



The Expression of MDM2 Gene Promoted Chondrocyte Proliferation in Rats with Osteoarthritis via the Wnt/ β -Catenin Pathway

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ABSTRACT

This study aimed to investigate the regulatory mechanism of *MDM2* gene expression on cartilage cell proliferation in Osteoarthritis (OA) rats. For this purpose, 22 SD rats were randomly divided into normal control (10 cases) and treated (12 cases) groups. Treated group was used for OA modelling with the modified Hulth method. After a week, RT-PCR was used to detect *MDM2* in cartilage tissue of rats, *Wnt 1*, *Wnt 3 a*, *Wnt 10 b* and β -*catenin* genes mRNA expression. Rat chondrocytes were isolated and cultured, and the recombinant eukaryotic expression vector pcDNA3.1 myc-siRNA-MDM2- β -catenin and co-expression plasmid pcDNA3.1 myc-siRNA-MDM2- β -catenin was used to transfect chondrocytes and the proliferation and related gene expression levels of the transfected chondrocytes were detected by MTT method and RT-PCR. The results showed that compared with the control group, *MDM2*, *Wnt 1*, *Wnt 3 a*, *Wnt 10 b* and β -*catenin* genes in OA rat cartilage constructed by Hulth method were increased ($p < 0.05$). The pcDNA3.1 myc- β -catenin transfection slowed down the proliferation of OA chondrocytes, different from the non-transfected OA group ($p < 0.001$), and increased *Wnt 1*, *Wnt 3 a*, *Wnt 10 b* and β -*catenin* genes expression compared with the Control group ($p < 0.05$), but did not affect the expression of *MDM2*. The transfection of siRNA-MDM2 was opposite to pcDNA3.1 myc- β -catenin. The co-expression plasmid pcDNA3.1 myc-siRNA-MDM2- β -catenin transfection did not affect the proliferation of OA chondrocytes. In general, the high expression of *MDM2* in OA rats restricts the proliferation of chondrocytes, which may be related to the main pathogenesis of the occurrence and development of OA *in vivo*, and the regulation of *MDM2* on the proliferation of chondrocytes may be achieved through the Wnt/ β -catenin pathway.

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Introduction

Osteoarthritis (OA) is a common chronic joint disease characterized by the degeneration of the articular cartilage, and the hyperplasia and ossification of the adjacent cartilage (1, 2). OA is more common in the middle-aged and elderly. It can cause physical disability and increase medical expenses (3, 4). OA often occurs due to different causes, and the abnormal molecular metabolism of joints is an important factor in the pathogenesis of OA (5, 6).

MDM2 (murine double minute clone 2) is an E3 ubiquitin ligase that encodes a negative regulator of the p53 tumour suppressor and has a variety of biological functions (7, 8). The amplification of *MDM2* gene has been found in a variety of malignancies (7, 9). In addition, the expression of *MDM2* promotes the inflammation of tissues, while the inhibition of the expression of *MDM2* has a strong

anti-inflammatory effect (10).

This study aimed to investigate the role of *MDM2* in osteoarthritis and deeply analysed the molecular regulation mechanism of *MDM2* on the occurrence and development of osteoarthritis.

Materials and Methods

Experimental Animals

Twenty-two SD rats (11 males and 11 females), weight 200~220 g, provided by Hunan Academy of Chinese Medicine, were kept at a room temperature of 20~25 °C.

OA Rat Modelling

From the 22 SD rats, 10 were randomly selected as a control group and the remaining 12 were used for OA modelling with the modified Hulth method (11, 12). The rats were weighed and 2% of pentobarbital

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sodium (30 mg/kg) was intraperitoneally injected for anaesthesia according to the body weight of each rat, then the medial collateral ligament was surgically cut off, the meniscus and the anterior and posterior cruciate ligaments were removed, and the inner and outer cortices were sutured, thus an OA rat model was built. To prevent infection, rats were injected with penicillin 2×10^5 U for 3 consecutive days after surgery.

Chondrocyte Isolation and Culture

After anesthetized with 2% pentobarbital sodium, the knee articular cartilage was taken from the rats in the two groups under sterile conditions and was washed 3 times with Hank's solution. Then, the cartilage tissue was cut into small cubes about 1 mm^3 in volume with a scalpel and these cubes were digested with 0.25% trypsin digestion solution at 37°C for 30 min, and the supernatant was aspirated. Then 0.05% collagenase was added to digest the cartilage cubes for 45 min and the supernatant was collected. After filtered through a woven wire sieve, the supernatant was centrifuged for 5 min (1500 r/min) and the supernatant was discarded. Ten percent calf serum was added and resuspended repeatedly, and inoculated in a culture flask, and then placed in a 5% CO_2 incubator at 37°C . The chondrocytes used for the experiment from the two groups were all in the 1st to 3rd generation.

Total RNA Extraction and RT-PCR

Total RNA was extracted from the cartilage tissue of successfully modelled OA rats and from the chondrocytes that were isolated and cultured, with the TRIzol (Invitrogen) cell lysis reagent. The mass and concentration of the RNA were measured using the Evolution 201 ultra-micro spectrophotometer, and the total RNA extracted above was reversely transcribed and purified using the TIANGEN Reverse Transcription Kit. Real-time quantitative detection was performed using the SYBR Green Master Mix real-time quantitative PCR kit. After the primer reaction finished, melting curve analysis was performed to evaluate the quality of the final PCR product, with β -actin as the internal reference. Primers involved in the PCR process were shown in Table 1.

Table1. RT-PCR primer list

Gene	Primer sequence
<i>MDM2</i>	Upstream primer: 5'-CTATCGGGTCACAGTCTA TCAGG-3' Downstream primer: 5'-AGTTCTACGAAGGG TCCAAC-3'
<i>Wnt-1</i>	Upstream primer: 5'-GGTGGGGCATCGTGAACATAG-3' Downstream primer: 5'-GGAGGTGATTGCGAAGATAAACG-3'
<i>Wnt-3a</i>	Upstream primer: 5'-AGACTATCTCTGTTCATG-3' Downstream primer: 5'-GCCCCGTATAGGCATTTCGA-3'
<i>Wnt10b</i>	Upstream primer: 5'-CTCTGGCCTTCTCTCTCAGTA-3' Downstream primer: 5'-GTAGGTAAAGGTGGCTGGGTAGT-3'
<i>β-catenin</i>	Upstream primer: 5'-GCCAGTGGATTCCGTAAGT-3' Downstream primer: 5'-TGAGTGGCTGTCTTTTACG-3'
<i>PCNA</i>	Upstream primer: 5'-TGCCGAGACCTCAGCCATATT-3' Downstream primer: 5'-GTTGCACTGGCTCATTCTCTCT-3'
<i>TGF-α1</i>	Upstream primer: 5'-GCCCTGGACACCAACTATTGC-3' Downstream primer: 5'-GCTGCACCTGCAGGAGGGCAC-3'
<i>β-actin</i>	Upstream primer: 5'-GTCAGGTCATCACTATCGGCAAT-3' Downstream primer: 5'-AGAGGTCTTTACGGATGTCAACGT-3'

Plasmid Construction

Plasmids pcDNA3.1myc- β -catenin (P- β -catenin), siRNA-MDM2 (S-MDM2) and co-expressed plasmid pcDNA3.1myc-siRNA-MDM2- β -catenin (PM β -catenin) were constructed by Sangon Biotech (Shanghai) Co., Ltd. The restriction enzyme cutting sites upstream and downstream of the plasmid pcDNA3.1myc were XbaI cutting site 5'-TGCTCTAGATTCACGGCTATCTTCAGGAG-3' and downstream EcoR I cutting site 5'-GGAA TTCTCA GTCTGA GTCA GGCCCTTCTG-3', respectively. After the double digest with XbaI and EcoR I, subcloning was carried out to connect β -catenin to the pcDNA 3.1 eukaryotic expression vector and thus to construct P- β -catenin recombinant plasmids. By S-MDM2 synthesis, the MDM2 siRNA oligonucleotide sequence and the p GCsilencer-U6 siRNA expression vector were connected, transformed and screened. The synthesized plasmids were double-digested with BamH I and Hind III, and were sequenced and identified. The restriction enzyme cutting sites upstream and downstream were introduced, namely Bgl II 5'-GCAGATCTTGCTTCGCGA2TGTACGGGCC-3' and Nru I 5'-CGTCGCGAGGGCTA TGA ACTAATGACCC-3'. Through the synthesis of co-expressed plasmid PM β -catenin, the above plasmids pcDNA3.1myc- β -catenin and siRNA-MDM2 achieved connection and construction by using the cutting sites of Bgl II and Nru I. Empty plasmids pcDNA3.1myc-negative control (P-NC) and

siRNA-negative control (S-NC) were constructed for the above P- β -catenin and S-MDM2 plasmids to exclude the impact of the plasmids.

Testing the Proliferative Activity of OA Chondrocytes with the MTT Assay

Take log-phase chondrocytes (cell density $5 \times 10^4 \text{ mL}^{-1}$) and aseptically inoculate into a 96-well cell culture plate with a cell suspension of $150 \mu\text{L}$ per well. Divide the wells into six groups (P- β -catenin group, P-NC group, S-MDM2 group, S-NC group, PM β -catenin group, and Control group) and five copies were set for each group. Add $20 \mu\text{L}$ of MTT ($5 \text{ g} \cdot \text{L}^{-1}$ PBS, freshly prepared) to each well and continue to incubate for 4 h. Aspirate the culture solution and discard it. Add $100 \mu\text{L}$ of DMSO to each well and shake for 10 min. Measure the absorbance values of the wells at 24 h, 48 h, 72 h and 96 h with a microplate reader.

Data Analysis and Processing

GraphPad Prism was used for statistical analysis and presented in each legend. The D'Agostino-Pearson normality test was used to verify whether the data followed a normal distribution. If the data followed a normal distribution, then the Student's t-test would be used for comparison between two groups, and the Tukey's multiple compare test would be used for comparison among multiple groups. If the data were not normally distributed, then the Mann-Whitney U test would be used for comparison between two groups, while the Kruskal-Wallis test and the Dunn's multiple comparison test would be used for comparison among three groups or more.

Results and discussion

The Association between the Expression of MDM2 Gene and Wnt/ β -Catenin Pathway-Related Genes in OA Rats

To explore the association between the expression of MDM2 gene and Wnt/ β -catenin pathway-related genes in OA rats, the RT-PCR method was used to detect the expression levels of MDM2 gene and Wnt/ β -catenin pathway-related genes in the cartilage tissue isolated from OA rats. The detection showed that MDM2 gene and Wnt/ β -catenin pathway-related genes (Wnt 1, Wnt 3 a, Wnt 10b and β -catenin) all had higher expression levels in the cartilage tissue of

OA rats (Figure 1). Among these genes, MDM2 and β -catenin contributed the most significant difference in expression (Figure 1A and E, $p < 0.001$). These findings showed that there was a positive correlation between the high expression of MDM2 and Wnt/ β -catenin pathway-related genes in the cartilage tissue of OA rats and the occurrence of OA, indicating that MDM2 and Wnt/ β -catenin pathway-related genes were possible potential molecular mechanisms of OA, and a potential link may exist between them.

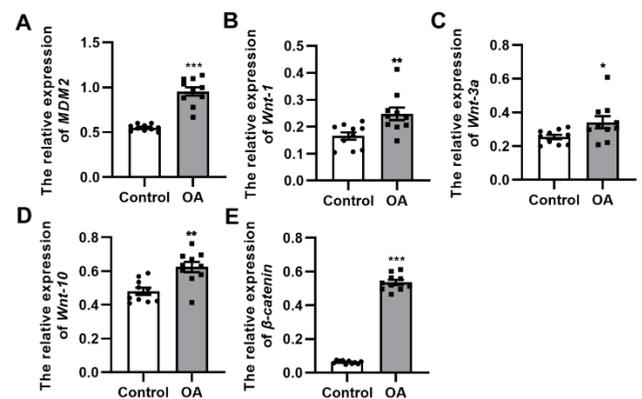


Figure 1. The expression levels of MDM2 gene and Wnt/ β -catenin pathway-related genes in the OA rat model built with the Hulth method; A: The expression level of MDM2 gene in the OA rat model. B: The expression level of Wnt-1 gene in the OA rat model. C: The expression level of Wnt-3a gene in the OA rat model. D: The expression level of Wnt-10b gene in the OA rat model. E: The expression level of β -catenin gene in the OA rat model, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$; Unpaired Student's t-test (A, D and E). Mann-Whitney U test (B and C). Error bars were used to express the Standard Error of the Mean (SEM).

P- β -Catenin Plasmids Inhibited the Proliferation of OA Chondrocytes without Affecting the Expression of MDM2

To verify the potential link between MDM2 and the Wnt/ β -catenin pathway, we constructed P- β -catenin plasmids to transfect OA chondrocytes and to specifically enhance the expression of β -catenin in OA chondrocytes. The verification showed that the enhanced expression of β -catenin (Figure 2A) inhibited the proliferation of OA chondrocytes (Figure 2B), but the expression of MDM2 was not affected (Figure 2C). Meanwhile, to verify the findings of the inhibition of proliferation of OA chondrocytes by P- β -catenin plasmids, we examined chondrocyte proliferation-related genes (PCNA and TGF- β 1) (13-15), and found that compared with the OA group, P- β -

catenin plasmids significantly reduced the expression of PCNA and TGF- β 1 genes (Figure 2D and E, $p < 0.001$). Therefore, the results showed that the high expression of β -catenin imposed no impact on MDM2, suggesting that MDM2 may be upstream of the Wnt/ β -catenin pathway and may affect the occurrence and development of OA via the Wnt/ β -catenin pathway.

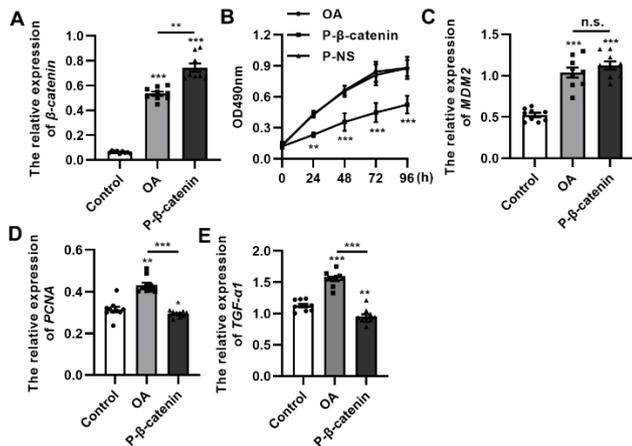


Figure 2. The in vitro transfection with P- β -catenin plasmids inhibited the proliferation of OA chondrocytes; A: P- β -catenin plasmids could increase the expression of β -catenin gene in OA chondrocytes. B: P- β -catenin plasmids inhibited OA chondrocyte proliferation. C: The high expression of β -catenin gene imposed no impact on the expression of β -catenin gene. D and E: The expression of cartilage proliferation genes PCNA and TGF- β 1 was inhibited. n.s. not significant, $**p < 0.01$, $***p < 0.001$. One-way ANOVA followed by the Tukey's multiple comparisons test (A, C, D and E). Chi-square test (B). Error bars were used to express the SEM.

The In Vitro Transfection with S-MDM2 Plasmid Promoted the Proliferation of OA Chondrocytes and Decreased the Expression of Wnt/ β -Catenin Pathway-Related Genes

To further verify the accuracy of the above findings, we used si-RNA plasmids to interfere with MDM2 expression and to explore whether it could affect the expression of Wnt/ β -catenin pathway genes. First, we detected the interference effectiveness of the S-MDM2 plasmids and found that S-MDM2 could effectively decrease the expression of MDM2 gene in OA chondrocytes (Figure 3A). It was found that interfering the expression of MDM2 could promote the proliferation of OA chondrocytes (Figure 3B) and promote the expression of chondrocyte proliferation genes PCNA and TGF- β 1 (Figure 3C and D), and

decrease the expression of Wnt/ β -catenin pathway-related genes (Figure 3E). These results indicated that MDM2 was upstream of the Wnt/ β -catenin pathway and regulated OA via the Wnt/ β -catenin pathway.

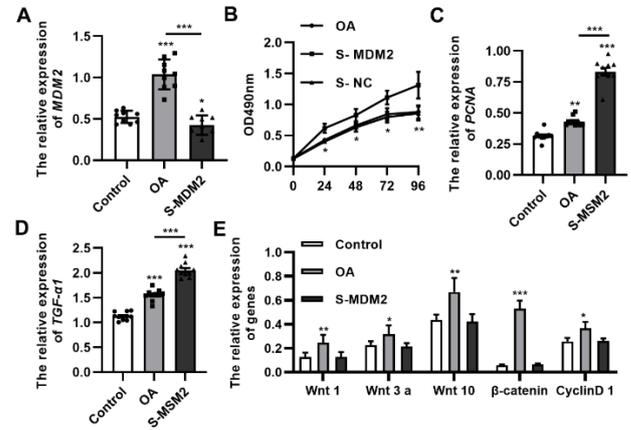


Figure 3. The in vitro transfection with S-MDM2 plasmids promoted OA chondrocyte proliferation; A: S-MDM2 plasmids inhibited the expression of MDM2 gene in OA chondrocytes. B: S-MDM2 plasmids promoted OA chondrocyte proliferation. C: The low expression of MDM2 gene reduced the expression of Wnt/ β -catenin pathway-related genes. D and E: S-MDM2 plasmids promoted the expression of PCNA and TGF- β 1 genes. n.s. not significant, $*p < 0.05$, $**p < 0.01$, $***p < 0.001$. One-way ANOVA followed by the Tukey's multiple comparison test (A, D and E); Chi-square test (B). Kruskal-Wallis test followed by the Dunn's multiple comparison test (C).

PM β -Catenin Plasmids Imposed No Impact on OA Chondrocyte Proliferation

To further verify the association between MDM2 and the Wnt/ β -catenin pathway, we constructed PM β -catenin plasmids to increase the expression of β -catenin while decrease the expression of MDM2. As a result, we found that the proliferation of OA chondrocytes cultured in vitro was not affected (Figure 4A), and compared with the OA group, the expression of Wnt/ β -catenin pathway-related genes and chondrocyte proliferation genes PCNA and TGF- β 1 was not affected (Figure 4B). This suggested that the low expression of MDM2 at the same time interfered with the promotion of β -catenin expression, indicating that MDM2 could both positively and negatively regulate the Wnt/ β -catenin pathway and affect OA cell proliferation.

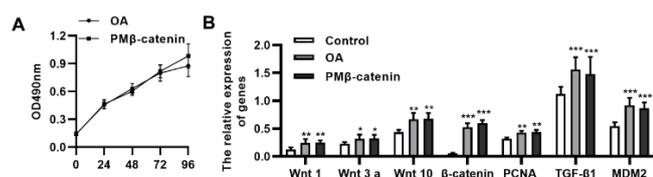


Figure 4. PMβ-catenin plasmids imposed no impact on OA chondrocyte proliferation

The Wnt family was involved in the transduction and regulation of various signals in vivo via the secretion of glycosylphospholipids by transcription helper activator β-catenin (16, 17). Mutations in the Wnt/β-catenin pathway are often associated with human congenital defects, cancer, and other immune-related diseases (18-20).

OA is a heterogeneous disease with unknown causes. It can be idiopathic, primary or related to known medical conditions or events; the major pathological changes occur in the hyaline cartilage structure with different degrees of synovitis. Inflammatory responses mediated by the Wnt/β-catenin signalling pathway play an important role in the occurrence of OA and have become a hotspot in OA research (16, 21, 22).

In this study, we explored the role and mechanism of MDM2 in the development of OA via the Wnt/β-catenin pathway. We found that (a) the expression of MDM2 increased in the cartilage of OA rats; (b) genes related to the Wnt/β-catenin pathway also had higher expression in different degrees; (c) by promoting the expression of β-catenin, the proliferation of chondrocytes and the expression of chondrocyte proliferation genes were inhibited; (d) S-MDM2 plasmids inhibited the expression of MDM2 and Wnt/β-catenin related genes. These efforts provide a new treatment strategy for OA treatment.

The Wnt/β-catenin signalling pathway is a strong stimulus for chondrocyte matrix degradation (23, 24). The activation of the Wnt signal also reduces the proliferation and differentiation of normal mesenchymal progenitor cells (MPCs) (25). MDM2 expression aggravates OA conditions by activating the Wnt/β-catenin signalling pathway (26-28). In previous studies, highly activated β-catenin expression has shown the ability to cause OA in adult mice (19, 26). Therefore, it is very meaningful to study how MDM2 is associated with the Wnt/β-catenin pathway and aggravates OA development.

Our data showed that compared with normal rats and their chondrocytes, OA rats and their chondrocytes had higher MDM2 levels, and the expression of Wnt/β-catenin signalling pathway-related genes was also increased in OA rats and their chondrocytes, while the excessive activation of β-catenin didn't change MDM2 levels. In addition, lowering the level of MDM2 also reduced the activity of Wnt/β-catenin signalling. These findings indicated that MDM2 played an important role in the occurrence and development of OA by stimulating the Wnt/β-catenin signalling pathway.

In conclusion, we demonstrated that MDM2 was an important regulatory gene that inhibited the proliferation of OA cartilage by up-regulating the Wnt/β-catenin signalling pathway. Our findings suggested that the development of a specific MDM2 inhibitor may help prevent or treat osteoarthritis.

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None.

Interest conflict

None.

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