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Low-expression of lncRNA-ANCR promotes tibial fracture healing via targeting RUNX2

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Abstract. – OBJECTIVE: To explore the role of long non-coding ribonucleic acid-anti-differentiation non-coding ribonucleic acid (lncRNA-ANCR) in tibial fracture healing in rabbits by regulating the runt-related transcription factor 2 (RUNX2) expression.

MATERIALS AND METHODS: A total of 60 healthy adult rabbits were evenly divided into Control group (n=20), Fracture group (n=20), and Lnc group (n=20). Then, RUNX2 transfection, Real Time Polymerase Chain Reaction (PCR) assay and relevant instruments were carried out and used to determine the differences in dry weight, bone mineral density, bone mechanical strength, and RUNX2 expression in tibiae among three groups of rabbits.

RESULTS: Comparison of the bone mineral density in rabbit tibiae among the three groups showed that the bone mineral density was significantly lower in Fracture group than that in Control group ($p<0.05$), and it was slightly higher in Lnc group than in Fracture group ($p<0.05$). The dry weight of the full-length tibiae in Fracture group was significantly decreased compared with that in Control group ($p<0.05$), and Lnc group had an increased dry weight of tibiae in comparison with Fracture group ($p<0.05$). The maximum load, flexural strength, elastic stress, elastic strain, elastic modulus, maximum stress, and maximum strain in Fracture group were lower than those in both Control group and Lnc group ($p<0.05$). Compared with those in Fracture group, the amount of new collagen was overtly increased in Lnc group, and that of mature collagen was decreased ($p<0.05$). The relative expression level of RUNX2 in tibial bone tissues was evidently lower in Fracture group than that in Control group ($p<0.05$), and it was markedly higher in Lnc group than that in Fracture group ($p<0.05$).

CONCLUSIONS: Down-regulating lncRNA-ANCR activates and triggers the expression of RUNX2 that facilitates the growth and metabolism of bone tissues to play an important role in the repair of bone tissues and promote the healing of the tibial fracture.

Key Words:
LncRNA-ANCR, RUNX2, Tibial fracture, Healing.

Introduction

Tibial fracture, accounting for 9.45% of the total fractures, is caused by severe impact, sprain, falling down, kick, impact, wheel crushing or slipping[1]. It is characterized by swelling, pain, and other symptoms of the shank. It is also able to result in dyskinesia and arthrorrhagia[2]. Fractures caused by various factors have slow healing and unsatisfactory healing performance, endangering the quality-life of people[3]. Currently, the treatment of fractures is one of the hottest topics in the bone fracture field. Runt-related transcription factor 2 (RUNX2) protein plays a vital role in the formation of osteoclasts, extracellular matrixes, bone tissues, and the differentiation of osteoblasts. Besides, RUNX2 protein participates in multiple signal transduction processes. As a transcription factor, RUNX2 is a major participant in the differentiation of stem cells into osteo-

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cytes and chondrocytes[4,5]. It acts on many bone metabolism-related cytokines to regulate the division and activity of osteoblasts, which is mainly manifested by the binding to osteoblast-specific cis-acting elements to promote the transcription and expression of osteocalcin and osteopontin[6,7]. Researchers have found that lncRNA-anti-differentiation non-coding RNA (ANCR) is involved in various biological processes and capable of effectively activating osteosarcoma cells and promoting their proliferation and differentiation[8,9]. However, there is no study on the regulatory effect of lncRNA-ANCR on bone cell metabolism. Therefore, this work aims to investigate the influence of lncRNA-ANCR on the healing of fractures by regulating the expression of RUNX2.

Materials and Methods

Experimental Grouping and Model Establishment

A total of 60 healthy adult rabbits weighing (2.3±0.2) kg bought from the Laboratory Animal Center, Chongqing Medical University were evenly divided into Control group (n=20), Fracture group (n=20), and Lnc group (fracture + lncRNA-ANCR inhibitor gavage, n=20). Once the rabbits were anesthetized with sodium pentobarbital (30 mg/kg), the anterolateral skin of tibiae was cut open to expose subcutaneous tissues. Then, the joint capsule was cut open to expose tibiae. Next, a wire saw was used to saw through tibiae and 100 g/L compound antibiotic (penicillin: gentamicin = 2:1) was dropped into the bone defect that was about 0.3 cm, followed by suture and binding up without external fixation. After modeling, the rabbits in Fracture group and Lnc group were intramuscularly injected with penicillin sodium for anti-infective treatment for 2 days (once per day, 50 IU per injection). In Lnc group, the rabbits were subjected to daily intragastric administration with experiment reagent at a dose of 1.6 g/kg, while those in Control group were not treated. This research was approved by the Animal Ethics Committee of Southwest Medical University Animal Center.

Specimen Collection

After modeling, the rabbits were anesthetized with 1% pentobarbital sodium at a dose of 0.1 g/kg (body weight). Then, the rabbits were sacrificed. and the bilateral tibiae were removed under sterile conditions. Next, the tibiae were carefully dissected, the volume was measured *via* the buoyancy method and the tibiae were cryopreserved in a refrigerator at –80°C.

Determination of Bone Mineral Density

A double-energy X-ray absorptiometer (Medilink, Paris, France) was utilized to measure the bone mineral density of bilateral tibiae in different groups of rabbits at different time points. The tibiae were scanned longitudinally to observe the morphology of proximal tibiae. Then, the epiphyseal line was moved to 4.0 mm and 13.0 mm for tomography.

Measurement of Bone Weight

The bilateral tibiae were taken out and the soft tissues attached to them were removed thoroughly. Next, the full-length tibia specimen was placed in an oven for drying at 110°C for 10 h, while the dry weight of the tibia was measured accurately using an analytical balance (accuracy: 0.0001 g).

Detection of Biomechanical Properties of Tibiae

Test tibia was collected from each group and the length and diameter were measured using a Vernier caliper. Next, both ends of the test tibia were embedded with resin and fixed. The test tibia was kept straight, the embedding depth was uniform, and the contact surface of the self-curing resin base and the testing machine was smooth. To avoid the collection of broken bone tissues during the three-point bending test and the drying of the test tibia during the test, the test tibia was wrapped with a thin plastic paper. The fixed test tibia was subjected to the three-point bending test using a universal tester (WDW-20; Tianchen Electronic, Shenzhen, China). The bending strength limit was measured and the bone strength was determined based on the maximum load and elastic *modulus*. The pressure loading rate was 2 mm/min. The tibia fixed at both ends was placed on the wedge bracket of the mechanical testing machine, with the concave side of the tibia faced up. Then, the pressure rod applied pressure to the middle part of the tibia at a constant speed from the top to the bottom until the test tibia broke. Lastly, the entire test procedures were recorded using a computer connected to the mechanical testing machine. Results were calculated based on bone strength indicators measured in each group.

Determination of Bone Histomorphology

The proximal metaphysis of bilateral tibiae in each group was taken, fixed with 10% neutral

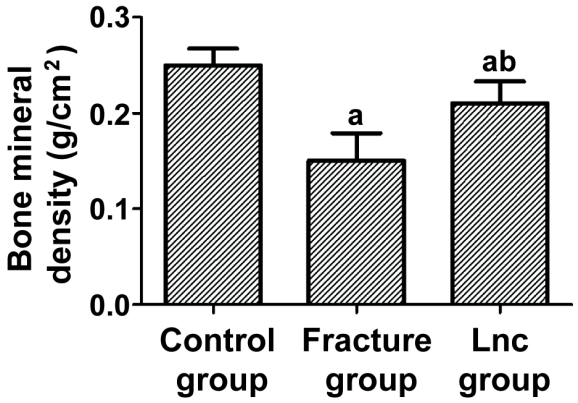

Figure 1. Comparison of bone mineral density among three groups of rabbits. Note: a: $p<0.05$ *vs.* Control group, b: $p<0.05$ *vs.* Fracture group.

formaldehyde, and decalcified with ethylenediaminetetraacetic acid (EDTA) decalcification solution. The tibia specimen was bisected at the coronal plane and dehydrated with gradient ethanol. Thereafter, bone tissues were treated and embedded with paraffin. Then, Masson staining assay was performed and the morphology of the bone was observed after sectioning for 6 times in a row.

RUNX2 Expression and Transfection

Eugonic rabbit BMSCs (cell density was 1×10^6 cells/mL) in Lnc group were taken and transfected with 5 mL medium and vector carrying RUNX2 for 3 consecutive days. During the transfection, the medium was changed every 24 h. After the transfection, the proportion of green fluorescence was observed using a fluorescence microscope and the transfection efficiency was analyzed.

Detection of RUNX2 Expression Level in Rabbits Through Real Time Polymerase Chain Reaction (PCR)

Real Time PCR assay was applied to detect the expression level of RUNX2 in rabbits as follows. RNAs were extracted from rabbit tibial bone tissues and reversely transcribed into cDNA. RT-PCR amplification was conducted using a SYBR Premix Ex Taq™ II kit (manufactured by TaKaRa, Otsu, Shiga, Japan). PCR conditions: 98°C for 6 min, 98°C for 28 s, 75°C for 30 s, and 80°C for 4 min, for a total of 55 cycles. Gene primer sequences are shown in Table I.

Statistical Analysis

Statistical Product and Service Solutions (SPSS) 19.0 software (SPSS Inc., Chicago, IL, USA) was used for processing all data in this paper. The *t*-test was used for comparisons of tibia dry weight, bone mineral density, bone mechanical strength, and Runx2 expression among Control group, Fracture group, and Lnc group. Univariate analysis was employed for analyses of data among different groups. Enumeration data were expressed as ($\chi\pm s$). $p<0.05$ indicated that the difference was significant.

Results

Comparison of Bone Mineral Density Among Three Groups of Rabbits

The comparison of bone mineral density in tibiae among three groups of rabbits revealed that the bone mineral density in tibiae was notably lower in Fracture group than that in Control group ($p<0.05$), while it was significantly higher in Lnc group than in Fracture group ($p<0.05$) (Figure 1).

Dry Weight of Tibiae in Three Groups of Rabbits

The dry weight of rabbit tibiae in full length was measured postoperatively and it was found that the dry weight of tibiae was decreased in Fracture group compared with that in Control group ($p<0.05$), while Lnc group exhibited a remarkably elevated dry weight of tibiae in comparison with Fracture group ($p<0.05$) (Figure 2).

Table I. Primer sequences.

Gene	Primer name	Primer sequences
RUNX2	Forward	ATGCATTTAAGATATGGTTGCC
	Reverse	TGGAGTTGGGAAACACTTGA
β-actin	Forward	GAGCTGTCTGCCTTGGTAGT
	Reverse	GCAGTCCTTCTGGCCCATAC

Table II. Comparison of bone mechanical strength among three groups of rabbits.

Bone mechanical strength	Control group	Fracture group	Lnc group
Maximum load (n)	63.10±9.64	40.00±6.22[a]	59.40±11.34[ab]
Elastic modulus (×10 Mpa)	3.90±0.16	1.98±0.08[a]	3.01±0.12[ab]
Flexural strength (Mpa)	142.30±6.35	69.60±6.83[a]	103.62±7.08[ab]
Elastic stress (Mpa)	52.10±7.34	24.60±4.65[a]	41.10±7.31[ab]
Elastic strain (%)	2.12±0.23	0.17±0.11a	1.53±0.22[ab]
Maximum stress (Mpa)	116.20±8.02	68.20±4.39[a]	96.57±7.36[ab]
Maximum strain (%)	3.84±0.24	1.24±0.33[a]	2.88±0.46[ab]

Note: [a]: $p<0.05$ *vs*. Control group, [b]: $p<0.05$ *vs*. Fracture group

Bone Mechanical Strength in Three Groups of Rabbits

The maximum load, elastic modulus, flexural strength, elastic stress, elastic strain, maximum stress, and maximum strain in Fracture group were lower than those in Control group and Lnc group ($p<0.05$) (Table II).

Histomorphology of Tibiae in Three Groups of Rabbits Detected

In Control group, the *trabecula* of the proximal tibia metaphysis was tightly connected and had no fractures, fewer pores, no abnormal increases in the volume of the medullary cavity and in the number of osteoclasts. In comparison with Control group, Fracture group showed a thicker cortex of bone. The *trabecula* had sparse structure and multiple fractures and was slender, with a significantly reduced degree of anastomosis between *trabeculae*, the enlarged volume of the medullary cavity, and a notably increased number of osteoclasts. There were certain improvements in Lnc group in comparison with those in Fracture group ($p<0.05$) (Figure 3). Under the Masson staining microscope, it could be seen that the new collagen was mostly stained blue, while the mature colla-

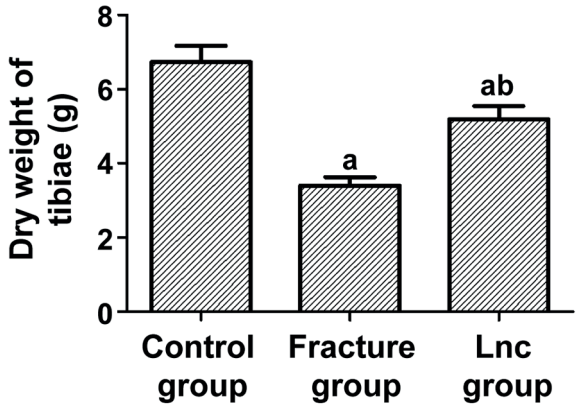

Figure 2. Comparison of dry weight of tibiae among three groups of rabbits. Note: a: $p<0.05$ *vs*. Control group, b: $p<0.05$ *vs*. Fracture group.

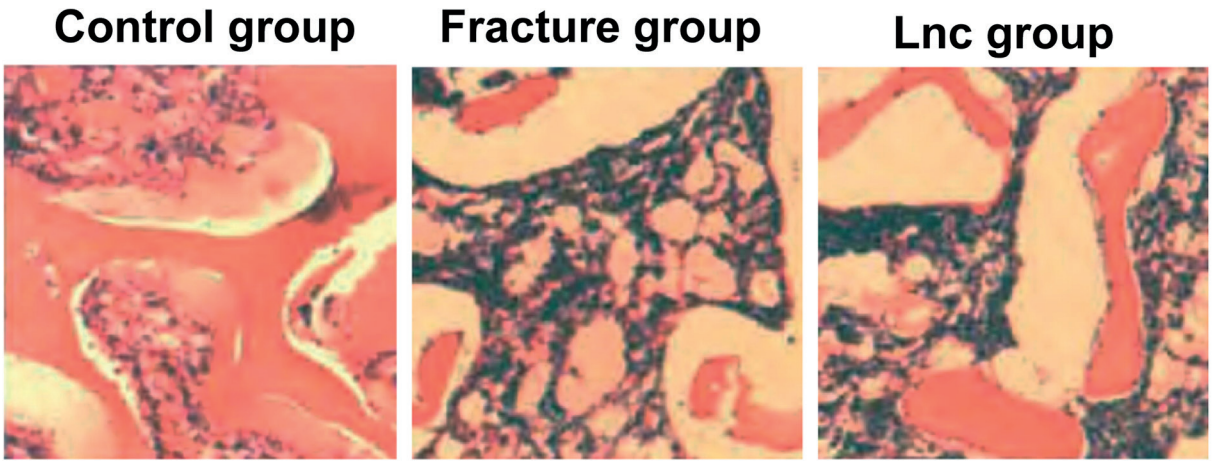

Figure 3. Comparison of morphology of tibial bone tissues among three groups of rabbits (magnification: 400×).

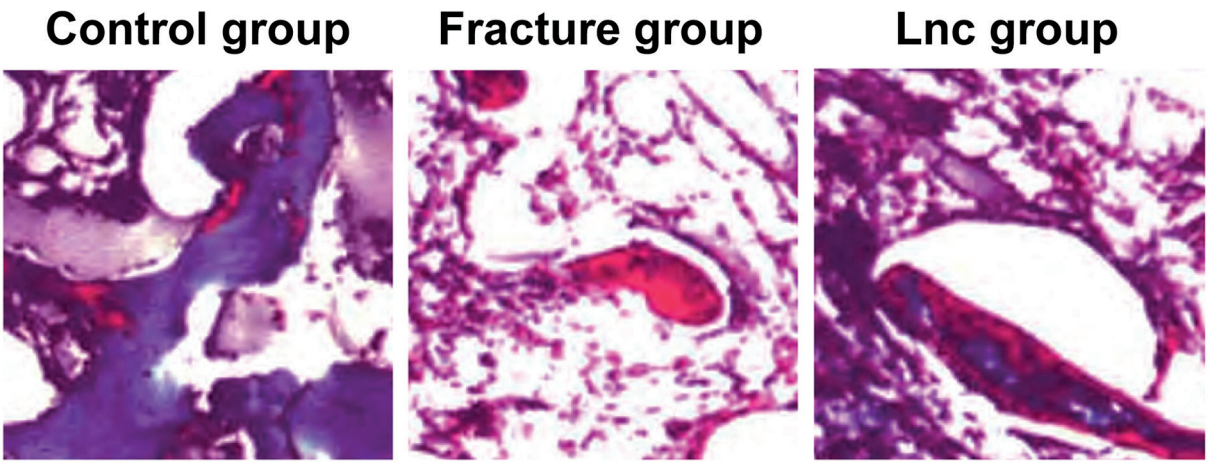

Figure 4. Comparison o f new collagen among three groups of rabbits (magnification: 400×).

gen was bright red. Results showed that compared with those in Fracture group, the amount of new collagen in bone was increased significantly in rabbit bone in Lnc group, while that of mature collagen was decreased (p<0.05) (Figure 4).

Expression of RUNX2 in Three Groups of Rabbits

The relative expression level of RUNX2 in tibial bone tissues was evidently lower in Fracture group than in Control group (p<0.05), and it was clearly increased in Lnc group compared with that in Fracture group (p<0.05) (Figures 5 and 6).

Discussion

Nonunion or delayed union often leads to problems including dyskinesia, postoperative pain, and other complications, seriously affecting the life and prognosis of patients[10]. With exacerbated ageing all over the world, fractures have become relatively severe problems worldwide and have strong impacts on the physical and mental health of the elderly[11]. Based on statistics, about 8% of patients with fractures in the world have delayed union and the healing of fractures is affected by factors such as trauma, energy, fracture end displacement, fracture type, and degree of injury of the surrounding soft tissues[12,13]. In this paper, rabbit models of tibial fracture were established to analyze the differences in tibial bone tissues among Control group, Fracture group, and Lnc group. Results revealed that the bone mineral density was clearly lowered in Fracture group compared with that in Control group (p<0.05), and it was increased in Lnc group compared with that in Fracture group (p<0.05). In comparison with Control group, Fracture group displayed a notable decreased dry weight of tibiae (p<0.05), while in comparison with Fracture group, Lnc group

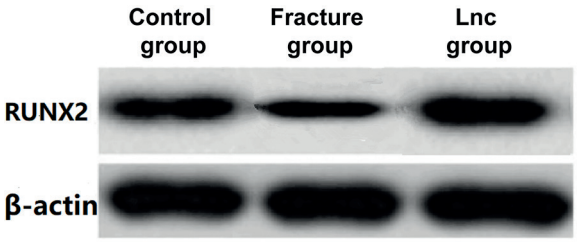

Figure 5. Protein expression of RUNX2 in three groups of rabbits.

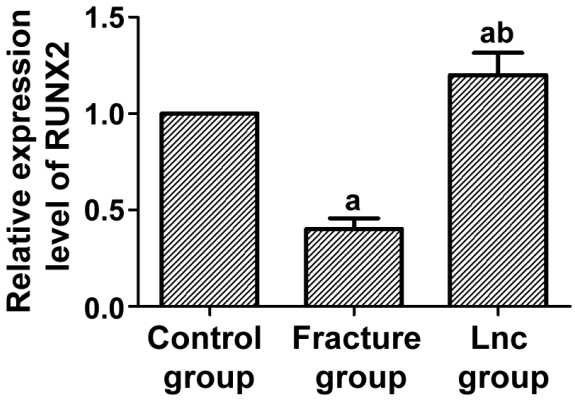

Figure 6. Comparison of relative expression level of RUNX2 among three groups of rabbits. Note: a: p<0.05 *vs.* Control group, b: p<0.05 *vs.* Fracture group.

exhibited a significantly elevated dry weight of tibiae ($p<0.05$). Research[14] has manifested that down-regulating lncRNA-ANCR facilitates the differentiation of periodontal ligament stem cells into osteoblasts, which is in line with the results of this study, suggesting that suppressing lncRNA-ANCR promotes the regeneration of bone cells and up-regulates the bone mineral density and dry weight of rabbit tibiae.

Here, it was found that compared with those in Control group and Lnc group, the maximum load, elastic modulus, flexural strength, elastic stress, elastic strain, maximum stress, and maximum strain were lowered in Fracture group ($p<0.05$). The cortex of bone was thicker in Fracture group than that in Control group. Besides, in Fracture group, the *trabecula* had sparse structure and multiple fractures and was slender, the degree of anastomosis between trabeculae was overtly reduced, the volume of the medullary cavity was enlarged, and the number of osteoclasts was evidently increased. In comparison with Fracture group, Lnc group had some improvements. The amount of new collagen in rabbit bone in Lnc group was significantly greater than that in Fracture group, while that of mature collagen was decreased. Reports[15,16] have found that the lowly-expressed lncRNA-ANCR has a close association with the microscopic three-dimensional structure of bone trabeculae, which can be used as an important index of bone mechanical strength and bone tissue weight bearing and compression resistance. Inhibiting lncRNA-ANCR is able to promote bone metabolism, increase the amount of new collagen in bone, and improve the mechanical strength of rabbit bone.

According to the results of this work, the relative expression level of RUNX2 in tibial bone tissues was significantly lower in Fracture group than that in Control group ($p<0.05$), while it was overtly higher in Lnc group than that in Fracture group ($p<0.05$), indicating that RUNX2 acts as a key regulator in fracture repair, determining the speed and quality of fracture repair. LncRNA-ANCR has an important regulatory effect on the expression of bone growth factor RUNX2[17]. RUNX2, an inducing factor widely existed in body tissues, can facilitate osteogenesis, chondrogenesis, the growth of bone tissues and the development of early bone tissues. Also, it can participate in the repair of bone defects and control the occurrence of certain bone diseases[18]. RUNX2 promotes the division and proliferation of osteoblasts, suggesting that this factor plays an all-important role in promoting fracture repair[19]. During the healing of fractures, bone formation, and refinement is a relatively balanced process[20]. The transcription of many genes relies on the regulation of RUNX2 which plays a vital role in the production and division of osteoblasts, division, and formation of chondrocytes, production, absorption of osteoclasts, and the formation of bone matrix proteins[21]. Moreover, RUNX2 has a highly specific effect on fracture healing. Repressing lncRNA-ANCR and inducing RUNX2 expression promote the healing of fractures. The influences of RUNX2 on the formation and differentiation of osteoblasts have been widely acknowledged and accepted. RUNX2 is a crucial factor in the differentiation of bone marrow mesenchymal stem cells (MSCs) into osteoblasts, while the expression of RUNX2 indicates the initiation of the above differentiation.

Conclusions

We found that lowering lncRNA-ANCR activates and triggers the expression of RUNX2 promoting the growth and metabolism of bone tissues, which is very important for bone tissue repair and facilitates the healing of the tibial fracture.

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Conflict of Interests

The Authors declare that they have no conflict of interests.

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