doi:10.1016/j.ymthe.2005.03.010

Cellular Manipulation of Human Embryonic Stem Cells by TAT-PDX1 Protein Transduction

Young Do Kwon,[1] Sun Kyung Oh,[1,2] Hee Sun Kim,[1,2] Seung-Yup Ku,[1,2] Seok Hyun Kim,[1,2] Young Min Choi,[1,2] and Shin Yong Moon[1,2,*]

[1]*Institute of Reproductive Medicine and Population, Medical Research Center, and* [2]*Department of Obstetrics and Gynecology, College of Medicine, Seoul National University, 28, Yeongeon-dong, Jongno-gu, Seoul 110-799, Korea*

**To whom correspondence and reprint requests should be addressed. Fax: +82 2 765 3881. E-mail: shmoon@plaza.snu.ac.kr.*

Available online 20 April 2005

Human embryonic stem cells (hESCs) are an *in vitro* model system for the study of human early development and a potential source for cell-based therapies. An efficient strategy for cellular manipulation of hESCs may be highly valuable for the analysis of gene function involved in human embryogenesis and the development of cell-based therapies via induced differentiation into particular cell types. However, plasmid transfection of hESCs has low efficiency and viral transduction may not be the method of choice for cell-based therapies due to genome integration. To overcome these limitations, we applied protein transduction technology that can transfer proteins into cells via direct penetration across the lipid bilayer. Here, we show that the FITC dye fused to the TAT protein transduction domain (PTD) was efficiently transferred into hESCs. In addition, the PDX1 transcription factor, which plays a central role in pancreatic development, was transferred into hESCs as a fusion form of TAT PTD. The transduced TAT-PDX1 activated its downstream target genes and induced insulin protein production in hESCs. These results demonstrate that protein transduction could be used in the cellular manipulation of hESCs and would provide a significant breakthrough for basic and therapeutic research in hESCs.

Key Words: human embryonic stem cells, protein transduction, PDX1

INTRODUCTION

Human embryonic stem cells (hESCs) are derived from the inner cell mass of blastocyst-stage embryos [1–3]. These cells can be maintained in an undifferentiated state for more than 1 year and can proliferate indefinitely in culture [1–4]. In addition, they retain the ability to differentiate into all cell types from the three germ layers [1–5]. Because of these properties, hESCs are expected to be an unlimited source for cell-based therapies of various diseases and a useful *in vitro* model system for the study of human early development. The practical applications of such basic and therapeutic potentials require the efficient manipulation of hESCs. Many strategies for genetic modifications, such as plasmid transfection and viral transduction, have been applied to hESCs [6–8]. However, plasmid transfection by chemical reagents or electroporation is known to have low transfer efficiency [6]. Lentiviral transduction may not be the appropriate method for therapeutic use due to genome integration [7,8]. To develop an effective tool that has high transfer efficiency and is compatible with therapeutic use in hESCs, here we used the protein transduction method.

Protein transduction was initially observed in the HIV-1 TAT protein, which was able to cross cell membranes directly and activate viral genes [9,10]. The protein's ability to transverse cell membranes resides in a stretch of 11 amino acids (residues 47–57) [11,12]. This highly basic TAT protein transduction domain (TAT PTD) is known to interact with negatively charged cell surface constituents and is rapidly internalized by lipid raft-dependent macropinocytosis [13]. The TAT PTD-mediated protein transduction has been used successfully to deliver a variety of molecules such as antigenic peptides [14], antisense oligonucleotides [15], and full-length proteins into cells [11,12,16]. Recent studies show that it could be utilized for a variety of therapeutic applications, including protection from ischemic brain injury [17,18], antigen presentation by dendritic cells [14], and modulation of NF-κB activity [19].

In this study, we report for the first time that TAT PTD-mediated protein transduction works with high efficiency in hESCs. We also demonstrate that as a fusion form of TAT PTD, the transduced PDX1 transcription factor, a crucial regulator for pancreatic development and

Copyright © The American Society of Gene Therapy
1525-0016/$30.00

insulin gene transcription [20,21], can elicit cellular changes in hESCs.

RESULTS AND DISCUSSION

Transduction of TAT-FITC Peptide into hESCs

To investigate whether TAT-mediated protein transduction occurs efficiently in hESCs, we treated three hESC lines, SNUhES2, SNUhES3, and SNUhES4, with free FITC as a control and TAT-FITC peptide at a final concentration of 50 μM for 1 h and then observed the cells under fluorescence confocal microscope. The results demonstrated that, whereas control free FITC did not enter the cells, TAT-fused FITC penetrated most of the cells in all three hESC lines (Fig. 1A). In addition, we formed embryoid bodies (EBs) from the three hESC lines and transduced them with TAT-FITC under the conditions mentioned above. Similar results were also obtained in EBs (Fig. 1B). To measure transduction efficiency quantitatively, we performed flow cytometry analysis using day 1 EBs. The results indicated that about 70% of cells in the EBs were transduced with TAT-FITC (Fig. 1C). To block artifactual increase in efficiency, we omitted the cell fixation step in the confocal microscopy and FACS analysis [22]. Previous reports demonstrated that nearly all cells were transduced with TAT-fusion proteins in ordinary animal cell lines such as HeLa and Jurkat [11,23]. In the case of EBs formed from hESCs, it seems that TAT-FITC could not reach deep inside of the EBs, possibly due to their aggregated structure. However, the transfer efficiency was much higher than that of genetic modification in hESCs [6,7] and therefore, the protein transduction method could be a more efficient transfer tool. Importantly, the protein transduction efficiencies showed similar levels in three hESC lines that were derived from different cryopreserved pronuclear-stage embryos and were established using different methods for isolation of the inner cell mass [3]. Consequently, protein transduction could be applied generally to nearly all of the hESC lines with high efficiency.

Inefficient Transduction of PDX1 Protein and Rapid Transduction of TAT-PDX1 Fusion Protein into hESCs

To deliver full-length proteins into hESCs, we isolated Pdx1 cDNA from a human cDNA library and cloned it into expression vectors pET21b and pPT1, which contains TAT PTD sequence. PDX1 and TAT-PDX1 proteins were purified in *Escherichia coli* BL21(DE3) using His-tag affinity chromatography (Figs. 2A and 2B). PDX1 itself contains an Antennapedia-like protein transduction domain and a study showed that PDX1 protein was able to enter a variety of cells [24]. To test whether PDX1 protein can be internalized into hESCs, we treated day 1 EBs from the SNUhES3 cell line with PDX1 at a final concentration of 0.2 μM and analyzed transduction

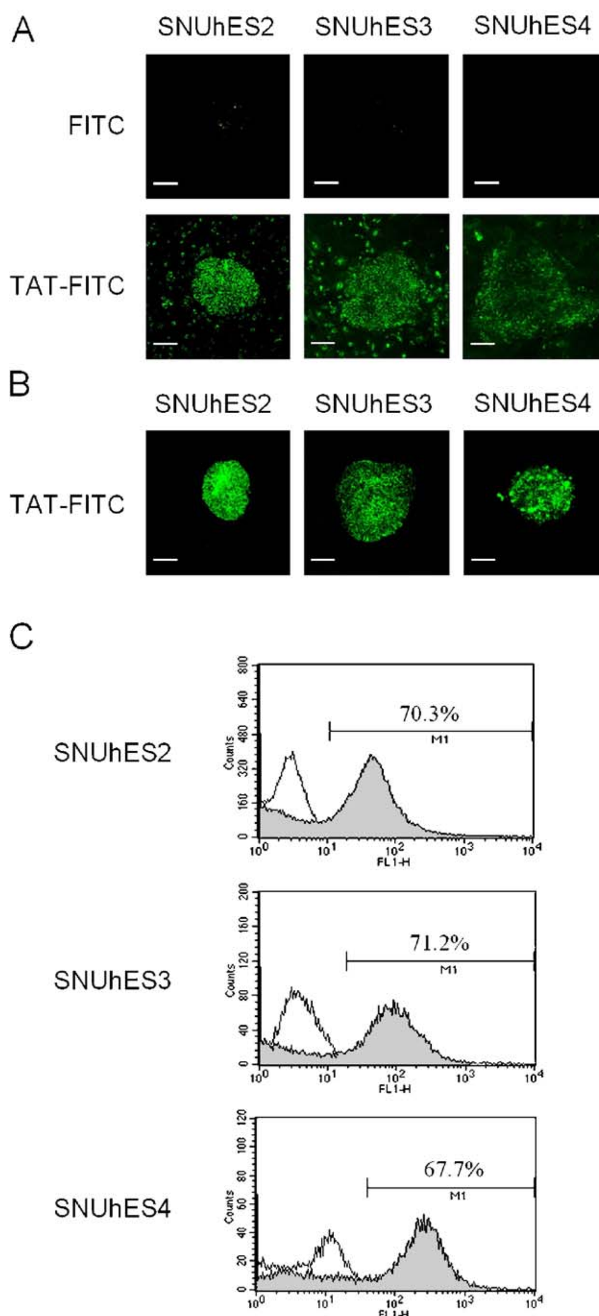

FIG. 1. Transduction of TAT-FITC peptide into three hESC lines. Fluorescence confocal microscopy of (A) day 4 colonies of hESCs and (B) day 1 EBs after treatment of control free FITC and TAT-FITC at the concentration of 50 μM for 1 h. (A) Original magnification, ×100; scale bars, 200 μm. (B) Original magnification, ×200; scale bars, 100 μm. (C) Flow cytometry analysis of day 1 EBs treated with control free FITC (open region) and TAT-FITC (gray region) at the concentration of 50 μM for 1 h. The EBs were treated with trypsin and the dissociated cells were washed with PBS and analyzed.

by western blotting using His-tag antibody. Contrary to previously reported results [24], positive signal was not detected during 24 h treatment (Fig. 2C). On the other

Copyright © The American Society of Gene Therapy

doi:10.1016/j.ymthe.2005.03.010

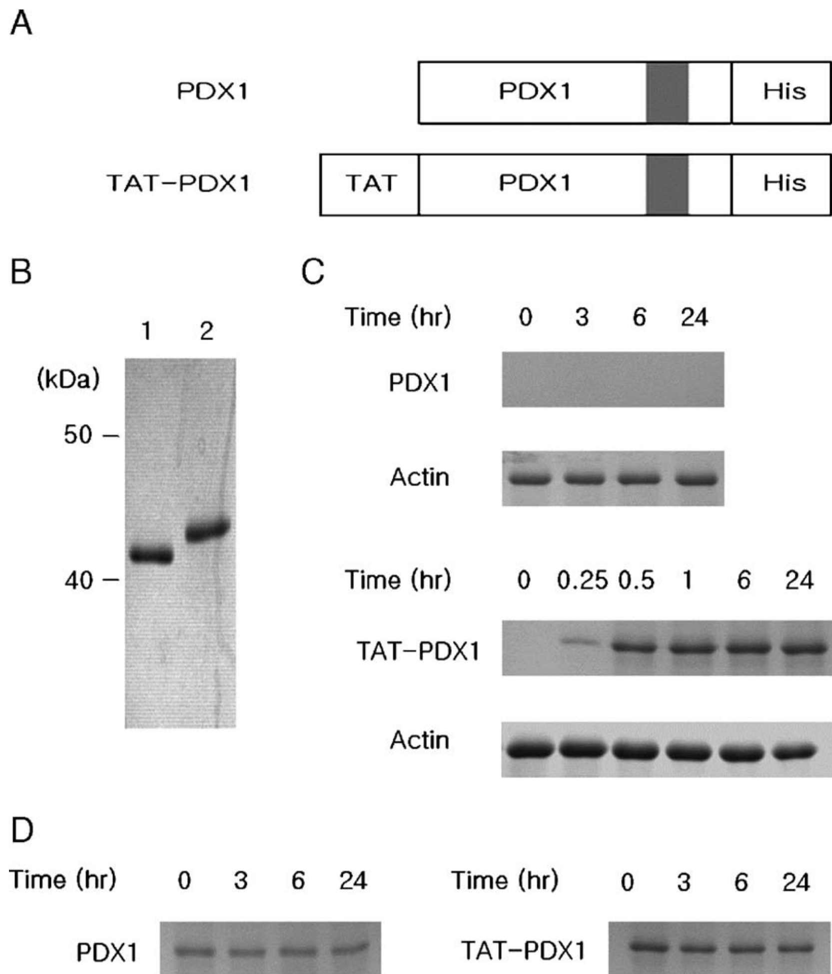

FIG. 2. Transduction of TAT-PDX1 protein into EBs. (A) Schematic representation of PDX1 and TAT-PDX1 proteins. Gray box represents the Antennapedia-like protein transduction domain in the PDX1 protein. (B) Purity of recombinant proteins. Purified proteins were run on a 10% gel and stained with Coomassie Brilliant Blue R solution. Positions of molecular weight markers are indicated. Lane 1, PDX1; lane 2, TAT-PDX1. (C) Western blotting of cell lysates of EBs treated with TAT-PDX1 or PDX1 protein at a concentration of 0.2 μM. Mouse anti-Penta · His HRP conjugate and the enhanced chemiluminescence system were used to probe the proteins. No signal was detected in the case of PDX1. (D) Stability of TAT-PDX1 and PDX1 proteins in culture medium. Day 1 EBs were treated with TAT-PDX1 or PDX1 protein at a concentration of 0.2 μM and the culture media were prepared at the indicated times. Western blotting was performed to detect the proteins.

hand, transduction of TAT-PDX1 fusion protein was detected at 15 min after adding the protein to culture medium and the level of the fusion protein in EBs was increased after that time (Fig. 2C). Both proteins were stable for at least 24 h in the culture medium (Fig. 2D). These results suggest that TAT-mediated protein transduction occurs efficiently in hESCs, but transduction of the Antennapedia-like PTD does not. This distinctive transduction efficiency between TAT and Antennapedia-like PTDs might be due to their different transduction mechanisms. A recent study on the mechanism of TAT-mediated protein transduction suggested that TAT PTD first interacts with cell surface lipid rafts containing sulfated proteoglycans and sialylated glycoproteins, and the PTD is rapidly internalized by macropinocytosis [13]. On the other hand, Antennapedia PTD, as Derossi and colleagues propose, interacts with negatively charged phospholipids, and accumulation of the peptide at the membranes subsequently results in formation of an inverted micelle that is able to transport the peptide to the cytoplasmic side [25]. We could infer from our finding and the previous studies that because of the unique properties of hESCs, such as strong cell-to-cell interactions and high content of extracellular matrix molecules [26], each PTD may interact differently with the membrane and extracellular matrix of hESCs, resulting in each PTD's distinctive transduction efficiencies.

The Transduced TAT-PDX1 Elicited Cellular Changes in hESC

To investigate whether the transduced TAT-PDX1 protein functions inside hESCs, we treated day 1 EBs with PDX1 as a control and TAT-PDX1 at a concentration of 0.2 μM for a week with daily exchange of medium. PDX1 transcription factor is known to activate many pancreas-specific genes such as insulin, glucokinase, GLUT2, IAPP, somatostatin, and Pdx1 itself [24,27]. Real-time RT-PCR against the downstream target genes of PDX1 was performed. The result showed an increase in insulin, IAPP, and Pdx1 mRNA levels, but not in GLUT2 mRNA level (Fig. 3A). No increase in GLUT2 mRNA level means

Copyright © The American Society of Gene Therapy

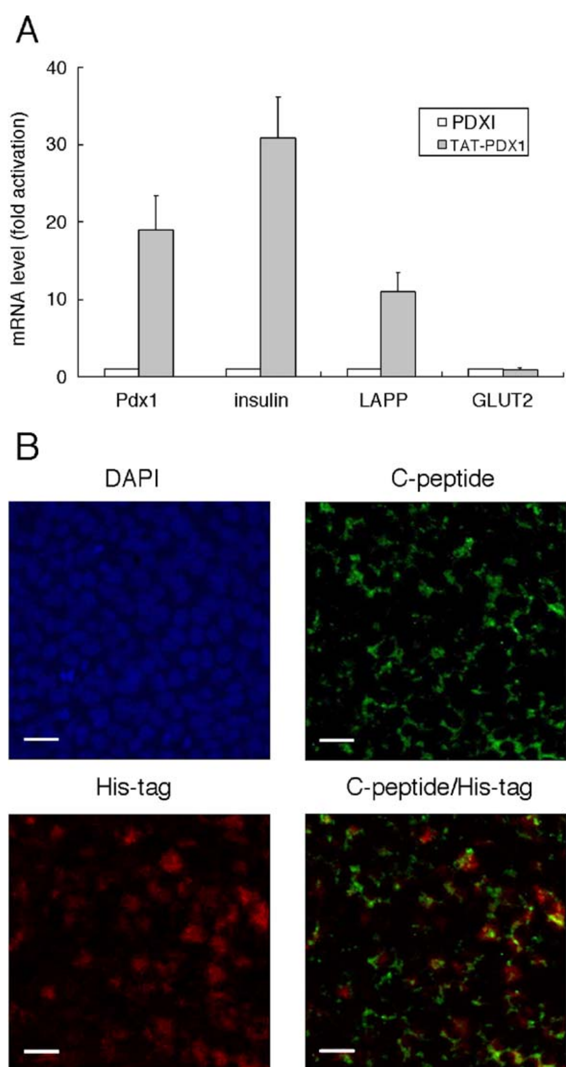

FIG. 3. Transcriptional activation of pancreatic genes by transduced TAT-PDX1 protein. (A) Real-time RT-PCR analysis of Pdx1, insulin, IAPP, and GLUT2 mRNA levels. Total RNA quantity was normalized to GAPDH mRNA level. Day 1 EBs were treated with TAT-PDX1 and control PDX1 for 1 week with daily exchange of medium. (B) Immunofluorescence staining with mouse anti-His-tag antibody and rabbit anti-human C-peptide antibody. Day 1 EBs were treated with TAT-PDX1 and PDX1 for 1 week and were attached to Matrigel-coated coverglasses. Original magnification, ×400; scale bars, 40 μm.

that the cells transduced by TAT-PDX1 might not be mature pancreatic cells. To obtain mature and functional pancreatic β cells, it seemed to require a longer period of culture and/or additional treatment with other differentiation-inducing factors such as Pax4, which can activate GLUT2 gene expression in mouse embryonic stem cells [28]. To determine the actual increase in insulin protein expression by transduced TAT-PDX1, we performed immunocytochemistry with antibodies against His-tag and C-peptide, an indicator of biosynthesis of insulin. Whereas we observed little fluorescence in the cells treated with control PDX1 protein (data not shown), the cells treated with TAT-PDX1 protein showed specific fluorescence signals for His-tag in the nucleus and for C-peptide in the cytoplasm (Fig. 3B). These RT-PCR and immunocytochemistry results demonstrated that the transduced TAT-PDX1 protein activated expression of its target genes and induced an actual increase in insulin protein level. Therefore, TAT-mediated protein transduction could be used for cellular manipulations in hESCs.

In conclusion, we showed for the first time a highly efficient TAT-mediated protein transduction and inefficient transduction of Antennapedia-like PTD in hESCs. In addition, we demonstrated that the transduced TAT-PDX1 was functional inside hESCs and generated insulin-producing cells from hESCs. Therefore, we generated a new cellular manipulation method in hESCs with high transfer efficiency and compatible with cell-based therapies owing to avoidance of genetic modification. This protein transduction technology could be used for delivery of a variety of proteins into hESCs and applied to cell-based therapies and the study of human early development.

Materials and Methods

Culture of hESCs. Three human embryonic stem cell lines, SNUhES2, SNUhES3, and SNUhES4, were used [3]. Undifferentiated hESCs were grown on mitotically inactivated STO cells in DMEM/F-12 supplemented with 20% knockout serum replacement, 1 mM glutamine, 1% nonessential amino acids, 4 ng/ml basic fibroblast growth factor (bFGF), 0.1 mM 2-mercaptoethanol, 50 U/ml penicillin, and 50 μg/ml streptomycin (all from GIBCO Invitrogen, San Diego, CA, USA). The hESC colonies were dissociated mechanically using a finely drawn out Pasteur pipette and then were transferred into new culture dishes. For EB formation, hESC colonies were treated with 2 mg/ml type IV collagenase (GIBCO Invitrogen) and the dissociated colonies were cultured in suspension without bFGF in petri dishes to allow aggregation and differentiation.

Peptide synthesis and protein purification. Synthetic TAT peptide was purchased from Peptron (Daejeon, Korea). The sequence was FITC-GGGGYGRKKRRQRRR. For generation of TAT-fusion protein, oligonucleotides 5′-GATCCCTATGGCAGGAAGAAGCGGAGACAGCGACGAAGAGG-3′ and 5′-AATTCCCTCTTCGTGGCTGCCGTCTCCGCTTCCTGCCATAGG-3′ were annealed and inserted into the *Bam*HI–*Eco*RI sites of pET21b to construct pPT1. Full-length PDX1 cDNA was amplified by PCR with primers 5′-CCCAAGCTTATGAACGGCGAGGAGCAGTAC-3′ and 5′-CCGCTCGAGTCGTGGTTCCTGCGGCCGCCG-3′, from the human pancreas RACE-ready cDNA library (Ambion, Austin, TX, USA) and inserted into the *Eco*RI–*Xho*I sites of pET21b and pPT1. BL21(DE3) *E. coli* cells (Novagen, Madison, WI, USA) containing the expression plasmids were induced for 15 h at 18°C with 0.5 mM IPTG and were sonicated. The supernatants were applied to NTA chelating agarose CL-6B (Peptron) and recombinant proteins were eluted with 0.5 M NaCl/20 mM Tris–HCl/20 mM imidazole/pH 5.9 buffer. The purity of eluted proteins was analyzed by SDS–PAGE and Coomassie Brilliant Blue R staining.

Flow cytometry. To measure the transduction efficiency of FITC-labeled TAT peptide, EBs were transduced with TAT-FITC at a concentration of 50 μM for 1 h and were dissociated by trypsin/EDTA treatment. After adding DMEM/F-12/10% FBS medium, the cells were centrifuged at 1000 *g* and resuspended and washed in 1 × D-PBS. Fluorescence analysis was performed with a FACScan Calibur (Becton–Dickinson, San Jose, CA, USA).

Copyright © The American Society of Gene Therapy

doi:10.1016/j.ymthe.2005.03.010

Western blotting. After being washed with PBS, the cells were solubilized in SDS–PAGE sample buffer. Cell extracts were run on a 10% gel and were blotted onto PVDF membranes (Amersham, Cardiff, Wales, UK). After being blocked at room temperature for 1 h in TBST with 3% BSA, the membranes were treated with Penta·His HRP conjugate antibody (Qiagen, Hilden, Germany) for detection of transduced protein. Also, the membranes were treated with mouse anti-actin antibody and then bovine anti-mouse IgG HRP conjugate antibody for normalization of cell extract quantity (all from Santa Cruz Biotechnology, Santa Cruz, CA, USA). After being washed with PBST, the membranes were probed using ECL Plus detection reagents (Amersham).

Real-time RT-PCR. Total RNA was isolated from hESCs using Trizol reagent (GIBCO Invitrogen) according to the manufacturer's instructions. First-strand cDNA was synthesized using the SuperScript system (GIBCO Invitrogen). Quantification of selected transcripts was performed using the Rotor-Gene 3000 system (Corbett, Australia) and QuantiTect SYBR Green PCR kit (Qiagen). PCR amplification conditions were 20 s denaturation at 94°C, 20 s annealing at 55°C, and 20 s extension at 72°C for all genes. The oligonucleotide primers used for PCR were as follows: GAPDH, 5′-AGCCACATCGCTCAGACACC-3′ and 5′-GTACTCAGCGGCCAGCATCG-3′; insulin, 5′-TACCTAGTGTGCGGGGAACGA-3′ and 5′-CCAGCTGGTA-GAGGGAGCAGA-3′; IAPP, 5′-TGTGCTCTCTGTTGCATTGAACC-3′ and 5′-TGGATCCCACGTTGGTAGATGA-3′; GLUT2, 5′-CGGCTGGTATCAG-CAAACCTG-3′ and 5′-CAGCAGCACAAGTCCCACTGA-3′; and PDX1, 5′-AAAGCTCACGCGTGGAAAGG-3′ and 5′-TCAACATGACAGC-CAGCTCCA-3′.

Immunocytochemistry. Protein-transduced EBs were attached to Matrigel-coated coverglasses. The cells were fixed with 4% paraformaldehyde in D-PBS buffer. After being blocked with 3% BSA in PBST for 1 h at room temperature, cells were incubated with mouse anti-His-tag antibody (Santa Cruz Biotechnology) and rabbit anti-human C-peptide antibody (Linco Research, St. Charles, MO, USA). The cells were then washed four times with PBST and were incubated with donkey anti-mouse IgG Alexa 594 conjugate and donkey anti-rabbit IgG Alexa 488 conjugate (all from Molecular Probes, Eugene, OR, USA) for 30 min at room temperature. All antibodies were used at 1:200. After being washed four times with PBST, the cells were observed under laser-scanning confocal microscopy (Bio-Rad, Hercules, CA, USA).

ACKNOWLEDGMENTS

The authors thank Yoon Young Kim for technical assistance and Lim Andrew Lee for comments on the manuscript. This research was supported by a grant (SC11011) from the Stem Cell Research Center of the 21st Century Frontier Research Program funded by the Ministry of Science and Technology, Republic of Korea.

RECEIVED FOR PUBLICATION JANUARY 6, 2005; ACCEPTED MARCH 16, 2005.

REFERENCES

1. Thomson, J. A., *et al.* (1998). Embryonic stem cell lines derived from human blastocysts. *Science* **282:** 1145 – 1147.
2. Reubinoff, B. E., Pera, M. F., Fong, C. Y., Trounson, A., and Bongso, A. (2000). Embryonic stem cell lines from human blastocysts: somatic differentiation in vitro. *Nat. Biotechnol.* **18:** 399 – 404.
3. Oh, S. K., *et al.* (2005). Derivation and characterization of new human embryonic stem cell lines, SNUhES1, SNUhES2 and SNUhES3. *Stem Cells* **25:** 211 – 219.
4. Amit, M., *et al.* (2000). Clonally derived human embryonic stem cell lines maintain pluripotency and proliferative potential for prolonged periods of culture. *Dev. Biol.* **227:** 271 – 278.
5. Itskovitz-Eldor, J., *et al.* (2000). Differentiation of human embryonic stem cells into embryoid bodies compromising the three embryonic germ layers. *Mol. Med.* **6:** 88 – 95.
6. Eiges, R., *et al.* (2001). Establishment of human embryonic stem cell-transfected clones carrying a marker for undifferentiated cells. *Curr. Biol.* **11:** 514 – 518.
7. Gropp, M., *et al.* (2003). Stable genetic modification of human embryonic stem cells by lentiviral vectors. *Mol. Ther.* **7:** 281 – 287.
8. 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.
9. Frankel, A., and Pabo, C. (1988). Cellular uptake of the Tat protein from human immunodeficiency virus. *Cell* **55:** 1189 – 1193.
10. Green, M., and Loewenstein, P. (1988). Autonomous functional domains of chemically synthesized human immunodeficiency virus Tat trans-activator protein. *Cell* **55:** 1179 – 1188.
11. Nagahara, H., *et al.* (1998). Transduction of full-length TAT fusion proteins into mammalian cells: TAT-p27^{Kip1} induces cell migration. *Nat. Med.* **4:** 1449 – 1452.
12. Schwarze, S. R., Ho, A., Vocero-Akbani, A., and Dowdy, S. F. (1999). In vivo protein transduction: delivery of a biologically active protein into the mouse. *Science* **285:** 1569 – 1572.
13. Wadia, J. S., Stan, R. V., and Dowdy, S. F. (2004). Transducible TAT-HA fusogenic peptide enhances escape of TAT-fusion proteins after lipid raft macropinocytosis. *Nat. Med.* **10:** 310 – 315.
14. Shibagaki, N., and Udey, M. C. (2002). Dendritic cells transduced with protein antigens induce cytotoxic lymphocytes and elicit antitumor immunity. *J. Immunol.* **168:** 2393 – 2401.
15. Astriab-Fisher, A., Sergueev, D., Fisher, M., Shaw, B. R., and Juliano, R. L. (2002). Conjugates of antisense oligonucleotides with the Tat and antennapedia cell-penetrating peptides: effects on cellular uptake, binding to target sequences, and biologic actions. *Pharm. Res.* **19:** 744 – 754.
16. Krosl, J., *et al.* (2003). In vitro expansion of hematopoietic stem cells by recombinant TAT-HOXB4 protein. *Nat. Med.* **9:** 1428 – 1432.
17. Asoh, S., *et al.* (2002). Protection against ischemic brain injury by protein therapeutics. *Proc. Natl. Acad. Sci. USA* **99:** 17107 – 17112.
18. Cao, G., *et al.* (2002). In vivo delivery of a Bcl-xL fusion protein containing the TAT protein transduction domain protects against ischemic brain injury and neuronal apoptosis. *J. Neurosci.* **22:** 5423 – 5431.
19. Kabouridis, P. S., Hasan, M., Newson, J., Gilroy, D. W., and Lawrence, T. (2002). Inhibition of NF-kappa B activity by a membrane-transducing mutant of I kappa B alpha. *J. Immunol.* **169:** 2587 – 2593.
20. Murtaugh, L. C., and Melton, D. A. (2003). Genes, signals, and lineages in pancreas development. *Annu. Rev. Cell Dev. Biol.* **19:** 71 – 89.
21. Grapin-Botton, A., Majithia, A. R., and Melton, D. A. (2001). Key events of pancreas formation are triggered in gut endoderm by ectopic expression of pancreatic regulatory genes. *Genes Dev.* **15:** 444 – 454.
22. Richard, J. P., *et al.* (2003). Cell-penetrating peptides: a reevaluation of the mechanism of cellular uptake. *J. Biol. Chem.* **278:** 585 – 590.
23. Fawell, S., *et al.* (1994). Tat-mediated delivery of heterologous proteins into cells. *Proc. Natl. Acad. Sci. USA* **91:** 664 – 668.
24. Noguchi, H., Kaneto, H., Weir, G. C., and Bonner-Weir, S. (2003). PDX-1 protein containing its own antennapedia-like protein transduction domain can transduce pancreatic duct and islet cells. *Diabetes* **52:** 1732 – 1737.
25. Derossi, D., *et al.* (1996). Cell internalization of the third helix of the Antennapedia homeodomain is receptor-independent. *J. Biol. Chem.* **271:** 18188 – 18193.
26. Kim, S. J., *et al.* (2005). Contribution of the PI3K/Akt/PKB signal pathway to maintenance of self-renewal in human embryonic stem cells. *FEBS Lett.* **579:** 534 – 540.
27. Hui, H., and Perfetti, R. (2002). Pancreas duodenum homeobox-1 regulates pancreas development during embryogenesis and islet cell function in adulthood. *Eur. J. Endocrinol.* **146:** 129 – 141.
28. Blyszczuk, P., *et al.* (2003). Expression of Pax4 in embryonic stem cells promotes differentiation of nestin-positive progenitor and insulin-producing cells. *Proc. Natl. Acad. Sci. USA* **100:** 998 – 1003.

Copyright © The American Society of Gene Therapy