

Original Article

Establishment of rat bone mesenchymal stem cell lines stably expressing Chondromodulin I

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Abstract: To study the role of Chondromodulin I (ChM-I) in inducing bone mesenchymal stem cells (MSCs) into chondrocytes, the plasmid expressing pcDNA3.1 (+) / ChM-I was constructed, and transfected into MSCs. Stable expression verified that ChM-I is upregulated in MSCs, which make it a useful preparation for studying the further role of ChM-I in inducing bone MSCs toward a chondrogenic I phenotype and for tissue engineering constructs. MSCs were obtained from adult Sprague Dawley (SD) rats using density gradient centrifugation. Cell surface antigens were detected by flow cytometry. The experimental groups were MSCs transfected with the plasmid pcDNA3.1 (+)/ChM-I. The control one was with the plasmid pcDNA3.1 (+) and the negative control one was with no plasmid. Transcription and translation were detected in the level of mRNA and protein respectively in order to identify the stability of the expression of ChM-I in cell lines by RT-PCR and western blot analysis. The results of RT-PCR showed that the mRNA level of ChM-I in the experimental group was six times higher than the control. The quantitative optical density analysis showed that the expression of ChM-I in the experimental group was significantly higher than the control by western blot. We conclude that we have established MSCs lines stably expressing ChM-I. This work lays the foundation of studying the potential role of the ChM-I in inducing BMSCs into chondrocytes.

Keywords: Chondromodulin-I, MSCs biological factors, plasmid pcDNA3.1 (+)/ChM-I, tissue engineering

Introduction

Defects of the cartilages of nose and ear are not uncommon in the practice of head and neck surgery, and plastic surgery. Excellent quality of repair is required at these anatomical sites as it can influence not only the function of the nose and ear but also esthetic appearance. Self-repair and regeneration of cartilage is inherently poor [1]. The best repair method is to preserve the structure and function of the nose. The field of tissue engineering cartilage is one potential approach to address this problem [2]. The importance of cell seeding, scaffold materials, and biological factors are key factors in achieving success.

To determine the biological factors that induce MSCs into chondrocytes, our group have showed the following in preliminary studies [3, 4]. Gene chip technology determined the differ-

ential expression of genes between rat MSCs, and hyaline and elastic cartilage. Bioinformatics results suggested that ChM-I may play a key role in the process of inducing MSCs to cartilage cells; The expression of ChM-I protein in hyaline and elastic cartilage chondrocytes was significantly higher than MSCs [5]. It has been shown that ChM-I has anti-angiogenic activity and has been suggested to inhibit endothelial cells from invading cartilage. ChM-I also promotes anchorage-independent growth of chondrocytes [6-8]. These observations suggest that Chondromodulin-1 may play an important role in inducing MSCs into chondrocytes accurately. To further study the role of the ChM-I in inducing MSCs into chondrocytes, we constructed the recombinant pcDNA3.1(+)/ChM-I expression vector and transfected MSCs. The stable expression of ChM-I cell line of rat MSCs was established. This is a tool for studying the further role of ChM-I in inducing bone MSCs toward a

chondrogenic I phenotype and for tissue engineering constructs

Materials and methods

Sprague-Dawley (SD) rats (Laboratory Animal Center, Dalian Medicinal University) received humane care in compliance with the "Guide for the Care and Use of Laboratory Animals" prepared by the Institute of Laboratory, Animal Resources, National Research Council, and published by the "Guide to the Care and Use of Experimental Animals" by the Chinese Council on Animal Care. TRIzol Reagent (Invitrogen, USA), RT-PCR kit (Takara, Japan), Ultrapure Agarose (GIBCO, USA), Ethidium Bromide (EB, USA), T4 DNA Ligase (Fermentus), HIND III, NOT I (NEB Company), DMEM (GIBCO, USA), percoll separator liquid (Promega, USA), FBS (GIBCO, USA).

Construction and identification of pcDNA3.1(+)/ChM-I expression vector

300 mg of rat cartilage was snap frozen in liquid nitrogen, ground to a frozen powder. Total RNA was extract by using Trizol reagent. The quality of RNA samples was assayed by the absorption value in 260 bp and 280 bp (value of OD260/280 between 1.8 to 2.0) and the yield of RNA was calculated according to 1 OD260 equals to 40 µg RNA. Integrity of the extracted RNA and contamination of DNA were determined by denaturation agarose electrophoresis for ChM-I PCR amplification (GenBank's Reception Number: AF051425). The primers for PCR were designed using National Center for Biotechnology Information (NCBI)'s site based on Rattus norvegicus ChM-I mRNA sequences from GenBank. The specific primers for ChM-I mRNA were designed by using Primer 5 software. The forward primer: GCAGACAAGCTTATGACAGA GAACTCGGACA, with a Hind III restriction enzyme cutting site being inducted. The reverse primer: GCA GAC GCG GCC GCT T ACA CCA TGC CCA AGA TG, with a NOT I restriction enzyme cutting site being inducted. The length of amplified segment was 1140 bp. PCR products were detected by electrophoresis in 1% agarose gel. The target segment was identified under Ultra-violet lamp. The target gene segment was retrieved and purified by using gel extraction kit. The PCR product and pcDNA3.1 linear plasmids of the HIND III and NOT I double enzyme digestion were connected overnight at 16 °C. Connected products were transformed into XL1-

Blue competent bacteria. Bacteria were transformed LB-Ampr+ flat at 37 °C by overnight incubation, and picked positive bacteria of the monoclonal growth to LB-Ampr+ liquid medium, cultured by shaking 200 rpm at 37 °C for 15 hours.

We used the plasmid extraction and purification kit to extract the plasmid in the bacteria. The extracted products were cut by enzymes at 37 °C according to the following enzyme cutting system: pcDNA3.1(+)/ChM-I plasmid (1.5 µg) 5 µl, HIND III 1 µl, NOT I 1µl, 10 × Buffer K 1 µl, 10 × BSA 2 µl, sterile water added up to 20 µl, enzyme cutting in water bath at 37 °C for 4 hours, then detected by electrophoresis in 1% agarose gel and sequenced.

Isolation, culture, and identification of MSCs

Male or female SD rats, 6-8 weeks old and 120-160 gms, were purchased from the Experimental Animal Center of Dalian Medicine University (Dalian, China). Rats were fed a standard rodent diet and water ad libitum according to the guidelines approved by the China Association of Laboratory Animal Care and the Institutional Animal Care in use Committee. Under anesthesia (pentobarbital sodium, 60mg/kg, IP), femurs were aseptically harvested, washed in a mixture of phosphate buffered saline (PBS) and antibiotics for 5 minutes, dissected of all soft tissue, transected at their epiphysis, and their marrow cavity rinsed repeatedly with a mixture of heparin and Dulbecco's minimum essential medium (DMEM). The harvested cells were collected and centrifuged at 1500 rpm for 10 minutes. Cell pellets were resuspended with DMEM, and 2 times percoll separator liquid with a density of 1.073 g/ml was added to the tube. After centrifugation at 2500 rpm for 30 minutes, the single nucleated cells layer were separated, the MSC layer was resuspended in DMEM, and centrifuged at 1500 rpm for 10 minutes. Following washes, the cells were placed in DMEM culture medium supplemented with 10% Fetal Bovine Serum, and cultured to the fourth passage. Cells were identified as MSC's by flow cytometry (FACS Calibur BD, USA).

pcDNA3.1 (+)/ChM-I plasmid transfection into MSCs mediated by lipofectamine 2000

Fourth passage MSCs at a confluence of 80-90%, grown in 6 cm dish, were used for trans-

fection. Medium was replaced with 2.5 ml of LG-DMEM in each dish, to prepare cells for transfection. Transfection was performed by following the Lipofectamine 2000 kit protocol. The cells were divided into three groups: 1). pcDNA3.1 (+)/ChM-I group: including adequate amounts of rat MSCs, 8 µg pcDNA3.1(+)/ChM-I plasmid, 16 µl Lipofectamine 2000, 3.5 ml LG-DMEM; 2). pcDNA3.1 (+) plasmid group: including adequate amounts of rat MSCs, 8 µg pcDNA3.1 (+) plasmid, 16 µl Lipofectamine 2000, 3.5 ml LG-DMEM; 3). Control group: 3.5 ml DMEM alone. Incubated cells were at 37 °C, 5% CO₂. Complete medium was changed after 6 hours, then after 48 hours, 200 µg/ml G418 selective medium was added. Medium was replaced every 3 days. When cells of the control group were non viable, the transfected groups were still viable in complete culture medium containing 200 µg/ml G418. The selected cells were cultured for passage and amplification until reaching 80-90% confluence in culture medium containing 100 µg/ml of G418. The cells of the third passage were characterized for further experiments.

Detection of ChM-I gene expression in target cells

To detect the expression of ChM-I at the transcriptional level, RT-PCR was used. Total RNA from three cell preparations was reverse transcribed in 10 µL of reaction mixture containing 1 µL of reverse transcriptase at 42 °C for 30 minutes. The following oligonucleotide primers: ChM-I (PCR product, 153 bp), forward primer: AAG CAG TGC TCC CTC TAC CA, reverse primer: CTC TCT CCT TCC TGC TGG TG; GAPDH (PCR product, 207 bp), forward primer: AGA CAG CCG CAT CTT CTT GT, reverse primer: CTT GCC GTG GGT AGA GTC AT. The cycling program was performed under the following conditions: 10 min at 95 °C, 35 cycles of 1 min at 94 °C, 30 seconds at 60 °C, and 1 min at 72 °C, 72 °C for 10 min. Amplified PCR products were analyzed on 2% agarose gels, and visualized by ethidium bromide staining. Analysis of absorbance was normalized by using the Biolimaging system. Relative expression of protein in each group was normalized by GAPDH expression. Each experiment was repeated six times.

Western blot analysis

To detect the expression of ChM-I protein, protein was extract from the cells as previously

described [5]. Protein concentration was measured using BCA protein kit (Beyotime Institute of Biotechnology, Jiangsu, China). Total cellular proteins (50 µg) were run onto SDS-PAGE with 12% separation gel, then protein was transferred to a nitrocellulose membrane, blocked with 5% dried milk at room temperature for 1 hour, incubated with anti-ChM-I antibody at a concentration of 1: 400 (Zhongshan golden bridge, Beijing, China) at 4 °C for overnight, washed with TBST, 10 min each for four times, and incubated with a peroxidate-conjugated secondary antibody goat anti-rabbit-IgG-ALP (Zhongshan golden bridge, China) conjugated at a concentration of 1:1000 at room temperature for one hour, washed with TBST, 10 min each for four times. Blots were stripped and reprobed with antibodies to anti-GAPDH (1: 1000) (Zhongshan golden bridge, China) to confirm equivalence in loading. The analysis of absorbance was done by using the Biolimaging system (UVP, USA).

Histochemistry and immunohistochemistry

Histochemical and immunohistochemical analyses were performed after 7 and 21 days for all groups pcDNA3.1 (+) plasmid group, pcDNA3.1 (+)/ChM-I plasmid group, MSCs group were separately placed into culture with medium containing TGF-β [10ng/ml]. Cultures were harvested in vitro and prepared for Toluidine blue dyeing and immunohistochemistry staining. For histochemical analyses, cells were rinsed twice with PBS, stained with 0.1% toluidine blue dyeing for 20 minutes. For immunohistochemical analyses, cells were rinsed twice with PBS, fixed with 4% paraformaldehyde for 20 minutes, primary antibodies for collagen type II (Zhongshan golden bridge, China) were used at 1:500 dilution for overnight at 4 °C, detected by the avidin-biotin-complex (ABC) method with diaminobenzidine as label (Vectastain ABC Elite Kit, Vector Laboratories, Burlingame, CA), and counterstained for 30 sec with hematoxylin. Controls omitted the primary antibody under identical conditions.

Statistical analysis

The data from RT-PCR and western blot were analyzed using the SPSS17.0 software package. Analysis of variance and LSD test were used to determine significant differences in multiple comparisons. P< 0.05 was considered statisti-

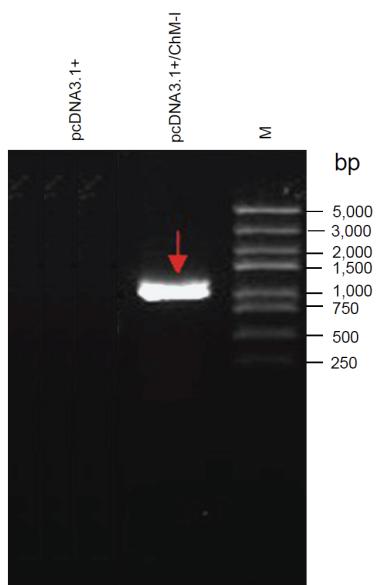


Figure 1. ChM-I expression by RT-PCR. The production of the RT-PCR was detected by 1% agarose gel electrophoresis. ChM-I strongly expressed in pcDNA3.1(+) / ChM-I group (around 1000 bp, with the red arrow). “M” indicated marker.

cally significant.

Results

ChM-I gene amplification by RT-PCR

The production of the RT-PCR was detected by 1% agarose gel electrophoresis. We showed that ChM-I strongly expressed in pcDNA3.1(+) / ChM-I group (around 1000 bp, with the red arrow) (**Figure 1**). We did not see any ChM-I expression in pcDNA3.1(+) group. “M” indicates marker.

Restriction enzyme analysis of positive clones

Digestion product of the plasmid in three positive clones (**Figure 2**, lane 1, 2, 3) by 1% agarose gel electrophoresis showed that the bacterial strains had been transfected by the recombinant plasmid, the red arrow is for the target gene fragment, the blue arrow for the plasmid vector. “M” indicates marker.

DNA sequencing of positive clones

The results of DNA sequencing in the positive clones showed that the length and sequence of

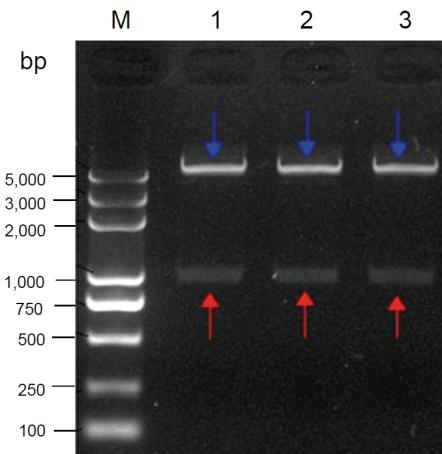


Figure 2. Restriction enzyme analysis of positive clones. Digestion products of recombinant plasmids in three positive clones (lane 1, 2, 3) by 1% agarose gel electrophoresis showed that the bacterial strains have been transfected by the recombinant plasmid, the red arrow is for the target gene fragment, the blue arrow for the plasmid vector.

ChM-I gene consistent with the theoretical prediction, and the reading frame was correct (**Figure 3**).

Growth and morphologic characteristic of MSCs

Five days after cell seeding (**Figure 4A**), those cells in suspension that underwent necrosis were removed. Selective cells grew in spindle, triangular, and polygonal shapes. Seven days after seeding, the cells entered the logarithm proliferative phase. Cell density reached 90% after culturing for 10-13 days (**Figure 4B**). Following passage of the third generation culture (**Figure 4C**), cell density reached 80% within 6 hours, and they grew faster than the original P1 generation. Some of the cells were shown long fusiform, like the fibroblast-like cells, growing densely and tightly packed, other cells seemed to grow in a whorl-like pattern.

After exposure to transfection medium for 6 hours, there were a large number of highly refractive vesicles within the cells, indicating that the combination of the liposome and plasmid had translocated into MSCs. **Figure 4D** showed 12 hours after transfection. After 48 hours, the cells were cultured in selection medium containing 200 µg/ml of G418. After 2 weeks, all of the control group cells had been killed. The remain-

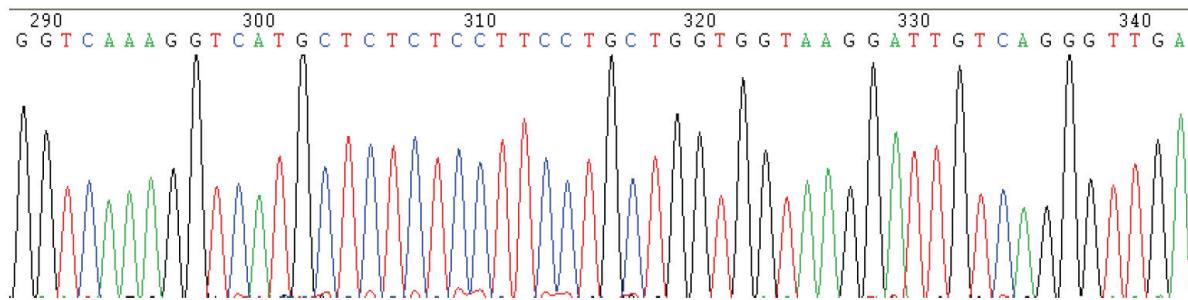


Figure 3. DNA sequence of ChM-I insert segment in recombinant plasmids. The results of DNA sequencing in the positive clones showed that the length and sequence of ChM-I gene consistent with the theoretical prediction, and the reading frame was correct.

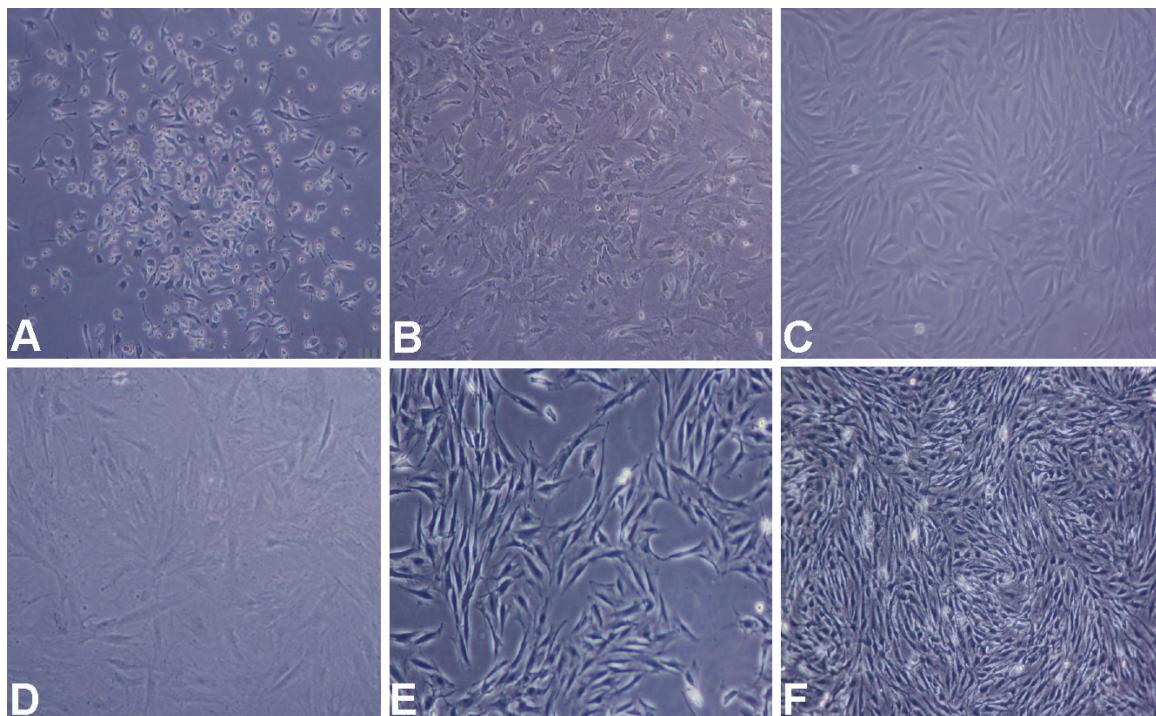


Figure 4. Growth and morphologic characteristic of MSCs. A. 5 days of primary culture, selective cells grew in spindle, triangular, and polygonal shapes. B. 13 days after seeding, the cells entered the logarithm proliferative phase. Cell density reached 90% after culturing for 10-13 day. C. Following passage of the third generation culture, cells grew faster than the original P1 generation. D. 12 hours after transfection. E. 3 weeks after selection. The remaining cells were fibroblast-like in appearance, long-spindle shaped. F. second generation after selective, cells grew vigorously and were densely arranged with a whorl-like geometry.

ing selected cells continued to thrive in the medium containing 200 µg/ml of G418, and proliferate. **Figure 4E** showed 3 weeks after selection. The remaining cells were fibroblast-like in appearance, long-spindle shaped. After passage, these cells grew vigorously and were densely arranged with a whorl-like geometry (**Figure 4F**).

Flow cytometric analysis of MSCs

Figure 5A was shown as negative control. The MSC positive surface antigen-CD90 and CD29-was 99.79% and 98.41% respectively (**Figure 5B**), while the rate of the negative markers-CD45 and CD11b/ c-was 2.77% and 2.51% respectively (**Figure 5C**).

Mesenchymal stem cell lines expressing Chondromodulin I

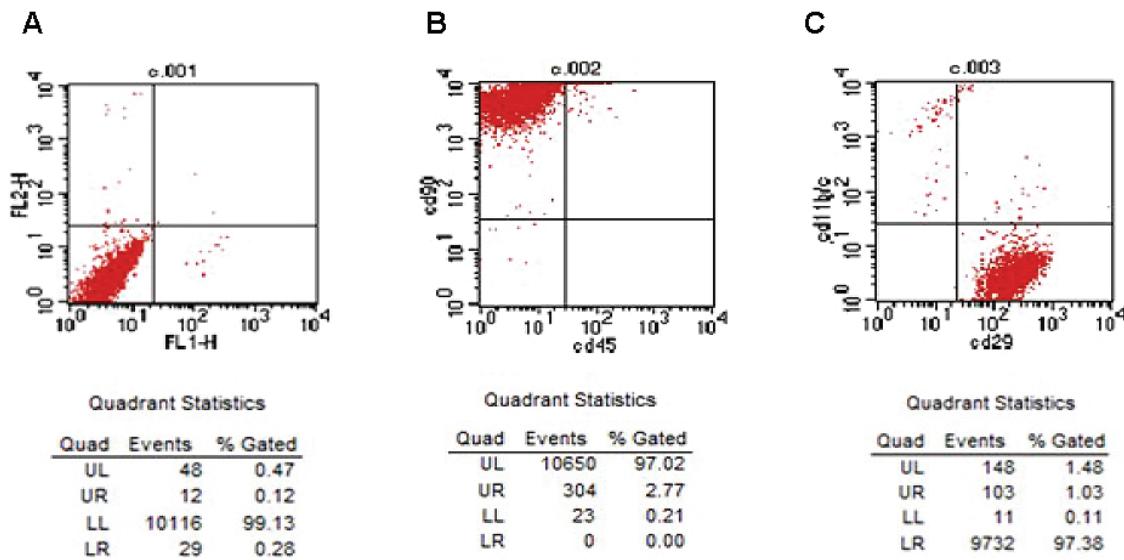


Figure 5. MSCs identified by flow cytometric analysis. A was shown as negative control. The MSC positive surface antigen-CD90 and CD29- was 99.79% and 98.41% respectively (B), while the rate of the negative markers-CD45 and CD11b/c-was 2.77% and 2.51% respectively (C).

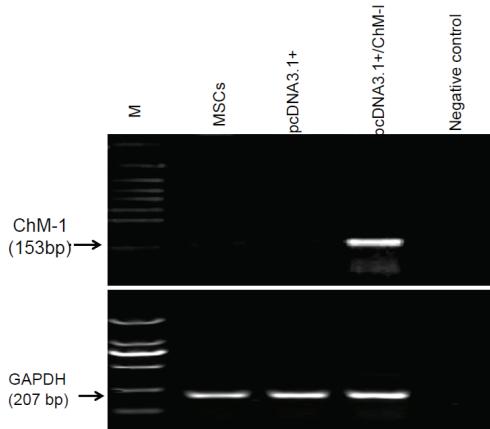


Figure 6. ChM-I transcription gene was analysis by RT-PCR. The expression level of gene ChM-I in stably transfected MSCs pcDNA3.1(+)/ChM-I was significantly higher than in the stable transfected MSCs of pcDNA3.1 (+) and nontransfected MSCs. The PCR products are 153 base pair (bp) for ChM-I. As an internal control, 207 bp bands for GAPDH were shown equal. “M” indicates marker. Negative control was water only.

Analysis of ChM-I transcription gene by RT-PCR

ChM-I transcription gene was analysis by RT-PCR. The expression level of gene ChM-I in

stably transfected MSCs pcDNA3.1+/ChM-I was significantly higher than in the stable transfected MSCs of pcDNA3.1 (+) and nontransfected MSCs, negative control was water only (**Figure 6**).

Analysis of the ChM-I protein by western blot

According to western blot, the quantitative optical density analysis showed that the average expression of ChM-I protein level in the MSCs of stable, transfected pcDNA3.1+/ChM-I was significantly higher comparing to those in the stable, transfected MSCs of pcDNA3.1 (+) and the nontransfected MSCs (* $p < 0.05$). However, the expression of ChM-I protein was not significant different between the stable transfected MSCs of pcDNA3.1 (+) and nontransfected MSCs ($p > 0.05$). Bar graphs show mean \pm SD of multiple samples of each type, normalized to GAPDH ($n=9$) (**Figure 7**).

Histochemical analysis of cartilage matrix

After 7 days in cultures, staining with toluidine blue dyeing revealed a homogeneous cell distribution in all groups (**Figure 8A**, 200 \times). The shape of cells changed from spindle in MSCs (a) and pcDNA3.1+ (b) groups to polygon in pcDNA3.1+/ChM-I (c) and pcDNA3.1+/ChM-I

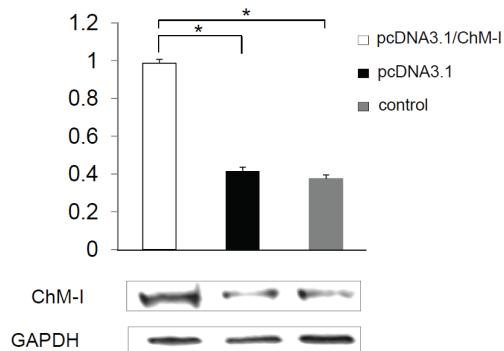


Figure 7. The results of western blot analysis: the quantitative optical density analysis showed that the average expression of ChM-I protein level in the MSCs of stable, transfected pcDNA3.1(+)ChM-I was significantly higher comparing to those in the stable, transfected MSCs of pcDNA3.1 (+) and the nontransfected MSCs (* $p < 0.05$). There was no different change between the stable transfected MSCs of pcDNA3.1 (+) and nontransfected MSCs ($p > 0.05$). Bar graphs show mean \pm SD of multiple samples of each type, normalized to GAPDH ($n=9$).

plus TGF- β (d) groups which had more specificity cartilage matrix secretion. Most MSCs differentiated into cells with a chondrocyte-like phenotype in pcDNA3.1+/ChM-I (c) and pcDNA3.1+/ChM-I plus TGF- β (d) groups comparing with MSCs (a) and pcDNA3.1+ (b) groups.

Immunohistochemistry analysis of collagen type II

For Immunohistochemical analysis of Collagen type II (Col II) after 7 days (**Figure 8B**), positive staining is indicated by the spotted brown-colored extracellular matrix and showed that Col II could be detected in the groups of pcDNA3.1+/ChM-I (g) and pcDNA3.1+/ChM-I plus TGF- β treatment (h), but not in the control groups of MSCs (e) and pcDNA3.1 (f), 200 \times .

Discussion

Cartilage tissue engineering requires a cell and scaffold composite to construct a living material that can be transplanted into human bodies. In this process, the regulation of cell growth is a key component. During cell division, growth factors can promote or inhibit the proliferation, migration and gene expression of cells. Therefore, proper growth factor selection, which can control the direction of targeted cell differentiation, is a challenging task.

Bone MSCs have been shown to represent 0.001% of the entire marrow cell population. MSCs are clonogenic and multipotent and are able to differentiate into bone, cartilage, hematopoietic supportive stroma, and adipocytes [9-17]. Ferrari et al. [18] reported that bone marrow-derived cells engrafted in injured skeletal muscle, demonstrated that a population in the bone marrow could colonize and possibly undertake a myogenic commitment. These characteristics make them ideally suited for a wide spectrum of clinical applications for repair of damaged or defective tissue. MSCs can be isolated primarily by adhesion to plastic, but could also be concentrated by Percoll gradient centrifugation [19]. A steady culture system for MSCs has been set up previously [20], and in this culture system, 3×10^4 primary cells can be obtained from each SD mouse, and the 10^8 cell number can be reached after four passages at the rate of 1:3. In this study, detecting the surface antigens with flow cytometry was used to identify cultured MSCs, and we examined four markers, and the cells shown in the result were CD90+, CD29+, CD45- and CD11b/c-, which are consistent with MSC phenotype.

Chondromodulin is a cartilage-derived growth factor, including ChM-I, ChM-II, ChM-III. Among these three, ChM-I is the first element to be a clear regulator of cartilage and has the strongest effect of a cartilage-derived growth factor. Hiraki Y [6] reported that ChM-I purified from fetal bovine cartilage markedly stimulated DNA synthesis of cultured growth-plate chondrocytes in the presence of basic fibroblast growth factor (FGF). Inoue [8] observed that ChM-I stimulates the colony formation of rabbit growth plate chondrocytes in agarose culture. ChM-I alone weakly stimulated the formation of chondrocyte colonies, but it markedly stimulated colony formation synergistically in the presence of an optimal dose of FGF-2. This effect was dependent on the dose of ChM-I. When the concentration of ChM-I is 1 ng/ml, it can be detected; when the concentration of ChM-I is 200 ng/ml, ChM-I shows the strongest activity. Hiraki [7] reported that ChM-I was identified as an angiogenesis inhibitor. In situ hybridization and immunohistochemical studies indicated that ChM-I is specifically expressed in the avascular zone of cartilage in developing bone, but not present in calcifying cartilage. Purified ChM-I inhibited DNA synthesis and proliferation of vascular endothelial cells as well as tube morphogenesis in vitro. In a recent study, ChM-I impairs the VEGF-A

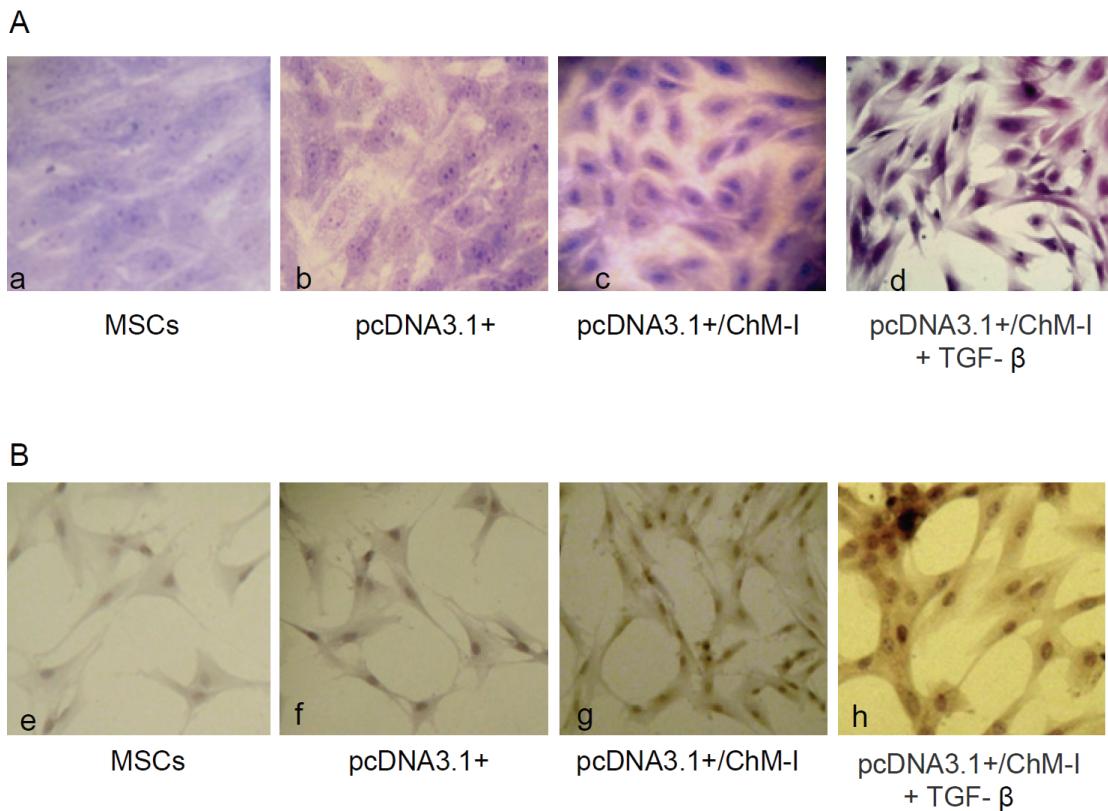


Figure 8. Histochemistry and immunohistochemistry staining after 7 days in culture. Histochemistry staining (A): The shape of cells changed from spindle in MSCs (a) and pcDNA3.1 (b) groups to polygon in pcDNA3.1(+)/ChM-I (c) and pcDNA3.1(+)/ChM-I plus TGF- β (d) groups. There was more specificity cartilage matrix secretion in pcDNA3.1(+)/ChM-I (c) and pcDNA3.1(+)/ChM-I plus TGF- β treated groups (d). Most MSCs differentiated into cells with a chondrocyte-like phenotype in pcDNA3.1(+)/ChM-I (c) and pcDNA3.1(+)/ChM-I plus TGF- β groups (d) comparing with MSCs (a) and pcDNA3.1(+) groups (b), 200 \times . Immunohistochemistry staining (B): Immunohistochemical analysis of Col II after 7 days. Positive staining was indicated by the spotted brown-colored extracellular matrix and showed that Col II could be detected in the groups of pcDNA3.1(+)/ChM-I and pcDNA3.1(+)/ChM-I plus TGF- β treatment (g and h), but not in the control groups of MSCs and pcDNA3.1 (e and f), 200 \times .

stimulated motility of endothelial cells by destabilizing lamellipodial extensions [21]. These results suggest that ChM-I plays a key role in cartilage formation and maintenance of cartilage avascular state. Combined with our report, we speculated that ChM-I may also play an important role in the induction of MSCs to chondrocytes during differentiation [5].

Eukaryotic expression vector pcDNA3.1, designed for the high performance of cDNA in mammalian cells [22], was used in our study to construct the stably effective gene expression vector system. The length of pcDNA3.1 is 5427 bp. pcDNA3.1 (+) vector has the following characteristics [23]: 1) because of containing human cytomegalovirus promoter and high expres-

sion in mammalian cells, it used a wide range; 2) due to vector containing multiple positive and negative internal Cloning sites, it facilitates cloning; 3) with the neomycin resistance gene, it facilitates selection for stable transfection of cell lines in the complete medium containing G418, because it free copy in cell line that latently infected sarcoma virus 40 (SV40) or expressed T antibody-based of SV40, it is used in broad scope of applications; 4) there is pcDNA3.1/CAT plasmid that can be selected as the pcDNA3.1 (+) plasmid transfection and expression of the positive control, therefore, it is easy to assay results; 5) pcDNA3.1 has the character of easy copy and stable. Based on the above advantages, pcDNA3.1 (+) vector has been widely used in various fields of medical

research work.

How to effectively transfer the target gene of pcDNA3.1/ChM-I vector into MSCs was the key point of this study. There are two useful gene transport vehicles, viral vector or liposome. Safety is a key concern as some incidents that have occurred have raised issues about the clinical application of viral vector [24, 25]. Liposomes are a type of lipid containing a hydrophilic inner core. When used as a gene transfer vector, liposomes have low transfection efficiency but a high safety. Liposomes have the following characteristics: they protect the DNA plasmid from degradation by nuclease; they are easy to manipulate; they have no immunogenicity; there is no limitation on the size of the exogenous gene. Therefore, positive ion Lipofectamine2000 was chosen as the transfer vector in this study. The transfer efficiency of liposomes is affected by the following parameters [26, 27]: the mix proportion of liposome and DNA; incubation time; the concentration of blood serum in medium. In our study, one day before transfection, a plate with 2×10^5 cells in 500 μl of growth medium was used so that the cells could be 90-95% confluent at the time of transfection in 6 cm well. Based on referred documents and tests from our preliminary experiment, the best ratio of DNA to liposome volume was DNA: LP2000 ($\mu\text{g}:\mu\text{l}$) = 4: 8 [28, 29]. Furthermore, serum-free DMEM cultivation should be used in the process of transfection. Because serum contains protein with negative charges and lipid with positive charge, which can reduce the ability of liposome contacting with DNA to form compound.

Differentiation into cartilage is typically characterized by expression of type II collagen, and aggrecan. In this study we demonstrate that the over expression of chondromodulin in MSC's led to increase in collagen type II synthesis. This is consistent with development of hyaline cartilage. Although we did not specifically look at aggrecan expression it can be inferred that aggrecan would be similarly increased since collagen type II has a very limited distribution and is found only in hyaline cartilage.

The expression ChM-I gene and protein were significantly up-regulated in the stable transfection of pcDNA3.1/ChM-I vector group. Taken together, recombinant plasmid pcDNA3.1/ChM-I was successfully constructed and transfected

into rat bone marrow mesenchymal stem cells. After G418 selection, expression of ChM-I has been stable in rat bone marrow mesenchymal stem cell cells. This work provided the foundation for further researching the function of ChM-I in inducing bone marrow mesenchymal stem cells into cartilage cells.

Acknowledgments

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