

# Long non-coding RNA TUG1 knockdown repressed the viability, migration and differentiation of osteoblasts by sponging miR-214

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**Abstract.** The maxillofacial region in the human body is susceptible to fracture and corresponding soft tissue injury. In the current study, the effect of long non-coding RNA (lncRNA) taurine upregulated gene 1 (TUG1) on maxillofacial fracture development was investigated. In total, 50 patients diagnosed with maxillary fracture and 50 healthy volunteers were enrolled in this study. Participants' TUG1 expression level in serum was measured using reverse transcription-quantitative (RT-q)PCR. After transfection with small interfering (si)-TUG1, microRNA (miR)-214 mimic, miR-214 inhibitor, bone morphogenetic protein 2 (BMP2) mimic or a combination, the biological behavior of osteoblasts was evaluated using MTT, Transwell assays, RT-qPCR, flow cytometry and western blot analysis. Recovery experiments were used to explore the potential mechanism. Results demonstrated that TUG1 expression was decreased in the serum of patients with maxillary fractures. Knockdown of TUG1 repressed viability, migration and differentiation and induced apoptosis of osteoblasts. StarBase v2.0 revealed that TUG1 served as a sponge for miR-214 and BMP2 is a direct target of miR-214. Altogether, it was revealed that TUG1 expression was decreased in patients with maxillary fractures and TUG1 knockdown repressed the biological process of osteoblasts by sponging miR-214.

## Introduction

The maxillofacial region is important in maintaining normal feeding, chewing, breathing and protecting the connected brain, cervical vertebra and other important anatomical structures.

As the most prominent part of the human body, the maxillofacial region is susceptible to fractures and corresponding soft tissue injuries (1,2). Maxillofacial fractures primarily include maxillary and mandible fractures. Patients with maxillary fractures experience damage to the maxillary sinus, which can affect the adjacent bone and tissue. If left untreated, fractures can impact facial appearance and occlusion (3,4). Maxillofacial fracture repair is a complex physiological process that is influenced by individual and environmental factors, as well as cytokines, somatic cells and endocrine factors (5,6). Osteoblast activity and differentiation also play an important role in the process of fracture healing (7).

Long non-coding RNA (lncRNA) is a class of transcripts >200 nt in length that regulate gene expression at epigenetic, transcriptional and post-transcriptional levels (8,9). Previous research has found abnormal lncRNAs to be involved in the regulation of osteoblasts activity. For example, maternally expressed gene 3 promotes osteoblast proliferation and differentiation during fracture healing (10); lncRNA AK016739 promotes osteogenic differentiation in skeletal disorders (11); and lncRNA Crnde mediates bone formation in animal models (12). The lncRNA taurine upregulated gene 1 (TUG1) was first identified in mouse retinal cells treated with taurine (13). Research on TUG1 focuses on its role in the development of malignant tumors (14,15). In the current study, the aim was to investigate the effect of TUG1 on maxillofacial fracture development.

## Materials and methods

**Patients.** Between January 2018 and December 2019, a total of 50 patients (range, 18-60 years old) diagnosed with maxillary fracture at The First Affiliated Hospital Xinjiang Medical University (Urumqi, China) were registered to participate in this study. There were 27 male and 23 female patients (age range, 18-60 years old; median, 43.25±5.32). The type and severity of maxillary fracture were classified according to the LeFort classification (16): type I (n=15), type II (n=25) and type III (n=10). During the same period, 50 healthy volunteers in the physical examination center were enrolled as controls. There were 27 male and 23 female volunteers (age range, 23-58 years old; median, 40.32±6.78). Written consent was

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obtained from all participants prior to enrollment, and the present study was approved by the Ethics Committee of The First Affiliated Hospital Xinjiang Medical University. Serum samples (5 ml) from participants were collected and centrifuged at 12,000 x g for 3 min at 37°C, and the supernatant was immediately frozen in liquid nitrogen for further analysis.

**Cell culture.** Mouse osteoblast cell line MC3T3-E1 (obtained from the Cell Bank of the Chinese Academy) was cultured in Dulbecco's modified Eagle's medium (DMEM) (Gibco; Thermo Fisher Scientific, Inc.) containing 10% Fetal Bovine Serum (FBS) (Invitrogen; Thermo Fisher Scientific, Inc.) and 1% penicillin/streptomycin under an atmosphere of humidified air and 5% CO<sub>2</sub> at 37°C.

**Cell transfection.** Small interfering RNA (siRNA) targeting TUG1 (si-TUG1; 20 nM), siRNA-negative control (si-NC; 20 nM), microRNA (miR)-214 mimic (30 nM), miR-NC (final concentration, 30 nM), miR-214 inhibitor (15 nM), NC inhibitor (15 nM), bone morphogenetic protein 2 (BMP2) mimics (30 nM) and NC mimic (30 nM) were synthesized by Shanghai GenePharma Co., Ltd. Transfection was performed using Lipofectamine<sup>®</sup> 2000 (Invitrogen; Thermo Fisher Scientific, Inc.) at 37°C for 48 h, after which the cells were harvested for reverse transcription-quantitative (RT-q)PCR analysis or western blotting. The sequences were shown as follows: si-TUG1 5'-GCUUGGCUUCUAUUCUGAAUCCUUU-3'; scrambled si-NC 5'-TTCTCCGAACGTGTCACGTTT-3'; miR-214 mimic 5'-ACAGCAGGCACAGACAGGCAGU-3'; scrambled miR-NC 5'-TTCTCCGAACGTGTCACGTTT-3'; miR-214 inhibitor 5'-ACGTGACACGTTCCGGAGAATT-3'; scrambled NC inhibitor 5'-CAGTACTTTTGTGTAGTACAA-3'; BMP2 mimic 5'-ACCCGCTGTCTTCTAGCGT-3'; and scrambled NC mimic 5'-ACGUGACACGUUCGGAGAATT-3'.

**Cell viability.** MC3T3-E1 cells (1x10<sup>5</sup> cells/well) with different transfections were seeded in 96-well plates and cultured for at 37°C for 0, 24, 48, 72 and 96 h. After cells were treated with 500 µg/ml MTT solution for 3 h at 37°C, 200 µl dimethylsulfoxide was added to dissolve precipitates. The optical density of each well was measured at 540 nm under a microplate spectrophotometer.

**Cell migration.** A total of 1x10<sup>5</sup> MC3T3-E1 cells were plated into the upper chambers containing 200 µl serum-free DMEM. DMEM containing 20% FBS was added to the lower chamber. Following incubation for 24 h at 37°C, the migrated cells were fixed with 100% methanol for 30 min and stained with 0.1% crystal violet for 30 min (both at room temperature). Finally, images were captured using an inverted microscope at x200 magnification (Olympus Corporation), and the mean number of migrated or invaded cells in five random microscopic fields per Transwell chamber was counted.

**Cell apoptosis assay.** After transfection, 1x10<sup>5</sup> harvested cells were washed two times with PBS and then were resuspended using 100 µl binding buffer. Transfected cells were double stained with propidium iodide and Annexin V-FITC (20 mg/ml; BD Pharmingen; BD Biosciences) at room temperature for 10 min in line with the manufacturer's instructions according

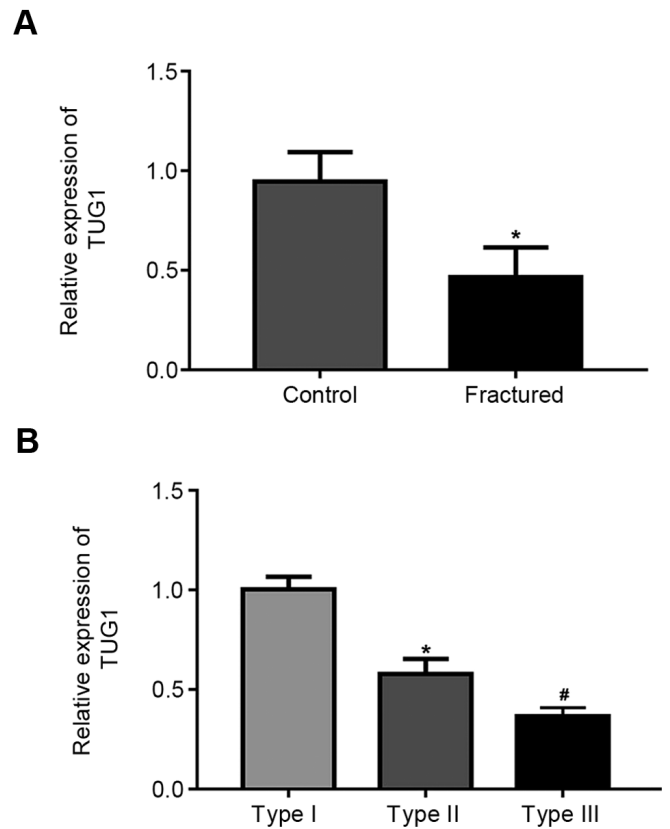


Figure 1. Decreased TUG1 expression levels in the serum of patients with maxillary fractures determined via reverse transcription-quantitative PCR. (A) TUG1 expression levels were significantly downregulated in patients with maxillary fractures compared with healthy controls. \*P<0.05 vs. control group. (B) TUG1 expression levels in serum samples from patients with Type I-III maxillary fractures. \*P<0.05 vs. type I group; #P<0.05 vs. type II group. TUG1, taurine upregulated gene 1.

to the manufacturer's protocol. Apoptosis was evaluated using a flow cytometer BD FACSCalibur<sup>™</sup> (BD Biosciences) and BD FACS<sup>™</sup> software (v1.0.0.650; BD Biosciences).

**Reverse transcription-quantitative (RT-q)PCR analysis.** Total RNAs in serums and MC3T3-E1 cells were isolated using TRIzol<sup>®</sup> reagent (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. cDNA synthesis was performed using the TaqMan MicroRNA Reverse Transcription Kit (Applied Biosystems; Thermo fisher Scientific, Inc.). qPCR was performed using the Mx3000p qPCR system (Agilent Technologies). GAPDH was an internal parameter of TUG1 and BMP2, while U6 was an internal parameter of miR-214. The following thermocycling conditions were used: Initial denaturation at 95°C for 10 min; followed by 40 cycles of denaturation at 95°C for 15 sec, and annealing at 60°C for 1 min; and a final extension of 10 min at 72°C. The sequences of primers the are as follows: TUG1 forward, 5'-TAGCAGTTC CCAATCCTTG-3' and reverse, 5'-CACAAATCCCATCA TTCC-3'; miR-214 forward, 5'-AGCATAATACAGCAG GCACAGAC-3' and reverse, 5'-AAAGGTTGTTCTCCACTC TCTCAC-3'; BMP2 forward, 5'-GCAAAGAAAAGGAAC GGACATT-3'; and reverse, 5'-AAAGGTTGTTCTCCACTC TCTCAC-3'; GAPDH forward, 5'-GGAGCGAGATCCCTC CAAAAT-3' and reverse, 5'-GGCTGTTGTCATACTTCT

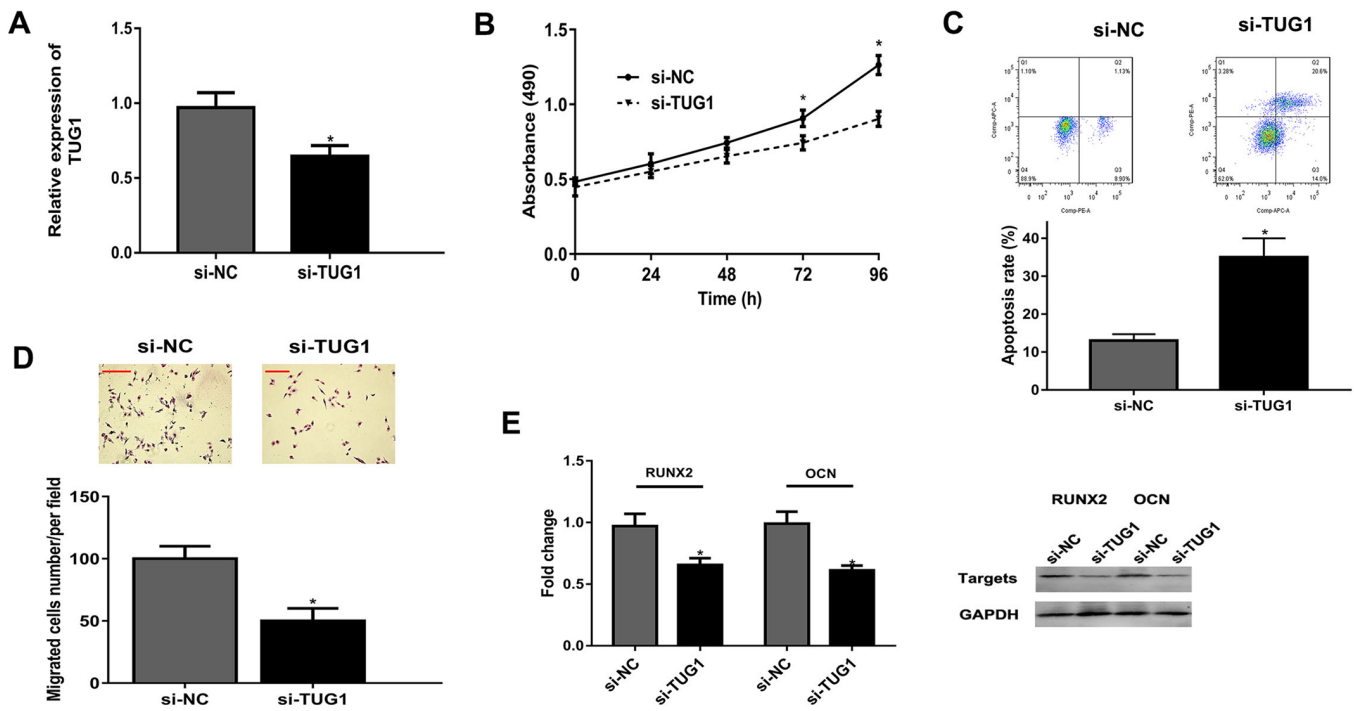


Figure 2. Knockdown of TUG1 represses viability, migration and differentiation and induces apoptosis of osteoblasts. (A) RT-qPCR results demonstrated that TUG1 expression was decreased in cells transfected with si-TUG1. (B) MTT assay showed that TUG1 knockdown suppressed the proliferation of osteoblasts at 72 and 96 h. (C) Flow cytometry revealed that the knockdown of TUG1 significantly increased the apoptosis rate of osteoblasts. (D) Transwell assay demonstrated that the knockdown of TUG1 inhibited the migration of cells. Scale bar, 20  $\mu$ m. (E) RT-qPCR and western blotting revealed that TUG1 knockdown significantly reduced the expression of differentiation-related RUNX2 and OCN at both mRNA and protein levels. \* $P$ <0.05 vs. si-NC group. TUG1, taurine upregulated gene 1; RT-qPCR, reverse transcription-quantitative PCR; si, small interfering; NC, negative control; RUNX2, runt-related transcription factor 2; OCN, osteocalcin.

CATGG-3'; U6 forward, 5'-CTCGCTTCGGCAGCACA-3'; and reverse, 5'-ACGCTTCACGAATTTGCGTGTGTC-3'. The relative expression was analysis using  $2^{-\Delta\Delta Cq}$  (17).

**Dual-Luciferase reporter assay.** The TUG1 fragments and BMP2 3'-UTR containing wild-type (WT) or mutant (MUT) miR-214 binding sites were obtained from Shanghai GenePharma Co., Ltd. Cells ( $1 \times 10^6$  per well) were co-transfected with 100 ng TUG1-WT, TUG1-MUT, BMP2-WT or BMP-2 MUT, and 100 nM miR-214 mimic or miR-NC using Lipofectamine<sup>®</sup> 2000. Following incubation for 48 h, luciferase activities were assessed using a Dual-Luciferase reporter assay kit (Promega Corporation), according to the manufacturer's protocol. Firefly luciferase activity was normalized to that of *Renilla* luciferase.

**Western blotting analysis.** Proteins were extracted from MC3T3-E1 cells using RIPA buffer (Sigma-Aldrich; Merck KGaA) containing a mixture of protease inhibitors. Total protein was quantified using a BCA protein assay kit (Pierce; Thermo Fisher Scientific, Inc.). Proteins (50  $\mu$ g per lane) were separated using 10% SDS-PAGE and electrotransferred to nitrocellulose membranes (EMD Millipore). The membranes were blocked with 5% non-fat milk at room temperature for 1 h. Subsequently, the membranes were incubated with the following primary antibodies at 4°C overnight: Anti-runt-related transcription factor 2 (RUNX2; 1:1,000; cat. no. ab192256), anti-osteocalcin (OCN; 1:1,000; cat. no. ab133612) and anti-GAPDH (1:1,000; cat. no. ab9485) all purchased from Abcam. The membranes were then incubated with anti-rabbit horseradish peroxidase-conjugated secondary antibody

(1:20,000; cat. no. ab6721; Abcam). Finally, the protein bands were visualized with an ECL kit (Cytiva) and analyzed using ImageJ v1.8.0 software (National Institutes of Health).

**Bioinformatics analysis.** The binding sites between miR-214 and TUG1, as well as miR-214 and BMP2 were predicted by StarBase v2.0 (<http://www.microrna.gr/IncBase>) and TargetScan software v7.1 ([http://www.targetscan.org/vert\\_71/](http://www.targetscan.org/vert_71/)), respectively.

**Statistical analysis.** All data are expressed as the mean  $\pm$  standard deviation. Differences between the two groups were analyzed using an unpaired Student's t-test. Multigroup comparisons were performed using a one-way ANOVA with a Tukey's post hoc test. All statistical analyses were performed using GraphPad Prism 5.0 (GraphPad Software, Inc.) and SPSS 14.0 (SPSS, Inc.). A statistically significant difference was defined as  $P$ <0.05.

## Results

**TUG1 expression is decreased in the serum of patients with maxillary fractures.** Results demonstrated that TUG1 expression was significantly downregulated in patients compared with healthy volunteers (Fig. 1A). Further, TUG1 expression significantly decreased with fracture severity (Fig. 1B).

**Knockdown of TUG1 represses viability, migration and differentiation and induces apoptosis of osteoblasts.** Following transfection of osteoblasts with si-TUG1, TUG1 expression was significantly decreased compared with the si-NC group (Fig. 2A).

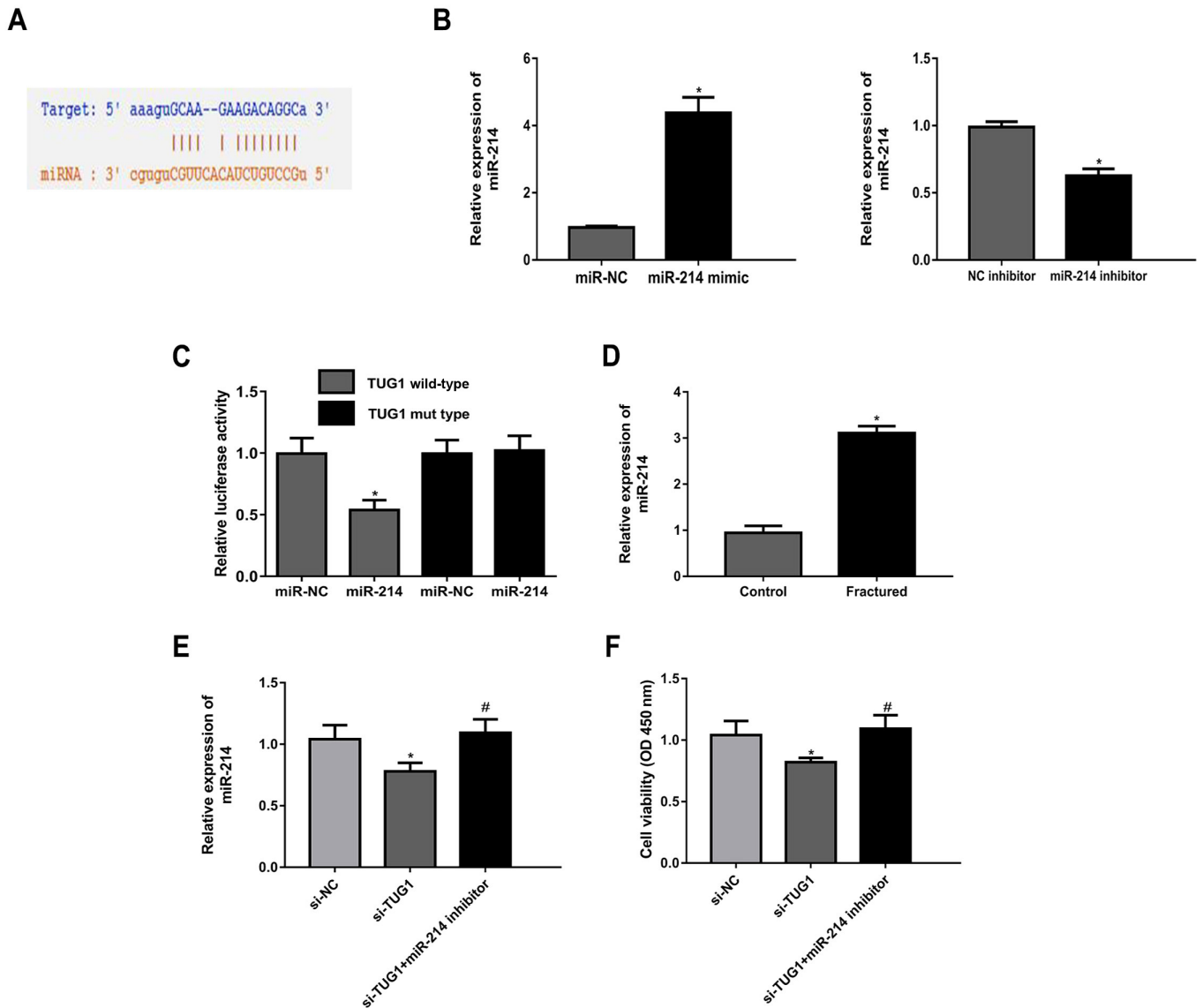


Figure 3. TUG1 acts as a sponge for miR-214. (A) StarBase v2.0 revealed that miR-214 had a potential binding site with TUG1. (B) The expression levels of miR-214 were increased in cells transfected with miR-214 mimic, while miR-214 expression was decreased in cells transfected with miR-214 inhibitor. \* $P < 0.05$  vs. miR-NC or miR inhibitor group. (C) Luciferase activity reporter assays demonstrated that miR-214 mimic inhibited luciferase activity in TUG1 wild-type. \* $P < 0.05$  vs. miR-NC group. (D) RT-qPCR demonstrated that miR-214 expression levels were detected at a significantly higher level in patients than in healthy controls. \* $P < 0.05$  vs. control group. (E) RT-qPCR revealed that TUG1 knockdown upregulated miR-214 expression, which was reversed by miR-214 inhibitor in cells. \* $P < 0.05$  vs. si-NC group; # $P < 0.05$  vs. si-TUG1 group. (F) MTT assay demonstrated that miR-214 inhibitor reversed the inhibitory effects of si-TUG1 in the viability of osteoblasts at 96 h. \* $P < 0.05$  vs. si-NC group; # $P < 0.05$  vs. si-TUG1 group. TUG1, taurine upregulated gene 1; miR, microRNA; RT-qPCR, reverse transcription-quantitative PCR; si, small interfering; NC, negative control.

MTT assay revealed that TUG1 knockdown significantly suppressed osteoblast proliferation at 72 and 96 h, compared with the si-NC group (Fig. 2B). Flow cytometry revealed knockdown of TUG1 significantly increased the apoptosis rate of osteoblasts (Fig. 2C). Knockdown of TUG1 inhibited osteoblast migration compared with si-NC (Fig. 2D). Finally, mRNAs and protein levels of differentiation-related RUNX2 and OCN were lower in the si-TUG1 group than that in si-NC group (Fig. 2E).

*TUG1 acts as a sponge for miR-214.* Most studies report that lncRNAs regulate gene expression by sponging miRNAs (18). Given this, StarBase v2.0 was used to predict the target microRNA binding with TUG1. It was demonstrated that miR-214 had a potential binding site with TUG1 (Fig. 3A).

The expression of miR-214 was increased in cells transfected with miR-214 mimic compared with miR-NC, while miR-214 expression was decreased in cells transfected with miR-214 inhibitor compared with the NC inhibitor (Fig. 3B). Luciferase activity reporter assays showed that miR-214 mimic inhibited the luciferase activity of TUG1 WT compared with TUG1 MUT (Fig. 3C). Further, miR-214 expression level was significantly higher in patients with maxillary fractures compared with controls (Fig. 3D). TUG1 knockdown upregulated miR-214 expression, which was reversed following co-transfection of cells with a miR-214 inhibitor (Fig. 3E). The results of the MTT assay indicated that si-TUG1 transfection resulted in a decrease in cell viability, and this effect was abrogated following co-transfection with miR-214 inhibitor (Fig. 3F).

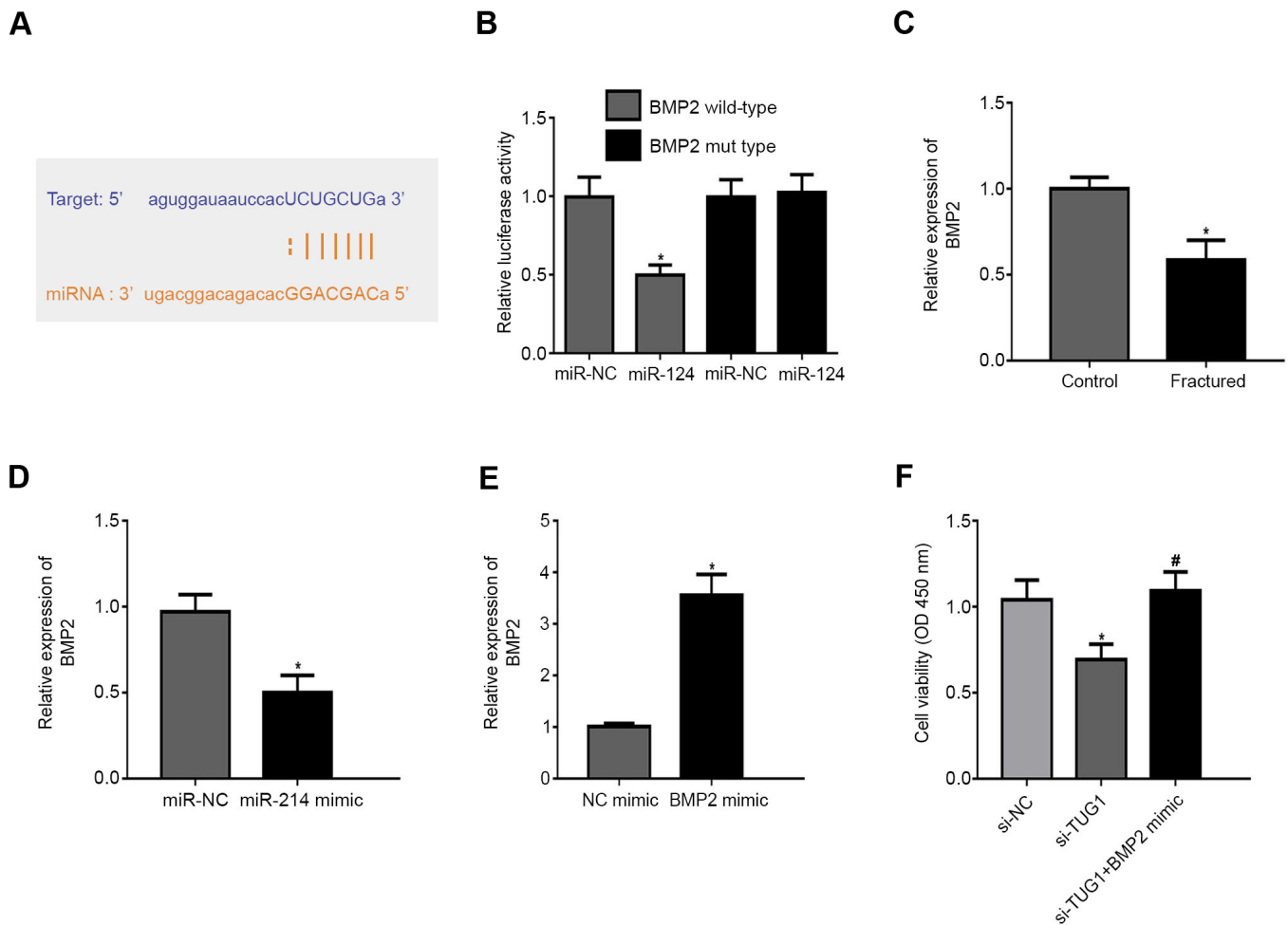


Figure 4. BMP2 is a direct target of miR-214. (A) TargetScan analysis demonstrated that the 3'UTR of BMP2 was highly conserved to bind with miR-214. (B) Luciferase activity reporter assays revealed that miR-214 mimic inhibited luciferase activity in BMP2 wild-type. \* $P < 0.05$  vs. miR-NC group. (C) RT-qPCR revealed that BMP2 expression levels were significantly lower in patients than in healthy controls. \* $P < 0.05$  vs. control group. (D) RT-qPCR demonstrated that the miR-214 mimic significantly inhibited BMP2 expression in cells. \* $P < 0.05$  vs. miR-NC group. (E) BMP2 mimic elevated the expression levels of BMP2 in cells. \* $P < 0.05$  vs. NC mimic group. (F) MTT assay demonstrated that the BMP2 mimic reversed the inhibitory effects of si-TUG1 in the viability of osteoblasts at 96 h. \* $P < 0.05$  vs. si-NC group; # $P < 0.05$  vs. si-TUG1 group. BMP2, bone morphogenetic protein 2; miR, microRNA; RT-qPCR, reverse transcription-quantitative PCR; si, small interfering; NC, negative control; TUG1, taurine upregulated gene 1; OD, optical density

*BMP2 is a direct target of miR-214.* TargetScan demonstrated 3'UTR of BMP2 was highly conserved to bind with miR-214 (Fig. 4A). In BMP2 WT, miR-214 mimic repressed the fluorescence (Fig. 4B). BMP2 expression was lower in patients with maxillary fractures compared with healthy controls (Fig. 4C). In addition, miR-214 mimic inhibited BMP2 expression compared with the NC (Fig. 4D). Transfection of cells with a BMP2 mimic resulted in increased BMP2 expression levels compared with the NC mimic (Fig. 4E). Notably, transfection of cells with si-TUG1 resulted in a decrease in cell viability and this was reversed following co-transfection with a BMP2 mimic (Fig. 4F).

## Discussion

Due to its complex anatomical structure, the diagnosis and treatment of maxillary bone injury is difficult. Maxillary bone injury can cause dysfunction and morphological deformities that severely impact quality of life. Given this, effective diagnosis and treatment methods are needed to not

only improve patients' facial appearance, but to reshape the occlusal relationship and peripheral structure (3,19). As a novel long non-coding RNA, TUG1 has been reported to participate in several types of orthopedic diseases including osteosarcoma (20), osteoarthritis (21) and intervertebral disc degeneration (22). In the present study, the role of TUG1 in the development of maxillary fractures was examined. It was revealed that TUG1 expression levels were decreased in the serum of patients with maxillary fractures. Recent studies have found TUG1 inhibition reduces osteoblast proliferation and differentiation (23,24). Similarly, TUG1 knockdown attenuated viability, migration and differentiation and induced apoptosis of osteoblasts in the present study. These data suggest TUG1 knockdown inhibits the activity of osteoblasts in fractures.

It is well known that lncRNAs play a regulatory role in gene expression by sponging miRNAs. It was hypothesized that miR-214 was the candidate target microRNA. Luciferase activity reporter assays confirmed an inhibition of luciferase activity in TUG1 WT. Other studies have found that miR-214

regulates bone formation by weakening osteoblast proliferation and differentiation (25,26). In the current study, increased miR-214 expression was observed in patients with maxillary fractures. Finally, miR-214 inhibitor reversed the repression of osteoblast proliferation by si-TUG1.

Studies have shown miRNAs participate in the regulation of gene expression through targeting 3'untranslated region (3'-UTR) of mRNAs (27). Subsequently, BMP2 was identified as the potential target gene of miR-214 in the present study. BMP2 is critical in stimulating bone formation during fracture healing (28,29). It induces osteoblast proliferation and differentiation (30,31). BMP2 expression was revealed to be lower in patients with maxillary fractures and miR-214 mimic inhibited BMP2 expression in osteoblasts. Finally, BMP2 mimic rescued the suppression of si-TUG1 in osteoblast proliferation.

The present study demonstrated that long noncoding RNA TUG1 promoted proliferation and differentiation of MC3T3-E1 cells. It could be suggested that an elevation in TUG1 levels is associated with later stages of maxillary fracture development, as an increased level of TUG1 is required in maxillary fracture repair. In the present study, the expression of TUG1 was only examined at early stages of maxillary fractures. To hypothesize, TUG1 expression would be expected to increase over time during the recovery of maxillary fracture. Thus, further studies will be used to explore the effect of TUG1 on the recovery of maxillary fracture.

In conclusion, decreased TUG1 expression levels were found in patients with maxillary fractures and TUG1 knock-down repressed the biological process of osteoblasts by sponging miR-214. Therefore, this study may provide some new information about maxillary fracture development.

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#### Availability of data and materials

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

#### Authors' contributions

ZY and MT designed the experiments. ZY and WA performed the experiments. AM analyzed the data. ZY and MT wrote the manuscript. ZY and MT confirm the authenticity of all the raw data. All authors have read and approved the final manuscript.

#### Ethics approval and consent to participate

The study was approved by the Medical Ethics Committee of The First Affiliated Hospital of Xinjiang Medical University (approval no. XJMU20171214; Urumqi, China). Written

informed consent was obtained from patients prior to enrollment.

#### Patient consent for publication

Not applicable.

#### Competing interests

The authors declare that they have no competing interests.

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