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MicroRNA-185 inhibits the growth and proliferation of osteoblasts in fracture healing by targeting PTH gene through down-regulating Wnt/β -catenin axis: In an animal experiment

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ABSTRACT

Fracture healing is a repair process of a mechanical discontinuity loss of force transmission, and pathological mobility of bone. Increasing evidence suggests that microRNA (miRNA) could regulate chondrocyte, osteoblast, and osteoclast differentiation and function, indicating miRNA as key regulators of bone formation, resorption, remodeling, and repair. Hence, during this study, we established a right femur fracture mouse model to explore the effect microRNA-185 (miR-185) has on osteoblasts in mice during fracture healing and its underlying mechanism. After successfully model establishment, osteoblasts were extracted and treated with a series of mimics or inhibitors of miR-185, or siRNA against PTH. Quantitative reverse transcription polymerase chain reaction (qRT-PCR) and western blot analysis were performed to determine the levels of miR-185, PTH, β-catenin and Wnt5b. Cell viability, cycle distribution and apoptosis were detected by means of MTT and flow cytometry assays. Dual luciferase reporter gene assay verified that PTH is a target gene of miR-185. Osteoblasts transfected with miR-185 mimics or siRNA against PTH presented with decreased levels of PTH, β-catenin and Wnt5b which indicated that miR-185 blocks the Wnt/β -catenin axis by inhibiting PTH. Moreover, miR-185 inhibitors promoted the osteoblast viability and reduced apoptosis with more cells arrested at the G1 stage. MiR-185 mimics were observed to have inhibitory effects on osteoblasts as opposed to those induced by miR-185 inhibitors. Above key results indicated that suppression of miR-185 targeting PTH could promote osteoblast growth and proliferation in mice during fracture healing through activating Wnt/β -catenin axis.

1. Introduction

Bone fracture is regarded as a significant health problem to the general public [1,2] any factors, such as age, gender, race, biology, etc. [3]. Some fractures frequently happened on childhood and young adulthood when they were doing sports, including tibia, fibula, and ankle [1]. Even though bone has a substantial capacity for repair and regeneration in response to injury, fracture healing is still a complex and multistage process aiming to response to injuries and finally restore bone function [4]. Fracture healing is a process of direct intramembranous healing or indirect intramembranous and endochondral healing, and indirect fracture healing occurred frequently than others [5]. Besides, bone fracture healing includes three phases including inflammation, bone formation and bone remolding, which all needs a timely sequence of interactions between mediators and cells [6]. Nevertheless, approximate 3–10% of fractures did not heal properly, leading to delayed union and non-union [7]. Thus, it is worthwhile to Figure out molecular mechanism of fracture healing.

As everyone know, Wnt axis acts as vital roles in skeletal

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development and homeostasis [8,9]. Recently, more and more researches have focused on the role of Wnt axis in fracture healing [10–12]. Wnt/β-catenin axiss involve with the bone formation, such as chondrogenesis, osteogenesis, and osteoclast formation [2,13]. As a canonical case, Wnt/β-catenin pathway promotes the formation of osteoblasts and chondrocytes that are principal cell types in the process of fracture healing [14,15]. PTH gene comprising 84 amino acid polypeptide can elevate related bone resorption [16]. Several studies reported that parathyroid hormone (PTH) gene mediated by Wnt axis, promoted both anabolic and catabolic in bone [14,17]. Moreover, a study had identified PTH was an anabolic medicine for osteoporosis and PTH effected on bone formation which regulated the development of a human recombinant peptide and the intermittent treatment improved fracture formation and enhanced mechanical strength in mice model [18].

Osteoblast differentiation is targeted by many factors, such as microRNAs (miRNAs) [19]. MiRNA plays a critical role in the different and complicated procedures during osteoblast differentiation [20]. MicroRNAs (miRNAs), consisted of 22 nucleotide, were a class of small, non-coding single-strand RNAs which regulate differently gene expression at post-transcriptional level [21–24]. Dong-Xu W et al. demonstrated that miR-185 can function as tumor suppressor via negative regulation of Wnt axis [25]. Collectively, we could find that there some relationship among miR-185, fracture healing, PTH gene, and Wnt axis but few researches were conducted to investigate the molecular mechanism of miR-185 in fracture healing via Wnt axis. Therefore, our study aimed to explore the influence of miR-185 on fracture healing by regulating PTH gene through Wnt/β -catenin axis in a mouse model.

2. Materials and methods

2.1. Establishment of fracture mouse model

Sixty six-week-old male C57BL/6 mice (ordinary class) were purchased from the Experimental Animal Center of Shanghai Jiaotong University School of Medicine, China. The mice were fed adaptively on the laboratory for 2 week. Health male mice (weighing 28 ± 2.78 g) were anesthetized by intraperitoneal injection of 2.5% Nembutal at a dose of 10 mg/kg. In deep anesthesia, mice were fixed in supine position and right knee was flexed to 90°. The outer side of the right knee was incised 1-cm longitudinally to expose distal femur and quadriceps tendon. Quadriceps tendon was moved to interior for adequate exposure of intercondylar groove of femur. Stainless steel in a diameter of 0.45 mm was stabbed into bone marrow and fixed the intramedullary nailing. The steel needle was cut and the needle tails were embedded into the skin to seal the wound. Nailed mice were moved to the table of the collision model along with placing nailed limbs on the flitch-plate. A weight of 500 g set was released from a height of 17 cm (adjust the falling height according to the size of mice) to result a right femoral fracture. After operation, all the mice were immediately taken X-ray using the Faxitron sample radiation system (model MX-20 DC12, Dalian Lindi Imp. & Exp. Co. Ltd, Dalian, China) on the same day to determine the modeling result [26,27]. All animal experiments were conducted with the approval of the Animal Ethics Committee of the Second Hospital of Lanzhou University.

2.2. Cells culture

After fracture model was established, mice were sacrificed to take the middle of the right femur. After washed by phosphate buffer saline (PBS) and D-Hank's fluid, aseptic femur was cut into 1-mm^3 pieces. The pieces were washed repeatedly with supernatant till they turned pale and then transferred into the tube containing 0.25% trypsin solution. Then, the bone pieces were digested in 25 mm HEPES (pH 7.4) containing 1 mg/ml collagenase I: II (1: 3) for 4 times and then further digested in EDTA and collagenase I: II, respectively. After digestive juice was discarded, the rest pieces were mixed with dulbecco's modified eagle medium (DMEM). Then the suspension was inoculated in a collagen-coated culture dish for culture, followed by placing the culture dish into an incubator with 5% CO_2 for a few days. The nutrient solution was changed after cells growth from the side of the bone block and then threw away bone blocks. The nutrient solution was change every two or three days. After 25-day culture till fusion generations, the cells were cultured in a basal medium to obtain undifferentiated osteoblasts. Then the third generation osteoblasts were selected for following experiment [28].

2.3. Cells transfection and grouping

Osteoblasts in the logarithmic growth phase were inoculated at 6-well plates. When cell density was increased to 30–50%, cells were transfected in accordance with the guidelines of lipofectamin-2000 (No. 11668019, Invitrogen Inc., Carlsbad, California, United States). A total of 250 μl non-serum-supplemented Opti-MEN was used to dilute 100 pmol miR-185 inhibitors, miR-185 mimics, miR-185 inhibitors + siPTH, siPTH and empty plasmid (the final concentration: 50 nM) [29,30], followed by mixing gently and incubating at room temperature for 5 min. A total of 250 μl non-serum-supplemented Opti-MEN was used to dilute 5 μl liPofectamin 2000, then mixed gently and incubated at room temperature for 5 min. The two kind of liquid were mix up. The mixed liquid was incubated at room temperature for 20 min and moved into culture plate. After cultured at 37 °C with 5% CO_2 for 6–8 h, cells were moved into complete medium for 24–48 h so as to continue the following experiments. Cells were divided into the blank (do not transfer any sequence), the negative control (NC) (transfected empty plasmid), the miR-185 mimics (transfected with miR-185 mimics), (transfected with miR-185 inhibitors), miR-185 inhibitors + siPTH (transfected with miR-185 inhibitors miR-185 inhibitors + siPTH) and siPTH groups (transfected siPTH). All the transfected cells were purchased from Shanghai Ji Ma Biological Company, shanghai, China.

2.4. Dual luciferase reporter gene assay

MicroRNA.org (a site of Biological prediction) was used to determine the target gene of miRNA. The relationship between PTH and miR-185 were verified by dual luciferase reporter gene assay. Firstly, recombinant plasmid of Luciferase Reporter Vector pPTH-Wt and pPTH-Mut were obtained to insert the 3′-untranslated region (3′-UTR) of PTH mRNA. The full-length 3′-untranslated region (UTR) of PTH gene were cloned and amplified. The polymerase chain reaction (PCR) product was cloned to the luciferase gene downstream of PmirGLO (No. E1330, Promega Corporation, Madison, WI, USA) and named pPTH-Wt. According to the Bioinformatics prediction of the binding site between miR-185 and the target gene, site-directed mutagenesis was utilized and PPTH-Mut vector was constructed. Promoter-Renilla luciferase reporter plasmid (pRL-TK) expressing renilla luciferase was served as an internal reference to adjust the transfection efficiency and the difference of the cell number. MiR-185 mimics and blank control (blank) were transfected into osteoblasts with luciferase reporter vector. The luciferase assay was performed according to the Promega Luciferase Assay System.

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