2 (1:500, Abcam), TAZ (1:500, LifeSpan Biosciences), MSX2 (1:125, Millipore), Runx2 (1:100, CST), ALP (1:3000, Abcam), OPN (1:500, Abcam), C/EBPα (1:5000, Abcam), PPARγ (1:1000, CST) and AP2 (1:1000, Millipore) respectively. Actin (1:500, Abcam) or GAPDH (1:1000, Cell Biolabs) on the same membrane was used as a loading control. Signals were revealed after incubation with anti-rabbit IgG secondary antibody (1:1500) or anti-mouse IgG secondary antibody (1:2000) coupled to peroxidase by using ECL (Amersham Biosciences). ELISA array for BMP2 protein was carried out according to the manufacturer's instructions (NeoBioscience, EHC172.96).

Data analysis

All results are presented as means ± SEM. Comparisons between groups were analyzed using *t*-tests (two-sided). Differences with *p* values of less than 0.05 were considered significant. The *p* values were corrected for multiple testing procedures and to control type I error rates.

Results

Expression of miRNA-17 family members during osteogenesis and adipogenesis of hADSCs

Human adipose-derived mesenchymal stem cells (hADSCs) were isolated and phenotyped. They were positive for adhesion molecules CD29, CD44, CD105 and CD166 (>95%) and negative for CD31, CD34, CD45 and HLA-DR (<5%) (Fig. 1A). Expression levels of a panel of 740 miRNAs were determined by high-throughput quantitative RT-PCR during either osteogenic differentiation or adipogenic differentiation. Results showed the expression levels of miRNA-17 family, including miR-17-5p, -106a and -20a were downregulated when hADSCs underwent differentiation toward osteogenic lineages, but upregulated during adipocyte differentiation (Fig. 1B). Downregulation of miR-17-5p and miR-106a during osteogenesis has been reported by Li et al. (2008). Therefore we speculated that miR-17-5p and miR-106a might be crucial regulators of osteogenic and adipogenic differentiation of hADSCs. These results were confirmed by miRNA specific qRT-PCR at days 0, 2, 4, 6, and 8 after either osteogenic or adipogenic differentiation of hADSCs (Fig. 1C).

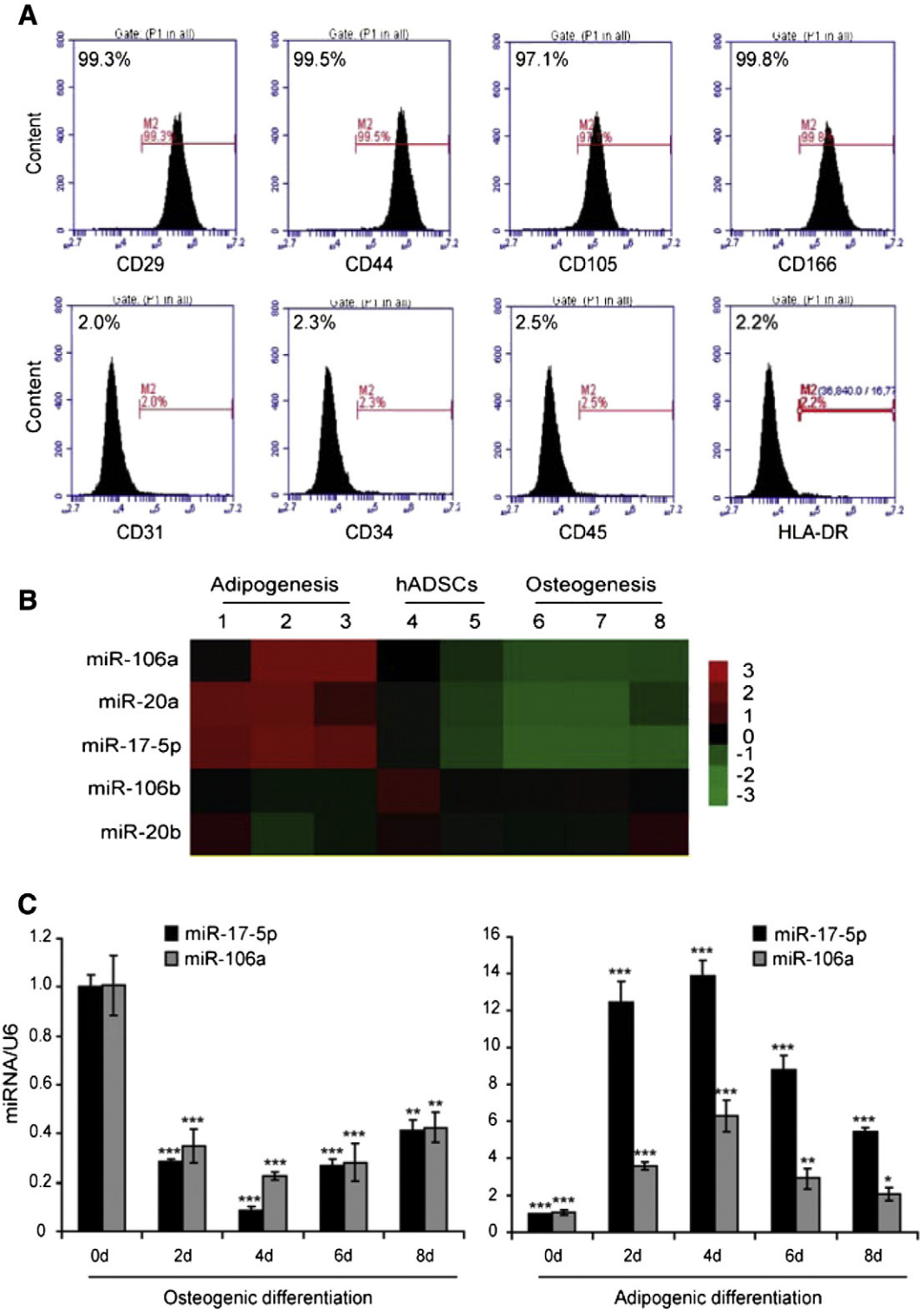

Figure 1 miR-17 family member expression profile during adipogenic and osteogenic differentiation of hADSCs. (A) Phenotypic analysis of hADSCs by flow cytometry. (B) miRNA profile during adipogenesis and osteogenesis of hADSCs was detected by TaqMan® Array Human MicroRNA Cards V3.0. Total RNA was extracted at day 3 or day 4 in hADSCs undergoing adipogenic and osteogenic differentiation, respectively. (C) Dynamic expression level of miR-17-5p and miR-106a in osteogenesis and adipogenesis of hADSCs was indicated by qRT-PCR. miR-17-5p is abbreviated as 17-5p, the others are similarly abbreviated. U6 was used for miRNA data normalization. Relative miRNA levels are represented as mean ± SEM, $n=3$. The significance difference is noted as ***, $p<0.001$; **, $p<0.01$; *, $p<0.05$, respectively.

Overexpression of miR-17-5p and miR-106a suppresses osteogenesis but promotes adipogenesis of hADSCs

To elucidate the roles of miR-17-5p and miR-106a during hADSCs differentiation, synthetic mimics of those miRNA were transfected into hADSCs. The concentration of 200 nM was used in the experiment because transfection efficiency was highest at this concentration at 24 h after transfection and the transfected hADSCs exhibited no morphological changes compared to naive or miR-NC (negative control)-transfected groups (Supplemental Fig. 1A). qRT-PCR analysis showed that the expression of miR-17-5p and miR-106a increased by 5.8-fold and 6.1-fold in hADSCs transfected with these miRNA's mimics respectively (Supplemental Fig. 1B). Next miR-17-5p and miR-106a-overexpressing hADSCs were induced to differentiate toward either adipogenic or osteogenic lineages. Results showed that overexpression of miR-17-5p and miR-106a significantly inhibited osteogenic differentiation as indicated by ALP activity, alizarin red staining for mineralization and expression of the osteogenic transcription factors *Runx2* and *OSX*, and osteoblast marker *ALP* and *OPN* at early stage, *OCN* at late stage (Fig. 2A–C). On the contrary, overexpression of these miRNAs promoted adipogenesis as indicated by oil red O staining and expression of adipocytic differentiation-related genes *C/EBPα*, *PPARγ*, *AP2* and *LPL* (Fig. 2D–F).

Inhibition of endogenous miR-17-5p and miR-106a promotes osteogenic differentiation and suppresses adipogenic differentiation of hADSCs

To further evaluate the roles of miR-17-5p and miR-106a on hADSC differentiation, miR-17-5p and miR-106a specific inhibitor (miR-17-5pI and miR-106aI) were transfected into hADSCs to inhibit endogenous expression. miRNA specific qRT-PCR showed that transfection of inhibitors of those miRNAs effectively inhibited their expression in hADSCs, compared with negative control inhibitor (miR-NCI)-transfected group (Supplemental Fig. 1C). hADSCs were then induced to differentiate toward osteogenic and adipogenic lineages. Treatment of hADSCs by either miR-17-5p or miR-106a inhibitor significantly increased ALP activity, alizarin red staining, expression of osteogenic transcription factors (*Runx2* and *OSX*) and marker genes (*ALP* and *OPN* at early stage, *OCN* at late stage) at mRNA and protein levels, indicating that downregulation of either miR-17-5p or miR-106a might promote osteogenesis of hADSCs (Fig. 3A–C). On the contrary, treatment of hADSCs by either miR-17-5p or miR-106a inhibitor significantly decreased oil red O staining, expression of adipogenic transcription factors (*C/EBPα* and *PPARγ*) and marker genes (*LPL* and *AP2*) at mRNA and protein levels, indicating that downregulation of either miR-17-5p or miR-106a might suppress adipogenesis of hADSCs (Fig. 3D–F).

BMP2 is the target of miR-17-5p and miR-106a

As a first step to investigate the mechanisms underlying the actions of the miR-17-5p and miR-106a on hADSCs differentiation, we focused on identifying the potential targets of miR-17-5p and miR-106a. Because miR-17-5p and miR-106a were downregulated during osteogenesis, we expected that the expression of their target genes should be concomitantly upregulated. qRT-PCR analysis showed that the expression levels of *BMP2*, *TAZ*, *MSX2*, *BMPR2*, *BMPR1B* and *Smad5* gradually increased when hADSCs were induced to differentiate toward osteogenesis, and decreased during adipogenesis (Fig. 4A and B). Especially, the expression patterns of *BMP2*, *TAZ* and *MSX2* were negatively correlative with the expression of miR-17-5p and miR-106a. *BMP2* had been proved to contribute to cell fate determination through regulating *TAZ* and *MSX2* (see in discussion), and bioinformatic analysis (TargetScan and PicTar) indicated that *BMP2* is a candidate target of miR-17 family members. Thus we focused on *BMP2* as a potential target of miR-17-5p and miR-106a. Indeed, overexpression of miR-17-5p and miR-106a had no effect on *BMP2* at mRNA level, but decreased BMP2 at protein level (Fig. 5A and B), indicating post-transcriptional regulation of BMP2 by those miRNAs. miR-17-5p and miR-106a, belonging to miR-17 family, have the same 'seed sequence', and regulate a similar set of genes (Lim et al., 2003; Ye et al., 2008). We further verified these results by using luciferase reporters which contained either wild-type (pRL-TK-BMP2-WT) or mutated (pRL-TK-BMP2-MUT) putative 3′UTR sequence for BMP2-binding (miR-17 family 'seed sequence') in the 3′UTR of the luciferase gene (Fig. 5C). Overexpression of miR-17-5p and miR-106a significantly suppressed luciferase activity of pRL-TK-BMP2-WT reporter plasmid but not that of pRL-TK-BMP2-MUT reporter plasmid (Fig. 5C). Inhibition of either miR-17-5p or miR-106a by co-transfection of their inhibitors (miR-17-5pI and miR-106aI, respectively) increased the luciferase activity of pRL-TK-BMP2-WT in hADSCs compared with inhibitor negative control (miR-NCI) (Fig. 5D).

miR-17-5p and miR-106a regulate lineage specification of hADSCs via BMP2

Furthermore, in order to confirm that the effect of miR-17-5p and miR-106a during hADSCs differentiation is mediated by *BMP2*, we used a specific siRNA (siBMP2) to knockdown endogenous *BMP2*. The efficiency of siRNAs for *BMP2* was verified by qRT-PCR and western blot (Fig. 6A). ALP assay, alizarin red staining for mineralization and qRT-PCR for lineage-related genes all indicated that downregulation of endogenous *BMP2* hampered osteogenesis. On the contrary, downregulation of endogenous *BMP2* promoted adipogenesis of hADSCs as indicated by oil red O staining for lipids and qRT-PCR for adipogenic lineage-related genes (Fig. 6B and C). These results were consistent with those seen in miR-17-5p and miR-106a mimic-transfected hADSCs. We then transfected siBMP2 into miR-17-5p depleted hADSCs and found that the effects of miR-17-5p inhibition on osteogenesis and adipogenesis of hADSCs could be significantly reversed by *BMP2* RNA interference, as demonstrated by ALP assay and alizarin red staining for mineralization and oil red O staining for lipids respectively (Fig. 6D).

To further confirmed that miR-17-5p and miR-106a promote adipogenesis and inhibit osteogenesis via BMP2 signal pathway, we examined the expression levels of TAZ, MSX2 and ID1, which were reported to be the downstream targets of BMP2 (Hong et al., 2005; Korchynskyi and ten Dijke, 2002;

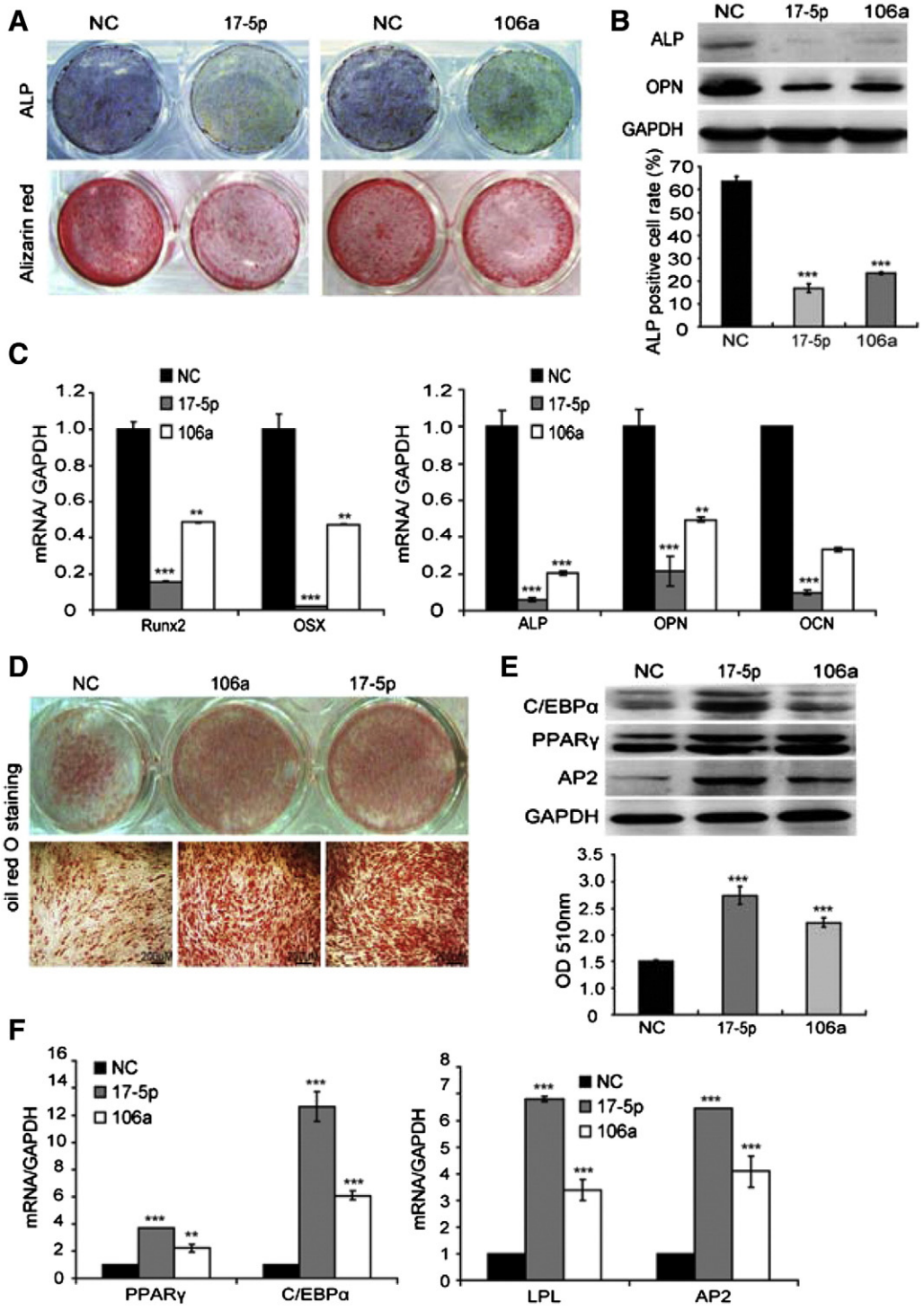

Figure 2 Overexpression of miR-17-5p and miR-106a in hADSCs inhibits osteogenesis and enhances adipogenic differentiation. (A) ALP and alizarin red staining for mineralization indicated the effect of miR-17-5p and miR-106a overexpression on hADSCs osteogenic differentiation for day 6 and day 14, respectively. (B) Runx2, ALP and OPN protein level were detected by western blot. ALP positive cell number was analysis by image pro plus 6.0 and normalized by total cell number. (C) miR-17-5p and miR-106a overexpression in hADSCs suppressed osteogenic gene transcription according to qRT-PCR at day 6 during osteogenesis. (D) Oil red O staining indicated the effect of miR-17 family overexpression on hADSCs adipogenic differentiation for day 8. (E) C/EBPα, PPARγ and AP2 protein level were detected by western blot. Oil red O staining was extracted by isopropanol and quantified by OD value at 510 nm wavelength. (F) Overexpression of miR-17-5p and miR-106a in hADSCs increased adipogenic gene transcription according to qRT-PCR at day 6 during adipogenesis. miRNA mimic's negative control is abbreviated as NC, the other analogy. Relative mRNA levels are represented as mean ± SEM. GAPDH was used for mRNA and protein data normalization. Data are presented as a mean with a standard error from three independent repeats. A Student's *T* test was performed for a control and the treatment. The significance difference is noted as ***, $p<0.001$; **, $p<0.01$; *, $p<0.05$, respectively.

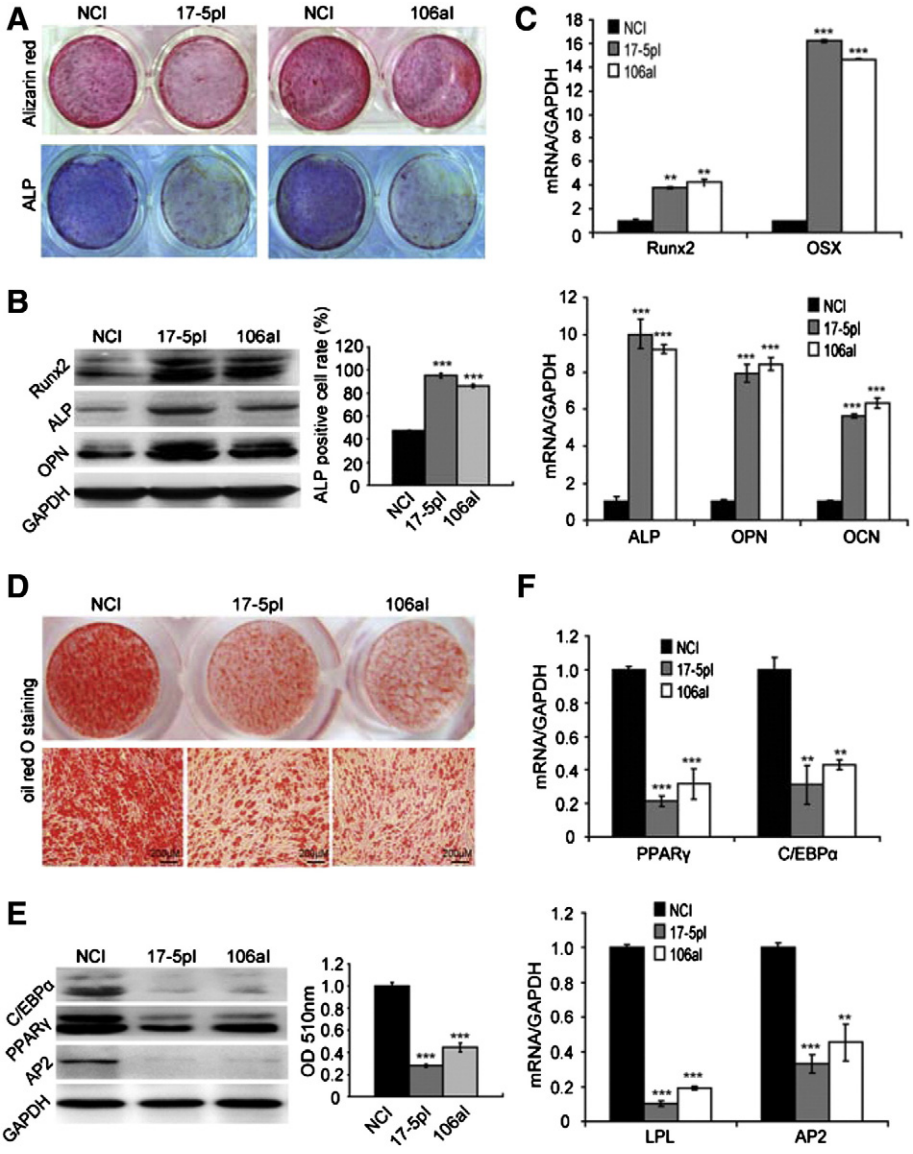

Figure 3 Inhibition of endogenous miR-17-5p and miR-106a in hADSCs increases osteogenesis and decreases adipogenic differentiation. (A) Downregulation of miR-17-5p and miR-106a expression in hADSCs promotes osteogenic differentiation as confirmed by ALP and alizarin red staining for day 6 and day 14, respectively. (B) Runx2, ALP and OPN protein level were detected by western blot. ALP positive cell number was analysis by image pro plus 6.0 and normalized by total cell number. (C) Downregulation of miR-17-5p and miR-106a expression in hADSCs promotes osteogenic gene levels as detected by qRT-PCR at day 6 during osteogenesis. (D) Oil red O staining indicated the suppressive affect of miR-17-5p and miR-106a downregulation on adipogenic differentiation of hADSCs for day 8. (E) C/EBPα, PPARγ and AP2 protein level were detected by western blot. Oil red O staining was extracted by isopropanol and quantified by OD value at 510 nm wavelength. (F) miR-17-5p and miR-106a downregulation in hADSCs decreases adipogenic gene transcription as confirmed by qRT-PCR at day 6 during adipogenesis. miRNA inhibitor's negative control is abbreviated as NCI, the other analogy. Relative mRNA levels are represented as mean ± SEM. GAPDH was used for mRNA and protein data normalization. Data are presented as a mean with a standard error from three independent repeats. A Student's *T* test was performed for a control and the treatment. The significance difference is noted as ***, $p<0.001$; **, $p<0.01$; *, $p<0.05$, respectively.

Lopez-Rovira et al., 2002; Matsubara et al., 2008). Western blot showed that the expression levels of TAZ and MSX2 decreased when BMP2 was downregulated by siBMP2, but the expression level of ID1 protein showed no significant change (date not show). miR-17-5p overexpression also decreased the protein levels of TAZ, MSX2 and osteogenic Runx2, and increased the protein levels of adipogenic C/EBPα and PPARγ, similar to downregulation of endogeneous *BMP2*. Again, miR-17-5p knockdown had opposite effects (Fig. 6E). These results suggested that the regulation of BMP2-induced cell fate specification between osteogenesis and adipogenesis of hADSCs by miR-17-5p and miR-106a is probably carried out via TAZ/MSX2-Runx2/PPARγ pathway (Fig. 7).

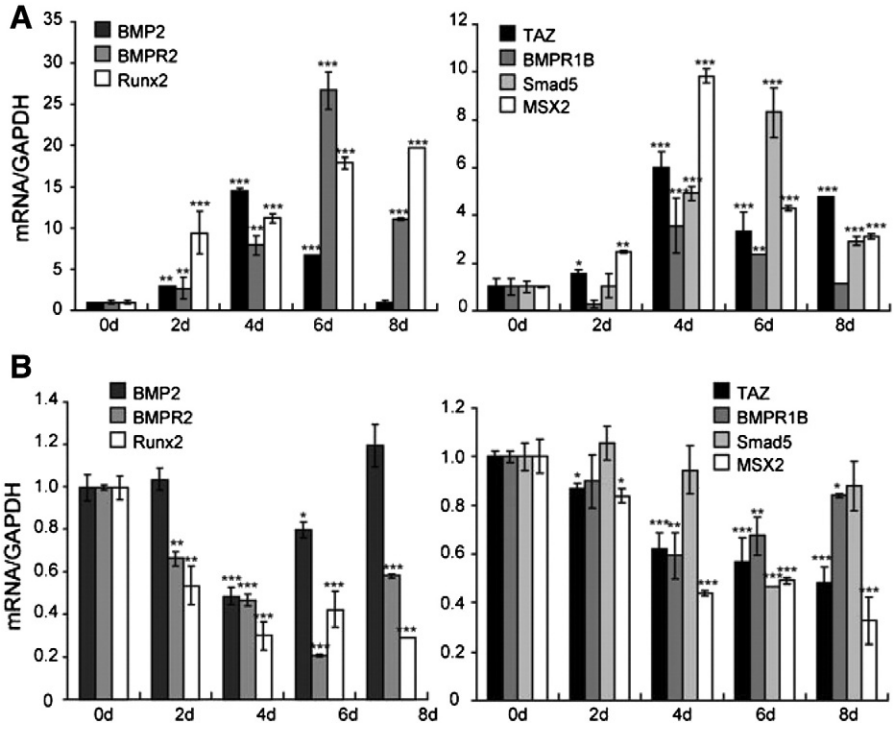

Figure 4 Expression pattern of BMP2/Smad5 pathway during osteogenic and adipogenic differentiation of hADSCs. (A and B) qRT-PCR analyzed the dynamic expression of *BMP2*/*Smad5* pathway components in hADSCs during osteogenic and adipogenic differentiation at 0, 2, 4, 6, and 8 days respectively. Relative mRNA levels are represented as mean ± SEM. *GAPDH* was used for mRNA data normalization. Data are presented as a mean with a standard error from three independent repeats. A Student's *T* test was performed for a control and the treatment. The significance difference is noted as ***, $p<0.001$; **, $p<0.01$; *, $p<0.05$, respectively.

Discussion

Osteogenesis and adipogenesis of MSCs maintain a homeostasis under physiological conditions. Once the homeostasis of osteogenic and adipogenic differentiation of MSCs are disrupted, disorders such as osteoporosis may occur. But what factors are involved and how they orchestrate to regulate the specification of cell fate remain elusive. Recently, Scheideler et al. (2008) found that gene repression to be most prevalent prior to commitment in both lineages, and computational analysis suggested that gene repression before commitment of MSCs is mediated by miRNAs.

In this study, we found that several members of miR-17 family including miR-17-5p and miR-106a were downregulated during differentiation of hADSCs toward osteogenic lineage, but upregulated during adipogenesis. Enhanced level of miR-17-5p and miR-106a promoted adipogenesis of hADSCs, and inhibited osteogenic differentiation, whereas knockdown one of them had opposite effects.

MiR-17 family is composed of six miRNAs (miR-17-5p, -20a, -106a, -20b, 106b, and -93) which are derived from miR-17-92 cluster and its two paralogues (Mendell, 2008), and have same 'seed sequence' that has been verified as an important element for mRNA targeting (Lim et al., 2003; Ye et al., 2008). Recently, several members of the miR-17 family have been identified to be specifically expressed in either undifferentiated or differentiating embryonic stem cells (Houbaviy et al., 2003; Suh et al., 2004; Tang et al., 2006). These miRNAs were also found to play an important role in early mammalian development, hematopoietic and adipocytic differentiation (Foshay and Gallicano, 2009; Garzon et al., 2006; Ventura et al., 2008; Wang et al., 2008). More recently, miR-20a was reported to be a regulator of osteogenic differentiation of human MSCs by co-regulating BMP signaling (Zhang et al., 2011c). Our data showed that other two important members of miR-17 family, miR-17-5p and miR-106a, can suppress osteogenic differentiation and promote adipogenic differentiation of hADSCs.

To elucidate the mechanisms underlying the actions of miR-17-5p and miR-106a on hADSCs differentiation, we detected the expression of osteoblastic and adipocytic related genes during hADSCs differentiation and found that the expression level of BMP2, TAZ and MSX2 mRNA peaked after 4 days of osteogenic induction and then gradually decreased, which is consistent with the recent finding that BMP2 was involved in the earliest steps of osteogenesis (Tsuji et al., 2006). These expression patterns were inversely correlated with those of miR-17-5p and miR-106a during hADSCs differentiation. As a member of TGFβ/BMPs pathway, BMP2 has been shown to be involved in osteogenesis, fracture healing, and bone formation (Cohen, 2002). BMP2 can determine the differentiation process of MSCs through activating Runx2-dependent gene transcription and repressing PPARγ-dependent gene transcription (Cheng et al., 2003; Foshay and Gallicano, 2009; Hong et al., 2005; Peng et al., 2004). Based on bioinformatics analysis that indicated *BMP2* is a potential target of miR-17-5p and miR-106a, we overexpressed

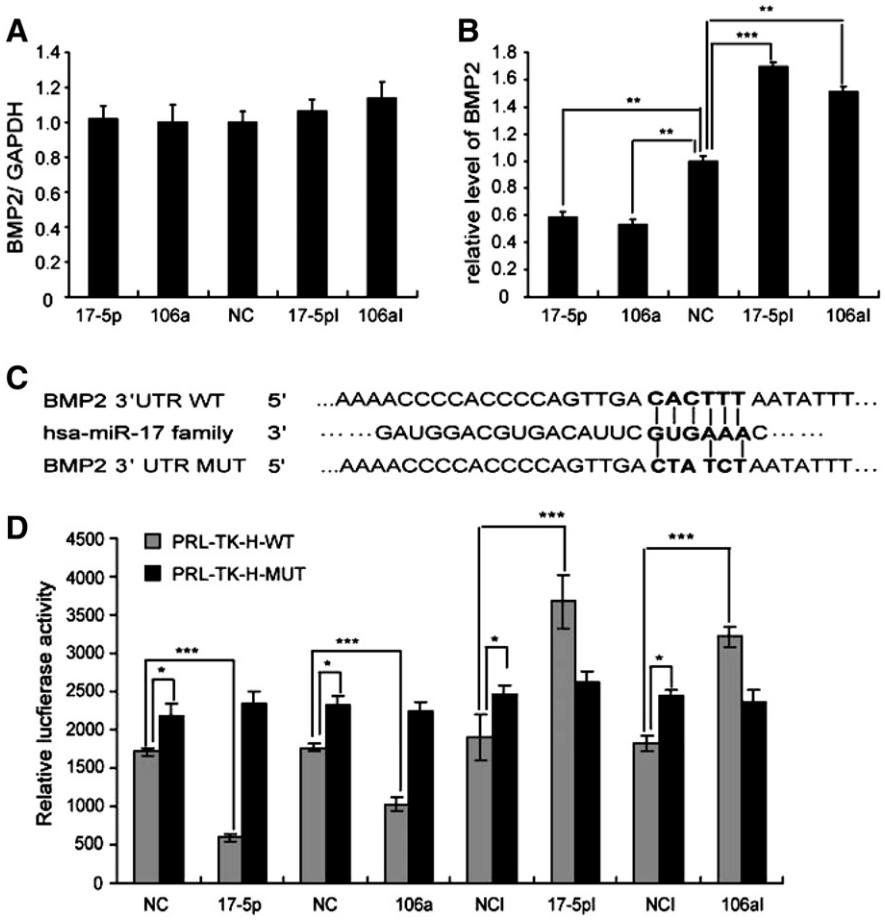

Figure 5 Prediction and confirmation of the direct target for miR-17-5p and miR-106a regulating hADSCs differentiation. (A) qRT-PCR detected the effect of miR-17-5p and miR-106a on the expression of *BMP2* mRNA level at indicated times. (B) ELISA array analyzed the BMP2 protein level on different treatment groups. (C) Target sequence and mutant of miR-17-5p and miR-106a on *BMP2* 3′UTR. (D) Identification of the relationship between miR-17-5p and miR-106a and *BMP2* using double luciferase reporter system. NC abbreviated as miR-NC transfected group; 17-5p: miR-17-5p transfected group; the other analogy. Relative mRNA/protein levels are represented as mean ± SEM. *GAPDH* was used for mRNA data normalization. Data are presented as a mean with a standard error from three independent repeats. A Student's *T* test was performed for a control and the treatment. The significance difference is noted as ***, $p<0.001$; **, $p<0.01$; *, $p<0.05$, respectively.

miR-17-5p and miR-106a and found that upregulation of either miR-17-5p or miR-106a decreased BMP2 expression at protein level. In addition, inhibition of either miR-17-5p or miR-106a increased BMP2 expression, strongly suggesting that *BMP2* is a direct target gene of miR-17-5p and miR-106a during osteogenic and adipogenic differentiation of hADSCs.

Then, we demonstrated that both miR-17-5p and miR-106a directly targeted the 3′UTR region of *BMP2* by luciferase reporter assay. Recently, miRNAs with similar 'seed sequence' have been reported to regulate a similar set of genes and have overlapping functions (Foshay and Gallicano, 2009; Ventura et al., 2008). In this study, we found that although miR-106a and miR-17-5p possess the same 'seed sequence', miR-106a mimic was less effective than miR-17-5p mimic in downregulating BMP2 in luciferase activity assay suggesting that nucleotide sequences other than 'seed sequence' may contribute to the difference of targeting efficiency in the same miRNA family. This might be one of the reasons that different members of miR-17 family showed different efficiency in regulating osteogenic and adipogenic differentiation. For instance, miR-17-5p can more effectively regulate adipogenesis and osteogenesis than miR-106a.

Furthermore, we found that downregulation of endogeneous *BMP2* suppressed osteogenesis and increased adipogenesis, similar to the effect of miR-17-5p or miR-106a overexpression. Moreover, inhibitory effects of miR-17-5p and miR-106a on osteogenesis and adipogenesis of hADSCs could be reversed by *BMP2* RNA interference. These results further indicated that miR-17-5p and miR-106a promote adipocyte differentiation and suppress osteogenesis directly by negatively regulating *BMP2*.

MSX2, *TAZ*, and *ID1* have been identified as candidate genes regulated by BMP2 and are involved in the switch between adipocyte and osteoblast differentiation. We found that miR-17-5p overexpression was accompanied by decreased expression levels of TAZ and MSX2, while ID1 showed no change, whereas miR-17-5p inhibitor increased the expression of those proteins, which was consistent with the effects of siRNA for *BMP2*. Those results confirmed that miR-17-5p and miR-106a mediated the commitment of hADSCs between adipogenesis and osteogenesis directly through targeting

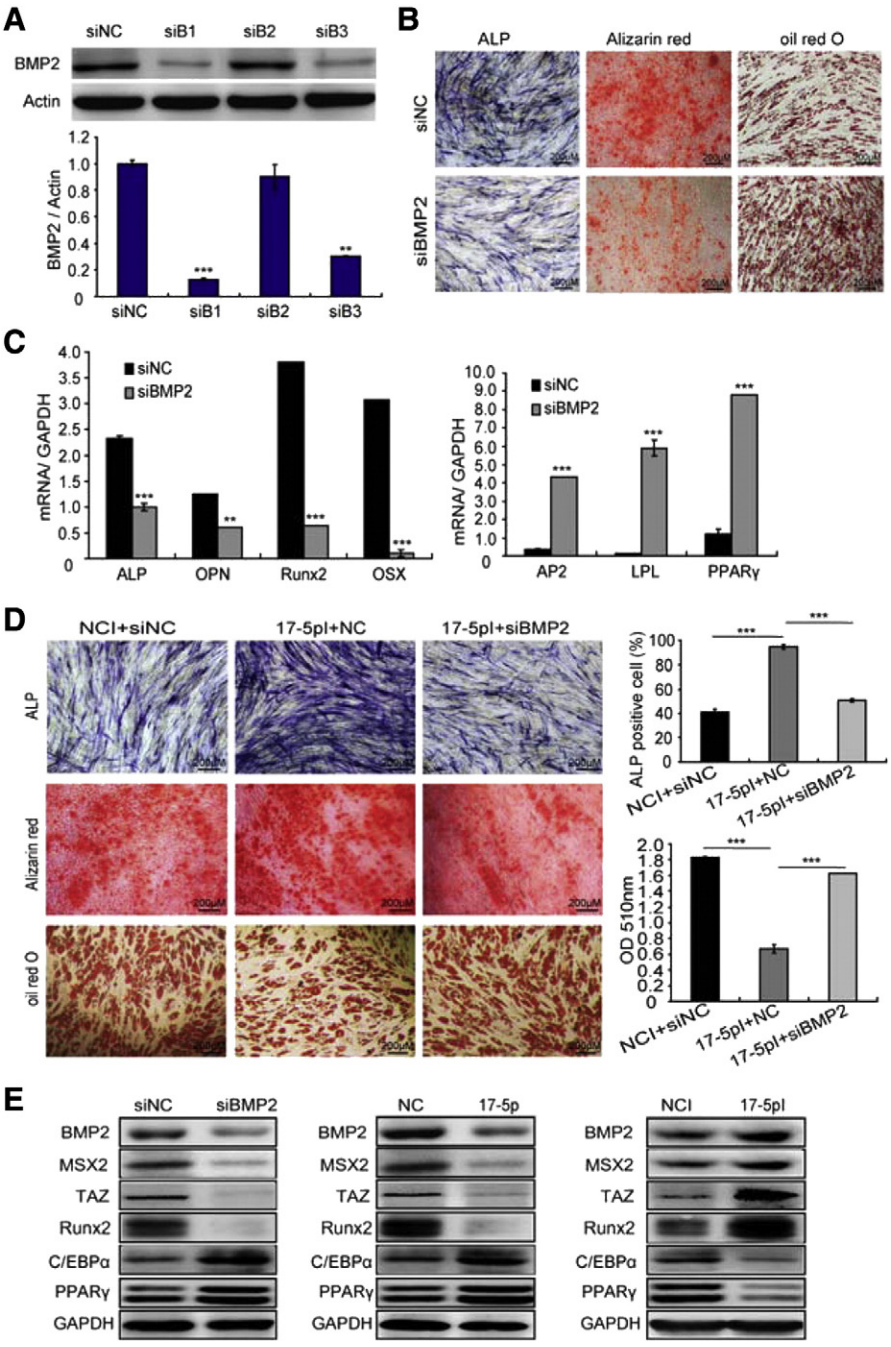

Figure 6 The effects of endogenous BMP2 downregulation in hADSCs are consistent with that of miR-17-5p and miR-106a overexpression on osteogenic and adipogenic differentiation. (A) The efficiency of *BMP2* siRNAs was confirmed by ELISA and western blot. Three pairs of siRNA, named as siB1, siB2, and siB3, and their negative control (siNC) were compared for the downregulated-efficiency of BMP2 protein. The first pair (siB1) was confirmed as the most efficient one and used in following experiments. (B) Downregulation of endogenous BMP2 in hADSCs suppressed osteogenic differentiation and increased adipogenic lineage, confirmed by ALP and alizarin red staining, and oil red O staining, respectively. (C) Expression level of osteogenic and adipogenic associated genes was verified by qRT-PCR during differentiation of siBMP2-transfected hADSCs. (D) Cotransfected siBMP2 with inhibitor of miR-17-5p reversed the effects of downregulation of miR-17-5p on hADSC differentiation indicated by ALP and alizarin red staining, and oil red O staining. ALP positive cell number was analysis by image pro plus 6.0 and normalized by total cell number. Oil red O staining was extracted by isopropanol and quantified by OD value at 510 nm wavelength. (E) Western blot confirmation that miR-17-5p targets the *BMP2*, followed by the decrease of its downstream. Relative mRNA/protein levels are represented as mean ± SEM. *GAPDH* was used for mRNA data normalization. GAPDH or Actin was used for protein data normalization. Data are presented as a mean with a standard error from three independent repeats. A Student's *T* test was performed for a control and the treatment. The significance difference is noted as ***, $p<0.001$; **, $p<0.01$; *, $p<0.05$, respectively.

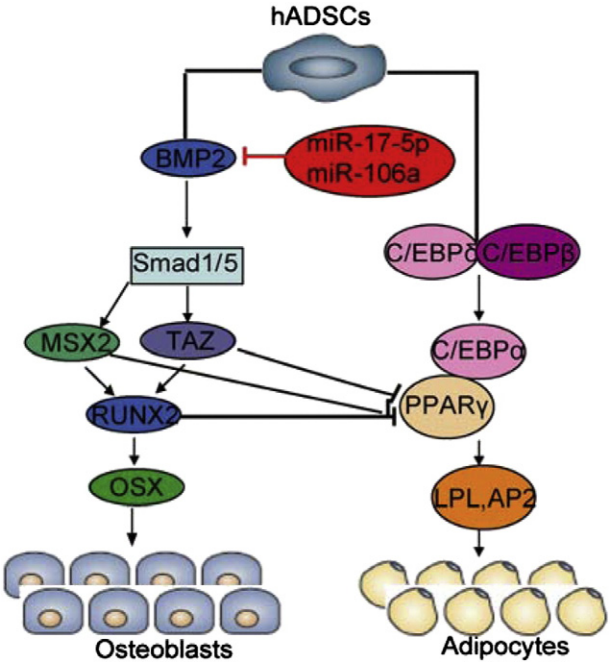

Figure 7 miR-17-5p and miR-106a regulate adipogenic and osteogenic differentiation of hADSCs by activating the TAZ/Msx2-Runx2/PPARγ pathway.

BMP2 and then participating in *MSX2*/*TAZ* regulated *Runx2* and *PPARγ* pathway. Our studies elucidate the mechanisms underlying selective induction of a tissue specific phenotype by miR-17-5p and miR-106a. Thus these findings could improve hADSCs-based cell therapy for osteogenesis- and adipogenesis-related disorders.

Conflict of interest

The authors declare no conflict of interest.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.scr.2012.11.007.

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