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Inhibition of long non-coding RNA TSIX accelerates tibia fraction healing *via* binding and positively regulating the SOX6 expression

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Abstract. – OBJECTIVE: Fragile fracture patients need to be treated with long-term fixation and the recovery process is slow. Several studies have shown that the fracture healing process is related to gene expression. We aimed to investigate the role of long chain non-coding RNA TSIX (lncRNA TSIX) on fracture healing after tibial fracture (TF) and explore the molecular mechanism underlying its action.

MATERIALS AND METHODS: The male C57BL/6J mice were used to construct TF models and osteoblasts were used as *in vitro* model. The proliferation, apoptosis, and osteogenesis-related genes of Col1a1, Col-II, and Col-X were detected to evaluate the role of lncRNA TSIX *in vivo* and *in vitro* after TF. Haematoxylin-eosin (HE) staining was conducted to confirm the fracture healing conditions.

RESULTS: We found that LncRNA TSIX expression in plasma of TF mice significantly upregulated in a time-dependent manner. Overexpression of lncRNA TSIX could significantly inhibit proliferation but promote apoptosis and regulate the osteogenesis-related genes expression by binding and positively regulate sex-determining region Y box 6 (SOX6) expression, while knockdown of lncRNA TSIX showed the opposite effect in osteoblastic cells. Inhibition of lncRNA TSIX could improve fracture healing after TF.

CONCLUSIONS: Taken together, our study supported that knockdown of lncRNA TSIX could promote the tibia fracture healing by binding and inhibiting the SOX6 expression. We suggest that lncRNA TSIX/SOX may be the potential targets for the treatment of TF.

Key Words:
Tibia fracture, Fracture healing, LncRNA TSIX, SOX6.

Introduction

Fracture is one of the major public health problems worldwide, which has a profound impact on social health and economy[1,2]. The healing process after fracture requires the coordination of various growth factors and other cytokines to regulate the activation of cells and the proliferation of osteoblasts[3,4]. The basic conditions for successful healing after fracture include proper mechanical fixation, proliferation of osteoblasts, and effective induction of osteogenic growth factors[5]. Although there are many strategies to improve the healing of fragile fractures to accelerate bone regeneration, there are some shortcomings in these treatments[6,7]. Therefore, we need to explore more effective and practical methods of bone regeneration.

Most RNAs in organisms can be encoded into proteins through transcription and translation, and then play important roles in various physiological metabolisms[8]. However, there are still some RNAs that cannot be translated into proteins, which we call non-coding RNAs[9]. Abnormal expression or mutation of non-coding RNA (ncRNA) could lead to the occurrence and development of many diseases[10,11]. Among these ncRNAs, miRNAs are associated with bone formation after fracture[12,13]. Consistently, miR-17 is involved in the regulation of osteoblast proliferation and differentiation, and may exert an important role in the development and maturation of bone[14,15]. Long-chain non-coding RNAs (lncRNAs) have been widely recognized to play important roles in many diseases, including tumors, cardiovascular diseases, immune

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diseases[8], and closely connected to bone-related diseases[16,17]. When we analyzed and screened the possible bone formation-related lncRNAs, we found a new lncRNA TSIX could promote osteoblast apoptosis i*n vivo* and *in vitro*[18]. We also found that the expression of lncRNA TSIX was up-regulated in a time-dependent manner after fracture, but whether lncRNA TSIX plays a role in the healing process after fracture needs further verification.

Fracture healing is a complex process requiring sufficient numbers of osteoblasts to proliferate and differentiate[19]. The proliferation and apoptosis of osteoblasts play a key role in bone healing[20]. In addition, in the process of fracture healing, osteogenesis-related genes, Col-1a1, Col-II, and Col-x are important indicators of osteogenesis[21]. Therefore, exploring the effect of lncRNA TSIX on the proliferation of osteoblasts after fracture and the effect of lncRNA TSIX on apoptosis and osteogenesis-related genes *in vitro* and *in vivo* will be an important way to elucidate the function of lncRNA TSIX in fracture healing.

In our present study, we determined the expression of lncRNA TSIX in blood at different time points after fracture and explored the role and molecular mechanism of lncRNA TSIX/SOX6 axis in TF, which might provide a theoretical basis for the treatment of fracture.

Materials and Methods

Ethics Statement

All experiments were approved by the Animal Ethics Committees of Shanghai General Hospital of Nanjing Medical University in accordance with the principles of animal protection and the relevant provisions of National Animal Welfare and Ethics.

Animal Experiments

Totally eighteen C57BL/6J male mice aged about 8 weeks old were included in the current study, obtained from the Laboratory Animal Center of Shanghai Institutes for Biological Sciences (Shanghai, China). Mice were maintained under appropriate barrier conditions under a 12-h light/dark cycle and received food and water. The tibia fraction (TF) was performed at the middle of the left tibia of mice. After anesthetization, left tibia of mice was exposed and transected middle of the tibia, a bone nail (0.45 mm in diameter) was then used to fix it and then closed the wound. Two weeks prior to the tibia fraction surgery, mice were injected adeno-associated virus-9 (AAV9) targeted LncRNA TSIX to silence its expression *vs.* negative control adeno-associated virus-9, followed tibia fraction surgery.

Cell Lines and Culture

MC3T3-E1 (the mouse osteoblastic cell line) used in the study was purchased from Cell Bank of Chinese Academy of Sciences (Shanghai, China) and cultured in a humidified incubator at 37°C with an atmosphere of 5% CO_2. Cells were cultured in α-Modified Eagle's Medium α-MEM medium (Gibco, Rockville, MD, USA) supplemented with 10% fetal bovine serum (FBS; Gibco, Rockville, MD, USA) with 100 U/mL penicillin.

Cell Transfection

LncRNA TSIX overexpression plasmid (2 μg/mL) or shRNA plasmids (2 μg/mL), SOX6 overexpression plasmid (2 μg/mL) or SOX6 siRNA (75 nM) *vs.* respective nonspecific control were transfected to cells using Lipo3000 (Invitrogen, Carlsbad, CA, USA) following the manufacturer's instructions.

MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyl Tetrazolium Bromide) Assay

Cell viability was detected using MTT (Dojindo Molecular Technologies, Kumamoto, Japan), according to the manufacturer's instructions. Osteoblastic cells were seeded in 96-well plates and then exposed to further treatment. Approximately 20 μL of MTT (5 mg/mL) regent were added to each well and then incubated at 37°C for an additional 4 h. Cell growth was analyzed at 24, 48 h after transfection. The optical density was measured at 570 nm by using a microplate reader (Thermo Fisher Scientific, Waltham, MA, USA).

Real Time Polymerase Chain Reaction (RT-PCR) Analysis

Sample RNA was isolated from whole cell lysate or specific subcellular fractions or serum using PARIS™ Kit (Applied Biosystems, Foster City, CA, USA). Complementary deoxyribose nucleic acid (cDNA) was converted by using Reverse transcriptase reactions were incubated in a PTC-200 thermal cycler (Bio-Rad, Hercules,

CA, USA) and performed for further quantitative real-time PCR with SYBR Green (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's instructions. The data were calculated as fold changes with the $2^{-\Delta\Delta Ct}$ method. The primers used in the qRT-PCR are shown in Table I.

Western Blotting Analysis

According to the manufacturer's instructions, Total protein of cells or tissues were prepared using radioimmunoprecipitation assay (RIPA) buffer (Solarbio, Beijing, China). Then, samples were separated by 10% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) gel and transferred onto polyvinylidene difluoride (PVDF) membranes (Millipore, Billerica, MA, USA). Membranes were then blocked with 5% bovine serum albumin (BSA) for 2 h and probed with primary and secondary antibodies. The following primary antibody was used in experiment: glyceraldehyde 3-phosphate dehydrogenase (GAPDH; Santa Cruz Biotechnology, Santa Cruz, CA, USA), BAX (Santa Cruz Biotechnology, Santa Cruz, CA, USA), BCL2 (Santa Cruz Biotechnology, Santa Cruz, CA, USA), SOX6 (Santa Cruz Biotechnology, Santa Cruz, CA, USA) overnight at 4°C. The band intensity was quantified by using an Odyssey infrared scanner (Li-Cor Biosciences Inc., Lincoln, NE, USA).

Luciferase Reporter Assay

To generate the lncRNA TSIX wt-luc vector, a fragment of the 3'UTRs of lncRNA TSIX contained the target site of SOX6 was obtained *via* PCR amplification and subsequently cloned into the pGL3-Basic Vector (Promega, Madison, WI, USA). MutaBest kit (TaKaRa, Otsu, Shiga, Japan) was then used to generate lncRNA TSIX mutant-luc vector. 48 h after transfection, luciferase activities were examined by using a Dual-Luciferase reporter assay system (Promega, Madison, WI, USA).

Terminal Deoxynucleotidyl Transferase-Mediated dUTP Nick-End-Labeling Assay (TUNEL) Assay

Cell apoptosis was examined using Terminal deoxynucleotidyl transferase-mediated dUTP nick-end-labeling assay (TUNEL) kit (Roche, Basel, Switzerland) according to the manufacturer's instruction. After TUNEL staining, images were captured by Fluorescence Microscope System (Carl Zeiss, Jena, Germany).

H-E Staining

After mice were sacrificed and the tibia after fracture was extracted and fixed in 10% neutral buffered formalin (NBF) for 3 days. After that, the samples were rinsed with deionized water and decalcified with 14% ethylene diamine tetraacetic acid (EDTA) solution for 14 days, with solution changed every other day. After decalcification, the samples were stained with HE and then observed under an optical microscope (Olympus, Tokyo, Japan).

Statistical Analysis

GraphPad Prism software 6.0 (La Jolla, CA, USA) was used for statistical analysis, and all data were presented as mean ± standard deviation (SD). Differences between two groups were analyzed by using the Student's *t*-test. Comparison between multiple groups was done using One-way ANOVA test followed by Post-Hoc Test (Least Significant Difference). $p<0.05$ was considered statistically significant.

Table I. List for primers used for qRT-PCR.

Gene	Forward Primer	Reverse Primer
Lnc TSIX	AGTCTGCCCCTGTTAATGGGATG	TGGCCTAAGTCGGATGGAATCCCT
U1	GGGAGATACCATGATCACGAAGGT	CCACAAATTATGCAGTCGAGTTTCCC
GAPDH	GGAGCGAGATCCCTCCAAAAT	GGCTGTTGTCATACTTCTCATGG
Col1a1	GCTCCTCTTAGGGGCCACT	CCACGTCTCACCATTGGGG
Col-II	CTCCCAGAACATCACCTACC	TCGTGCAGCCATCCTTCAGG
Col-X	AGGCTACCTGGATCAGGCTTC	ACATTCTTTTCAGCCTACCTCC
18S	AAGCCTGGTAAGTGGCCGTA	TGGCTGAACTGGGTAACGTGA
SOX6	CGCGCCCTTGAATGCGTAGG	GGTCCTGAGTCCGTAGAATGT

Results

LncRNA TSIX Expression in Plasma of Tibia Fracture Mice Significantly Upregulated in a Time-Dependent Manner

To investigate the expression of lncRNA TSIX after fracture in plasma, RT-PCR analysis was used to detect the expression of lncRNA TSIX in plasma of tibial fracture mice and healthy control group at different time points. The results showed that the expression of lncRNA TSIX increased significantly at the 7-day and elevated in a time-dependent manner after fracture treatment in mice (Figure 1A). In addition, we used nuclear plasmid separation experiments and found that lncRNA TSIX was mainly expressed in the cytoplasm by RT-PCR analysis (Figure 1B).

Functional Analysis of LncRNA TSIX in the Osteoblastic Cells

To explore the function of lncRNA TSIX during osteogenesis, we constructed overexpression and knockdown vector of lncRNA TSIX. The expression of lncRNA TSIX was significantly upregulated or downregulated in osteoblastic cells after transfection with overexpression or knockdown vector of lncRNA TSIX, respectively (Figure 1C). MTT assay results revealed that overexpression of lncRNA TSIX significantly inhibited cell proliferation, while inhibition of lncRNA TSIX significantly promoted cell proliferation in osteoblastic cells (Figure 1D). Then, Western blot analysis and TUNEL assay results demonstrated that overexpression of lncRNA TSIX promotes osteoblastic cell apoptosis, but inhibition of lncRNA TSIX attenuates osteoblastic cell apoptosis (Figure 1E-1F). Lastly, we detected effect of lncRNA

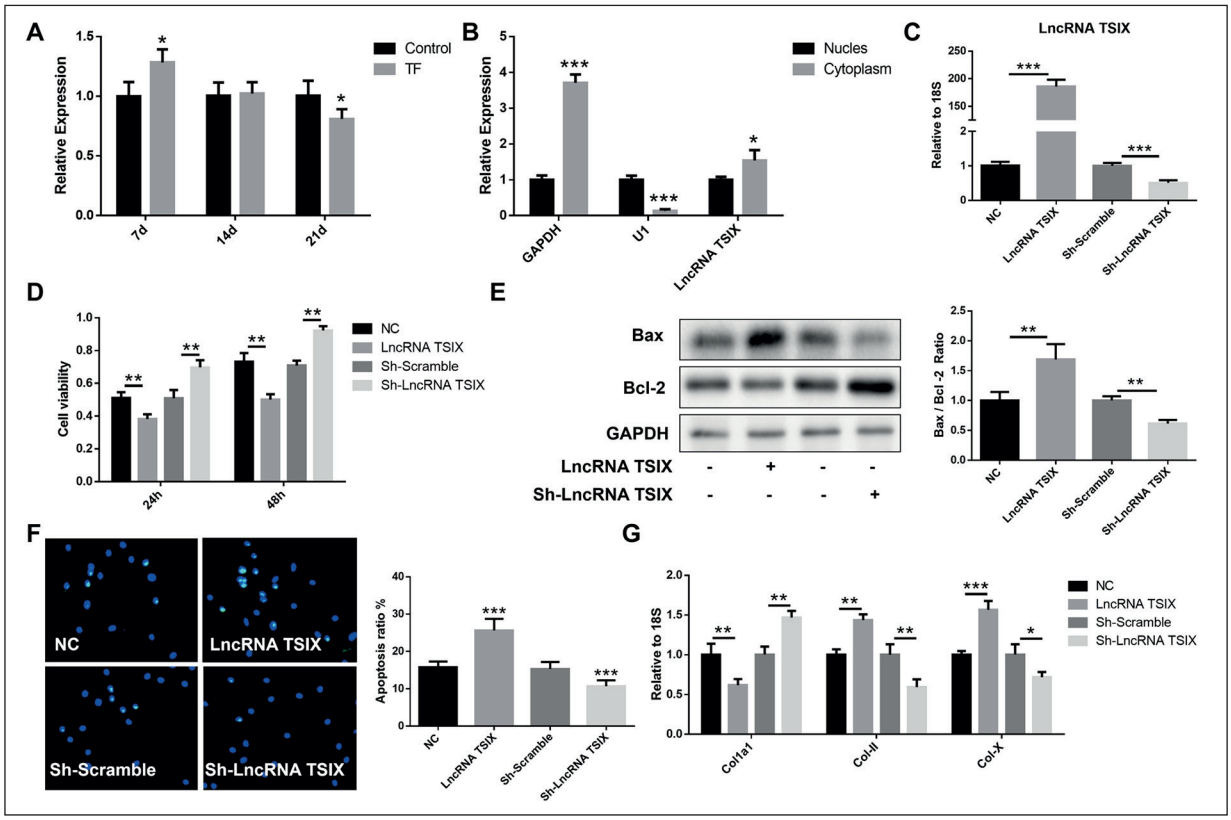

Figure 1. Biological features of LncRNA TSIX. **A,** Relative expression of LncRNA TSIX at different time after tibia fraction in mice was analyzed via serum detection (n=6). **B,** Location of LncRNA TSIX was examined via extracting specific subcellular fractions from MC3T3-E1 cell line (n=6). **C-G,** Function of LncRNA TSIX was examined after overexpression or inhibition. **C,** Relative expression of LncRNA TSIX was verified via real-time PCR after transfection of overexpression plasmid or shRNA plasmids (n=6). **D,** LncRNA TSIX inhibited cell proliferation but its inhibition showed promoting cell proliferation (n=6). **E-F,** LncRNA TSIX promoted cell apoptosis and its inhibition exerted reverse effect on apoptosis (n=6) (400×). **G,** Effect of LncRNA TSIX on the osteogenesis-related genes were examined *via* q-PCR analysis (n=6). *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$; *vs.* respective control.

TSIX on the osteogenesis-related genes. Overexpression of lncRNA TSIX could significantly inhibit Col1a1 expression but promote Col-II and Col-X expression, while knockdown of lncRNA TSIX showed the opposite effect in osteoblastic cells (Figure 1G).

SOX6 is the Target of LncRNA TSIX

Previous studies have shown that lncRNA TSIX could directly target binding downstream genes and regulate the protein expression to exert biological function. We further used bioinformatics analysis to predict the possible protein target of lncRNA TSIX. The predict results showed that SOX6 possessed binding sites with lncRNA TSIX**.** Dual-Luciferase reporter assay indicated that the relative activity was increased while reduced in wt group in cells co-transfected with SOX6 and overexpression or knockdown lncRNA TSIX, whereas no changes determined in mut group (Figure 2A). Lastly, we further wondered that lncRNA TSIX can positively regulate SOX6 expression by RT-PCR and Western blot analysis (Figure 2B-2C).

Knockdown of SOX6 Mediates the Role of LncRNA TSIX in the Osteoblastic Cells

To investigate whether SOX6 mediates the role of lncRNA TSIX during osteogenesis, we constructed siRNA of SOX6. The expression of SOX6 was significantly decreased in osteoblastic cells after transfection with SOX6 siRNA (Figure 3A). Moreover, we further co-transfected lncRNA TSIX overexpression vector and SOX6 siRNA in osteoblastic cells. MTT assay results showed that lncRNA TSIX significantly inhibits the proliferation of osteoblasts and could be partially restored by SOX6 siRNA (Figure 3B).

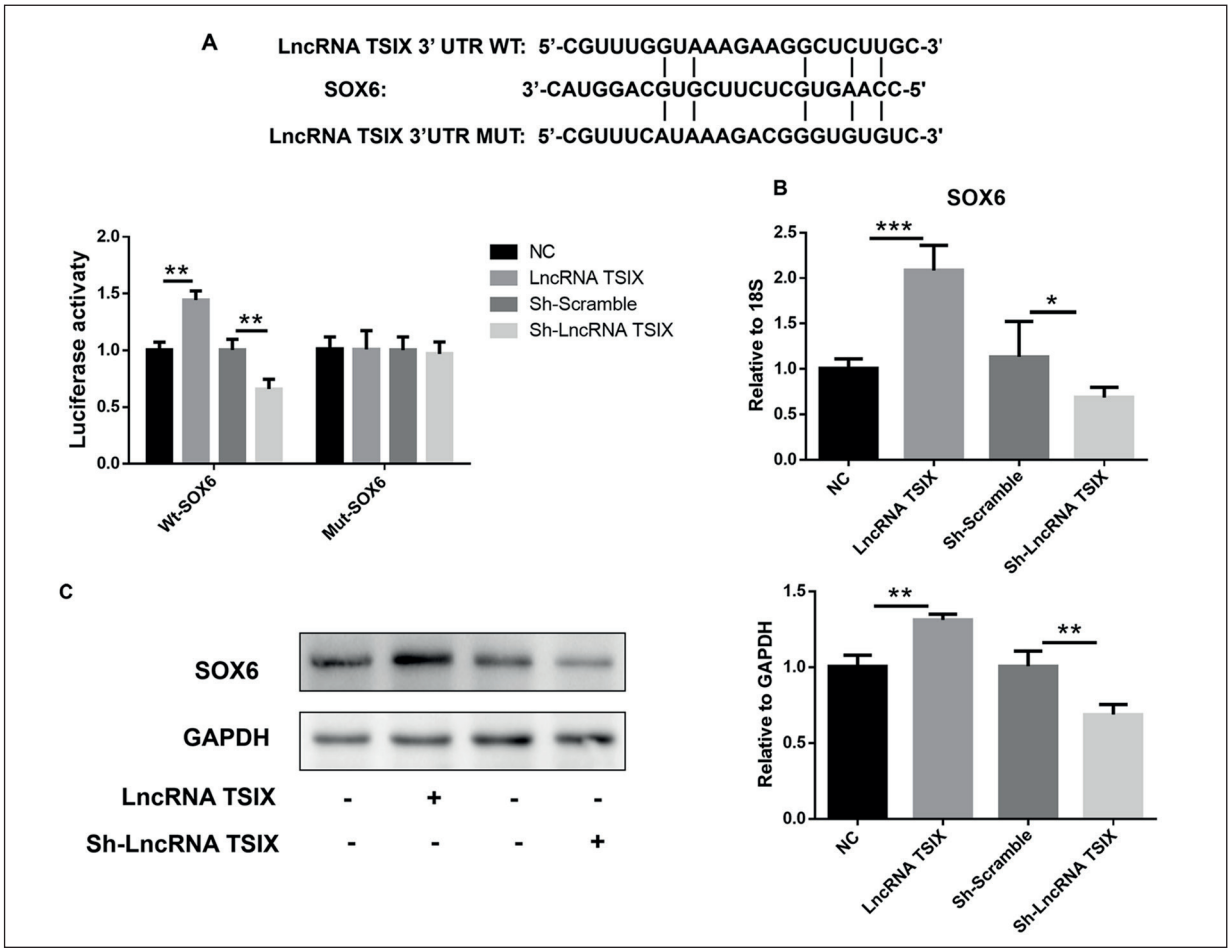

Figure 2. LncRNA TSIX performed its function *via* regulating SOX6. **A,** Luciferase reporter assay indicated SOX6 is the target of LncRNA TSIX. **B-C,** Q-PCR analysis and Western blot analysis showed LncRNA TSIX positively regulated the expression of SOX6 (n=6). *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$; *vs.* respective control.

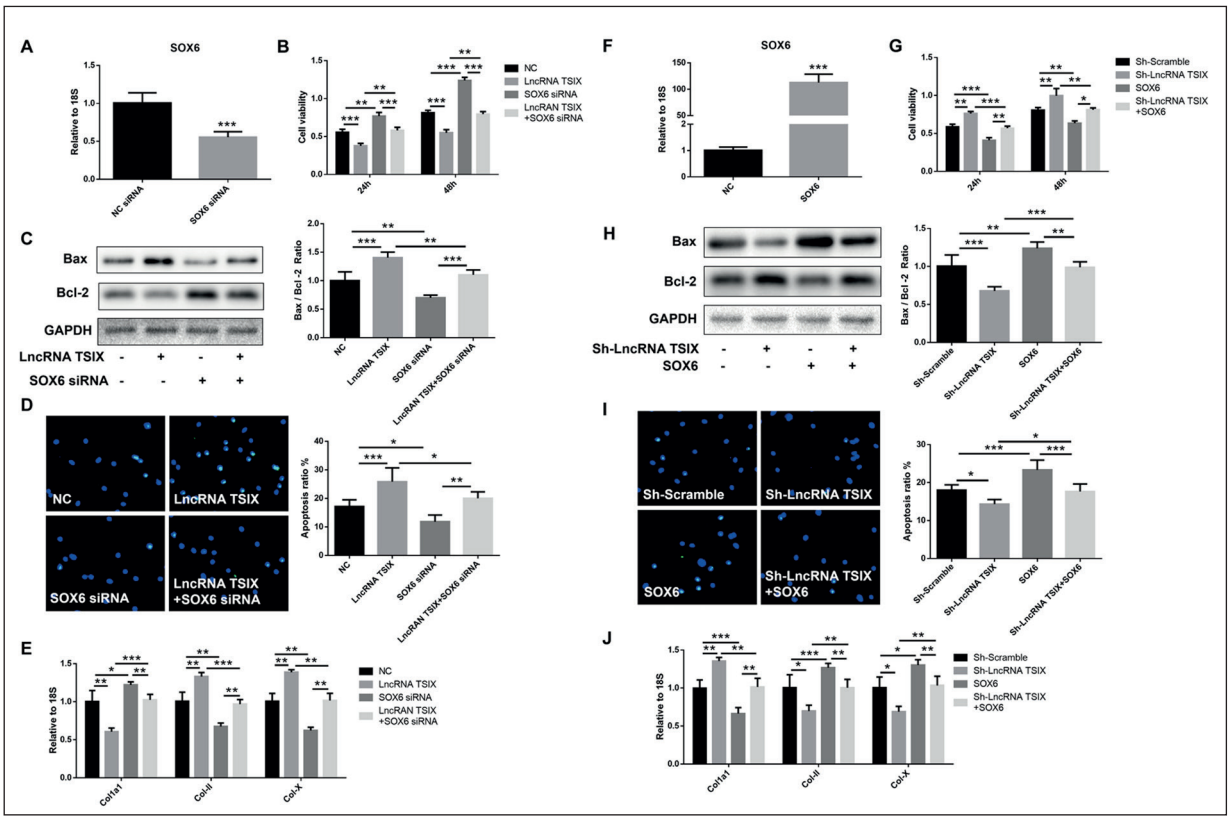

Figure 3. A-E, Inhibition of SOX6 abolished effect of LncRNA TSIX. **A,** RT-PCR analysis detected the efficacy of interfering SOX6 (n=6). **B,** SOX6 siRNA improved cell viability and diminished effect of LncRNA TSIX (n=6). **C-D,** Western blot analysis and TUNEL staining indicated SOX6 inhibition decreased apoptosis and attenuated LncRNA TSIX induced cell apoptosis (n=6) (400X). **E,** SOX6 siRNA reversed effect of LncRNA TSIX on the osteogenesis-related genes (n=6). **F-J,** Overexpressing SOX6 inhibited effect of interfering LncRNA TSIX. F. RT-PCR analysis revealed efficacy of SOX6 overexpressed plasmid (n=6). **G,** SOX6 decreased cell viability and diminished effect of inhibiting LncRNA TSIX (n=6). **H-I,** Western blot analysis (n=6) and TUNEL staining (n=6) exhibited SOX6 promoted cell apoptosis and declined effect of inhibiting LncRNA TSIX (n=6) (400×). **J,** SOX6 reversed effect of inhibiting LncRNA TSIX on the osteogenesis-related genes (n=6). *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$; *vs.* respective control.

Western blot analysis and TUNEL assay results wondered that overexpression of lncRNA TSIX promotes osteoblastic cell apoptosis, while could blocked by SOX6 siRNA (Figure 3C-3D). Consistent with the above results, overexpression of lncRNA TSIX could significantly inhibit Col1a1 expression but promote Col-II and Col-X expression and could be reversed after SOX6 siRNA treatment (Figure 3E).

Overexpression of SOX6 Reverses the Function of LncRNA TSIX in the Osteoblastic Cells

To further verify the role of SOX6-mediated lncRNA TSIX in osteoblasts, we constructed an overexpressed SOX6 vector. We first used SOX6 overexpression plasmid to elevate the SOX6expression (Figure 3F). Then, the lncRNA TSIX siRNA vector and SOX6 overexpression plasmid were co-transfected in osteoblastic cells. MTT assay results showed that the promotion of lncRNA TSIX inhibition on osteoblast proliferation was significantly reversed by SOX6 overexpression (Figure 3G). The results of RT-PCR and Western blot analysis showed that the inhibition of lncRNA TSIX on osteoblast apoptosis was significantly blocked by SOX6 overexpression (Figure 3H-5I). Lastly, the regulation of lncRNA TSIX inhibition on osteoblast-related genes was significantly restored by SOX6 overexpression (Figure 3J).

Inhibition of LncRNA TSIX Promoted Tibia Fraction Healing In Mice

To study the role of lncRNA TSIX in the process of fracture healing *in vivo*, we pre-admin-

istered lncRNA TSIX knockdown adenovirus *via* caudal vein 14 days before the construction of TF mice. LncRNA TSIX RNAi significantly inhibited the upregulation of lncRNA TSIX in TF mice (Figure 4A). The results of RT-PCR and Western blot analysis also showed that the up-regulated SOX6 expression in TF model was also inhibited after lncRNA TSIX RNAi treatment (Figure 4B-4C). Knockdown of lncRNA TSIX significantly inhibits TF-induced apoptosis in peri-fracture tissues detected by western blot analysis (Figure 4D). Furthermore, inhibition of lncRNA TSIX enhanced the Col1a1 gene expression inhibited by TF and inhibited the Col-II and Col-X gene expression induced by TF (Figure 4E). Similarly, HE staining results revealed that on the 21 day after TF, the callus was at the reconstruction stage and multiple relic woven bones existed in the TF. In lncRNA TSIX RNAi group, the callus remained reparative, the shape of callus was more compact and regular, there were more residual woven bones, more callus cartilage and callus around the fracture site (Figure 4F). These data suggest that lncRNA TSIX RNAi may promote TF mice fracture healing and advance functional recovery.

Discussion

At present, the most common theory of fracture healing is that the blood at the fracture site becomes the healed bone[22]. There are several ways to restore damaged bones, and the remarkable feature of bone repair is that there is no fibrous scar formation, which indicates that this process is also a form of tissue regeneration[23]. Nevertheless, delayed and nonunion rates are highest in fracture cases, usually associated with severe trauma or loss of surrounding muscle tissue[24]. In the process of fracture healing, the proliferation and apoptosis of osteoblasts directly affect the speed of fracture healing. The proliferation of osteoblasts increased significantly, while the apoptosis decreased significantly, which were important factors in the acceleration of healing. It is very important to find out the factors and genes that could regulate the proliferation and apoptosis of osteoblasts in the process of fracture healing. Therefore, in our study, we explored and demonstrated that inhibition of lncRNA TSIX could promote fracture healing *via* binding and positive regulating the SOX6 expression through *in vivo* and *in vitro* experiments.

LncRNAs expression could regulate cell proliferation and apoptosis in many diseases, including bone-related diseases[8]. Liu et al[25] have demonstrated that lncRNA MEG3 improves TF healing *via* inhibiting the Wnt/β-catenin signaling pathway. To explore the role of lncRNA TSIX in TF, we first constructed a model of tibial fracture in mice and found that the expression of lncRNA TSIX in blood increased significantly after tibial fracture. The proliferation level of osteoblasts after fracture was significant elevated in the early stage and decreased gradually in the later stage. The change of lncRNA TSIX expression in our study was negatively correlated with the proliferation curve of osteoblasts after tibial fracture. We further constructed lncRNA TSIX overexpression and knockdown vectors and found that overexpression of lncRNA TSIX could significantly inhibit the proliferation while promote apoptosis of osteoblasts. In addition, *in vivo* and *in vitro* experiments have proved that knocking down of lncRNA TSIX could enhance proliferation but reduce apoptosis, and regulate the expression of osteogenesis-related genes, and finally promote fracture healing. These data indicate that lncRNA TSIX is a functional non-coding RNA during fracture healing after tibial fracture.

In investigating the potential mechanism of lncRNA TSIX's function in tibial fracture, we predicted the genes that could potentially bind to lncRNA TSIX through biological software. Through further analysis of these genes, we focused on and validated the relationship between SOX6 and lncRNA TSIX. SOX6, a member of the SOX transcription factor family, has been proved to be involved in cell proliferation and apoptosis in many diseases[26,27]. Uusitalo et al[28] have shown that SOX6 mediated the proliferation of osteoblasts and fracture healing after fracture. In our present study, we observed that lncRNA TSIX could bind and positively regulate the expression of SOX6. Through further rescue experiments, we found that SOX6 could mediate the effect of lncRNA TSIX on osteoblast proliferation, apoptosis and healing after tibial fracture. These results demonstrated that inhibition of lncRNA TSIX promotes cell proliferation and healing after tibial fracture by binding and positively regulating SOX6 expression.

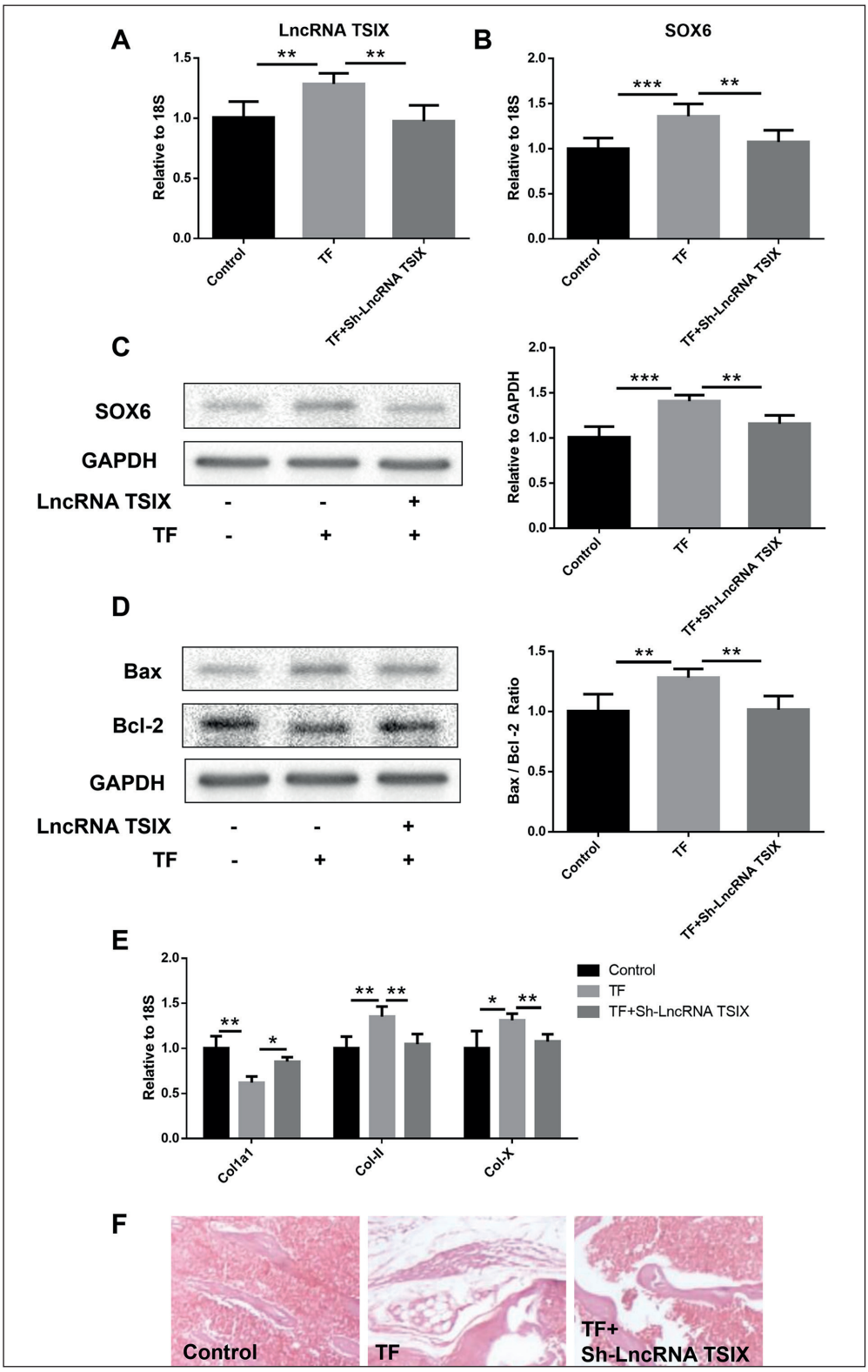

Figure 4. ShRNA of LncRNA TSIX promoted tibia fraction healing in mice. **A,** RT-PCR analysis determined expression of LncRNA TSIX in per group (n=6). **B-C,** Interference of LncRNA TSIX decreased expression of SOX6 induced by tibia fraction (n=6). **D,** Inhibiting LncRNA TSIX diminished TF induced apoptosis (n=6). **E,** LncRNA TSIX inhibition reversed expression of osteogenesis-related genes resulted from TF (n=6). **F,** H-E staining showed LncRNA TSIX interference promoted tibia fraction healing (200×). *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$; *vs.* respective control.

Conclusions

In summary, we proposed and proved the hypothesis that lncRNAs may play important roles in fracture healing. The main novelty of this study was that we first identified the expression changes and potential functions of lncRNA TSIX in the process of fracture healing. We confirmed that knockdown of lncRNA TSIX silencing enhances the proliferation, inhibits apoptosis, and regulates the osteogenesis-related gene expression after tibial fracture by binding and inhibiting the expression of SOX6. Our study suggests that lncRNA TSIX/SOX may have a crucial effect on fracture healing process after fracture. It provides a new insight for fracture healing treatment in the future.

Conflict of Interest

The Authors declare that they have no conflict of interests.

Acknowledgements

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