SCIENTIFIC ARTICLE

Effects of Cartilage Oligomeric Matrix Protein on Bone Morphogenetic Protein-2-induced Differentiation of Mesenchymal Stem Cells

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Objective: To investigate the effect of overexpression of cartilage oligomeric matrix protein (COMP) on bone morphogenetic protein-2 (BMP-2) induced osteogenic and chondrogenic differentiation of mesenchymal stem cells (MSCs). In this study, we used liposomes to transfect MSCs with plasmid encoding COMP and then induced the transfected MSCs to differentiate in osteogenic and chondrogenic differentiation media containing BMP-2.

Methods: MSCs transfected with plasmid DNA encoding recombinant human COMP were induced to differentiate into osteocytes and chondrocytes by BMP-2. Real-time polymerase chain reaction (PCR) assays of osteogenesis-related markers (collagen type I alpha 1, runt-related transcription factor 2, osteopontin, bone gla protein) and chondrogenesis-related markers (collagen type II alpha 1, sry-related high-mobility group box 9, Aggrecan) was performed to evaluate the process of cell differentiation. Cell differentiation was evaluated by alkaline phosphatase (ALP) and Alizarin red S stains for osteogenic differentiation and alcian blue staining for chondrogenic differentiation.

Results: Real-time PCR assay showed significantly greater COMP expression by MSCs when COMP gene had been transfected into the cells ($P < 0.01$). Overexpression of COMP down-regulated expression of osteogenesis-related markers and up-regulated expression of chondrogenesis-related markers. ALP staining and Alizarin red S staining were weakened, whereas alcian blue staining was enhanced.

Conclusion: Overexpression of COMP inhibits BMP-2-induced osteogenic differentiation and promotes BMP-2-induced chondrogenic differentiation. These findings may provide new insights for cartilage tissue engineering. The experiments in the present study were all *in vitro*, which has potential limitations. Further *in vivo* studies to investigate the effects of COMP in animal models are necessary, which will be the next step in our research.

Key words: Bone morphogenetic protein-2; Cartilage oligomeric matrix protein; Chondrogenic differentiation; Mesenchymal stem cells; Osteogenic differentiation

Introduction

As is well known, hyaline cartilage protects bones from wear. Even though it is a durable type of tissue, once hyaline cartilage has been injured, it has very limited ability to self-repair¹. Tissue transplantation, one of the major available treatment options, has produced short-term positive results in

terms of improvement in symptoms; however, the long-term results are still uncertain²⁻⁴. More importantly, transplantation of tissues such as osteochondral allograft and autologous cartilage has the major limitations of insufficient supply and donor injury. Therefore, cartilage tissue engineering using stem cells and gene technique is being investigated for new

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insights into treatment of cartilage injury. Given the an insufficient supply of autologous chondrocytes for cartilage tissue engineering, mesenchymal stem cells (MSCs) provide an optimal choice with their broad origins, easy accessibility and ability to self-replenish and be stimulated to differentiate into different specialized cells, such chondrocytes, adipose cells and osteocytes⁵⁻¹¹. MSCs are widely used as ideal seed cells in various areas, including cartilage tissue engineering¹². Since Evans *et al.* first reported gene transfer to cartilage tissues^{13,14}, clinical application of gene therapy for cartilage repair has attracted considerable attention. Gene modification techniques have potential application in promoting proliferation and chondrogenic differentiation of MSCs for long-term regulation of cartilage repair^{15,16}.

Many factors have been identified as participating in the differentiation of MSCs, including various transcription and growth factors such as sry-related high-mobility group box 9 (SOX9), bone morphogenetic proteins (BMPs), transforming growth factor- β (TGF- β), and vascular endothelial growth factor^{17,18}. BMPs, which are especially effective for bone regeneration and repair, have proven ability to induce ectopic bone formation¹⁹⁻²¹. Of these, BMP-2 is of particular interest. BMP-2 belongs to TGF- β , which is known to induce proliferation and chondrogenic differentiation²²⁻²⁵. At the same time, BMP-2 is also known to induce osteogenic differentiation of MSCs and stimulate endochondral ossification; it has been approved for clinical use in management of tibial nonunion by the USA Food and Drug Administration²⁶⁻²⁸. Because of its dual effects, in that it both inhibits osteogenic differentiation and promotes chondrogenic differentiation, BMP-2 is of particular interest for cartilage repair.

Overexpression of cDNAs encoding cartilage-specific extracellular matrix such as cartilage oligomeric matrix protein (COMP) can reportedly be used to maintain the cartilage phenotype²⁹. COMP, which belongs to the thrombospondin family, is a large disulfide-bonded homopentameric glycoprotein and one of the most important non-collagenous proteins in cartilage, ligament, tendons and synovium³⁰⁻³². COMP is also a sensitive marker for the state of differentiation of articular primary chondrocytes and COMP concentration in serum is a biomarker of arthritis^{33,34}. In addition, mutations in the COMP gene that are responsible for at least two types of osteoarthritis, multiple epiphyseal dysplasia and pseudoachondroplasia, have been identified^{35,36}. COMP functions as a type of signaling protein, interacting with various molecules, including receptor complexes at the cell surface, and playing a vital role in the adhesion, proliferation and differentiation of cells³⁷⁻⁴⁰. Also functioning as a type of integrin ligand, COMP can recognize and bond integrin $\alpha 5 \beta 1$ and initiate the subsequent signal transduction to direct cell behavior and fate⁴¹. Du *et al.* reported that COMP inhibits calcification of vascular smooth muscle cells by interacting with BMP-2⁴². COMP also reportedly has multiple binding sites for the TGF- β family of proteins⁴³, suggesting that it plays a vital role in cell fate. However, little attention has been focused on the effects of COMP on stem cell differentiation

and it is still not clear whether overexpression of COMP affects BMP-2 induced cell differentiation of MSCs.

The aim of this study was to investigate the effects of overexpression of COMP gene on BMP-2-induced cell differentiation of MSCs and the synthesis of related differentiation products *in vitro*. In this study, we used liposomes to transfect MSCs with plasmid encoding COMP and then induced the transfected MSCs to differentiate in osteogenic and chondrogenic differentiation media containing BMP-2.

Materials and Methods

Materials

Human recombinant bone morphogenetic protein 2 was obtained from Pepro Tech (Rocky Hill, NJ, USA). pIRES-hrGFP-1a plasmid encoding recombinant human COMP was obtained from Sangon Biotech (Shanghai, China). The plasmid was amplified in *Escherichia coli* and purified by using a plasmid purification kit (Bioer, Hangzhou, China) according to the manufacturer's instructions. The cell line was cultured in complete Dulbecco's modified Eagle's medium (DMEM; Gibco, Carlsbad, CA, USA), supplemented with 10% fetal bovine serum (Gibco), 100 U/mL penicillin and 100 mg/mL streptomycin (Sigma, St Louis, MO, USA), and then maintained at 37 °C in a humidified 5% CO₂ atmosphere. MSCs, corresponding stem cell products and cell staining reagents were obtained from Cyagen (Guangzhou, China), except when otherwise noted.

Cell Culture

Sprague-Dawley rat mesenchymal stem cells were cultured in Sprague-Dawley rat MSC growth medium at 37 °C in a humidified 5% CO₂ atmosphere. The medium was changed every 3 days and cells were detached with trypsin-ethylenediaminetetraacetic solution at 80% to 90% confluence for re-seeding. MSCs at passage five were used in the experiments.

Cell Transfection

Mesenchymal stem cells in logarithmic growth phase were re-seeded (initial density 1×10^4 cells/cm²) into 6-well plates in MSC growth medium. The transfection procedure was performed according to the manufacturer's instructions for Lipofectamine LTX and PLUS reagent (Invitrogen, CA, Carlsbad, USA) at 80% confluence. Namely, 2 μ g of plasmid DNA was diluted with Opti-MEM (Life Technologies, Carlsbad, CA, USA) and mixed with 2 μ L PLUS reagent and 6 μ L LTX reagent to a final volume of 500 μ L. The mixture was then incubated at room temperature for 30 min before transfection. Medium was replaced with different kinds of medium after transfection for 6 h according to the experimental design. Fresh medium was replenished every 4-5 days. The experiment was performed on cells transfected with plasmid encoding target gene (COMP group); cells transfected with empty plasmid (LIPO group); and non-transfected cells (control).

TABLE 1 Primer oligonucleotide sequences in this study

Gene	Oligonucleotides (5'–3')	Gene Bank ID
GAPDH	F:GACATGCCGCTGGAGAAAC R:AGCCCAGGATGCCCTTAGT	NM_017008.4
RUNX2	F:GCGTCCTATCAGTTCCAAT R:CAGCGTCAACACCATCATT	NM_001278483.1
OPN	F:CTTGGCTTACGGACTGAGG R:GCAACTGGGATGACCTTGAT	NM_012881
BGP	F:CAAGTCCACACAGCAACTC R:CCAGGTCAGAGAGGAGAAT	NM_013414.1
COMP	F:CCCAACTCAGACCAGAAGGA R:GTCACAAGCATCTCCACAA	NM_000095.2
SOX9	F:GACGTGCAAGCTGGGAAAGT R:CGGCAGGTATTGGTCAAACTC	XM_001081628.3
Col2a1	F:CGCCACGGTCCTACAATGTC R:GTCACCTCTGGGTCTCTGTTTAC	NM_012929.1
AGG	F:TGGCATTGAGGACAGCGAAG R:TCCAGTGTGTAGCGTGTGGAATAG	NM_022190.1
Col1a1	F:GACATGTTCAAGCTTTGTGGACCTC R:GGGACCCCTTAGGCCATTGTGTA	NM_053304.1

Cell Differentiation and Detection of Related Gene Expression

According to the instructions, BMP-2 was added to DMEM supplemented with 10% fetal bovine serum, 100 U/mL penicillin and 100 mg/mL streptomycin at a final concentration of 50 ng/mL. The medium was replaced with the DMEM mixture described above after transfection for 6 h. Cells were harvested at appropriate time intervals for subsequent real-time polymerase chain reaction (PCR) analysis.

The total RNA of the MSCs harvested at the desired time points was extracted using TRIZOL reagent (Invitrogen) according to the manufacturer's protocol. Reverse transcription reactions were carried out using Prime Script RT Reagent Kit (Takara, Dalian, China) according to the manufacturer's instructions. Real-time PCR was performed using SYBR Premix Ex Taq (Takara) according to the manufacturer's instructions. Target genes were normalized to glyceraldehyde 3-phosphate dehydrogenase (GAPDH) expression and all sample values were calculated by the $2^{-\Delta\Delta C_t}$ method. Table 1 shows the primer sequences used in this study.

Osteogenic Staining

The medium was replaced with osteogenic differentiation medium (Cyagen) containing 50 ng/mL BMP-2 after transfection for 6 h and cell staining performed at the desired time points. Cells that had been cultured in osteogenic differentiation medium for 7 days were stained using a BCIP/NBT Alkaline Phosphatase Color Development Kit (Boster, Wuhan, China) according to the protocol for alkaline phosphatase (ALP) staining.

On day 14 after osteogenic differentiation, alizarin red S staining for calcium precipitation was performed. Briefly, the cells were fixed with 4% formaldehyde solution for 30 min, followed by washing twice with phosphate-buffered saline (PBS). The cells were then stained with alizarin red S for 5 min. After rinsing with PBS another two times, the staining of the mineralized nodules was observed under a light microscope (DMIL; Leica, Heidelberg, Germany).

Chondrogenic Staining

The medium was replaced with chondrogenic differentiation medium (Cyagen) containing 50 ng/mL BMP-2 after transfection for 6 h and cell staining performed at the desired time points.

On day 21 after chondrogenic differentiation, alcian blue staining for proteoglycans synthesized by chondrocytes was performed. The cells were washed with PBS, fixed with 4% formaldehyde solution for 30 min and washed again with PBS, then stained with alcian blue solution for 30 min and washed with distilled water. The cells were then observed under a light microscope.

Statistical Analysis

The results are expressed as mean $\pm$ standard deviation (SD). One-way analysis of variance was performed to evaluate the significance of the observed differences between the study groups. $P < 0.05$ was considered statistically significant.

Results

Target Gene Expression

Real-time PCR assay was performed to quantify expression of COMP in MSCs using GAPDH as a reference gene for normalization. As shown in Fig. 1, MSCs in the COMP group expressed the COMP gene significantly more strongly than those in LIPO group ($P < 0.01$). Expression decreased with

Fig. 1 Relative mRNA expression of target gene COMP was analyzed by real-time PCR at different time points (3, 7, 14 days). The values were normalized to GAPDH ($n = 3$, $**P < 0.01$).

Fig. 2 Relative mRNA expression of Col1a1, RUNX2, OPN and BGP was analyzed by real-time PCR. The values were normalized to GAPDH ($n = 3$, $*P < 0.05$, $**P < 0.01$).

time, dramatically less expression occurring on day 7 than on day 3.

Osteogenesis-related Gene Expression and Osteogenic Staining

To investigate differentiation of MSCs at the gene level, real-time PCR was performed to quantify mRNA expression of related genes on days 3, 7 and 14. The findings for osteogenesis-related genes are shown in Fig. 2. Compared with that in the LIPO group, expression of osteopontin (OPN) was significantly down-regulated at all assessed time points in the COMP group ($P < 0.05$). Even though there was decreased expression of collagen type I alpha 1 (Col1a1), runt-related transcription factor 2 (RUNX2) and bone gla protein (BGP) in the COMP group at an early stage of induction (day 3) ($P < 0.05$), a final significant up-regulation of expression appeared in the COMP group at a late stage of induction (days 7, 14) compared with that in the LIPO group ($P < 0.05$), which is in agreement with the results of osteogenic staining. Osteogenic differentiation was assessed by ALP staining and alizarin red S staining. The intensity and the area of ALP staining was less in the COMP group than in the LIPO group, indicating lower ALP activity (Fig. 4A). Alizarin red S staining was also weaker in the COMP group at the same time (Fig. 4B).

Chondrogenesis-related Gene Expression and Chondrogenic Staining

Expression of chondrogenesis-related genes as assessed by real-time PCR is shown in Fig. 3. An opposite trend occurred in the chondrogenic differentiation assay. Compared with that in the LIPO group, expression of Aggrecan (AGG) was up-regulated in the COMP group at all assessed time points ($P < 0.05$). Expression of collagen type II alpha 1 (Col2a1) increased in both groups with time and, compared with that in the LIPO group, there was significant up-regulation in the COMP group on days 7 and 14 ($P < 0.05$). There was also a trend toward greater expression of SOX9 in the COMP group than in the LIPO group, the difference being significant only on day 7 ($P < 0.05$). In parallel with the gene expression findings above, alcian blue staining was enhanced in the COMP group compared with the LIPO group, indicating accumulation of more proteoglycans (Fig. 4C).

Discussion

Because of the multiple effects of growth factors such as BMP-2 and according to abundant animal experiments, it is important to augment chondrogenic induction for cartilage tissue engineering. Indeed, the use of gene delivery for cartilage

Fig. 3 Relative mRNA expression of Col2a1, SOX9 and Aggrecan was analyzed by real-time PCR. The values were normalized to GAPDH ($n = 3$, * $P < 0.05$, ** $P < 0.01$).

repair was pioneered in animal studies^{13,14}. The success of these experiments provided new insights into the novel approach of gene therapy for cartilage injury. Gene therapy has the advantages of high specificity and long-term effect, both of which traditional treatment methods lack. It has been reported that the delivery and expression of cDNAs encoding cartilage-specific extracellular matrix such as COMP may be used to maintain the cartilage phenotype²⁹. Therefore, this study investigated a combination of overexpression of COMP and BMP-2-induced cell behavior to investigate the interaction between these two factors.

To investigate whether the target gene COMP could successfully express in MSCs and to quantify changes in expression with time, real-time PCR assays of COMP were performed. Significantly stronger expression of COMP mRNA was detected in MSCs in the COMP group than in the LIPO group, indicating successful transfection of target gene. Expression exogenous genes transfected by liposomes reportedly reaches a peak after 3 days of culture and cannot be sustained for a long time because of loss of the exogenous gene^{44,45}. We detected decreased expression with time, a dramatic decrease being noted on day 7 compared with that at day 3, which is in agreement with previous studies.

To further investigate the effect of overexpression of COMP on BMP-2-induced osteogenesis by MSCs, real-time PCR of osteogenesis-related gene markers was employed. RUNX2 is a key transcription modulator of osteoblast differentiation and plays a vital role in regulating osteoblasts maturation, which can be stimulated by BMP-2⁴⁶⁻⁴⁸. Further, studies have shown that RUNX2 can up-regulate expression of factors involved in bone matrix formation, such as Col1a1, OPN, BGP, fibronectin and so on^{49,50}. In our study, up-regulated expression of osteogenesis-related gene markers occurred at an early stage of induction in the COMP group, whereas reduced expression of these gene markers was observed in the COMP group at a later stage of induction. We postulate that there a type of negative feedback regulation may exist: this possibility requires more research because few studies have focused on it. The weakening of alizarin red S and ALP staining that we observed in the COMP group also indicates suppression of mineralization of the MSCs and reduced activity of ALP, which is an important enzyme in the process of osteoblast maturation associated with overexpression of COMP. Du *et al.* have demonstrated that COMP can bind directly to BMP-2 through the C terminus, inhibit BMP2 receptor binding and block BMP2 osteogenic signaling⁴². Our results are consistent with these findings, which clarify the mechanism of action of COMP. It has also been reported that COMP enhances osteogenesis by directly binding and activating BMP-2⁵¹. Similarly to Du *et al.*, this study also found that COMP binds to BMP-2 and intervenes in the related signal pathway. However, their study investigated ectopic bone formation in the C2C12 cell line and in a rat model, which is quite different from the model we used to examine gene transfection way; this may explain the difference in our findings. These researchers also found that BMP-2 binds to COMP in a dose-dependent manner, which may also have contributed to the difference. Our results suggest that overexpression of COMP can inhibit BMP-2-induced osteogenic differentiation of MSCs.

Meanwhile, we assessed chondrogenic differentiation by real-time PCR and alcian blue staining. We observed consistent up-regulation of expression of Col2a1, AGG and SOX9 mRNA in the COMP group compared with the LIPO group. SOX9, a transcription factor that belongs to the SOX proteins family, is considered a key transcription factor for chondro-

Fig. 4 Staining of MSCs in the COMP, LIPO and control groups. (A) ALP staining of MSCs after osteogenic induction for 7 days; (B) Alizarin red S staining of MSCs after osteogenic induction for 14 days; (C) Alcian blue staining of MSCs after chondrogenic induction for 21 days.

genesis²⁵. Both Col2a1 and AGG are essential components of extracellular matrix in cartilage and are important molecular markers of chondrogenesis. The up-regulation of these markers suggests enhancement of the chondrogenic process. In addition, alcian blue staining was enhanced in the COMP group, indicating augmentation of chondrogenesis. COMP is a signaling protein that includes many domains, such as its C-terminals, RGD domains and T3C5 domains^{39,40}. COMP reportedly has multiple binding sites for the TGF- β family of proteins and activates TGF- β -dependent transcriptional activity⁴³. This may explain the finding of enhanced chondrogenesis in our study because BMP-2 belongs to the TGF- β family and may be activated by COMP. Another study has shown that ectopic COMP expression enhances several early aspects of chondrogenesis induced by BMP-2⁵². The results of our chondrogenic differentiation assay are in agreement with

this study: they suggest that overexpression of COMP can promote BMP-2-induced chondrogenic differentiation of MSCs.

Conclusions

In this study, we investigated the effects of overexpression of COMP on BMP-2-induced osteogenic and chondrogenic differentiation of MSCs. Our findings suggest that overexpression of COMP inhibits BMP-2-induced osteogenic differentiation and promotes BMP-2-induced chondrogenic differentiation, which may provide new insights for cartilage tissue engineering. The experiments in the present study were all *in vitro*, which has potential limitations. Further *in vivo* studies to investigate the effects of COMP in animal models are necessary, which will be the next step in our research.

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