Original Article

LncRNA TUG regulates osteogenic differentiation of bone marrow mesenchymal stem cells via miRNA-204/SIRT 1

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Abstract

Objective: To explore the regulation of LncRNA TUG /miRNA-204/SIRT1 pathway on osteogenic differentiation of bone marrow mesenchymal stem cells (BMSCs), so as to provide a new theoretical basis for the clinical treatment of osteoporosis. **Methods**: Detect changes of LncRNA and miRNA expression predicted in post-differentiation BMSCs with Western blot and qPCR tests. Verify the regulatory relationship between LncRNA and miRNA, miRNA and SIRT1 through the luciferase reporter assay. Transfect recombinant plasmids with LncRNA and their shRNA or transfected miRNA mimics and inhibitors. **Results**: According to the bioinformatic prediction, LncRNA TUG/miR-204 affected the regulation of SIRT1 on osteogenic differentiation of BMSCs, which were consistent with the results of luciferase reporter assay, namely, there are direct regulation targets between LncRNA TUG and miR-204, miR-204 and SIRT1. Overexpression and knockdown experiments revealed that LncRNA TUG overexpression/knockdown down/up-regulated miR-204 expression, which otherwise increased/decreased SIRT1 levels, and was positively correlated with osteogenic differentiation of BMSCs. Conversely, miR-204 was negatively correlated with LncRNA TUG and SIRT1, and negatively regulated osteogenic differentiation. **Conclusion**: This study found the direct regulatory relationship of LncRNA TUG/miR-204/SIRT1 during the osteogenic differentiation of BMSCs, and revealed that SIRT1 positively regulates the osteogenic differentiation of BMSCs, which provides a theoretical basis and potential therapeutic targets for a series of osteogenic differentiation-related diseases including osteoporosis.

Keywords: BMSCs, LncRNA TUG, miR-204, Osteoporosis, SIRT1

Introduction

In recent years, osteoporosis has become a common chronic metabolic disease with increasing prevalence. It seriously affects the life and health of patients, as well as bone fragility and fracture risk, which is also affected by genetic and environmental factors[1-3]. Osteoblasts play an important role in bone formation and bone development, most of which are derived from bone marrow mesenchymal stem cells (BMSCs), a kind of cells with multi-directional differentiation potential. It can induce differentiation into osteoblasts and adipocytes under certain conditions, and is widely used in tissue engineering and genetic engineering[4,5]. At present, the clinical treatment of osteoporosis mainly includes vitamin D, calcium, physical exercise, reasonable diet and so on, which cannot effectively control the occurrence and development of osteoporosis. Hence, it is of great clinical significance to find an effective treatment for osteoporosis[6].

With the increase of age and the decrease of hormone level, the expression of inflammatory factors is increasing, which affects the survival of osteoblasts in bone marrow microenvironment, breaking the dynamic balance between osteoblasts and osteoclasts, with a large loss of sclerotin, eventually causing osteoporosis. Many evidence suggests that the epigenetic modifications can be applied to explain the potential mechanism of association between genetic and environmental factors with osteoporosis and increased

risk of fracture, and therefore, the exploration of epigenetic inheritance for this process is expected to be a key in the treatment of osteoporosis[7,8].

Sirtuin 1 (SIRT1) interacts with proteins such as transcription factors, c-myc, NF-κB, IGFBP1, p300, and p53 in various signal transduction pathways. And it participates in the neuroprotection, cell senescence and apoptosis, glycolipid metabolism, inflammatory oxidative stress response and so on, playing its regulatory function on genes. Studies have confirmed that SIRT1 is an important positive regulator of osteoblast formation and bone mass, which can be activated by kinases such as adenosine monophosphate-activated protein kinase, JNK1 and casein kinase 2, as well as small molecular drugs such as resveratrol[9]. These kinases and drugs affect bone metabolism, and studies have authenticated that SIRT1 signaling pathway can also directly regulate bone metabolism, but the specific regulatory mechanism remains unclear. Therefore, our study took SIRT1 as the entry point of the osteogenic differentiation regulatory pathway, in order to explore and improve the regulatory mechanism of SIRT1 on osteogenic differentiation.

In order to explore the regulatory mechanism of SIRT1 on osteogenic differentiation, our research focused on the epigenetic regulators that play important roles in gene expression, biological life processes and bone differentiation of cells, including microRNAs (miRNAs) and long non-coding RNAs (LncRNAs)[10]. Among them, miRNA is widely studied, as a class of non-coding single-stranded RNA molecules with the length as about 22 noncoding single-stranded RNA molecules of ribonucleic acid. It participates in the occurrence and development of many diseases by complementing the untranslated region of target gene mRNA at the post-transcriptional level, combined with the inhibition of target gene expression[11]. Reportedly, some miRNAs can be involved in bone formation, bone development, and then participate in the occurrence and development of bone related diseases. For example, some researchers have found that knockdown miR-185 can regulate BMP/Smad pathways, promote osteogenic differentiation, inhibit bone loss in patients with osteoporosis[12]. miR-103a and miR-660 are related to bone structure in patients with osteoporosis[13]. miR-204 is involved in Runt transcription-related factor 2 (Runx2) / ALP/BMP2 signaling pathways, which regulates the differentiation of BMSCs, inhibit osteoporosis caused by the imbalance due to osteogenic differentiation of BMSC[14].

As studies on LncRNA continue, LncRNA TUG1 may negatively regulate miR-204 to reduce inflammation and insulin resistance by promoting SIRT1 pathway has been revealed. Other studies have proposed the role of Lnc RNA TUG1 in promoting osteoblast proliferation and differentiation by luohan V[15]. However, specific mechanism of LncRNA TUG on osteogenic differentiation of BMSCs has not been elucidated, and its downstream targets need to be further verified.

Hence, based on the above theoretical basis, this study revealed that the regulatory function of LncRNA TUG/miRNA -204/SIRT1 during osteogenic differentiation may contribute to identifying potential therapeutic targets for osteoporosis. Therefore, according to the above theoretical basis, this study aims to explain the regulatory function of LncRNA TUG/miRNA-204/SIRT1 pathway in osteogenic differentiation of BMSCs, with the specific experimental schemes as follows:

Details of experimental methods

BMSCs cultivation

BMSCs were purchased from Procell, China, which were cultured with a special complete medium (Procell, Wuhan, China) to human BMSCs containing 10% FBS, mesenchymal stem cell growth additive, 1% glutamine and 1% double antibody. Then, the cells were cultivated in the incubator (Thermo, CA, USA) with 5%CO_2 at 37°C[16].

Induction in osteogenic differentiation of BMSCs

Human BMSCs (Catalogue Number HUXMA-01001; Cyagen Biosciences, CA, USA) were cultured in the humid atmosphere containing 10% FBS (Gibco, Thermo Fisher Scientific, Inc, CA, USA), DMEM of 100U/ml penicillin and 100U/ml streptomycin (HyClone, UT, USA), with 5% CO_2, at 37°C. To induce osteogenic differentiation, the cells were cultured in DMEM containing 10% FBS, dexamethasone (100 nM; Sigma-Aldrich, MO, USA), with ascorbic acid 2-phosphate (200 μM; Sigma-Aldrich, MO, USA) and β-glycerophosphate (10 mM; Sigma-Aldrich, MO, USA) at 37°C for 15 days, changing the culture media every three days[17].

Prediction of potential binding sites

Potential binding sites between miR-204 and TUG1, miR-204 and SIRT1 were predicted with network tools, such as StarBase (http://starbase.sysu.edu.cn/index.php) and Targetscan (http://www.targetscan.org/vert_72/)[18].

Determination of luciferase activity

The luciferase kit was purchased from Beyotime Biological Co., Ltd (Nanjing, China). BMSCs were inoculated into a 24-well plate, which were transfected with calcium phosphate after 24 h, to transfect all required DNA. After abandoning the culture, the cells were washed with PBS once, added 120 μL lysate to each hole, placed at 4°C for 10 min, with centrifugation at 13000 rpm for 5 min, to take the supernatant. 20 μL lysate was mixed with 10 μL luciferase test reagent well, which was immediately detected for fluorescent signals with sepctrafluor plus, to analyze the relative activity of luciferase after fluorescence calibration[19].

Detection of alkaline phosphatase (ALP) activity

ALP kit was purchased at Beyotime Biological Co., Ltd. (Nanjing, China). 100 μL substrate buffer and 20 μL samples were added to a 96-well microporous plate, incubated at 37°C for 15 min after fully oscillating on the
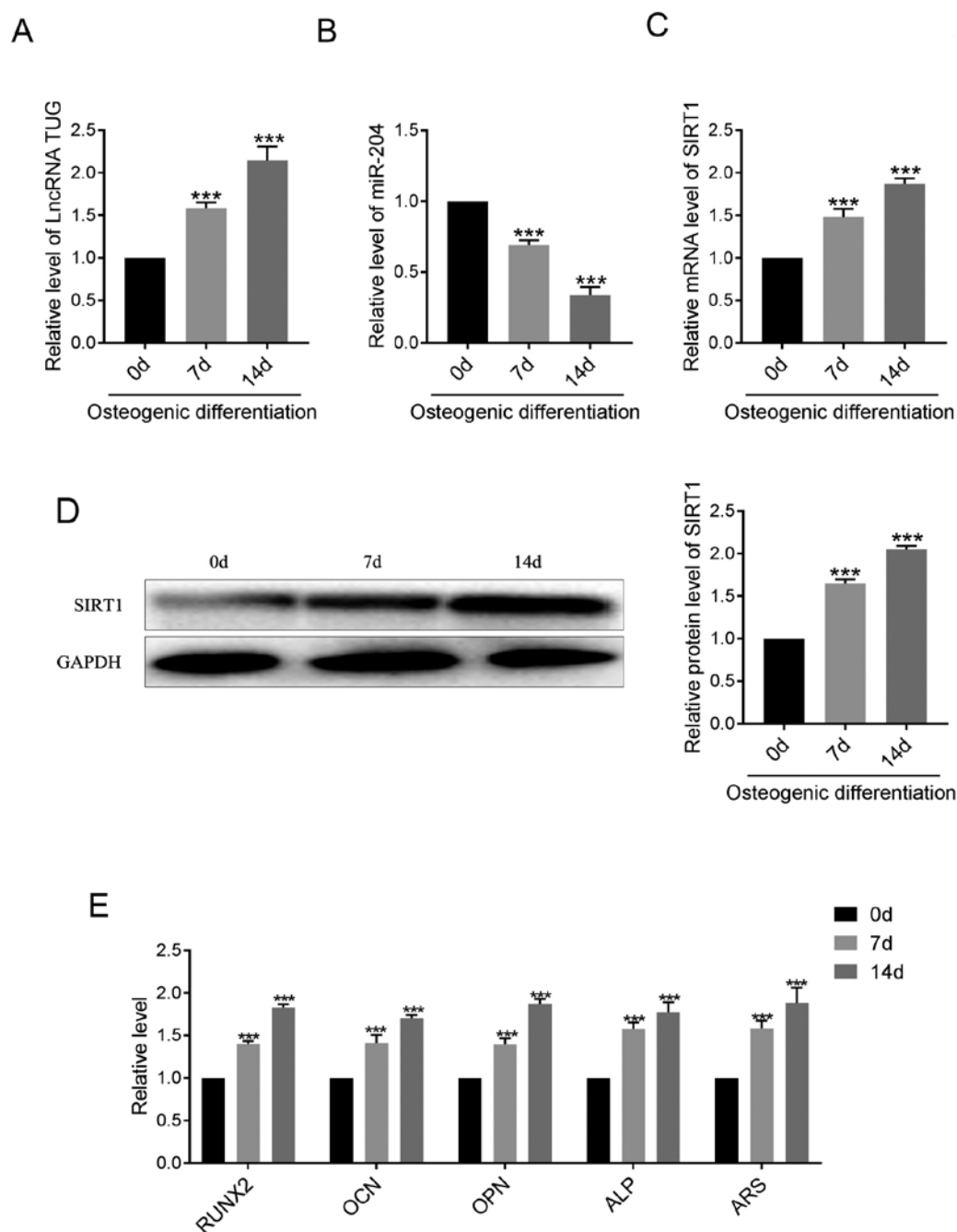
Figure 1. Changes of LncRNA TUG, miR-204 and SIRT1 after osteogenic differentiation of BMSCs. A: 0d was used as a comparison control. Changes of markers, such as Runx2, OCN, OPN, ALP and ARS, for osteogenic differentiation of BMSCs detected before induction (0d), the 7th day after induction (7d), and the 14th day after induction (14d), n=3, ***P<0.001; B-E: 0d was used as a comparison control. Changes of LncRNA TUG, miR-204 and SIRT1 mRNA levels before and after osteogenic differentiation detected with real-time PCR; Changes of SIRT1 proteins detected with Western blot methods, n=3, ***P<0.001.

microplate vibrator for 1 min. 80 μL reaction termination solution was added, and measured with enzyme labeling instrument for absorption values at 405 nm after fully oscillating on the microplate vibrator for 1 min. The detection of blank control and standards were carried out with the same method[20].

Alizarin red staining (ARS)

ARS kit was purchased from Beyotime Biological Co., Ltd. (Nanjing, China). BMSCs were aspirated the culture solution, and washed with PBS three times. 1 ml 10% CPC (10mM Na2HPO4, prepared pH 7.0 10% CPC) was
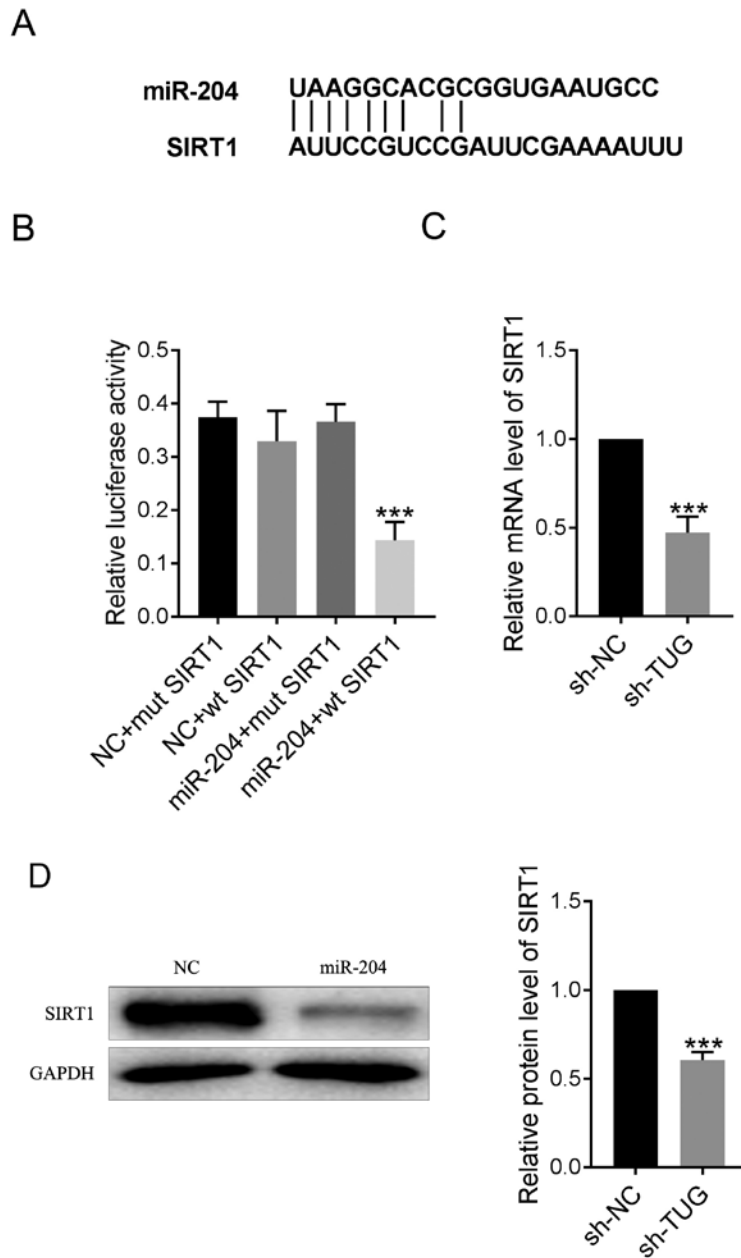
Figure 2. Direct regulation of SIRT1 expression by miR-204 in BMSCs. A: Sites of interaction between miR-204 and SIRT1 mRNA predicted with the bioinformatics tool STARBASE; B: Targeted regulation effect of miR-204 on SIRT1 mRNA in BMSCs and the sites with interaction detected with the luciferase reporter, n=3, ***P<0.001; C, D: Changes of SIRT1 mRNA and protein levels detected with real-time PCR and Western blot, n=3, ***P<0.001.

added to each hole of a 6-hole plate, shook on the shaker for 15 min to 1 h. The eluted CPC was transferred into the EP tube. The cells were diluted 20 times with 10% CPC, and determined with an enzyme marker at 562 nm of wavelength, with 10% CPC to zero. When the measured value is between 0-1.8, it shows a better linear, and the standard curve can be drawn[21].

RNA extraction and qPCR amplification

According to previous studies, total RNAs were extracted from cells with TRIzol reagent (Invitrogen, CA, USA), and were also extracted from chondrocytes with TRIzol reagent. High volume cDNA reverse transcription kit (Applied Biosystems, CA, USA; Cargo number: 4368814) was also adopted according to the instructions. The levels of LncRNA TUG1,

miR-204, SIRT1, Runx2, osteopontin (OPN), osteocalcin (OCN), GAPDH and U6 were determined with SYBR Green and ABI 7500 fast real-time PCR system (Applied Biosystems, CA, USA). GAPDH and U6 were taken as internal controls. All experiments were repeated three times[22].

Western blot analysis

Protein extraction and Western blot analysis were performed referring to the literature step by step. Antibody: SIRT1, Runx2, OPN, OCN (1:1000, diluted, ab109520, abcam, USA), GAPDH (1:1000, diluted, ab8245, abcam, USA). The polyclonal goat anti-rabbit antibody (Cell Signaling Technology, MA, USA) and Western blotting detection system (Odyssey, LI-COR, LN, USA) were used for detection[22].

Cellular transfection

BMSCs were inoculated on the 6-hole plate with a inoculation density of 2×10^6 cells/hole. Before transfection, serum-free culture media was used to culture for 24 h. Lipofect 2000 transfection reagent was used to transfect 100nM pGL6-NC, pGL6-TUG1, sh-NC, sh-TUG1, miR-204-NC, miR204, AMO-204, AMO-NC (Santa Cruz technology, TX, USA; Cargo Number: sc-29428, USA), the transfection was operated according to the product instruction. After 6 h of transfection, the transfected culture media was replaced with the conventional culture media. After 48 h of transfection, the cells were harvested for *in vitro* experimental[23].

Statistical analysis

Data were analyzed with GraphPad Prism 7.0 software. All results were expressed as the mean ± Standard Error of the Mean (SEM). The statistical comparison was conducted by t test or one-way ANOVA between the two groups, and the statistically significant difference was set to $P<0.05$. "Relative level" means paired-comparison of each parallel group in the following results.

Results

Changes of LncRNA TUG, miR-204 and SIRT1 after osteogenic differentiation of BMSCs

In order to study the osteogenic differentiation of BMSCs in osteoporosis, we induced osteogenic differentiation of BMSCs, and detected the changes of markers, such as the changes of Runx2, OCN, OPN, ALP and ARS in osteogenic differentiation. Figure 1A demonstrates the establishment of the induction on osteogenic differentiation of BMSCs in this study. The changes of LncRNA TUG, miR-204 and SIRT1 expression were detected before osteogenic differentiation of BMSCs and on the 7th and 14th days after osteogenic differentiation of BMSCs with real-time PCR, Western blot and other methods. As shown in Figure 1B-E, LncRNA TUG1 expression was continuously up-regulated and miR-204 was gradually down-regulated with the induction of osteogenic differentiation, while the levels of SIRT1 mRNA and proteins were gradually up-regulated, suggesting a correlation between LncRNA TUG, miR-204 and SIRT1 with osteogenic differentiation of BMSCs.
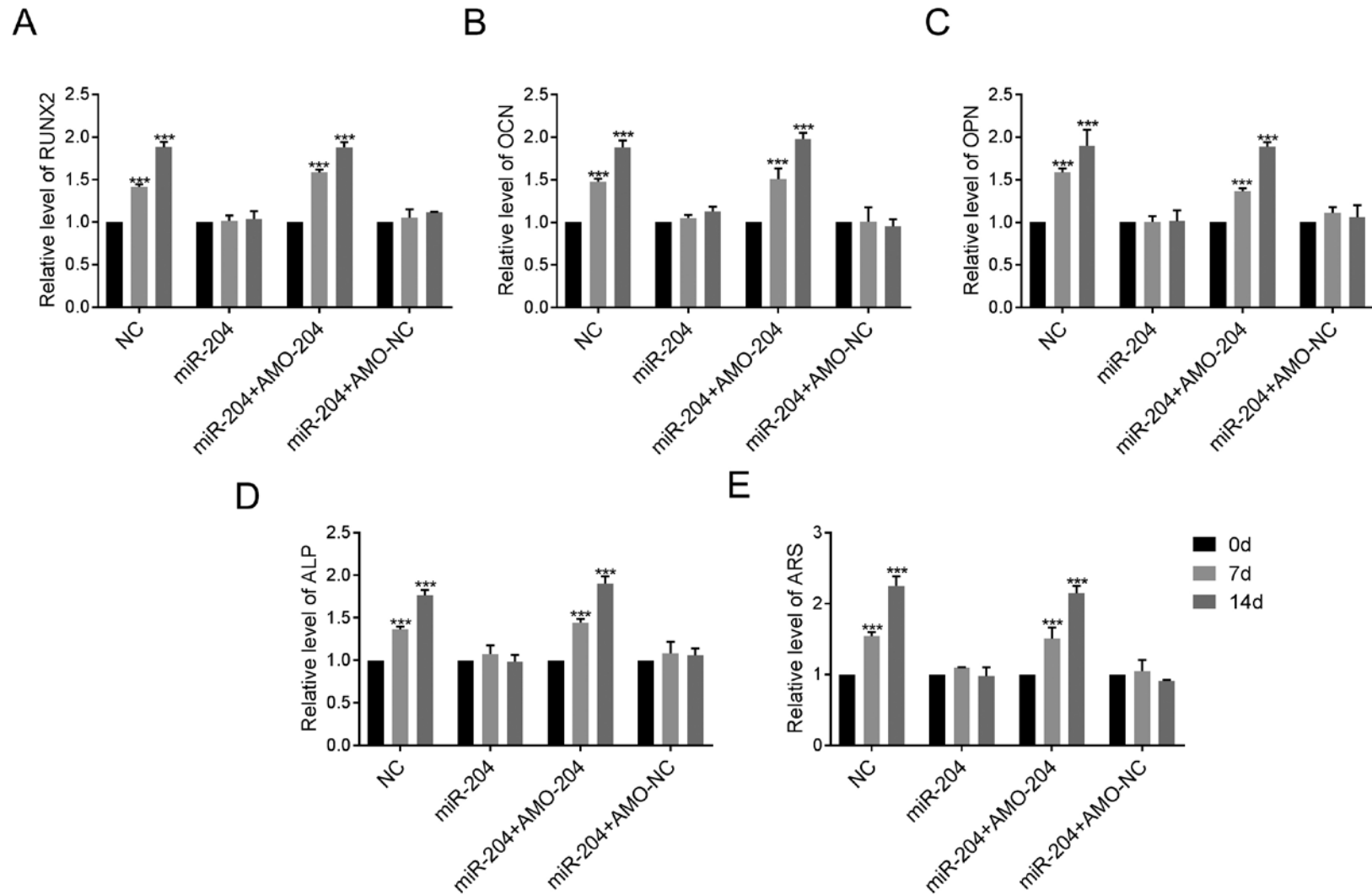
miR-204/SIRT1 regulating osteogenic differentiation in BMSCs

The sites where miR-204 interacted with SIRT1 mRNA were predicted with bioinformatics tool STARBASE (Figure 2A), so we're assuming that MiR-204 affects the process of osteogenic differentiation of BMSCs by regulating the transcription of SIRT1. To test this hypothesis, we first explored whether it has a direct target with double luciferase reporter. As shown in Figure 2B, through the analysis of double luciferase activity, miR-204 overexpression can significantly reduce SIRT1-WT activity compared with its negative control group (NC), but overexpression of miR-204 mimics has no significant effect on SIRT1-MUT activity. Meanwhile, overexpression of miR-204 mimics can also significantly inhibit the expression of SIRT1 mRNA and proteins (Figure 2C-D). These results suggest that SIRT1 is a direct target of miR-204, and may be negatively regulated.

To verify miR-204/SIRT1 regulation on osteogenic differentiation of BMSCs, we transfected miR-204, miR-204+AMO-204 in BMSCs, and compared them with negative control group as a reference. As shown in Figure 3, 7 and 14 days after the introducing of osteogenic differentiation, miR-204 significantly inhibited the expression of SIRT1, Runx2, OPN and OCN, and the levels of ALP and ARS, while the inhibitor AMO-204 increased the protein levels of these osteogenic differentiation markers, neutralized the effect of miR-204 on osteogenic differentiation, indicating that miR-204 is indeed the upstream of SIRT1, which can mediate the regulation on osteogenic differentiation of BMSCs.

Direct regulatory relationship between LncRNA TUG and miR-204 in BMSCs

Similarly, the sites where LncRNA TUG interacted with miR-204 were predicted with bioinformatics tool StarBase (Figure 4A), and the interaction between the two was verified by luciferase reporter assay (Figure 4B). The effects of knockdown LncRNA TUG expression in BMSC on osteogenic differentiation were explored through transfecting shRNA or its negative control shRNA (sh-NC) of LncRNA TUG. As shown in Figure 4C, after sh-TUG, miR-204 was up-regulated, with down-regulation of SIRT1 expression. The expression of osteogenic differentiation markers, such as Runx2, OCN and OPN decreased significantly, the levels of ALP and ARS were significantly down-regulated. To verify LncRNA/miR-204 regulation on osteogenic differentiation of BMSCs, we transfected miR-204 analogues and LncRNA overexpression plasmids at the same time. As shown in Figure 4D, LncRNA TUG and miR-204 were co-transfected, which hindered miR-204 regulation on osteogenic differentiation of BMSCs, suggesting that LncRNA TUG is really a upstream regulator of miR-204 in BMSCs.

Figure 3. Osteogenic differentiation of BMSCs mediated by miR-204. Regulation of BMSCs before and after osteogenic differentiation explored with Western blot, ALP and ARS methods, n=3, *** P=0.001.
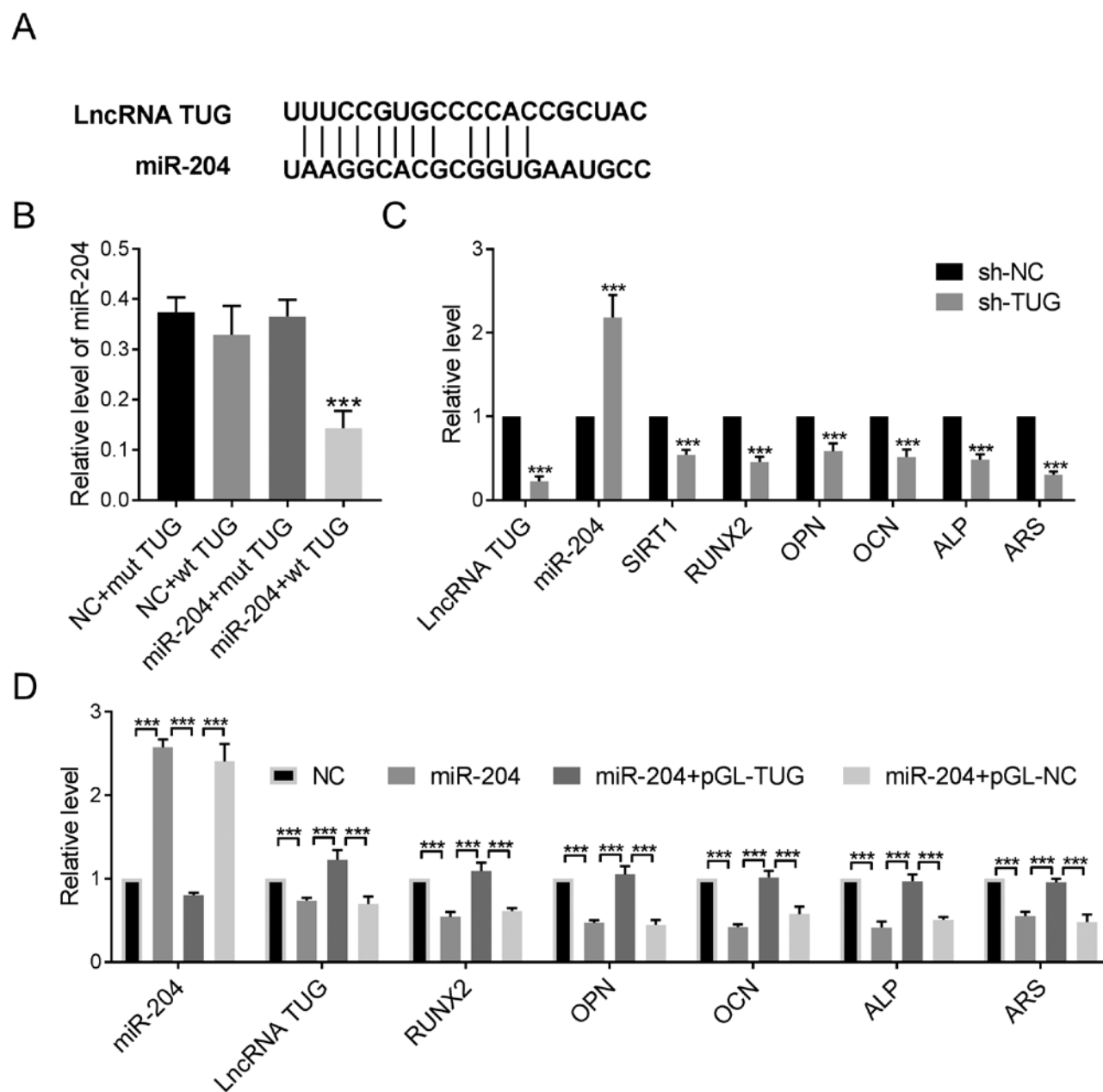
Figure 4. LncRNA/miR-2O4 regulating on osteogenic differentiation of BMSCs. A: Sites where LncRNA TUG interacted with miR-2O4 were predicted with the bioinformatics tool StarBase; B: Targeted regulation effect of miR-2O4 on LncRNA TUG in BMSCs and the sites with interaction detected with the luciferase reporter, n=3, ***P<0.OO1; C: sh-NC was used as a comparison control. Effects of shRNA of LncRNA TUG on miR-2O4, SIRT1, and markers of osteogenic differentiation detected with real-time PCR, Western blot, ALP and ARS methods, n=3, ***P<0.OO1; D: NC was used as a comparison control. Changes in miR-2O4, SIRT1, and markers of osteogenic differentiation detected with real-time PCR, Western blot, ALP and ARS methods, n=3, ***P<0.OO1.

As shown above, our study verified the regulatory relationship of LncRNA/miR-2O4/SIRT1 in BMSCs, clarified the effects of this pathway on osteogenic differentiation of BMSCs, and revealed the important mechanism of LncRNA-miRNA-mRNA in osteoporosis, which provides a new research theory for the basic research in this field and a new therapeutic target and intervention mechanism for the treatment of osteoporosis.

Discussion

In this study, we had three findings: 1) During the osteogenic differentiation of BMSCs, the expression of LncRNA TUG1 was up-regulated, the expression of miR-204 was down-regulated, and the expression of SIRT1 was up-regulated; 2) LncRNA TUG1 interacted with miR-204 and negatively regulated the expression of miR-204; miR-204 specifically bound to SIRT1 and negatively regulates the expression of SIRT1; 3) LncRNA TUG1 promoted osteogenic differentiation by regulating the expression of miR-204/SIRT1, confirming the role of LncRNA TUG1/miR-204/SIRT1 pathway in the osteogenic differentiation of BMSCs.

Bone marrow mesenchymal stem cells, BMSCs, are multifunctional cells that can differentiate into different types of connective tissue cells, namely osteoblasts, chondrocytes, adipocytes, myoblasts and so on. BMSCs can be used for tissue engineering repair or replacement of tissues and organs, which can be differentiated into adipogenic, cartilaginous, myogenic, neurogenic and osteoblast lineage pathways. *In vitro* osteoblast differentiation of BMSCs is crucial for the progress in bone regeneration and osteoporosis. Adipocytes in bone marrow increase in age-related osteoporosis. As we all know, osteoporosis is associated with postmenopausal estrogen deficiency, which is one of the most common causes of age-related bone loss. Hormone replacement therapy (HRT) can inhibit endocrine-deficient postmenopausal osteoporosis and reduce the incidence of fractures, but adverse side effects of these drugs have recently been identified. HRT can increase the risk of breast cancer and endometrial cancer, accompanied by other adverse side effects, including fluid retention, headache, mood swings, and depression, significantly reducing women's quality of life. Therefore, more effective, safer and selective treatment strategies are needed to osteoporosis. Hence, the research on the pathogenesis of osteoporosis lays a theoretical foundation for the treatment of osteoporosis[25-29].

To date, LncRNAs and miRNAs are considered to play a key regulatory function in the biological process of many diseases, which may have good diagnostic, therapeutic and prognostic value for various chronic metabolic diseases. Non-coding RNA is a key regulator of osteogenic induction of stem cells[30,31]. There is growing evidence that LncRNA can combine specific miRNA, further give play to its regulatory function by combining targeted mRNA. Some studies have reported that LncRNA-miRNA-mRNA regulatory pathways can regulate osteogenic differentiation, which in turn affects many orthopaedic diseases including osteoporosis[32,33]. MicroRNAs (miRNAs) are approximately 22 nucleotides long, which play a role in gene regulation during posttranscriptional regulation. Recently, a report indicated that miR-214 can regulate osteogenic differentiation and bone formation, and high levels of miR-214 are associated with low bone formation. Moreover, MiR-214 can inhibit osteoblastic function by targeting ATF4[34]. MiR-204 can also be up-regulated during osteoclast formation, and promote osteoclast formation.

In addition, miR-204 can also attenuate osteogenic differentiation of mesenchymal stem cells[35,36]. Our study predicted a binding site between miR-204 and SIRT1 by bioinformatics. Besides, there has been no report elucidating miR-204 regulation on osteogenic differentiation of BMSCs through direct regulation of the signaling pathway. Long non-coding RNAs (LncRNAs) are over 200 nucleotides long, which are involved in multiple cellular processes, and can regulate osteogenic differentiation that has been reported in other studies. LncRNA TUG is an antisense LncRNA on human TUG sites to promote epithelial cell proliferation and epithelial-mesenchymal transition that has been reported as well. It also mediates the growth of hepatocellular carcinoma as competing endogenous RNA (ceRNA)[37]. Recently, there have also been reports showing that it can promote osteogenic differentiation by activating Wnt/β-catenin signaling pathways to alleviate osteolytic[38]. Through literature and bioinformatics analysis, the direct interaction between miR-204 and LncRNA TUG can be inferred. However, whether TUG can regulate osteogenic differentiation by neutralizing miR-204 has not been reported, which need to be further clarified.

SIRT1 silencing information regulator 1 is a highly conservative NAD$^+$ dependent protein deacetylases, also known as anti-aging enzyme. SIRT1 can interact with forkhead-box transcription factors (FOXO) 1/3/4, c-myc, NF-κB, IGFBP1, p300, p53 and other proteins, which is involved in neuroprotection, cell senescence, apoptosis, glycolipid metabolism, inflammatory oxidative stress, acting with its regulatory function on genes. Given above functions, SIRT1 has attracted wide attention of researchers in various disciplines. In addition to being widespread in mature tissues, SIRT expresses high in fetal brain tissues and adult brain tissues, skeletal muscle, kidney and heart. Because of the differences of cells in the organism, SIRT1 location is different, some only express in the nucleus, some only express in the cytoplasm, and some express in both of them. SIRT1 plays an important role in maintaining genome stability, repairing DNA damage, regulating mitosis, antioxidant stress, anti-inflammatory response, anti-apoptosis and other life activities. It can interact with different substrates, playing different biological functions. Research indicates that SIRT1 can inhibit the cell death induction mechanism and promote cell survival by enhancing the expression of FOXO target genes and changing the cell cycle retention or arrest in the process of anti-oxidative stress. SIRT1 either directly or indirectly regulates the cell cycle, indicating that SIRT1 is involved in the cell development cycle, as a certain role. Studies have found that SIRT1 can regulate insulin secretion and indirectly regulate glucose and lipid metabolism by directly binding to the decoupling protein promoter gene and inhibiting UCP2 expression. SIRT1 protein activity is known to be modulated by resveratrol and nicotinamide, which activate and inhibit SIRT1, respectively, and SIRT1 activation reduces adipocyte formation during mesenchymal stem cell osteoblast

differentiation. In previous studies, treatment with SIRT1 inhibitor nicotinamide down-regulated bone-specific matrix compounds, suggesting that SIRT1 seems to be a regulator of mesenchymal stem cell differentiation into osteoblasts. Additionally, nicotinamide-treated pre-osteoblasts down-regulated bone-specific matrix components, with apoptosis, while activating SIRT1 can reduce adipocyte differentiation, and increase osteoblast differentiation. This differentiation is accompanied by the expression of the osteoblastic transcription factor Runx2, which leads to early initiation of osteoblast differentiation procedures. SIRT1 can promote osteoblast differentiation by directly regulating Runx2 and other factors, as well as the NCoR and PPAR-c of nuclear receptor co-inhibitors[39-44]. Even so, for osteogenic differentiation, the upstream regulation network of SIRT1 is still incomplete, there are still a lot of research vacancies. Consequently, the exploration of key regulatory factors upstream of SIRT1 is the theoretical basis for perfecting SIRT1 as a potential therapeutic target for osteoporosis.

Through the study, we determined the regulatory role of SIRT1 in the process of osteogenic differentiation, and further improved the regulatory network of SIRT1-related osteogenic differentiation process. Our study complemented the upstream regulatory pathway of SIRT1, an important regulator of bone differentiation, and validated the effect of disrupting this regulatory pathway on bone differentiation. As shown in result section 4, exogenous interference upstream of SIRT1 can significantly affect the osteogenesis regulation of BMSCs, suggesting that the targeted regulation of LncRNA TUG or/and miR-204 can be a potential target for future clinical treatment.

In addition, based on our research, we can also provide bolder inspiration for future research directions: 1) Whether the treatment methods for non-orthopaedic diseases that can affect LncRNA TUG or/and miR-204 can affect the related diseases of abnormal osteogenic differentiation; 2) Can the tissue or serum levels of LncRNA TUG and miR-204 be used as diagnostic criteria to indicate disease progression? Joint exploration and clarification are still needed.

Authors' contributions

XO designed the study and drafted the manuscript. YD and LY were responsible for the collection and analysis of the experimental data. FX and XY revised the manuscript critically for important intellectual content. All authors read and approved the final manuscript.

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