Mol. Cells *28*, 515-520, December 31, 2009
DOI/10.1007/s10059-009-0146-4

Molecules and Cells

A New Method for Transduction of Mesenchymal Stem Cells Using Mechanical Agitation

Jin-O Park, Sung-Hoon Park, and Seong-Tshool Hong*

Applications of bone marrow-derived mesenchymal stem cells in gene therapy have been hampered by the low efficiency of gene transfer to these cells. In current transduction protocols, retrovirus particles with foreign genes make only limited contact with their target cells by passive diffusion and have short life spans, thereby limiting the chances of viral infection. We theorized that mechanically agitating the virus-containing cell suspensions would increase the movement of viruses and target cells, resulting in increase of contact between them. Application of our mechanical agitation for transduction process has increased the absorption of retrovirus particles more than five times compared to the previous static method without changing cell growth rate and viability. The addition of a mechanical agitation step increased transduction efficiency to 42%, higher than that of any other previously-known static transduction protocol.

INTRODUCTION

Mesenchymal stem cells are the progenitor cells of connective tissues such as bone, cartilage, hematopoiesis-supporting stroma, and adipocytes (Ahmed et al., 2007; Chamberlain et al., 2007). It has also been shown that mesenchymal stem cells can differentiate into cells of non-connective tissues such as neurons and muscle (Schultz and Lucas, 2006; Yang et al., 2006; Yoo et al., 2008). This multipotency and self-renewability have suggested their use as genetic vehicles for *ex vivo* gene therapy intended to achieve reliable, high-level, long-term, tissue-specific and regulated expression of foreign genes (Daga et al., 2002; Koda et al., 2007; Lee et al., 2008; Rose et al., 2003).

One of the technical challenges in using mesenchymal stem cells for *ex vivo* gene therapy is to increase the efficiency of foreign gene transfer. Typical efficiencies of gene delivery to mesenchymal stem cells are 5-10%, achieved by either electroporation or transfection (Cai et al., 2002; Ding et al., 1999; Eiges et al., 2001; Lakshmipathy et al., 2004; Peister et al., 2004). The transduction efficiency of mesenchymal stem cell using adenovirus was 90% at multiplicity of infection (MOI) of 50 (Liu et al., 2008). However, the cells infected with adenovirus were transiently transducted cells. Transduction through retroviral vector-based gene delivery systems was developed to overcome this obstacle and shown up to 50% transduction efficiency (Ding et al., 1999; Ma et al., 2003; Zaehres et al., 2005). Recently, primary $CD34^+$ peripheral blood stem cells, K562, were shown to have transduction efficiency as high as 90% at MOI of above 20 (Olga et al., 2003). However, those cells might be at a higher risk for malignant transformation because the frequency of insertional mutagenesis may directly correlate with the number of integrated vector copies. Moreover, complicated procedures such as concentration of the virus, repetitive infection, or superior selection are needed to accomplish this level of foreign gene transfer efficiency. Developing new method to obtain the highest possible transduction efficiency using lowest MOI might be the key for the success of future applications.

In current transduction protocols, virus particles passively diffuse through the liquid culture medium to reach their target cells which are layered on the bottom of a culture dish (Chuck and Palsson, 1996). The contact between viruses and target cells is limited in these methods. Increasing the chance of contact between virus particles and their target cells would increase the chance of gene transfer, thereby promoting transduction efficiency. One simple way to increase contact between viruses and target cells is through mechanical agitation. Shaking of virus-containing cell suspensions will increase the movement of viruses and target cells, resulting in more frequent contact between them. Suspended target cells have a better chance of making physical contact with virus particles than adherent target cells because of the possibility for three-dimensional contact between cells and viruses. Based on this hypothesis, we developed a new experimental method for retroviral transduction of primary cells.

MATERIALS AND METHODS

Cell isolation

Rat mesenchymal stem cells were isolated by flushing the femurs of two-month-old female SD rats (Damool Bioscience Co., Korea) with phosphate-buffered saline (PBS). Pure rat mesenchymal stem cells were isolated from the rat bone marrow culture by their plastic-adherent properties, counted and plated in T-75 culture flasks at 2×10^5 cells/cm^2 in DMEM/High glucose

Department of Microbiology and Immunology and Institute for Medical Science and Research Center for Industrial Development of BioFood Materials, Chonbuk National University Medical School, Chonbuk 561-712, Korea
*Correspondence: seonghong@chonbuk.ac.kr

Received February 26, 2009; revised September 8, 2009; accepted September 10, 2009; published online November 19, 2009

Keywords: high transduction efficiency, mechanical agitation, mesenchymal stem cell, retrovirus, transduction

(Hyclone, USA) with 10% FBS (Hyclone), 2 mM L-glutamin (Invitrogen, USA), 100 μg/ml streptomycin, and 100 U/ml penicillin (Sigma, USA). Non-adherent cells were removed through a medium change after 24 h, and adherent cells were then cultured, replacing the medium every three days. The quality of the rat mesenchymal stem cells used in these experiments was confirmed by testing for mesenchymal stem cell lineage markers by staining with anti-integrin β1 and anti-CD54 using a standard protocol (Prockop, 1997). When the cells reached 80% confluence, the cultures were digested with TrypLE Express (Invitrogen), and cells were then seeded at 8×10^3 cells/cm^2 in a T-75 flask to be used either for gene transduction or for further culture and subculture. Cells from the fourth through eighth passages were used for all of the retroviral transduction experiments.

Phenotypic assay

Isolated rat mesenchymal stem cells were characterized using a rat mesenchymal stem cell characterization kit (Millipore, USA). For immunostaining, the rat mesenchymal stem cells were plated on a 10 mm diameter coverslip at a density of 2×10^5 cells/cm^2. After one day, the coverslip was fixed with 4% paraformaldehyde for 20 min and blocked with 5% goat serum and 0.3% Triton X-100 in PBS for 2 h. After blocking, the coverslip was double-stained with rabbit anti-integrin β1 (CD29) at a dilution of 1:500 and mouse anti-fibronectin at a dilution of 1:1,500. Hematopoietic cell surface markers (CD14 and CD45) were also used for staining at a dilution of 1:1,000 and 1:100, respectively. After staining the coverslip with primary antibody for 2 h, Alexa fluor 568-conjugated goat anti-rabbit IgG and Alexa fluor 488-conjugated goat anti-mouse IgG at a dilution of 1:500 (Invitrogen) were stained for visualization (Prockop, 1997). For Hoechst 33342 (Invitrogen) counterstaining, slides were incubated with Hoechst 33342 (1 μg/ml) after incubation with the secondary antibody for 10 min. Fluorescence was visualized using a Carl Zeiss LSM510 Meta microscope.

Plasmid manipulation

The pCAG-EGFP expression vector (Addgene, USA) was chosen as the source of the gene encoding for enhanced green fluorescent protein (EGFP). The EGFP gene was subcloned into the pMSCVneo retroviral vector (Clontech, USA), generating a retroviral vector pMSCVneo-EGFP, in which the expression of EGFP is controlled by the promoter in the LTR and a neomycin resistance gene encoding for neomycin phosphotransferase is controlled by the SV40 promoter. pMSCVneo-EGFP was amplified in DH5α *E. coli* (RBC, Taiwan) and purified using the Nucleobond plasmid midi kit (Macheret-nagel, Germany).

Virus production

The retroviral vector encoding EGFP was prepared by transfecting the EGFP-expressing retroviral plasmid pMSCVneo-EGFP into the packaging cell line PT67 (Clontech) using Lipofectamine 2,000 (Invitrogen). The retrovirus-packaging cells were cultured in DMEM/High glucose (Hyclone) containing 10% fetal bovine serum (FBS) (Hyclone), 100 μg/ml streptomycin, 100 U/ml penicillin (Sigma), 2 mM L-glutamine (Invitrogen) and 6 μg/ml G418 (Sigma). At 80% confluence, the media was removed and the packaging cells were cultured further in fresh DMEM with 10% FBS, streptomycin, penicillin, and L-glutamine at the aforementioned concentrations. After two weeks of selection, 20 stable clones were chosen and transferred to individual wells. After determining viral titers by infecting NIH 3T3 cells and calculating the number of colonies expressing EGFP, two clones were chosen. The medium containing retrovirus (Retro-EGFP) was collected from a selective PT67 clone, filtered through a 0.45 μm cellulose acetate filter (Millipore) and stored at -70°C until use. All transductions were performed at MOI of 4.0.

Virus-cell binding and immunofluorescence assays

The amount of virus bound on the cell surface was quantified by indirect immunofluorescence using a 1,420 multilabel counter (Perkin-Elmer, Finland) described previously (Kwon et al., 2003). Briefly, 1 ml of virus was mixed with rat mesenchymal stem cells (2×10^4 cells) under various speeds of mechanical agitation (0, 20, 40, 80, 100 rpm) or without shaking, and the cells were collected every 10 min for 1 h. The cells were then centrifuged at 16,000 × *g* for 15 s, the supernatant was discarded, and the cells were washed once with 1 ml of ice-cold wash buffer (PBS containing 10% goat serum). The cells were resuspended in 250 μl of mouse monoclonal antibody 10A1 (Santa Cruz) and incubated at 4°C for 1 h. They were then washed again with 1 ml of cold wash buffer and resuspended in 100 μl of wash buffer containing 0.5 μg of Alexa fluor 488-conjugated goat anti-mouse IgG (Invitrogen) for 30 min at 4°C. After a final wash, they were fixed in 300 μl of 4% paraformaldehyde in PBS. The fluorescence intensity of the cells was analyzed on a 1,420 multilabel counter (Perkin-Elmer).

Optimum mechanical agitation conditions

The effect of mechanical agitation in cytotoxicity and cell proliferation was determined by measuring the reduction of MTT [3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] to formazan. The plate containing rat mesenchymal stem cells, 2×10^4 cells, was mechanically agitated on a rocker (SLS4, Seoulin, Korea) at 0, 20, 40, 80, 100 rpm at 37°C in 5% CO_2 for 1 h or 4 d for test about cell proliferation. After incubation, the cells in 96-well plates were washed twice with PBS, and MTT (100 μg/0.1 ml of complete medium) was added to each well. The cells were incubated at 37°C for 2 h, and DMSO (100 μl was added to dissolve the formazan crystals. The absorbance rate of each well optical density (*OD* value) was measured at 540 nm using E-MAX micro-well reader (Molecular Devices, USA).

Mechanically agitated transduction

For transduction of the retrovirus into rat mesenchymal stem cells, pure rat mesenchymal stem cells were trypsinized (0.1 ml/cm^2) for 3 min at 37°C. Trypsin was inactivated by the addition of growth medium (DMEM) containing 10% FBS. The cell suspension was adjusted to contain 5×10^5 cells/ml. A 1 ml aliquot of the trypsinized rat mesenchymal stem cells was directly mixed with 1 ml virus stock in the presence of 6 μg/ml polybrene (Calbiochem, USA) and seeded in a six-well plate (Falcon, USA). The plate containing the mixture of rat mesenchymal stem cells and virus was mechanically agitated on a rocker (SLS4, Seoulin, Korea) at 20 rpm for 50 min while incubating at 37°C under 5% CO_2. After mechanical agitation of the mixture of rat mesenchymal stem cells and virus, the plate was incubated at 37°C under 5% CO_2 for 24 h and the supernatant containing virus particles was replaced with fresh growth medium. After an 86 h incubation, EGFP fluorescence in the transduced rat mesenchymal stem cells was observed with a fluorescence microscope (TE2000-S, Nikon, Japan).

Detection of transduction efficiency

For quantitative flow cytometry studies by fluorescence-activated cell sorting using a FACSCalibur instrument (Becton Dickinson, USA), cells were harvested with TrypLE Express (Invitrogen) and resuspended in PBS containing 2 mM EDTA

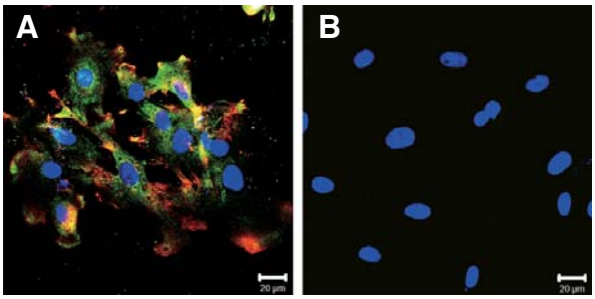


Fig. 1. Fluorescence microscope images of rat mesenchymal stem cells. The cells were stained with two antibodies for extracellular matrix molecules then with anti-integrin β1 (CD29) and anti-fibronectin antibodies. Alexa fluor 568-conjugated goat anti-rabbit IgG and Alexa fluor 488-conjugated goat anti-mouse IgG secondary antibodies bound to these primary antibodies, allowing for the identification of rat mesenchymal stem cells (A). Hematopoietic cell surface markers (anti-CD14 and CD45) were stained as a negative control (B). The cell nuclei were visualized with Hoechst stain (blue). Images were captured using a confocal microscope (LSM510, Carl Zeiss).

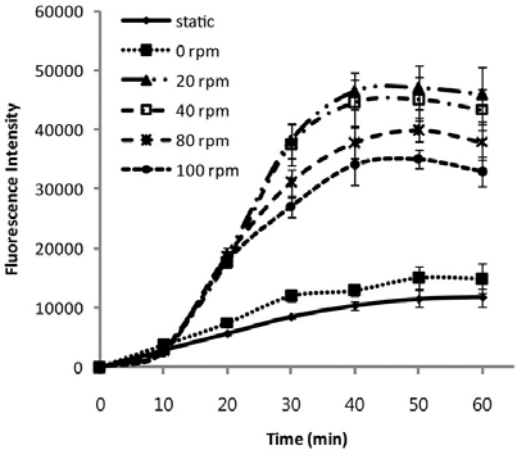


Fig. 2. Effect of mechanical agitation on virus-cell binding. After rat mesenchymal stem cells were mixed with the virus at different rpm for different times, the amount of virus bound to the cell surfaces was quantified by indirect immunofluorescence using a 1420 multilabel counter. Time course profiles of the virus-cell binding ratio are plotted. Triplicate data were taken, with standard deviations shown by the bars.

and 5% bovine serum albumin to be analyzed. Untransduced cells were used to evaluate the percentage of positive cells.

Multilineage differentiation assay

Transduced rat mesenchymal stem cells were plated in 24-well plates to determine whether the transduced mesenchymal stem cells (2×10^5 cells) maintained their multipotentiality. The wells were incubated for three weeks in either commercial osteogenic medium (Lonza, Switzerland) or adipogenic medium (Millipore) to induce differentiation into osteocytes and adipocytes, respectively. The medium was replaced every three days for 20 d. To confirm osteogenic differentiation, cells were washed with PBS, fixed in 100% ice-cold methanol for 10 min, and stained for 15 min with 1 ml of Alizarin red on a shaker at room temperature. Cells were then washed with water five times. To confirm adipocyte differentiation, cells in adipogenic medium were washed with PBS and fixed for 1 h with 10% formalin. Cells were stained for 15 min with a diluted Oil red-O solution.

Statistical analyses

All of the experimental data were obtained in triplicate and are presented as means ± standard deviations. Statistical comparison by analysis of variance was done at a significance level (P) of < 0.05 based on Student's t-test.

RESULTS

Characterization of rat mesenchymal stem cells

The rat mesenchymal stem cells had elongated fibroblast-like morphologies and reached confluence in T-75 flasks after seven days. The cells also stained positive with the cell-surface markers integrin β1 (CD29) and fibronectin but not with hematopoietic cell surface markers (CD14 and CD45) (Fig. 1). These observations indicate that the cultured cells were typical mesenchymal stem cells.

Effect of mechanical agitation on virus-cell binding

We measured the proportion of viruses bound to cells to confirm whether this mechanical agitation process affected virus adsorption to cells. Virus adsorption was analyzed using an indirect immunofluorescence assay which quantified the number of viral particles bound to cells. As expected, the amount of virus on the cells transduced with 20 rpm mechanical agitation for 50 min was nearly five times higher than the amount of virus on rat mesenchymal stem cells that were not agitated during transduction (Fig. 2). This result could not exclude the possibility that mechanical agitation also enhanced penetration of the virus into the cell or the reverse transcription step in cytoplasm. However, mechanical agitation increased the chance of transduction because penetration and subsequent steps were enhanced by virus adsorption to the cell.

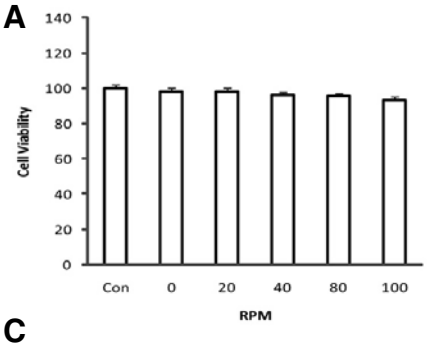
Growth kinetics and cytotoxicity of rat mesenchymal stem cells agitated on a rocker

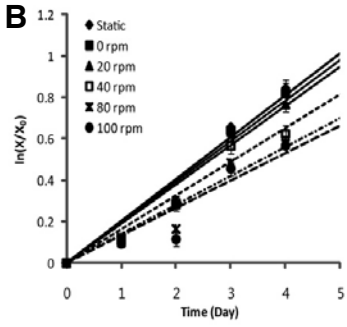
To optimize transduction conditions, the mesenchymal stem cells were agitated on a rocker under several different conditions - adhered cells, suspended cells, or cells rocked at 0, 20, 40, 80 or 100 rpm. Target cells should be in the exponential growth phase to get maximum retroviral transduction efficiency, as retroviruses can only transduce actively dividing cells. Also, the growth rate should be tested to determine whether the mechanical agitation could stimulate the growth of rat mesenchymal stem cells to get correct transduction efficiency. The specific growth rate (μ) of rat mesenchymal stem cells can be calculated from the equation $X = X_0 \mu t$, where X is the cell number at an arbitrary time t and X_0 is the initial cell density.

According to our MTT results, mechanical agitation does not affect the viability of adhered rat mesenchymal stem cells. However, the viability of suspended rat mesenchymal stem cells decreased above 80 rpm, but was as high as 98% after the 20 rpm mechanical agitation step. So even though mechanical agitation seemed to inhibit the growth rate of rat mesenchymal stem cells, the specific growth rate (μ) corrected for cell viability was not changed (Fig. 3).

Transduction efficiency by mechanical agitation

As expected, fluorescence microscope images of the rat mesenchymal stem cells transduced with Retro-EGFP showed that this suspended cell transduction method with 20 rpm mechanical agitation for 50 min allowed high transduction efficiency of





C

	Adhered cells	Mechanical agitation with Suspended cells (RPM)				
		0	20	40	80	100
Cell viability (%)	100	98.2±2.1	98.2±1.8	95.9±1.8	95.9±2.5	93.5±2.5
μ/h	0.008408	0.008158	0.007883	0.006788	0.005704	0.005504

Fig. 3. Effect of mechanical agitation on growth kinetics of rat mesenchymal stem cells. To measure cell viability, cells (2×10^4 cells/well on 96-well plates) were cultured for 24 h after mechanical agitation for 50 min (A). To measure growth kinetics, the number of cells (X) was determined at 0, 1, 2, 3 and 4 days after inoculation (2×10^4 cells/well on 96-well plates). μ was determined from each slope for a least square linear fit of ln (X/X_0) to t (B) and numerical representations of the cell viability and growth rate are shown (C). Control cells were cultured without mechanical agitation. Cell viability and growth rate were measured by MTT assay.

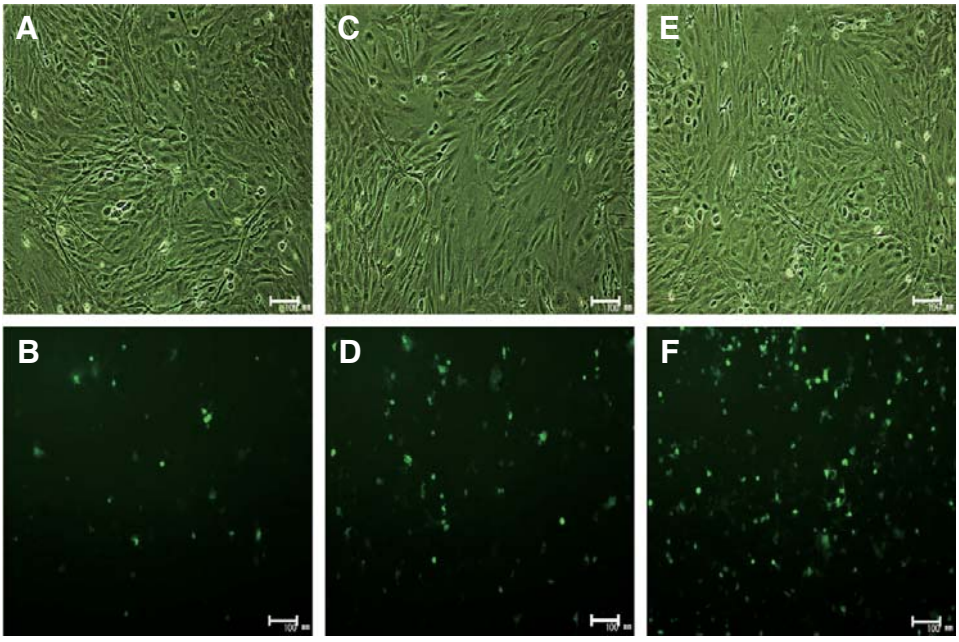


Fig. 4. Fluorescence microscope images of rat mesenchymal stem cells. After 86 h, transduced rat mesenchymal stem cells were observed for EGFP fluorescence with a fluorescence microscope (TE2000-S, Nikon); (A, B) phase contrast and fluorescence image of adherent rat mesenchymal stem cells transduced using the static method; (C, D) phase contrast and fluorescence image of adherent rat mesenchymal stem cells transduced with 20 rpm mechanical agitation for 50 min; (E, F) phase contrast and fluorescence image of suspended rat mesenchymal stem cells transduced with 20 rpm mechanical agitation for 50 min.

rat mesenchymal stem cells (Fig. 4).

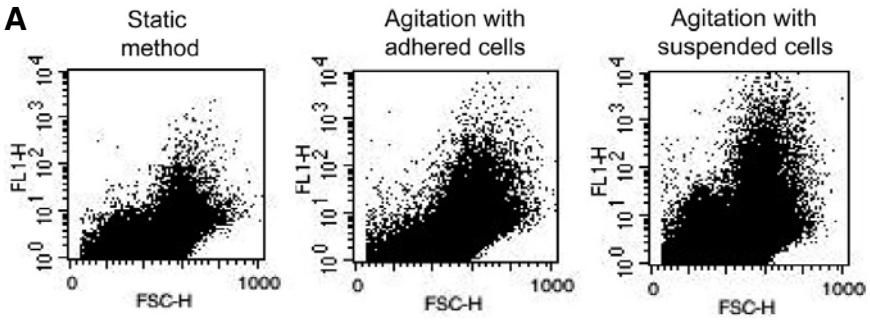
The exact transduction efficiency of rat mesenchymal stem cells was measured by FACSCalibur. Since a viral target cell monolayer such as that grown on a surface in a culture flask is subject only to flows parallel to the solid surface, the penetration distance of the virus will depend on the monolayer's thickness. This hydrodynamic limitation was overcome by the agitation method established in this study. To increase the chances of direct contact between the viruses and the target cells, rat mesenchymal stem cells were detached from the flask and suspended in the incubation media. The cells were suspended just before transduction, and then the suspended cells were incubated with virus particles under optimum conditions, as mentioned above. As expected, our method including simultaneous mechanical agitation of viruses with suspended cells showed the highest transduction efficiency, up to 42%, about 2.5 fold higher than the efficiency of the static method (Fig. 5). However, the transduction efficiency did not increase in proportion to the fraction of viruses adsorbing to cells.

Differentiation of transduced rat mesenchymal stem cells

As indicated in Fig. 6, the transduced rat mesenchymal stem cells differentiated into osteocytes and adipocytes. No difference was observed between transduced and control rat mesenchymal stem cells either in the osteogenic or adipogenic media. The results suggest that rat mesenchymal stem cells are multipotent even after application of this mechanical agitation method.

DISCUSSION

Recently, retroviral vectors have often been used for gene transfer into humans and animals for gene therapy and the establishment of transgenic animals because these viral vec-



B

	Static method	Simultaneous mechanical agitation	
		Adhered cells	Suspended cells
% EGFP (+) cells	17 ± 2.7	30 ± 1.4	43 ± 2.5

Fig. 5. Mechanical agitation enhanced the transduction efficiency of EGFP-carrying retrovirus. (A) The representative FACS plots of rat mesenchymal stem cells after transduction with Retro-EGFP using the static method (left panel), mechanical agitation of viruses with adhered cells (middle panel) and simultaneous mechanical agitation of retroviruses with suspended cells (right panel). (B) Numerical representation of the transduction efficiencies of EGFP retrovirus under the static protocol versus the new agitation protocol. The transduction efficiency is defined as the percentage of cells expressing EGFP as measured using FACSCalibur. The mean percentage of GFP-positive cells is presented as the average of three independent transduction experiments (+/- SEM, n = 3).

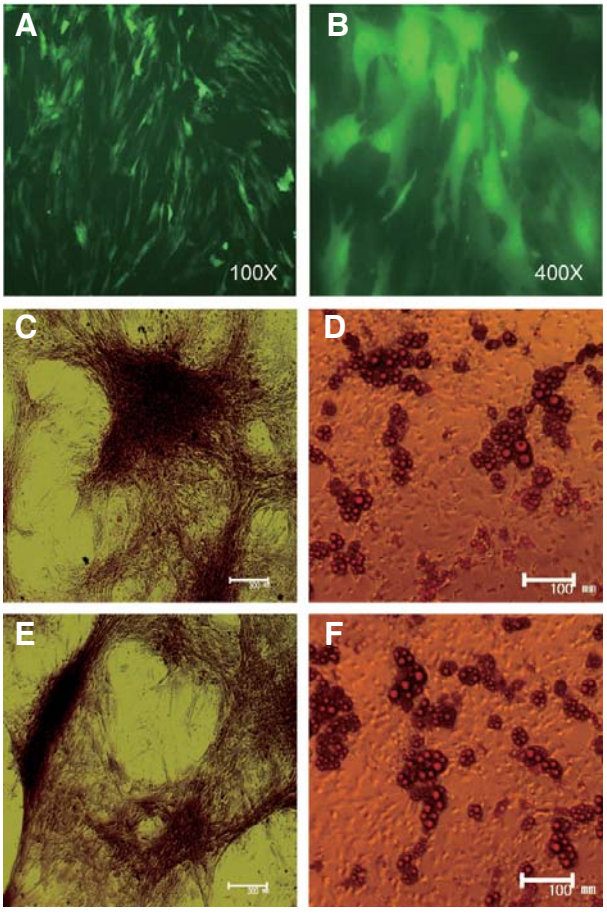


Fig. 6. Microscopic images of rat mesenchymal stem cells transduced with EGFP retrovirus. Transduction of rat mesenchymal stem cells with our protocol (A, B) (100× and 400× magnification); Alizarin Red S staining of rat mesenchymal stem cells transduced with the Retro-EGFP (C) and normal rat mesenchymal stem cells (E) on day 20 after osteocyte induction. Microscope images of Oil-Red-O staining of rat mesenchymal stem cells transduced with the Retro-EGFP (D) and normal rat mesenchymal stem cells (F) on day 20 after adipocyte induction.

tors show a high efficiency of gene transfer and a stable integration of the transgene into the host genome. For gene transfer, the establishment of an easy and efficient procedure for transduction with high efficiency is important. In static protocols, the viral particles make contact with the cells by undergoing Brownian movements and passively diffusing through the cell culture media (Chuck and Palsson, 1996; Chuck et al., 1996). This Brownian-limited motion is one of the main obstacles to transduction. This new protocol was developed on the hypothesis that the efficiency of retroviral transduction would be increased if virus particles had increased contact with the target cells. A mechanical agitation step was implemented during the incubation of viruses with target cells to increase the chance of physical contact between viruses and cells. This simple addition of a mechanical agitation step overcame the limitations on time and distance for capture imposed by Brownian motion.

In conclusion, this mechanical agitation method led to the higher transduction efficiency than that of any current static transduction method. It was effective even at low viral concentration, eliminating many of the concerns associated with the production of high-titer virus stock. The method can potentially be applied to a variety of other transduction protocols with slight adjustments to agitation time and speed. This protocol will contribute to various *ex vivo* gene therapies and *in vitro* gene transfer experiments for primary cells.

ACKNOWLEDGMENTS

This research was supported by Research Center for Industrial Development of BioFood Materials in Chonbuk National University, Jeonju, Korea. The center is designated as a Regional Innovation Center appointed by the Ministry of Knowledge Economy, Jeollabuk-do Provincial Government and Chonbuk National University.

REFERENCES

Ahmed, N., Dreier, R., Gopferich, A., Grifka, J., and Grassel, S. (2007). Soluble signalling factors derived from differentiated cartilage tissue affect chondrogenic differentiation of rat adult marrow stromal cells. Cell Physiol. Biochem. *20*, 665-678.

Akira, T. (2005). High temperature condition enhances retrovirus infection to cell. J. Health Sci. *51*, 96-100.

Cai, S., Ma, Q., Yu, X., Dang, G., and Ma, D. (2002). Expression of human VEGF(121) cDNA in mouse bone marrow stromal cells.

Chin. Med. J. (Engl) *115*, 914-918.
Chamberlain, G., Fox, J., Ashton, B., and Middleton, J. (2007). Concise review: mesenchymal stem cells: their phenotype, differentiation capacity, immunological features, and potential for homing. Stem Cells *25*, 2739-2749.
Chuck, A.S., and Palsson, B.O. (1996). Consistent and high rates of gene transfer can be obtained using flow-through transduction over a wide range of retroviral titers. Hum. Gene Ther. *7*, 743-750.
Chuck, A.S., Clarke, M.F., and Palsson, B.O. (1996). Retroviral infection is limited by Brownian motion. Hum. Gene Ther. *7*, 1527-1534.
Daga, A., Muraglia, A., Quarto, R., Cancedda, R., and Corte, G. (2002). Enhanced engraftment of EPO-transduced human bone marrow stromal cells transplanted in a 3D matrix in non-conditioned NOD/SCID mice. Gene Ther. *9*, 915-921.
Ding, L., Lu, S., Batchu, R., Iii, R.S., and Munshi, N. (1999). Bone marrow stromal cells as a vehicle for gene transfer. Gene Ther. *6*, 1611-1616.
Eiges, R., Schuldiner, M., Drukker, M., Yanuka, O., Itskovitz-Eldor, J., and Benvenisty, N. (2001). Establishment of human embryonic stem cell-transfected clones carrying a marker for undifferentiated cells. Curr. Biol. *11*, 514-518.
Kwon, Y.J., Hung, G., Anderson, W.F., Peng, C.A., and Yu, H. (2003). Determination of infectious retrovirus concentration from colony-forming assay with quantitative analysis. J. Virol. *77*, 5712-5720.
Koda, M., Kamada, T., Hashimoto, M., Murakami, M., Shirasawa, H., Sakao, S., Ino, H., Yoshinaga, K., Koshizuka, S., Moriya, H., et al. (2007). Adenovirus vector-mediated *ex vivo* gene transfer of brain-derived neurotrophic factor to bone marrow stromal cells promotes axonal regeneration after transplantation in completely transected adult rat spinal cord. Eur. Spine J. *16*, 2206-2214.
Lakshmipathy, U., Pelacho, B., Sudo, K., Linehan, J.L., Coucouvanis, E., Kaufman, D.S., and Verfaillie, C.M. (2004). Efficient transfection of embryonic and adult stem cells. Stem Cells *22*, 531-543.
Lee, G.S., Kim, G.S., Sheih, J.H., and Moore, M. (2008). Forced expression of HoxB4 enhances hematopoietic differentiation by human embryonic stem cells. Mol. Cells *25*, 487-493.
Liu, X.H., Bai, C.G., Xu, Z.Y., Huang, S.D., Yuan, Y., Gong, D.J., and Zhang, J.R. (2008). Therapeutic potential of angiogenin modified mesenchymal stem cell: Angiogenin improves mesenchymal stem cells survival under hypoxia and enhances vasculogenesis in myocardial infarction. Microvasc. Res. *76*, 23-30.
Ma, Y., Ramezani, A., Lewis, R., Hawley, R.G., and Thomson, J.A. (2003). High-level sustained transgene expression in human embryonic stem cells using lentiviral vectors. Stem Cells *21*, 111-117.
Olga, S.K., Anke, W., Klaus, K., Birgit, S., Axel, R.Z., Christopher, B., and Boris, F. (2003). Dose finding with retroviral vectors: correlation of retroviral vector copy numbers in single cells with gene transfer efficiency in a cell population. Gene Ther. *12*, 3934-3937.
Peister, A., Mellad, J.A., Wang, M., Tucker, H.A., and Prockop, D.J. (2004). Stable transfection of MSCs by electroporation. Gene Ther. *11*, 224-228.
Prockop, D.J. (1997). Marrow stromal cells as stem cells for non-hematopoietic tissues. Science *276*, 71-74.
Rose, T., Peng, H., Shen, H.C., Usas, A., Kuroda, R., Lill, H., Fu, F.H., and Huard, J. (2003). The role of cell type in bone healing mediated by *ex vivo* gene therapy. Langenbecks Arch. Surg. *388*, 347-355.
Schultz, S.S., and Lucas, P.A. (2006). Human stem cells isolated from adult skeletal muscle differentiate into neural phenotypes. J. Neurosci. Methods *152*, 144-155.
Wang, Y., Chen, X., Zhu, W., Zhang, H., Hu, S., and Cong, X. (2006). Growth inhibition of mesenchymal stem cells by aspirin: involvement of the WNT/beta-catenin signal pathway. Clin. Exp. Pharmacol. Physiol. *33*, 696-701.
Yang, L.Y., Huang, T.H., and Ma, L. (2006). Bone marrow stromal cells express neural phenotypes *in vitro* and migrate in brain after transplantation *in vivo*. Biomed. Environ. Sci. *19*, 329-335.
Yoo, S.W., Kim, S.S., Lee, S.Y., Lee, H.S., Kim, H.S., Lee, Y.D., and Suh-Kim, H. (2008). Mesenchymal stem cells promote proliferation of endogenous neural stem cells and survival of newborn cells in a rat stroke model. Exp. Mol. Med. *40*, 387-397.
Zaehres, H., Lensch, M.W., Daheron, L., Stewart, S.A., Itskovitz-Eldor, J., and Daley, G.Q. (2005). High-efficiency RNA interference in human embryonic stem cells. Stem Cells *23*, 299-305.