Long non-coding RNA taurine upregulated gene 1 is downregulated in osteoporosis and influences the osteogenic differentiation of bone marrow mesenchymal stem cells

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ABSTRACT

Background. With aging, an imbalance in bone remodeling leading to increased bone resorption and decreased bone formation is thought to contribute to osteoporosis. Osteoblastic differentiation of bone marrow mesenchymal stem cells (BMMSCs) plays a vital role in the pathogenesis of osteoporosis. However, the detailed molecular mechanisms of osteoporosis remain incompletely understood. Given that long non-coding RNA taurine upregulated gene 1 (lnc TUG1) plays a critical role in the osteogenic differentiation, and microRNA-23b (miR-23b) as a putative sponge for lnc TUG1 has upregulated expression in osteoporosis. Therefore, this study investigated the roles of TUG1/miR-23b in osteoporotic pathology.

Material and Methods. TUG1 and miR-23b expression in the plasma of osteoporotic patients were evaluated by quantitative real-time PCR (qRT-PCR). The osteogenic differentiation in human BMMSCs was evaluated by qRT-PCR, western blot, Alizarin red staining after knockdown of TUG1 by small interfering RNA (siRNA) treatment.

Results. Decreased expression of TUG1 and increased expression of miR-23b evident in the plasma of patients with osteoporosis than in that of age- and sex-matched healthy controls. Additionally, increased miR-23b expression inhibited runt-related transcription factor 2 (RUNX2), osteocalcin, and osteopontin expression and reduced calcified nodule formation based on the results of qRT-PCR, western blot, and Alizarin Red S staining.

Conclusion. The study for the first time reported that silence of lncRNA TUG1 significantly suppressed the osteogenic differentiation of BMMSCs possibly by targeting the miR-23b/RUNX2 signaling pathway. This mechanism of TUG1/miR-23b/RUNX2 signaling within the osteogenic differentiation of BMMSCs might provide new insight for the development of lncRNA-directed diagnostic and therapeutic strategies for osteoporosis.

Keywords Osteoporosis, LncRNA TUG1, miR-23b, Osteogenic differentiation, TUG1/miR-23b/RUNX2 signaling pathway

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INTRODUCTION

The reductions in bone mass and structural integrity that occur in osteoporosis cause the bones to become fragile and more susceptible to fracture (Kanis, 1994). Accordingly, approximately 8.9 million osteoporotic fractures were suffered by the estimated 200 million elderly women with osteoporosis in 2006 and 2007 (Johnell & Kanis, 2006). The ever-increasing incidence of osteoporosis-related fractures has serious negative impacts on the health and quality of life of the elderly population, particularly elderly women, and consequently, increases the burden on public health services. Pathogenesis of osteoporosis mainly involves disruption of the coordinated balance between the bone resorption activity of osteoclasts and bone formation by osteoblasts (Manolagas, 2000; Moerman et al., 2004). An additional mechanism involves the bone marrow mesenchymal stem cells (BMMSCs), which can differentiate into osteoblasts, chondrocytes, myocytes, or adipocytes. Multiple studies have demonstrated that alteration of the reciprocal balance between the adipogenic and osteogenic differentiation of BMMSCs is a key pathogenic driver of osteoporosis (Zhou et al., 2008; Yang et al., 2018). BMMSCs from 26-month-old mice (old mice) showed a significantly reduced ability for osteogenic differentiation, as well as reduced osteoblast numbers and low bone mineral density (BMD) (Moerman et al., 2004). A clinical study by Shen et al. (2012) revealed an inverse relationship between the amount of bone marrow adipose tissue and BMD, suggesting that adipose tissue accumulation in bone marrow at the expense of osteoblast formation is responsible for low BMD (Shen et al., 2012). These findings all imply that the imbalance between the adipogenic differentiation and osteogenic differentiation of BMMSCs plays a crucial role in elderly development of osteoporosis. The weakened osteogenic differentiation ability of BMMSCs may responsible for osteoporosis in the elderly.

Long non-coding RNAs (lncRNAs) are RNAs that exceed 200 nucleotides in length and lack significant open reading frames. LncRNAs are involved in various stages of cellular development and differentiation, and previous studies have identified certain lncRNAs involved in the regulation of osteogenesis by BMMSCs (Song et al., 2015; Wang et al., 2015). The results of these studies indicate that dysregulation of lncRNAs could be associated with the development and progression of disease. Taurine up-regulated gene 1 (TUG1) is a lncRNA with 6.7 kb nucleotides that is located at chromosome 22q12 and was first shown to contribute to photoreceptor development in the rodent retina (Yu et al., 2018). Increasing evidence indicates that lncRNAs act as a ‘sponge’ to titrate microRNAs (miRNAs) and thereby participate in post-transcriptional processing. Cai et al. (2017) reported that TUG1 inhibited miR-299, leading to enhanced tumor-induced angiogenesis in human glioblastoma (Cai et al., 2017). Additionally, Katsushima et al. (2016) showed that TUG1 bound miR-145 in the cytoplasm of glioma cells (Katsushima et al., 2016). Recently, the study found that knockdown of TUG1 significantly inhibits osteoclast formation in the context of calcific aortic valve disease while TUG1 enhanced osteogenic differentiation via bounding miR-204-5p (Yu et al., 2018). In addition, He et al. (2018) demonstrated that TUG1 expression enhanced the osteogenic differentiation of periodontal ligament stem cells via activation of homolog A (He et al., 2018). These findings indicate that TUG1 plays...
a critical role in the osteogenic differentiation of stem cells. However, whether TUG1 acts as a molecular sponge for miRNAs in the context of osteoporosis has yet to be established, prompting us to investigate the interaction between TUG1 and miRNAs in osteoporosis. The previous study showed up-regulated expression of miR-23b in osteoporosis (Chen et al., 2016). Deng et al. (2017) revealed that miR-23b was involved in TNF-α-mediated reduction of BMSC osteogenesis by targeting runt-related transcription factor 2 (RUNX2). Moreover, TUG1 was predicted to bind to miR-23b by bioinformatics software (Rnahybrid: https://bibiserv.ccbio.uni-bielefeld.de/rnahybrid/). Therefore, the aim of this study was to analyze the involvement of TUG1 in osteoporosis and to explore the interaction between TUG1 and miR-23b in the osteogenic differentiation of human BMMSCs. We found that IncRNA TUG1 was significantly reduced in patients with osteoporosis compared with sex- and age-matched healthy controls. Subsequent functional studies showed that knockdown of TUG1 leads to suppression of osteogenic differentiation of hBMMSCs. Mechanistically, miR-23b inhibited osteoblastic differentiation of hBMMSCs by directly suppressing the translation of RUNX2. TUG1 could act as a molecular sponge for miR-23b. This study of the biological role of TUG1/miR-23b/RUNX2 signaling in the osteogenic differentiation of hBMMSCs may provide insight for the development of IncRNA-directed therapeutics in osteoporosis. TUG1 could inhibit miR-23b, which resulted in the suppression of RUNX2 might be reversed and would promote the osteogenic differentiation of hBMMSCs.

**METHODS AND MATERIALS**

**Clinical samples**

Blood samples (5 ml) were extracted from 28 female osteoporosis patients and 25 sex- and age-matched healthy controls who were recruited at The Sixth Affiliated Hospital of Kunming Medical University from 2018–2019. Osteoporosis was diagnosed by dual-energy X-ray absorptiometry (T score ≤−2.5 standard deviation). The inclusion criteria were: (1) having been in menopause for >2 years and diagnosed with osteoporosis; (2) availability of complete medical records; and 3) well acquainted with the experimental procedure and willing to participate. Patients were excluded if they: (1) had a history of abnormal metabolism disease or multiple other diseases; (2) had previously used bisphosphonate, teriparatide, denosumab, hormonal therapy, or thyroxine; or (3) failed to cooperate with researchers. This study was written approved by the ethics committee of Yuxi Municipal People’s Hospital (Approval number: 2019kmykdx6h62). All study participants signed the informed consent form.

**Cell culture**

hBMMCSs (Procell Life Science & Technology Co., Ltd., China) were seeded at a density of 3×10^5 cells per 100-mm plastic dish and maintained in complete Dulbecco’s Modified Eagle’s Medium with low glucose (DMEM; Procell Life Science&Technology Co., Ltd.) containing 10% fetal bovine serum (FBS; Gibco, Thermo Fisher Scientific Inc., MA, USA) and 100 U/ml penicillin/streptomycin/neomycin (PSN; Gibco, Thermo Fisher Scientific Inc.) at 37 °C in a 5% CO₂ humidified atmosphere. And the medium was changed every
other 4 days. To stimulate osteogenic differentiation, the cells were cultured in osteogenic induction medium containing 10% fetal bovine serum, 100U/ml PSN, 50 µg/mL ascorbic acid (Merck, Germany), 5 mmol/L β-glycerophosphate (Sigma Co., St. Louis, MO, USA) and 10 nmol/L dexamethasone (Sigma Co.). Similarly, the osteogenic induction medium was changed every other 4 days.

**Knockdown of TUG1 expression via RNA interference**

LncRNA TUG1-specific small interfering RNA (siRNA) and non-specific siRNA (siRNA-NC) were synthesized by Genecreate (Wuhan Genecreate Biological Engineering Co., Ltd., China) using the previously published primer sets (Jiang et al., 2017). The sequences of the three TUG1 siRNAs were as follows: siTUG1-1: CAGUCCUGUGAUUUAGACGUU; siTUG1-2: CCCAGAAGUUGUAAGUUCACCUUGA; siTUG1-3: CAGCUGUAACAUCAUCUUUA; and siRNA-NC: UUCUCCGAACGUGACGUACGUTT. hBMSCs were transiently transfected with each siRNA using the Lipofectamine 2000 Reagent Kit (Invitrogen; Thermo Fisher Scientific Inc.) following the manufacturer’s instructions. After 24 h, the cells were harvested to determine the knockdown efficiency of each siRNA via quantitative real-time polymerase chain reaction (PCR).

**Cell transfection**

si-TUG1 and miR-23b mimics, as well as their corresponding negative controls (NCs) including miR-23b mimic NC and siRNA NC, were purchased from Wuhan Genecreate Biological Engineering Co., Ltd. hBMSCs in 6-well plates were transfected with 50 pmol si-TUG1, 50 nM miR-23b, or their NCs using Lipofectamine 2000 according to the manufacturer’s instructions. The transfected cells were subsequently maintained for an additional 48 h in complete DMEM prior to the induction of osteogenic differentiation. The sequences for siTUG1 and siNC were presented above, and those for the miR-23b mimic and miR-23b NC were: 5′-AUCACAUUGCCAGGGAUACCAC-3′ and 5′-UUCUCCGAACGUGUCACGUTT-3′, respectively. The efficiency of transfection was confirmed by real-time PCR.

**RNA extraction and quantitative real-time PCR**

Total RNA was extracted from sample blood and cultured cells using TRIzol reagent (Invitrogen, Thermo Fisher Scientific Inc.) according to the manufacturer’s instructions. Then 1 µg of total RNA was reverse transcribed into a final volume of 20 µl using random primers and the RevertRra Ace qRT-PCR Kit (FSQ-101). For quantitative real-time PCR (qRT-PCR) analysis, qRT-PCR was conducted by mixing SYBR Green PCR Master (SYBR Premix Ex Taq TM, TaKaRa, Japan) with 1 µl of the complementary DNA and primers, and the analysis was carried out using the ABI 7500 RT-PCR system (Applied Biosystems, Foster City, CA, USA) according to the manufacturer’s instructions. The results were analyzed using the 2−ΔΔCt method and normalized to the expression level of glyceraldehyde-3-phosphate dehydrogenase (GAPDH). The primer sequences for these genes are presented in Table 1.
Table 1  Sequences of gene primers used for quantitative real-time RT-PCR analyses.

| Gene   | Primer sequence (5′–3′) |
|--------|-------------------------|
| mir-23b| F:CTCAACTGGTGTCGTGGAGTCGGCAATTCAGTTGAGGTGGTAAT  
          | R:ACACTCCACGCTGGAGATCACATTGCCAGGGAT |
| LncRNA | F:GGATGAAGTAGTGAAGCAAGAGTAGG  
          | R:CGGTGGTAAAGGAAGAGTAGTGTAGC |
| TUG1   | F:TGTCATGGCGGGTAACGAT  
          | R:AAACGGGTATGAGTCAAGGTCGAA |
| RUNX2  | F:GTGCAGAGTCCAGCAAGGTAAT  
          | R:AAGCGGTATAATGGTCAAGGGTAAT |
| OCN    | F:TTGCAGTGACAGCTCTGCACTGAC  
          | R:TCAGCCAACTCGTCACAGTC |
| OPN    | F:GCCACAGCATCTGGGTATTGGTAAT  
          | R:GCCATACGCGCCACAGTTCAC |
| GAPDH  | F:ACACAGGCCTCAAGATCTAGCTAGC  
          | R:GCCATACGCGCCACAGTTCAC |
| U6     | F:CTCGCTTCGGCCAGCAAC  
          | R:AACCGCTAGGAATTGTGGGTC |

Notes.
- mir-23b, microRNA-23b; RUNX2, runt-related transcription factor 2; OCN, osteocalcin; OPN, osteopontin; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; U6, U6 small nuclear 1 (RNU6-1).

Western blot analysis

hBMMSCs were lysed using radioimmunoprecipitation assay (RIPA) protein extraction buffer (Aspen Biopharma Labs, India) supplemented with 10 µl/ml protease inhibitor cocktail (Roche). The concentrations of extracted proteins were quantified using a BCA protein assay kit (Aspen Biopharma Labs), and 40 µg of protein from each sample was fractionated via sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) and transferred to a 0.45-µm polyvinylidene fluoride (PVDF) membrane. The membranes were then incubated with primary antibodies of anti-RUNX2 (dilution of 1:1000, Abcam, ab23981) and anti-GAPDH (dilution of 1:10000, Proteintech, 60004-1-Ig) at 4 °C overnight, followed by reaction with the horseradish peroxidase (HRP)-conjugated secondary antibody (dilution of 1:2000, Proteintech, SA00001-1) at 4 °C for 1 h. Target protein bands were visualized using an enhanced chemiluminescence (ECL) kit (Aspen Biopharma Labs) and exposed to Kodak XBT-1 film.

Alizarin Red S (ARS) staining

The osteogenic differentiation of BMMSCs was evaluated by staining of mineralized matrix nodules with ARS. After hBMMSCs were cultured in the osteogenic induction medium for 14 days, they were fixed with 4% formaldehyde at room temperature for 15 min. After washing two times with phosphate-buffered saline (PBS), the fixed cells were exposed to 0.2% ARS solution (Beijing Solarbio Science and Technology Co., Ltd.) for 5 min, followed by thorough washing with PBS. Stained calcium mineral deposits were visualized and scored under a microscope.
Dual-luciferase reporter assay
hBMMSCs were cultured in 96-well plates for 24 h before transfection with a mixture of miR-23b mimics or miR-NC and TUG1 wild-type or mutant TUG1 fragment using Lipofectamine 2000 according to the manufacturer’s instructions. The mutant TUG1 fragment was constructed such that the amplified full-length TUG1 sequence was cloned into the plasmid vector pGL3-basic. Luciferase activity was determined using a Dual Luciferase Reporter Gene Assay Kit (Beyotime Biotechnology, Co., Ltd., China), and relative luciferase activity was normalized against Renilla luciferase activity.

Statistical analysis
SPSS Statistics software (SPSS Inc., Chicago, IL, USA) was used for all data analysis. Data for continuous variables are expressed as the mean ± standard deviation (SD). Comparisons were performed using Student’s test or the Mann–Whitney test, as appropriate. All hypotheses were two-tailed and \( P \leq 0.05 \) was considered to be significant.

RESULTS
LncRNA TUG1 expression is reduced in osteoporosis patients
To investigate whether lncRNA TUG1 and miR-23b expression were altered in osteoporosis, qRT-PCR was performed to detect TUG1 expression and miR-23b in the plasma of 28 female osteoporotic patients and 25 normal healthy control women matched for age. The ages of patients with osteoporosis ranged from 51–69 years, with a mean age of 60.8 ± 6.86 years. The ages of healthy controls ranged from 47–58 years, with a mean age of 50.1 ± 3.65 years. No significant differences in age, weight, and height were found between the osteoporotic patients and healthy controls. The qRT-PCR results showed that plasma levels of TUG1 expression was significantly lower in osteoporotic patients than in healthy controls, in contrast, miR-23b showed the opposite expression in osteoporotic patients (Fig. 1).

Silencing of LncRNA TUG1 suppresses the osteogenic differentiation of hBMMSCs
The above results indicated that lncRNA TUG1 may have a role in osteoporosis. To further determine the effect of lncRNA TUG1 on the osteogenic differentiation of BMMSCs, we conducted an in vitro loss-of-function experiment in hBMMSCs. We first tested the knockdown efficiency of three TUG1-specific small interfering RNAs (siTUG1-1, siTUG1-2, and siTUG1-3). siTUG1-1 was found to be most efficient and then used in further experiments (Fig. 2A). Subsequently, to investigate whether LncRNA TUG1 played roles in osteogenic phenotype, hBMMSCs were transfected with siTUG1-1 and siRNA-NC before culture in osteogenic medium. The osteogenic phenotype of the hBMMSCs was then evaluated by observing the activity of markers of osteoblastic differentiation (RUNX2, osteocalcin, and osteopontin) and calcified nodule formation. As illustrated in Figs. 2D–2F, silencing of TUG1 with siTUG1-1 in hBMMSCs downregulated the expression of RUNX2, osteocalcin, and osteopontin compared with the expression levels of these markers in hBMMSCs transfected with siRNA-NC. In addition, after knockdown of TUG1, hBMMSCs
LncRNA TUG1 expression was downregulated and miR-23b upregulated in the plasma of patients with osteoporosis compared with levels in sex- and age-matched healthy controls. (A) qRT-PCR results for lncRNA TUG1 expression showed significantly lower expression in osteoporotic patients compared with healthy controls ($p < 0.01$). (B) qRT-PCR results for miR-23b expression showed significantly higher expression in osteoporotic patients compared with healthy controls ($p < 0.01$).

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These results indicated that TUG1 played a supportive role in the osteogenic differentiation of hBMMSCs.

**LncRNA TUG1 interacts directly with miR-23b**

A recent study by Ramirez-Salazar et al. showed that miR-23b was highly expressed in patients with osteoporosis and might serve as a biomarker for the diagnosis of osteoporosis (Ramirez-Salazar et al., 2018). To investigate whether TUG1 directly acted as a molecular sponge for miR-23b, we transfected hBMMSCs with a mutant TUG1 fragment, miR-NC or TUG1 wild-type along with miR-23b mimics using a firefly dual-luciferase reporter system. As shown in Fig. 3, co-transfection of hBMMSCs with miR-23b mimics and TUG1 wild-type reduced the luciferase expression level compared with that in cells co-transfected with TUG1 wild-type and miR-NC, mutant TUG1 and miR-NC, or mutant TUG1 and miR-23b mimics, suggesting that TUG1 directly interacted with miR-23b.

**miR-23b expression reduces the RUNX2 levels in hBMMSCs**

To explore whether miR-23b was involved in the regulation of the osteogenic differentiation of hBMMSCs, cells were transfected with miR-23 mimics or miR-23b NC, and then the intracellular miR-23b content was analyzed by qRT-PCR. As presented in Fig. 4A, transfection with the miR-23b mimics led to a significant increase in the miR-23b level, whereas transfection with miR-23b NC had no significant effect on miR-23b expression. At the same time, the lncRNA TUG1 level was decreased significantly in hBMMSCs transfected with miR-23b mimics compared with that in wild-type cells, suggesting that lncRNA TUG1 acted as a molecular sponge for miR-23b (Fig. 4B).

The involvement of RUNX2 in bone and cartilage development is well accepted, and a study by Chen et al. (2016) demonstrated that RUNX2 gene expression was inversely
Figure 2  (A–G) Knockdown of TUG1 suppressed osteoblastic differentiation of hBM-MSCs in vitro. (A) Knockdown of TUG1 in hBM-MSCs via siRNA transfection. (B) Alizarin Red S staining of mineralized matrix nodule formation in hBM-MSCs. (C) Quantification of Alizarin Red stain using Image J software. The graph showed the area of Alizarin Red stain positive region of the two groups. (D–G) hBM-MSCs were transfected with siTUG1-1 or siRNA-NC and then cultured in osteogenic induction medium for 7 days to stimulate osteoblastic differentiation. mRNA levels of osteocalcin (OCN), osteopontin (OPN), RUNX2 and miR-23b were measured by qRT-PCR. GAPDH expression was used for normalization. All tests were performed at least three times. Data are presented as mean ± SD, ** p < 0.01, *** p < 0.001.

Figure 3  (A and B) TUG1 directly interacted with miR-23b. (A) Representation the lnc RNA TUG1 and miR-23b binding sites by BIBISERV. (B) Luciferase activity was detected in hBM-MSCs after co-transfection with miR-23b mimics or negative control (miR-23b NC) and reporter plasmids containing a mutant TUG1 fragment (TUG1-Mut) or wild-type TUG1 (TUG1-WT) via the firefly dual-luciferase reporter system. The normalized luciferase activity in the miR-NC+TUG1-WT group was used as the relative luciferase activity. All tests were performed at least three times. Data are presented as mean ± SD, ** p < 0.01.
correlated with miR-23b expression during the osteogenic differentiation of C2C12 myoblasts (Chen et al., 2016). To determine whether miR-23b was involved in the regulation of RUNX2 expression in hBMMSCs, we performed qPCR and western blot analyses to evaluate the expression of RUNX2 in hBMMSCs after transfection with miR-23b mimics, miR-23b NC, or no transfection (wild-type cells). As shown in Figs. 4C–4E, RUNX2 mRNA and RUNX2 protein levels were significantly reduced upon miR-23b overexpression. Together, the results of these analyses suggested that miR-23b played a critical role in osteoblastic differentiation via targeting of RUNX2.

**TUG1 plays a key role in regulation of miR-23b/Runx2 network of BMMSCs**

We then explored the role of TUG1 in regulation of miR-23b/Runx2 signaling of BMMSCs. Firstly, we explored whether RUNX2 was a direct target of miR-23b in the regulation of osteoblastic differentiation of BMMSCs. As presented in Figs. 5A–5C, the RUNX2 mRNA and protein levels were significantly downregulated in hBMMSCs co-transfected with miR-23 mimics, in comparison to levels in hBMMSCs co-transfected with miR-23 NC. Moreover, we performed ARS staining to investigate the osteogenic phenotype of the transfected hBMMSCs. Staining for calcium deposition in hBMMSCs co-transfected with miR-23 mimics was significantly reduced compared with that among cells co-transfected with miR-23 NC (Figs. 5D&5E). In conclusion, the above results indicated that miR-23b expression reduced mineralized matrix nodule formation among hBMMSCs via direct
Figure 5 (A–E) TUG1 plays key role in regulation of miR-23b/Runx2 network of BMMSCs. hBMMSCs were transfected with si-TUG1, miR-23b mimics, miR-23b NC and then cultured in osteogenic induction medium for 7 days to stimulate osteoblastic differentiation. (A & B) mRNA and protein levels of RUNX2 in hBMMSCs, as analyzed by qRT-PCR and western blotting. The quantification of protein expression using Image J software. And the protein expression of the two groups was calculated according to the gray values of the protein bands. (C) Alizarin Red S staining analysis of mineralized matrix nodule formation in hBMMSCs. Quantification of Alizarin Red stain using Image J software. The graph showed the area of Alizarin Red stain positive region of the two groups. Data are presented as mean ± SD, *** p < 0.001.


dimensions: 612.0x792.0

suppression of RUNX2 translation, and TUG1 play crucial roles in regulation of miR-23b/Runx2 network of BMMSCs.

DISCUSSION
Numerous lncRNAs, including lncRNA-H19, HIF1a-AS1, MEG3, and MALAT1, have been shown to be involved in the regulation of osteoblast differentiation (Wu et al., 2018). However, the detailed biological role and underlying molecular mechanism of lncRNAs in osteoporosis remain to be fully elucidated. The aim of this study was to investigate the role of lncRNA TUG1 in osteoporosis-related pathways within hBMMSCs. Decreased TUG1 expression and increased miR-23b evident in the plasma of patients with osteoporosis than in that of age- and sex-matched healthy controls. Subsequent loss-of-function experiments indicated that inhibition of TUG1 could suppressive the osteoblastic differentiation of hBMMSCs, as evidenced by decreased expression of osteoblastic differentiation markers including RUNX2, osteocalcin, and osteopontin as well as reduced calcified nodule formation.

LncRNAs can act as molecular sponges for miRNAs to regulate the activities of specific miRNAs (Cesana et al., 2011). We used a firefly dual-luciferase reporter system, as well as bioinformatic prediction analysis, to investigate the potential interaction between lncRNA TUG1 and miR-23b, a miRNA previously found to influence osteoblast formation. We...
found that overexpression of miR-23b decreased the expression of TUG1, with our results showing an inverse correlation between miR-23b expression and TUG1 expression in hBMMSCs. From these results, we proposed that TUG1 could act as a sponge for miR-23b in hBMMSCs and thereby influenced the osteoblastic differentiation of these cells.

RUNX2, a primary osteoblast transcription factor, was reported to be closely involved in the osteoblastic differentiation of BMMSCs in osteoporosis (Komori, 2006). RUNX2 directs multipotent mesenchymal cells toward an osteoblastic lineage and promotes calcification-related protein (osteocalcin and osteopontin) expression, contributing to the formation of calcium deposits (Johnson, Leopold & Loscalzo, 2006). Related research found that LncRNA XIXT upregulated RUNX2 by absorbing miRNA-30a-5p, and thus induced hBMSCs osteogenesis to alleviate osteoporosis (Zhang, Du & Dong, 2019). Another study confirmed that LncRNA HOTAIRM1 promotes osteogenesis by regulating JNK/AP-1 signaling-mediated RUNX2 expression (Fu et al., 2019). In a recent study, TUG1 was shown to promote osteoblastic differentiation in calcific aortic valve disease by sponging of miR-204-5p and consequent upregulation of RUNX2 gene expression (Yu et al., 2018). In addition, a large number of studies have found that TUG1 influences osteoblast proliferation and differentiation by regulating multiple signaling pathways and gene expression (Zhang et al., 2019; Liu et al., 2019). In the present study, we found that miR-23b overexpression following transfection with miR-23b mimics led to reduced mineralized matrix nodule formation in hBMMSCs via targeting of RUNX2 gene expression. In addition, TUG1 directly interacted with miR-23b and acted as a molecular sponge of miR-23b. These data indicated that LncRNA TUG1 indeed plays a critical role in osteoporosis. Moreover, TUG1 may serve as a potential therapeutic target for treating osteoporosis.

This study for the first time revealed that silencing of LncRNA TUG1 suppressed the osteoblastic differentiation of hBMMSCs possibly by targeting the miR23b/RUNX2 signaling pathway. The mechanism by which TUG1/miR-23b/RUNX2 signaling regulated the osteogenic differentiation of BMMSCs in osteoporosis remains to be investigated. Further studies are needed to elucidate the potential link between TUG1 and miR-23b/RUNX2 signaling in osteoblast differentiation in the context of osteoporosis.

In conclusion, LncRNA TUG1 expression was downregulated, and miR-23b was upregulated in the plasma of osteoporotic patients compared with levels in healthy controls. In vitro experiments showed that miR-23b inhibited the osteoblastic differentiation of hBMMSCs via direct suppression of RUNX2 translation. LncRNA TUG1, acting as a miR-23b sponge, might reverse the suppression of RUNX2 and thus promote the osteogenic differentiation of hBMMSCs. Together these data suggested that TUG1 might represent a novel therapeutic target for osteoporosis.

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ADDITIONAL INFORMATION AND DECLARATIONS

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Competing Interests
The authors declare there are no competing interests.

Author Contributions
• Zhaowei Teng conceived and designed the experiments, performed the experiments, analyzed the data, prepared figures and/or tables, authored or reviewed drafts of the paper, and approved the final draft.
• Yun Zhu, Qinggang Hao, Xiaochao Yu, Yirong Teng and Qiaoning Yue performed the experiments, analyzed the data, prepared figures and/or tables, and approved the final draft.
• Xiguang Zhang and Sheng Lu conceived and designed the experiments, analyzed the data, prepared figures and/or tables, authored or reviewed drafts of the paper, and approved the final draft.

Human Ethics
The following information was supplied relating to ethical approvals (i.e., approving body and any reference numbers):
The Six Affiliated Hospital of Kunming Medical University granted Ethical approval to carry out the study within its facilities (Ethical Approval number: 2019kmykdx6h62).

Data Availability
The following information was supplied regarding data availability:
Raw data are available as Supplemental Files.

Supplemental Information
Supplemental information for this article can be found online at http://dx.doi.org/10.7717/peerj.11251#supplemental-information.
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