Original Paper

MicroRNA-10a Influences Osteoblast Differentiation and Angiogenesis by Regulating β -Catenin Expression

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Key Words

MicroRNA-10a • Osteoblast differentiation • Angiogenesis • β -catenin • Bone regeneration

Abstract

Background/Aims: Elucidation of the molecular mechanisms governing osteoblast differentiation and angiogenesis are of great importance for improving the treatment of bone-related diseases. In this study, we examined the role of microRNA (miR)-10a in the differentiation of MC3T3-E1 cells and pro angiogenic activity of mouse umbilical vein endothelial cells (MUECs). **Methods:** The murine pre-osteoblast cell line MC3T3-E1 and MUECs were used in the experiment. After transfected with miR-10a mimics or inhibitors, with or without LiCl pretreatment, the miR-10a, ALP, Runx2, Osx, OC and Dlx5 expression were assessed by RT-PCR. MC3T3-E1 cells were cultured with BMP2 to differentiate into bone cells, osteogenic differentiation of MC3T3-E1 cells were detected by ALP and ARS staining. Cell viability were analyzed by MTT and the protein expression of β -catenin, LEF1, cyclinD1, MMP2, and VEGF were detected by Western blotting; VEGF and VE-cadherin release were assessed by ELISA, and the migration of MUECs, as well as tube formation were also detected. **Results:** MiR-10a expression was obviously down-regulated during osteogenic differentiation. Overexpression of miR-10a inhibited osteogenic differentiation of MC3T3-E1 cells, effectively decreasing MUECs proliferation, migration, VEGF expression, VE-cadherin concentrations, and tube formation *in vitro*, whereas miR-10a silence enhanced those processes. Further mechanism assays demonstrated that overexpression of miR-10a reduced the β -catenin at both protein and transcription level, while pretreatment with Wnt signaling activator LiCl partially attenuated the suppression effects of miR-10a overexpression on osteoblast differentiation and angiogenesis. **Conclusion:** Our findings imply that miR-10a plays a suppressive role in osteoblast differentiation of MC3T3-E1 cells and pro angiogenic activity of MUECs by regulating the β -catenin expression, representing a novel and potential therapeutic target for the treatment of bone regeneration-related diseases.

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Introduction

Bone defects caused by traumas, congenital malformations or diseases cause enormous social and economic burdens, which seriously affect people's quality of life worldwide [1]. The main issue in the treatment and prevention of these diseases remains how to promote osteogenesis. Among the many factors of bone regeneration, osteoblast differentiation and angiogenesis play the most important roles [2, 3]. Reports show that inhibiting osteoblast differentiation significantly induced bone loss in a mouse model [4]. Furthermore, angiogenesis, which provides nutrition during skeletal development, also has an important part in bone formation [5]. Thus, elucidation of the molecular mechanisms governing osteoblast differentiation and angiogenesis are of great importance for improving the treatment of bone-related diseases. There are a wide variety of stimuli and signal pathways taking part in the process of bone regeneration. In terms of skeletal homeostasis and bone repair, the Wnt pathway is among the most attractive targets for such therapeutic interventions. There is now a substantial literature supporting the role of Wnt signaling in skeletogenesis and a growing appreciation for the functions of Wnt in regulating stem cell and skeletal cell behaviors [6, 7]. Reports show that Wnt signals regulate bone mass by perpetuating the self-renewal and proliferation of stem cells and inducing differentiation of osteoprogenitor cells [8, 9]. Therefore, targeting the Wnt pathway may provide novel insights into the mechanisms underlying bone formation.

MicroRNAs (miRNAs) are a family of non-coding RNAs that are 18-22 nucleotides in length and inhibit target gene expression by inducing mRNA degradation or by blocking protein translation [10]. Recently, several miRNAs have been reported by many researchers to regulate skeletal homeostasis and osteoblastogenesis, either positively or negatively, through multiple signaling pathways [11, 12]. For example, several miRNAs were shown to influence stem cell differentiation into osteocytes, to repress osteogenic differentiation of adipose-derived stromal cells [13], to mediate BMP-2 dependent osteoblast proliferation and differentiation [14], or to contribute to angiogenesis [15]. Among the miRNAs, miR-10a has been shown to be involved in many pathological processes, such as tumor cell proliferation and migration, as well as for angiogenic processes [16]. Recently, numerous studies characterize miR-10a as a biomarker for injury, such as kidney injury [17] and cardiac injury [18]. However, its effect and the underlying mechanism involved in the progression of bone regeneration remains unclear.

Here, we demonstrated that miR-10a was down-regulated in BMP2-induced osteoblast differentiation of MC3T3-E1 cells. Its roles in pro angiogenic activity were investigated through the use of MUVECs. Furthermore, the functional pathway in which miR-10a participated was also explored.

Materials and Methods

Antibodies and reagents

All substances were purchased from Gibco (Grand Island, NY), if not otherwise mentioned. A-minimal essential medium (AMEM) and RPMI 1640 medium were bought from Abcam (Cambridge, MA). LiCl (Wnt/ β -catenin signaling pathway activator) was bought from Calbiochem (San Diego, CA). Rabbit anti-LEF1, ALP, β -catenin, GSK3- β , cyclin D1, MMP2 and VEGF polyclonal antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA). Anti- β -catenin and β -actin antibodies were bought from Sigma (St. Louis, MO). HRP-conjugated goat anti-mouse and anti-rabbit antibodies were from Abcam (Cambridge, MA).

Cell lines and cell culture

The murine pre-osteoblast cell line MC3T3-E1 and mouse umbilical vein endothelial cells (MUVECs) were obtained from CHI Scientific, Inc. (Massachusetts, USA) and were cultured according to the established protocols. Briefly, MC3T3-E1 cells were cultured in a proliferation medium containing the α -minimal essential medium (Amem) supplemented with 10% fetal bovine serum (FBS), 2 mM of L-glutamate, 100

U/ml of penicillin, and 100 µg/ml of streptomycin. Cells at passages 16 were used for this study. MUVECs were grown in RPMI 1640 medium containing vascular endothelial growth factor (VEGF), heparin, 20% fetal bovine serum (FBS) (Atlanta Biologicals), 1% ascorbic acid 100 U/ml penicillin, and 100 mg/ml streptomycin. The fourth to sixth passage MUVECs in actively growing conditions were used for the experiments. All cells were cultured at 37°C in an incubator (Life Technologies, Baltimore, MD) containing 5% CO₂. Osteoblastic differentiation was induced by the addition of 300 ng/ml BMP2 for 7d [19].

Oligonucleotide and siRNA transfection

Cells were seeded into six-well plates, and were then transfected with miR-10a mimics, miR-10a inhibitors, or miR controls (50 nM, GenePharma) using Lipofectamine. Briefly, the MC3T3-E1/MUVEC cells were seeded at a concentration of 2×10^7 /well. Then, a miR-10a mimic/inhibitor was added when the cells reached about 60% confluence. The cells were incubated with 40 Nm mimic/inhibitors or their matched negative controls (mimic-nc/inhibitor-nc) for 48 h. Then, the RT-PCR analysis was performed to evaluate the transfected effect.

GSK3-β siRNA (Sigma-Aldrich) transfection was performed according to the manufacture's instruction. Briefly, 1 µg of vector was incubated with 50 µl of serum-free medium for 5 min (Solution A), and 2 µl of Lipofectamine 2000 was incubated with serum-free medium for 5 min (Solution B). Solution A was mixed with solution B, and incubated for 20 min. After incubation, cells were added to the mixture. Cells were then harvested at 48 hours after transfection for further analysis. The efficiency of GSK3-β siRNA was confirmed by Western blotting analysis.

β-catenin Plasmid Construction and Cell Transfection

Total RNA was isolated from experimental cells using TRIzol reagent (Invitrogen, Carlsbad, CA, USA), based on the manufacturer's instructions. The cDNA was synthesized by reverse transcription of total RNA, using the PrimeScript RT reagent kit (Takara, Dalian, China) with oligo-dT primers, according to the manufacturer's protocol. Then, the open reading frame of DDAH1 cDNA was cloned and inserted into the pcDNA3.1 vector (Invitrogen, Carlsbad, CA), namely pcDNA3.1-β-catenin expression vector. The pcDNA3.1 vector alone transfected cells were used as negative control. For cell transfection, cells were cultured to 60% confluence, cell transfection was performed using the FuGENE HD transfection reagent (Roche, Indianapolis, IN) methods as suggested by the manufacturer.

3-(4, 5-Dimethylthiazol-2-yl)-2, 5-diphenyltetrazoliumbromide (MTT) assay analysis

MC3T3-E1 cells and MUVECs viability were determined by MTT assay. The cells transfected with miR-10a mimic/inhibitor and the corresponding negative control were planted in the 96-well plates and cultured under regular conditions until they reached 80% confluence. Then, the culture medium was discarded and fresh medium containing MTT (5 mg/ml in PBS, 150 µl/well, Sangon, Shanghai, China) was added followed by incubation with cells for an additional four hours. Then, 150 µl of DMSO (Sigma) was added per well and was shaken gently for 10 min to dissolve the formazan. Absorbance at 570 nm was determined using an ELISA reader. Each cell's viability assay was performed in quadruplicate and repeated three times and the cell viability was expressed as percent-ages of the value of normal cells.

Transwell migration assay

The BioCoat migration chamber was purchased from BD Biosciences (Bedford, MA). Transwell migration assays were performed with 8-µm porous chambers according to the manufacturer's instructions. A total of 1×10^6 MUVECs in 0.5 ml culture medium were implanted into the upper chamber. The bottom well contained 0.6 ml of Dulbecco's modified Eagle's medium with 10% fetal bovine serum. After culturing for 24 h, the cells in the upper and lower chambers were stained with 0.4% trypan blue, followed by air-drying; the membrane was then stained with crystal violet. The number of migrating cells was then counted using a microscope under five fields ($\times 200$).

RT-PCR

Total RNA was extracted using Unizol Reagent according to the manufacturer's instructions (Invitrogen, Carlsbad, CA, USA). About 5 µg of RNA from each sample was used as a template for Cdna synthesis with a reverse transcription kit (Fermentas, St. Leon-Rot, Germany). An equal volume of Crna product was used in

Table 1. Sequences of primers

Targeted gene	Sense primer (5'-3')	Antisense primer (5'-3')
ALP	CCG ATG GCA CAC CTG CTT	GAG GCA TAC GCC ATC ACA TG
OC	AGC AGC TTG GCC CAG ACC TA	TAG CGC CGG AGT CTG TTC ACT AC
Runx2	CAC TGG CGG TGC AAC AAG A	TTT CAT AAC AGC GGA GGC ATT TC
OSX	CCT CTG CGG GAC TCA ACA AC	TGC CTG GAC CTG GTG AGA TG
Dlx5	GCC CCT ACC ACC AGT ACG	TCA CCA TCC TCA CCT CTG
VE-Cadherin	ACG GAC AAG ATC AGC TCC TC	TCT CTT CAT CGA TGT GCA TT
LRP5	CGT GAT TGC CGA TCT C	TTC CGG CCG CTA GTC TTG T
cyclin D1	AAC AGA TTG AAG CCC TTC T	ATC TCA GTG GTA TTT GTG A
VEGF	GAA GTC CCA TGA AGT GAT CCA G	TCA CCG CCT TGG CTT GTC A
β -catenin	ATC ACT GAG CCT GCC ATC TG	GTT GCC ACG CCT TCA TTC C-3
GSK3- β	CCT GGT GCT GGA CTA TGT	CAA GAG GTT CTG TGG TTT AA
β -actin	AGG AGC AAT GAT CTT GAT CTT	TGC CAA CAC AGT GCT GTC T
miR-10a	ACC CTG TAG ATC CGA ATT TGT G	
U6 snRNA	TGG CCC CTG CGC AAG GAT G	

the PCR. The primers used for amplification are listed in Table 1. The PCR conditions comprised 35 cycles of denaturation at 94°C for 30 s, annealing at 54°C for 30 s, and polymerization at 72°C for 30 s. Aliquots of the PCR products were electrophoresed on 1.5% agarose gels, and PCR fragments were visualized by ethidium bromide staining. Real-time PCR experiments for each gene were performed on three separate occasions. B-actin and U6 SnRNA were used for internal control of Mrna or miRNA, respectively (Table 1).

Western blotting

Expressions of LEF1, ALP, β -catenin, cyclin D1, MMP2 and VEGF were analyzed by Western blotting using commercially available antibodies. Cells were lysed in cell lysis buffer (Beyotime, Haimen, China) and the protein concentration was measured using the Bradford method. A total of 20-30 μ g of protein was loaded on 12% sodium-dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gels and blotted onto polyvinylidene fluoride (PVDF) membranes. The membranes were blocked by 2.5% nonfat milk for 1 h at 37°C, and were then incubated with primary antibodies against LEF1 (1:2000), ALP (1:1000), cyclin D1 (1:1000), MMP2 (1:1000), VEGF (1:1000), β -catenin (1:1000), GSK3- β (1:1000) and β -actin (1:2000) at 4°C overnight. After incubation with HRP-conjugated secondary antibody for 1 h at room temperature, protein bands were visualized using an enhanced chemiluminescence detection system. Densitometry values were normalized to levels of β -actin and the control lane in each blot.

Alkaline phosphatase staining (ALP) and alizarin red staining (ARS)

ALP staining was used to evaluate the osteoblast phenotype. The ALP staining was performed at 7 d after drug treatment using an ALP staining kit (Nanjing Jiancheng Bioengineering Institute, China) according to the manufacturer's instructions. ARS was performed at 12 d to detect the osteoblast calcification after BMP2 and miR-10a mimic/inhibitor pretreatment. Cells in 24-well plates were washed with PBS, fixed in 95% ethanol for 10 min, washed with distilled water, and then were stained by alizarin red solution (1 g Tris and 0.1 g alizarin red (Bio Basic Inc., Canada) in 100 ml ultrapure water, adjusting the Ph to 8.3 by Hcl for 30 min at 37°C. After being washed with distilled water twice, the cells were photographed.

Tube formation assay

To assess the ability of MUVECs to integrate in the vascular structure, a Matrigel tube formation assay was performed. A 48-well culture plate was coated with Matrigel plus 100 ng/ml VEGF. The MUVECs cultured with the test compounds were seeded in the Matrigel-coated plate at concentrations of 5×10^4 cells per well. After 24 h, the vascular structural tube formed by MUVECs was fixed and stained with the Diff-Quik® staining set (DADE BEHRING, Inc., Newark, DE), according to the manufacturer's instructions. After washing three times with PBS, the average length of the tube branches in four areas of each sample was assessed under an inverted light microscope.

ELISA analysis

The levels of VEGF and VE-cadherin concentrations in the culture medium were analyzed using a commercially available enzyme linked immunosorbent assay (ELISA; Yanjin Biotechnology Co., Shanghai, China) according to the manufacturer's procedure; the absorbance was taken at 450 nm using a microplate reader (Biorad, Hercules, USA). All of the samples were analyzed in duplicate.

Statistical analysis

The quantitative data was expressed as mean $\pm$ SD. Statistical analysis was carried out using a one-way analysis of variance (ANOVA) followed by a Bonferroni test for multiple groups, or a Student's t-test between two groups (using SPSS 13.0 software; SPSS Inc., Chicago, USA). Differences with a p value less than 0.05 were regarded as statistically significant.

Results

MiR-10a inhibits BMP2-induced differentiation of MC3T3-E1 cells

To gain an insight into the role of miR-10a in osteoblastic differentiation, we first examined the osteogenic-related proteins, alkaline phosphatase (ALP), runt-related transcription factor 2 (Runx2), as well as miR-10a mRNA expression during the osteoblast differentiation over 7 days (cultured with 300ng/ml BMP2). The results showed a time-dependent increase of ALP and Runx2 expression, and a significantly decrease of miR-10a mRNA expression. As shown in Fig. 1A, the expression of ALP and Runx2 began rise at 48 h and reach a peak at the 7th day. While miR-10a was significantly decreased by more than 50% at the 7th day, compared with that of untreated control MC3T3-E1 cells, suggesting that miR-10a might play a critical role in osteoblastic differentiation.

To further investigate the role of miR-10a during osteoblast differentiation, we stably transfected MC3T3-E1 cells with miR-10a mimics or inhibitors and respective negative control to reinforce the effect of miRNAs. Afterwards, the osteogenesis ability was examined. According to the RT-PCR analysis, a 3-fold increase of mature miR-10a expression was shown in MC3T3-E1 cells 48 h post-transfection of miR-10a mimics, and a 2-fold decrease was found after transfection of the miR-10a inhibitor (Fig. 1B). MiR-10a target, PTEN was used to control whether the mimic and the inhibitor is working. Results show that the oligonucleotide was successfully transfected (Fig. 1C). The results of the MTT assay exhibited an approximate 50% decrease in cell viability after miR-10a mimic transfection, but more than 2-fold increase in MC3T3-E1 cells after miR-10a inhibitor transfection compared to the controls (Fig. 1D). The osteogenic induction was conducted at d0 after transfection. After induction by BMP2 for 7 d, ALP staining showed a significantly lessened difference in the miR-10a mimic group compared with the mimic-negative control (mimic-nc) group, whereas, after inhibition of miR-10a, the outcome was totally inversed (Fig. 1E upper). The alizarin red staining at d12 showed the same tendency at the matrix mineralization level (Fig. 1E lower), the cells without BMP2 treatment were also assessed at the same time point as controls. Results show that without BMP2 treatment, miR-10a overexpression also inhibits the spontaneous differentiation of MC3T3-E1 cells, while miR-10a silencing promoted the spontaneous differentiation of MC3T3-E1 cells. However, this changes is inconspicuous compare with the cells treated with BMP2. Key osteogenic-specific genes' (ALP, Runx2, osterix (Ox), osteocalcin (OC), and distal-less homeobox5 (DLX5)) Mrna expression was examined by RT-PCR after 48 h induction. The results showed that the osteoblast differentiation-related genes' (ALP, Runx2, OSX, OC, and DLX5) Mrna expression were significantly down-regulated after miR-10a mimic transfection, but up-regulated after the inhibition of miR-10a in MC3T3-E1 cells (Fig. 1F and G). Therefore, these results suggested that overexpression of miR-10a may result in osteogenesis inhibition, while down-regulation of miR-10a promoted osteogenesis.

Fig. 1. MiR-10a inhibits BMP2-induced MC3T3-E1 osteogenic differentiation. The MC3T3-E1 cells were treated with BMP2 (300 ng/ml) for 7 days. The mRNA level of ALP, Runx2 and miR-10a were measured by RT-PCR (A). After transfecting with a miR-10a mimic/inhibitor or mimic/inhibitor-negative control (nc) for 48 h, the mRNA expression of miR-10a was analyzed (B). PTEN, a known miR-10a target, was used to control whether the mimic and the inhibitor is working (C). MC3T3-E1 cell viability was assessed by MTT (D). The osteoblast phenotype was evaluated by ALP staining and the osteoblast calcification was detected by ARS staining (E). Osteoblast differentiation-related genes, ALP, Runx2, OSX (F), and OC, DLX5 (G), were analyzed using RT-PCR. β -actin and U6 SnRNA were used as a loading control. Data are presented as mean $\pm$ SD. The * symbol indicates differences from their respective controls, at $p < 0.05$.

MiR-10a suppresses cell viability, migration and tube formation in MVECs

To determine whether miR-10a could influence angiogenesis, miR-10a mimics and inhibitors were individually transfected into MVECs and their effects were assessed by

Fig. 2. MiR-10a suppresses MUVECs' viability, migration and tube formation *in vitro*. MiR-10a levels in MUVECs was analyzed by using RT-PCR after transfection with miR-10a mimic or inhibitor or mimic/inhibitor-negative control (nc) for 48 h. U6 was used as a loading control (A). Cell viability was assessed by MTT (B). MUVECs' migration was confirmed via a transwell assay (C). An ELISA assay was used to test VEGF (D) and VE-cadherin (E) concentrations in the culture medium. The matrigel tube formation assay was performed to analyze the ability of MUVECs to integrate into the vascular structure (F). The * symbol indicates differences from their respective controls, at $p < 0.01$ and $p < 0.05$, respectively.

observing cell viability, migration, release of VEGF, VE-Cadherin and tube formation. RT-PCR analysis showed a nearly 3-fold increase of mature miR-10a expression after miR-10a mimic transfection, as well as a 3-fold decrease after transfection of miR-10a inhibitor in

Fig. 3. MiR-10a triggered the activation of the Wnt signaling pathway. After pretreatment with the miR-10a mimic or inhibitor or mimic/inhibitor-negative control (nc), the β -catenin mRNA expression of both MC3T3-E1 cells and MUVECs were assessed using RT-PCR (A), and the corresponding protein levels were determined using Western blot (B) and were quantized (C). The protein expression of key osteogenic-specific genes, LEF1 (D), and angiogenesis related genes, cyclin D1, MMP2, and VEGF (E), were also analyzed. Data are presented as mean $\pm$ SD. The symbol * indicates differences from the controls, at $p < 0.05$.

MUVECs (Fig. 2A). The results of the MTT assay exhibited an approximate 40% decrease in cell viability after miR-10a mimic transfection, but a 2-fold increase in MUVEC viability after miR-10a inhibitor transfection compared to the controls (Fig. 2B).

We further analyzed whether miR-10a could influence the chemo taxis of MUVECs using a transwell migration assay. As shown in Fig. 2C, miR-9 mimic pretreatment induced about a 50% decrease in cell migration compared to the controls. In contrast, transfection of miR-10a inhibitor induced a 2-fold increase in MUVEC migration. The results suggested that miR-10a overexpression effectively suppressed the MUVECs migration, impeding angiogenesis, while the silence of miR-10a might have facilitated angiogenesis. VEGF and VE-cadherin are known to be the key elements of angiogenesis. An ELISA assay confirmed the significant decrease in both VEGF and VE-cadherin levels in the culture medium after pretreatment with a miR-10a mimic. In contrast, transfection of the miR-10a inhibitor reduced the concentrations of VEGF and VE-cadherin (Fig. 2D and E). Further tube formation assays suggested that pre-miR-10a treatment significantly decreased the total tube length, while the miR-10a silence effectively promoted a total tube length *in vitro* compared with the controls (Fig. 2F), implying that miR-10a was associated with angiogenesis.

MiR-10a down-regulates β -catenin expression in both transcription and protein levels

The Wnt/ β -catenin signaling pathway was found to play an important role in cell proliferation, differentiation and bone regeneration. To further explore the mechanism involved in miR-10a-regulated osteoblast differentiation and angiogenesis, we analyzed the β -catenin expression in both the mRNA and protein levels via RT-PCR and Western blotting.

The results showed that miR-10a overexpression decreased the β -catenin mRNA expression of MC3T3-E1 cells; meanwhile, elevated β -catenin levels were observed by miR-10a inhibition. Furthermore, the protein expression of β -catenin was also markedly decreased after miR-10a pre-treatment and was increased by miR-10a inhibition. The same effect was observed in the MUECs, as expected. The miR-10a overexpression significantly down-regulated both the β -catenin mRNA expression and its protein level (Fig. 3A, B and C). The protein expression of key osteogenic-specific gene LEF1, as well as angiogenesis-related genes, cyclin D1, MMP2, VEGF, which are downstream molecules of the Wnt signaling pathway, was also detected. As expected, miR-10a overexpression significantly brought down the protein level of LEF1 in MC3T3-E1 cells (Fig. 3D). Meanwhile, the inhibitory effect on cyclin D1, MMP2, and VEGF expression triggered by miR-10a overexpression was also determined in the miR-10a mimic groups (Fig. 3E). In contrast, transfection of the miR-10a inhibitor effectively up-regulated the expression of those proteins underlying the Wnt/ β -catenin signaling pathway. These results suggested that the miR-10a might trigger osteoblast differentiation and angiogenesis by regulating β -catenin expression.

MiR-10a regulated osteoblastic differentiation and angiogenesis by regulating β -catenin expression

To further delineate the effect of the Wnt/ β -catenin signaling pathway on miR-10a-triggered osteoblast differentiation and pro angiogenic activity, the Wnt inhibitor LiCl was used. RT-PCR analysis revealed that miR-10a overexpression down-regulated β -catenin mRNA expression in both MC3T3-E1 and MUECs cell lines, while this suppressing effect was markedly impeded after LiCl pretreatment (Fig. 4A). The same effects can be seen in the protein expression of β -catenin assessed by Western blot (Fig. 4B and C). Furthermore, the down-regulation of ALP, Runx2, and Osx, as well as OC and DLX5, mRNA induced by miR-10a mimic in MC3T3-E1 cells was also reversed when pretreatment by LiCl (Fig. 4D and E). Moreover, the decrease in MUECs' viability (Fig. 4F) and migration (Fig. 4G) induced by miR-10a up-regulation was also dramatically attenuated following LiCl stimulation. Importantly, LiCl treatment also blocked miR-10a-induced suppression of tube formation (Fig. 4H). Interestingly, when pretreatment control cells with LiCl in the absence of miR-10a mimic transfection, the β -catenin protein expression, as well as ALP, Runx2, Osx, OC, DLX5 mRNA levels, and cell migration, tube formation has been significantly increased compare with the control group. Moreover, as LiCl is not very specific and also Gsk3- β has many other targets [20]. Therefore, all these results were confirmed by Gsk3- β knock down and also β -catenin overexpression. And LiCl as well as β -catenin overexpression has also been used to investigate whether miR-10a can still suppress osteoblast differentiation in the presence of Wnt activation. Results in Fig. 5 showed that in the presence of Wnt activation miR-10a can partially suppress osteoblast differentiation. These results indicate that miR-10a may suppress osteoblastic differentiation and angiogenesis by regulating β -catenin expression.

Discussion

In the present study, we demonstrated a negative role of miR-10a during osteoblast differentiation and angiogenesis *in vitro*. First, we provided evidence that miR-10a expression was decreased during the BMP2-induced osteoblast differentiation. Further analysis demonstrated that overexpression of miR-10a inhibited MC3T3-E1 cell differentiation into osteoblasts and pro angiogenic activity in MUECs, while miR-10a silence promoted the process of osteoblast differentiation and pro angiogenic activity. The mechanism assay confirmed that miR-10a regulated osteoblast differentiation and pro angiogenic activity by regulating β -catenin expression. These findings suggest a potential important role of miR-10a in regulating the process of bone regeneration.

Recently, miRNAs have been shown to be involved in the regulation of cellular behaviors, including differentiation and development, metabolism, proliferation, apoptosis, viral

Fig. 4. MiR-10a inhibited osteoblastic differentiation and angiogenesis by regulating β -catenin expression. After pretreatment of MC3T3-E1 cells and HUVECs with LiCl for 48 h, the mRNA expression of β -catenin was analyzed using RT-PCR (A). Corresponding protein levels were analyzed by Western blotting (B) and were quantized (C). The protein levels of ALP, Runx2, Osx (D), and OC, DLX5 (E) in MC3T3-E1 cells, as well as cell viability (F), migration (G) and tube formation (H) of HUVECs, were also assessed. β -actin served as the loading control. The results are presented as mean $\pm$ SD. The symbol * indicates differences from their respective controls, at $p < 0.05$. The symbol # indicates differences from the miR-10a mimic group, at $p < 0.05$.

infection and tumor genesis [21, 22], and dysregulation of miRNAs can result in a myriad of pathological states [23]. Recently, dozens of miRNAs are emerging as important negative

Fig. 5. β-catenin was involved in miR-10a induced osteoblastic differentiation and angiogenesis suppression. After pretreatment of MC3T3-E1 cells (A) and MUVECs (B) with GSK3-β siRNA and/or pcDNA3.1-β-catenin for 48 h, the mRNA expression of GSK3-β and β-catenin was analyzed using RT-PCR. Corresponding protein levels were analyzed by Western blotting (C). The protein levels of ALP, Runx2, Osx (D), and OC, DLX5 (E) in MC3T3-E1 cells, as well as cell viability (F), migration (G) and tube formation (H) of MUVECs, were also assessed. β-actin served as the loading control. The results are presented as mean ± SD. The symbol * indicates differences from their respective controls, at $p < 0.05$. The symbol # indicates differences from the miR-10a mimic group, at $p < 0.05$.

or positive regulators of posttranscriptional gene expression and are considered critical for osteogenesis [24, 25]. Among of them, miR-10a has recently been involved in the progression

of several diseases, such as kidney or cardiac injury [18, 26]. Reports show that miR-10a is up-regulated in NSCLC and may promote cancer by targeting PTEN [27]. MiR-10a also targets Bcl-6 and constrains the plasticity of helper T cells [28]. In addition, studies have shown that miR-10a can suppress angiogenesis in tumor tissues [29]. During the process of bone regeneration, osteoblastic differentiation and angiogenesis are both key elements, and thus, we explored the effects of miR-10a in bone regeneration.

In our study, miR-10a expression was significantly decreased in the process of BMP2-induced osteoblastic differentiation, which is consistent with the previous study [30]. In the process of bone regeneration, ALP activity and calcification level are considered to be markers of osteogenic lineage differentiation. In this study, after miR-10a mimic transfection, ALP staining indicated a down-regulated osteoblast phenotype, and ARS staining confirmed a reduction of calcification in MC3T3-E1 cells. In contrast, transfection of the miR-10a inhibitor reversed those above-mentioned results. The transcription factors Runx2 and Osx are crucial early markers of osteogenic lineage. OC is a major component of the bone extracellular matrix synthesized exclusively by osteoblastic cells [31] and DLX5 acts as the early BMP-responsive transcriptional activator needed for osteoblast differentiation [32]. In the current study, miR-10a overexpression effectively brings down the mRNA levels of ALP, Runx2, OSX, OC, and DLX5, while the silence of miR-10a up-regulated the expression of those osteogenic related genes. All of those results show that miR-10a may inhibit bone regeneration by suppressing osteoblast differentiation.

Angiogenesis is a key component of bone regeneration or repair. New blood vessels bring oxygen and nutrients to the highly metabolically active regenerating callus and serve as a route for bone precursor cells to reach the injury site. A variety of studies in transgenic and gene-targeted mice have demonstrated the importance of angiogenesis in fracture healing [33]. Proliferation and migration of MUVECs are closely related to angiogenesis. To clarify miR-10a function during bone regeneration, its role in angiogenesis was then investigated. Our data revealed that overexpression of miR-10a inhibited the cell viability of MUVECs, whereas inhibition of the miR-10a function enhanced the MUVECs' viability. Simultaneously, the migration capability of MUVECs, which was considered to be an important characterization for angiogenesis, was also decreased by miR-10a mimic transfection and increased by miR-10a inhibitor pretreatment.

Angiogenesis is regulated by a variety of growth factors, such as vascular endothelial growth factor (VEGF) and vascular endothelial cadherin (VE-cadherin) [34]. In this study, overexpression of miR-10a effectively decreased VEGF and VE-cadherin concentrations in the culture medium, while miR-10a silence increased both VEGF and VE-cadherin levels, suggesting that miR-10a might suppress angiogenesis. Tube formation plays an important role in angiogenesis. Studies have shown that inducing apoptosis in tube-forming endothelial cells can effectively suppress angiogenesis [35]. Just as expected, further analysis of tube formation ability showed a notable decrease in the formation of capillary tubes after miR-10a mimic preconditioning, but an obvious increase after miR-10a inhibitor transfection was also found. Together, these results indicate that miR-10a might play a negative role in bone regeneration based on the inhibition effects of angiogenesis.

In terms of skeletal homeostasis and bone repair, the Wnt/ β -catenin pathway is among the most attractive targets for such therapeutic intervention. Studies found that the activating mutations of the Wnt function in humans caused high bone mass phenotypes, such as van Buchem disease, whereas inactivating mutations caused osteopenic diseases, such as osteoporosis pseudoglioma syndrome [36]. These findings raise the possibility that modulation of the Wnt pathway is a viable path for improving bone formation. More importantly, the Wnt signaling in endothelial cells accounts for the regulation of angiogenesis *in vitro* [37]. In the current study, we found that miR-10a suppressed the Wnt/ β -catenin signaling pathway by decreasing β -catenin expression. Here, interestingly, we found miR-10a not only decreased the protein expression of β -catenin but also affecting its transcription. As one previous study show that down-regulated STAT3 expression can result in reduced β -catenin mRNA and protein levels [38], and another study reported that BAMBI knockdown

can significantly decrease the expression of β -catenin mRNA and suppress metastasis of gastric cancer cells [39]. However, miR-10a silencing may reverse cisplatin resistance in human lung cancer cell line via the TGF- β /Smad2/STAT3/STAT5 pathway [40]. Thus, we speculate that miR-10a may indirectly affect the β -catenin mRNA expression by targeting STAT3 and/or BAMBI, and this will be further investigated in our future research. We also observed that LiCl with miR-10a mimic pretreatment brings up the mRNA expression of β -catenin compared with the miR-10a mimic group, but there was no statistical difference between the cells only treated with LiCl and controls. We speculate that LiCl as a metal ion preparations, may effecting the transfection efficiency or function realization of the miR-10a mimic.

LEF1 was reported to promote osteoblast differentiation, cyclinD1 focused on cell proliferation, MMP2 was effectively associated with cell migration, and VEGF was one of the key elements for vascular formation. To further analyze the role of Wnt/ β -catenin in miR-10a induced pro-differentiation and angiogenesis, we analyzed the expression of osteogenic-related protein LEF1, and angiogenesis-related protein cyclinD1, MMP2, and VEGF, which were located downstream of the Wnt/ β -catenin signaling pathway. As expected, miR-10a mimic transfection significantly down-regulated the protein levels of LEF1, cyclinD1, MMP2, and VEGF, while miR-10a inhibitor reversed these results. Then, we inhibited the signaling by using the Wnt signaling activator, LiCl. The results showed that pretreatment with LiCl effectively attenuated the suppression effects of osteoblast differentiation in MC3T3-E1 cells that were induced by miR-10a overexpression. Consistently, Wnt activation also brings up the MUECs' viability and migration, as well as tube formation, *in vitro*. All results above were further confirmed by Gsk3- β knock down and also β -catenin overexpression. These results suggested that miR-10a might regulate osteoblast differentiation and angiogenesis by regulating β -catenin expression.

In summary, miR-10a was identified as a novel regulator in osteoblast differentiation and angiogenesis through regulating β -catenin expression *in vitro*. Therefore, this study may provide new insights into the possibility of miR-10a and miR-10a being potential therapeutic targets in the management of bone regeneration-related diseases.

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Disclosure Statement

The authors have declared that no competing interests exist.

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