Cell-penetrating peptide (CPP)-conjugated proteins is an efficient tool for manipulation of human mesenchymal stromal cells

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Delivery of proteins has been regarded as the safest and most useful application in therapeutic application of stem cells, because proteins can regulate gene expression transiently without any genomic alteration. However, it is difficult to accurately measure efficiency or quantity of intracellular protein uptake. Here, we performed a comparison study of cell-penetrating peptide (CPP)-conjugated protein delivery system using seven arginine and Streptolysin O (SLO)-mediated system. To compare CPP- and SLO-mediated protein delivery systems, we used GFP and ESRRB protein, which is known to regulate pluripotency-related genes, for delivery into human bone marrow stromal cells (hBMSCs) and human testicular stromal cells (hTSCs). We found that CPP-conjugated protein delivery was more efficient, lower cytotoxicity, and higher biological activity than SLO-mediated protein delivery system. These results suggest that delivery of CPP-conjugated proteins is an efficient tool for introducing biologically active proteins into cells and may have important implications in clinical cell-based therapy.
large pores in the plasma membrane of mammalian cells and may provide the possibility of exogenous protein delivery into the cytosol\(^{15}\). Therefore, SLO is being used widely as a material for the delivery of exogenous protein in many fields\(^{16–18}\). In fact, several reports regarding SLO-mediated cellular extracts and protein delivery were reported recently in the stem cell research field\(^{19,20}\).

In this study, we synthesized green fluorescent protein (GFP) and estrogen-related receptor \(\beta\) (ESRRB) with or without CPP-conjugation, and both proteins were transported into hBMSCs and human testicular stromal cells (hTSCs) using these two different protein delivery systems. GFP was used to estimate protein transduction efficiency, cytotoxicity, and intracellular protein uptake rate. In addition, we used ESRRB, which is known to interact with pluripotency-related factors such as OCT4, SOX2, and NANOG to analyze the biological activity.

### Results

#### Purification of CPP-conjugated and non-conjugated GFP and ESRRB proteins.

The ESRRB- and GFP-expressing vectors were constructed by cloning the cDNAs of both factors into the pET-20b vector for purification of the recombinant proteins. Each expression vector construct was transformed into BL21(DE3)pLysS competent cells, and recombinant proteins were obtained from the soluble fraction. CPP-GFP was confirmed by Coomassie Brilliant Blue staining and detected by a GFP-specific antibody at the molecular weight (MW) of 29 kDa (Fig. 1B). GFP was detected at the MW of 27 kDa because the 7× arginine and 6× histidine sequence was removed. In comparison with the purchased commercial GFP (Atgen), R7-GFP was well enriched. These results demonstrated that the protein purification system was optimized well. CPP-ESRRB and ESRRB were purified using the same procedure as for CPP-GFP. CPP-ESRRB and ESRRB were purified as pure proteins and confirmed by Coomassie Brilliant Blue staining and Western Blot. A specific band was detected at approximately 50 kDa (Fig. 1B). The MW of the ESRRB protein was 1 kDa smaller than that of R7-ESRRB because the 7× arginine sequence was removed.

#### Comparison of the protein uptake efficiency using two different protein delivery systems.

To examine the intracellular protein uptake of the two delivery systems, we performed the following experiments. Suspended hTSCs and hBMSCs were treated with 10 \(\mu\)g of CPP-GFP for CPP-conjugated protein delivery system or with 10 \(\mu\)g of GFP for 1 hr after SLO-mediated pore formation for 50 min for SLO-mediated protein delivery system; the protein uptake efficiency was then compared. In terms of uptaking percentage of cells, we observed a high efficiency of protein delivery both of two different protein delivery systems, nearly 99%. Hence, we performed amount quantification of total intracellular protein uptake by confocal laser microscopy analysis. As shown in Figure 2A, CPP-conjugated GFP was delivered efficiently into hTSCs and hBMSCs. However, the SLO-mediated GFP showed lower delivery efficiency compared with CPP-conjugated GFP. Semi-quantitative analysis of GFP uptake was performed by measuring

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**Figure 1 | Characterization of recombinant proteins.**

A) Schematic diagrams of recombinant proteins with or without the CPP (R7)-conjugated vectors. (B) Identification of recombinant protein (GFP and ESRRB) and R7-conjugated protein (R7-GFP and R7-ESRRB) by Coomassie Brilliant Blue staining and Western blotting using specific antibodies against GFP and ESRRB. Full-length gel and blot images are available in Supplementary Figure 4. M, molecular weight marker.
the intensity of the intracellular GFP signal using ImageJ software (NIH). The intensity of the CPP-GFP signal was over 2-fold that of the SLO-mediated GFP signal in hTSCs and hBMSCs (Fig. 2B).

The intracellular distribution of GFP was examined in high magnification images, and the GFP signal was observed in the nuclei and cytosol (Fig. 2A). Difference in quantity of the two delivery methods was confirmed by Western blot. Lane 1, non-treated control; Lane 2, mock protein control (GFP and ESRRB); Lane 3, CPP-conjugated protein (R7-GFP and R7-ESRRB); Lane 4, SLO-mediated protein (GFP and ESRRB). All samples were normalized to α-Tubulin. Full-length blot images are available in Supplementary Figure 4. Relative intensities are shown for both GFP (D) and ESRRB (E). Data are presented as means ± SEM of three replicates. Different superscripts represent significant differences (p < 0.05).

In vitro cytotoxicity assay. We evaluated the cytotoxicity of the two protein delivery systems using two different assays. First, we performed a cell viability assay. Live cells were detected with calcein-AM (green signal), and dead cells were detected with ethidium homodimer-1 (red signal) (Fig. 3A). The viability of the CPP-conjugated protein delivery system was 90.0% ± 1.26 in hTSCs and 85.9% ± 1.10 in hBMSCs, compare to the control. However, the viability of the SLO-mediated protein delivery system was 84.0% ± 0.70 in hTSCs and 76.4% ± 0.85 in hBMSCs, indicating that the pore-forming toxin significantly reduces cellular viability (Fig. 3B).

Second, we investigated cell apoptosis by the TUNEL assay. The CPP-conjugated protein delivery system induced apoptosis in only a few cells, but the SLO-mediated protein delivery system induced apoptosis in over 4% and 10% of cells (Fig. 3C).

Comparison of the biological activity of ESRRB delivered cells using two different protein delivery systems. Finally, to compare the biological activity of CPP-ESRRB and SLO-mediated ESRRB delivery, hTSCs and hBMSCs were treated, and the cells were
collected 24 hr after delivery. The biological activity of ESRRB was measured as the alteration of the expression levels of pluripotency-related genes using qRT-PCR. The expression levels of OCT4, SOX2, and NANOG showed a significant increase in the CPP-ESRRB delivered cells compared with cells treated by SLO-mediated ESRRB-delivery (Fig. 4A, B). In addition, to measure biological activity of ESRRB in other way, proliferation rate of control, CPP-conjugated and SLO-mediated ESRRB delivered hTSCs and hBMSCs were calculated. The groups of CPP-ESRRB delivered hTSCs and hBMSCs showed a significantly increased proliferation rate (Fig. 4C). Also, differentiation potential was examined to detect biological activity of delivered ESRRB. All three groups of hTSCs and hBMSCs were induced in vitro differentiation into three mesodermal lineage cells; adipogenic, chondrogenic, and osteogenic cells. In adipogenic differentiation, we confirmed elevated expression level of C/EBPα and PPARγ which are adipogenic-specific markers in both CPP-conjugated and SLO-mediated ESRRB delivered cells compare to control cells. Importantly, CPP-ESRRB delivered hTSCs and hBMSCs showed significantly higher expression levels in both markers than SLO-mediated ESRRB delivered cells (Fig. 4D). In case of chondrogenic differentiation, COMP and SOX9 were used as chondrogenesis-specific markers, and CPP-ESRRB delivered hTSCs showed much higher expression level in COMP compares to control and SLO-mediated ESRRB delivered cells. Besides, hBMSCs showed significantly increased efficiency in SOX9 expression level in both CPP- and SLO-mediated ESRRB delivered cells, but significantly higher expression was detected in CPP-ESRRB delivered cells than SLO-mediated ESRRB delivered cells (Fig. 4E). However, we could not find any significant difference in osteogenesis differentiation in all groups (Fig. 4F). Collectively, delivery of the large CPP-ESRRB protein was shown to be efficient and result in measurable levels of biological activity.

Discussion
CPP has been considered as a safe, convenient, and useful tool for stem cell manipulation by introduction of exogenous protein, and applied in many research, pharmaceutical, and clinical fields. Many researchers have studied using various CPP and there were reports that oligoarginine-conjugated p53 protein can be transduced into cancer cells to inhibit their growth. However, a few studies were conducted for assessment of CPP-conjugated protein delivery efficiency. In this study, therefore, we performed a comparison assay of two different protein delivery systems using human stromal cells to evaluate the efficiency of those tools, which are CPP- and SLO-mediated protein delivery systems. We have found that the CPP-conjugated protein delivery system has a significantly higher protein uptake efficiency compared to SLO-mediated protein delivery system. Also, CPP-conjugated protein caused few cell damages while SLO showed reduction of cell viability causing apoptotic cell death. The effect of delivered CPP-conjugated protein showed better results than SLO-mediated delivered protein. The bottom line is that...
CPP-conjugated protein delivery system is regarded as a better than SLO-mediated protein delivery system. Nevertheless, although oligoarginine is a tremendous protein delivery tool, there is a limitation that individual proteins must be manipulated to link to the CPP domain. SLO is a useful bacterial toxin and a very simple method for exogenous protein delivery, but the SLO-mediated protein delivery system was shown to result in a lower protein uptake rate and higher cytotoxicity. Because the

Figure 4 | ESRRB induced expression level of pluripotency-related genes, proliferation, and in vitro differentiation efficiency. (A) Quantitative real-time RT-PCR and (B) RT-PCR analysis of pluripotency-related genes. Expression levels of OCT4, SOX2, and NANOG in hTSCs and hBMSCs were analyzed 24 hr after R7-ESRRB or SLO-mediated ESRRB delivery. Each gene was normalized to β-actin as a housekeeping control [1: ESRRB(Mock), 2: R7-ESRRB, 3: SLO(Mock), 4: SLO ESRRB]. (C) Altered proliferation rate of ESRRB delivered hTSCs and hBMSCs were calculated as fold change of population doubling number. In vitro differentiation analysis of ESRRB delivered cells was performed by quantitative real-time RT-PCR of (D) adipogenesis-specific markers C/EBPα and PPARγ; (E) chondrogenesis-specific markers COMP and SOX9; and (F) osteogenesis-specific markers COL-I and RUNX2. Data are presented as means ± SEM of four (A), six (B), three (C), and four (D–F) replicates. * p < 0.05.

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SLO-mediated protein delivery system has the advantage that individual proteins do not need to be manipulated, it has been widely used for delivery of undefined proteins, such as cellular protein extract. The two different protein delivery systems can be used for their appropriate purposes in many cases. However, protein delivery for clinical applications in stem cell research fields requires defined proteins that are known to regulate the expression of specific genes so that they can control the appropriate cellular properties. Otherwise, unwanted side effects are a considerable risk.

The characteristics of mesenchymal stem cells (MSCs) revolve around multipotency and self-renewal. So, MSCs have great potential as cell therapeutic materials considering their characteristics of self-renewal, proliferation, and differentiation into various cell types. While MSCs have been spotlighted as an attractive tool for tissue repair, they also possess the potential to maintain stemness, thus, MSCs quality was improved, thus proliferation and differentiation potential were enhanced. These results demonstrated that the expression of pluripotency-related genes can maintain the stemness of MSCs and this type of manipulation could increase efficiency of clinical application required a good quality of MSCs.

In the present study, ESRBB as a defined protein was used to regulate the expression levels of pluripotency-related genes in human mesenchymal stromal cells. ESRBB is an orphan nuclear receptor and is an important factor involved in the maintenance of self-renewal and pluripotency. It mediates the reprogramming of somatic cells to pluripotent stem cells in conjunction with OCT4 and SOX2. Here, we have shown that ESRBB protein delivery into hTSCs and hBMSCs increased the OCT4, SOX2 and NANOG expression levels. CPP-conjugated ESRBB delivery presented a more efficient uptake and a more significant increase in the expression levels of OCT4, SOX2 and NANOG than SLO-mediated ESRBB delivery. Furthermore, cytotoxicity was shown to be low in the CPP system. CPP-ESRBB delivery may be a good tool for maintenance of the hMSC populations and can thus make them more available for clinical applications. CPP-conjugated proteins can be successfully delivered in adherent cells; therefore, CPP-ESRBB can be used easily as a medium supplement. Continuous CPP-ESRBB intracellular delivery may elevate the proliferation and differentiation potential of human stromal cells. However, in the present study, a single treatment of CPP-ESRBB was performed in suspended human stromal cells because the purpose of the study was to evaluate and compare the CPP- and SLO-mediated delivery systems as potential tools in hMSCs manipulation.

Although oligoarginine is a good tool for protein delivery, it is not as effective as viral gene transfer. However, there was a recent report that protein uptake efficiency was increased using oligoarginine with pyrenebutyrate. Therefore, a safe, convenient, and selective defined protein delivery system must be developed for further clinical application in stem cell research. Our results suggest that CPP-conjugated protein delivery is an excellent tool for biologically active, defined protein delivery and may have important clinical applications for the use of stem cells in regenerative medicine.

**Methods**

**Construction of a protein expression vector and purification of proteins.** To generate pure protein for intracellular delivery, a protein expression vector was constructed as previously described. Briefly, we modified the pET-20b vector with 7 arginines (R7) at the N-terminal as the CPP sequence and 6× His-tag at the C-terminal. We removed the CPP sequence for the proteins used for SLO-mediated delivery. The gene of interest could easily be inserted into pET-20b using Ndel, XhoI, and BamHI restriction sites, which facilitate the manipulation of various open reading frames (ORFs; Fig. 1A). The constructed vectors were transformed into BL21(DE3) pLYsS competent cells (Stratagegen Inc., La Jolla, CA) and cells were cultured in LB medium and induced with 1 mM isopropyl-1-thio-β-D-galactopyranoside (IPTG, Sigma-Aldrich, St. Louis, MO). Cells were lysed with NP-10 (50 mM NaH2PO4 (Sigma Aldrich), 300 mM NaCl (Sigma Aldrich), 10 mM spermidine (Sigma Aldrich) and 2% (w/v) benzamidine (2 mg/ml, Basel, Switzerland). Proteins in the soluble supernatant were purified by His-Tagged Recombinant Protein Purification - His60 Ni Resin (ChonTech laboratories, CA, USA) according to the manufacturer’s instructions. GFP recombinant protein without the R7 domain and 6× His-tag was purchased from Aigen (agtp0302; Seoul, South Korea). The identity of the purified protein was confirmed by mass spectrometry. Brilliant Blue R520 staining and Western blotting with specific primary antibodies: anti-GFP (AB3080; Millipore, Billerica, MA) and anti-ESRBB (sc-47662; Santa Cruz Biotechnology, Santa Cruz, CA).

**Culture of hTSCs and hBMSCs.** We used the hTSCs and hBMSCs. The hTSCs were obtained from excised human testes and were isolated the CD34+/73+ cells and cultured as previously described. hTSCs have fibroblastic morphology, similar to hBMSCs (Fig. S1). The hTSCs were positive for CD34, CD73, class I major histocompatibility (MHC) antigens (HLA ABC), CD29, CD44, CD90, CD105, and CD166 and were negative for CD14, CD31, CD45, HLA DR, TRA-1-60, SSEA3, TRA-1-81, c-Kit, CD33, and CD140 (Fig. S2A). This study was approved by the Institutional Review Board of the CHA Gangnam Medical Center, Seoul, South Korea. The hBMSCs were purchased from Lonza (PT2501) and positive for CD105, CD90, CD73, CD166 (data not shown), CD29 (data not shown) and CD44 (data not shown) and were negative for CD14 (data not shown), c-Kit, CD34, CD45, HLA-DR, and SSEA4, as determined by flow cytometry (Fig. S2B). Lonza, Walkersville, MD.

The hBMSCs were expanded and cultured in DMEM/F12 (Gibco-BRL) supplemented with 10% FBS (Gibco-BRL) and 100 U/ml penicillin G, and 100 μg/ml streptomycin (Gibco-BRL). Cells were maintained in T75 flasks (Nunc, Roskilde, Denmark) at 37°C in a 5% humidified CO2 incubator and subcultured every 4–5 days. In this study, we used the cultured cells at passage 4–6.

**Direct delivery of exogenous protein into hTSCs and hBMSCs using CPP-conjugated and SLO-mediated systems.** GFP and ESRBB proteins were delivered into suspended hTSCs and hBMSCs. Cells released with 0.05% trypsin-EDTA (Hyclone) were collected and washed with Hank’s balanced salt solution (HBSS) without Ca2+ and Mg2+ (Gibco-BRL) and total 7.5 × 103 cells were collected for each experimental sample. In the case of SLO-mediated delivery, cells were left in HBSS with 230 mg SLO, for 50 min at 37°C, and tubes were inverted to mix thoroughly. Then, tubes were incubated on ice for 5 min. After centrifugation, perforated cells were collected and resuspended in 200 μl HBSS. The protein of 10 μg of GFP or ESRBB were treated for 1 hr at 37°C. After washing with HBSS, rescaling of pores was induced by the addition of 2 mM CaCl2, in 1 ml growth medium for 2 hr.

In the CPP-conjugated protein delivery system, cells were suspended in 200 μl HBSS with 10 μg of CPP-GFP or CPP-ESRBB and incubated for 1 hr at 37°C. After that, cells were washed with an acidic buffer to remove the CPP-protein that was absorbed on cell surface. Cells were washed twice for 30 sec with cold 0.2 M glycine buffer containing 500 μl of 0.15 M NaCl (pH 3.0), followed by DPBS wash. GFP protein was used for visualization of protein delivery, and we evaluated the protein transduction efficiency, intracellular protein uptake, and localization. Therefore, GFP or CPP-GFP protein-treated cells were sampled immediately and assessed. The purpose of using ESRBB was to provide a functional assay to estimate the biological effects of proteins. Thus, ESRBB or CPP-ESRBB protein-delivered cells were seeded onto 6-well dishes, and after 24 hr in culture, the cells were collected for further experiments.

**Confocal laser microscopic analysis.** After protein delivery using CPP-GFP or SLO-mediated GFP, each sample was split at a count of 1 × 106 cells and fixed in 4% paraformaldehyde (PFA). The cells were attached to glass slides by cytospin at 1000 rpm for 5 min. All samples were counterstained with 4',6-diamidino-2-phenylindole (DAPI; Sigma Aldrich). All images were captured with a Carl Zeiss LSM 510 META confocal laser-scanning microscope (Carl Zeiss, Jena, Germany). The intensity of GFP was measured using ImageJ software (NIH; http://rsb.info.nih.gov/ij).
RNA extraction, RT-PCR, and Real-time RT-PCR. To quantify expression levels of pluripotency-related genes (OCT4, SOX2, NANOG) after ESRRB delivery, real-time RT-PCR was performed. Total RNA was extracted from cells using TRIzol reagent (Invitrogen Carlsbad). First strand cDNA was synthesized with 2 µg of each RNA using a PrimeScript 1st strand cDNA synthesis kit (TakaraBio, Shiga, Japan) according to the manufacturer’s instructions. Quantitative real-time RT-PCR was performed using iQ™ SYBR Green supermix (Bio-Rad Laboratories) on a Bio-Rad IQ5 real-time PCR machine. The primer sequences for the genes are listed in supplementary table 1. Each gene was normalized to 18S rRNA as a housekeeping gene.

Proliferation assay. ESRRB was delivered into hTSCs and hBMSCs using R7-ESRRB or the SLO-mediated protocol, and then each cell line was seeded in a 24×100 mm2 well plate at a density of 2×10^4 cells/well. After 5 days, each group of cells was collected and the number of cells in each group was counted manually. Then, those cells were used to perform protein delivery and seeded onto T75 flask nick-end labeling (TUNEL) assay, The In Situ Cell Death Detection Kit, TMR red (Roche, Mannheim, Germany) was used to label DNA strand breaks with TMR red. Cells were fixed with 4% PFA and attached to glass slides by Cytospin. All images were photographed using an optical microscope (Eclipse TE-2000 U, Nikon, Tokyo, Japan).

Statistical analysis. All experiments were performed in at least three independent experiments, and the results are expressed as the mean ± standard error. Statistical analyses were performed using the one-way ANOVA test and followed by Student’s t-test if necessary. A p value of p < 0.05 was considered statistically significant.

1. Liu, Y. et al. Delivery of intact transcription factor by using self-assembled supramolecular nanoparticles. Angew Chem Int Ed Engl 50, 3058–62 (2011).
2. O’Malley, J., Woltjen, K. & Kaji, K. New strategies to generate induced pluripotent stem cells (iPSCs) and induced differentiation. Angew Chem Int Ed 50, 5038–62 (2011).
3. Venkatesan, J. K. & O’Malley, J. A. The role of the transcription factor OCT4 in the regulation of pluripotency. Cell Mol Life Sci 79, 2077–92 (2012).
4. Wada, J. S. & O’Brien, S. A. Protein transduction technology. Curr Opin Biotechnol 13, 52–6 (2002).
5. Patel, L. N., Zaro, J. L. & Shen, W. C. Cell penetrating peptides: intracellular pathways and pharmacological perspectives. Pharm Res 24, 1977–92 (2007).
6. Schwarz, S. R., Woerle, J., Veroec-Akbani, A. & Dowdy, S. F. In vivo protein transduction: delivery of a biologically active protein into the mouse. Science 285, 1569–72 (1999).
7. Wada, J. S. & Dowdy, S. F. Protein transduction technology. Curr Opin Biotechnol 13, 52–6 (2002).
8. Patel, L. N., Zaro, J. L. & Shen, W. C. Cell penetrating peptides: intracellular pathways and pharmacological perspectives. Pharm Res 24, 1977–92 (2007).
9. Astriab-Fisher, A., Sergeev, D., Fisher, M., Shaw, B. R. & Juliano, R. L. Conjugates of antisense oligonucleotides with the Tat and antenapedia cell-penetrating peptides: effects on cellular uptake, binding to target sequences, and biological actions. Pharm Res 19, 744–54 (2002).
10. Crombez, L. et al. Targeting cyclin B1 through peptide-based delivery of siRNA prevents tumour growth. Nucleic Acids Res 37, 4559–69 (2009).
11. Jarver, P. & Langel, U. Cell-penetrating peptides—a brief introduction. Biochim Biophys Acta 1758, 260–3 (2009).
12. Madani, F., Lindberg, S., Langel, U., Futaki, S. & Gárdlund, A. Mechanisms of cell-penetrating peptide uptake of cell-penetrating peptides. J Biophys 2011, 414729 (2011).
13. Jarver, P. & Langel, U. The use of cell-penetrating peptides as a tool for gene regulation. Drug Discov Today 9, 395–402 (2004).
14. Jo, J. et al. Regulation of differentiation potential of human mesenchymal stem cells by intracytoplasmic delivery of coactivator-associated arginine methyltransferase 1 protein using cell-penetrating peptide. Stem Cells 30, 1703–13 (2012).
15. Bhakdi, S. et al. Staphylococcal alpha-toxin, streptolysin-O, and Escherichia coli hemolysin: prototypes of pore-forming bacterial cytolsins. Arch Microbiol 165, 73–9 (1996).
16. Waley, L. et al. Delivery of proteins into living cells by reversible membrane permeabilization with streptolysin-O. Proc Natl Acad Sci U S A 98, 3185–90 (2001).
17. Ogino, S. et al. Observation of NMR signals from proteins introduced into living mammalian cells by reversible membrane permeabilization using a pore-forming toxin, streptolysin O. J Am Chem Soc 131, 10843–5 (2009).
18. Fu, H., Ding, J., Flatter, B. & Gao, B. Investigation of endogenous antigen processing by delivery of an intact protein into cells. J Immunol Methods 335, 90–7 (2008).
19. Cho, H. et al. Induction of pluripotent stem cells from adult somatic cells by protein-based reprogramming without genetic manipulation. Blood 116, 896–910 (2010).
20. Labovsky, V. et al. Cardiomyogenic differentiation of human bone marrow mesenchymal cells: Role of cardiac extract from neonatal rat cardiomyocytes. Differentiation 79, 93–101 (2010).
21. Inoue, M. et al. p53 protein transduction therapy: successful targeting and inhibition of the growth of the bladder cancer cells. Eur Urol 49, 161–8 (2006).
22. Takenobu, T. et al. Development of p53 protein transduction therapy using membrane-permeable peptides and the application to oral cancer cells. Mol Cancer Ther 1, 1043–9 (2002).
23. Bianco, P., Robey, P. G. & Simmons, P. J. Mesenchymal stem cells: historical context, concepts, and assays. Cell Stem Cell 2, 313–9 (2008).
24. Brooke, G. et al. Therapeutic applications of mesenchymal stromal cells. Semin Cell Dev Biol 18, 846–58 (2007).
25. Garcia-Gomez, J. L. et al. Mesenchymal stem cells: biological properties and clinical applications. Expert Opin Biol Ther 10, 1453–68 (2010).
26. Hayrick, L. The Limited in Vitro Lifetime of Human Diploid Cell Strains. Exp Cell Res 37, 614–36 (1965).
27. Banzi, A. et al. Proliferation kinetics and differentiation potential of ex vivo expanded human bone marrow stromal cells: Implications for their use in cell therapy. Exp Cell Res 314, 1075–85 (2008).
28. Baxter, M. A. et al. Study of telomere length reveals rapid aging of human marrow stromal cells following in vitro expansion. Stem Cells 22, 673–82 (2004).
29. Go, M. J., Takenaka, C. & Obgushi, H. Forced expression of Sox2 or Nanog in human bone marrow derived mesenchymal stem cells maintains their expansion and differentiation capabilities. Exp Cell Res 314, 1147–54 (2008).
30. Liu, T. M. et al. Effects of ectopic Nanog and Oct4 overexpression on mesenchymal stem cells. Stem Cells Dev 18, 1013–22 (2009).
31. Han, J. et al. Nanog reverses the effects of organismal aging on mesenchymal stem cell proliferation and myogenic differentiation potential. Stem Cells 30, 2746–59 (2012).
32. Feng, B. et al. Reprogramming of fibroblasts into induced pluripotent stem cells with orphan nuclear receptor Zebb. Nat Cell Biol 11, 197–203 (2009).
33. Hitsuda, T. et al. A protein transduction method using oligo-arginine (3R) for the delivery of transcription factors into cell nuclei. Biomaterials 33, 4665–72 (2012).
34. Choi, W. Y. et al. Isolation and characterization of novel, highly proliferative human CD34/CD73-double positive testis-derived stem cells. Stem Cells Dev 20, 2746–59 (2011).
35. Kaneyama, S. et al. Acid wash in determining cellular uptake of Fab/cell-permeating peptide conjugates. Biopolymers 88, 98–107 (2007).
36. Livak, K. J., & Schmittgen, T. D. Analysis of relative gene expression data using the 2(-Delta Delta C(T)) Method. Methods 25, 402–8 (2001).
Author contributions
J.J. and D.L. conceived and designed the experiments. J.J., S.H. and W.C. performed experiments and analyzed the results. J.J., S.H. and D.L. discussed the results and wrote the manuscript. D.L. advised the experiments and revised manuscript.

Additional information
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