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Research on the Mechanism of miR-142-5p's Promotion of the Function and Mineralization of Osteoblasts by Down-regulating the Expression of WWP1 Gene

Lei Zhang[1], Wenrong Song[2], Jiangtao Song[1*]

Abstract

Objective: To study the mechanism of miR-142-5p's promotion of the function and mineralization of osteoblasts by down-regulating the expression of WWP1 gene.

Methods:a fracture model of wild-type SPF mice was established, and the expression of miR-142-5p in callus tissues of mice was detected by real-time quantitative PCR. The osteoblastic cell line SV40 was cultured in vitro. The SV40 cells were treated with agonistmiR-142-5p, agonist control, antagonist-miR-142-5p and antagonist control reagents. miR-142-5p-acquired and miR-142-5p-deficientSV40 cell modelswere constructed, and a blank control group was established. miR-142-5p was transfected respectively, and its effects on ALP mRNA, OC mRNA, matrix mineralization and mRNA and protein of the target gene WWP1 were analyzed.

Result:The levels of miR-142-5p in callus tissues on 7, 14, 21 and 28d were detected after modeling by quantitative PCR in real time and the results showed that miR-142-5p expression was significantly enabled during fracture healing, peaked at 21d after modeling, and marginally decreased at 28d. In the agonist miR-142-5p group, the levels of ALP mRNA, OC mRNA, and alizarin red S (ARS) were meaningfully higher than in the agonist control group, and The levels in the antagonist-miR-142-5p group for ALP mRNA, OC mRNA and ARS quantification were substantially lower than those in the antagonist control group. There was no substantial difference in WWP1 mRNA expression among four groups, the expression of WWP1 protein in the agonist miR-142-5p group was significantly lower than in the agonist control group; the expression of WWP1 protein in the antagonist-miR-142-5p group was meaningfully higher than in the antagonist control group.

Conclusion: the activation of the expression of miR-142-5p in callus tissues during fracture healing is closely related to the activity of osteoblasts, and supports the function and mineralization of osteoblasts. The mechanism may be related to miR-142-5p's down-regulation of EEP1 gene at the post-transcriptional level.l

Keywords: miR-142-5p, WWP1 gene, Osteoblast, Mineralization, Mechanism.

1. Introduction

1.Department of Orthopedics, Hanchuan People's Hospital, Hanchuan, PR China
2.Department of Endocrinology, Hanchuan People's Hospital, Hanchuan, PR China
**Lei Zhang, Hanchuan People's Hospital, No.1, Renmin Avenue, Hanchuan City, Hubei Province, China*
Email:taomumei899902@163.com

Fracture refers to the complete or partial fracture of the continuity of bone structure. Most patients have fractures at one site, and a few have multiple fractures, which are characterized by wideinvolvement range, complex etiology, diversified types and indefinite injury sites (Elizabeth

M. et al., 2017).There are mainly three causes of fracture: direct violence, indirect violence and cumulative strain, and the clinical signs includedeformity, abnormal movement, bony crepitus and bone rubbing sensation.In addition, patients often suffer from extensive soft tissue injury, massive bleeding or complicated visceral injury, which future results in shock (Matthew M. et al., 2018). Although with a strong fracture healing ability, most patients recover their original function. But still 5%~10% of fracture healing is obstructed and accompanied by different degrees of sequelae, leading to physical and mental disorders in patients and increases their economic burden (Takehito Ono & Hiroshi Takayanagi, 2017).Fracture healing refers to the local repair of traumas of bone tissues. A natural healing process includes three phases: inflammatory phase, repair phase and remodeling phase. This process is not only affected by the patients' own factors and environmental factors, but also influenced bymicro factors, such ascytokines andendocrine.Relevant data show that bone formation dominated by osteoblasts and bone resorptioncontrolled by osteoclasts play an important role in the reconstruction process of bones (Pieracci F.M. et al., 2017).The differentiation and maturation of osteoblasts involve a variety of growth factors and cytokines, and are closely related toa variety of signaling pathways and expressions of relevant genes.MiRNAisa kind of endogenous, which exerts various important regulatory effects in cells (Angela Jones et al., 2017). In recent years, miRNAsrelated to the regulation of the differentiation of osteoblasts and osteoclastshave been discovered successively. For this reason, this study attempts to analyze the mechanism of miR-142-5p's promotion of the function and mineralization of osteoblasts.

2. Materials and Methods

2.1. Experimental reagents and instruments

The osteoblastic cell line SV40 was obtained from the Chinese Cell Bank, the Trizolreagent was purchased from Invitrogen, USA; the paraformaldehyde was bought from Tianjin Damao Chemical Reagent Factory; the absolute ethanol, isopropanol and hydrochloric acid were purchased from Sinopharm Chemical Reagent Co., Ltd.; the chloroform was bought from Tianjin Fuyu Fine Chemical Co., Ltd.; the PBS was purchased from Beijing Zhongshan Jinqiao Biological Technology Co., Ltd.; the liver, kidney and bone alkaline phosphatase (ALP) kit and osteocalcin (OC) kit were purchased from USCN Life Science;theRIPA lysis buffer was purchased from Beijing Applygen Technologies; the WWP1 monoclonal antibody, β-actin monoclonal antibody and secondary antibody labeled were bought from Santa Cruz, USA.

The laminar flow cabinet and carbon dioxide cell incubator were purchased from Forma Scientific, USA; the electronic scalewas purchased from Sartorius, Germany; the bench-tophigh-speed refrigerated centrifuge purchased from Beckman, USA; the PCR cycler was purchased from Biometra, Germany; the spectrophotometer was purchased from Thermo Fisher Scientific, USA; the ELIASA was purchased from Bio-Tek, USA; the electrophoresis system was purchased from Bio-Rad, USA and the pipette was purchased from Eppendorf, Germany.

2.2. Establishing a fracture model of experimental mice

48 wild-type SPF mice,male, aged 10~11 weeks and weighed 24~28g were selected. They were provided by Laboratory named as Beijing Vital River that is owned by the Animal Technology Co., Ltd. and raised by the Animal Experimental Center of our hospitalataroom temperature of 22~24℃, with a humidity of 50%~60%. Clean and adequate amount of water and food were given. The mice can drink water and take food freely. After 5 days of adaptive feeding, the mice were anesthetized using 5% chloral hydrate.The body hair on the skin of lateral right femur was pruned, and the operation area on the lateral right femur was exposed. After disinfection, a 1 mm skin incision was made near the knee joint using scissors, and the patellar tendon was exposed.Using a syringe with needle 26g, the patellar tendon was broken throughalong the longitudinal axis of bone, the greater trochanter of femur was also broken through along the contourinside the femoral marrow cavity, a 2 mm-long needle was retained at the patellar tendon. A 1cm-long skin incision was made in the posterolateralright femur of mice, and the femur was exposed. The middle part of femoral shaft was cut open. If the needle in the marrow can be seen onthe section, the modelwas successfully established. An appropriate amount of penicillin powder was sprinkled on the operation area, the skin was disinfected using 75% alcohol and the incision was sutured.

2.3. Culturing the osteoblastic cell line SV40

The osteoblastic cell line SV40 was placed in α-MEN culture medium which contained 10% FBS, 100U/ml penicillin and 100U/ml streptomycin and cultured in a 5%CO2 incubator at 37℃. When the cells reached a confluence of 80% on the bottom, digestion and passage were carried out.Mineralization medium containing 50μg/ml ascorbic acid and 100Mβ sodium glycerophosphate was added to the cells.The SV40 cells were treated using agonist miR-142-5p, agonist control, antagonist -miR-142-5p and antagonist control reagents respectively, miR-142-5p-acquired and miR-142-5p-deficient SV40 cell models were constructed, and a blank control group was established.

2.4. Test method

①The expression of miR-142-5p in callus tissues was detected by real-time quantitative PCR,the total RNA of callus tissues frozen by liquid nitrogen was extracted withTrizolreagent, according to the instructions of reverse transcription kit. With total RNA as the template, cDNA was synthesized by reverse transcription. After reverse transcription, the cDNA product was removed immediately, cooled on the ice, diluted 3 times, stored in a -20 ℃ refrigerator andset aside. With cDNAas the template, the primer of miR-142-5pwas amplified. With β-actin as the internal control, the primer was prede natured at 95℃ for 3min, at 95℃ for 12 sec, at 62℃ for 55sec and amplified for 40 cycles. The relative quantification of miR-142-5p was done with Ct method(the data were analyzed using the formula of $2^{-\triangle\triangle CT}$).

②The effect of transfected miR-142-5pon the changes in the expressions of ALP mRNA and OCmRNA in the osteogenic cell line SV40 were detected by real-time quantitative PCR. The method and steps were the same as①.

③The effect of transfected miR-142-5p on the matrix mineralization of the osteoblast SV40: after being induced by mineralization solution for 21 days, the cells in each group were stained by alizarin red S (ARS). The ARS quantificationwas measured using a spectrophotometer.

④The effect of transfected miR-142-5pon the mRNA and protein of the target gene WWP1 of the osteoblast SV40: the detection of the mRNAof WWP1 was the same as①. The expression of the protein of WWP1was detected usingthe Western blot method. The SV40 cells in each group were collected. The protein was extracted using the Bradford assay, according to the instructions of protein quantification kit, and the concentrationof protein was tested. SDS buffer was added before electrophoresis, placed in 100 ℃ water bath for 10min and polyacrylamide gel electrophoresis was conducted. After the protein was isolated, it was electrotransferred to PVDF membrane and blocked for 1h. β-actin antibody at dilution of 1:200and WWP1 antibody at dilution of1:500were added at incubated at 4 ℃ overnight. A corresponding secondary antibody was added and incubated at the room temperature for 2h.The gray of the protein band was analyzed using Quantity Onesoftware.

2.5. Statistical method

All data in this study were analyzed using SPSS20.0 software package. All measurement data were expressed as ($\bar{x}\pm s$). The means of two groups were compared using an independent sample t-test, and the means of multiple groups were comparedusingvariance analysis. $P < 0.05$ was considered as statistically significant.

3. Result

3.1. Expression of miR-142-5p in callus tissues

The levels of miR-142-5p in callus tissues on 7, 14, 21 and 28d after modeling were detected by real-time quantitative PCR and the results showed that expression of miR-142-5p was significantly activated during fracture healing, peaked on 21d after modeling, and slightly decreased on 28d.See Figure 1.

3.2. The effect of transfected miR-142-5pon the changes in the expressions of ALP mRNA and OC mRNA in the osteogenic cell line SV40

The results of real-time quantitative PCR showed that the levels of ALP mRNA and OC mRNA in the agonist -miR-142-5p group were significantly higher than those in the agonistcontrol group ($P < 0.05$). See Table 1.

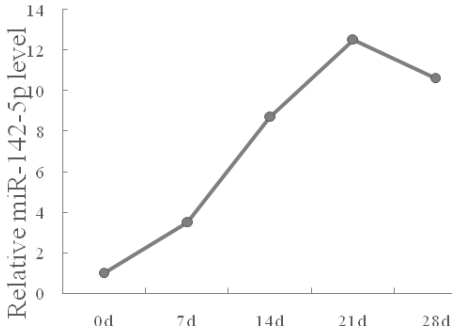

Figure 1: **Expression of miR-142-5p in callus tissues**

Table 1: **The effect of transfected miR-142-5pon the changes in the expressions of ALP mRNA and OC mRNA in the osteogenic cell line SV40 (x±s)**

Group	ALP mRNA	OC mRNA
Blank control	1.00±0.02	1.00±0.03
Agonist -miR-142-5p	2.26±0.24*	2.80±0.13*
Agonist control	1.07±0.03	1.05±0.04
Antagonist -miR-142-5p	0.42±0.01#	0.36±0.02#
Antagonist control	1.01±0.04	1.02±0.03

Note: *P＜0.05 compared with the agonist control group;#P＜0.05 compared with the antagonist control group

3.3. The effect of transfected miR-142-5p on the matrix mineralization ofthe osteoblast SV40

After being induced by mineralization solution for 21 days, the osteoblasts in each group were stained by alizarin red S (ARS), and the results showed that the ARS quantification in the agonist -miR-142-5p group was meaningfully higher than that in the agonist control group (P < 0.05), and the ARS quantification in the antagonist -miR-142-5p group was meaningfully slower than that in the antagonist control group (P < 0.05).See Figure 2.

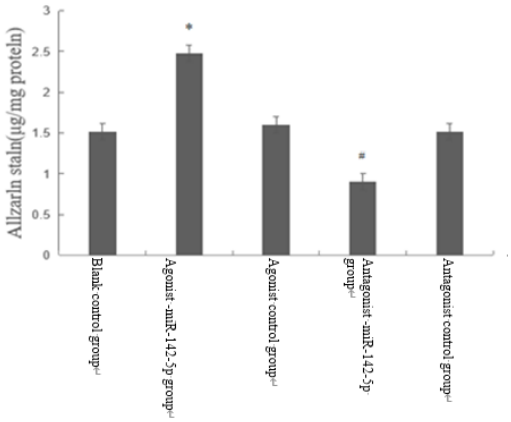

Figure 2: **The effect of transfected miR-142-5p on the matrix mineralization ofthe osteoblast SV40**

Note: $^{*}P$＜0.05 compared with the agonist control group; $^{\#}P$＜0.05 compared with the antagonist control group

3.4. The effect of transfected miR-142-5pon the mRNA and protein of the target gene WWP1 of the osteoblast SV40

The results of real-time quantitative PCR showed that there was no significant difference in the expression of WWP1 mRNA among four groups(P > 0.05); The expression of WWP1 protein in the agonist miR-142-5p group was significantly lower than that in the agonist control group (P < 0.05); the expression of WWP1 protein in the antagonist -miR-

142-5p group was meaningfully higher than that in the antagonist control group (P < 0.05).See Figures 3 and 4.

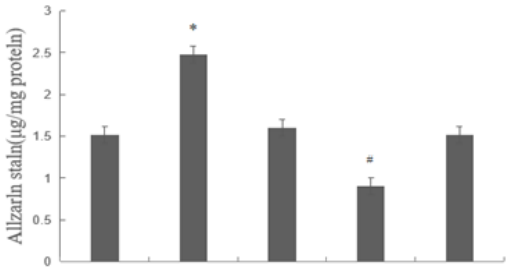

Figure 3: **The effect of transfected miR-142-5p on the mRNA of the target gene WWP1of the osteoblast**

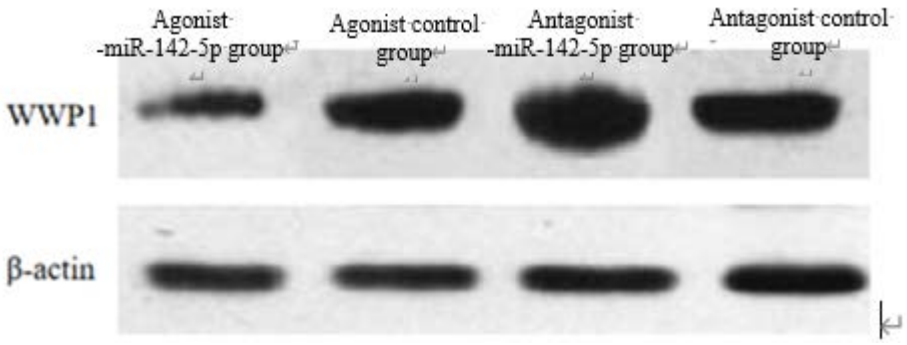

Figure 4: **The effect of transfected miR-142-5pon the protein of the target gene WWP1of the osteoblast**

4. Discussion

Fracture is the most common disease in orthopedics in clinical practice. The bone tissues are regenerative tissues. The fracture healing is a complex dynamic process that involves histology, endocrinology and biomechanics. Although general fractures can be cured by surgical approaches, still some fracture patients experience bone un-union and delayed healing due to improper treatment or other reasons, which is always a Gordian knot and huge challenge confronting clinical traumatic orthopedic surgeons (Gabriela Rover et al., 2017)(Gaume J. et al., 2017). Therefore, it is of great significance to explore factors related to fracture healing for the clinical treatment of bone healing. Relevant data have showed that besides the bone tissues proper, nerve tissues and neuropeptides contained in them are also closely related to the prognosis of fractures (Young-Chang Park et al., 2017)(J. Reinoso et al., 2017).The nature of fracture healing is a continuous process of bone destruction, removal and regeneration based on the regeneration of periosteal osteoblasts. Osteoblasts and osteoclasts interplay to regulate bone resorption/bone formation (PanagiotisAnagnostis et al., 2018). MiRNAis a kind of endogenous and non-coding single-stranded RNA, widely present in eukaryotes, with obvious cell specificity and tissue specificity. It can degrade the mRNA of target genes rapidly and effectively or inhibit the translation of target protein and has a broad application prospect in genetic treatment and the diagnosis and treatment of autoimmune disease[11].In recent years, miRNAs related to the regulation of the differentiation of osteoblasts and osteoclasts have been discovered successively. Runx2 and its downstream molecule Osterix are important specific transcription factors that regulate the differentiation of osteoblasts. miRNA can activate or inhibit bone differentiation through their interactions (Julie Massart et al., 2017).

In this study, to prove the correlation between miRNA-related factors and bone healing, the author established a fracture model of mice. The levels of miR-142-5p in callus tissues on 7, 14, 21 and 28d after modeling showed that expression of miR-142-5p was significantly activated during fracture

healing, peaked on 21d after modeling, and slightly decreased on 28d.This suggested that the activation of the expression of miR-142-5p was closely related to the activity of osteoblasts. The preosteo blasts of SV40 mice were transfected using miRNA agonist miR-142-5p and antagonist miR-142-5p, which had gone through special chemical modification. miR-142-5p-acquiredandmiR-142-5p-deficient models were constructed. The levels of ALO and OC in the cells were detected to explore the functions of miR-142-5p, and it was found that the levels of ALPmRNA, OC mRNA and ARS quantification in the agonist -miR-142-5p group were meaningfully higher than those in the agonist control group ($P < 0.05$), and the levels of ALPmRNA, OC mRNA and ARS quantification in the antagonist -miR-142-5p group were significantly lower than those in the antagonist control group, suggesting that miR-142-5p can support the function and mineralization of osteoblasts.

Located at 8q21, WWP1 gene is a member of the E3 ubiquitin ligase family. Generally speaking, ubiquitin ligase can ubiquitinate the protein of objective protein and form a polyubiquitin chain (Satoko Kojima et al., 2017). Existing studies have shownthatWWP1 can participate in a variety of cell signaling pathways and the formations of tumors through ubiquitination (Davis K.A., 2017). Some scholars find through animal studies that after the WWP1gene of mice is knocked out, the activity and bone formation of osteoblasts are significantly improved, which can get involved in the occurrence of osteoporosis by promoting the ubiquitination of the positive regulator of the differentiation of osteoblasts (Sheila McCormick, 2018).In this study, by analyzing the effect of transfected miR-142-5pon the mRNA and protein of the target gene WWP1 of the osteoblast SV40, this author found that WWP1 protein in the agonist -miR-142-5p group was meaningfully lower than that in the agonist control group, andWWP1 protein in the antagonist-miR-142-5p group was significantly lower than that in the antagonist control group ($P < 0.05$). Therefore, we speculated that miR-142-5p's support of the function and mineralization of osteoblasts maybe associated with the regulation of WWP1 expression.

In summary, the activation of the expression of miR-142-5p in callus tissues during fracture healing is closely related to the activity of osteoblasts, and supports the function and mineralization of osteoblasts. The mechanism may be related to miR-142-5p's down-regulation of EEP1 gene at the post-transcriptional level.

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