대한해부학회지 제36권 제5호 2003 The Korean J. Anat. 36(5), 381~387 2003

The Efficient Gene Delivery into Human Mesenchymal Stem Cells Using Retroviral Vectors

Sung Soo Kim[1], Bum Jun Kim[1], Haeyoung Suh-Kim[1,2]*

Department of [1]Anatomy, School of Medicine,
[2]Brain Disease Research Center, Ajou University, 442-749, Suwon, Korea

Abstract

Human mesenchymal stem cells (hMSCs) are multipotent stem cells that can differentiate into several mesenchymal lineage cells. In this study, we established an efficient method for gene delivery into these cells. Non-viral transfection reagents that were commercially available yielded 5% efficiency. In contrast, a retroviral vector yielded more than 46% transduction, which was further increased to 90% by repetitive infection. Retroviral transduction did not alter the multipotency of hMSCs. Thus, the cells retained the potential to differentiate into adipogenic, chondrogenic, or osteogenic lineages.

The conditions established in this study will contribute to development of trans-differentiation methods of hMSCs into non-mesodermal lineage cells and thereby facilitate their possible use as vehicles for autologous transplantation in both cell and gene therapy for various diseases.

Key words : Mesenchymal stem cells, Gene therapy, Retrovirus, Transfection

INTRODUCTION

Mesenchymal stem cells (MSCs) are non-hematopoietic cells in bone marrow and referred to as plastic-adherent cells, colony forming-unit fibroblasts, and bone marrow stromal cells. MSCs have potentials to differentiate into mesodermal lineage cells such as osteocytes, chondrocytes, adipocytes, and myocytes (Pittenger *et al*., 1999; Makino *et al*., 1999). Recently, MSCs have attracted a lot of attention because of their ability to differentiate into non-mesodermal lineage cells and their possible use as vehicles for autologous transplantation in both cell and gene therapy for various diseases.

MSCs are easily isolated from the bone marrow using their plastic-adherent properties. Isolated MSCs are capable of extended proliferation in culture. Previous studies have shown that several factors may be required for the growth of MSCs and the maintenance of their multipotentiality. Daily feeding or growth factors increased growth kinetics of MSCs (Bruder *et al*., 1997) although the effects of growth factors vary between species and experimental conditions. Kuznesov and coworkers (1997) showed that PDGF and TFG-β but not bFGF and EGF stimulated colony formation of human mesenchymal stem cells (hMSCs) whereas Gronthos and Simmons (1995) demonstrated that PDGF or EGF but not bFGF and TGF-β stimulated colony formation of hMSCs. In contrast, other groups showed that addition of bFGF increased growth kinetics of hMSCs (Muraglia *et al*., 2000). Taken together, factors that increase self-renewal and preserve the multipotential of hMSCs may differ in various experimental conditions.

It has been suggested that mesenchymal stem cells can be utilized as vehicles for beneficial genes due to their high migration potential to the injury (Chen *et al*., 2001, 2003) or tumor sites (Studeny *et al*., 2003). However, their use in clinical studies has been hampered by low gene transfer efficiencies. Efficient gene transfer into stem cells is a prerequisite for various gene therapeutic strategies. Therefore, *in vitro* expansion of hMSCs in a more immature state and efficient gene transfer into expanded

* 교신저자: Haeyoung Suh-Kim
Tel: 82-31-219-5033, Fax: 82-31-219-5039; hysuh@ajou.ac.kr

cells are the most important requirement for gene and cell therapy using these cells.

In this study, we report that retroviral transduction is highly effective in delivering genes into hMSCs.

MATERIALS AND METHODS

Isolation and culture of hMSCs

Bone marrows were obtained from the iliac crests of six normal individuals ranging in age from 10 to 15 years old and undergoing bone marrow harvests for the future allogenic transplantation. Informed consent was obtained from all donors and approximately 5 milliliters of bone marrow aspirates were taken from the donors. Aspirates were layered over 5 ml of HISTOPAQUE-1077 (Sigma, St. Louis, MO). After centrifugation at 400 × g for 30 min, the mono-nucleated cell layer was obtained from the interface and suspended in Hank's balanced salt solution (HBSS; GibcoBRL, Grand Island NY). The cells were centrifuged again at 1,500 × g for 15 min and resuspended in DMEM supplemented with 10% FBS, 100 IU/ml penicillin and 100 μg/ml streptomycin. The cells were plated at a density of $1 \sim 2 \times 10^7$ nucleated cells per 100 mm dish and incubated at 37°C in humidified atmosphere containing 95% air and 5% CO_2. The non-adherent cell population was removed after 4 hours by changing the medium and the adherent cells were continuously grown for 1 ~ 2 weeks. After the culture reached to 80 ~ 90% confluence, the cells were harvested with 0.25% trypsin and 1 mM EDTA for 2 min at 37°C and transferred to fresh plates with 1 : 20 dilution, which was counted as the first passage. The remaining cells were slowly frozen and stored in liquid nitrogen.

In vitro differentiation of cultured hMSCs

To determine the potential for hMSCs to differentiate into the chondrogenic, osteogenic and adipogenic lineages, the cells were grown in DMEM supplemented with 10 ng/ml bFGF, 10% FBS, 100 IU/ml penicillin and 100 μg/ml streptomycin to the 8th passage. Differentiation to adipogenic, chondrogenic, and osteogenic lineages was induced according to the procedures described by Pittenger and co-workers (Pittenger *et al.*, 1999). After 2 ~ 3 weeks of culture, the differentiated cells were fixed with 3% formaldehyde. Adipocytes were detected by staining the lipid droplets in the cell using 0.3% Oil red-O staining for 10 min. Osteocytes were detected by calcium phosphate deposits after *von* Kossa staining. Briefly, cells were fixed with ethanol and stained with 5% silver nitrate for 1 hour. After rinsing with distilled water, the cells were incubated in 5% sodium thiosulfate for 2 min to allow precipitation of insoluble black silver granules around calcium phosphate. Staining for alkaline phosphatase was performed for 1 hour at 25°C using 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium alkaline phosphatase substrate (BCIP/NBT; Sigma, St. Louis, MO) as recommended by the manufacturer. During reaction, culture dishes were protected from drying and direct light. The cells were rinsed with deionized water, and air-dried prior. Chondrocytes were detected by alcian blue staining. Briefly, the pellets were embedded in paraffin, cut into 5 μm sections. The sections were fixed with 3% formaldehyde and stained with 0.5% alcian blue 8GX (Sigma, St. Louis, MO) in 3% acetic acid (pH 2.5) solution for 15 min and then counter-stained with 0.1% nuclear fast red solution (Sigma, St. Louis, MO) for 5 min.

Cell transfection and retroviral transduction

A commercially available plasmid pCMV-β-gal (Stratagene, West Cedar Creek, TX) was amplified using a CsCl gradient centrifuge and used for transfection. Human MSCs were seeded 24 h before transfection so that they could reach 80% confluence on the day of transfection. All experiments were done in triplicate. LipofectAMINE (Invitrogen Corp., Carlsbad, CA) and FuGENE 6 (Roche Diagnostics Corp., Indianapolis, IN) were used for transfection according to the manufacturers' instructions. Calcium phosphate, DEAE-dextran and polybrene were used as previously described (Sambrook and Russell, 2001). A retroviral vector encoding β-galactosidase was prepared by transfecting a lacZ expressing retroviral plasmid, pMSCV-puro/lacZ (Clontech, Palo Alto, CA) into a packaging cell line, PA317. The virus was obtained from the medium, diluted with growth medium containing polybrene (Sigma, St. Louis, MO) to a final concentration of 8 μg/ml, and added to hMSCs (MOI = 100) that were plated at approximately 3,000 cells/cm^2 and maintained in growth medium overnight. After 6 hours, the medium was replaced with fresh growth medium containing bFGF (20 ng/ml). For repetitive transduction, the cells were subject to the same procedure twice as described above on the 2nd and 3rd day. Two days after the final transduction, the cells were fixed with 4% paraformaldehyde and stained with X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside; USB Corp., Cleveland, OH) for 10 hours at 37°C.

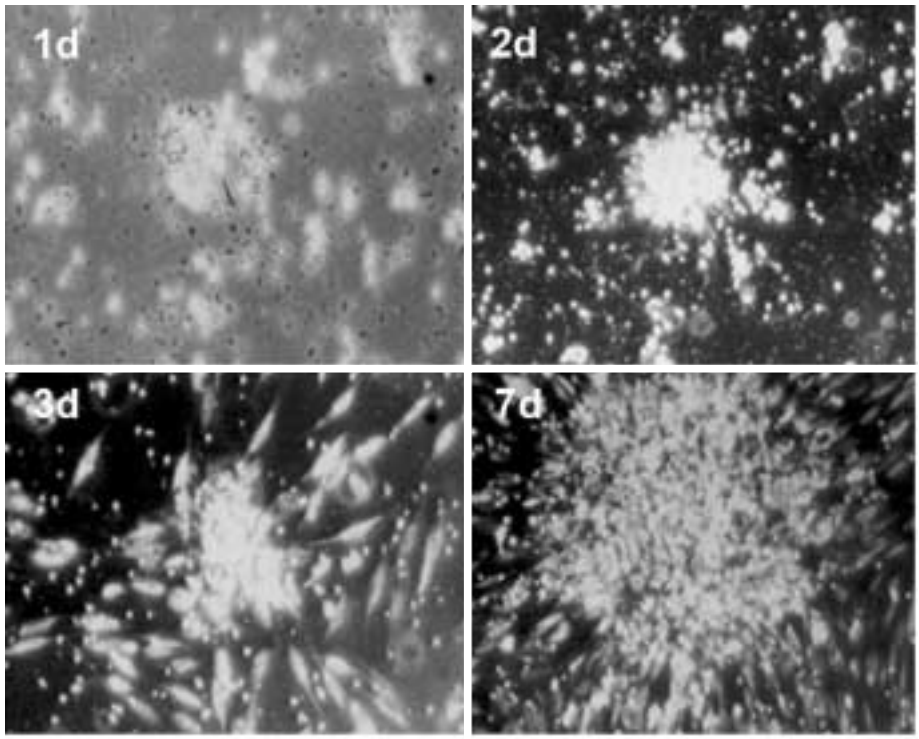

Fig. 1. Isolation and culture of hMSCs *in vitro*. Mononucleated cells were isolated from bone marrow aspirates by Ficoll-Hypaque gradient fractionation and plated in 100-mm dishes at $1\sim2\times10^7$ cells per dish with DMEM supplemented 10% FBS, 100 IU/ml penicillin, and 100 μg/ml streptomycin. The non-adherent cell population was removed after 4 hours and the adherent layer washed once with fresh media; the cells were then continuously cultured for 1~2 weeks. Photomicrographs are representative of hMSCs cultures at 1, 2, 3, and 7 days after plating.

RESULTS

Isolation of hMSCs from bone marrow

Nucleate cells were isolated from the bone marrow by a Ficoll-Hypaque density gradient centrifugation and plated on to plastic dishes in DMEM supplemented with 10% FBS. The non-adherent cells were removed and the adherent cells were continuously grown for 1~2 weeks. Colony forming capacity of hMSCs was detectable between 2nd and 3rd day. The colonies consisted of a homogeneous population of small spindle-shaped cells were very pronounced in a week and the size of colonies increased over time by 14 days in culture (Fig. 1). Daily inspection of the cultures indicated that each colony resulted from the proliferation of a single cell (Fig. 1). Usually 1×10^7 nucleated cells yielded 10~20 colonies composed of 100~1,000 cells per colony. The cells were trypsinized and transferred to fresh plastic plates, where the cells exhibited a fibroblastic morphology and seemed to be homogeneous in appearance. The fibroblastic morphology was maintained through continuous passage of the cells. During the process, the culture reached to 90% confluent between 7 and 10 days after plating.

Multipotency of hMSCs

To determine the multipotency of *in vitro* expanded hMSCs, the cells of the 8th passage were used for induction into mesodermal lineages according to the previously described methods (Pittenger *et al*., 1999). Expression of specific phenotypes was analyzed by histochemical methods. After induction for 1~2 weeks, prominent cytoplasmic lipid droplets appeared (Fig. 2A). Multiple treatments resulted in more than 60% of the cells to adopt the adipocyte fate and the lipid vacuoles continued to increase over the period, coalesced, and eventually filled the entire cell body. To promote chondrogenic differentiation, hMSCs were gently centrifuged and allowed to form a pelleted micromass. The cells were incubated in DMEM supplemented with TGF-β1 for 3 weeks. The cell pellet developed a multi-layered matrix-rich morphology. The stained sections showed the homogeneous matrix and lacunae with mature chondrocytes. The matrix reflects the presence of extracellular substance, suggesting cartilage

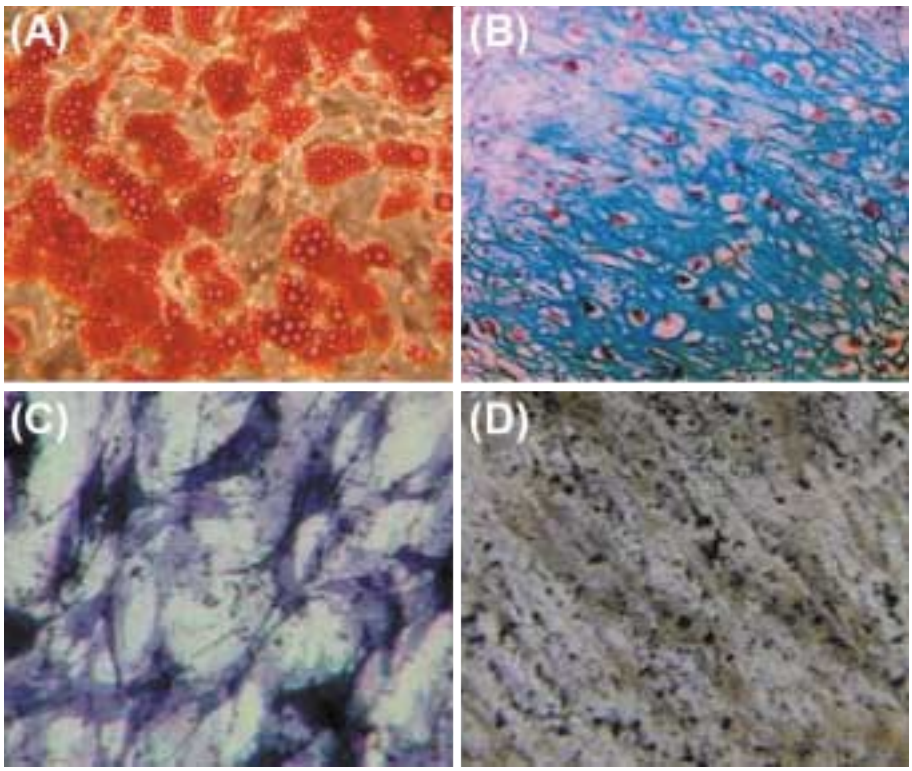

Fig. 2. Differentiation of expanded hMSCs into mesenchymal lineage cells *in vitro*. hMSCs from the 8th passage were induced to differentiate into mesenchymal lineage cells as described in the 'Material and Methods'. (A) Adipogenesis was visualized with staining of lipid vacuoles with oil-red O. (B) Chondrogenesis was demonstrated with increased proteoglycan rich extracellular matrix by alcian blue staining. (C) Osteogenic differentiation of hMSCs was demonstrated by the increased alkaline phosphatase activity and (D) the accumulation of minerals stained by *von* Kossa method.

formation. The polyaniline alcian blue characteristics of chondroitin sulfate were used to verify the presence of cartilage-specific matrix, which was clearly shown as blue (Fig. 2B). Osteogenic differentiation of hMSCs over a period of 21 days was demonstrated by the increase in alkaline phosphatase (Fig. 2C). The cells were fixed and incubated in the presence of BCIP/NBT alkaline substrate. More than 60% cells were alkaline phosphatase positive and developed insoluble purple precipitates. Commitment to Osteocytes was also verified by the method of *von* Kossa (Fig. 2D). Calcium minerals were accumulated in the form of crystalline hydroxyapatite and stained with silver.

Gene transfer into hMSCs

Gene transfer efficiencies of five established nonviral methods were evaluated using a plasmid DNA encoding β-galactosidase. The numbers of β-galactosidase positive cells were less than 5% with LipofectAMINE, calcium phosphate, DEAE-dextran, or polybrene as DNA carriers. Fugene 6 yielded 8.3±2.1% of β-galactosidase-positive cells (Fig. 3A). We also examined the transduction efficacy of retrovirus using a retroviral vector encoding β-galactosidase, pMSCV-puro/lacZ. The virus was packaged in PA317 cells and harvested from the medium. The virus was mixed with polybrene and introduced to hMSCs grown in the presence of bFGF (MOI = 100). The β-galactosidase positive cells were 46±3.6% after the single transduction (Fig. 3B). The number of β-galactosidase positive cells were increased to ~90% by two more transductions for the consecutive two more days (Fig. 3C). To determine viral transduction might alter the multipotency of hMSCs, the cells transduced with the retrovirus were subject to differentiation processes. The cells were able to differentiate into adipocytes, chondrocytes, and osteocytes (Fig. 3D-G). The results suggest that hMSCs are multipotent after retroviral transduction.

DISCUSSION

Several properties of hMSCs, such as less invasive methods for obtaining the cells, simple propagation procedures, high migra-

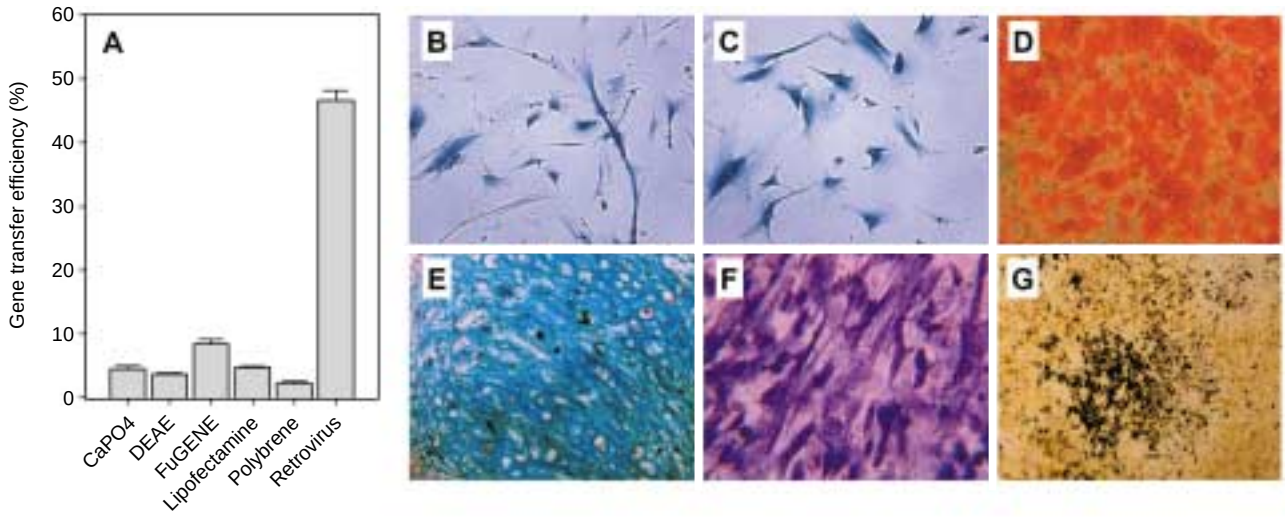

Fig. 3. Comparison of gene transfer methods into hMSCs. (A). Gene transfer efficiencies were evaluated using a plasmid DNA or retrovirus encoding β-galactosidase. The plasmid was transfected into hMSCs according to manufactures' recommendations or methods described in the 'Materials and Methods'. The virus was mixed with polybrene and introduced to hMSCs grown in the presence of bFGF (MOI = 100). The Gene transfer efficiencies were calculated by counting the X-gal positive cells per total number of cells. Data are shown as the averages ±SE of three independent experiments. (B) Light microscopy of X-gal stained hMSCs after single transduction and (C) after two more transduction for the consecutive 2 more days. (D-G) The cells transduced with the retrovirus were induced to differentiate into mesenchymal lineage cells as described in the 'Material and Methods'.

tion to the injury or tumor sites, make these cells attractive candidates as cellular vehicles for gene or cell therapy. To develop hMSCs as therapeutic agents, methods for efficient gene transfer to the cells are prerequisite.

Numerous previous reports have demonstrated that hMSCs are relatively easy to expand *in vitro* and then they become free of hematopoietic precursors after two or three passages *in vitro*. (Friedenstein *et al*., 1987; Kuznetsov *et al*., 1997; Bruder *et al*., 1997). The rapid propagation of the hMSCs is dependent on the presence of growth factors in the culture. The growth rate is approximately 3 times faster in the presence of bFGF compared to its absence (data not shown). The results are consistent with the previous studies performed with osteogenic precursors or marrow stromal cells, which are now presumed to be mesenchymal stem cells (Pitaru *et al*., 1993; Lennon *et al*., 1995; Martin *et al*., 1997; Zhang *et al*., 2002).

We compared the strategies for gene delivery into hMSCs. Transfection of hMSCs using non-viral methods yielded 5∼8% efficiency (Fig. 3). In contrast, the transduction efficiency using a retroviral vector yields 46% efficiency in the presence of bFGF (Fig. 3B). Multiple consecutive transductions of hMSCs yielded about 90% efficiency (Fig. 3C). Since retroviruses only infect proliferating cells, the presence of bFGF may facilitate transduction of retroviral vectors as well.

The hMSCs after retroviral transduction still retain the potential to differentiate into adipocytes (Fig. 3D). Differentiation into adipocytes of hMSCs is the evidence for multipotency since marrow stromal cells easily lose the adipogenic potential first and then become osteochondrogenic progenitors (Muraglia *et al*., 2000).

In this study, we have described a method that efficient gene transfer to hMSCs. The conditions established in this study will contribute to utilize hMSCs as vehicles or therapeutic sources for autologous transplantation in both cell and gene therapy for various diseases.

ACKNOWLEDGEMENTS

We thank Dong-A Pharmaceutical Inc. for the kind gift of bFGF.

This research was supported by a grant (M10016000038-02B1600-03510) form Life Phenomena, and Function Group and a grant (M103KV010010-03K2201-01010) of the 21st Century Frontier Research Program, funded by the Ministry of Science and Technology, Republic of Korea; and by KOSEF through Brain Disease Research Center at Ajou University.

REFERENCES

Bruder SP, Jaiswal N, Haynesworth SE : Growth kinetics, self-

renewal, and the osteogenic potential of purified human mesenchymal stem cells during extensive subcultivation and following cryopreservation, *J Cell Biochem 64: 278-94, 1997.*

Chen J, Li Y, Katakowski M, Chen X, Wang L, Lu D, Lu M, Gautam SC, Chopp M : Intravenous bone marrow stromal cell therapy reduces apoptosis and promotes endogenous cell proliferation after stroke in female rat, *J Neurosci Res 73: 778-86, 2003.*

Chen J, Li Y, Wang L, Zhang Z, Lu D, Lu M, Chopp M : Therapeutic benefit of intravenous administration of bone marrow stromal cells after cerebral ischemia in rats, *Stroke 32: 1005-1, 2001.*

Friedenstein AJ, Chailakhyan RK, Gerasimov UV : Bone marrow osteogenic stem cells: *in vitro* cultivation and transplantation in diffusion chambers, *Cell Tissue Kinet 20: 263-72, 1987.*

Gronthos S, Simmons PJ : The growth factor requirements of STRO-1-positive human bone marrow stromal precursors under serum-deprived conditions *in vitro*, *Blood. 85: 929-40, 1995.*

Kuznetsov SA, Friedenstein AJ, Robey PG. Factors required for bone marrow stromal fibroblast colony formation *in vitro*, *Br J Haematol 97: 561-70, 1997.*

Lennon DP, Haynesworth SE, Young RG, Dennis JE, Caplan AI : A chemically defined medium supports *in vitro* proliferation and maintains the osteochondral potential of rat marrow-derived mesenchymal stem cells, *Exp Cell Res. 219: 211-22, 1995.*

Makino S, Fukuda K, Miyoshi S, Konishi F, Kodama H, Pan J, Sano M, Takahashi T, Hori S, Abe H, Hata J, Umezawa A, Ogawa S : Cardiomyocytes can be generated from marrow stromal cells *in vitro*, *J Clin Invest. 103: 697-705, 1999.*

Martin I, Muraglia A, Campanile G, Cancedda R, Quarto R : Fibroblast growth factor-2 supports ex vivo expansion and maintenance of osteogenic precursors from human bone marrow, *Endocrinology. 138: 4456-62, 1997.*

Muraglia A, Cancedda R, Quarto R : Clonal mesenchymal progenitors from human bone marrow differentiate *in vitro* according to a hierarchical model, *J Cell Sci 113: 1161-6, 2000.*

Pitaru S, Kotev-Emeth S, Noff D, Kaffuler S, Savion N : Effect of basic fibroblast growth factor on the growth and differentiation of adult stromal bone marrow cells: enhanced development of mineralized bone-like tissue in culture, *J Bone Miner Res 8: 919-29, 1993.*

Pittenger MF, Mackay AM, Beck SC, Jaiswal RK, Douglas R, Mosca JD, Moorman MA, Simonetti DW, Craig S, Marshak DR : Multilineage potential of adult human mesenchymal stem cells, *Science 284: 143-7, 1999.*

Sambrook J, Russel DW : Molecular Cloning A Laboratory Manual, 3rd Ed, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, *2001.*

Studeny M, Marini FC, Champlin RE, Zompetta C, Fidler IJ, Andreeff M : Bone marrow-derived mesenchymal stem cells as vehicles for interferon-beta delivery into tumors, *Cancer Res 62: 3603-8, 2002.*

Walsh S, Jefferiss C, Stewart K, Jordan GR, Screen J, Beresford JN : Expression of the developmental markers STRO-1 and alkaline phosphatase in cultures of human marrow stromal cells: regulation by fibroblast growth factor (FGF)-2 and relationship to the expression of FGF receptors 1-4, *Bone 27: 185-95, 2000.*

Zhang X, Sobue T, Hurley MM : FGF-2 increases colony formation, PTH receptor, and IGF-1 mRNA in mouse marrow stromal cells, *Biochem Biophys Res Commun 290: 526-31, 2002.*

〈초 록〉

레트로바이러스 벡터를 이용한 사람 중간엽 줄기세포로의 효율적인 유전자전달

김 성 수[1], 김 범 준[1], 서 해 영[1,2]*

아주대학교 의과대학 [1]해부학교실, [2]뇌질환연구센터

사람의 중간엽 줄기세포(human mesenchymal stem cell, hMSC)는 여러 중배엽 유래의 세포로 분화할 수 있는 다능성세포로서, 저자 등은 시험관내에서 장기간 배양한 세포에 효과적으로 유전자를 전달할 수 있는 방법에 관해 연구하였다. 일반적인 방법에 의한 hMSC로의 유전자전달 효율은 5% 정도였고, 레트로바이러스를 이용할 경우 46%의 효율을 나타내었다. 또한 반복적인 레트로바이러스 감염에 의해 유전자 전달효율은 90%로 향상되었고, 레트로바이러스 감염에 의한 세포의 분화능력에는 변화가 없었다. 본 연구에서 설정된 방법은 hMSC를 이용한 trans-differentiation 방법의 개발 및 여러 질환치료 목적으로 자가이식이 가능한 세포의 공급원으로서 사용이 가능할 것으로 생각된다.

찾아보기 낱말 : 중간엽 줄기세포, 유전자치료법, 레트로바이러스, 감염