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LncRNA HAGLR accelerates femoral neck fracture healing through negatively regulating miRNA-19a-3p

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Abstract. – OBJECTIVE: This study aims to uncover the function of long non-coding RNA (lncRNA) HAGLR in the healing process of femoral neck fracture and the underlying mechanism.

PATIENTS AND METHODS: Expression levels of HAGLR, microRNA-19a-3p (miRNA-19a-3p) and TGFBR2 in fractured femoral neck tissues and adjacent normal tissues were detected by quantitative Real Time-Polymerase Chain Reaction (qRT-PCR). Regulatory effects of HAGLR on viability, apoptosis, migration, and protein levels of BALP and Osteocalcin in MC3T3-E1 cells were determined. Dual-Luciferase reporter gene assay was conducted to assess the binding in HAGLR/miRNA-19a-3p/TGFBR2. In addition, relative levels of TGFBR2, p-smad2, p-smad3, and RUNX2 in MSCs influenced by HAGLR were detected.

RESULTS: HAGLR was downregulated in fractured femoral neck tissues. Knockdown of HAGLR reduced viability and migration, enhanced apoptotic rate, as well as downregulated BALP and Osteocalcin in MC3T3-E1 cells. HAGLR served as miRNA-19a-3p sponge, and miRNA-19a-3p directly targeted 3′-untranslated region (3′-UTR) of TGFBR2. Knockdown of HAGLR downregulated expressions of TGFBR2, p-smad2, p-smad3, and RUNX2 in MC3T3-E1 cells, indicating the inhibited TGF-β pathway.

CONCLUSIONS: LncRNA HAGLR/miRNA-19a-3p/TGFBR2 regulatory loop accelerates the healing process of femoral neck fracture by inhibiting the TGF-β pathway.

Key Words:
HAGLR, MiRNA-19a-3p, TGFBR2, Bone fracture.

Introduction

Fractures are considered to be the most common trauma diseases in the world[1]. Fracture healing is a complex process, in which mechanical forces are necessary for bone tissue regeneration[2]. Fractures would lead to walking restrictions, depression, loss of independence, and chronic pain. Almost all fracture patients require long-term treatment and healing in the hospital[3-5]. Various growth factors and cytokines are involved in the healing process of bone fractures, which are responsible for regulating cell activation and osteoblast proliferation[6]. There are four necessary conditions for successful fracture healing: Proper mechanical environment, osteoblasts, bone scaffolds, and growth factors that effectively induce osteogenesis[7]. Although there are many strategies contributing to accelerate bone regeneration, many defects and limitations are existed[8]. Effective and practical bone regeneration strategies are needed to be developed.

Long non-coding RNAs (LncRNAs) are transcripts with over 200 nt long and lack protein-encoding function. They used to be by-products of RNA polymerase II transcription without any biological functions. Later, lncRNAs are found to be able to regulate chromatin structure, gene expressions, and disease development[9,10]. LncRNAs are also involved in the process of fracture healing. Liu et al[11] showed that epigenetic silence of lncRNA MEG3 promotes the healing of tibiofibular fracture by activating the Wnt/β-catenin pathway. Gong et al[12] found that long non-coding RNA H19 promotes the osteogenic differentiation of rat ectomesenchymal stem cells via Wnt/beta-catenin signaling pathway. LncRNA HAGLR locates between HOXD1 and HOXD3 gene in the HOXD cluster. HAGLR is reported to be critical in tumor progression[13].

MicroRNAs (miRNAs) are single-chain, non-coding RNAs spanning 20-25 nucleotides[14]. By specifically binding 3'UTR of target mRNAs, miRNAs could negatively regulate them at post-transcriptional level and thus influence cellular behaviors[15]. Relevant studies[16,17] have demonstrated the role of miRNAs in bone formation. MiRNA-19a-3p is a member of miR-17-92 cluster. Dysregulation of miRNA-19a-3p is closely linked to many tumor diseases[18-21].

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In this paper, we mainly uncovered the role of HAGLR in regulating the process of femoral neck fracture *via* mediating osteoblast behaviors.

Patients and Methods

Sample Collection

Forty femoral neck fracture patients were enrolled in this study. Fractured bone tissues and adjacent normal tissues were harvested and preserved in liquid nitrogen. Severity and subtype of femoral bone fracture were evaluated according to the Pauwels classification, including type I (n = 12), type II (n = 13), and type III (n = 15). Patients and their families in this study have been fully informed. This investigation was approved by the Ethics Committee of Ningbo Medical Center Lihuili Eastern Hospital.

Cell Culture and Transfection

Mouse osteoblasts MC3T3-E1 provided by Cell Bank (Shanghai, China) were cultured in Dulbecco's Modified Eagle's Medium (DMEM) containing 10% fetal bovine serum (FBS; Life Technologies, Gaithersburg, MD, USA) and maintained in a 37°C, 5% CO_2 incubator. Medium was replaced every 2-3 days. At 80-90% confluence, cell transfection was conducted using Lipofectamine™ 2000 (Invitrogen, Carlsbad, CA, USA). Transfected cells for 48 h were harvested for functional experiments.

Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR)

TRIzol reagent (Invitrogen, Carlsbad, CA, USA) was applied for isolating cellular RNA. Complementary deoxyribose nucleic acid (cDNA) was obtained by reverse transcription of 2 μg RNA using cDNA synthesis kit (TaKaRa, Otsu, Shiga, Japan) and amplified on the MiniOpticon qPCR determination system (Bio-Rad Laboratories, Hercules, CA, USA). Relative level was calculated using $2^{-\Delta\Delta CT}$ method. HAGLR, F: 5'-TCTGAAAGAAGGACCAAAGTAA-3' and R: 5'-ATTCAAGGGACAGTCACAGG-3'; MiRNA-19a-3p, F: 5'-GGGGGGGTGTGCAAATCT-3' and R: 5'-GTGCGTGTCGTGGAGTCG-3'; TGFBR2, F: 5'-TCGAAAGCATGAAGGACAACG-3' and R: 5'-AGCACTCAGTCAACGTCTCAC-3'.

Western Blot

Radioimmunoprecipitation assay (RIPA; Beyotime, Shanghai, China) was applied for isolating cellular protein. Protein sample was quantified by bicinchoninic acid (BCA) method (Pierce, Rockford, IL, USA) and underwent electrophoresis. Protein was transferred on a polyvinylidene difluoride (PVDF) membranes (Millipore, Billerica, MA, USA), and blocked in phosphate-buffered saline (PBS) containing 5% skim milk for 2 h. Subsequently, membranes were reacted with primary antibodies at 4°C overnight and secondary antibodies for 2 h. Band exposure was achieved by enhanced chemiluminescence (ECL) and analyzed by Image Software (NIH, Bethesda, MD, USA).

Dual-Luciferase Reporter Gene Assay

HAGLR (wt-HAGLR and mut-HAGLR) and TGFBR2 (wt-TGFBR2 and mut-TGFBR2) vectors were constructed by GenePharma (Shanghai, China). After co-transfection of luciferase vector and miRNA-19a-3p mimic/negative control for 48 h, the relative luciferase activity was determined.

Cell Counting Kit-8 (CCK-8)

Cells were inoculated in a 96-well plate at 80% confluence. Viability was determined at the established time points using CCK-8 kit (Dojindo Laboratories, Kumamoto, Japan). Absorbance at 450 nm was recorded for plotting the viability curve.

Transwell

5×10^4 cells were applied in the upper side of the transwell chamber (Corning, Corning, NY, USA) pre-coated with 200 mg/mL Matrigel. In the bottom side, 600 μL of medium containing 20% FBS was applied. After 48 h of incubation, cells penetrated to the bottom side were fixed in 4% paraformaldehyde for 20 min, stained with crystal violet for 20 min and counted using a microscope. The number of penetrating cells was counted in 5 randomly selected fields per sample (magnification: 40×).

Flow cytometry

3×10^5 cells were washed with PBS for three times. Cells were dual-stained with Annexin-V-FITC and subjected to flow cytometry (FACSCalibur; BD Biosciences, Detroit, MI, USA). Apoptotic rate was analyzed using the FlowJo software.

Statistical Analysis

Statistical Product and Service Solutions (SPSS) 19.0 (IBM, Armonk, NY, USA) was used for data analyses. Data were expressed as mean ± standard deviation. The Student's *t*-test was applied for analyzing differences between the two groups. $p<0.05$ was considered statistically significant.

Results

HAGLR Was Downregulated in Fractured Femoral Neck Tissues

Totally, 40 paired cases of fractured femoral neck tissues and adjacent normal bone tissues were harvested. It is shown that HAGLR was downregulated in fractured femoral bone tissues (Figure 1A). In addition, based on the fracture severity, HAGLR level was found to be gradually downregulated in femoral neck fracture cases with type II and III compared with those with type I (Figure 1B). We believed that HALGR was involved in the process of bone fracture healing.

Knockdown of HAGLR Attenuated Proliferative and Migratory Abilities, and Induced Apoptosis in Osteoblasts

To elucidate the role of HAGLR in bone fracture, si-HAGLR was conducted and its transfection efficacy was tested in MC3T3-E1 cells (Figure 2A). Transfection of si-HAGLR greatly reduced viability and migratory cell number in MC3T3-E1 cells (Figure 2B, D). In addition, apoptosis was stimulated in MC3T3-E1 cells with HAGLR knockdown (Figure 2C). Subsequently, regulatory effects of HAGLR on osteoblast activities were assessed by detecting expression levels of BALP and Osteocalcin. The mRNA levels of BALP and Osteocalcin were downregulated in MC3T3-E1 cells transfected with si-HAGLR (Figure 2E).

HAGLR Sponged miRNA-19a-3p

Through the Targetscan prediction, miRNA-19a-3p was identified to be the direct target of HAGLR (Figure 3A). Furthermore, Dual-Luciferase reporter gene assay showed that overexpression of miRNA-19a-3p inhibited luciferase activity in wild-type HAGLR vector, verifying the binding between HAGLR and miRNA-19a-3p (Figure 3B). MiRNA-19a-3p was lowly expressed in fractured femoral neck tissues (Figure 3C). Moreover, miRNA-19a-3p level was upregulated after transfection of si-HAGLR in MC3T3-E1 cells (Figure 3D). Notably, reduced viability in osteoblasts with HAGLR knockdown was partially reversed by co-silence of miRNA-19a-3p (Figure 3E). It is suggested that miRNA-19a-3p was responsible for HAGLR-regulated healing process of the femoral neck.

MiRNA-19a-3p Directly Targeted 3'UTR of TGFBR2 and HAGLR Inhibited the TGF-β Pathway

Similarly, the direct target of miRNA-19a-3p was predicted in Targetscan and TGFBR2 was selected. Luciferase vectors of wild-type and mutant-type TGFBR2 were constructed according to the binding sequences in the promoter regions of miRNA-19a-3p and TGFBR2 (Figure 4A). Later, the Dual-Luciferase reporter gene assay confirmed the binding between miRNA-19a-3p and TGFBR2 (Figure 4B). In fractured femoral neck tissues, TGFBR2 was downregulated (Figure 4C). In addition, protein level of TGFBR2 was downregulated in MC3T3-E1 cells overexpressing miRNA-19a-3p (Figure 4D). It is shown that knockdown of HAGLR downregulated expressions of TGFBR2, p-smad2, p-smad3, and RUNX2 in MC3T3-E1 cells, indicating the inhibited the TGF-β pathway (Figure 4E-H).

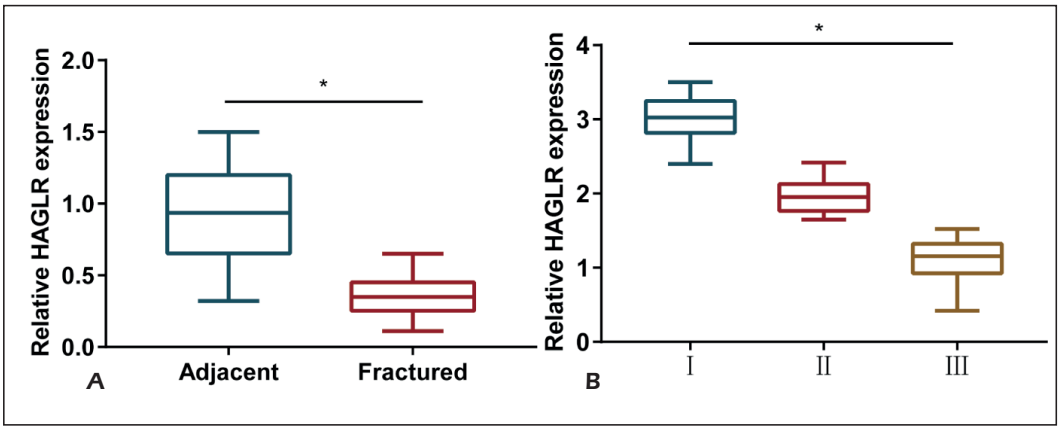

Figure 1. HAGLR was downregulated in fractured femoral neck tissues. **A**, HAGLR levels in fractured femoral neck and adjacent bones. **B**, HAGLR levels in femoral bone fracture patients with type I, II and III.

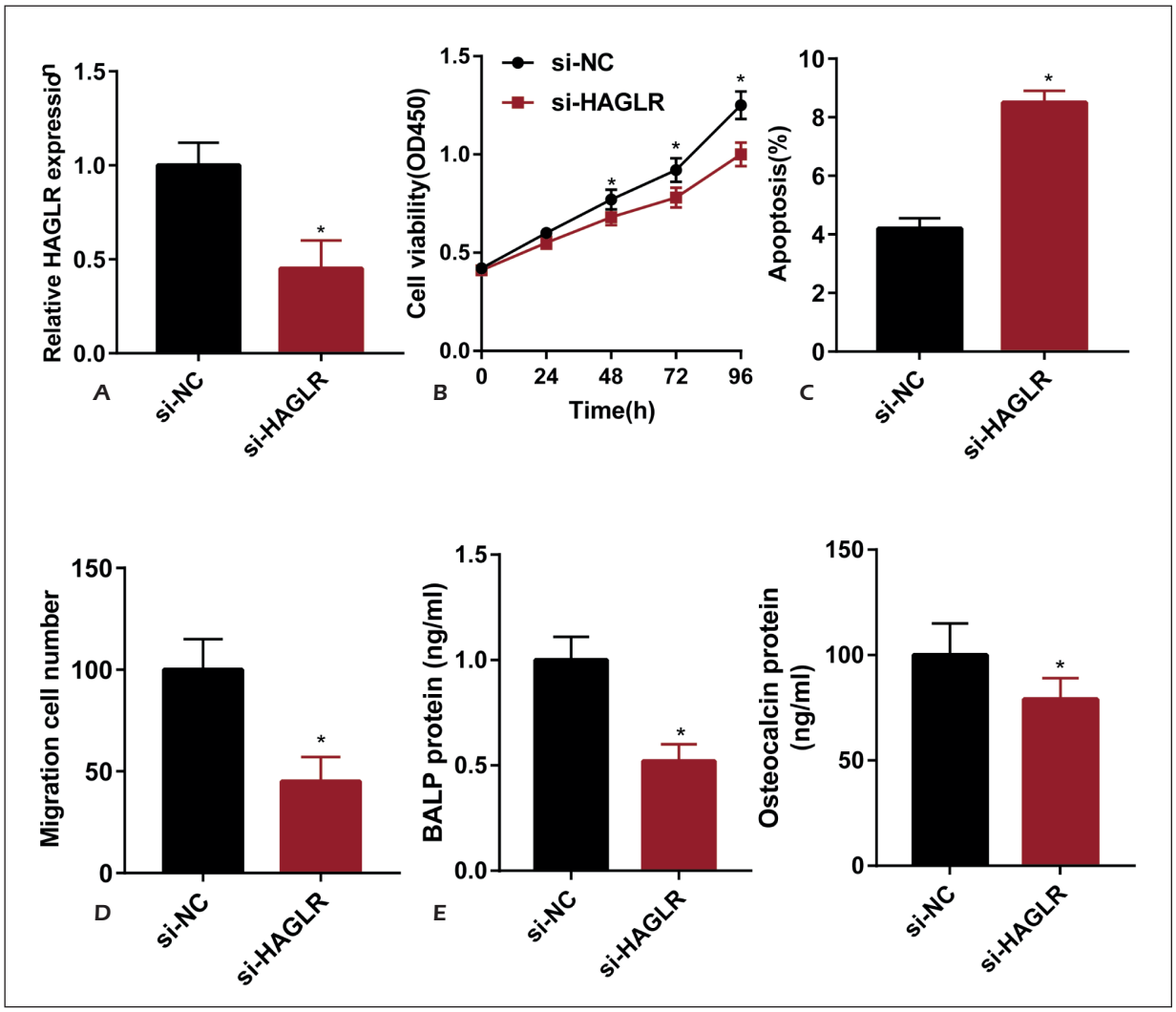

Figure 2. Knockdown of HAGLR attenuated proliferative and migratory abilities, and induced apoptosis in osteoblasts. **A**, Transfection efficacy of si-HAGLR in MC3T3-E1 cells. **B**, Viability in MC3T3-E1 cells transfected with si-HAGLR or si-NC. **C**, Apoptosis in MC3T3-E1 cells transfected with si-HAGLR or si-NC. **D**, Migratory cell number in MC3T3-E1 cells transfected with si-HAGLR or si-NC (magnification: 40×). **E**, Expression levels of BALP and Osteocalcin in in MC3T3-E1 cells transfected with si-HAGLR or si-NC.

Discussion

Fracture healing is a complex process that regulates the activation, proliferation, and differentiation of local mesenchymal stem cells or progenitor cells by precise growth factors and cytokine sequences[1,22]. However, 5-10% fracture patients experience nonunion or delayed healing[23]. Improvement of fracture healing rate and avoidance of fracture nonunion are research focuses nowadays[24].

Critical roles of lncRNAs in fracture healing have been identified[25]. In this paper, HAGLR was found to be downregulated in fractured femoral neck tissues. Its level was associated with bone fracture type, suggesting that HAGLR may be a hallmark for femoral neck fracture. Moreover, functional experiments showed that silence of HAGLR attenuated viability and migratory ability, induced apoptosis, and downregulated osteoblast activity in MC3T3-E1 cells.

Serving as a ceRNA, lncRNA sponges miRNA to suppress its expression[26], and attenuates the inhibitory effects of miRNA on the downstream genes[27,28]. Liang et al[29] illustrated that lncRNA H19 sponges miRNA-141 and miR-22 to upregulate RUNX2, thus accelerating osteogenesis in hMSCs through activating the Wnt path-

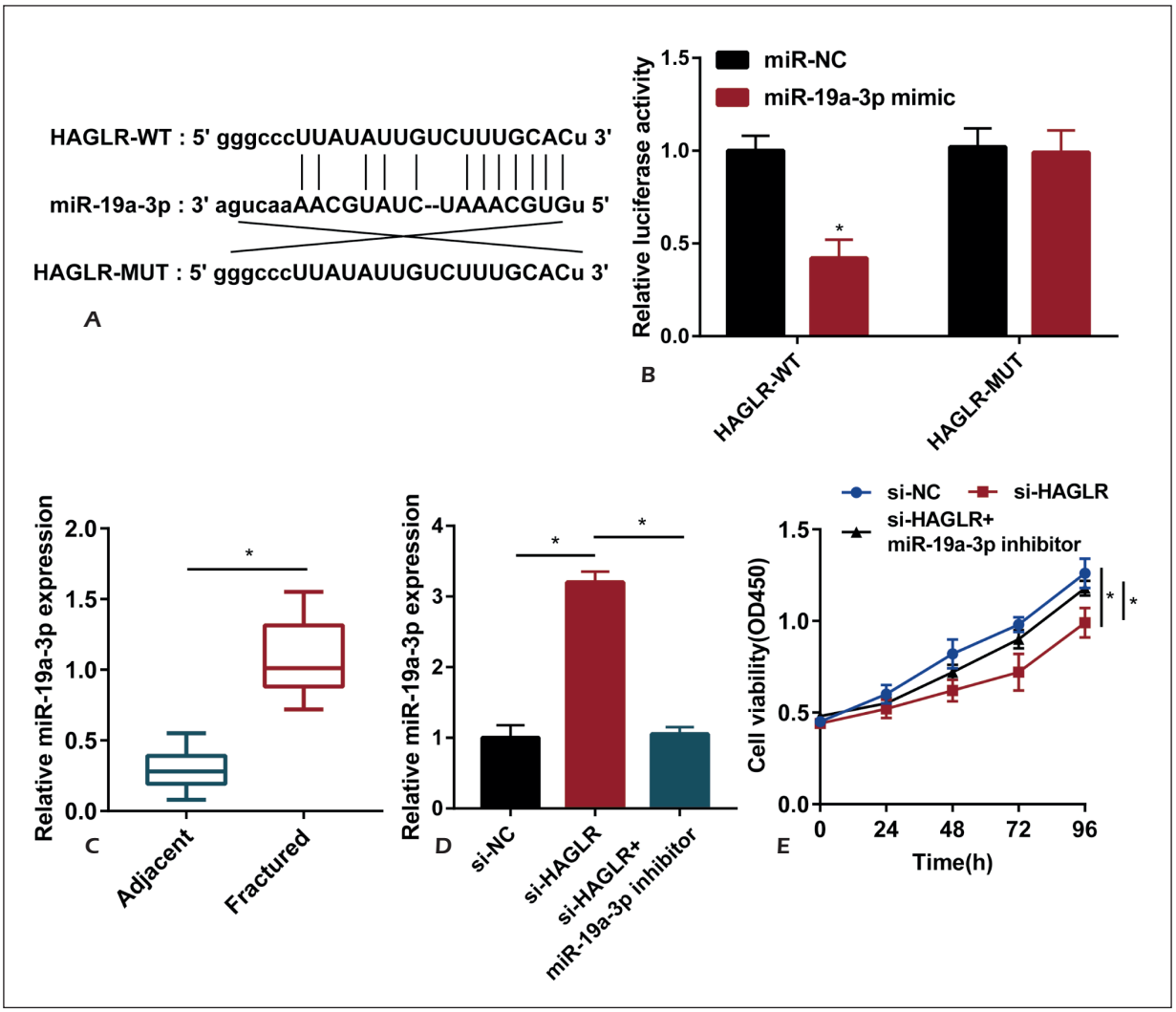

Figure 3. HAGLR sponged miRNA-19a-3p. **A**, Binding sequences in the promoter regions of HAGLR and miRNA-19a-3p. **B**, Luciferase activity in MC3T3-E1 cells co-transfected with miRNA-19a-3p mimic/NC and HAGLR-WT/HAGLR-MUT. **C**, MiRNA-19a-3p levels in fractured femoral neck and adjacent bones. **D**, MiRNA-19a-3p levels in MC3T3-E1 cells transfected with si-NC, si-HAGLR or si-HAGLR+miRNA-19a-3p inhibitor. **E**, Viability in MC3T3-E1 cells transfected with si-NC, si-HAGLR or si-HAGLR+miRNA-19a-3p inhibitor.

way. Wu et al[30] demonstrated that 0.5 Hz mechanical stretching for hBMSCs leads to upregulation of H19, which further sponges miR-138 to stimulate osteogenesis in BMSCs. Our findings verified that miRNA-19a-3p was the potential target of HAGLR, which was upregulated in fractured femoral neck tissues. Moreover, miRNA-19a-3p directly bound 3'UTR of TGFBR2 and negatively regulated TGFBR2 level.

TGF-β signaling is able to suppress cell proliferation, embryogenesis, and bone remodeling[31]. Potential influences of TGF-β on phenotypes of osteoblasts and osteoclasts have been well explored[32,33]. In bone repair, TGFβ-2 is a vital mediator[34]. The activation of TGF-β is induced by intracellular Smad and non-Smad-associated genes[35]. In this paper, TGFBR2 was downregulated in fractured femoral bones. Overexpression of miRNA-19a-3p markedly inhibited TGFBR2 level in MC3T3-E1 cells. Furthermore, the knockdown of HAGLR could downregulated TGFBR2, p-smad2, p-smad3, and RUNX2 in MC3T3-E1 cells. As a result, the downregulation of HAGLR was capable of inhibiting the TGF-β pathway, which was responsible for accelerating femoral neck fracture healing.

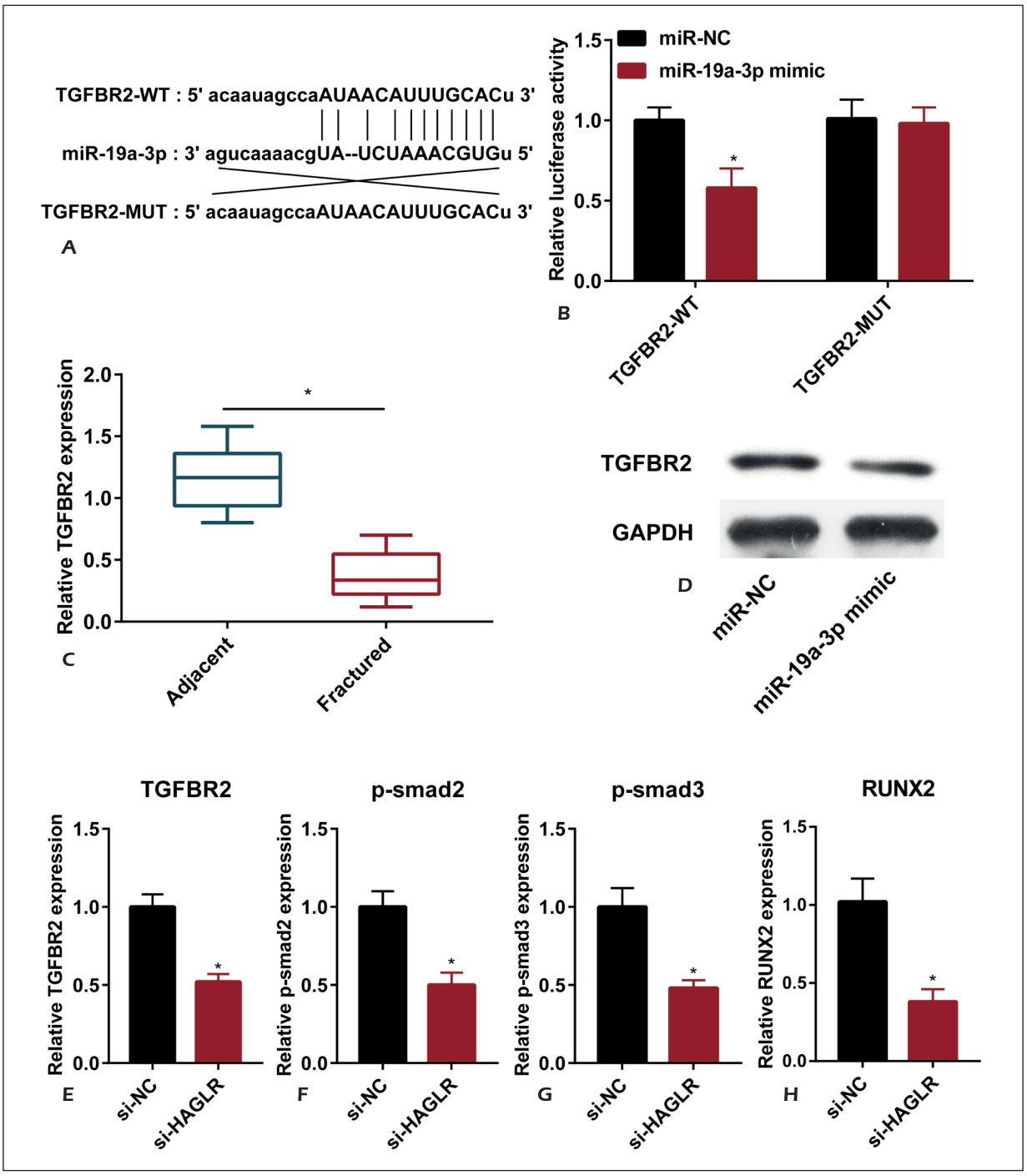

Figure 4. MiRNA-19a-3p directly targeted 3'UTR of TGFBR2 and HAGLR inhibited the TGF-β pathway. **A**, Binding sequences in the promoter regions of miRNA-19a-3p and TGFBR2. **B**, Luciferase activity in MC3T3-E1 cells co-transfected with miRNA-19a-3p mimic/NC and TGFBR2-WT/TGFBR2-MUT. **C**, TGFBR2 levels in fractured femoral neck and adjacent bones. **D**, Protein level of TGFBR2 in MC3T3-E1 cells transfected with miR-NC or miRNA-19a-3p mimic. E-H, Expression levels of TGFBR2 (**E**), p-smad2 (**F**), p-smad3 (**G**) and RUNX2 (**H**) in MC3T3-E1 cells transfected with si-NC or si-HAGLR.

Conclusions

Shortly, lncRNA HAGLR/miRNA-19a-3p/TGFBR2 regulatory loop accelerates bone fracture healing by inhibiting the TGF-β pathway, which could be utilized as therapeutic targets for clinical treatment of femoral neck fracture.

Conflict of Interests

The Authors declare that they have no conflict of interests.

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