LncRNA MEG3 promotes osteogenesis of hBMSCs by regulating miR-21-5p / SOD3 axis

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Background: This study aimed to investigate the role of long non-coding (Lnc) RNA MEG3 on the osteogenesis of human bone marrow mesenchymal stem cells (hBMSCs). Materials and Methods: The binding of miR-21-5p to LncRNA MEG3 and SOD3 was determined using luciferase reporter assay; fluorescence quantitative PCR was used to detect the expression of LncRNA MEG3 at different induction times. hBMSCs were transfected with LncRNA MEG3 overexpression vector and induced for osteoblasts for 14 days. Alkaline phosphatase (ALP) staining, and alizarin red staining were used to detect bone differentiation; immunofluorescence assays were used to detect the expression of SOD3 and COL2A1. Results: Luciferase reporter assay revealed that miR-21-5p bond to LncRNA MEG3 and SOD3. Flow cytometry analysis showed that hBMSCs were highly pure. After osteogenic induction for 14 days, compared with the control group, the overexpression of LncRNA MEG3 significantly increased the activity of ALP and enhanced the formation of calcium nodules in hBMSCs. The overexpression also increased the expression of COL2A1 and SOD3 significantly (P<0.05). Conclusions: LncRNA MEG3 can promote the osteogenesis and bone regeneration of hBMSCs and increasing the expression of SOD3 and COL2A1 via targeting the miR-21-5p/SOD3 axis

Keywords: LncRNA MEG3, miR-21-5p, SOD3, hBMSC, osteogenesis

INTRODUCTION

Trauma, infection, congenital diseases, and tumors may cause bone defects and the repair of large bone defects is still a challenging issue in clinical practice (Cui et al., 2018). At present, the filling materials for bone defects mainly include autogenous, allogeneic, xenogeneic and artificial bone. Autologous bone transplantation, especially autogenous bone transplantation with microvascular anastomosis to reconstruct blood vessels, can minimize adverse recipient reactions, and change the process of creeping substitution to direct fracture healing. The disadvantage of autologous bone transplantation is that the amount of bone to be taken is limited and it may cause a series of potential complications at the donor site such as trauma (Li et al., 2019; Verbeek et al., 2019). Compared with other seed cells, human bone marrow mesenchymal stem cells (hBMSCs) have a number of advantages. They are easy to obtain, can be cultured in vitro for quick proliferation, have excellent adhesive ability, and could maintain typical characteristics of stem cells even if the donors are aged. In addition, hBMSCs are not immunogenic (de Boeck et al., 2010; Liu et al., 2018a; Wu et al., 2018).

The treatment and rehabilitation of bone diseases are very important in clinical medicine. The occurrence of bone diseases is often due to the disturbance to dynamic balance in the bone. There are three cell types in bone tissue: osteoblasts, osteoclasts, and osteocytes. Among them, osteoblasts are the main functional cells for bone formation, responsible for synthesis, secretion, and mineralization of bone matrix. Osteoblasts mainly come from bone marrow-derived stroma cells (BMSCs). Differentiation of osteoblasts from BMSCs has been widely studied for the treatment of bone diseases (Yang et al., 2012; Zou et al., 2021).

Long noncoding RNA (LncRNA) is a class of RNAs with a length of more than 200 nucleotides. It is located in the cytoplasm or the nucleus but does not encode proteins. Its expression is highly tissue-specific (Kawasaki et al., 2018). In recent years, it has been found that LncRNA is involved in many biological processes, such as maintaining homeostasis in cells and tissues (Zhang et al., 2018). In the immune system, LncRNA regulates the differentiation, activation, proliferation, apoptosis, and cytokine expression of T lymphocytes (Li et al., 2018; Xiong et al., 2018). LncRNA MEG3 is an imprinted gene located on human chromosome 14q32.3 (Zhou et al., 2017) and is shown to block tumor progression by inhibiting angiogenesis (Liu et al., 2018c), indicating that it has a crucial role in angiogenesis that is closely related to the development of osteoarthritis (Gordon et al., 2010). Similarly, Su et al. showed that the expression of MEG3 in the cartilages of patients with osteoarthritis was significantly down-regulated, and its expression level was negatively correlated with the level of vascular endothelial growth factor (VEGF), suggesting that MEG3 may participate in the occurrence and development of osteoarthritis through regulating angiogenesis (Su et al., 2015a). However, there are few studies on the role of LncRNA MEG3 in osteogenesis.

MicroRNA (miRNA) is a group of short non-coding RNA with a length of 21-23 nucleotides, which play an important regulatory role in many biological processes (Lichner et al., 2013). MiRNA can inhibit the expression of proteins or induce the degradation of mRNA through
complementary pairing with the base of the target gene at specific site to participate the regulation of gene expression (Zhao et al., 2017). The miRNA-21 family is a highly conserved miRNA family, which regulates apoptosis, differentiation, vascular remodeling and insulin synthesis (Balaban et al., 2005; Horton Jr. et al., 2006; Sun et al., 2013). miR-21-5p is the precursor of 5-miRNA-21. The role of miR-21-5p in the differentiation of osteoblasts remains largely unknown. Recent studies have shown that in liver adipogenesis, MEG3 reduces excessive lipid deposition, likely via regulating the expression of LRP6 by binding with miR-21 (Su et al., 2015b). In cancer cells, one of the main targets of miR-21 is superoxide dismutase 3 (SOD3). Increased SOD3 protein level was observed during chondrogenesis of BMSCs. The overexpression of SOD3 increased the collagen type II A1 level (COL2A1), a chondrogenic marker, promoting chondrogenesis of BMSCs (Shi et al., 2019a). On the other hand, knockout of the SOD3 gene inhibited the expression of COL2A1. However, whether MEG3 plays a role in osteoblast differentiation through miR-21-5p remains to be further investigated.

Taken together, how LncRNA regulates the differentiation of stem cells into osteoblasts is still largely unclear and the mechanism underlying MEG3 mediated-osteogenesis of BMSCs needs to be explored. Based on the previous studies, we speculated that LncRNA MEG3 may promote the osteogenesis of hBMSCs by regulating the miR-21-5p/SOD3 axis to promote osteogenesis. The findings would provide insights into the role of LncRNA MEG3 on osteogenesis of hBMSCs for the repair of bone defects and cures for molecular therapy of bone injury.

MATERIALS AND METHODS

Experimental cells

hBMSCs HUM-iCell-s011 (http://www.icellbionscience.com/cellDetail/916) were purchased from Biohippo, USA and cultured in iCell Primary Mesenchymal Stem Cell Culture System (cat no. PriMed-iCell-012, iCell, USA) at 37°C in CO₂ incubator. Human embryonic kidney HEK203 cells were obtained from Thermo, USA.

Reagents and instruments

Fetal bovine serum (FBS) (04-007-1A) was obtained from Biologo, USA; dexamethasone (SD9530), vitamin C (A8100), alkaline phosphatase staining solution (G1481) and alizarin red S (G8550) were purchased from Solarbio, USA; Lipofectamine 3000 (Transfection Reagent, L3000015) was obtained from Invitrogen, USA; double luciferase reporter gene detection kit (RG027) was a product of Beyotime Biotech, Beijing; CD29-PE (Biogen 303003), CD44-PE-CY7 (Biogen 338185), CD45 FITC (Biogen 368507), CD90 FITC (Biogen 328107), CD105 APC (Biogen 323207) and CD73 PERCP/CY5.5 (Biogen 344013 ) were purchased from Biologo, USA; lentivirus vectors empty and LncRNA MEG3 over-expression vector were obtained from ZGLAB, Shenzhen; Trizon reagent (CW0580), ultrapure RNA Extraction Kit (CW0581M), miRNA qPCR Assay Kit (CW2142S), miRNA Purification Kit (CW0627S) and miRNA cDNA Synthesis Kit (CW2141S) were obtained from Cwbiotech, Beijing; MitoSOX Red Mitochondrial Superoxide indicator (M36008) was obtained from Thermo Fisher Scientific, USA; HiScript II qRT SuperMix for qPCR (+gDNA wiper) (R223-01) was purchased from Vazyme, USA; 2×SYBR Green PCR Master Mix (A4004M) was obtained from Lifeint, Beijing; SOD3 (DF7753) and collagen II (AF0135) were purchased from Affinity, USA. Microscopes (CX41) and fluorescence microscope (CKX53) were products of Olympus, Japan; cytometer Novocye (NovoCyte 2060R) was purchased from Essen Biology, Hangzhou; real time fluorescent PCR (CFX connect) was obtained from Biorad, Shanghai.

Luciferase reporter assay

The assays were carried out as previously described (Huang et al., 2019a; Teoh-Fitzgerald et al., 2012). There were seven groups including (1) control that did not receive any treatment (control), (2) LncRNA MEG3, (3) dual luciferase reporter vector with LncRNA MEG3 + mimic NC (negative control), (4) dual luciferase reporter vector with LncRNA MEG3+mir-21-5p mimic, (5) dual luciferase reporter vector with SOD3, (6) dual luciferase reporter vector with SOD3+mimic NC, and (7) dual luciferase reporter vector with SOD3+miR-21-5p mimic. The cells were digested, counted, and diluted according to the needs of the experiment. Cells (8×10⁴ per well) were evenly seeded and cultured in the incubator until they were completely adherent to the wall. When the cell reached 70% of confluence, the medium was changed to low serum medium (5% FBS). The cells were transfected with dual luciferase reporter vectors. HEK 293 T cells (3.5×10⁴ ) were transfected with pRL-SV40 containing different 3′-UTR sequences from LncRNA MEG3 and SOD3 using Lipofectamine 2000. The transfections were carried out in the presence and absence of 25 nM hsa-mir-21-5p mimics whose sequence is AGUUGUAGU

Characterization of hBMSCs

At 90% confluency, cells were harvested, washed with PBS twice and digested with trypsin for 3 min and suspended in complete culture medium containing 10% FBS. The cells were pelleted by centrifugation at 1500 rpm for 5 min and re-suspended in 50 µL PBS and added with 5 µL each of antibodies against CD29-PE, CD44-PE-CY7, CD45 FITC, CD73 PERCP/CY5.5, CD90 FITC and CD105 APC. After gently mixing, they were incubated at room temperature in the dark for 15 min, centrifuged to remove excessive dyes and loaded on to a flow cytometer (NovoCyte 2060R, from ACEA Biosciences Inc, USA.) for analysis.

Osteoblast induction

hBMSCs were cultured in DMEM medium containing 10% FBS and antibiotics (penicillin and streptomycin, 100 µg/ml) and were subcultured when the confluence reached 80–90%. 24 h after the subculture, the medium
was replaced with fresh osteoblast induction medium (PoliMed-iCell-012+10% FBS+penicillin and streptomycin (100µg/ml)+100 nM/L. dexamethasone+50 mg/L vitamin C+10 mM/L β-glycerophosphate sodium). The medium was refreshed every three days and cells were harvested on day 7 and 14 for LncRNA MEG3 expression analysis.

Real time fluorescent quantitative PCR (qRT-PCR)

Total RNA was isolated from cultured hBMSCs using the Trizol Reagent according to manufacturer’s instructions and reversely transcribed to cDNA for mRNA expression analysis using miRNA cDNA Synthesis kit according to manufacturer’s protocols. HiScript II qRT SuperMix for qPCR was used for quantification of cDNA according to manufacturer’s protocols. Normalization was done with GAPDH and U6. The PCR was carried out in a total volume of 10 µl containing 1.5 µl of diluted and pre-amplified cDNA, 10 µl of 2×SYBR Green PCR Master Mix and 1 µl of each fluorescence probe. The cycling conditions were 50ºC for 2 min, 95ºC for 10 min followed by 45 cycles, each one consisting of 10 s at 95ºC and 30 s at 58ºC. Samples were run in triplicate and the mean value was calculated for each case. The data were managed according to the previously described protocol (Livak & Schmittgen, 2001). The primers used for qRT-PCR are listed in Table 1.

Transfection

At confluency of 60%, cells were harvested and transfected with lentivirus and Lipofectamine 3000 at final concentration of 10 µg/ml according to manufacturer’s protocols. The cells were either transfected with empty vector (Vector) or LncRNA MEG3 overexpression vector (LncRNA MEG3) or not transfected (Control). After cultured at 37ºC in 5% CO₂ incubator for 12 hours, fresh complete culture medium was added, cells were cultured for 48 h and then harvested for analysis or used for osteoblast induction.

Alkaline phosphatase (ALP) assay

ALP assay was carried out as described (Sun et al., 2018). Briefly, after transfection and cultured for 4 days in the osteoblast induction medium, hBMSCs were harvested, washed with PBS for 3 times and fixed in 4% paraformaldehyde for 10–15 min. The cells were then washed with PBS and stained in ALP incubation solution at 37ºC for 5–15 min according to the manufacturer’s protocols and photographed under microscope.

Alizarin red staining

The fixed hBMSCs were washed with PBS for 3 times and dried. 1% Alizarin red staining solution was added, and cells were immersed for 5–10 min. After washing with distilled water, the cells were spread onto slides and photographed under microscope.

Immunofluorescence assay

Cells were fixed in 4% paraformaldehyde for 10-15 min and rinsed 3 times with PBS for 3 minutes each time. The cells were cleared with 0.5% Triton X-100 (in PBS) at room temperature for 20 min and washed with PBS for 3 times, 5 min each time. After blocking with 5% BSA at 37°C for 30 min, antibodies against SOD3 (1:250) and collagen II (1:250) were added and the slides were incubated overnight at 4°C. Then, the slides were immersed in PBS for three times, three minutes each and added with diluted (1:200) fluorescent secondary antibody against Cy3. After incubation at 37°C for 45 minutes and counter-strained with DAPI in dark for 5 min, images were captured using a fluorescence microscope.

Statistical analysis

SPSS 20.0 software was used for statistical analysis. The measurement data were expressed as means ± S.D., and single factor analysis of variance was used for comparison using the Tukey test as post post-hoc test. All assays were performed with triplicate in three independent experiments. A value of \( P<0.05 \) was considered statistically significant.

RESULTS

miR-21-5p bound to LncRNA MEG3 and SOD3

To assess the binding of miR-21-5p to LncRNA MEG3 and SOD3, we first assessed the transfection efficiency of LncRNA MEG3 in hBMSCs. RT-PCR analysis showed that after transfection with LncRNA MEG3 overexpression vector, the levels of LncRNA MEG3

| Primer | Sequence |
|--------|----------|
| LncRNA MEG3 F | CCCACCAACATACAAAGCAG |
| LncRNA MEG3 R | TGAGCATAGCAAAGGTCAGG |
| GAPDH F | TGACTTCAACAGCAGACACCC |
| GAPDH R | CACCCTGTTGCTGTAGCCAAA |
| U6 F | GCCCGGGAGACACATATACTAAAAT |
| U6 R | CGCTTCAGAATTGCGGTTCAG |

*denotes \( P<0.05 \) vs control group.
mRNA were significantly increased (Fig. 1A), suggesting that the transfection is successful. On the other hand, the mRNA levels of miR-21-5p were significantly downregulated (Fig. 1B) in the transfected cells, indicating that LncRNA MEG3 may interact with miR-21-5p. Dual-luciferase reporter assay showed that when miR-21-5p was co-transfected with LncRNA MEG3 or SOD3, the luciferase activities were significantly reduced, indicating that miR-21-5p is able to bind to LncRNA MEG3 and SOD3 (Fig. 1C and D).

Characteristics of hBMSCs

In order to detect the purity of primary hBMSCs, flow cytometry was used to analyze the phenotypic markers CD29, CD44, CD45, CD73, CD105 and CD90. Results revealed that 97.21% and 97.70% of the cells were positive for CD29 and CD44 (Fig. 2), and only 0.43% cells were positive for CD45, demonstrating that the purity of hBMSCs is high. Also, microscopy studies showed that the cells had typical morphology of MSCs (Fig. 3). In addition, assessments with phenotypical CD73, CD105 and CD90 markers showed that these markers were expressed in 99.86%, 99.98% and 99.94% hBMSCs, further confirming that the cells had high purity.

Osteoblast induction reduced LncRNA MEG3 expression

Expression of LncRNA MEG3 in osteoblast differentiation process was analyzed. qRT-PCR analysis showed that 7 days after osteoblast induction, the mRNA levels of LncRNA MEG3 were similar between the control and induced hBMSCs (Fig. 4). However, when assessed on the 14th day, the mRNA level in the induced hBMSCs was significantly reduced \((P < 0.05)\), suggesting that LncRNA MEG3 might play a role in osteogenic differentiation of hBMSCs.

To explore the effect of LncRNA MEG3 on osteogenic differentiation of hBMSCs, we overexpressed LncRNA MEG3 in the cells. ALP assay and alizarin red staining showed that ALP activities and formation of calcium nodules were remarkably increased in the hBMSCs overexpressing LncRNA MEG3 as indicated by enhanced staining (Fig. 5).

LncRNA MEG3 overexpression enhanced osteogenic differentiation

To elucidate the mechanism of LncRNA MEG3-mediated enhanced osteoblast differentiation, the expressions of SOD3 and COL2A1 in the hBMSCs were assessed. The results showed that SOD3 level was increased in the process of bone formation in hBMSCs. As showed above, SOD3 could bind to miR-21-5p in the dual luciferase assays. Therefore, we investigated the effect of LncRNA MEG3 overexpression on SOD3 protein level. In addition, overexpression of SOD3 can increase the level of COL2A1. Therefore, in order to further confirm the effect of LncRNA MEG3 overexpression on the osteogenic differentiation of hBMSCs at the protein level.
and explore the effect of LncRNA MEG3 on proteins downstream miR-21-5p/SOD3, we investigated the effect of LncRNA MEG3 overexpression on the level of COL2A1. Immunofluorescence assays showed that compared with the control, overexpression of LncRNA MEG3 significantly increased the expression of COL2A1 and SOD3 by 1.6 and 3.3 folds ($P<0.05$, Fig. 5), respectively, demonstrating that overexpression of LncRNA MEG3 up-regulates the expression of SOD3 and COL2A1 during the osteogenic differentiation of hBMSCs.

**DISCUSSION**

Treatments of bone defects based on BMSC have been widely used for bone repair. BMSC is a clonogenic cell population that is characterized by self-renewal capacity and differentiation potential into osteoblasts. It can be induced to differentiate into bone, cartilage, tendon, fat, and other cells under specific conditions. BMSC is easy to isolate and can be proliferated in vitro, therefore it is an ideal seed cell source for tissue engineering. Inducers and microenvironment are important for BMSC to differentiate into osteoblast (Liu *et al.*, 2018b). Studies have shown that TGF-$\beta$ superfamily can promote ossification of BMSC in vitro and in vivo (Wang *et al.*, 2010) and BMSC has the ability to transform into bone-like cells (Zhou *et al.*, 2019). More recently, studies have shown that other bioactive factors such as fibroblast growth factor-2 (FGF-2) and bone morphogenetic protein-2 (BMP-2) are capable of up-regulating the expression of osteogenic-related gene markers and subsequent osteogenic differentiation of BM-MSC (Gromolak *et al.*, 2020), and that TGF-$\beta 1$ as a multifunctional cytokine belonging to the transforming growth factor superfamily also plays an important role in the differentiation of BMSCs (Lv *et al.*, 2020; Zhang *et al.*, 2020). It is generally believed that BMSCs express membrane antigens such as CD44 and CD29, but not CD45. In this study, the expression of CD44, CD29 and CD45 was detected by flow cytometry, and it was found that in most hBMSCs CD29 and CD44 were positive, while CD45 was negative. The results demonstrated that the purity of primary hBMSCs is high.

Some LncRNAs have been identified as diagnostic or prognostic markers as well as potential therapeutic targets. In addition, they are also considered as endogenous miRNA sponges (Rapicavoli *et al.*, 2013). LncRNA MEG3 is an important regulatory factor, which regulates at the transcriptional, post transcriptional and epigenetic levels to impact the occurrence of diseases (Wu *et al.*, 2020). LncRNA MEG3 also plays an important role in regulating the differentiation of BMSCs (Wang *et al.*, 2017). During liver adipogenesis, LncRNA MEG3 could alleviate excessive lipid deposition, which may be achieved through its binding with miR-21 to regulate the expression of LRP6 (Huang *et al.*, 2019b). The main targets of miR-21 are SOD3 and TNFz. MiR-21 reduces the level of SOD2 by directly down-regulating SOD3, leading to increased metabolism of hydrogen peroxide by superoxide produced by endogenous basic activity or exposure to ionizing radiation (IR), and enhanced infrared-induced cell transformation (Zhang *et al.*, 2012). During the osteogenesis of BMSCs SOD3 protein was found increased (Nightingale *et al.*, 2012). In this study, BMSCs were induced to form osteoblasts. During this process, the expression of LncRNA MEG3 decreased significantly at 14 days, suggesting that LncRNA MEG3 might play role in osteogenesis of BMSCs. To confirm this, LncRNA MEG3 was overexpressed in the hBMSCs, which resulted in increased SOD3 level and decreased miR-21-5p level. In addition, luciferase reporter assays showed that miR-21-5p could bind with LncRNA MEG3 and SOD3, leading to significantly reduced luciferase activity, indicating that miR-21-5p has binding sites with LncRNA MEG3 and SOD3 and this pathway might be therefore involved in osteogenic process of BMSCs.

ALP is an enzyme functioning in osteoblasts and its activities can reflect the differentiation degree of cells to osteoblasts (Panteghini & Pagani, 1989; Wu *et al.*, 2012; Wei *et al.*, 2017). This enzyme plays a key role in calcium deposition. Increased ALP activity is an important indication that BMSCs have differentiated to osteoblasts. In order to further explore the effect of overexpression of LncRNA MEG3 on the osteogenic differentiation, we examined the ALP activity and the formation of calcium nodules in induced hBMSCs using ALP and alizarin red staining. The results showed that LncRNA MEG3 could promote ALP activity and the formation of calcium nodules, indicating that LncRNA MEG3 promotes the differentiation of BMSCs into osteoblasts.

The SOD3 gene is the last identified SODS gene. It is located on human chromosome 4. SOD3 is essential for the clearance of ROS in the extracellular matrix. ROS overexpression plays an important role in the development of osteoarthritis (Loeser *et al.*, 2016). Earlier studies have shown that SOD3 is significantly increased in the process of bone formation from BMSCs (Shi *et al.*, 2019b) and deficiency of SOD3 aggravates collagen-induced arthritis (Ross *et al.*, 2004). When the SOD3 gene is knockout, the expression of collagen type-α 1 chain (COL2A1) is suppressed. On other hand, overexpression of SOD3 increases the level of osteogenic marker COL2A1. When SOD3 is knockdown, the level superoxide anion is increased, indicating that SOD3 can promote bone formation of BMSCs (Shi *et al.*, 2019b). Our results showed that the overexpression of LncRNA MEG3 promotes the expression of SOD3.
and COL2A1, indicating that LncRNA MEG3 may target miR-21-5p/SOD3 axis to induce bone formation in hBMSCs and confirming that SOD3 and COL2A1 are osteogenic markers.

CONCLUSION

Our work demonstrates that LncRNA MEG3 is the target of miR-21-5p and SOD3; it could up-regulate SOD3 and COL2A1 expressions via miR-21-5p and promote osteogenic differentiation of BMSCs via regulating the miR-21-5p/SOD3 axis.

DEclarations

Ethics approval and consent to participate

This work does not involve human or animal study.

Consent for publication

Not applicable

Availability of data and material

The datasets used during the current study are available from the corresponding author on reasonable request.

Competing interests

The authors declare that they have no competing interests

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Authors’ contributions

SW, GX and NL designed the study. SW, GX, RN, ZP, MX and NL drafted the manuscript. SW, GX, RN, ZP, MX and NL designed the study. SW, GX, RN, ZP, MX and NL drafted the manuscript. SW, GX, RN, ZP, MX and NL drafted the manuscript. SW, GX and NL collected the data and performed analysis. RN, ZP, MX and NL drafted the manuscript. All authors read and approved the final version of the manuscript.

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