## Original Article
# Long non-coding RNA SNHG7 promotes the fracture repair through negative modulation of miR-9

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**Abstract:** Fracture is the most common disease in the orthopedics. Long non-coding small nucleolar RNA host gene 7 (SNHG7) has been confirmed to enhance cell proliferation and decrease cell apoptosis in many cancers. However, the role of SNHG7 in skeletal fracture remains largely to be elucidated. In the current study, we observed SNHG7 was down-regulated in femoral neck fracture tissues. In addition, SNHG7 knockdown inhibited proliferation and migration, induced apoptosis, reduced activity in osteoblast cells in vitro. Bioinformatics analysis revealed SNHG7 acts as a molecular sponge for miR-9 and MiR-9 directly targets with 3'-UTR of TGFBR2. Furthermore, SNHG7 knockdown repressed the TGF-β signaling pathway. Taken together, this study manifested SNHG7 promotes bone repair in femoral neck fracture, and may serve as a potential target for enhancing bone formation.

**Keywords:** Femoral neck fracture, osteoblast cells, SNHG7, miR-9, TGF-β signaling pathway

## Introduction

Skeletal fracture is the loss of bone mechanical integrity, besides, it also includes local soft tissue and vascular damage. Fracture is the most common disease in the orthopedics due to increasing incidences of trauma, tumor excision and other deformities [1, 2]. Bone tissue belongs to regenerative tissues, and the fracture healing is a complex system in which various cells and cytokines involve in repairing injured bones with the final aim of restoring skeletal function [3]. During this process, maintenance of osteoblast differentiation and activity is crucial [4]. The differentiation and activity of osteoblasts involved various hormones, growth factors and cytokines, which mediated the regulation of the gene expression related to osteoblast differentiation by different signal transduction pathways.

LncRNAs are a class of transcripts that are longer than 200 nucleotides in length and have no protein-coding potential. Evidence has increasingly shown that lncRNA could regulate the gene expressions in cell cycle, cell differen-

tiation and apoptosis [5-7]. LncRNA SNHG7 (long non-coding small nucleolar RNA host gene 7) is located on chromosome 9q34.3 in length of 2176 bp [8]. Previous studies have confirmed that SNHG7 could enhance cell proliferation and decrease cell apoptosis in many cancers [9-11]. However, the role of SNHG7 in skeletal fracture remains largely to be elucidated.

MicroRNAs (miRNAs) are a class of small non-coding single-stranded RNA (around 22 nucleotides in length) that participate in a variety of biological processes, such as cell survival, proliferation, apoptosis, differentiation, cell cycle progression and migration [12-14]. Research has noticed that miRNAs could regulate the process of osteoblastic bone formation [15-17]. Nevertheless, the molecular mechanism of miR-9 in skeletal fracture still remains to be fully investigated.

In the current study, we first investigated the expression of SNHG7 and further explore whether SNHG7 could regulate the biological processes of osteoblasts in femoral neck frac-

ture, which might conduct a new strategy for the research of skeletal fracture.

## Materials and methods

### Patients

From January 2015 to December 2016, 90 eligible patients with fresh femoral neck fractures were recruited in Tianjin Third Central Hospital and Tianjin Hospital. The type and severity of femoral neck fracture was evaluated in accordance with the Pauwels classification: type I (n=32), type II (n=30) and type III (n=28). Inclusion criteria for eligibility: patients aged 18-60 years old; no history of hip disease; unilateral femoral neck fracture. The exclusion criteria: severe osteoporosis; pathological fracture; old fracture (more than 14 days); autoimmune diseases. All participants obtained informed consent, and the current study was approved by the ethics committee of Tianjin Third Central Hospital. The bone samples from fracture area and normal area were stored at liquid nitrogen immediately after surgery for further analysis.

### Cell culture

The mouse pre-osteoblastic cell line MC3T3-E1, purchased from the Chinese Academy of Sciences CellBank (Shanghai, China), was cultured in minimum essential medium α (Gibco), supplemented with 10% fetal bovine serum, penicillin at 100 U/ml, and streptomycin at 100 μg/ml. To induce osteoblastic differentiation, cells were cultured in the medium containing ascorbic acid at 50 mg/L (Invitrogen) and 10 mM β-glycerophosphate (Sigma, St Louis, MO, USA). All cultures were maintained in a humidified 5% $CO_2$ atmosphere at 37°C.

### Cell transfection

Cell transfection was performed with Lipofectamine 2000 reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. Cells were seeded into 6-well plates (200,000 cells/well), and then transduced with si-SNHG7, miR-9 inhibitor or their parental negative controls (NCs). Forty-eight hours after transfection, cells were used for the tests.

### Cell proliferation assay

Cells (10,000 cells/well) were plated into a 96-well plate, and incubated for 0, 24, 48 and 72 h. Subsequently, 0.5 mg/ml MTT was stained for 4 h, and then 200 μl of dimethylsulfoxide (DMSO) was added to dissolve precipitates. The optical density (OD) of each well was measured at 490 nm using an enzyme immunoassay analyzer.

### Cell migration assay

Cells at density of $1 \times 10^5$ per well were respectively seeded into the upper chamber filled with serum-free DMEM. After 24 hours incubation, cells adhered on the upper surface of membrane were removed by cotton swabs, while the cells on the other side of the membrane were stained with 0.1% crystal violet. Five random fields were counted for each well.

### Cell apoptosis assay

Cell apoptosis was measured by flow cytometry following the manufacturer's instructions for FITC Annexin-V Apoptosis Detection Kit (BD, Franklin Lakes, NJ, USA). Cells were resuspended in flow cytometry binding buffer after washed in PBS twice, and then incubated with Annexin V-FITC and propidium iodide (PI) for 15 min at room temperature avoiding from light. Afterwards, the apoptosis cells were immediately measured by FACSCalibur (Becton-Dickinson, CA, USA).

### Real time quantitative PCR analysis

The total RNA was extracted from tissues and cells using Trizol reagent (Invitrogen, Shanghai, China) according to the manufacturer's instructions. Complementary DNA (cDNA) was synthesized using the High-Capacity cDNA Reverse Transcription Kits (Thermo Fisher Scientific) and MicroRNA Reverse Transcription Kit (Thermo Fisher Scientific), respectively. And the relative expression was analyzed using 2-ΔΔCT method with normalization to GAPDH and U6 snRNA, respectively.

### Luciferase reporter assay

Wild and mutant reporter plasmids of SNHG7 (wt-SNHG7 and mut-SNHG7) and TGFBR2 (wt-TGFBR2 and mut-TGFBR2), which contained miR-9 binding sites, were synthesized by GenePharma (GenePharma, Shanghai, China). Cells were seeded into plates and transfected with involved oligonucleotides (the SNHG7 and TGFBR2 wild-type or mutant reporter vector,
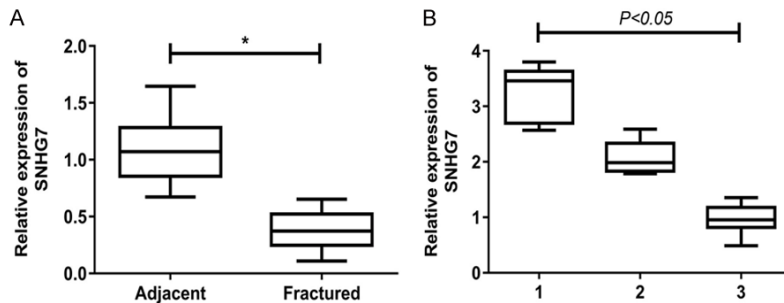
**Figure 1.** SNHG7 was down-regulated in femoral neck fracture tissues. A: The level of SNHG7 in fractured tissues. B: The levels of SNHG7 in different types of femoral neck fracture. *P<0.05 compared to control group.

GraphPad (vision 6.0, USA). Data were expressed as the mean ± standard deviation. P-value less than 0.05 indicated a statistically significant difference.

## Results

### SNHG7 was down-regulated in femoral neck fracture tissues

In order to explore the role of SNHG7 in the development of femoral neck fracture, we analyzed the expression of SNHG7 in femoral neck fracture tissues adjacent normal tissues using qRT-PCR. As shown in **Figure 1A**, the level of SNHG7 was down-regulated in fractured tissues significantly. Besides, SNHG7 levels were significantly decreasing in patients with type II and type III femoral neck fracture (**Figure 1B**).

### SNHG7 knockdown inhibited proliferation and migration, induced apoptosis, reduced activity in osteoblast cells in vitro

To investigate the role of SNHG7 in the presence of fracture, we stably established SNHG7 silencing via siRNA transfection in MC3T3-E1 (**Figure 2A**). MTT assay showed that knockdown of SNHG7 suppressed the proliferation of MC3T3-E1 cells significantly (**Figure 2B**). Quantitative analysis of apoptosis by flow cytometry revealed that silencing of SNHG7 markedly augmented apoptosis in cells compared with the non-transfected cells (**Figure 2C**). Cell migration assay revealed significant differences in two cell lines transfected with si-SHNG7 or si-NC (**Figure 2D**). In addition, we evaluated the role of SNHG7 in osteoblast activity. Functionally, the levels of BALP and osteocalcin were remarkably lower in cells transduced by si-SNHG7 compared with the control (**Figure 2E**).

### SNHG7 acts as a molecular sponge for miR-9

Increasing evidence had illustrated that lncRNAs could regulate the expression of miRNAs [18]. Bioinformatics analysis was used to predict the candidate targets of microRNAs binding with SNHG7. Results indicated that miR-9 was a potential miRNA target for SNHG7. The
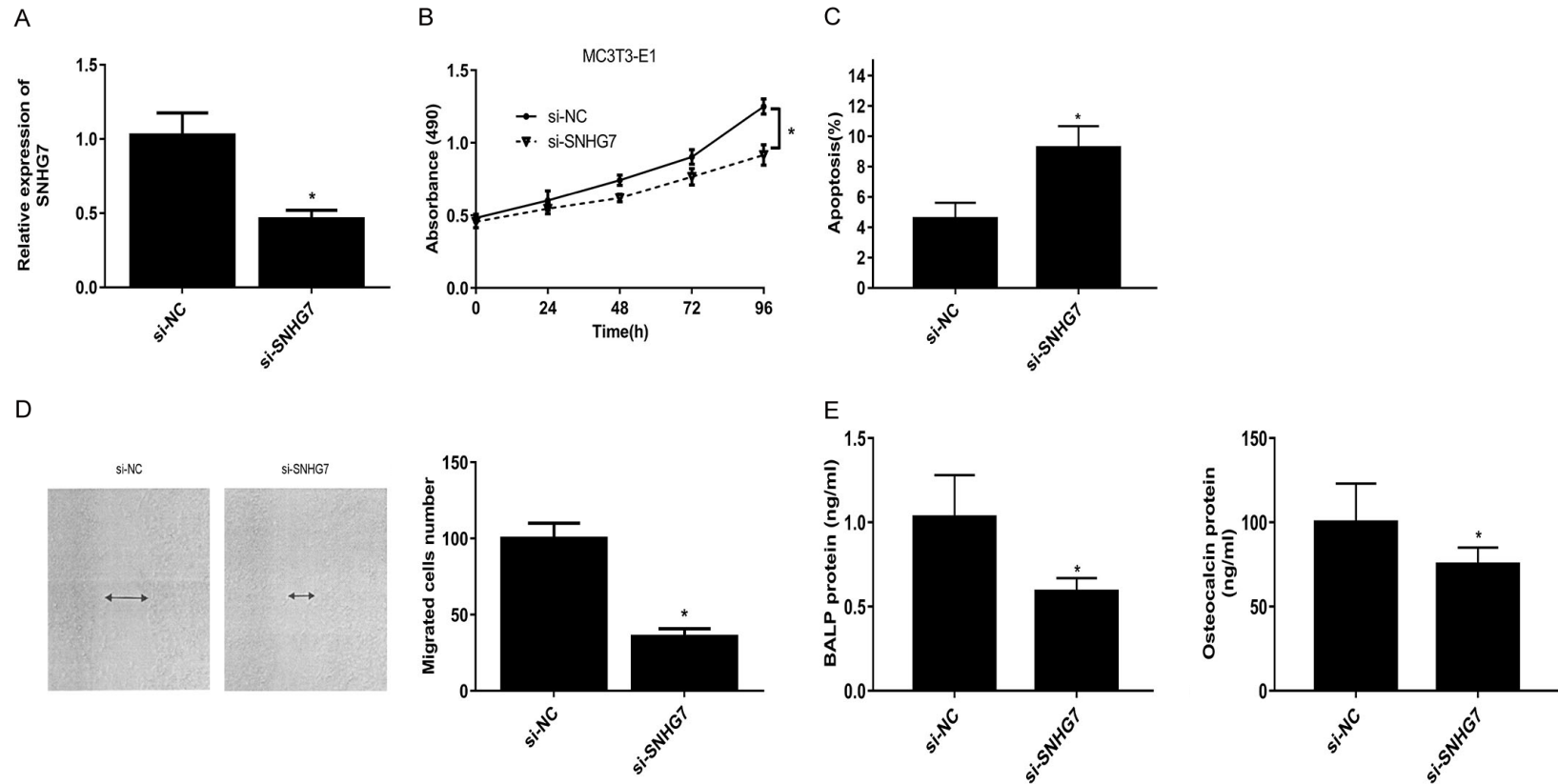
miR-9 mimic or negative control) mixed with Lipofectamine 2000 reagent. Luciferase activity was detected after transfection 24 h using Dual-Luciferase Reporter System (Promega, Madison, WI, USA). Renilla luciferase activities were used as the internal control for the normalization of firefly luciferase activity.

### Bone-specific alkaline phosphatase and osteocalcin ELISA analysis

After digested by trypsin, cells were re-suspended in minimum essential medium α (Gibco), supplemented with 10% fetal bovine serum. Subsequently, the supernatant was taken and the concentration of BALP and osteocalcin were measured with an ELISA kit according to the manufacturer's instructions (USCN Life Science).

### Western blotting analysis

Proteins were extracted from cultured cells by RIPA buffer containing a mixture of protease inhibitors, and then transferred to polyvinylidene fluoride (PVDF) membrane by SDS-PAGE. The membrane was blocked with 5% nonfat milk in PBS and then incubated with anti-TGFBR2, anti-p-smad2, anti-p-smad3 or anti-Runx2 in PBS overnight. Then the secondary antibodies were employed to incubate the PVDF membranes conjugated with horseradish peroxidase for 1 h. Blots were processed with an enhanced chemiluminescence kit (Santa Cruz Biotechnology), and exposed to the film.

### Statistical analysis

All calculations were performed using SPSS 17.0 software (PSS, Inc., Chicago, IL, USA) and

**Figure 2.** SNHG7 knockdown inhibited proliferation and migration, induced apoptosis, reduced activity in osteoblast cells in vitro. A: SNHG7 silencing was established via siRNA transfection in MC3T3-E1 cells. B: MTT assay showed that knockdown of SNHG7 suppressed the proliferation of MC3T3-E1 cells significantly. C: Quantitative analysis of apoptosis by flow cytometry revealed that silencing of SNHG7 markedly augmented apoptosis in cells. D: Cell migration assay revealed significant differences in two cell lines transfected with si-SHNG7 or si-NC. E: The levels of BALP and osteocalcin were remarkably lower in cells transduced by si-SNHG7. *$P<0.05$ compared to control group.
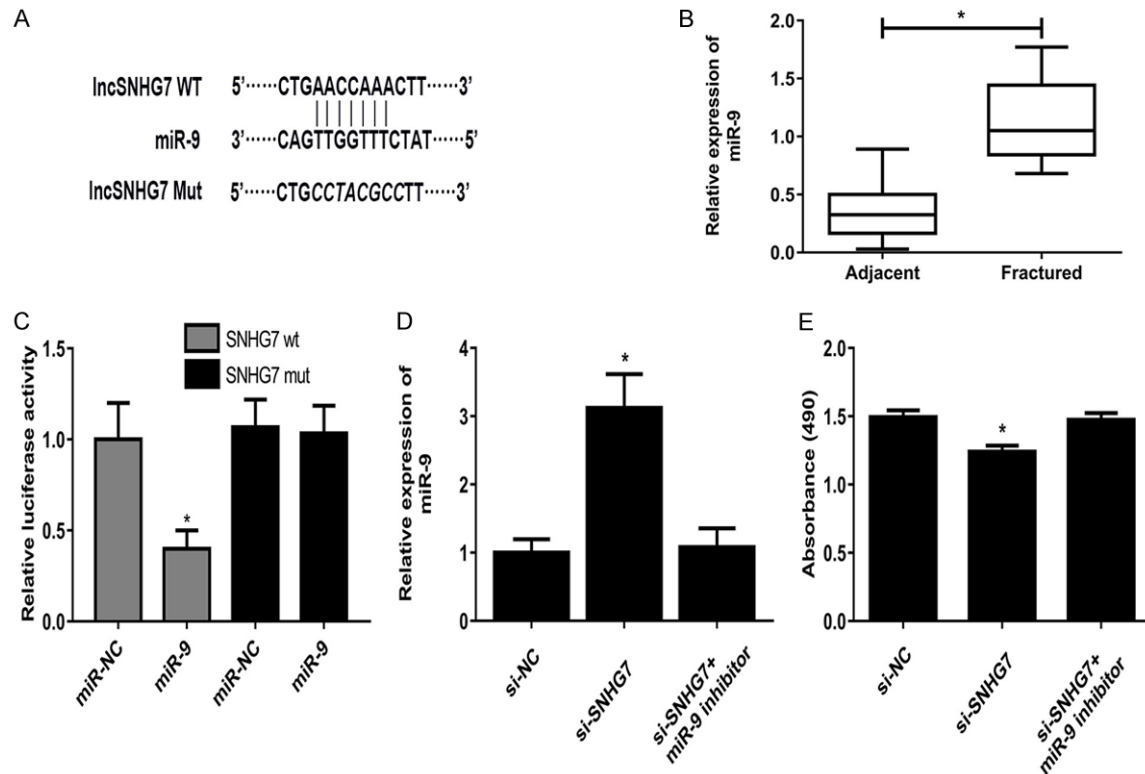
**Figure 3.** SNHG7 acts as a molecular sponge for miR-9. A: The predicted binding sites within miR-9 and 3'-UTR of SNHG7. B: Expression of miR-9 in fractured tissues. C: Luciferase reporter assay showed the miR-9 inhibited the luciferase activity of cells transduced with wild type SNHG7 plasmid. D: After MC3T3-E1 cells were transfected with si-SNHG7, miR-9 expression levels were significantly up-regulated which was reversed by miR-9 inhibitor. E: MTT assay revealed that miR-9 inhibitor could rescue the suppression by si-SNHG7 in the proliferation of cells. *$P<0.05$ compared to control group.
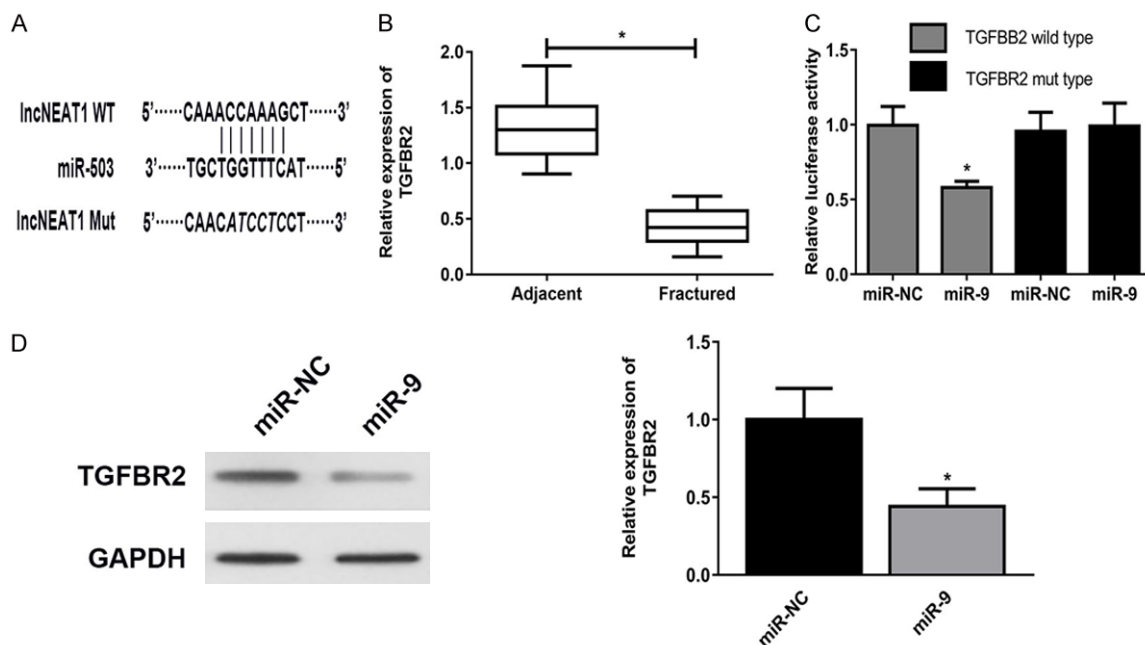


**Figure 4.** MiR-9 directly targets with 3'-UTR of TGFBR2. A: The predicted binding sites within miR-9 and 3'-UTR of TGFBR2. B: Expression of TGFBR2 in fractured tissues. C: Luciferase reporter assay showed the decreasing fluorescence within miR-9 and TGFBR2 wild type. D: TGFBR2 expression in cells transfected with miR-9 mimic at the mRNA and protein levels. *$P<0.05$ compared to control group.
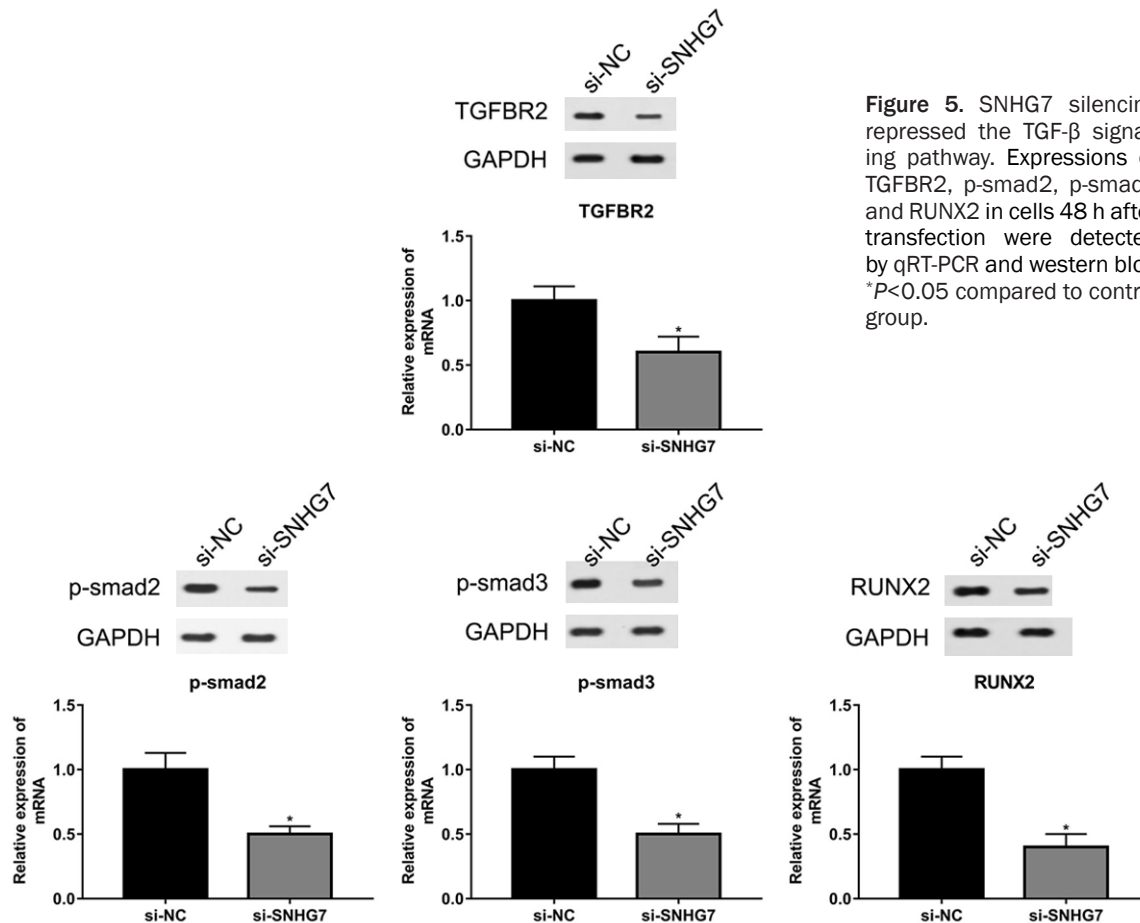
**Figure 5.** SNHG7 silencing repressed the TGF-β signaling pathway. Expressions of TGFBR2, p-smad2, p-smad3 and RUNX2 in cells 48 h after transfection were detected by qRT-PCR and western blot. $^*P<0.05$ compared to control group.

3'-UTR binding sites can be seen in **Figure 3A**. MiR-9 level was higher in fractured tissues significantly (**Figure 3B**). Luciferase activity reporter assays showed that miR-9 inhibited the luciferase activity of cells transduced with wild type SNHG7 plasmid (**Figure 3C**). Moreover, after MC3T3-E1 cells were transfected with si-SNHG7, miR-9 expression levels were significantly up-regulated which was reversed by miR-9 inhibitor (**Figure 3D**). MTT assay revealed that miR-9 inhibitor could rescue the suppression by si-SNHG7 in the proliferation of MC3T3-E1 cells 96 hours later (**Figure 3E**).

*MiR-9 directly targets with 3'-UTR of TGFBR2*

We examined computationally predicted targets of miR-9 using bioinformatics analysis. Results revealed 3'UTR of TGFBR2 was highly conserved to bind with miR-9 (**Figure 4A**). TGFBR2 level was significantly lower in fractured tissues than it in normal adjacent tissues (**Figure 4B**). Luciferase reporter assay validated miR-9 targeted the 3'UTR of TGFBR2 mRNA

in cells, suggesting miR-9 has inhibitory effects on TGFBR2 expression via interaction with the 3'-UTR of TGFBR2 (**Figure 4C**). Moreover, as shown in **Figure 4D**, overexpression of miR-9 level obviously downregulated TGFBR2 expression in MC3T3-E1 cells at the mRNA and protein levels. Overall, our study discovered that miR-9 suppressed the expression of TGFBR2 by binding with the 3'-UTR.

*SNHG7 silencing repressed the TGF-β signaling pathway*

As shown in **Figure 5**, statistically significant differences were found in the expressions of TGFBR2, p-smad2 and p-smad3 among the different groups 48 h after transfection. Compared with the control, si-SNHG7 significantly suppressed TGFBR2, p-smad2 and p-smad3 expression in cells. RUNX2, the target gene of smad2/3, is a key transcription factor associated with osteoblast differentiation. PCR and western blot demonstrated si-SNHG7 significantly inhibited RUNX2 expression.

## Discussion

The incidence of femoral neck fractures is significantly increased in young or middle-aged populations with the rise in high-energy trauma such as traffic injuries and injuries [19]. Although lncRNAs are verified to be emerging as important regulatory molecules in carcinogenesis [20-22], only a few studies focused on the role of lncRNAs in the osteogenic differentiation. For instance, Lin Zhu et al. observed downregulated ANCR promotes osteoblast differentiation [23], Wenzhuo Zhuang et al. found MEG3 promotes osteogenic differentiation of mesenchymal stem cells from multiple myeloma patients [24], Yiping Huang et al. reported long noncoding RNA H19 promotes osteoblast differentiation by deriving miR-675 [25]. However, the role of SNHG7 in skeletal fracture remains elusive. In our study, we first demonstrated SNHG7 was down-regulated in femoral neck fracture tissues. Furthermore, SNHG7 downregulation was correlated with fracture type, implying SNHG7 may act as one of the biomarkers of femoral neck fracture. Moreover, knockout of SNHG7 could suppress the proliferation and migration of osteoblast cells. Quantitative analysis of apoptosis by flow cytometry revealed that silencing of SNHG7 markedly augmented apoptosis in cells and lowered osteoblastic activity.

An increasing number of studies demonstrated that lncRNAs might function as a ceRNA or a molecular sponge in modulating miRNA [20, 26]. Therefore, we predicted the candidate targets of microRNAs binding with SNHG7using bioinformatics analysis. And we discovered that miR-9 was a potential miRNA target for SNHG7. Mounting evidence suggested miRNAs may involve in bone formation and osteoblast differentiation [27, 28]. In this study, we observed miR-9 level was higher in fractured tissues significantly and miR-9 inhibitor could rescue the suppression by si-SNHG7 in the proliferation of MC3T3-E1 cells.

LncRNAs played a part in ceRNA networks and lncRNA-miRNA-mRNA crosstalk. Subsequently, we investigated the downstream molecular mechanism regulated by miR-9. The results showed miR-9 has inhibitory effects on TGFBR2 expression by binding to the 3'-UTR. TGFβ has been reported to play an important role in the regulation of osteoblasts and osteoclasts [29]. Kazuhiro Takeyama, et al. observed inhibition of TGFβ signaling impaired migration and differentiation of osteoblasts during fracture healing [30]. In our study, we detected TGFBR2 level was significantly lower in fractured tissues and overexpression of miR-9 obviously downregulated TGFBR2 expression in MC3T3-E1 cells. TGFβ-2 signaling is essential for bone formation. The activation of TGFBR2 could regulate downstream smad family proteins phosphorylation and gene expression [31]. Results showed SNHG7 knockout significantly suppressed TGFBR2, p-smad2 and p-smad3 expression in cells. Meanwhile, as the target gene of smad2/3, RUNX2 level was decreasing. These data suggested SNHG7 silencing could repress the TGF-β signaling pathway.

## Conclusion

The current study suggests SNHG7 plays as an important role in femoral neck fracture repair, and may serve as a potential target for enhancing bone formation of femoral neck fracture.

## Acknowledgements

## Disclosure of conflict of interest

None.

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