

BMP2 promotes chondrocyte proliferation via the Wnt/ β -catenin signaling pathway

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Abstract. Bone morphogenetic protein 2 (BMP2), a member of the transforming growth factor- β (TGF- β) superfamily, plays a key role in the induction of the differentiation of mesenchymal cells into chondrocytes to form cartilage tissue. However, it is not clear whether BMP2 regulates the proliferation of chondrocytes. In the present study, the effect of BMP2 on the proliferation of chondrocytes and its underlying mechanism was investigated. Chondrocytes isolated from the knee of SD rats were cultured and identified using toluidine blue staining. The second generation chondrocytes were collected and stimulated with or without BMP2 for 48 h. Cell viability was analyzed using the MTT assay. mRNA and protein expression levels of β -catenin, GSK-3 β , Dvl1 and Cyclin D1 were detected using real-time RT-PCR and Western blotting, respectively. The cell cycle distribution of the chondrocytes was analyzed by flow cytometry. BMP2 stimulation was found to significantly increase cell viability. In addition, following BMP2 treatment, β -catenin, Cyclin D1 and Dvl1 expression was significantly increased, whereas GSK-3 β expression was significantly decreased. Moreover, the percentage proportion of chondrocytes in the G0/G1 phase was significantly decreased, whereas that in the S phase was significantly increased. The results indicate that BMP2 promotes chondrocyte proliferation via the Wnt/ β -catenin signaling pathway.

Introduction

Osteoarthritis (OA) is a chronic degenerative disease characterized by a basic pathology of cartilage degradation caused by the mutual influence of mechanical and biological factors (1,2). Cartilage is composed of chondrocytes that produce a large amount of extracellular matrix (ECM), including type II

collagen and proteoglycan (PG) (3). Chondrocytes rapidly respond to changes in the articular microenvironment and regulate the dynamic equilibrium between the degradation and synthesis of the ECM, which is crucial to the maintenance of cartilage function (4,5). Therefore, the functional changes of chondrocytes contribute to the degradation of the articular cartilage, and thus to the pathogenesis of OA.

The maturation process of chondrocytes is closely regulated by a variety of growth factors and environmental conditions (6,7). The Wnt/ β -catenin signaling pathway is one of the pathways that play an important role in chondrogenesis. The activation of the Wnt/ β -catenin signaling pathway is initiated when Wnt proteins bind to cell-surface receptors of the Frizzled family, causing the receptors to activate Dishevelled family proteins, such as Dvl1. The activated Dishevelled (DSH) then inhibits a protein complex that is composed of Axin, GSK-3 β and the protein APC. The Axin/GSK-3 β /APC complex normally promotes the proteolytic degradation of β -catenin. The inhibition of the Axin/GSK-3 β /APC complex results in the accumulation of cytoplasmic β -catenin, which causes some β -catenin to translocate into the nucleus, where it interacts with TCF/LEF family transcription factors to promote the expression of specific genes, such as Cyclin D1 (8,9). Cyclin D1 is a positive regulator of G1/S transition and one of the key restriction points in the cell cycle. Therefore, upregulation of Cyclin D1 expression facilitates cell cycle progression and promotes chondrocyte proliferation.

Promoting the proliferation of chondrocytes is an efficient treatment for delaying the progression of cartilage degradation. Previously, we reported that millimeter wave therapy potentially exerts its therapeutic effect on OA by enhancing chondrocyte proliferation (10). Recent studies have focused on growth factors, which have been found to enhance cell proliferation and ECM synthesis *in vitro* and *in vivo*. In most studies on the regeneration of transplantation-ready cartilage, recombinant growth factors have been tested individually or in combination. For example, bone morphogenetic protein 2 (BMP2) has been used to promote proliferation and differentiation in primary and subcultured chondrocytes (11,12). The purpose of the present study was to examine whether BMP2 enhances chondrocyte proliferation via the Wnt/ β -catenin signaling pathway *in vitro*. The effects of BMP2 on β -catenin, GSK-3 β , Dvl1 and Cyclin D1 in chondrocytes were observed

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to explore the mechanisms by which BMP2 regulates the Wnt/ β -catenin signaling pathway and promotes chondrocyte proliferation at the cellular and molecular levels.

Materials and methods

Materials and reagents. Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS) and trypsin-EDTA were purchased from Hyclone (USA). Type II collagenase and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were purchased from Sigma-Aldrich (USA). The SYBR fluorescence quantization kit and TRIzol reagent were purchased from Invitrogen Corporation (USA). Rabbit anti-rat β -catenin, GSK-3 β , Dvl1, Cyclin D1, β -actin and HRP-IgG secondary goat anti-rabbit antibodies were purchased from Santa Cruz Inc. (USA). The cell cycle assay kit was provided by Becton Dickinson (USA). Primer synthesis was performed by Shanghai Sangon Biological Engineering Technology Services, Ltd. (China). Rat BMP2 was obtained from Beijing Boao Sen Bio-Technology Co., Ltd. (China). Toluidine blue was purchased from Sinopharm Chemical Reagent Co., Ltd. (China).

Animals. Forty-eight 4-week-old male SD specific pathogen-free (SPF) rats were used in this study, including six for primary culture and the identification of chondrocytes and ten for BMP2 stimulation experiments. All experiments were performed in triplicate. The animals were provided by the Shanghai Slack Laboratory Animal Co., with qualified certificate number SCXK (Shanghai) 2007-0005. The treatment of the laboratory animals complied with the Guidelines for the Care and Use of Laboratory Animals 2006, administered by the Ministry of Science and Technology, China (13).

In vitro culture of chondrocytes. The SD rats were sacrificed and soaked in 75% alcohol for 5 min. The knee joint was separated and the cartilage removed under sterile conditions. The cartilage was rinsed in PBS and in DMEM three times. The cartilage was sectioned into 1-mm³-thick slices and placed in dishes containing 0.2% type II collagenase, then transferred to a 37°C incubator. The supernatant was collected every 60 min and centrifuged at 800 rpm for 5 min to obtain the cell pellet. This step was repeated four times. The cells were re-suspended in DMEM complete culture medium (containing 10% FBS, 50 mg/l vitamin C and 100 U/ml each of ampicillin and streptomycin). The cells were then filtered through 200-mesh stainless steel filters and counted with a blood cell count plate to adjust the concentration of the cell suspension to 2-3x10⁵ ml. The cells were seeded in flasks and cultured at 37°C in a 5% CO₂ incubator. The primary cultured cells were observed under an inverted microscope and passaged upon reaching 80% confluence. The second generation of chondrocytes was identified using toluidine blue staining.

Experimental groups. The passage 2 chondrocytes were seeded into 6-well plates at a density of 2.0x10⁵ cells/well in 2 ml 10% FBS DMEM, cultured for 24 h, then starved for 24 h in serum-free DMEM medium. The cells were divided into four groups respectively stimulated with BMP2 at concentrations of 0, 5, 10 or 20 ng/ml for 24 or 48 h.

Identification of chondrocytes using toluidine blue staining. The second generation chondrocytes were seeded on cover slips and cultured for 72 h. The cells were washed with PBS, fixed in 4% neutral formalin for 30 min and stained with 1% toluidine blue for 30 min. The slips were rapidly washed in ethanol, dried, placed on slides and sealed. Cell morphology was observed using a phase-contrast microscope (Olympus, Japan). Images were captured at a magnification of x200.

Evaluation of cell viability by MTT assay. Chondrocyte viability was assessed by the MTT colorimetric assay. The passage 2 chondrocytes were seeded into 96-well plates at a density of 1.0x10⁴ cells/well in 0.1 ml 10% FBS DMEM, cultured for 24 h and then starved for 24 h in serum-free DMEM medium. The chondrocytes were then treated with final BMP2 concentrations of 0, 5, 10 or 20 ng/ml for 24 or 48 h. The medium was then removed and the cells were washed once with PBS, then 20 μ l of 0.5% MTT solution was added to each well followed by incubation for 4 h at 37°C. Subsequently, the MTT solution was removed by aspiration, and 150 μ l DMSO was added to each well. The 96-well plates were placed on a shaker for 10 min and the optical density (OD) value of each well was measured at 570 nm using an ELISA reader (Bio-Tek, Model EXL800, USA).

Real-time PCR detection of β -catenin, GSK-3 β and Dvl1 mRNA expression in chondrocytes. After treatment of the chondrocytes with BMP2, total RNA was isolated with Trizol reagent according to the manufacturer's instructions. RNA (1 μ g) was reverse transcribed into cDNA as a template for real-time PCR amplification. The primer sequences and the sizes of the amplicons were as follows: β -catenin, sense 5'-TCT AGT GCA GCT TCT GGG TT-3', antisense 5'-GAT GGC AGG CTC GGT AAT G-3' (184 bp); GSK-3 β , sense 5'-CTG CCC TCT TCA ACT TTA CC-3', antisense 5'-TAT TGG TCT GTCCACGGTCT-3' (159 bp); Dvl1, sense 5'-TCAC CGA CTC TAC CAT GTC C-3', antisense 5'-ATA CGA TCT CCC GAA GCA C-3' (258 bp); and, as an internal reference, β -actin, sense 5'-CGT TGA CAT CCG TAA AGA CC-3', antisense 5'-GGA GCC AGG GCA GTA ATC T-3' (108 bp). The conditions for the real-time PCR amplification reaction were 94°C for 4 min, 94°C for 30 sec, 52°C for 40 sec and 72°C for 45 sec, for 40 cycles. The dissolution curve was analyzed to determine the specificity of the real-time PCR amplification. The expression levels of the target and reference genes were detected first. To compare differential gene expression among the groups, the 2^{- $\Delta\Delta$ Ct} method was used. The control group was considered the standard sample, and the experimental group was considered the test sample. The calculation method was as follows: i) the reference gene was used to calibrate the target gene expression in the control and experimental groups according to the equation Δ Ct (standard sample) = target gene (Mean Ct) - reference gene β -actin (Mean Ct), Δ Ct (test sample) = target gene (Mean Ct) - reference gene β -actin (Mean Ct); ii) the normalization of Δ Ct in the standard and test samples was calculated as $\Delta\Delta$ Ct = Δ Ct (test sample) - Δ Ct (standard sample); iii) differential gene expression was calculated using the formula 2^{- $\Delta\Delta$ Ct}.

Western blot detection of β -catenin, GSK-3 β , Dvl1 and Cyclin D1 protein expression in chondrocytes. Following

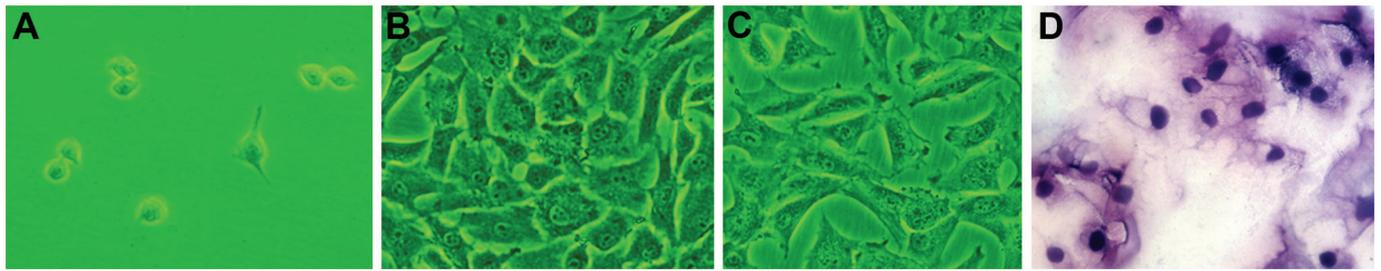


Figure 1. Morphological assessment and identification of chondrocytes (magnification, x200). (A) The initial seeded cells were small and rounded and began to adhere after 24 h. (B) Following 8 days of culture, the cells grew in an irregular 'paving stone' shape. (C) On day 3, the second generation cells were circular and polygonal-shaped. (D) Toluidine blue-staining results of the second generation chondrocytes on day 3.

Table I. MTT assay of chondrocyte viability.

| BMP2 (ng/ml) | Before stimulation | Stimulation (24 h) | Stimulation (48 h) |
|--------------|--------------------|--------------------|------------------------|
| 0 | 0.27±0.05 | 0.34±0.05 | 0.42±0.03 |
| 5 | 0.28±0.08 | 0.36±0.07 | 0.46±0.05 |
| 10 | 0.26±0.06 | 0.37±0.06 | 0.48±0.04 ^a |
| 20 | 0.27±0.07 | 0.38±0.08 | 0.49±0.06 ^a |

^aP<0.05 compared with BMP2 0 ng/ml.

BMP2 stimulation, total protein was extracted from the second generation chondrocytes according to the manufacturer's instructions, and the protein concentration was determined using the BCA assay. A 25- μ l sample (containing 20 μ g total protein) was loaded onto a 12% SDS-polyacrylamide gel, electrophoresed, and then transferred to a PVDF membrane. The membrane was blocked in 3% bovine serum albumin in Tris-buffered saline (TBS) for 2 h at room temperature. The primary rabbit antibodies against β -catenin, GSK-3 β , Dvl1, Cyclin D1 and β -actin were added and incubated with the membrane overnight at 4°C, then the membrane was washed three times with TBS/Tween 20. A secondary antibody against rabbit conjugated to horseradish peroxidase was added to the membrane, followed by incubation at room temperature with gentle agitation for 2 h. Lastly, the membrane was washed three times for 10 min per wash with TBS/Tween 20. The bands were visualized using an enhanced chemiluminescence (ECL) kit, followed by exposure to X-ray film. The intensity of the bands was analyzed using a Fluor-S gel imaging system. The data are presented as the ratio of the target gene to β -actin.

Cell cycle analysis of chondrocytes by fluorescence-activated cell sorting (FACS). Following BMP2 stimulation, the passage 2 chondrocytes were digested, collected and re-suspended in PBS. The cell concentration was adjusted to 1x10⁶ ml following centrifugation. Solutions A, B and C were added according to the manufacturer's instructions prior to sample detection. The cells were analyzed using Cellquest software. Modfit software was used to analyze the DNA content and to count the chondrocyte number in the G0/G1, S and G2/M phases. The chondrocyte proliferation index was calculated according to the formula: proliferation index (PI) = (S+G2/M)/(G0/G1+S+G2/M).

Statistical analysis. SPSS13.0 statistical software was used to process the data, which are presented as the mean \pm standard deviation. Differences among the four groups were compared using one-way analysis of variance (ANOVA), and multiple comparisons were performed with the SNK-q test. P<0.05 was considered statistically significant.

Results

Morphological observation and identification of chondrocytes. The initial seeded cells were small and rounded, and began to adhere after 24 h. The cells gradually increased in size and spread to form pseudopods (Fig. 1A). After 8 days of culture, the cells grew into clusters and were gradually integrated into a single layer in an irregular 'paving stone' shape (Fig. 1B). The second generation chondrocytes had a regular morphology, uniform size and high proliferation rate (Fig. 1C). After 3 days of culture, toluidine blue-staining induced the formation of purple metachromatic granules within and around the cells. The nuclei were dark blue and round or oval-shaped (Fig. 1D).

Effect of BMP2 on chondrocyte viability. Cell viability was measured using the MTT assay. As shown in Table I, there were no significant differences between the various groups of chondrocytes before or after stimulation for 24 h. However, after stimulation for 48 h, the OD values of the chondrocytes in the groups treated with 10 and 20 ng/ml BMP2 were significantly higher than those in the 0 ng/ml group (P=0.035, P=0.023), suggesting that BMP2 increases cell viability.

Effect of BMP2 on β -catenin, GSK-3 β , Dvl1 and Cyclin D1 expression. Following BMP2 treatment for 48 h, the mRNA and protein expression of β -catenin, GSK-3 β and Dvl1 was respectively determined by real-time PCR and Western blotting.

The results of real-time PCR for mRNA expression in the chondrocytes are shown in Table II. β -catenin mRNA was significantly higher in the groups treated with 10 or 20 ng/ml BMP2 as compared to the 0 ng/ml group (P=0.029, P=0.015). Dvl1 mRNA was significantly higher in the groups treated with 10 or 20 ng/ml BMP2 as compared to the 0 ng/ml group (P<0.001) and 5 ng/ml group (P=0.040, P=0.013). GSK-3 β mRNA was significantly lower in the 20 ng/ml BMP2 group as compared to the 0 g/ml group (P=0.043).

The results of Western blotting for protein expression in the chondrocytes are shown in Table III and Fig. 2. β -catenin protein was significantly higher in the groups treated with 10 or

Table II. mRNA expression of β -catenin, GSK-3 β and Dvl1 in chondrocytes.

| BMP2 (ng/ml) | β -catenin | GSK-3 β | Dvl1 |
|--------------|------------------------------|------------------------------|--------------------------------|
| 0 | 1.00 \pm 0.00 | 1.00 \pm 0.00 | 1.00 \pm 0.00 |
| 5 | 1.08 \pm 0.10 | 0.98 \pm 0.04 | 1.09 \pm 0.05 |
| 10 | 1.14 \pm 0.11 ^b | 0.97 \pm 0.05 | 1.19 \pm 0.09 ^{a,c} |
| 20 | 1.16 \pm 0.14 ^b | 0.96 \pm 0.04 ^b | 1.21 \pm 0.12 ^{a,c} |

^aP<0.01; ^bP<0.05 compared with BMP2 0 ng/ml. ^cP<0.05 compared with BMP2 5 ng/ml.

Table III. Protein expression of β -catenin, GSK-3 β , Dvl1 and Cyclin D1 in chondrocytes.

| BMP2 (ng/ml) | β -catenin | GSK-3 β | Dvl1 | Cyclin D1 |
|--------------|-------------------------------|------------------------------|--------------------------------|------------------------------|
| 0 | 0.43 \pm 0.07 | 0.52 \pm 0.07 | 0.39 \pm 0.06 | 0.48 \pm 0.05 |
| 5 | 0.48 \pm 0.07 | 0.48 \pm 0.07 | 0.42 \pm 0.05 | 0.51 \pm 0.06 |
| 10 | 0.58 \pm 0.08 ^{ad} | 0.43 \pm 0.06 ^b | 0.55 \pm 0.06 ^{a,c} | 0.56 \pm 0.05 ^b |
| 20 | 0.61 \pm 0.09 ^{ad} | 0.42 \pm 0.05 ^b | 0.57 \pm 0.07 ^{a,c} | 0.57 \pm 0.07 ^b |

^aP<0.01; ^bP<0.05 compared with BMP2 0 ng/ml. ^cP<0.01; ^dP<0.05 compared with BMP2 5 ng/ml.

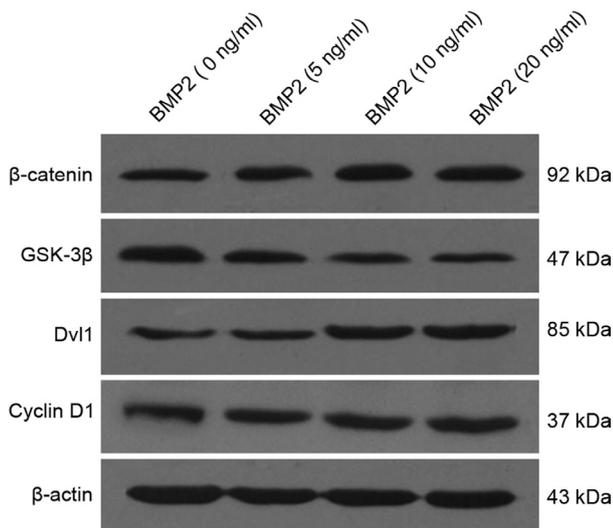


Figure 2. Effect of BMP2 on the expression of β -catenin, GSK-3 β , Dvl1 and Cyclin D1 in the chondrocytes. Chondrocytes were stimulated with BMP2 for 48 h and Western blotting was performed to detect the expression of β -catenin, GSK-3 β , Dvl1 and Cyclin D1 proteins in the chondrocytes.

20 ng/ml BMP2 as compared to the 0 ng/ml group ($P=0.004$, $P=0.001$) and 5 ng/ml group ($P=0.032$, $P=0.011$). Dvl1 protein was significantly higher in the groups treated with 10 or 20 ng/ml BMP2 as compared to the 0 ng/ml group ($P\leq 0.001$) and 5 ng/ml group ($P\leq 0.001$). Cyclin D1 protein was significantly higher in the groups treated with 10 or 20 ng/ml BMP2 as

Table IV. Flow cytometry cell cycle analysis of chondrocytes.

| BMP2 (ng/ml) | G0/G1 phase | S phase | G2/M phase | Proliferation (PI) |
|--------------|-------------------------------|-------------------------------|------------------|-------------------------------|
| Be. S | | | | |
| 0 | 92.68 \pm 2.83 | 4.10 \pm 1.89 | 3.22 \pm 1.21 | 7.32 \pm 2.83 |
| 5 | 91.66 \pm 2.29 | 4.51 \pm 1.51 | 3.83 \pm 1.32 | 8.34 \pm 2.29 |
| 10 | 92.36 \pm 3.17 | 4.37 \pm 1.85 | 3.27 \pm 1.51 | 7.64 \pm 3.17 |
| 20 | 91.58 \pm 3.13 | 4.65 \pm 2.28 | 3.77 \pm 1.45 | 8.42 \pm 3.13 |
| Af. S | | | | |
| 0 | 75.14 \pm 4.85 | 14.92 \pm 3.36 | 9.94 \pm 3.24 | 24.86 \pm 4.85 |
| 5 | 72.62 \pm 4.48 | 15.72 \pm 3.47 | 11.66 \pm 2.51 | 27.38 \pm 4.48 |
| 10 | 68.68 \pm 5.29 ^a | 19.47 \pm 4.72 ^a | 11.85 \pm 3.04 | 31.32 \pm 5.29 ^a |
| 20 | 67.39 \pm 5.19 ^a | 19.79 \pm 3.75 ^a | 12.82 \pm 2.96 | 32.61 \pm 5.19 ^a |

^aP<0.05 compared with BMP2 0 ng/ml.

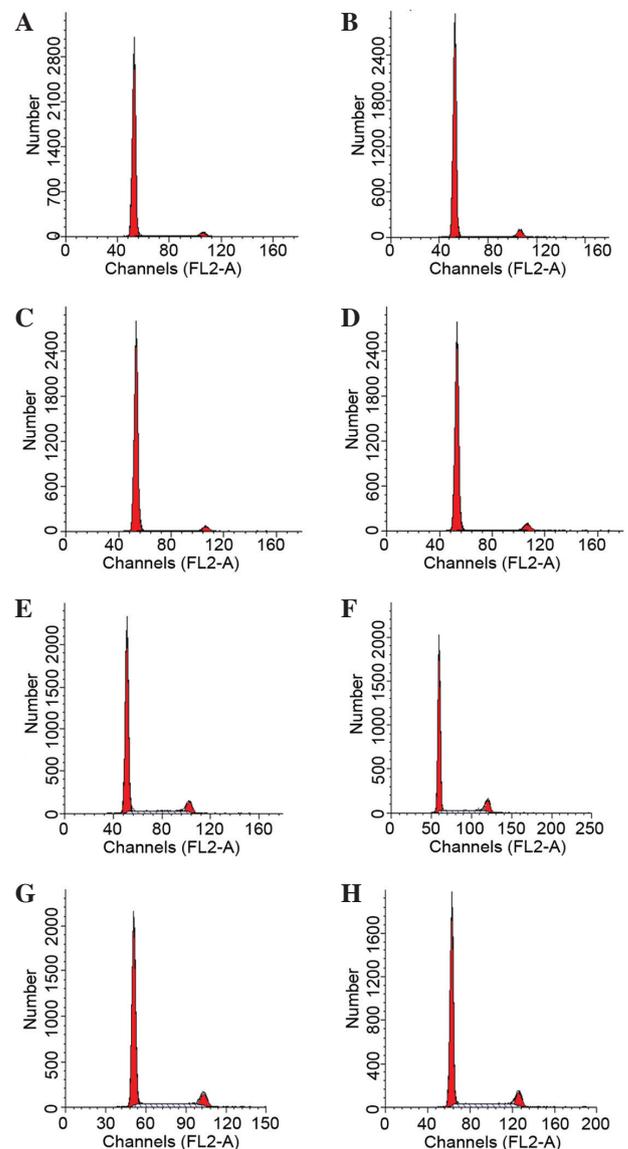


Figure 3. Effect of BMP2 on the cell cycle of the chondrocytes. Cells were starved for 24 h before stimulation with BMP2 at concentrations of (A) 0 ng/ml, (B) 5 ng/ml, (C) 10 ng/ml, or (D) 20 ng/ml; or for 48 h at concentrations of (E) 0 ng/ml, (F) 5 ng/ml, (G) 10 ng/ml, or (H) 20 ng/ml. Following BMP2 treatment, the chondrocytes were collected and stained with PI, then subjected to FACS analysis.

compared to the 0 ng/ml group ($P=0.033$, $P=0.012$). GSK-3 β protein was significantly lower in the BMP2 20 ng/ml group as compared to the 0 ng/ml group ($P=0.043$).

Taken together, the data indicate that BMP2 upregulates β -catenin, Cyclin D1 and Dvl1, and downregulates GSK-3 β .

Effect of BMP2 on the cell cycle of chondrocytes. The G1/S transition is one of the two main checkpoints used by cells to regulate cell cycle progression and thus cell proliferation. We therefore investigated the effect of BMP2 on G1/S progression in the chondrocytes via PI staining followed by FACS analysis. As shown in Table IV and Fig. 3, the percentage proportion of cells was significantly lower in the G0/G1 phase in the groups treated with 10 or 20 ng/ml BMP2 as compared to the 0 ng/ml group ($P=0.036$, $P=0.014$), and was significantly higher in the S phase in the groups treated with 10 or 20 ng/ml BMP2 as compared to the 0 ng/ml group ($P=0.021$, $P=0.016$). The proliferation of cells was therefore significantly increased in the groups treated with 10 or 20 ng/ml BMP2 as compared to the 0 ng/ml group ($P=0.036$, $P=0.014$), indicating that BMP2 stimulates chondrocyte proliferation by promoting cell cycle G1/S progression.

Discussion

The Wnt/ β -catenin signaling pathway plays a key role during the early stages of cartilage formation (14,15). The Wnt/ β -catenin pathway regulates the DNA-binding proteins of the TCF/LEF family, mainly in regard to the stability of β -catenin in the cytoplasm. Intracellular β -catenin levels are maintained by the balance between degradation and antagonist proteins (16,17). The degradation proteins consist of the APC complex, including GSK-3 β , Axin and APC. The antagonist proteins include Dsh, CK1 ϵ and GSK-3 β -binding protein (GBP) (18). In normal unstimulated cells, the Wnt/ β -catenin signaling pathway is inactivated. Most β -catenin is attached to the cytoskeleton through binding to membrane cadherins to mediate the adhesion of the same type of cells. BMP2 is a member of the TGF- β superfamily and a multiple regulator of cell growth and differentiation. BMP2 is capable of activating the Wnt/ β -catenin pathway, upregulating intracellular β -catenin levels, promoting the translocation of β -catenin to the nucleus, and activating Cyclin D1 to facilitate chondrocyte cycle progression (19,20). In the present study, chondrocytes were stimulated with BMP2 for 24 or 48 h, and were observed to exhibit significant proliferation ability after 48 h of stimulation.

BMP2 upregulates the expression of Wnt3a, a secreted ligand for members of the Frizzled family of receptors. This in turn increases the ability of BMP2 to induce the formation of cartilage. Exogenous Wnt3a upregulates β -catenin and Smad4, both of which synergistically enhance the inducing effect of BMP2. At the same time, BMP2-activated Wnt3a also binds the Frizzled receptor to activate Dvl1. The released GBP binds axin-associated GSK-3 β , thus inhibiting GSK-3 β -mediated β -catenin phosphorylation/degradation. Unphosphorylated β -catenin accumulates in the cytoplasm and translocates to the nucleus to impair the activity of the complex TCF/LEF family and the GRO and GBP inhibitory proteins. As a consequence, activated Cyclin D1 facilitates the G1/S transition, thus promoting the proliferation of chondro-

cytes (21,22). One checkpoint exists in the late G1 phase, and once the cells pass through this checkpoint, they are no longer dependent on an exogenous stimulation signal for proliferation, thus acquiring the capacity to independently complete the cell cycle (23). The DNA content of the cells reflects the specific processes of cell growth and proliferation. G0 cells are in a quiescent state, G1 cells are in a phase of pre-DNA synthesis, DNA is synthesized in the S phase, and G2 cells become tetraploid. Cells in the G2 phase reserve energy for mitosis (M phase). Our study showed that, following stimulation of chondrocytes with BMP2 for 48 h, the percentage of G0/G1 cells was reduced while the S phase cells and the proliferation index were increased. The expression of Dvl1, β -catenin and Cyclin D1 was significantly increased, while GSK-3 β expression was significantly reduced.

In conclusion, BMP2 promotes chondrocyte proliferation by upregulating the expression of Dvl1, β -catenin and Cyclin D1 and downregulating the expression of GSK-3 β .

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