

Effect of N-cadherin on Chondrogenic Differentiation of Bone Marrow-Derived Mesenchymal Stem Cells through Wnt Signaling Pathway

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ABSTRACT

This study aimed to compare and analyze the effect of N-cadherin on chondrogenic differentiation of bone marrow-derived mesenchymal stem cells (BMSCs) and to explore the related mechanism, so as to provide a novel theoretical basis for the clinical work of articular cartilage injury regeneration and repair. For this purpose, the experimental animals were clean grade SD rats (aged 5-6 weeks, weighing 180-250g). Alcian blue staining was carried out to observe the induced chondrogenesis following N-cadherin inhibition. The specific role of N-cadherin in the Wnt signaling pathway and chondrogenic differentiation of BMSCs was detected by Western blot; while the effect of N-cadherin on the molecular level changes of β -catenin in the cytoplasm was evaluated by fluorescence quantitative real-time PCR (qRT-PCR). In addition, immunoprecipitation (IP) was used for the verification of the interaction between N-cadherin and β -catenin. Results showed that under the light microscope, 90% of the BMSCs at the third generation, 90% of the cells were fused. Alcian blue staining showed that the green staining area in the BMP2 induction group was large and dense, while that in the N-cadherin inhibition group and blank control group was small and sparse. Western blot revealed that N-cadherin and SOX9 were significantly developed in the BMP2 induction group, but Wnt3a was not significantly developed. While in the N-cadherin inhibition group, the development of Wnt3a was obvious, yet without evident development of N-cadherin and SOX9. The qRT-PCR indicated that the relative mRNA expression of Wnt3a was significantly increased in the N-cadherin inhibition group ($P < 0.05$). However, no obvious difference was observed in the mRNA expression of β -catenin between the BMP2 induction group and the N-cadherin inhibition group ($P > 0.05$). Western blot indicated that in the BMP2 induction group; there existed the development of β -catenin, significant development of phos-GSK-3 β and total GSK-3 β , but no obvious development of Wnt3a. In the N-cadherin inhibition group, there was significantly enhanced development of Wnt3a and β -catenin than that before, blurred development of phos-GSK-3 β than that before, and also obvious development of total GSK-3 β with little change from before. N-cadherin promoted the expression of β -catenin mostly in the cell membrane, but only a few in the cytoplasm and nucleus. Additionally, verification by IP showed that N-cadherin and β -catenin were developed on N-cadherin and β -catenin bands, suggesting an interaction between N-cadherin and β -catenin. According to these results, N-cadherin can ultimately promote chondrogenic differentiation of BMSCs by inhibiting the Wnt signaling pathway.

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Introduction

Articular cartilage injury is the most common cartilage injury in military training leading to joint dysfunction (1). Cartilage tissue is composed of a small number of chondrocytes and a large number of specific extracellular matrices in the surrounding area, which has unique physiological and biomechanical

characteristics (2, 3). However, due to the absence of blood vessels, nerves and lymph supply, cartilage tissue relies mainly on the nourishment of joint synovial fluid for nutrition, which results in a high difficulty to regenerate after injury (4, 5). In this regard, the repair of articular cartilage injury has always been a difficult issue in the field of

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orthopedics. Autologous chondrocyte transplantation is the result of the further development of cartilage injury repair technology (6). Nevertheless, the directly transplanted chondrocytes are not only prone to apoptosis and difficult to survive but also will be inactivated owing to the dedifferentiation (7). In recent decades, with the progress of cell biology, various seed cells with germinal function have been gradually paid attention to in the treatment of cartilage injury (8-10).

Bone marrow-derived mesenchymal stem cells (BMSCs) are pluripotent stem cells that exist in many human tissues and can differentiate into bone, cartilage, muscle and adipose tissue (11). It not only supports and regulates hematopoiesis *in vivo*, but also can be distributed in a variety of tissues and organs *in vivo* (12). Moreover, BMSCs have multi-directional differentiation potential and can differentiate into osteoblasts, fibroblasts, reticular cells, adipocytes, and endothelial cells (13, 14). It may facilitate the introduction and stable expression of foreign genes, which have been recognized to be an excellent choice of seed cells (15, 16). Acting as a type of important seed cells in the field of tissue engineering, BMSCs are expected to provide hope for regenerative medicine. Many scholars are committed to repairing osteochondral defects by differentiation and regeneration of BMSCs into chondrocytes (17, 18). However and significantly, as it has been described above, the feature of multi-directional differentiation potential possessed by BMSCs can not only promote their differentiation into chondrocytes but also into osteoblasts *in vivo*, revealing an unstable and uncertain therapeutic effect of BMSCs. Accordingly, over the years, scholars have tried to control the differentiation direction of BMSCs to avoid this instability and uncertainty (19). It has been documented that the differentiation direction of BMSCs is related not only to extracellular factors (20) but also to the cell microenvironment and the intracellular response to external stimuli (21).

Furthermore, the signaling pathway can transfer extracellular molecular signals into cells through the cell membrane to play an effect, which is a bridge to communicate extracellular factors, cell microenvironment and cell biological effects (22). Recent studies have shown that the activation of the Wnt signaling pathway also plays an important role in

the directional differentiation of BMSCs (23, 24). As a highly conserved signaling pathway in evolution, the Wnt pathway is one of the key signal transduction pathways in animal development. It is involved in cell proliferation, differentiation, apoptosis and cell localization control (25). β -catenin is a pivotal molecule of the classical Wnt signaling pathway (26). When the extracellular signal molecule of Wnt protein in the Wnt signaling pathway is stimulated by non-inhibitory stimulation, it can release signals to directly inhibit the activity of GSK-3 β directly and hence prevent its impact on the phosphorylation of β -catenin (27). On the other hand, the combination of Wnt protein and its receptor can also prevent the combination of β -catenin with APC, GSK-3 β and Axin degradation complex to form a new complex without biological effects, and finally promote the aggregation of β -catenin in the cytoplasm (28). Consequently, when there is a certain amount of β -catenin aggregated, it can initiate the expression of target genes and play a biological role. It is hence proposed here that regulating the Wnt signaling pathway may be one of the possible ways to affect the differentiation direction of BMSCs.

N-cadherin is mainly expressed in neurons, fibroblasts and other mesenchymal cells. Overexpression of N-cadherin protein can induce cell movement and promote cell migration and invasion (29). At the same time, the existing experiments also found that the mRNA level of N-cadherin was closely related to the level of molecules in the Wnt signaling pathway (30). In other words, N-cadherin may affect the chondrogenic differentiation of BMSCs by regulating the Wnt signaling pathway (31). However, at present, it is still unclear with respect to the result of this effect and its corresponding mechanism. Meanwhile, N-cadherin, as an existing regulatory factor in the physiological state of BMSCs natural repair of cartilage injury, may be well known for other roles of BMSCs in the process of natural repair of cartilage injury in the past (32). While it may also "quietly" affect the Wnt signaling pathway, and then alter the process of chondrogenic differentiation of BMSCs. Undoubtedly, the study of this naturally occurring self-regulation may be beneficial to a more comprehensive understanding of the chondrogenic differentiation of mesenchymal stem cells.

With respect to the aforementioned interpretation, this study was carried out to compare and analyze the effect of N-cadherin on chondrogenic differentiation of BMSCs and to explore the related mechanism, so as to provide a novel theoretical basis for the clinical work of articular cartilage injury regeneration and repair. Our study proposed a hypothesis that N-cadherin could inhibit the activation of the Wnt signaling pathway. Indeed, the Wnt signaling pathway can exert an inhibitory role in the chondrogenic differentiation of BMSCs (23, 24). Therefore, our study speculated that N-cadherin might ultimately promote chondrogenic differentiation of BMSCs by inhibiting Wnt signaling pathway.

Materials and Methods

Experimental materials and instruments

Dimethyl sulfoxide (DMSO; Sigma, USA); SD rats (both male and females; the Animal Center of Academy of Military Medical Sciences); inverted phase-contrast microscope (Olympus, Japan); CO₂ incubator, qPCR instrument, ultra-clean workbench, and fluorescence microscope were used.

Isolation and culture of rat BMSCs

The experimental animals were clean grade SD rats (aged 5-6 weeks, weighing 180-250g), which were purchased from the Animal Center, Academy of Military Medical Sciences. The rats were killed by cervical dislocation, and the tibia and femur were taken after their sacrifice immediately. After sampling, both ends of the long bone were cut off; the cells in the medullary cavity were then pushed into a 50ml centrifuge tube from one end of the long bone with a complete culture medium containing heparin. The next step was centrifugation at the centrifugal speed of 1500rpm for 4 min. With the supernatant discarded, the middle layer mononuclear cells were collected for cell re-suspension. After that, the collected cell suspensions were inoculated in the Petri-dish, and cultured in a 5% CO₂ incubator at 37°C. The third generation of cells was selected for subsequent experiments.

Observation of the induced chondrogenesis following N-cadherin inhibition by Alcian blue staining

The collected cells as described above were randomly divided into three groups of BMP2 induction group, N-cadherin inhibition group and blank control group according to different experimental protocols. To be specific, in the BMP2 induction group, BMSCs were cultured in a chondrogenic induction medium containing BMP2; BMSCs in the N-cadherin inhibition group were added to chondrogenic induction medium containing BMP2 and co-cultured with N-cadherin inhibitor ADH1, and BMSCs in the blank control group were cultured in common medium. Cells were washed after 21 d of culture, and then 1% Alcian blue solution was added for 30 min of staining. Corresponding results were observed under the microscope. This experiment was conducted to identify whether the inhibition of N-cadherin played a role in enhancing differentiation or a role of weakening (ineffective).

Observation of the specific role of N-cadherin in Wnt signaling pathway and chondrogenic differentiation of BMSCs by Western blot

BMSCs were cultured in BMP2 chondrogenic induction medium as well as the combined medium of chondrogenic induction medium and ADH1 for 21 d. The protein expressions of Wnt3a, N-cadherin and SOX9 were observed by Western blot. Meanwhile, BMSCs in the blank control group, BMP2 induction group and N-cadherin inhibition group were cultured in BMP2 chondrogenic induction medium for 21 d, respectively. Following the extraction of proteins, the expressions of Wnt3a, β -catenin, phos-GSK-3 and total GSK-3 were also detected by Western blot. In terms of the process, BMSCs were washed three times with PBS and then transferred into the EP tube for centrifugation at 1000rpm for 3 min. After centrifugation, the supernatant was discarded and the collected cell samples were fully lysed by using RIPA for the extraction of proteins. Furthermore, Pierce® BCA Protein Assay Kit was utilized for the detection of the concentrations of proteins according to the method of BCA. Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) was used to transfer the membrane to a plate containing TBST solution for decolorization at room temperature. The 5% skimmed milk with TBST solution was added for sealing of the membrane on the shaking table for 2h. The primary antibodies were

diluted with TBST solution to an appropriate concentration and incubated at room temperature for 1~2h. The secondary antibody diluent was prepared by the same method and incubated with the membrane at room temperature for 1~2h. Then chemiluminescence development was carried out for 5~10min. The residual fixing solution was rinsed off with tap water, followed by drying at room temperature. The slices were scanned or photographed, and the molecular weight and net value of optical density of the target protein bands were analyzed by the gel image processing system.

In addition, BMSCs of the blank control group, BMP2 induction group and N-cadherin inhibition group were cultured in BMP2 chondrogenic induction medium for 21 d, and the contents of β -catenin in the cell membrane, cytoplasm and nucleus were detected by Western blot according to the same steps as above. Histone 3, GAPDH and Na⁺/K⁺ATPase were used as markers of nuclear protein, cytoplasmic protein and membrane protein, respectively. Quantity-One software was used to analyze gel images and to detect IA values.

Observation of the effect of N-cadherin on the molecular level changes of β -catenin in the cytoplasm by fluorescence quantitative real-time PCR (qRT-PCR)

BMSCs of the BMP2 induction group and N-cadherin inhibition group were cultured in BMP2 chondrogenic induction medium for 21 d, respectively. The mRNA levels of Wnt3a and β -catenin, two central proteins of the Wnt classical signaling pathway, were detected by qRT-PCR. The cells were washed twice with pre-cooled PBS, centrifuged at 4°C for 5 min, and the supernatant was discarded. After that, the collected cells were added with 1ml Trizol to fully lyse the cells until they were clear. Thermo NANODROP 2000 was used to measure the concentration and purity of RNA. The total RNA solution was blown evenly and kept at 85°C for 5 min to denature the RNA. The RNA reverse transcription reaction was carried out on the ice. Afterward, an amount of 20 μ L reverse transcription system was diluted 10 times as the template for qPCR. An amount of 4 μ L diluted cDNA was added to each well and then centrifuged at 1000rpm for 1min. The steps for qPCR were denatured at 95°C for 5 min;

amplification at 95°C for 10s, at 60°C for the 20s, in a total of 50 cycles; and then melting at 95°C for 10s, at 60°C for 10s; and cooling at 40°C for 30s.

Verification of the interaction between N-cadherin and β -catenin by immunoprecipitation (IP)

With the cells harvested, an appropriate amount of cell IP lysis buffer (including protease inhibitor) was added for cell lysis on ice or at 4°C for 30min, followed by centrifugation at 12000 \times g for 30min, and collection of the supernatant. A small amount of lysate was collected for Western blotting analysis, and the remaining lysate was added with 1 μ g corresponding antibodies and 10~50 μ L protein A/G-beads for incubation overnight at 4°C. After the IP reaction, cells were centrifuged at 3000 \times g for 5min at 4°C, and protein A/G-beads were precipitated to the bottom of the tube. With the absorption of the supernatant carefully, the protein A/G-beads were washed with 1mL lysis buffer 3 ~ 4 times. Finally, 15 μ L 2 \times SDS sample loading buffer was added and heated at 100 °C for 10min, which was then loaded for SDS-PAGE for Western blotting analysis.

Statistical Analysis

All data in this experiment were expressed as mean \pm standard deviation ($\bar{x}\pm$ SD), and were analyzed by using SPSS version 19.0 statistical software (International Business Machines Corp., Armonk, New York, USA). The comparison of data between groups was realized with t-test. $P<0.05$ defined that the difference was statistically significant.

Results and discussion

Isolation and culture of rat BMSCs *in vitro*

The morphology of BMSCs in SD rats at different growth stages was observed under an optical microscope. Under the light microscope, the distribution of irregular short spindled cells was observed on the first day of culture (Figure 1A). At the third generation, 90% of the cells were fused, and the cell morphology was basically the same. It was a long spindled fibroblastic morphology, arranged regularly and grew in a vortex (Figure 1B).

Observation of the induced chondrogenesis following N-cadherin inhibition by Alcian blue staining

BMSCs were cultured for 21 d, after which Alcian blue staining was performed in the three groups of the blank control group, BMP2 induction group and N-cadherin inhibition group. Under the microscope, it was found that the green staining area in the BMP2 induction group was large and dense, while that in the N-cadherin inhibition group and blank control group was small and sparse (Figure 2A, and Figure 2B). These results suggest that the addition of ADH1 during chondrogenic induction will reduce the production of proteoglycan, that is, reduce the production of cartilage tissue.

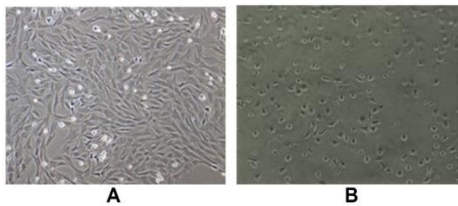


Figure 1. Morphology of rat BMSCs ($\times 100$) after isolation and culture in vitro; (A) Morphology of rat BMSCs on the first day of culture, showing the distribution of irregular short spindled cells; (B) Morphology of rat BMSCs after third passage showing the fusion of 90% of the cells.

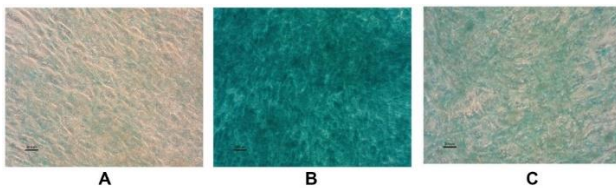


Figure 2. Observation of the induced chondrogenesis following N-cadherin inhibition by Alcian blue staining; (A) The glycosaminoglycan production of cartilage matrix in BMP2 induction group was more than that in the blank control group; (B) The glycosaminoglycan production of cartilage matrix in N-cadherin inhibition group was less than that in BMP2 induction group.

Observation of the specific role of N-cadherin in Wnt signaling pathway and chondrogenic differentiation of BMSCs by Western blot

BMSCs in the BMP2 induction group and blank control group were cultured for 21 d, the protein was extracted, and the expressions of Wnt3a, N-cadherin and SOX9 were detected by Western blot. It was observed that N-cadherin and SOX9 were significantly developed in the BMP2 induction group, but Wnt3a was not significantly developed; The GAPDH development was obvious, indicating that the results were effective (Figure 3).

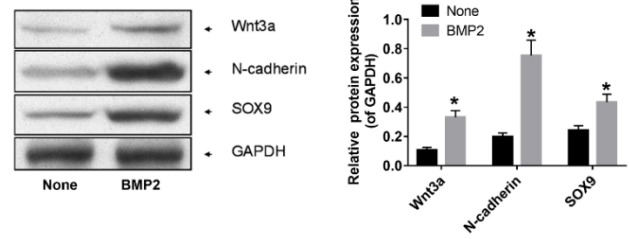


Figure 3. The expressions of Wnt3a, N-cadherin and SOX9 in BMSCs by Western blot; BMSCs in the blank control group (marked as "None" in the figure) and BMP2 induction group (marked as "BMP2" in the figure) were cultured for 21 d to detect Wnt3a, N-cadherin and SOX9, with GAPDH as the internal reference.

Similarly, BMSCs in the BMP2 induction group and N-cadherin inhibition group were cultured for 21 d, the protein was extracted, and the expressions of Wnt3a, N-cadherin and SOX9 were detected by Western blot. Corresponding results revealed that in the BMP2 induction group, there was significant development of N-cadherin and SOX9, but no obvious development of Wnt3a. While after the addition of N-cadherin inhibitor ADH1, that was, in the N-cadherin inhibition group, the development of Wnt3a was obvious yet without evident development of N-cadherin and SOX9 (Figure 4).

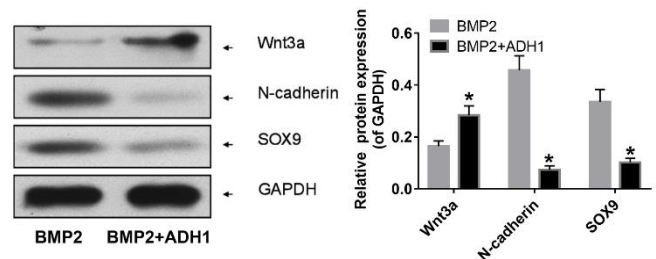


Figure 4. The expressions of Wnt3a, N-cadherin and SOX9 in BMSCs after the inhibition of N-cadherin by Western blot; BMSCs in BMP2 induction group (marked as "BMP2" in the figure) and N-cadherin inhibition group (marked as "BMP2+ADH1" in the figure) were cultured for 21 d to detect Wnt3a, N-cadherin and SOX9, with GAPDH as the internal reference.

Observation of the effect of N-cadherin on the molecular level changes of β -catenin in the cytoplasm by fluorescence qRT-PCR

According to the detection results of qRT-PCR, the relative mRNA expression of Wnt3a was significantly increased following a combined treatment of BMP2 induction and the suppression of N-cadherin, with a statistical difference ($P < 0.05$). However, no obvious

difference was observed in the mRNA expression of β -catenin between the BMP2 induction group and the N-cadherin inhibition group ($P>0.05$). The results are shown in Figure 5.

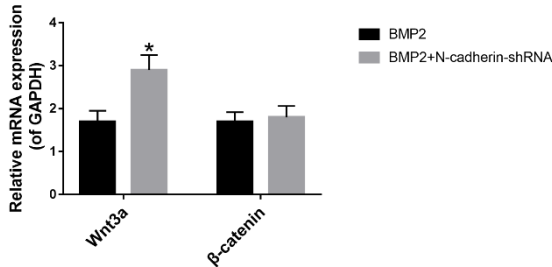


Figure 5. The detection of Wnt3a and β -catenin by qPCR to evaluate the effects of N-cadherin on mRNA expression of β -catenin in the cell cytoplasm; The relative mRNA expression of Wnt3a was increased significantly following a combined treatment of BMP2 induction and the suppression of N-cadherin (BMP2+N-cadherin-shRNA), with no obvious difference observed in β -catenin.

Observation of the specific role of N-cadherin on β -catenin degradation-related proteins by Western blot

Further Western blot experiment indicated that in the BMP2 induction group; there existed the development of β -catenin, significant development of phos-GSK-3 β and total GSK-3 β , but no obvious development of Wnt3a. At the same time, in the N-cadherin inhibition group, there was a significantly enhanced development of Wnt3a and β -catenin than that before, blurred development of phos-GSK-3 β than that before, and also obvious development of total GSK-3 β with little change from before (Figure 6).

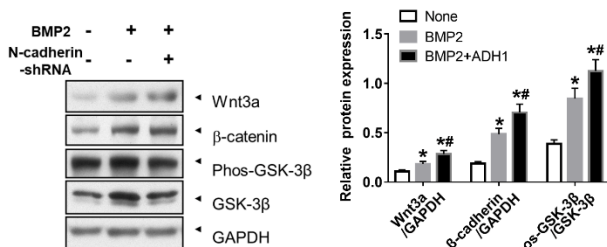


Figure 6. The detection of Wnt3a, β -catenin, phos-GSK-3 β and total GSK-3 β by Western blot to evaluate the effects of N-cadherin on β -catenin degradation related protein, with GAPDH as the internal reference; Following a combined treatment of BMP2 induction and the suppression of N-cadherin, there were significantly enhanced development of Wnt3a and β -catenin, blurred development of phos-GSK-3 β , and also obvious development of total GSK-3 β .

Observation of the effect of N-cadherin on the entry of β -catenin into the cytoplasm by Western blot

Based on the above experimental findings, we further shifted our attention to the localization of β -catenin in BMSCs, as shown in Figure 7. Western blot was used to detect the expression of β -catenin in the cell membrane, cytoplasm and nucleus. Na⁺/K⁺ATPase is a cell membrane-specific integrin, while Histone 3 and GAPDH are unique proteins in the nucleus and cytoplasm, respectively. With reference to the expression of the nuclear protein, cytoplasmic protein and membrane protein markers of Histone 3, GAPDH and Na⁺/K⁺ATPase, corresponding results revealed that N-cadherin promoted the expression of β -catenin mostly in the cell membrane, but only a few in the cytoplasm and nucleus.

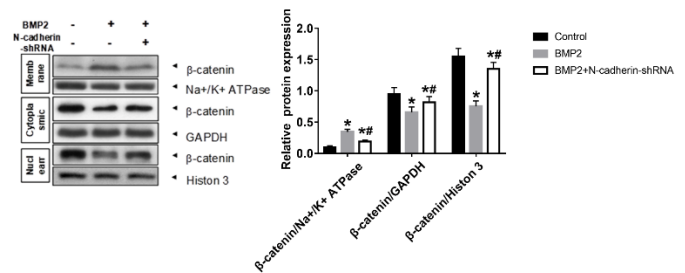


Figure 7. The detection of Wnt3a, β -catenin, phos-GSK-3 β and total GSK-3 β by Western blot to effects of N-cadherin on the expression of β -catenin in the cell membrane, cytoplasm and nucleus by Western blot, with GAPDH as the internal reference; N-cadherin promoted the expression of β -catenin mostly in the cell membrane, but only a few in the cytoplasm and nucleus.

Verification of the interaction between N-cadherin and β -catenin by IP

In order to verify the interaction between N-cadherin and β -catenin, this part of the experiment intervened BMSCs with N-cadherin inhibition and β -catenin inducement, harvested cell proteins and carried out IP experiment. The protein lysate was taken to detect the expression level of exogenous proteins (input). As displayed in Figure 8, both N-cadherin and β -catenin were developed on N-cadherin and β -catenin bands, suggesting the existence of interaction between N-cadherin and β -catenin.

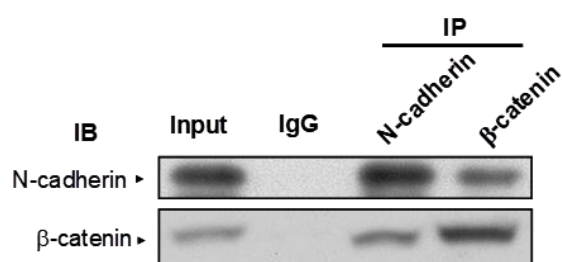


Figure 8. Validation of the interaction between N-cadherin and β -catenin by immunoprecipitation; The protein lysate was taken to detect the expression level of exogenous proteins (input). There was an interaction between N-cadherin and β -catenin since both N-cadherin and β -catenin were developed on N-cadherin and β -catenin bands.

In order to promote and benefit the clinical practice of articular cartilage injury regeneration and repair, our study focused on exploring the effect of N-cadherin on chondrogenic differentiation of BMSCs and its possible mechanism. Finally, our study confirmed the hypothesis proposed above that N-cadherin might exert an inhibitory effect on Wnt signaling pathway to promote chondrogenic differentiation of BMSCs.

As is known to all, BMSCs are derived from mesoderm and ectoderm in early development and have strong proliferation ability and multi-directional differentiation potential (12, 13). However, multiple studies in the past have documented that BMSCs are regulated by many factors including cytokines, chemical factors (11), physical factors (33) and genes (34, 35) in order to achieve their ideal directional differentiation effect. As an important mechanism to communicate the intracellular and extracellular environment, the signaling pathway plays an important role in regulating the directional differentiation of BMSCs.

The Wnt signaling pathway is involved in cell proliferation, differentiation, apoptosis and cellular localization control (22), among which β -catenin is the central molecule of the classical Wnt signaling pathway (26). When the Wnt signaling pathway is activated, it can inhibit the degradation of β -catenin in the cytoplasm through two aspects. On the one hand, it can activate the Disheveled (DSH) protein-rich in the PDZ domain in the cytoplasm and release signals to directly inhibit the activity of GSK-3 β , so that it cannot phosphorylate β -catenin (25). On the other hand, the combination of Wnt protein and receptor can also cause Axin instability, which eventually leads

to the obstacle to the formation of APC-GSK-3 β -Axin degradation complex, so as to prevent the combination of β -catenin with APC, GSK-3 β and Axin degradation complex to form a new complex without biological effects, inhibit the phosphorylation of β -catenin in the complex, and finally promote the aggregation of β -catenin in the cytoplasm (28, 36). When the aggregation of β -catenin reaches a certain amount, it can start the expression of target genes and play a biological role. At the same time, phosphorylated GSK-3 β (phos-GSK-3 β) can also result in the phosphorylation of β -catenin (37). After that, the phosphorylated β -catenin will be degraded by proteasome through ubiquitination pathway, resulting in the maintenance of β -catenin level in the cytoplasm at a quite low level, and hence inhibiting the Wnt signaling pathway.

Cadherins Superfamily molecules are calcium-dependent cell adhesion molecules with many members, including E-cadherin, N-cadherin, P-cadherin, VE-cadherin, etc. (38). Cadherins can be expressed in most normal tissues and cells, which, however, exhibit significantly different expression profiles by various cells. Among them, N-cadherin is mainly expressed in neurons, fibroblasts and other mesenchymal cells (29). In the past, it was thought that N-cadherin mainly induced cell movement to promote cell migration. However, recent experiments have shown that N-cadherin can affect the growth of osteoblasts through the Wnt signaling pathway (39). For instance, Haÿ et al. (40) reported in their study that in osteoblasts, N-cadherin can compete with Wnt signaling molecules to bind LRP5/6, so as to inhibit the activation of the Wnt signaling pathway through the classical pathway of the Wnt signaling pathway. In addition, during the co-culture of BMP2 and BMSCs, it was also found that the level of N-cadherin mRNA was closely related to the level of molecules in the Wnt signaling pathway (41). In this regard, N-cadherin may regulate the Wnt signaling pathway. However, we are concerned about whether this regulation also exists in the chondrogenic differentiation of BMSCs and their exact role.

In this study, BMSCs of β -catenin inducement and N-cadherin inhibition group were cultured in a chondrogenic induction medium for 21 days respectively. Western blot showed that N-cadherin could not affect the transcriptional production of β -

catenin. Therefore, the upregulation of the protein level of β -catenin after the inhibition of N-cadherin could only be resulted from the presence of post-transcriptional changes of β -catenin after inhibiting N-cadherin, that is, promoting the accumulation of β -catenin protein. It can be suggested that N-cadherin can inhibit the accumulation of β -catenin protein in the cytoplasm. Accordingly, N-cadherin mainly causes the change of the number of β -catenin protein in the cytoplasm, rather than the cascade reaction in the nucleus, that is, the active site is in the upstream of β -catenin protein.

The upstream of β -catenin protein to induce the change of the number of β -catenin protein in the cytoplasm can be explained by two main ways, namely, affecting the degradation of β -catenin; and promoting the transfer of β -catenin to other structural parts within cells. Western blot in our study showed that the phosphorylation level of GSK-3 β decreased and the expression of β -catenin protein increased; while the inhibition of N-cadherin can reduce the level of phosphorylated GSK-3 β . In other words, N-cadherin can promote the phosphorylation of GSK-3 β , while the silencing of N-cadherin produces no significant effect on the total protein level of GSK-3 β . These results support that phosphorylation (activation) of GSK-3 is the main site where N-cadherin regulates Wnt signaling pathway. In short, N-cadherin can promote the phosphorylation of GSK-3 β , and the phosphorylated GSK-3 β can further degrade β -catenin, resulting in reduced accumulation of β -catenin in the cytoplasm.

Western blot was used to further detect the expression of β -catenin in the cell membrane, cytoplasm and nucleus. It was noticed that N-cadherin could promote the accumulation of β -catenin in the cell membrane, and reduce its amount in the cell cytoplasm and nucleus. We would like to find out the reason why N-cadherin could contribute to the accumulation of β -catenin in the cell membrane. Therefore, our further study carried out IP to confirm that there was an interaction between N-cadherin and β -catenin. It suggests that N-cadherin can form a polymer with β -catenin and migrate into the membrane components which hence result in its accumulation in the membrane.

It can be interpreted as follows that on the one hand, N-cadherin can exert a promoting role in the

phosphorylation of GSK-3 β , which can further stimulate the degradation of β -catenin and reduce its accumulation in the cell cytoplasm, so that β -catenin cannot reach the amount of giving play to its biological effect. On the other hand, N-cadherin can form a polymer with β -catenin that can be stored in the cell membrane, which further reduces the amount of β -catenin in the cytoplasm. Synergistically, the superposition and coordination of the two effects maintain a fairly low level of β -catenin in the cytoplasm and prevent β -catenin from initiating the subordinate reaction. In other words, N-cadherin inhibits the activation of the Wnt signaling pathway. At the same time, research in the past has documented that the Wnt signaling pathway can regulate the chondrogenic differentiation of BMSCs negatively. With respect to the above, the inhibition of the Wnt signaling pathway by N-cadherin actually promotes chondrogenic differentiation of mesenchymal stem cells. It has also been verified by the results of this study.

However and indeed, there are some limitations in this study. Firstly, our study was carried out based on *in vitro* experiment merely. Indeed, *in vitro* test alone is still inadequate seriously which is not enough to completely represent the *in vivo* test. The combination of both *in vivo* and *in vitro* tests may be important to enhance the reliability and credibility of the findings of our study. Secondly, our study was conducted based on a relatively simple experimental design. In-depth research of the regulatory role of gene expression may provide insights in developing effective approaches to initiate chondrocyte differentiation and maintain the phenotype of articular cartilage for a long time. In order to address this issue, it is necessary to coordinate the expression of multiple genes through the application of a complex stem cell regulatory system in a future study, which may provide the possibility of gene therapy for stem cell-derived cartilage repair. In addition, our future study may emphasize a comprehensive analysis of the characteristics of N-cadherin and Wnt and their advantages and disadvantages in cartilage differentiation, which may lay a good foundation for the preparation of efficient cartilage repair materials and the performance of *in vivo* experiments.

Conclusions

In conclusion, the present study suggests that N-cadherin can play an inhibitory role in activating the Wnt signaling pathway by suppressing the activities of hub factors of this pathway. Meanwhile, considering that the Wnt signaling pathway can inhibit chondrogenic differentiation of BMSCs. Our study's findings support that by inhibiting the Wnt signaling pathway, targeting N-cadherin can contribute to chondrogenic differentiation of BMSCs, which may have a promising therapeutic function in articular cartilage injury regeneration and repair.

Ethics approval and consent to participate

Not applicable

Consent for publication

Not applicable

Availability of data and material

Not applicable.

Competing interests

The authors declare that they have no competing interests.

Funding

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Authors' contributions

QF designed the study and was a major contributor in writing the manuscript, ZM and LY evaluated and refined the details of the study, SX, LH, ZJ, YB, LC and QW performed the experiment and analyzed the data. All the authors checked and revised the manuscript before submitting it.

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Not applicable.

Interest conflict

None.

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