Original Research

MiR-145 regulates osteogenic differentiation of human adipose-derived mesenchymal stem cells through targeting *FoxO1*

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Impact statement

Researching on ASCs was a promising strategy to study osteogenic differentiation. The regulatory role of miR-145 on hASCs osteogenic differentiation remained partially explored.

Our study revealed a novel mechanism of the osteogenic differentiation process and suggested that miR-145 and its target gene *FoxO1* may be potential targets for the therapy of human osteogenic-related disorders.

Abstract

In this study, we aimed to investigate the expression of miR-145 before and after hASCs osteogenic differentiation. We also intended to explore the influence of the target relationship between miR-145 and *FoxO1* on osteogenic differentiation. Dual-luciferase reporter gene assay and real-time PCR were used to confirm the target relationship between miR-145 and *FoxO1*. Furthermore, the modulatory effects of miR-145 and *FoxO1* on hASCs osteoinductive differentiation were measured by real-time PCR , Western blot, ALP staining, ARS staining, and cell immunofluorescence assay. After osteogenic differentiation, miR-145 was gradually down-regulated, while *FoxO1* was up-regulated in hASCs. MiR-145 could directly target *FoxO1* 3′UTR. *FoxO1* was negatively regulated by miR-145. After osteoinductive differentiation, BSP, Ocn, and OPN expression was lowered with the overexpression of miR-145 or the knockdown of *FoxO1*. Furthermore, ALP and ARS staining assay results showed weakened ALP activity and extracellular matrix calcification. When overexpressing miR-145 and FoxO1 simultaneously, no obvious change in ALP activity and extracellular matrix calcification was seen. MiR-145 could suppress hASCs osteoinductive differentiation by suppressing *FoxO1* directly.

Keywords: miR-145, osteogenic differentiation, human adipose-derived stem cells, FoxO1

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Introduction

Mesenchymal stem cells (MSCs), a population of self-renewing multipotent cells, have been widely used experimentally for tissue engineering due to their strong ability to differentiate into bone, cartilage, and fat under appropriate induction conditions.[1] Human mesenchymal stem cells (hMSCs) are derived from various tissues, including bone marrow and adipose tissues. HMSCs have multilineage differentiating potentials.[2] Human adipose-derived stem cells (ASCs) also have multilineage differentiation potentials and they are prospective ancestor cells for bone regeneration.[3,4] Interests in the therapeutic potential of stem cells isolated from adipose tissue, called ASCs has grown due to their abundant sources and easy access for some clinical uses which avoided further manipulation, suggesting a more favorable tissue source than bone marrow.[5–8] Thus, researching on ASCs was a promising strategy to study osteogenic differentiation.

MicroRNAs (miRNAs) are a kind of endogenous small non-coding RNAs that function as post-transcriptional regulators by binding to 3′UTRs of target messenger RNAs.[9] With the increasing application of miRNAs in the treatment and monitoring of different diseases, miRNAs have become an important tool in biological and medical research. miRNAs are involved in osteogenic differentiation of stem cells.[4] For instance, miR-34 inhibited osteogenic proliferation and differentiation in mice by targeting *SATB2*.[10] MiR-138 was reported down-regulated during osteogenic differentiation and negatively regulating the osteogenesis of hMSCs.[11] Other studies showed that miR-141, miR-31, and miR-200a were negative regulators of the

differentiation of bone marrow stromal cells.[12,13] Of our particular interest, miR-145 has also been found to have influence on bone differentiation. For instance, Jia *et al.*[14] reported that miR-145 suppressed osteogenic differentiation by targeting *Sp7*. In addition, Fukuda *et al.*[15] determined that miR-145 regulated osteoblastic differentiation by targeting the transcription factor *Cbfb*. However, the regulatory role of miR-145 on hASCs osteogenic differentiation remained partially explored.

FoxO1 is the main regulator of redox balance among the three predominant members of the FoxO family (FoxO1, FoxO3a, and FoxO4). FoxO1 could stimulate proliferation and differentiation as well as inhibits apoptosis of osteogenic lineage cells.[16] Previous studies have shown the target relationship between miRNA and *FoxO1* as well as the effect of their interaction on osteogenic differentiation process. For instance, miR-182 could act as a FoxO1 inhibitor to antagonize osteogenic differentiation.[16,17] In addition, miR-615–3p could hinder the osteogenic differentiation of human lumbar ligamentum flavum cells by suppressing *GDF5* and *FoxO1*.[18] However, the modulating regulation between miR-145 and FoxO1 as well as the role of FoxO1 in hASCs needs further investigation.

Herein, we aimed at elucidating the effect of miR-145 and FoxO1 on hASCs osteogenic differentiation and the regulatory mechanisms between them. In the present study, we detected the dynamic expression of miR-145 and FoxO1 before and after osteogenic differentiation. Our findings indicated that miR-145 was involved in the efficient regulation of hASCs osteogenic differentiation through targeting FoxO1. This evidence prompted us to conclude that miR-145 may serve as a novel therapeutic target for hASC osteogenesis-related disorders.

Materials and methods

Cell cultivation and transfection

hASCs HMSC-ad cell line (Sciencell, USA) was cultured in Dulbecco's modified Eagle's medium (Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (Gibco), 100 units/ml penicillin, and 100 μg/ml streptomycin (Gibco) in 5% CO_2 in a humidified incubator at 37°C. pcDNA3.1 plasmids were used to construct FoxO1 overexpression plasmids. The synthesis of miR-145 mimics, control mimics, pcDNA3.1-FoxO1 and pcDNA3.1-control plasmids was purchased from Invitrogen (Carlsbad, CA, USA). The synthesis of *FoxO1* siRNA was conducted by Shanghai Sangon Company. The synthetics were transfected using Lipofectamine, 2000 (Invitrogen, Carlsbad, CA, USA).

Osteoblast differentiation induction

To induce osteoblast differentiation, HMSC-ad cells were plated in six-well plates (1×10^5 cells/well, 2 ml medium per well) and cultured normally. After five days cultivation (to 80%∼90% confluence), the media were replaced with osteoblasts-specific induction medium, the low glucose of Dulbecco's Modified Eagle's Medium with 10% FBS, 10 mM β-glycerophosphate, 0.1 uM dexamethasone, and 0.2 mM antiscorbic acid (Sigma–Aldrich, Louis, MO, USA) to induce osteoblast differentiation. Von Kossa staining was conducted after two weeks.

Table 1. RT-qPCR primer sequence.

Name	Primers
MiR-22	F: 5′-GCAGTTCTTCAGTGGCAA-3′
MiR-22	R: 5′-GAATACCTCGGACCCTGC-3′
U6	F: 5′-CTCGCTTCGGCAGCACA-3′
U6	R: 5′-AACGCTTCACGAATTTGCGT-3′
FoxO1	F: 5′-TTAGCCAGTCCAACTCGG-3′
FoxO1	R: 5′-TCTTGACCATCCACTCGTA-3′
GAPDH	F: 5′-TGGTCACCAGGGCTGCTT-3′
GAPDH	R: 5′-AGCTTCCCGTTCTCAGCC-3′

RT-qPCR

The total RNA was extracted from hASCs osteodifferentiated induction cells with TRIzol reagent (Life Technologies corporation, Gaithersburg, MD, USA) and reverse-transcribed into cDNA using PrimeScript II 1st Strand cDNA Synthesis Kit (TaKaRa, Tokyo, Japan). RT-qPCR was performed using SYBR Premix Ex Taq (TaKaRa, Tokyo, Japan). U6 was used as an endogenous normalization control for miR-145, while *GAPDH* was used as a *FoxO1* mRNA control. The relative level of RNA was quantified using the $2^{-\Delta\Delta Ct}$ method. The primers were synthesized by Shanghai Sangon Company and are listed in Table 1.

Western blot

Quantification of total protein was performed using Bradford protein assay (Bio-Rad, Hercules, CA, USA). Proteins were resolved by SDS-PAGE and transferred to a PVDF membrane using the iBlot Dry Blotting Transfer System (Life Technologies corporation, Gaithersburg, MD, USA). After blocking in PBS with 5% milk and 0.1% Tween for 2 h, the membrane was incubated with primary antibodies for goat anti-FoxO1, anti-Ocn, anti-BSP, anti-OPN (1:1000 dilution) (Santa Cruz Biotechnology, Santa Cruz, CA, USA) or goat anti-GAPDH polyclonal antibody (1:1000) (GenScript, Nanjing, China) at 4°C overnight and washed and incubated with secondary antibodies horseradish peroxidase labeled anti-goat (Abcam, Cambridge, MA, USA) for 1–2 h. After incubation, PVDF membrane was washed with PBS before placing in a darkroom. Moderate amount of DAB substrate was applied and the membranes were put in the darkroom for exposure. Images were obtained using a chemiluminescent image analyzer (LAS-4000 mini; Fujifilm) and cumulative optical density value of each target band was semi-quantified by ImageJ software (NIH). GAPDH was used as a loading control to quantify the relative protein level.

Alkaline phosphatase staining and alizarin red S staining

Seven or fourteen days after osteogenic induction, DMEM media were discarded. Cells were washed three times with PBS and fixed with 4% paraformaldehyde. The cell layer

was subsequently washed and dried in air. Reaction solution was prepared using an alkaline phosphatase (ALP) Assay Kit (Beyotime Biotechnology, Shanghai, China). After reaction at 37°C for 1 h, purplish red granules formed were detected under an optical microscope; 7 or 14 days post osteogenic differentiation, cells were fixed with ethanol for 10 min. Lastly, the cells were treated with 0.1% alizarin red S (ARS)-Tris-Hcl (pH 8.3) at 37°C for 1 h. To quantify calcium deposition, cells were washed with distilled water and dried in air. Absorbance was measured under an optical microscope.

Dual-luciferase reporter assay

The FoxO1 3′UTR fragment containing the predicted potential miR-145-binding sites was amplified and pGL3-WT plasmids were constructed by PCR method. pGL3-Mut was generated by site-directed mutagenesis, replacing the first six ribonucleotides of the miR-145 complementary sequence. HEK293T cells were grown in 24-well plates and cotransfected with miR-145 mimics and pGL3-WT (miR-WT group), miR-145 mimics and pGL3-Mut (MiR-Mut group), control mimics and pGL3-WT (NC-WT group), control mimics and pGL3-MuT (NC-Mut group). The media were replaced after 6 h; 24 h after cotransfection, the Dual-Luciferase Reporter Gene Test Kit (Beyotime Biotechnology, Shanghai, China) was used to detect the luciferase activities in different groups. The firefly and the renal luciferase reagent were added to detect the luciferase activity of each group.

Statistical analysis

Affymetrix Data Mining Tool version 3.1 (DMT 3.1) and scripts called within R environment (http://www.r-project.org/) were utilized to screen differentially expressed mRNAs and miRNAs. The filtration criteria were: Log2 (Fold Change) >2 and $P < 0.001$. MiRNA analysis was conducted using GSE72429 and GPL16770. MRNA analysis was conducted using GSE63754 and GPL17077 platform. Three osteogenic differentiation hASCs and three normal hASCs without osteogenic differentiation were selected to screen out differentially expressed miRNAs and mRNAs, respectively. The results represented the mean of three independent experiments and data were presented as mean ± standard deviation ($x \pm s$). Comparisons between two groups were analyzed by Student's *t* test. For the testing among multiple groups, a one-way analysis of variance (ANOVA) was conducted. Statistical analysis was performed using GraphPad Prism 6.0 software. *P*-values < 0.05 were considered to be statistically significant.

Results

FoxO1 was highly expressed after hASCs osteogenic differentiation

Three osteogenic HMSC-ad cell lines and three normal HMSC-ad without osteogenic differentiation cell lines were selected to screen out differentially expressed mRNAs. Among the 16 upregulated mRNAs with significant differences in osteoinductive HMSC-ad cell lines, there was FoxO1. The expression of FoxO1 in osteoinductive HMSC-ad cell lines was 2.29-fold higher in osteoinductive differentiation cell lines (Figure 1(a) and (b)). The expression of FoxO1 was gradually upregulated with the progression of osteogenic differentiation (Figure 1(c)). The protein expression of FoxO1 increased in hASCs after osteoinductive differentiation. Meanwhile, overexpression of FoxO1 (FoxO1 cDNA) and silence expression of FoxO1 (FoxO1 siRNA) was successfully constructed (Figure 1(d)).

FoxO1 promoted hASCs osteogenic differentiation

The result of RT-qPCR and Western blot was consistent. The expression of BSP, Ocn, and OPN in silence expression of FoxO1 group was downregulated since the fourth day and was more significant with the progression of osteogenic differentiation ($P < 0.01$). A remarkably opposite trend was observed in overexpression of FoxO1 group ($P < 0.001$) (Figure 2(a) and (b)).

ALP staining and ARS staining showed that on days 7 and 14, ALP activity and calcification were lowered in FoxO1 siRNA group, but enhanced in FoxO1 cDNA group ($P < 0.05$) (Figures 2(c) and 3(d)). Collectively, these results revealed that FoxO1 could promote hASCs osteogenic differentiation.

MiR-145 was low expressed during hASCs osteogenic differentiation

Three osteogenic HMSC-ad cell lines and three normal HMSC-ad without osteogenic differentiation cell lines were selected to screen out differentially expressed miRNAs. Microarray analysis showed that miR-145 was low expressed in all three HMSC-ad osteogenic differentiation cell lines (Figure 3(a)). The expression of miR-145 in HMSC-ad was 2.09-fold lower after osteoinductive differentiation. MiR-145 expression was downregulated since the fourth day and was more significant along with the progression of osteogenic differentiation. Significant statistical difference was detected on day 14 (Figure 3(c)). MiR-145 and *FoxO1* mRNA expression was detected in six cell lines on day 7 and 14 after osteo-differentiation induction, and the correlation analysis results showed that there was a significantly negative correlation between them ($P < 0.01$) (Figure 3(d)).

MiR-145 targeted the 3′UTR region of *FoxO1*

According to the database TargetScan, the binding site of miR-145 was located between bases 770 and 776 of the *FoxO1* 3′-UTR, suggesting that *FoxO1* might be the target of miR-145 (Figure 4(a)). The luciferase reporter gene assay was conducted in 293T cells to verify their binding relationship. The result revealed that the luciferase activity of cells cotransfected with miR-145 mimics and pGL3-WT was significantly lower, whereas miR-145 mimics had no such effect on the fluorescence of the Mut recombinant plasmid (Figure 4(b)). Taken together, these results revealed that miR-145 directly targeted *FoxO1* 3′UTR.

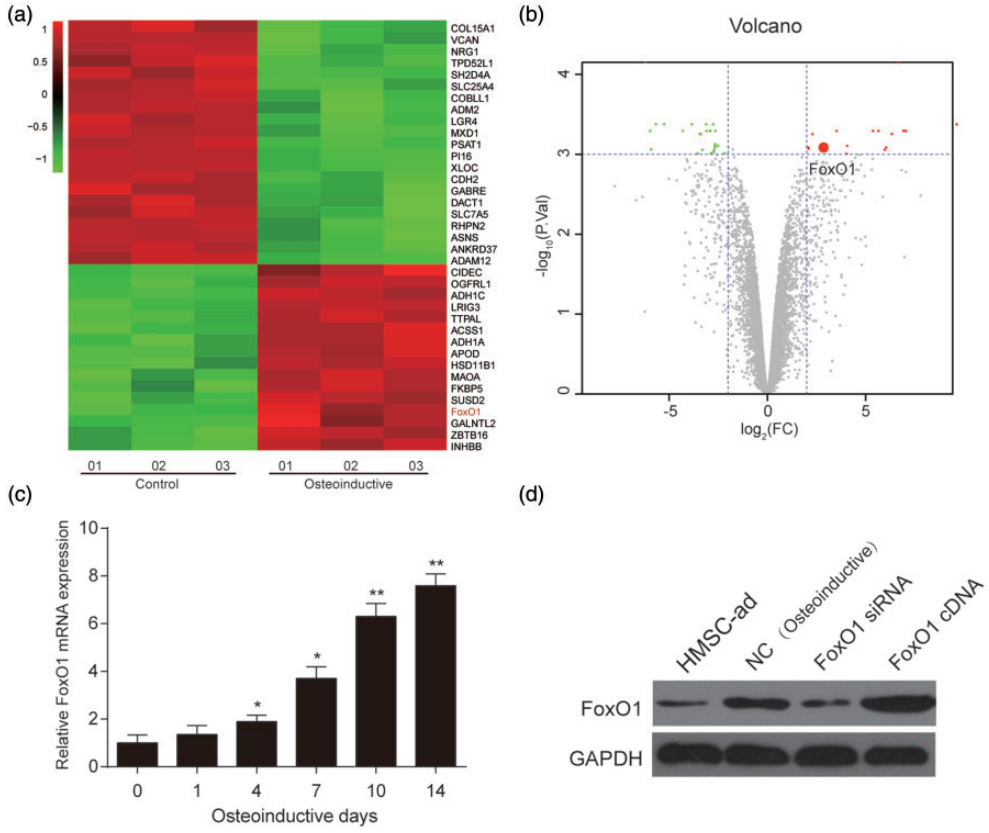

Figure 1. The expression of *FoxO1* was up-regulated during hASCs osteogenesis. (a) Heat map revealed the *FoxO1* was higher expressed after hASCs osteogenic differentiation. (b) Volcano plot showed the *FoxO1* was highly expressed after hASCs osteogenic differentiation. (c) *FoxO1* expression was up-regulated gradually along with the osteoinductive differentiation. (d) *FoxO1* expression increased after osteoinductive differentiation in HMSC-ad cells. $^{*}P < 0.05$, $^{**}P < 0.01$, compared with day 0. (A color version of this figure is available in the online journal.)

MiR-145 prevented hASCs osteogenic differentiation via suppressing *FoxO1* expression

The protein expression of the cells after osteoinductive differentiation was detected on day 7 and 14. The protein expression of BSP, Ocn and OPN showed identical tendencies on day 7 and 14. In FoxO1 cDNA cells, BSP, Ocn, and OPN levels were high, whereas in miR-145 mimics cells, their levels were lower than in NC cells (Figure 5(a)). APL activity and ARS activity (calcification) were optimal in FoxO1 cDNA group, while the worst in miR-145 mimics group ($P < 0.05$) (Figure 5(b) and (c)). The above results demonstrated that miR-145 inhibited expression of osteogenic differentiation protein markers BSP, Ocn, and OPN via suppressing *FoxO1*, preventing hASCs osteogenic differentiation.

Discussion

We have uncovered that miR-145 was down-regulated during hASCs osteogenic differentiation and it directly regulated *FoxO1* expression. Importantly, the dynamic expression of miR-145 and FoxO1 led to efficient modulation of hASCs osteogenic differentiation. Our findings would contribute to the comprehension of the regulatory network on hASCs osteogenic differentiation, and provided the evidence that miR-145 might be developed as a potential therapy target for osteogenic-related diseases.

MiRNAs had been detected dysregulated expression and verified to act as an important role during osteogenic differentiation of various types of human cells. For example, miR-615–3p was downregulated in many human cell lineages such as human BMSCs and osteoblasts during osteogenic differentiation.[18] MiR-145 was also downregulated during osteogenic differentiation in C2C12 (mouse myoblast) and MC3T3-E1 (mouse preosteoblast) cells,[14] which was in accordance with our result. Our findings showed that miR-145 was low expressed after hASCs osteogenic differentiation, indicating that miR-145 contributed to the prevention of hASCs osteogenic differentiation. Similarly, recent studies also have identified that miR-145 could regulate osteoblast differentiation by targeting transcription factors, core-binding factor subunit beta (Cbfb) and osterix (Osx).[19] These findings underpinned the potential of systemic miR-145 therapy as a promising strategy by regulating the process of osteogenic differentiation.

MiRNAs have been discovered to regulate *FoxO1* expression directly and negatively during osteogenic differentiation according to several recent studies. For instance, Yin *et al.*[18] found miR-615–3p suppressed osteogenic differentiation by directly inhibiting *GDF* and *FoxO1* in human lumbar ligamentum flavum cells. Kim *et al.*[16]

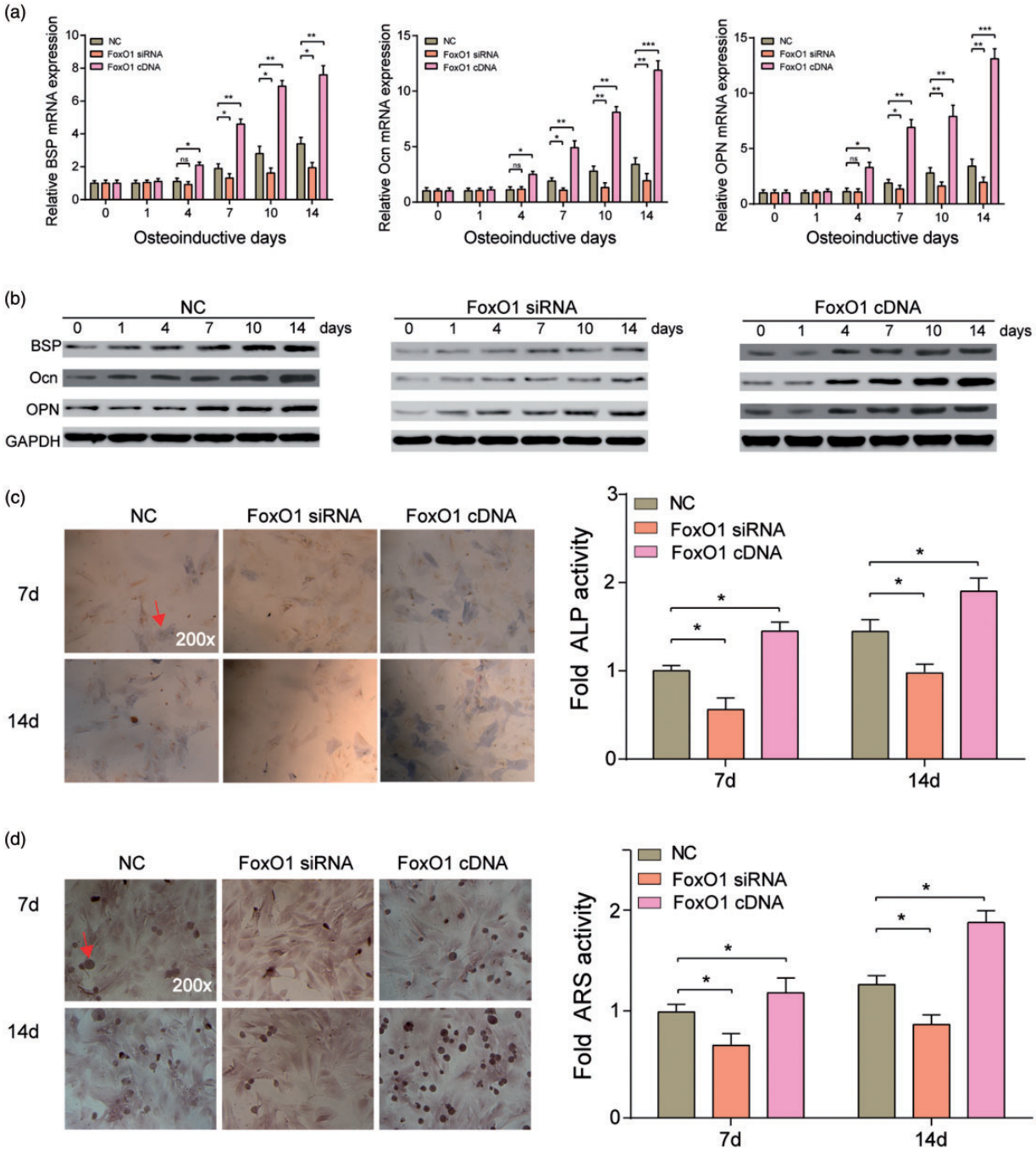

Figure 2. *FoxO1* promoted hASCs osteogenesis. (a–b) RT-PCR and Western blot results showed that *BSP*, *Ocn*, and *OPN* mRNA expression in *FoxO1* siRNA group were much lower than in NC group after osteoinductive differentiation, whereas those in *FoxO1* cDNA group were much higher than in NC group after osteoinductive differentiation, especially four days after the differentiation induction. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ compared with NC group. (c and d). ALP and ARS staining results showed that HMSC-ad osteogenesis differentiation after osteoinduction was much stronger in *FoxO1* cDNA group yet much weaker in *FoxO1* siRNA group at either time point (day 7 and day 14). $^{*}P < 0.05$ compared with NC group. (A color version of this figure is available in the online journal.)

identified *FoxO1* as a target of miR-182 in C3H10T1/2 MSCs and MC3T3E1 preosteoblasts, negatively regulating osteoblast differentiation. The target relationship of miR-145 and *FoxO1* in other medical processes was explored. A recent study showed that miR-145 was frequently down-regulated in human bladder cancer and suppressed tumor formation by directly inhibiting *FoxO1* functioning.[20] However, the regulation of miR-145 targeting *FoxO1* in hASCs remained to be elucidated. In the current study, we found *FoxO1* a potential target of miR-145 and confirmed that miR-145 targeted the 3′UTR region of *FoxO1* directly. Hence, based on previous studies, we believe that *FoxO1* plays a key role during osteo-differentiation. *FoxO1* can be targeted by various miRNAs during osteogenic differentiation, yet its suppression by miR-145 in human ASCs has not been elucidated until the present study. We thus speculated that miR-145 might reduce bone differentiation by suppressing *FoxO1* directly.

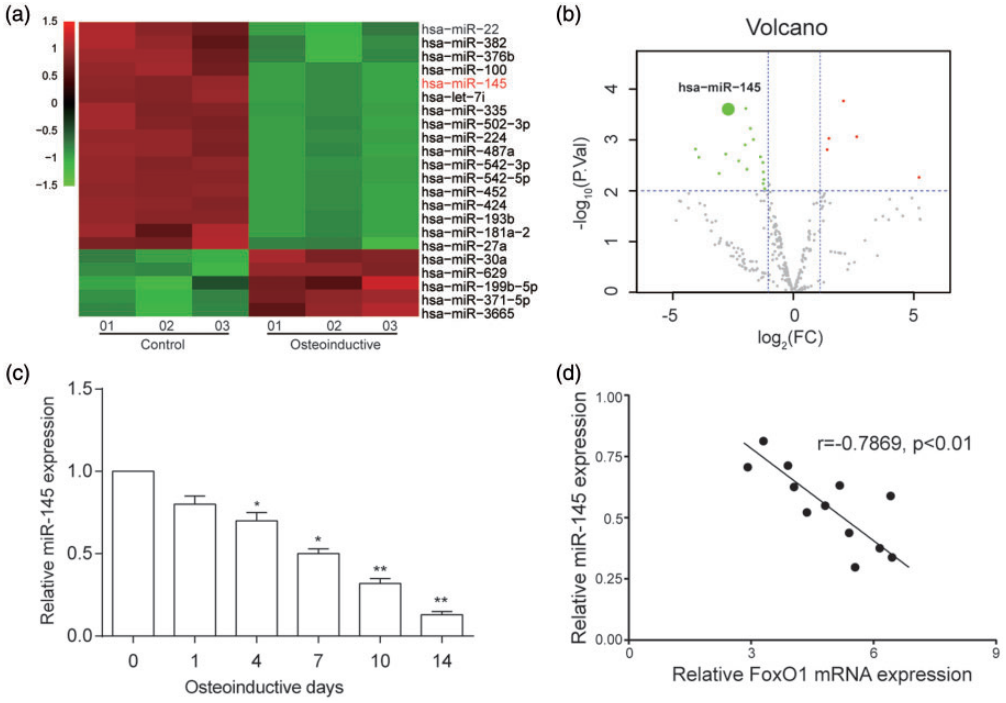

Figure 3. MiR-145 expression was down-regulated during hASCs osteogenesis. (a) Heat map revealed the miR-145 was lower expressed after hASCs osteogenic differentiation. (b) Volcano plot showed the miR-145 was lower expressed after hASCs osteogenic differentiation. (c) MiR-145 expression was downregulated gradually along with osteoinductive differentiation. (d) There is a significantly negative correlation between miR-145 and *FoxO1* expression. $^{*}P < 0.05$, $^{**}P < 0.01$ compared with day 0. (A color version of this figure is available in the online journal.)

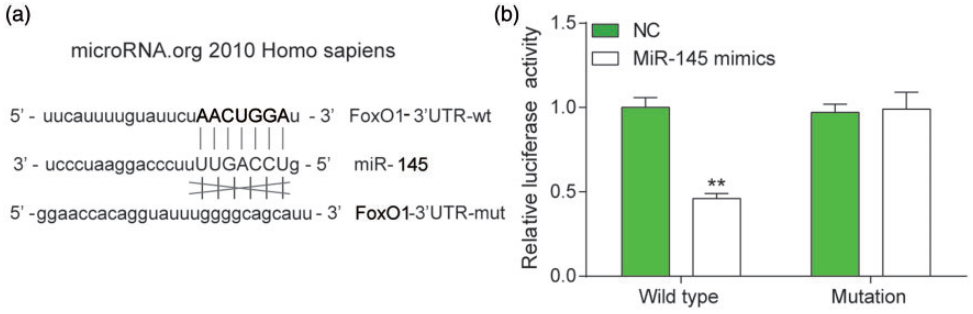

Figure 4. MiR-145 targeted *FoxO1* 3′UTR. (a) The binding sites of miR-145 and *FoxO1* as well as the putative binding relationship were illustrated. (b) Dual luciferase reporter gene assay results demonstrated that the luciferase activity in miR-145 mimics + wild type group was significantly weaker than in NC + wild type group. $^{**}P < 0.01$ compared with NC group. (A color version of this figure is available in the online journal.)

FoxO1 has revealed high expression during osteogenic differentiation. For instance, *FoxO1* mRNA levels remarkably increased after 14 days during osteoblast differentiation.[21] Teixeira *et al.*[22] approved that *FoxO1* activity and expression increased during osteogenic differentiation. Coincidentally, we found that *FoxO1* was highly expressed after hASCs osteogenic differentiation, suggesting that *FoxO1* may induce hASCs osteogenic differentiation. Similarly, *FoxO1* has recently been reported to inhibit osteoblast differentiation, indirectly suggesting that *FoxO1* has a positive effect on promoting differentiation.[21] Yin *et al.*[18] previously proved that knockdown of *FoxO1* could inhibit the osteogenic differentiation of human lumbar ligamentum flavum cells. However, the role of *FoxO1* in differentiation of mesenchymal cells was also unraveled in recent studies. It has been shown that activation of *FoxO1* prevented mesenchymal cells from differentiating into fat or muscle cells.[22] It was possible that this apparent discrepancy was due to the approach used or that *FoxO1* had different effects on osteogenic differentiation depending on the degree.

It was known that one gene can be targeted by multiple miRNAs, and in turn, one miRNA can also regulate multiple target genes. A limitation of this study was the fact that no other genes were taken into consideration, compromising the credit of the target relationship between miR-145 and *FoxO1*. The lack of animal experiments in our study should also be noted.

Collectively, our findings unveiled a novel mechanism of the osteogenic differentiation process and suggested that

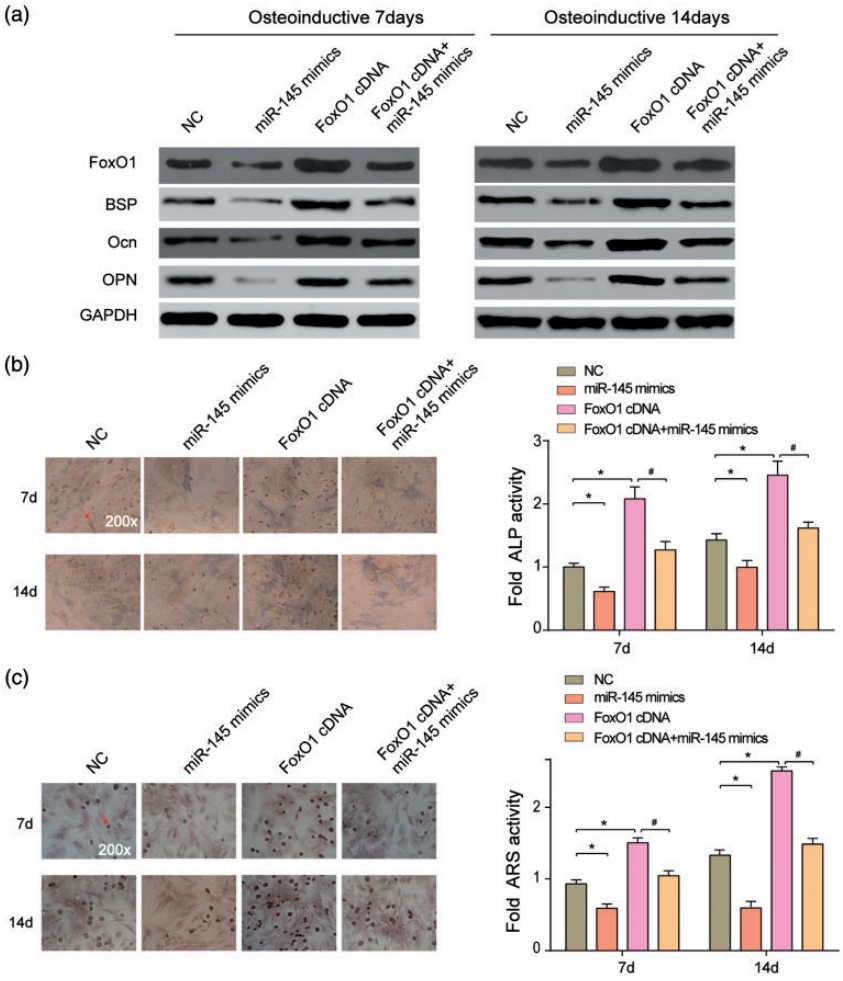

Figure 5. MiR-145 inhibited *FoxO1* to suppress hASCs osteogenesis. (a) FoxO1, BSP, Ocn, and OPN expression in miR-145 mimics group was much lower, whereas those in *FoxO1* cDNA group was much higher than in NC group. There was no significant difference between the NC group and the (b–c) ALP and ARS staining results demonstrated that miR-145 mimics significantly reduced HMSC-ad osteogenesis differentiation after osteoinduction and *FoxO1* cDNA significantly increased HMSC-ad osteogenesis differentiation. The simultaneous transfection of *FoxO1* cDNA and miR-145 mimics showed no significant difference from the NC group. $^{*}P < 0.05$, compared with NC group; $\#P < 0.05$, compared with miR-145 mimics group. (A color version of this figure is available in the online journal.)

miR-145 and its target gene *FoxO1* may be potential targets for the therapy of human osteogenic-related disorders.

Authors' contributions: All authors participated in the design, interpretation of studies and analysis of data and review of manuscript; Lugang Zhou, Yujie Sun and Tian He conducted the experiments; Hao Su, Jianqiang Ni and Peng Shi supplied critical reagents; Wei Hao and Hongzhi Liu drafted the manuscript; Xin Wang critically revised the manuscript and received the grant.

Approval of final manuscript: all authors.

DECLARATION OF CONFLICTING INTERESTS

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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