

Effect of the human insulin-like growth factor 1 gene transfection to human umbilical cord blood mesenchymal stem cells

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ABSTRACT

الأهداف: مراقبة تأثير تعداد عامل النمو للجينات البشرية والذي يشبه الأنسولين 1 (hIGF) في دم الحبل السري للإنسان الخلايا الجذعية المتعلقة باللحمة المتوسطة (hUCB-MSCs) عن طريق النواقل غير الفيروسية.

الطريقة: أجريت هذه الدراسة في مستشفى التابعة لجامعة تشينغداو وتشينغداو، والصين من يونيو 2012م إلى مايو 2013م. حصد 12 عينة من hUCB، وعزلت الخلايا اللمفاوية المتوسطة، ومن ثم زرعت. وكشفت عن سطح مستضد في الخلايا الجذعية المتعلقة باللحمة المتوسطة من قبل التدفق الخلوي. البلازميد المؤتلف pIRES2 بالكامل محسنة بروتينات الفلورية الخضراء hIGF-1 (EGFP) ثم تعددت إلى الخلايا الجذعية المتعلقة باللحمة المتوسطة من قبل X-treme GENE HP DNA كاشف التعداد. ثم، لوحظ EGFP مع المجهر الفلورسنت العكسي في نقاط زمنية مختلفة. ثم استخدمت انزيم المرتبط المناعي لفحص تحديد تركيز البروتين hIGF-1 في supernatants. استخدمت الفحص المجهر المناعي وتفاعل البوليميراز المتسلسل المنتسخة العكسية RT-PCR للكشف عن التعبير hIGF-1 في الدم الحبل السري للإنسان الخلايا الجذعية المتعلقة باللحمة المتوسطة. وكشفت الكولاجين من النوع الثاني صبغة الكيمياء الهيستولوجية المناعية.

النتائج: كانت كفاءة التعداد $28.74 \pm 7.31\%$. ازدادت CD34، CD45، CD90، CD105، CD146 (CD) وبينما انخفضت ANTI-DR. أكدت نتائج الفحص المجهر المناعي و RT-PCR التعبير عن الجينات hIGF-1. أظهر تركيز البروتين hIGF-1 في supernatants مستوى الذروة في $34.63 \pm 1.61 \text{ ng/ml}$ 48 ساعة بعد التعداد. أثبت التحليل المناعي لتعداد hUCB-MSCs أن الكولاجين من النوع الثاني يمكن التعبير عنه بشكل إيجابي.

الخلاصة: يمكن تعدد الجينات البشرية النمو الذي يعبر عن الأنسولين IGF-1 في دم الحبل السري للإنسان الخلايا الجذعية المتعلقة باللحمة المتوسطة (hUCB-MSCs) وعلى مستوى عالٍ من الكولاجين من النوع الثاني.

Objectives: To observe the effect of transfecting the gene human insulin-like growth factor (hIGF)-1 into human umbilical cord blood mesenchymal stem cells (hUCB-MSCs) via non-viral vector.

Methods: This study was performed in the Affiliated Hospital of Qingdao University, Qingdao, China from June 2012 to May 2013. Twelve hUCB samples were harvested, and isolated in lymphocyte separation medium, and then cultured. Surface antigen expression in MSCs was detected by flow cytometry. Recombinant plasmid pIRES2-enhanced green fluorescent protein (EGFP)-hIGF-1 was transfected into MSCs by X-treme GENE HP DNA transfection reagent. Then, EGFP was observed with reverse fluorescent microscope at different time points. Enzyme-linked immunosorbent assay was used to determine the hIGF-1 protein concentration in supernatants. Immunofluorescence microscopy and reverse transcription polymerase chain reaction were used to detect the expression of hIGF-1 in the hUCB-MSCs. Expression of type II collagen was detected by immunohistochemistry staining.

Results: Transfection efficiency was $28.74 \pm 7.31\%$. The cluster of differentiation (CD)90, CD105, CD146, and CD146 expression increased CD34, CD45, and anti-HLA-DR expression decreased. Results of immunofluorescence microscopy and RT-PCR confirmed expression of the hIGF-1 gene. The hIGF-1 protein concentration in the supernatants showed a peak level at $34.63 \pm 1.61 \text{ ng/ml}$ 48 hours after transfection. Immunohistochemical analysis of transfected hUCB-MSCs proved that type II collagen could be expressed positively.

Conclusion: Human IGF-1 gene can be transfected into hUCB-MSCs, and expressed at a high level with subsequent expression of type II collagen.

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Chondral and osteochondral lesions of the knee are notoriously difficult to treat due to the poor healing capacity of articular cartilage, ultimately causing disabling pain, and early osteoarthritis.¹ The treatment of chondral or osteochondral in the weight-bearing region of joints is a therapeutic challenge.² Mesenchymal stem cells (MSCs) have drawn interest in regenerative medicine because they are highly proliferative and differentiate in multiple specialized lineages under defined conditions. Scaffold technology combined with MSCs has been used to treat hyaline cartilage defects previously.³ In recent years, tissue engineering was proven to be a suitable method for repairing bone defects, as well as cartilage defects.⁴ Growth factors, especially the human insulin-like growth factor (hIGF)1 have a pivotal role in the stimulation of chondrocyte and undifferentiated cells, which is important for homeostasis.⁵ Introduce protein of hIGF-1 to MSCs, or chondrocytes cultured in vitro was revealed to stimulate the proliferative and metabolic actions, promotes the synthesis of proteoglycan, and type-II collagen.⁶ However, any injected protein will be cleared rapidly from the joint, and it is difficult to maintain a prolonged exposure due to the chondroinductive factor with a one-time delivery of the protein.⁷ Cells carrying a transgene for specific proteins may solve this problem.⁸ In this study, we constructed the bicistronic eukaryotic vector pIRSES2-enhanced green fluorescent protein (EGFP)-hIGF-1, and transfected it into human umbilical cord blood mesenchymal stem cells (hUCB-MSCs) by X-treme GENE HP DNA transfection reagent to test whether the hIGF-1 gene with marker could be expressed. Cell supernatants were then examined for secretion of downstream cytokines and morphological changes of hUCB-MSCs following transfections observed.

Methods. According to the hIGF-1 cDNA sequence data (X00173) published at GenBank, the primer design software Premier 5.0 was used to design primers containing XhoI/EcoRI restriction site fragment. Sequence of the primers were: Upstream primer 5'-GCC TCG AGG AAG ATG CAC ACC ATG TCC TC-3';

Downstream 5'-GCG AAT TCA TAC ATC CTG TAG TTC TTG TTT C-3'. The complementary (c)DNA for hIGF-1 was amplified from a human hepatocyte cDNA library (Clontech Inc, CA, USA) by polymerase chain reaction (PCR). The amplified products were ligated to the pMD18-T simple Vector (Takara Biotechnology, Ohtsu, Shiga, Japan), and the recombinant pMD18-T-hIGF-1 plasmid was sequenced. The plasmid that contained the correct hIGF-1-cDNA product was selected, and cloned into the bicistronic eukaryotic vector pIRSES2-EGFP (Roche Company, Basel, Switzerland) containing a marker gene and the internal ribosomal entry site. Then the recombinant pIRSES2-EGFP-hIGF-1 plasmid was transfected into HEK293 cells to test its expression. The marker gene expression of EGFP was observed with a reverse fluorescent microscope.

This study was performed in the Affiliated Hospital of Qingdao University, Qingdao, China from June 2012 to May 2013. This research was carried out in compliance with the Helsinki Declaration, and was approved by the research ethics committee of the Affiliated Hospital of Qingdao University. Patients were informed before any procedure was carried out. The material was used for scientific research only after informed consent was signed.

There are 12 volunteers involved in the sampling, and residual hUCB (approximately 50 ml) was obtained from the cord of full-term deliveries in 20 ml tubes preloaded with 2 ml of heparin for MSC culture, with informed consent from the mother. The umbilical cord blood was treated with phosphate buffered saline (PBS) (1:1) to produce a cell suspension, and then mixed with lymphocyte separation medium (3:1). The mixture was centrifuged at 2000 rpm for 30 minutes. After centrifugation, the monocytes were extracted and treated with Dulbecco's Modified Eagle's Medium (DMEM)-F12 media containing 1% penicillin/streptomycin, and 10% fetal bovine serum (Hyclone, UT, USA) to produce a cell suspension. The cell suspension was seeded in 25 cm² culture bottles at a density of 4×10³ cells/cm². The cells were incubated at 37°C in 5% CO₂ and 95% air at 100% humidity. After 72 hours, the media was changed, and then changed every 3 days after that. When the MSC colonies reached 70-80% confluency, they were trypsinized and reseeded into 3 new bottles. The passage 3 cells were used for this study. Trypsinized MSCs were suspended in PBS containing 5% fetal bovine serum at a concentration of 2×10⁶/ml before they were used. Then the cells were incubated at 4° for 30 minutes with conjugated antibodies to cluster of differentiation (CD)90-

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fluorescein isothiocyanate (FITC), CD105-FITC, CD146-FITC, CD34-PE, CD45-PE, and anti-HLA-DR. Cells were centrifuged at 1500 rpm for 5 minutes. Supernatants were removed and cells were washed twice in PBS. Control cells were stained with a FITC-conjugated mouse immunoglobulin (Ig)G isotype, or a phycoerythrin (PE)-conjugated mouse IgG isotype at 4°C for 30 minutes. The cells were analyzed by flow cytometry. Twenty-four hours before the transfection, cells in log phase were trypsinized by 0.25% trypsin (Hyclone, UT, USA), and seeded onto 24-well plates with 3×10^4 cells per well. At 80-90% confluency, the cells were washed twice in PBS. Following manual instructions for X-treme GENE HP DNA transfection reagent (Clontech, CA, USA), DNA, and transfection reagent were added to each well in a 1:3 ratio, and 450 μ l of serum-free DMEM-F12 media without penicillin/streptomycin was added to each well, and mixed by gentle shaking. The cells were incubated in a 37°C incubator in 5% CO₂. The expression of green fluorescent protein (EGFP) in the cells was observed using fluorescence microscope at different time points following transfection, and the transfect ratio calculated. The transfection efficiency formula used was:

$$\text{Transfection efficiency} = \frac{\text{EGFP - positive cells number}}{\text{counted cells number}} \times 100\%$$

The enzyme-linked immunosorbent assay (ELISA) for hIGF-1 was performed using cell culture supernatant from 48-well plates collected at different time points after transfection. The supernatant was centrifuged at 2000 rpm for 15 minutes before testing. A 96-well ELISA plates were treated with the assay reagents as described in the manufacturer's protocol. Absorbance was read at 450 nm, and the values measured were calculated to indicate hIGF-1 concentration by utilizing the standard curve available

in the assay kit. Each time point was detected 5 times. Immunofluorescence was used to detect the expression of hIGF-1 in the cells, 72 hours after transfection. The cells transfected with pIRES2-EGFP-hIGF-1 in 6-well plates were washed 3 times, and fixed with paraformaldehyde for 20 minutes at room temperature, then washed again. Cells were permeated with 0.3% Triton for 10 minutes, blocked with 10% goat serum, and incubated at 37°C for 30 minutes. The serum was removed, and then the plates were incubated overnight with rabbit anti-hIGF-1 primary antibody (Yueyan Biotechnology, Shanghai, China) at 4°C. Plates were washed with PBS and incubated with goat anti rabbit IgG-FITC (Yueyan Biotechnology, Shanghai, China). After 60 minutes incubation at 37°C, the plates were washed, and observed under an inverted fluorescence microscope at 480 nm. One week after transfection, the total RNA was extracted from hUCB-MSCs with, or without hIGF-1 gene transfection using TRIzol reagent mixed with chloroform, and then precipitated with 100% isopropanol. The RNA was reverse transcribed into cDNA. The expression of hIGF-1 was detected by RT-PCR. The RT-PCR was performed using hIGF-1 primers, and an internal control was carried out using GAPDH primers. The protocol for the PCR reaction system used was as follows: denaturation (94°C, 2 minutes), 35 cycles of denaturation (94°C, 30 seconds), annealing (54°C, 30 seconds), extension (72°C, one minute), and final extension (72°C, 10 minutes). The RT-PCR products were run on a 1.2% agarose gel. Two weeks after transfection, the cells were washed with PBS, and fixed using acetone for 10 minutes at 4°C, and incubated with 3% H₂O₂ for 10 minutes. They were then washed with PBS again, and permeated with 0.3% Triton for 10 minutes, blocked with 10% goat serum, and incubated at 37°C for 30 minutes. The serum was

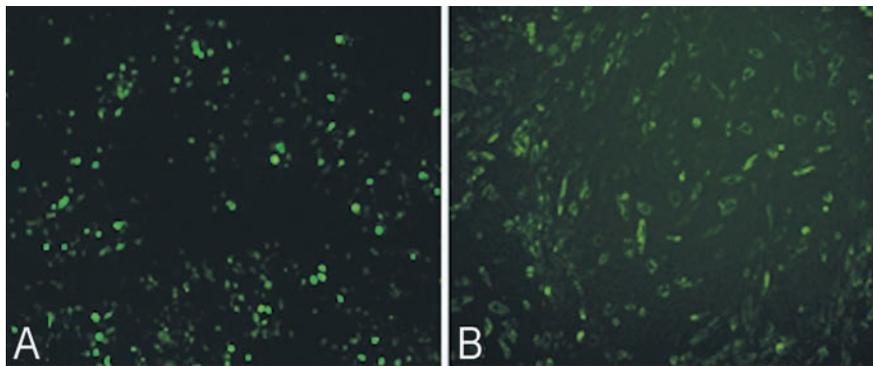


Figure 1 - Green fluorescent protein can be observed after 48 hours of transfection in: A) HEK293 cells, and B) human umbilical cord blood mesenchymal stem cells under fluorescence microscope.

removed, and slides were incubated overnight with rabbit anti-human Collagen type II Antibody (Boster Biotechnology, Wuhan, China) at 4°C. Slides were washed with PBS and incubated with goat anti-rabbit IgG. After a 60-minute incubation at 37°C, the slides was treated with 3,3'-Diaminobenzidine (DAB) and hematoxylin reagent, and observed under an inverted microscope.

Statistical analysis. The Statistical Package for Social Sciences version 17 for Windows (SPSS Inc, Chicago, IL, USA) was used for data analysis. The data were summarized as the mean \pm standard deviation measurements. Paired T-test was used to check for the difference of the hIFG-1 protein concentration between the experimental and matched group. The significance was determined at $p < 0.05$.

Results. The fragments inserted into the pMD-18T simple vector were analyzed by sequencing, and

results were consistent with the published sequence in GeneBank (X00173). Double enzyme digestion with XhoI and EcoRI separated on an agarose gel showed a band at approximately 410 base pair (bp) consistent with the size of hIFG-1 cDNA. Green fluorescence was observed under an inverted fluorescence microscope, and the expression was nearly 67% (Figure 1A). After transfection, the expression of green fluorescent protein in the cells was observed under a fluorescence microscope at different time points. The green fluorescent can be observed 8 hours after transfection, and the expression of green fluorescence protein reached maximum intensity 48 hours after transfection. The transfection efficiency within the hUCB-MSCs was $28.74 \pm 7.31\%$ at 48 hours after transfection (Figure 1B). Few umbilical cord blood-derived mononuclear cells attached after 24 hours of incubation, and appeared round, or short spindle-shaped. After 3 days of culture in vitro, dispersed small colonies of MSCs were observed with

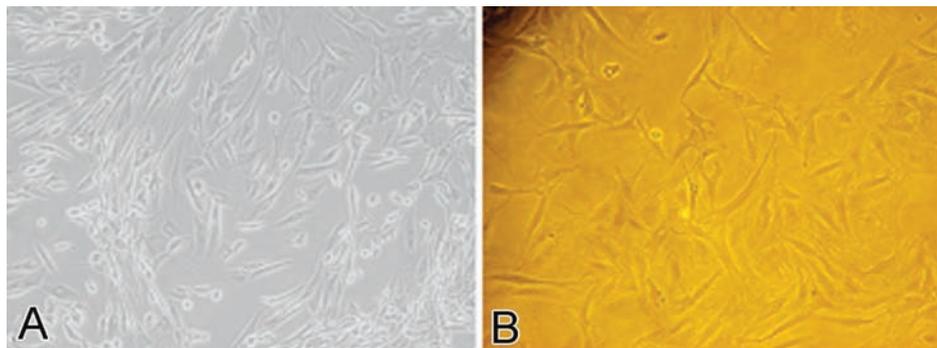


Figure 2 - An image showing the cellular morphology of human umbilical cord blood mesenchymal stem cells before (A), and after gene intervention (B). Cells enlarged and more polygonal morphologies existed after transfection.

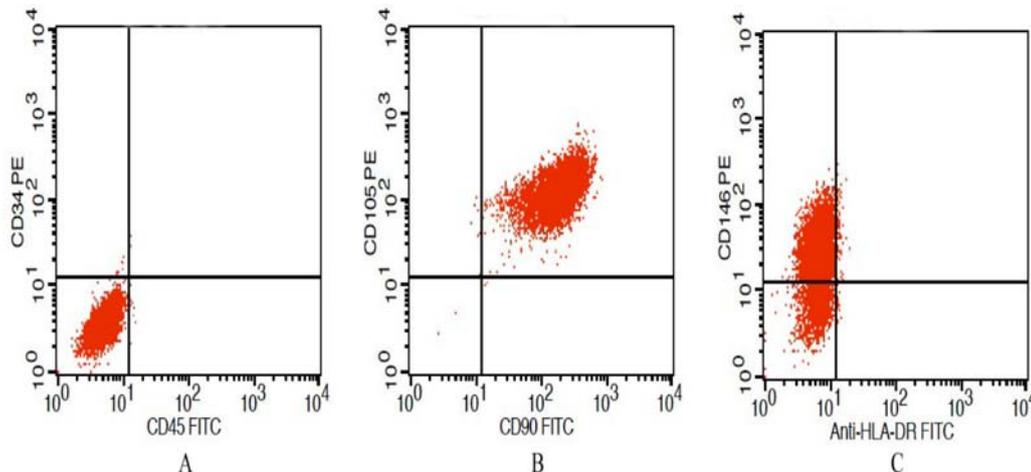


Figure 3 - Surface markers of human umbilical cord blood mesenchymal stem cells were detected with flow cytometry analysis. The cells expressed CD90, CD105, and CD146 positively, but CD34, CD45, and anti-HLA-DR were expressed negatively. FITC - fluorescein isothiocyanate

long spindle-shaped morphology and radiated growth. The cells reached 70-80% confluence after 7-8 days in culture (Figure 2A). After transfection, cells enlarged and more polygonal cellular morphologies were observed after transfection (Figure 2B). Fluorescence-activated cell sorting analysis was used to identify cell surface markers on hUCB-MSCs at the third passage. The cells were positive for CD90, CD105, and CD146, which were specific for stem cells, but negative for hematopoietic and endothelial markers including CD34, and CD45. The cells were also negative for anti-HLA-DR (Figure 3).

Table 1 - Paired T test of the hIGF-1 protein concentration between experimental group(n=12) and control group(n=12) at every time point.

Time point	Experimental group (ng/ml)	Control group (ng/ml)	P-value
Before transfection (t)	3.26 ± 0.72	3.25 ± 0.23	0.901
6 hours (h) after t	4.12 ± 0.33	3.11 ± 0.13	<0.001
12 h after t	7.46 ± 0.41	3.24 ± 0.27	<0.001
24 h after t	17.56 ± 0.80	3.27 ± 0.06	<0.001
48 h after t	34.63 ± 1.61	3.36 ± 0.26	<0.001
72 h after t	22.34 ± 0.97	3.26 ± 0.21	<0.001

The ELISA for hIGF-1 was used to test the amount of hIGF-1 secreted into the supernatants after transfection at different time points. The hIGF-1 in the supernatants of untransfected cells was 3.26 ± 0.72 ng/ml (n=12), and increased up to 4.12 ± 0.33 ng/ml (n=12) 6 hours after transfection. Gradually increasing with time, the concentration peaked at 34.63 ± 1.61 ng/ml after 48 hours. This increase was similar to differences seen in fluorescence under the microscope. Paired T test was used to examine the hIGF-1 protein concentration between the experimental and control group at every time point (Table 1). We can see that the hIGF-1 protein concentrations were significantly higher than the control group at every time point after transfection (n=12, $p < 0.001$). The PCR analysis results showed that the expression of hIGF-1 mRNA was increased in hUCB-MSCs transfected with pIRES2-EGFP-hIGF-1 after one week compared to untransfected hUCB-MSCs. The electrophoresis gel revealed that the hIGF-1 mRNA was approximately 410 bp (Figure 4). Immunofluorescence was used to detect the expression of hIGF-1 in the hUCB-MSCs after transfection. The inspection of hIGF-1 in the cytoplasm of hUCB-MSCs

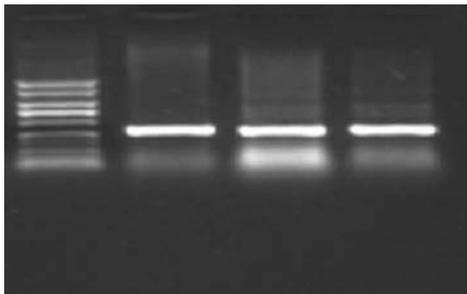


Figure 4 - The result of real-time polymerase chain reaction for the expression of human insulin-like growth factor-1.



Figure 5 - Immunofluorescent result for detection of the human insulin-like growth factor-1 in the human umbilical cord blood mesenchymal stem cells revealed positive particles within the cytoplasm.

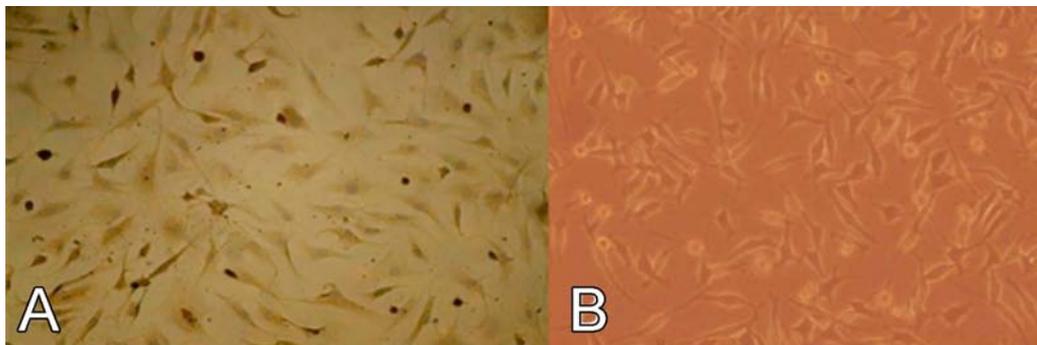


Figure 6 - Immunohistochemical staining for type II collagen demonstrated: A) brown staining in the transfected cells, and B) proliferation and appearance of the cells changed after transfection.

displayed a large quantity of hIGF-1 positive cells (Figure 5). The result of immunohistochemical analysis for transfected hUCB-MSCs showed that the hIGF-1 protein secreted could stimulate the cells to express type II collagen, the typical cartilage element (Figure 6).

Discussion. Repair of hyaline cartilage defects remains a challenging problem in joint surgery. Whenever these defects were left untreated, the knees would be swelling and painful, and eventually lead to occurrence of osteoarthritis.⁹ The treatment of chondral or osteochondral in the weight-bearing region of the joints is a therapeutic challenge.¹⁰ Currently, tissue engineering has emerged as an excellent approach to repair bone and cartilage defects.¹¹ Mesenchymal stromal cells are highly proliferative cells that are able to home to, and engraft in injured or other specific tissues, and then differentiate into functional osteoblasts, chondrocytes, and/or adipocytes. Bone marrow mesenchymal cells are thought to be an attractive source for cell-based therapies and tissue engineering.¹² However, an article reported that bone mesenchymal stem cell (BMSC)/progenitor cell number and differentiation capacity decrease in an age-dependent manner.¹³

Umbilical cord blood (UCB) is considered one of the most abundant sources of non-embryonic stem cells.¹⁴ The collection of mesenchymal stem cells from UCB that is discarded at the time of birth is an easier, less expensive, and a non-invasive method for collecting MSCs from the bone marrow,¹⁵ and ethical issues associated with UCB may be relatively practical. Another important characteristic of UCB-MSCs is that they are less immunogenic and more primitive than MSCs isolated from some other tissue sources.¹⁶ When stimulated in vitro, the chondrogenesis of hUCB-MSCs could be acquired by adding special growth factors into the medium, creating a high-density environment.^{17,18} The hIGF-1 is one of the most important growth factors in cartilage development and homeostasis. The hIGF-1 has been reported to stimulate the synthesis of type-II collagen and aggrecan, which are the major components of the cartilage.¹⁹ The hIGF-1 can also enhance chondrocyte metabolism, and maintain differentiated chondrocyte morphology.²⁰ However, there are many problems when injecting hIGF-1 protein into the human body directly, such as the short half-life of the growth factor in biologic systems, and the potential induction of immunologic rejection. Due to the short half-life, repeated application is necessary to maintain high enough levels for treatment, and thus, very expensive.²¹ To overcome this, gene transfer approaches, which are already used for treatment of arthritis can be adopted to provide sustained synthesis

of growth and differentiation factors. This would promote chondrogenic differentiation of MSCs and increase matrix synthesis within cartilage lesions, and ultimately lead to improved repair.²²

The key to gene cloning is the vector, which determines whether the exogenous gene can be expressed steadily after being transfected into cells. The pIRSES2-EGFP vector contains an internal ribosome entry site and EGFP clone regions. There are internal ribosome entry sites between multiple cloning sites and a GFP coding gene, which allow co-expression of the exogenous gene and EGFP simultaneously.²³ The selection of the gene transfer method improves the clinical effectiveness.

In this study, we successfully constructed a bicistronic eukaryotic vector pIRSES2-EGFP-hIGF-1. The hUCB-MSCs were isolated with plastic adherence, and the density gradient centrifugation method. We have used flow cytometry to characterize the cell surface protein expression of the hUCB-MSCs. The flow cytometry analysis results showed that the cells positively expressed CD90, CD105, and CD146 but did not express CD34, CD45, and anti-HLA-DR. Then, the pIRSES2-EGFP-hIGF-1 was transfected into hUCB-MSCs by X-treme GENE HP DNA transfection reagent in vitro. The transfection efficiency was $28.74 \pm 7.31\%$ at the 48 hour time point. Although this efficiency was lower than the viral vector transgenic method, this method could avoid the adverse effects of viral vectors, such as host immune response and mutagenic potential when used in vivo. Immunofluorescence and PCR analysis results showed that hIGF-1 gene was expressed successfully in transfected hUCB-MSCs. The electrophoresis gel revealed that the hIGF-1 mRNA was approximately 410 bp. We found the concentration of hIGF-1 secreted in the supernatants 48 hours after transfection were at a high level of 34.63 ± 1.6 ng/ml. Concentration of hIGF-1 in the medium was at 50 ng/ml, which could optimally stimulate the matrix deposition and maturation of cartilage specific matrix.²⁴ The immunohistochemical results showed that hIGF-1 protein secreted by the transfected hUCB-MSCs could stimulate them to express major cartilage matrix protein type II collagen.

There are several limitations in the present study. Firstly, the transfection efficiency of the non-viral gene transfer was relatively low compared to virus vector systems. Transgenic vectors are usually comprised of viral and non-viral vectors. Viral vectors have been the popular transgenic method because of their high efficiency.²⁵ However, the limitations of viral vectors are that they induce the host's immune response, and their mutagenic potential is limited to its clinical application.

To avoid such adverse effects, the liposome-mediated transfer has been used extensively.²⁶ Secondly, the concentration of the hIGF-1 secreted in the transfected cell supernatant is relatively low. The concentration of hIGF-1 in the supernatants after 7 days was still higher than that in untransfected cells. This time period and concentration may be sufficient for this cytokine to play a role in future clinical applications. The third limitation is that the fluorescence quenching limits the observation of EGFP expression. The EGFP is a wild-type GFP, with selective fluorescence, and can be highly expressed in mammalian cells. Although the target gene was still expressed, some expression of EGFP diminished with time. The fourth limitation of this research is that the chondrogenesis and proliferation, as well as the differentiation was not determined more in detail.

In conclusion, we successfully constructed a bicistronic eukaryotic vector pIRSES2-EGFP-hIGF-1, which could be transfected into hUCB-MSCs by X-treme GENE HP DNA transfection reagent in vitro. Fluorescence microscopy, RT-PCR, ELISA, immunofluorescence, and immunohistochemistry were used to verify vector expression and function, and these results may be useful for further modification to improve therapeutics that repair bone and cartilage defects.

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