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NOTE

Combination of cytomegalovirus enhancer with human cellular promoters for gene-induced chondrogenesis of human bone marrow mesenchymal stem cells

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High-expression plasmid vectors for human mesenchymal stem cells (MSCs) were constructed by combination of cytomegalovirus immediate-early enhancer with cellular promoters. MSCs transfected with the vector showed higher transgene production of a cytokine, which increased the differentiation level to chondrocytes.

[**Key words:** Cartilage regeneration; Gene therapy; Human mesenchymal stem cell; Nonviral-transfection; CMV enhancer; Human cellular promoter]

Mesenchymal stem cells (MSCs) in adult bone marrow have been shown to give rise to multiple mesodermal tissue types, including bone and cartilage. MSCs can be easily isolated from bone marrow aspirates (1). Therefore, MSCs are an attractive cell source for cartilage regenerative medicine (2).

Some clinical applications of transplantation of undifferentiated MSCs into full-thickness articular cartilage defects in humans have been reported (3), which resulted in stable fibrocartilage tissue formation at the defect site. Therefore, generation of a stable hyaline-rich cartilage tissue using MSCs is desired (4).

Chondrogenesis is induced by transforming growth factor-β3 in the culture of MSCs (TGF-β3) (5,6). Therefore, transplantation of MSCs transfected with the TGF-β3 gene was supposed to be effective to generate hyaline-rich cartilage, because it can be expected that genetically modified MSCs produce chondrogenic inductive transgene products such as TGF-β3 in the transplantation site and these cytokines induce hyaline-rich chondrogenic differentiation of transplanted MSCs by autocrine or paracrine effects.

Here, highly efficient and high-safety transfection methods into MSCs are essential to use genetically modified MSCs for above-mentioned clinical applications. Although viral transfection methods have been shown highly transfection efficiency, they have several drawbacks including acute inflammatory responses. On the other hand, nonviral methods such as Lipofectamine have markedly low transfection efficiency (<5%) for human MSCs. Therefore, we adopted a modified electroporation method, nucleofection, which shows high efficiencies exceeding 70% for human MSCs (7). Moreover, a high-expression plasmid in human MSCs is essential, and the design of a plasmid vector is important to achieve a high transgene expression level. The gene expression level after introduction generally depends on the promoter sequence on the plasmid vector. However, a comparative study of expression efficiencies of several types of promoter in human MSCs has not been well performed so far.

The promoters that regulate highly expressed genes in MSCs, such as the fibronectin promoter (FNp), collagen, type1 α1 chain promoter (Col1A1p), collagen, type1 α2 chain promoter (Col1A2p), and eukaryotic translation elongation factor1 α1 chain promoter (EF-1αp) (8) were supposed to be capable of expressing these genes effectively. In addition, the use of a cellular promoter together with a viral transcriptional enhancer element in plasmid vectors provides a promising strategy to enhance expression of transgenes (9,10).

In this study, we constructed various hybrid promoters, which are combinations of cytomegalovirus immediate-early enhancer (CMVE) and several cellular promoters (i.e., the FNp, Col1A1p, Col1A2p, or EF1-α1p). The expression efficiency of the TGF-β3 gene using these hybrid promoters were then compared in correlation with the stimulation of differentiation of MSCs to chondrocyte.

pCMVp/hMGFP, consisting of the CMV promoter driving green fluorescent protein (GFP) expression, was constructed by inserting a GFP from the phMGFP vector (Promega, Madison, USA) into the *Eco*RV/*Xba*I site (under control of the CMV promoter) of the multiple cloning site of the pcDNA3.1 (+) vector (Invitrogen, CA, USA). pCMVp/hTGF-β3, consisting of the CMV promoter driving human TGF-β3 (hTGF-β3) expression, was generated by inserting hTGF-β3 gene into the *Bam*HI/*Eco*RI site of the pcDNA3.1 (+) vector. Human TGF-β3 gene was amplified by PCR from complementary DNA prepared using RNA from human chondrocyte using specific primers (Table 1). pFNp/hMGFP was generated by substituting a 0.23-kb fragment of a human FN promoter sequence (−203 to +28) [1786 to 2017: NG012196, GenBank] for the CMV promoter at the *Bgl*II/*Eco*RV site of pCMVp/hMGFP. The FN promoter fragment was amplified by PCR from human genomic DNA using specific primers (Table 1). pCol1A1p/hMGFP was generated by substituting a 0.27 -kb fragment

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TABLE 1. Primer sequences used in amplification reaction of promoters and hTGF-β3 cDNA.

Primers	Sequence
FNp forward	5'-GAAGATCTTCTTTTGTTCGC-3'
FNp reverse	5'-TTTTACCCTGTGCAGCACAG-3'
Col1A1p forward	5'-GAAGATCTTCACCACAGCAC-3'
Col1A1p reverse	5'-AAAACCCCGAGGAGAAACTC-3'
Col1A2p forward	5'-TATCGCGAGGAGATCTGCAAATTCTG-3'
Col1A2p reverse	5'-GGAATTCCTGCAGTCGTGGCCAGTAC-3'
EF-1αp reverse	5'-CGGGATCCCGTCACGACACCTG-3'
SYNp forward	5'-CTGCAGAGGGCCCTGCGTAT-3'
SYNp reverse	5'-CGCCGCAGCGCAGATGGTCG-3'
CMVE forward	5'-GAAGATCTTCATTAGTTCATAGCC-3'
CMVE reverse	5'-AACAAACTCCCATTGACGTC-3'
hTGF-β3 cDNA forward	5'-CGGGATCCCGATGAAGATGCACTTGC-3'
hTGF-β3 cDNA reverse	5'-TCAGCTACATTTACAAGACTTCACC-3'

of a human Col1A1 promoter sequence (−243 to +24) [58 to 325: AF017178, GenBank] for the CMV promoter at the *Bgl*II/*Eco*RV site of pCMVp/hMGFP. The Col1A1 promoter sequence was amplified by PCR from human genomic DNA using specific primers (Table 1). pCol1A2p/hMGFP was generated by substituting a 0.45-kb fragment of a human Col1A2 promoter sequence (−378 to +58) [1915 to 2348: AF004877, GenBank] for the CMV promoter at the *Bgl*II /*Eco*RV site of pCMVp/hMGFP. The human Col1A2 promoter sequence was excised from the pGVα2 (I) vector (a gift from Dr. Ryuichiro Hata of Kanagawa Dental College; Kanagawa, Japan) and amplified by PCR using specific primers (Table 1). pEF-1αp/hMGFP was generated by substituting a 1.4-kb fragment of a human EF-1α promoter sequence (from 331 to 1718 bp of pTraser-EF/BsdA vector (Invitrogen, CA, USA)) for the CMV promoter at the *Nru*I/*Kpn*I site of pCMVp/hMGFP. pSYNp/hMGFP was generated by substituting a 0.4-kb fragment of a human synapsin (SYN) promoter sequence [1889 to 2289: M55301, GenBank] for the CMV promoter at the *Bgl*II/*Eco*RV site of pCMVp/hMGFP. The SYN promoter sequence was amplified by PCR from human genomic DNA using specific primers (Table 1).

GFP expression hybrid promoter vectors, which are combinations of CMVE and cellular promoters (i.e., pCMVE-FNp/hMGFP, pCMVE-Col1A1p/hMGFP, pCMVE-Col1A2p/hMGFP, and pCMVE-EF-1αp/hMGFP) were generated by inserting the fragment of a CMVE sequence (−554 to −130) (11) into the *Bgl*II site (restriction site located upstream of all cellular promoters) of pFNp/hMGFP, pCol1A1p/hMGFP, pCol1A2p/hMGFP, and pEF-1αp/hMGFP, respectively. The CMVE sequence was amplified by PCR from pcDNA3.1 (+) using specific primers (Table 1). TGF-β3 expression hybrid promoter vectors (i.e., pCMVE-FNp/hTGF-β3, pCMVE-Col1A1p/ hTGF-β3, pCMVE-Col1A2p/hTGF-β3, and pCMVE-EF-1αp/hTGF-β3) were generated by substituting fragments of CMVE-FNp, CMVE-Col1A1p, CMVE-Col1A2p, and CMVE-EF-1αp for the CMV promoter at the *Nru*I/*Bam*HI site of pCMVp/hTGF-β3, respectively. All plasmid vectors used in this study were isolated and purified using an EndoFree® Plasmid Maxi kit (Qiagen, MD, USA).

MSCs were isolated from bone marrow aspirate obtained by routine iliac crest aspiration from a human donor (19-year-old male), as previously reported (12). The percentage of $CD90^+CD166^+$ cells which were reported to be markers of MSCs was approximately 98% (data not shown) (13). All subjects enrolled in this study were approved by our institutional committee on human research, as required by the study protocol. MSCs (6.0×10^5 cells) were transfected with 1.0 pmol (4.0–5.0 μg) of a plasmid vector using amaxa Nucleofector II ® (Lonza, Köln, Germany) in accordance with the manufacturer's protocol. Briefly, the cells harvested by trypsinization were resuspended at a concentration of 6.0×10^6 cells/ml in 100 μl of human MSC Nucleofector® solution (Lonza) and mixed with a plasmid vector (4.0–5.0 μg) in an amaxa electroporation cuvette (Lonza). Immediately after electroporation using the U23 program, cells were inoculated onto a 6-well plate (Corning) at a cell density of 6.5×10^4 cells/cm^2 using DMEM supplemented with 20% FCS. After adhesion of transfected cells in a 6-well plate for 24 h, the MSCs (2.5×10^5 cells) harvested by trypsinization in 15-ml centrifuge tube (Sumitomo Bakelite, Tokyo) were centrifuged at 1000 rpm for 5 min to promote 3D aggregate formation. The FCS-containing medium was then replaced with 0.5 ml of a chondrogenic medium consisted of DMEM containing 100 μg/ml pyruvate (Wako Pure Chemicals, Osaka), 50 μg/ml ascorbate 2-phosphate (Wako), 40 μg/ml proline (Wako), 39 ng/ml dexamethasone (ICN Biomedicals, Irvine, CA, USA), 100 ng/ml insulin-like growth factor-I (IGF-I; Peprotech, Rocky Hill, NJ, USA), and 1% ITS™-Premix (BD biosciences, MA, USA) supplemented with or without recombinant human TGF-β3 (10 ng/ml, Peprotech). The cells in the centrifuge tube were incubated under an atmosphere of 5% CO_2 at 37 °C for 2 weeks, during which the media were changed every 7 days (3D culture).

2 days after the transfection, GFP expression efficiency was analyzed using a flowcytometer (EPICS XL; Beckman Coulter, CA, USA) using a FITC filter. The mean fluorescence intensity calculated from GFP-positive cells was determined as the expression efficiency. MSCs used in this study showed approximately 60% of transfection efficiency (% of GFP-expressing population) (data not shown).

The concentration of hTGF-β3 in the culture supernatant was determined by ELISA (Quantikine Immunoassay kits, R&D Systems, Minneapolis, MN, USA).

The mRNA expression level of aggrecan gene as a cartilage marker gene was determined using RT-PCR as previously reported (12).

To compare the activity of various promoters in MSCs, GFP-expressing vectors under the control of six different promoters (CMVp, FNp, Col1A1p, Col1A2p, EF-1αp, and SYNp) were constructed. MSCs were transfected with these vectors and their expression efficiency was analyzed using a flowcytometer after 48 h of transfection (Fig. 1). The expression of GFP by MSCs transfected with pSYNp/hMGFP was not detected. On the other hand, the expression of GFP by MSCs transfected with pFNp/hMGFP, pCol1A1p/hMGFP, pCol1A2p/hMGFP, and pEF-1αp/hMGFP was markedly high, although the expression efficiencies were at most 30% that with pCMVp/hMGFP.

Therefore, the hybrid promoters that were combinations of CMVE with FNp, Col1A1p, Col1A2p, or EF-1αp were employed and significantly increased expression efficiency (FNp, 2.7-fold; Col1A1p, 5.7-fold; Col1A2, 2.7-fold, and EF-1α, 2.4-fold). Notably, the

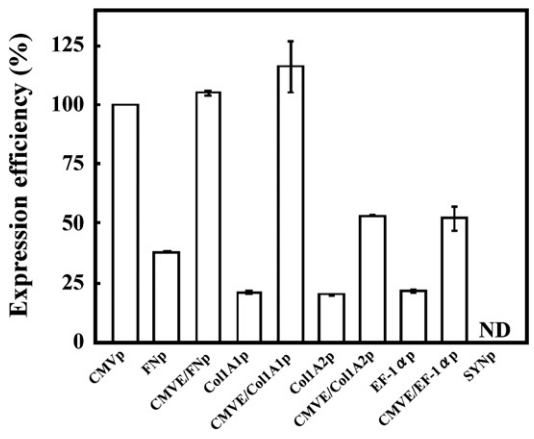

FIG. 1. Comparison of GFP expression using various promoters in MSCs. MSCs were transfected with GFP-expressing vectors using various promoters, and 48 h after transfection, the mean fluorescence intensity of GFP was analyzed using a flowcytometer. Expression efficiency was calculated using the mean fluorescence intensity of CMVp as a control. ND: not detected.