

# H19 promotes the proliferation of osteocytes by inhibiting p53 during fracture healing

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**Abstract.** – **OBJECTIVE:** We explored the possible mechanism underlying the expression change of H19 during fracture healing.

**MATERIALS AND METHODS:** A total of 18 male SD mice aged from 6-8 weeks old (18.5-24.6 g) were selected to establish tibial fracture models. The left tibia undergoing sham surgery was considered as the control group, and the right tibia undergoing sawing treatment was considered as the experimental group. The control tibia and fracture tibia from three mice were harvested at six time points after operation, respectively. QRT-PCR was utilized to detect the changes of H19 and p53 mRNA expression.

**RESULTS:** Compared with the control group, the expression of H19 in the experimental group was significantly increased at 4, 8, and 12 d. However, there was no significant difference in the expression of H19 between the experimental group and the control group at 16, 20, and 24 d.

The proliferation of chondrocytes and osteoblasts from mouse and human was significantly inhibited, and the apoptosis was significantly increased after interference of H19. As p19 plays important roles in diverse biological process, we detected the expression level of p19 after interference of H19. In addition, knockdown of H19 significantly up-regulated the expression of p53 in osteoblast cell lines, while the down-regulation of p53 expression reversed the proliferation of osteoblasts.

**CONCLUSIONS:** H19, as a molecular marker for promoting fracture healing, promote the proliferation of osteocytes by inhibiting the expression of p53.

*Key Words:*

Fracture healing, H19, p53.

## Introduction

According to statistics, about 10% of fracture patients have delayed union or nonunion and required further treatment<sup>1</sup>. Fracture nonunion or delayed healing is one of the major complications

of fractures, which brings physical and psychological distress to patients and their families<sup>2</sup>. The best therapy for delayed union or nonunion currently is autologous bone graft<sup>3,4</sup>. However, the suitable implantable bone in the human body is very limited, and the incidence of adverse reactions in the donor bone will increase after transplantation<sup>5</sup>. Currently, the fracture treatment with less damage and fewer complications is in urgent need. Long non-coding RNA (lncRNA) is a class of RNA longer than 200 nt without protein-coding function<sup>6</sup>. Previous studies were mostly focused protein encoding genes, whereas non-coding RNAs were thought as “junk RNA” or “noise” in life processes. However, with the rapid development and application of high-throughput sequencing technologies during the last decade, more and more researches have shown that lncRNAs can regulate protein-coding genes expression at epigenetic, transcriptional and post-transcriptional level, thus affecting a series of biological processes<sup>7-9</sup>. In addition, lncRNAs can also serve as molecular markers for disease diagnosis and target of therapy<sup>10</sup>.

H19 (H19, imprinted maternally expressed transcript) is located on the human chromosome Chr11p15.5. Recent studies<sup>12-14</sup> have shown that its exon region also encodes a small RNA, miR-675<sup>11</sup>. H19 is abundant in embryonic development, but its expression is decreased in tissues other than skeletal muscle after birth. The high expression of H19 is found to be associated with tumor cell proliferation, apoptosis and metastasis in many oncological diseases<sup>15</sup>. Meanwhile, H19 is involved in cell proliferation and tissue repair in several diseases of the skin and skeletal muscle<sup>16</sup>. However, H19 functions in fracture healing have not been elucidated yet. This study aims to elucidate whether and how H19 functions in fracture healing.

## Materials and Methods

### Animals

A total of 18 male SD (Sprague Dawley) mice (18.5-24.6 g) aged 6-8 weeks old were anesthetized by intraperitoneal injection of 1% sodium pentobarbital at a dose of 1 mL/kg (there was no significant difference in age, sex and weight of mice). After that, sham operation was performed on the left tibia of the mice, and the right tibia of mice in treatment group was sawn off (to cause stable fracture) and completely matched with the intramedullary fixation needle. The left and right tibia tissues of three mice were collected on the 4, 8, 12, 16, 20, and 24 d after operation, respectively. The samples were stored in -80°C refrigerator. This study was approved by the Animal Ethics Committee of Nanjing Medical University Animal Center.

### Cell Culture

Chondrocytes (ATDC5), osteoblasts (MC3T3-E1), human articular chondrocytes (HC-a) and osteoblasts (hFOB1.19) were purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA). The cells were cultured in DMEM (Dulbecco's modified Eagle medium) medium containing 10% FBS (fetal bovine serum), 100 U/mL penicillin and 100 µg/mL streptomycin (Invitrogen, Carlsbad, CA, USA), and then placed in an incubator at 37°C with 5% CO<sub>2</sub>. The medium was changed every 2-3 days and cells were passaged when fusion degree reached 80-90%.

### qRT-PCR

We used TRIzol to extract total RNA and reverse transcription was performed according to the instructions of PrimeScript RT reagent Kit (TaKaRa, Dalian, China). Diluted cDNA and a certain amount of primers, premix and ultrapure water were mixed into a 20 µL reaction system. ABI 7500 FAST Real-time PCR instrument was used for cDNA amplification. The expression level of the target gene was calculated using the 2<sup>-ΔΔCT</sup> method. Primers used in qRT-PCR were as follows: H19: 5'-GCGGGTCTGTTTCTT-TACTTC-3' (forward), 5'-TTTCATGTTGTGG-GTTCTGG-3' (reverse); GAPDH: 5'-GGAC-CAATACGACCAAATCCG-3' (forward), 5'-AGCCACATCGCTCAGACAC-3' (reverse); p53: 5'-CCTCAGCATCTTATCCGAGTGG-3' (forward), 5'-TGGATGGTGGTACAGTCAGA-GC-3' (reverse). Small interference sequences

used in transfection were as follows: si-H19-01: 5'-GGAGAGTTAGCAAAGGTGACATCTT-3'; si-H19-03: 5'-AGAGTTAGCAAAGGTGACA-TCTTCT-3'; si-scramble: 5'-GGATGATCGA-AGATGAGACTAGCTT-3'; Si-p53: 5'-CUA-CUCCUGAAAACAACG-3'.

### Western Blot

Total protein from cells was extracted using a cell lysate (RIPA) containing protease. After the measurement of protein concentration by Nano-Drop 2000 micro spectrophotometer, the protein was denatured with 5× loading buffer. The appropriate amount of protein samples and protein marker was separated by SDS-PAGE (sodium dodecyl sulphate-polyacrylamide gel electrophoresis) gel electrophoresis. After transferred from the gel to the PVDF (polyvinylidene difluoride) membrane, the protein samples were incubated with primary and secondary antibodies sequentially. Finally, the membranes were exposed and photographed.

### Cell Counting Kit-8 (CCK-8) Assay

Cells were seeded in 96-well plates and synchronized for 12 h after cell adherence. 6 replicate wells were set up for each sample and the total reaction volume per well was 200 µL. Each well was added with 20 µL of CCK-8 reaction solution and then incubated at 37°C for 2 h in the dark. After shaking for 10 min on a micro sample shaker, absorbance of each well at 450 nm was measured with a microplate reader.

### Flow Cytometry

After cell supernatants were collected into labeled tubes, EDTA-free trypsin was used to digest the cells. The cell suspension was centrifuged and washed twice with phosphate buffered saline (PBS), and 200 µL of binding buffer containing calcium ions were added to the tubes after centrifugation. 5 min prior to incubation in the dark, 10 µL of Annexin V-FITC fluorescent probe and 5 µL of PI (propidium iodide) were added to the tubes. FL1 and FL3 dual channel wavelength detection was performed to access the apoptosis of cells.

### Statistical Analysis

We used statistical product and service solutions 17.0 software (SPSS Inc., Chicago, IL, USA) for statistical analysis. All data were expressed as mean ± SD, and independent sample *t*-test was used to analyze the difference between two groups. *p*<0.05 was considered statistically significant.

## Results

### ***H19 Expression Was Increased in Fracture Tissues in Fractured Animal Models***

In order to study the physiological changes and molecular mechanisms during fracture healing, we compared the tissues at different stages of fracture healing with normal bone tissues. We performed qRT-PCR to detect the expression of H19 in the left and right tibia of mice at 4, 8, 12, 16, 20, and 24 d after operation. The results revealed that the expression of H19 in the experimental group was significantly increased at 4, 8, and 12 d compared with the control group, while there was no significant difference in the expression of H19 between the experimental group and the control group at 16, 20 and 24 d (Figure 1 A).

### ***H19 Promoted the Proliferation of Osteocytes and Inhibited Cell Apoptosis***

Fracture healing can be divided into three phases, including the hematoma machine evolution, the formation of the original callus and callus transformation<sup>17</sup>. Among them, the proliferation and differentiation of chondrocytes and osteoblasts are the basic and key events of fracture healing<sup>18-19</sup>. Previous studies have reported that high expression of H19 is associated with the scar hyperplasia of skin, as well as the proliferation and differentiation of skeletal muscle. Accordingly, we hypothesized that elevated H19 expression during fracture healing is correlated with the proliferation of chondrocytes and osteoblasts.

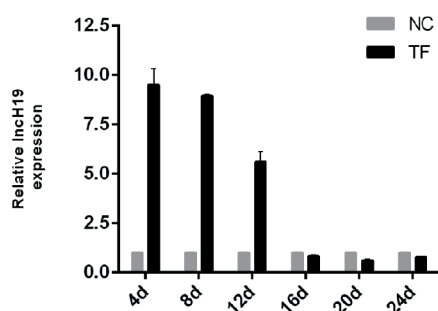
Small interfering RNA (siRNA) targeting H19 gene used in our study significantly reduced H19

expression in chondrocytes and osteoblasts (Figure 2A, F). Knockdown of H19 in chondrocytes and osteoblasts remarkably inhibited the cell proliferation and enhanced the apoptosis, while overexpression of H19 markedly increased the proliferation of both chondrocytes and osteoblasts from mouse and human (Figure 2 B-E, H-J). Above results indicated that H19 expression was up-regulated early in fracture healing and might play an essential role in promoting proliferation and inhibiting apoptosis in osteoblasts and chondrocytes.

### ***H19 Promoted the Proliferation of Osteocytes by Inhibiting the p53 Gene***

It is generally accepted that lncRNAs are involved in various biological activities of cells at epigenetic, transcriptional and post-transcriptional levels<sup>20</sup>. Yang et al<sup>13</sup> reported that H19 inhibited the activity of downstream targets of p53 by directly binding to this protein. The p53 gene was previously identified to block the progression of cell cycle and inhibit cell proliferation by enhancing the transcription of different genes<sup>21</sup>. Therefore, we measured the mRNA and protein levels of p53 in cells transfected with si-H19 or plasmids overexpressing H19. The results showed that the expression of p53 was significantly up-regulated after H19 overexpression (Figure 3A) and *vice-versa* (Figure 3B).

To further explore whether p53 plays a crucial role in H19 promoting osteoblast proliferation, we performed p53 interference in human osteoblast cell line hFOB1.19 with knockdown of H19. We found that the proliferation of hFOB1.19 cells could still be rescued when the expression of p53 was down-regulated to the level of that in the control group, even if the expression of H19 was disrupted (Figure 3C). In addition, the inhibition of p53 expression alone significantly enhanced the proliferation of hFOB1.19 cells. Our results demonstrated that up-regulated expression of H19 promoted the proliferation of osteocytes during fracture healing by inhibiting the expression of p53.

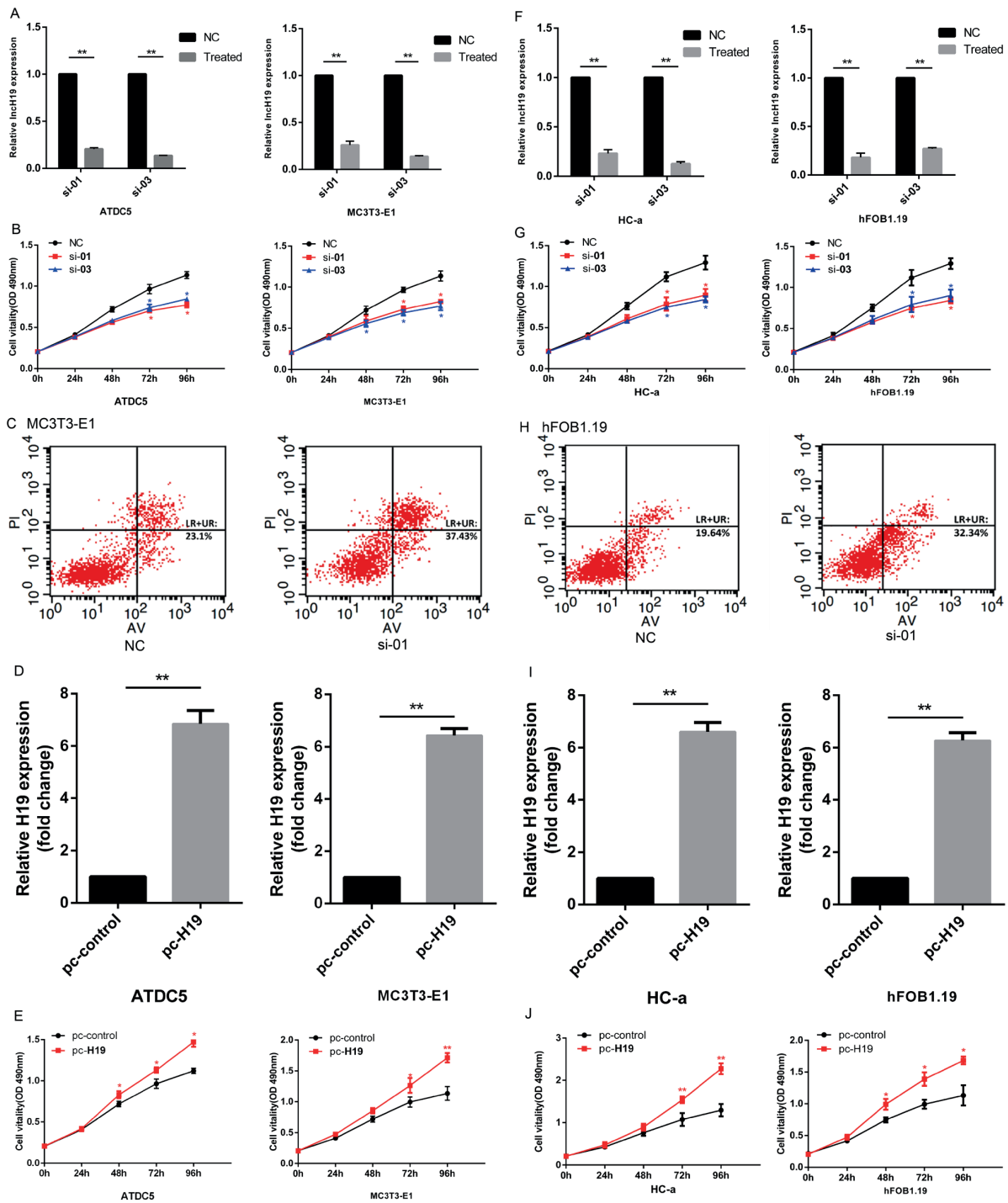


**Figure 1.** Relative expression of H19 in mouse tibia. The expression of H19 in the experimental group was significantly increased at 4, 8 and 12 d compared with the control group, while there was no significant difference in the expression of H19 between the experimental group and the control group at 16, 20 and 24 d.

## Discussion

This study first reported that the expression of lncRNA H19 was increased in the early stage of fracture healing, but no significant change was observed in the later stage. Most of the previous researches<sup>22</sup> were focused on the effect of H19 in tumorigenesis, while its role in normal and im-

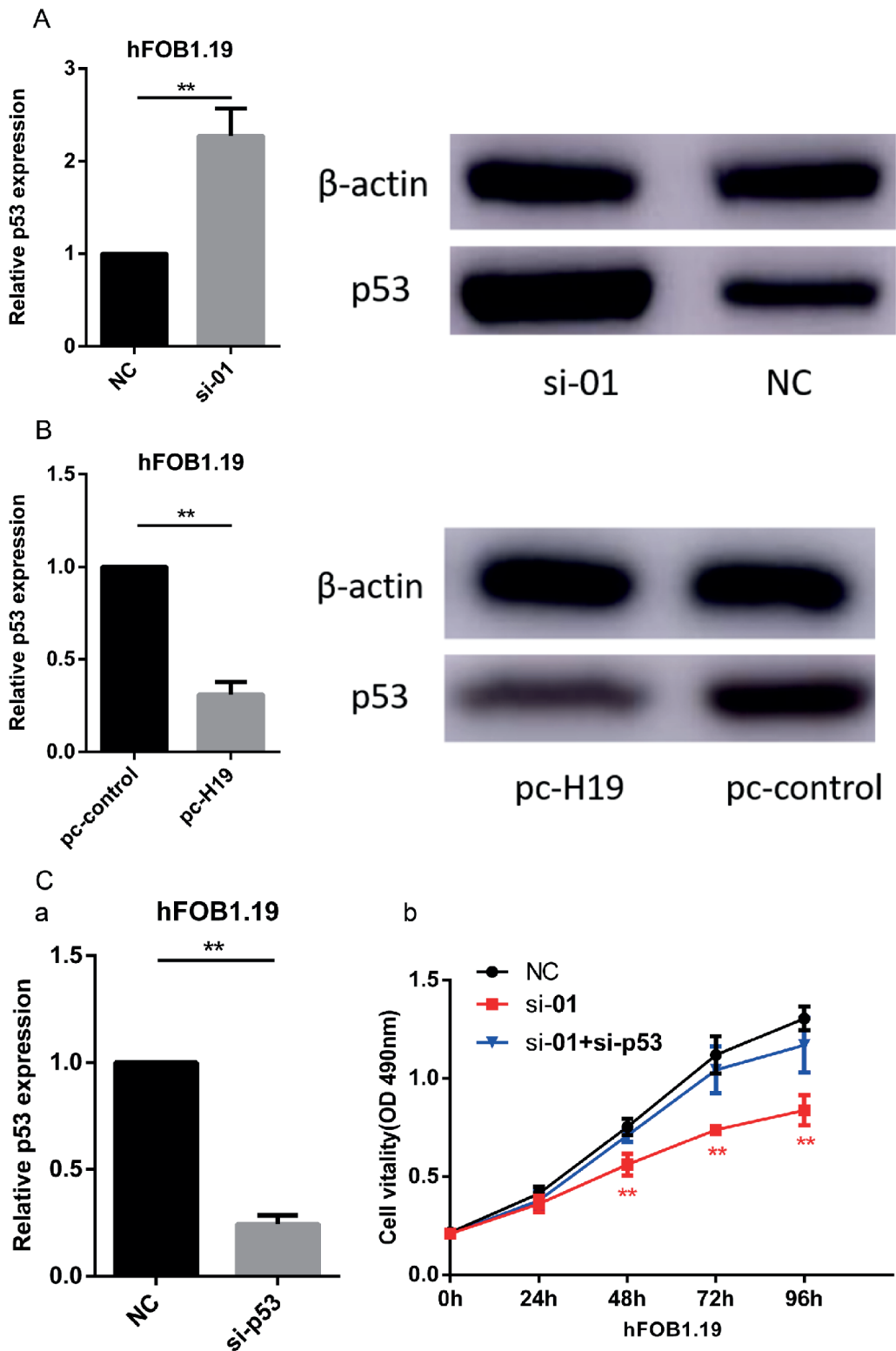
## H19 promotes the proliferation of osteocytes by inhibiting p53 during fracture healing



**Figure 2.** H19 promoted the proliferation of osteocytes and inhibited their apoptosis. **A** and **F**, The expression of H19 was significantly decreased in mouse and human osteoblast cell lines after transfection with siRNA-H19. **B** and **G**, CCK-8 assay showed that the proliferation of mouse and human osteoblast was significantly inhibited after knockdown of H19. **C** and **H**, Flow cytometry results showed that the apoptosis of MC3T3-E1 cells and hFOB1.19 cells was significantly increased after knockdown of H19. **D** and **I**, H19 expression was significantly increased in mouse and human osteoblasts transfected with pc-H19. **E** and **J**, CCK-8 assay showed that the proliferation of mouse and human osteoblast was increased after transfection of pc-H19.

paired tissues was rarely reported. In addition, few studies have been done on fracture healing

because human bone tissues at the healing phase are difficult to obtain. Here, we introduced a



**Figure 3.** H19 promoted the proliferation of osteocytes by inhibiting the p53 gene. *A*, P53 expression was significantly up-regulated at mRNA and protein levels after knockdown of H19. *B*, P53 expression was significantly down-regulated at both RNA and protein levels after H19 overexpression. *C*, Down-regulation of p53 rescued cell proliferation inhibited by H19.

mouse tibial fracture model. Tibial fracture, a kind of stable fracture, can effectively reduce the differences between groups<sup>23</sup>. Through the interference and overexpression of H19 in human and mouse cells, we believe that H19 is greatly involved in the proliferation of osteocytes.

It was reported that p53 can significantly inhibit cell proliferation and promote apoptosis through the downstream target gene Bax. However, there is little research on its relationship with H19. In the present study, the expression of p53 changed inversely with the expression of H19. Osteoblast proliferation induced by inhibition of H19 could be rescued by downregulating p53 expression. All of these results comprehensively demonstrated that H19 promoted the proliferation and inhibited the apoptosis of osteocytes by inhibiting the expression of p53 during fracture healing.

Further research is urgently needed to explore why H19 drops to the normal level in the later period. The mechanism by which cells regulate H19 expression may explain the physiological changes occurred during normal tissue injury repair. In addition, whether H19 and p53 act on osteocytes via indirect or direct effects or both is still unknown.

## Conclusions

We found that H19 and fracture healing are closely related, and H19 could promote bone cell proliferation and inhibit apoptosis by down-regulating the expression of p53, which also suggested that H19 may serve as a new therapeutic target for delayed union or non-union of fracture.

## Conflict of Interest

The Authors declare that they have no conflict of interest.

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