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MiR-204 promotes fracture healing via enhancing cell viability of osteoblasts

N. ZHANG[1], R.-F. ZHANG[2], A.-N. ZHANG[3], G.-X. DONG[1], N. SUO[1], Z.-P. WU[1], Y.-M. LIU[1], L.-T. WANG[1]

[1]Department of Orthopedics, Harrison International Peace Hospital, Hengshui, China
[2]Department of Orthopedics, the 89th Hospital of People's Liberation Army, Weifang, China
[3]Medical Insurance Management Section, Weifang Maternal and Child Health Care Hospital, Weifang, China

Ning Zhang and Rongfeng Zhang contributed equally to this work

Abstract. – OBJECTIVE: To investigate the effects and related mechanisms of miR-204 on fracture healing.

MATERIALS AND METHODS: Mouse osteoblastic cell line MC3T3-E1 was used in our experiment. Three groups were established to investigate the potential function between miR-204 and osteoblastic cells: miR-NC group (negative control), miR-204 mimics group (MC3T3-E1 cells transfected with miR-204 mimics) and miR-204 mimics + inhibitor group (MC3T3-E1 cells transfected with miR-204 mimics and inhibitor). After incubation, cell viability, activity of caspase-3, and migration ability of MC3T3-E1 cells, were measured. Further, the expression levels of Runt-related transcription factor 2 (RUNX2) and Osterix (OSX) were detected and analyzed.

RESULTS: Compared with miR-NC group, the cell viability and migration ability of MC3T3-E1 cells were enhanced while the activity of caspase-3 was respectively mitigated. Besides, the expression level of RUNX2 and OSX was increased by treatment of miR-204 mimics. However, these variations of the indicators were reversed by the intervention using miR-204 inhibitor.

CONCLUSIONS: We revealed the promotion effect of miR-204 on fracture healing, indicating that miR-204 could be a potential therapeutic target for the treatment of a fracture.

Key Words
Fracture healing, miR-204, Osteoblasts, Runt-related transcription factor 2 (RUNX2), Osterix (OSX).

Introduction

With the social-economic development and aging of population, osteoporosis has become a major disease affecting the health of all human beings. Fracture is one of its severest complications. Moreover, fracture is also one of the most common clinical diseases in orthopedics, seriously affecting the patient's quality of life and even threatening the life once it occurs[1-3]. A complex and highly-regulated bone regeneration process will be triggered by fracture, so as to restore the original structure and function of bone. Therefore, investigating the molecular biological mechanism in the fracture healing process is helpful to improve fracture healing and reduce disability and mortality rates due to fracture.

The bone regeneration process consisting of old bone removal and new bone formation is constantly repeated in normal bone tissues, and the osteoblast/osteoclast function coupling is the basis for maintaining the bone remodeling[4]. The damaged bone cells will stimulate the activity of osteoclasts when the microfracture occurs, resulting in bone resorption in the damaged area and initiating bone remodeling[5]. After the old bone resorption, the procollagen synthesized by osteoblasts will enter the extracellular space, forming the type I collagen network. Moreover, the procollagen deposits on the ossein rack in the form of calcium phosphate crystal, producing the mineralized bone matrix, thereby completing the bone remodeling[6]. Therefore, osteoblast plays an important role in fracture healing process.

Micro ribonucleic acids (miRNA) are a kind of single-stranded, non-coding RNAs with over 25 nucleotides in length. MiRNAs can exert their regulatory roles via complementary pairing of 3'UTR, thus degrade or inhibit the translation of target mRNAs at the post-transcriptional level[7]. Besides, miRNA regulates the gene expression at the post-transcriptional level, thus playing important roles in physiological, developmental and

Corresponding Author: Litao Wang, MM; e-mail: znasyzs@163.com

pathological processes. Differentiation and function of osteoblasts are also regulated by miRNA[8-10].

In this experiment, the regulatory effect of miR-204 on fracture healing was investigated, in order to elucidate the interaction mechanism of miRNA and protein-coding genes on the function of osteoblasts, and lay a foundation for the regulation of biological behavior of chondrocytes using miRNA in clinic, which is expected to provide valuable clues for the treatment of fracture.

Materials and Methods

Cell Culture and Treatment

Mouse osteoblastic cell line MC3T3-E1 was purchased from the Chinese Academy of Sciences (Shanghai, China). All cells were incubated in α-modified minimal essential medium (α-MEM, Gibco, Grand Island, NY, USA) complemented with 10% fetal bovine serum (FBS), 100 mg/mL streptomycin and 100 IU/mL penicillin (Invitrogen, Carlsbad, CA, USA) in 5% CO_2 cell culture incubator.

Cell Transfection and Treatment

miR-204 mimics and inhibitor were synthesized and transfected to osteoblastic cells to analyze the potential biological function of miR-204. Next, the three groups were established to study the potential relevance between miR-204 and osteoblastic cells: miR-NC group (negative control), miR-204 mimics group (MC3T3-E1 cells transfected with miR-204 mimics) and miR-204 mimics + inhibitor group (MC3T3-E1 cells transfected with miR-204 mimics and inhibitor). All the stuff was purchased from RiboBio and transfected by using lipofectamine RNAiMAX (Life Technologies, Gaithersburg, MD, USA) according to the manufacturer's instructions.

Measurement Cell Viability

When cells grew to the logarithmic growth phase, MC3T3-E1 cells were harvested and inoculated into 96-well plates at a density of 2 x 10^3 cells for 48 hours. MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) solution (5 mg/mL, MultiSciences, Hangzhou, China) was appended to each well after 4-h incubation. Then, 150 μL of dimethyl sulfoxide (DMSO) were added to each well for solubilizing the formazan formed. 30 min later, the absorbance was measured using a microplate reader (Bio-Rad, Hercules, CA, USA) at 490 nm.

Measurement of Caspase-3 Activity

Caspase-3 activity assay kit (Beyotime, Shanghai, China) was used to detect the activity of Caspase-3, according to manufacturer's protocols. Osteoblastic cells were lysed, then 40 μg cytosolic protein were incubated with reaction buffer for 1 h at 37°C. The samples were measured with a microplate reader (Biotek, Winooski, VT, USA) at an absorbance of 405 nm.

Cell Migration Assay

Cell migration was performed by transwell assay: after cells were counted, 5×10^5 cells were added into the chamber and 500 μL serum-free medium were added into the upper chamber, while 800 μL Roswell Park Memorial Institute 1640 (RPMI 1640) medium (HyClone, South Logan, UT, USA) containing 10% fetal bovine serum were added into the lower chamber. After cell migration for 12-18 h, the culture solution in both upper and lower chambers was discarded, the filter membrane was washed with pre-heated phosphate-buffered saline (PBS), and PBS was gently blown and beaten to clean the lower surface of filter membrane. 600 μL 4% paraformaldehyde were added into the lower chamber, so that the lower surface of filter membrane was immersed into it to fix cells for 15 min. After the fixing solution was discarded, the transwell chamber was placed upside down, so that the lower surface of filter membrane faced upwards and air-dried naturally. After drying, several drops of Giemsa stain were added directly on the lower surface of filter membrane of the inverted transwell chamber for 10 min. After the filter membrane was washed with distilled water, cells not migrating on the surface were wiped off using a cotton ball, followed by observation under an inverted microscope and counting.

Quantitative Reverse Transcriptase-Polymerase Chain Reaction (qRT-PCR)

Total RNA was procured by TRIzol Reagent (Invitrogen, Carlsbad, CA, USA) in accordance with the manufacturer's protocol. SYBR green qPCR assay (Invitrogen, Carlsbad, CA, USA) was used to measure the level of Runt-related transcription factor 2 (RUNX2), Osterix (OSX) expression and endogenous controlled by glyceraldehyde 3-phosphate dehydrogenase (GAPDH). TaqMan miRNA assays (Applied Biosystems, Waltham, MA, USA) were used to measure the level of miR-204 expression normalized to miRNA U6. The primer sequences were as

the follows: miR-204: CTGTCACTCGAGCTGCTGGAATG, R: ACCGTGTCGTGGAGTCGGCAATT; RUNX2: F: ACCAGCAGCACTCCATATCTCTAC, R: CTTCCATCAGCGTCAACACCATC; OSX: F: CCCCACCTCTTGCAACCA, R: CCTTCTAGCTGCCCACTATTTCC; U6: F: GCTTCGGCAGCACATATACTAAAAT, R: CGCTTCAGAATTTGCGTGTCAT; GAPDH: F: CGCTCTCTGCTCCTCCTGTTC, R: ATCCGTTGACTCCGACCTTCAC.

Western Blot

Osteoblastic cells were harvested and lysed using radioimmunoprecipitation assay (RIPA) buffer (Beyotime, Shanghai, China). The total protein was extracted, and its concentration was determined according to instructions of the bicinchoninic acid (BCA) protein concentration kit (Thermo Fisher Scientific, Waltham, MA, USA). The same amount of total protein was separated *via* sodium dodecyl sulfate polyacrylamide gel electropheresis (SDS-PAGE), transferred onto a nitrocellulose membrane, sealed with 5% skim milk and incubated with rabbit anti-rat RUNX2, OSX and β-actin primary antibodies (1:1000) at 4°C overnight. Next, the membrane was washed 3 times by tris-buffered saline with Tween-20 (TBST) (Beyotime, Shanghai, China), anti-rabbit secondary antibody (coupled by horseradish peroxidase) was added and incubation for 2 h, followed by development *via* enhanced chemiluminescence (ECL, Thermo Fisher Scientific, Waltham, MA, USA), exposure in gel imaging system, fixation and observation of results. With β-actin as an internal reference, the relative changes in protein expression were detected.

Statistical Analysis

Statistical analysis was performed with a Student's *t*-test or *F*-test. Comparison between groups was done using One-way ANOVA test followed by Post-Hoc Test (Least Significant Difference). All *p*-values were two-sided and $p<0.05$ were considered significant and analyzed by Prism 6.02 software (La Jolla, CA, USA).

Results

The Expression of miR-204 was Up-regulative by Mimics Intervene

As shown in Figure 1, compared with miR-NC group, the expression level of miR-204 significantly increased after the intervened with mimics. However, the results from mimics + inhibitor group indicated that the inhibitor could counteract the effect of mimics and inhibit the miR-204 expression (Figure 1).

MiR-204 Promoted Cell Viability of Osteoblasts

To examine the effect of miR-204 on the viability of osteoblasts, we took MTT assay to detect the cell viability. The results from the MTT assay revealed that the cell viability was remarkably increased after up-regulation of miR-204 expression, whereas down-regulating miR-204 expression level by inhibitor could markedly suppress the cell viability (Figure 2A).

MiR-204 Inhibited Caspase-3 Activity of Osteoblasts

The activity of caspase-3 was measured in our experiment. Caspase-3 is a characteristic sign of apoptosis[11]. Our results indicated that caspase-3 activity was significantly decreased by intervened with miR-204 mimics; however, the activity was found increased in the mimics + inhibitor group (Figure 2B).

MiR-204 Stimulated the Migration Ability of Osteoblasts

Migration is a key factor in cell proliferation. In the transwell experiments, we got the results that the migration ability of osteoblastic cell was increased by up-regulation of miR-204 with mimics meanwhile the migration ability was limited by inhibitor (Figure 3).

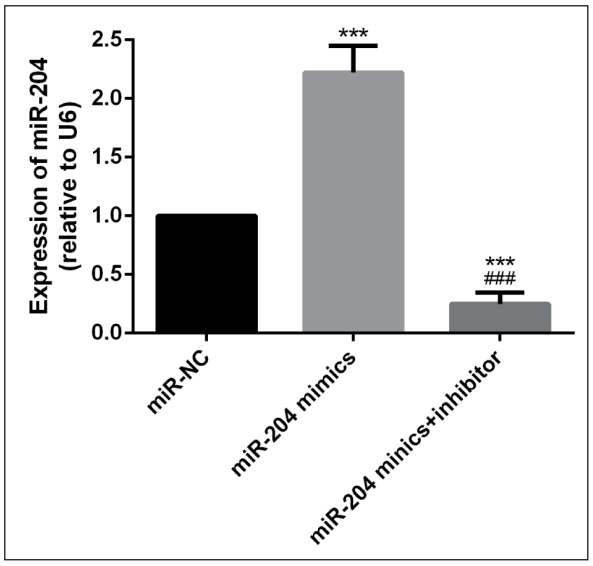

Figure 1. The expressions of miR-204 after transfection with mimics and inhibitor (***$p<0.001$ compared with miR-NC group, ###$p<0.001$ compared with miR-mimics group).

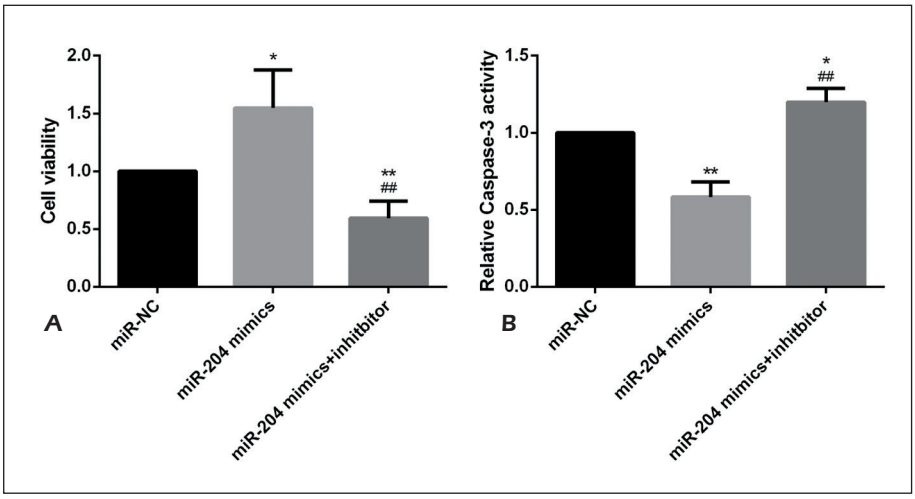

Figure 2. The effect of miR-204 on the cell viability and activity of caspase-3 of osteoblasts. Up-regulation of miR-204 improved the cell viability and decrease the activity of caspase-3, while down-regulation of miR-204 could inhibit cell viability and enhance the activity of caspase-3.(*p<0.05, **p<0.01 compared with miR-NC group, $^{\#\#}p$<0.01 compared with miR-mimics group).

MiR-204 Increased the Expression Level of RUNX2 and OSX

Recent study[12] has demonstrated that RUNX2 and OSX directly impact the transcription of osteoblasts. The qRT-PCR and Western-blot experiments in our study declared that the expression level of RUNX2 and OSX was substantial increased after intervention with miR-204 mimics. However, the effect of miR-204 was counteracted by addition of miR-204 inhibitor (Figure 4).

Discussion

Fracture healing is influenced by a variety of factors, and a complex and precisely regulated healing process will be triggered immediately after fracture, so as to restore the original structure and function of bone tissues. The function of osteoblast exerts a crucial effect in fracture healing. After fracture, on the one hand, mesenchymal stem cells are recruited to the fracture site and differentiated

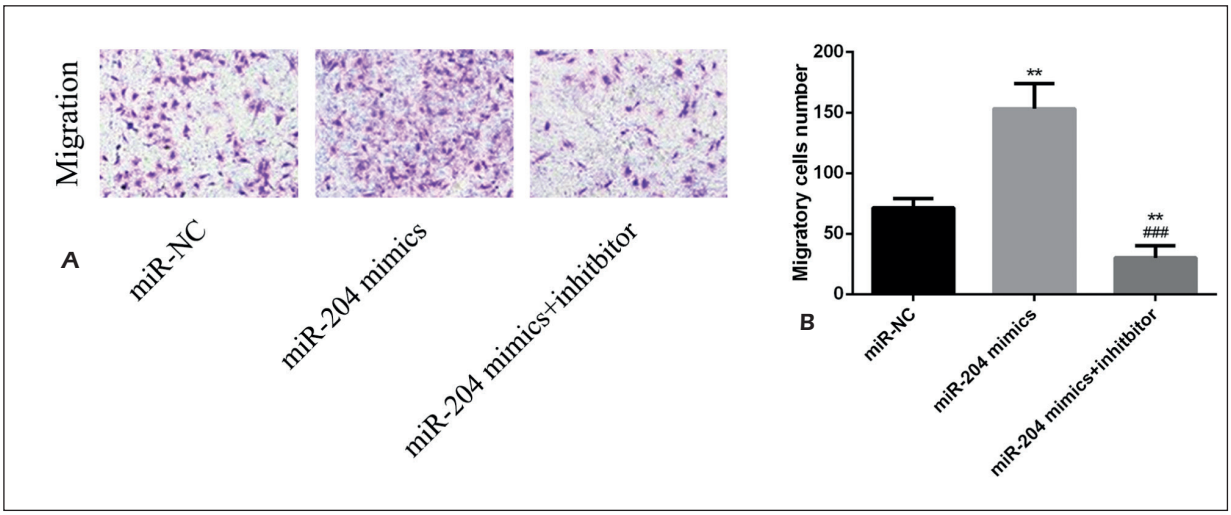

Figure 3. MiR-204 accelerated the migration of osteoblasts. **A**, The metastasis of osteoblasts was analyzed using transwell assay and detected by microscope (× 200). **B**, Statistical analysis of A. Data were presented as means ± standard deviations. (**p<0.01 compared with miR-NC group, $^{\#\#\#}p$<0.001 compared with miR-mimics group).

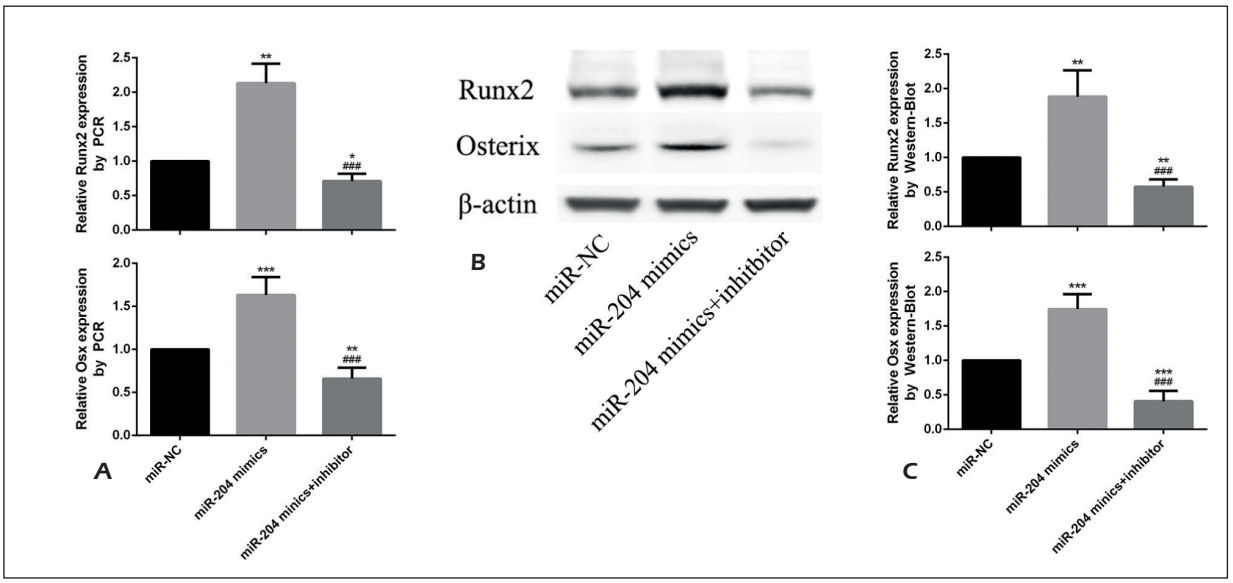

Figure 4. The effect of miR-204 on the expression of RUNX2 and OSX. **A**, Expression of RUNX2 and OSX determined by PCR. **B**, Protein expression of RUNX2 and OSX determined by Western blot. **C**, Statistical analysis of B. Data were presented as means ± standard deviations (**p<0.01, **p<0.001 compared with miR-NC group, $^{\#\#}p$<0.001 compared with miR-mimics group).

into osteoblasts under the action of such cytokines as platelet-derived growth factor (PDGF), fibroblast growth factor (FGF), insulin growth factor (IGF) and bone morphogenetic proteins (BMPs). Then, osteoblasts are involved in and regulate the formation and mineralization of bone matrix through synthesizing and secreting bone matrix type 1 collagen and non-collagen proteins, such as osteocalcin, osteopontin and bone sialoprotein, thereby forming new bones[13]. On the other hand, osteoblasts, through secreting RANKL, induce the differentiation and maturation of osteoclasts, and initiate the bone modeling process, ultimately forming bone tissues after healing[14]. The function of osteoblasts is regulated by various cytokines, among which Runt-related transcription factor 2 (RUNX2) and Osterix (OSX) are the two most important transcription factors.

RUNX2 is regarded as a major transcription factor of osteoblasts, which can bind to the cis-acting element that encodes the osteocalcin gene promoter region[15]. There is a lack of osteoblasts in the bone of RUNX2-deficient mice[16,17]. Inactivation and mutation of human RUNX2 can cause the cleidocranial dysplasia[18]. Besides, RUNX2 regulates various genes determining the osteoblast phenotype, and it can induce the expressions of many osteoblast marker proteins, such as osteocalcin[19]. Other than in osteoblasts, forcing the RUNX2 expression in fibroblasts of mice is enough to induce the expressions of osteoblast marker proteins, such as type I collagen, osteocalcin or bone sialoprotein. Compared with that in control group, more MSCs could be produced in mice with high expression of RUNX2[20]. Similarly, after marrow stroma cells of rats transfected with RUNX2 retroviral vector were inoculated into the 3D polycaprolactone scaffold, the production of mineralized matrix could be twice that in control group[21]. RUNX2 can stimulate osteoblast differentiation and promote the expressions of alkaline phosphatase and osteocalcin *in vitro* in human adipose-derived MSCs[22].

OSX, also known as Sp7, is the second transcription factor, besides RUNX2, necessary for the osteoblast differentiation, and it contains the zinc finger protein, which belongs to the transcription factor SP family. Moreover, OSX is specifically expressed in osteoblasts, which is also necessary for bone formation[23]. RUNX2 can be expressed in mice with OSX knockout, whereas OSX cannot be expressed in mice with RUNX2 deficiency, suggesting that OSX is located in the downstream pathway of RUNX2[23]. RUNX2, in fact, can promote the expression of OSX through directly binding to its promoter[24]. The formation of new skull in mice with OSX overexpression was 5 times that in control group[25]. Furthermore, the expressions of alkaline phosphatase and osteocalcin are significantly increased in human umbilical cord blood MSCs with OSX overexpression, and bone regeneration in nude mice can also be increased[26].

miRNAs are small endogenous non-coding single-stranded RNAs, which, through binding to the 3'UTR or CDS region of the target gene mRNA, exert its post-transcriptional regulation effect. miRNAs can regulate various signaling pathways/transcription factors related to the osteoblast differentiation. For example, miR-2861, through inhibiting histone deacetylases (HDACS) expression, can promote the bone morphogenetic protein-2 (BMP-2)-induced differentiation of ST2 cells into osteoblasts[27]. MiR-3960 promotes the osteoblast differentiation *via* inhibiting the target gene HOXA2[28], MiR-335-Sp promotes the osteogenic differentiation through inhibiting Dkk-1 and activating Wnt signal[29]. Besides, Kapinas et al[30] found that miR-29a promotes the osteoblast differentiation through down-regulating inhibitors of classic Wnt signal, such as Dkk-1, Kremen2 and secreted frizzled-related protein.

In order to study the role of miR-204 on function of osteoblasts, they were transfected with miR-204 to analyze the biological effects. Firstly, we found that overexpression of miR-204 could enhance cell viability and inhibit caspase-3 activity in osteoblasts. Furthermore, the expression level of RUNX2 and OSX, which has promoted the effect in chondrogenic differentiation, was significantly raised in mimics group. All the effects of miR-204 on function of osteoblasts could be suppressed by miR-204 inhibitor.

The molecular mechanisms about the commitment and maturation of osteoblasts in fracture healing have been well understood in the past decade. Moreover, increasing evidence has demonstrated that miRNAs can regulate the expression of many fracture healing-related transcription factors[31]. Therefore, better understanding of the interaction of miRNAs with osteoblast functions can advance the development of therapies for bone diseases.

Conclusions

In the present study, miR-204 plays an important role in the function of osteoblasts, and its mechanism may depend on regulating the expression of two osteoblast transcription factors: RUNX2 and OSX. Our results revealed that miR-204 could be a potential therapeutic target for the fracture healing.

Conflict of Interests:
The authors declared no conflict of interest

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