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DNA-binding Cell-penetrating Peptide-based TRAIL Over-expression in Adipose Tissue-derived Mesenchymal Stem Cells Inhibits Glioma U251MG Growth

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Abstract. *Background/Aim: Genetic manipulation of stem cells using non-viral vectors is still limited due to low transfection efficiency. We investigated whether the DNA-binding cell-permeation peptides (CPP) can enhance the transfection efficiency of non-viral vectors in adipose tissue-derived mesenchymal stem cells (ASCs) and whether ASCs over-expressing TRAIL through CPP can inhibit the growth of glioma U251MG cells in vitro and in vivo. Materials and Methods: ASCs were genetically engineered to over-express TRAIL by using CPP, pCMV3-TRAIL and lipid-based transfection reagents (X-tremeGENE). Results: The transfection efficiency of ASCs increased by approximately 7% using CPP; 53.9% of ASCs were transfected and TRAIL expression in ASCs increased by approximately 3 times compared to X-tremeGENE alone. ASCs over-expressing TRAIL using CPP inhibited growth of glioma U251MG cells both in vitro and in the U251MG xenograft model. Conclusion: CPP can be used as an enhancer for genetically manipulating ASCs and tumor treatment.*

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As a pro-apoptotic gene, tumor necrosis factor (*TNF*)-related apoptosis-inducing ligand (*TRAIL*) induces tumor- or infected cell-specific apoptosis through interaction with two agonistic TRAIL receptors, TRAIL-R1/DR4 and TRAIL-R2/DR5 (1, 2). Although TRAIL is effective in tumor regression, including colorectal cancer (3), glioblastoma (4), and NSCLC (5), the short half-life of TRAIL in the plasma due to rapid clearance by the kidneys shows poor bioavailability (6).

Mesenchymal stem cells (MSCs) have tumor tropism and can act as a double-edged sword that promotes or suppresses tumor growth (7, 8). Owing to the tumor tropism of MSCs, they have been used as a vehicle to deliver anticancer drugs, including cancer therapy genes (*e.g.* suicide genes, cytokines, and pro-apoptotic genes) for cancer treatment (9). Moreover, MSCs as a vehicle increase expression of carried genes locally in the tumor microenvironment. MSCs expressing TRAIL have been applied to several preclinical models of sarcoma (10), lung cancer metastasis (11), renal cancer (12), colorectal cancer (13), lymphomas (14), and breast cancer (15).

Viral or non-viral vector systems are used for the genetic engineering of MSCs (16). Retrovirus, lentivirus, adenovirus, and adeno-associated viruses have been extensively used as viral vectors for gene delivery into MSCs (16-18). Retroviral or lentiviral vectors, which are integrating viral vectors, can be used to correct genetic pathology or to continuously express target genes throughout the patient's lifespan, but adenovirus can be used for non-inherited diseases or diseases

requiring the transient expression of therapeutic genes (19). Viral vectors have high gene transfer efficiency, but have problems related to potential immunogenicity and insertional mutagenesis (18, 20). Single or combinations of cationic lipids, surfactants, peptides, polysaccharides, nanoparticles (gold, magnetic iron), and synthetic polymers have been used for genetic manipulation when using non-viral vectors (21, 22). Non-viral vectors are preferable for expressing the therapeutic gene for a short period, but transfection efficiency using such vectors is lower than that of viral vectors (23).

Previously, we reported that adipose tissue-derived mesenchymal stem cells (ASCs) cultured at high density, express TRAIL and inhibit the growth of several cancer cell lines, including Huh7, H460, MCF-7, and MDA-MB-231 (24-26). However, the tumor-suppressive capability was insignificant in an H460 xenograft model (25). Therefore, we investigated whether the DNA-binding cell permeation peptide (CPP) can enhance the transfection efficiency of non-viral vectors in ASCs and whether ASCs over-expressing TRAIL (ASC-TRAIL) through CPP can inhibit the growth of glioma U251MG *in vitro* and *in vivo*.

Materials and Methods

Cell culture. This study was approved by the Institutional Review Board of Yonsei University Wonju College of Medicine (CR319048). Human adipose tissues were obtained from three healthy donors (24-38 years of age) who provided written informed consent through elective liposuction procedures under anesthesia at the Wonju Severance Christian Hospital (Wonju, Republic of Korea). The adipose tissue-derived mesenchymal stem cells (ASCs) were isolated using a modified protocol as described by Zuk *et al*. (27). Briefly, after removing the contaminated blood cells and local anesthetics with phosphate-buffered saline (PBS) washing, the adipose tissues were digested with 0.075% type IA collagenase (Sigma-Aldrich, St. Louis, MO, USA) in PBS to obtain mononuclear cells. The mononuclear cells (5×10^6) were seeded in 100 mm culture dishes with low-glucose Dulbecco's Modified Eagle's Medium (DMEM; Gibco, Rockville, MD, USA) containing 10% fetal bovine serum (FBS; Gibco) and penicillin/streptomycin. After 2 days, the non-adherent cells were removed by changing the media. The ASCs at passage 3 to 5 were used for this experiment. The human glioma cell line U251MG was purchased from the Korean Cell Line Bank (Seoul, Republic of Korea). The U251MG cells were maintained in DMEM (Gibco) supplemented with 10% FBS and penicillin/streptomycin. The U251MG and ASCs were indirectly co-cultured using a Transwell plate.

Synthesis of CPP and analysis of its binding to the supercoiled plasmid. The DNA-binding CPP was ordered from Peptron, Inc. (Daejeon, Korea). Briefly, CPP peptides were synthesized by Fmoc solid-phase peptide synthesis using ASP48S (Peptron) and purified by the reverse phase Prominence HPLC (Shimadzu, Kyoto, Japan) using a Vydac Everest C18 column (250 mm × 22 mm, 10 μm, HiChrom, Berkshire, UK). Elution was carried out with a water-acetonitrile linear-gradient (10-75% (v/v) of acetonitrile) containing 0.1% (v/v) trifluoroacetic acid. Molecular weights of the purified peptides were confirmed using LC/MS (Shimadzu). To confirm the binding affinity of CPP with supercoiled plasmids, 1 μg of pCMV3-GFP (SinoBiological, Wayne, PA, USA) and CPP (0-10 μg) were incubated in 10 μl of DNA binding buffer (50 mM Tris-HCl, pH 7.5, 10 mM $MgCl_2$, 250 mM KCl) for 15 min at room temperature (20 to 25˚C). The complex of pCMV3-GFP with CPP was loaded in 1% agarose gel, separated by electrophoresis in TAE buffer (40 mM Tris-HCl, 20 mM acetic acid, 1 mM EDTA), and visualized using a ChemiDoc XRS+ system (Bio-Rad, Hercules, CA, USA).

ASC transfection. The X-tremeGENE HP DNA transfection reagents (Roche, Basel, Switzerland) were used to transfect ASCs with pCMV3-TRAIL (SinoBiological). Briefly, ASCs were seeded in a 100 mm culture dish at a density of 5.2×10^5 cells and cultured for 24 h. About 10 μg of pCMV3-TRAIL were diluted with 1 ml of serum-free DMEM and incubated with 20 μl of X-tremeGENE HP reagents with or without 5 μg of CPP for 20 min at room temperature (20 to 25˚C). Then, the transfection complex was added dropwise to the cells. The cells were incubated for 3 days to express TRAIL and the conditioned medium (CM) was recovered by centrifugation and then stored at –80˚C until further analysis. The transfection efficiency with X-tremeGENE HP reagents with or without CPP was determined by using pCMV3-GFP (SinoBiological) and the GFP-positive cells were evaluated using a flow cytometer.

MTT assay. The U251MG cells were plated at 2×10^4 cells/cm^2 in 96-well plates. After 24 h, the U251MG cells were treated with CM obtained from ASC and ASC-TRAIL cultures for an additional 48 h, and then 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, Sigma) dissolved in phosphate-buffered saline (PBS) was added to each well (final concentration, 5 mg/ml) and incubated at 37˚C for 2 h at room temperature (20-25˚C). The MTT formazan was dissolved in 100 μl DMSO and incubated for a further 15 min with shaking before the optical density of each well was read at 570 nm using a microplate reader (Molecular Devices, San Jose, CA, USA).

Immunoblotting. The cells were lysed in sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer (62.5 mM Tris-HCl, pH 6.8; 1% SDS; 10% glycerol; and 5% β-mercaptoethanol). Protein samples were boiled for 5 min, subjected to SDS-PAGE, and transferred to an immobilon polyvinylidene difluoride membrane (Millipore, Billerica, MA, USA). The membrane was blocked with 5% skim milk in Tris-HCl buffered saline containing 0.05% Tween 20 and then incubated with primary antibodies against caspase 3, cleaved caspase 3, and PARP (1:2,000, Cell Signaling Technology, Danvers, MA, USA), TRAIL (1:1,000, R&D Systems, Minneapolis, MN, USA), and GAPDH (1:1,000, Santa Cruz Biotechnology, Dallas, TX, USA). This was followed by treatment with peroxidase-conjugated secondary antibodies (1:2,000, Santa Cruz Biotechnology). The membrane was treated with EZ-Western Lumi Pico or Femto (DOGEN, Seoul, Republic of Korea) and visualized using a ChemiDoc XRS+ system (Bio-Rad).

Apoptosis assay. The phycoerythrin (PE)-Annexin-V apoptosis detection kit I (BD Biosciences, San Jose, CA, USA) was used according to the manufacturer's instructions. The cells were harvested, washed twice with cold PBS, and resuspended in binding buffer. Cells were stained with

PE-Annexin-V and 7-aminoactinomycin D (7-AAD) for 15 min at room temperature (20-25˚C) in the dark. The cells were then analyzed without washing on a flow cytometer (BD FACSAria III) within 1 h.

Animal studies. All the animal experiments were performed according to institutional guidelines and approved by the Institutional Animal Care and Use Committee of Yonsei University Mirae Campus at Wonju (YWCI-202012-N-006). Five-week-old athymic nude mice were purchased from Central Lab Animal Inc. (Seoul, Republic of Korea). Before transplantation, the U251MG or ASCs were washed with PBS thrice, once with Hank's Balanced Salt Solution (HBSS; Sigma-Aldrich), and then resuspended in HBSS for transplantation into the nude mice. The U251MG (2×10^6 cells/mice) and ASCs (2×10^6 cells/mice) were mixed just prior to co-injection. The cells were suspended in 100 μl HBSS and subcutaneously injected into the flanks of nude mice. The sham groups were injected only with U251MG (2×10^6 cells/mice). Three weeks later, mice were euthanized in a CO_2 chamber; thereafter, the tumors were excised and photographed, and the tumor weights were measured using an electronic balance.

Statistical analysis. Data are presented as the mean±standard deviation (SD) or standard error (SE) of the mean. To compare the group means, the Student's *t*-test and one-way analysis of variance were performed, followed by Scheffe's test. A p-value <0.05 was considered significant.

Results

Binding affinity of CPP for pCMV3-GFP. The DNA-binding CPP peptides are composed of 24 amino acids with a cell-penetrating domain, a linker, and a DNA-binding domain (Figure 1A). To confirm that the synthesized CPP bind to the pCMV3-GFP plasmid, 1 μg of pCMV3-GFP was incubated with different doses of CPP (0-10 μg) for 15 min. The supercoiled pCMV3-GFP plasmid (SC, 1 μg) almost disappeared because it bound with 1 μg of CPP for 15 min and then retarded in the well (Figure 1B). To determine the time required for the optimal binding of CPP and pCMV3-GFP, 1 μg of pCMV3-GFP and 0.5 μg CPP were incubated for 0-2 h. About 50% of the supercoiled pCMV3-GFP disappeared after 15 min and SC was rarely observed after 30 min (Figure 1C). Therefore, it was confirmed that 1 μg of pCMV3-GFP could bind to 1 μg of synthesized DNA-binding CPP within 15 min, suggesting that the synthesized CPP has proper DNA-binding capability.

Enhancement of the transfection efficiency of ASCs with plasmid vectors by DNA-binding CPP. Since the transfection efficiency using a non-viral vector is reported to be low in MSCs (0-40%) (28), we investigated whether CPP can increase the transfection efficiency of non-viral vectors in ASCs. First, the expression levels of TRAIL were determined in ASCs, which were transfected with pCMV3-TRAIL, X-tremeGENE HP transfection reagents, and/or CPP. In the presence of CPP, ASCs over-expressed TRAIL (ASC-TRAIL) and the amount of TRAIL expression increased by more than 2.2 times; and when 1 μg of CPP per 2 μg of pCMV3-TRAIL was used, the expression of TRAIL increased the most by 3 times (Figure 2A). In ASC-TRAIL, TRAIL secreted into the culture medium was also increased in the presence of CPP, and when 1 μg of CPP was used, secreted TRAIL was increased by about 37.6% and the amount of secreted TRAIL was 754.21±15.35 pg/ml compared to the control at 548.51±12.22 pg/ml (Figure 2B). In addition, flow cytometry analysis indicated that the transfection efficiency of the pCMV3-GFP in the presence of CPP was increased by about 7%, from 53.88±5.01% to 46.71±3.19% in the case of using only X-tremeGENE (Figure 2C). Therefore, when CPP is used together with X-tremeGENE HP and a non-viral vector, it is possible to further increase gene expression by increasing the transfection efficiency of ASCs.

Cytotoxicity of U251MG by ASC-TRAIL. To determine whether ASC-TRAIL exhibit cytotoxicity against U251MG glioma cells, these cells were incubated for 2 days with conditioned medium (CM) obtained from ASC or ASC-TRAIL. CM of ASC and ASC-TRAIL induced approximately 5.78±0.03% and 29.27±0.03% cytotoxicity in U251MG cells, respectively (Figure 3A). In addition, CM of ASCs and ASC-TRAIL induced cleavage of caspase 3 and PARP in U251MG cells, and the levels of cleaved caspase 3 and PARP were higher in U251MG cells treated with CM from ASC-TRAIL than those of cells treated with CM from ASCs (Figure 3B).

Next, we analyzed the death of U251MG cells following indirect co-culture with ASC or ASC-TRAIL cells. In indirect co-culture, ASCs reduced apoptosis of U251MG cells, while ASC-TRAIL increased the rate of death in U251MG cells (Figure 3C). These results suggest that ASC-TRAIL can exhibit anti-cancer effects by inducing apoptosis of U251MG cells.

Anti-tumor effects of ASC-TRAIL in the xenograft animal model. Although ASCs cultured at high density are able to express TRAIL and induce cell death in H460 lung cancer cells *in vitro*, insignificant inhibition of tumor growth was observed in a xenograft animal model (25). To investigate whether ASC-TRAIL transfected using CPP show antitumor activities, nude mice (n=5, each) were injected with U251MG only, U251MG+ASC, or U251MG+ASC-TRAIL subcutaneously. After cell transplantation, the increase in tumor size was very slow in both U251MG only and U251MG+ASC-TRAIL groups, compared to the U251MG+ASC group. On the 21st day after transplantation, the nude mice were euthanized, and the tumor weight was measured (Figure 4). The weight in the U251MG and U251MG+ASC groups was 0.17±0.04 gram and 0.60±0.09 gram; the latter was about 353% heavier than the former. However, the weight in the U251MG+ASC-TRAIL group was 0.03±0.03 grams, which was 15% of that in the

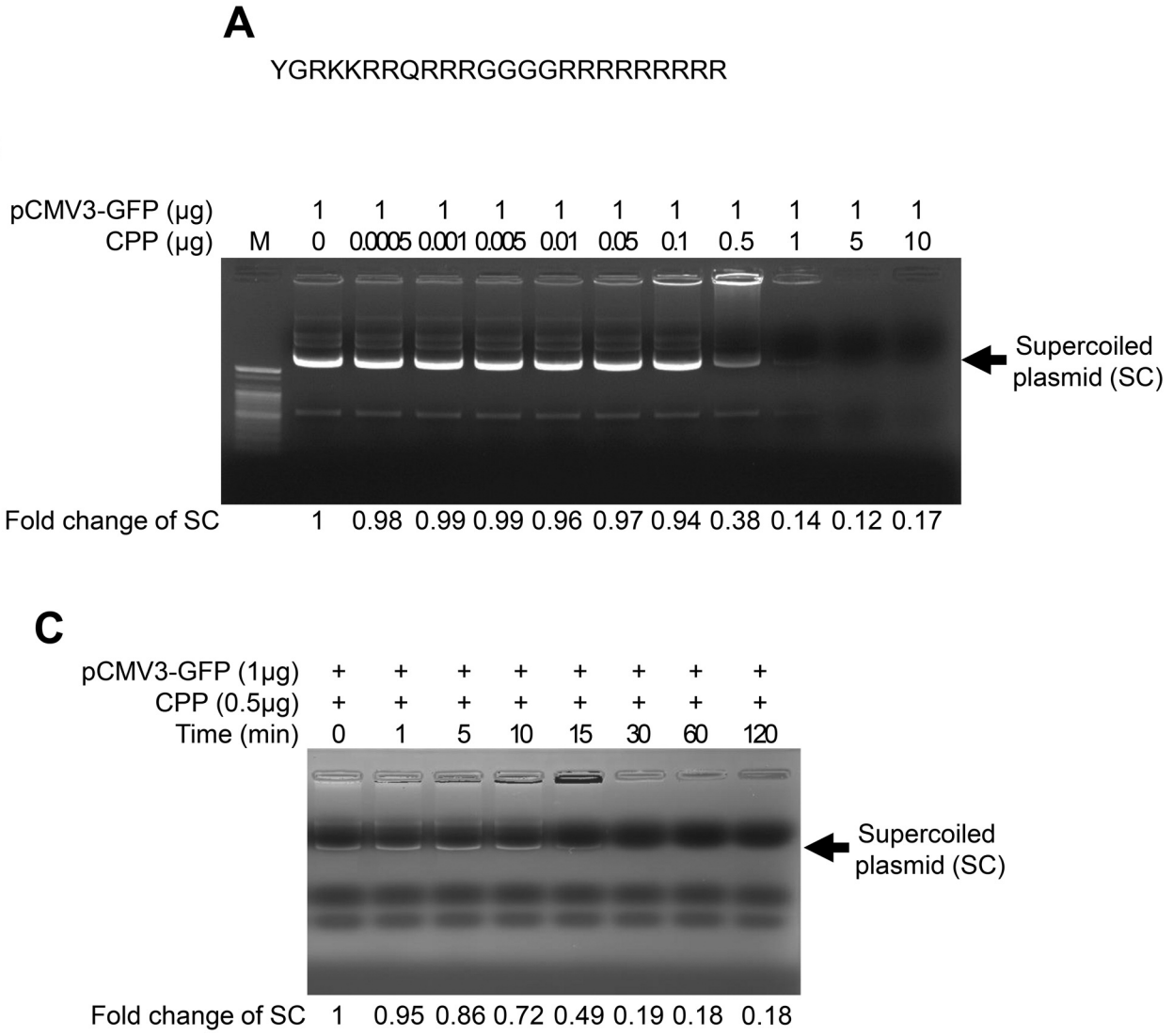

Figure 1. *The binding affinity of cell-permeation peptides (CPP) for pCMV3-GFP. (A) Amino acid sequences of CPP. (B and C) Binding affinity of CPP and pCMV3-GFP. One μg of pCMV3-GFP was incubated with CPP (0-10 μg) for 15 min in 10 μl of DNA binding buffer. The binding affinity was judged by the degree of reduction in a supercoiled plasmid (SC). One microgram pCMV3-GFP bound to 1 μg of CPP by about 86% affinity within 15 min (B). The time required for optimal binding was determined as the time that SC decreased by 50% after incubating 1 μg of pCMV3-GFP with 0.5 μg of CPP (C).*

sham control (Figure 4B). Therefore, the naive ASCs can promote tumor growth, whereas ASC-TRAIL can inhibit tumor growth in the U251MG xenograft model, suggesting the use of ASC-TRAIL in tumor treatment.

Discussion

Due to the low efficiency (0-40%) of the prevalent transfection methods using non-viral vectors in MSCs, viral vectors (high transfection efficiency, >80%) are widely used to genetically engineer MSCs in preclinical and clinical studies. However, although viral vectors have high gene transfer efficiency and enable stable expression, they have potential immunogenicity and insertional mutagenesis problems (18, 20). For the genetic modification of MSCs using non-viral vectors, electroporation, cationic liposomes, and polymers have shown transfection efficiency of about 40%, 2-35%, and 25-40%, respectively, which is lower than that of viral vectors (>80%). Human ASCs could be transfected with high efficiency (>80%) by using linear polyethyleneimine and enhancers, which are a mixture of histone deacetylase 6 inhibitor and fusogenic lipids (29). Moreover, solid gold

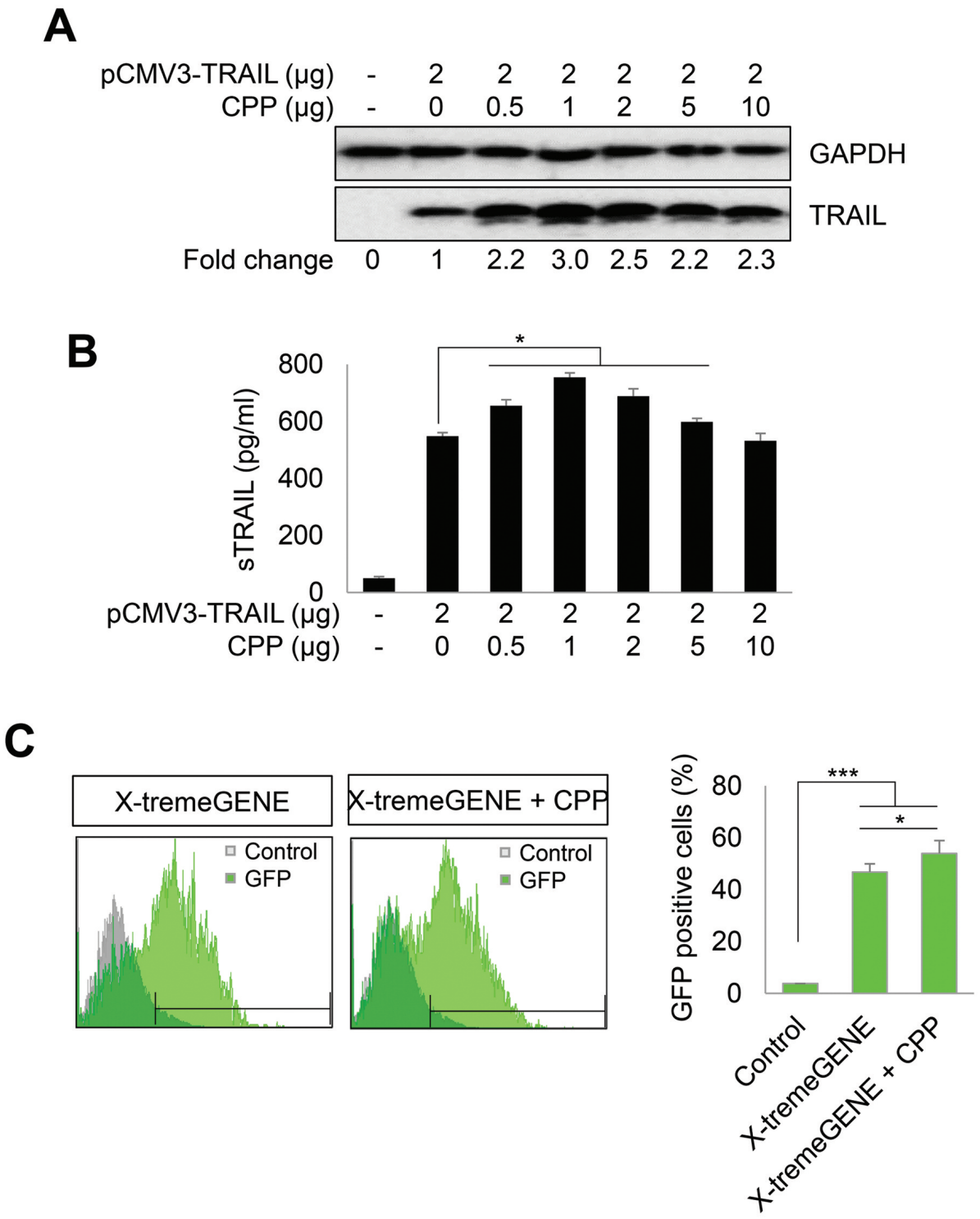

Figure 2. *TRAIL expression in adipose tissue-derived mesenchymal stem cells (ASCs) and transfection efficiency in the presence of cell-permeation peptides (CPP). (A) TRAIL expression in ASC-TRAIL. ASCs were transfected with pCMV3-TRAIL and CPP for 3 days and then TRAIL expression was detected by immunoblotting. (B) TRAIL secretion levels of ASC-TRAIL. The ASC-TRAIL was transfected in the presence of different doses of CPP (0-10 μg) for 3 days and then soluble TRAIL was analyzed in the conditioned medium (CM) by ELISA. The data are expressed as the mean±SD from three independent experiments. *p≤0.05. (C) The transfection efficiency in ASCs treated with the pCMV3-GFP and/or CPP. The rate of green fluorescence protein (GFP)-positive cells was analyzed by flow cytometry. The data are expressed as the mean±SD from three independent experiments. *p≤0.05; ***p≤0.001.*

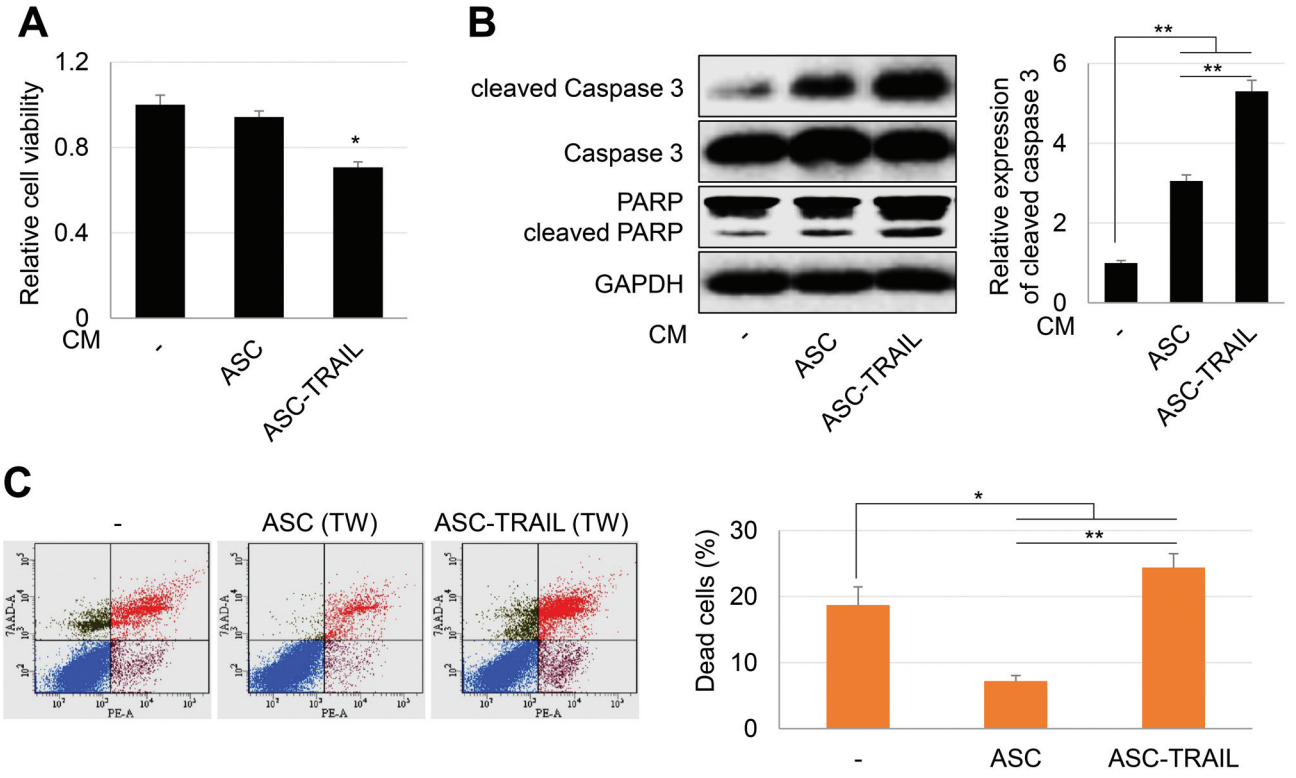

Figure 3. *Death of U251MG cells by adipose tissue-derived mesenchymal stem cell (ASC)-TRAIL and its conditioned medium (CM). (A) Viability of U251MG cells exposed to CM obtained from ASC and ASC-TRAIL. U251MG cells were cultured with CM obtained from ASC and ASC-TRAIL for 2 days and then cell viability was determined by the MTT assay. Data are shown as the mean±SD from four independent experiments. *p≤0.05. (B) Caspase 3 activation by CM obtained from ASC and ASC-TRAIL. U251MG cells were treated with CM from ASC and ASC-TRAIL for 2 days and cleaved caspase 3 levels in U251MG cells were detected by immunoblotting. Data are shown as the mean±SD from three independent experiments. **p≤0.01. (C) Death of U251MG cells indirectly co-cultured with ASC and ASC-TRAIL. After indirect co-culture for 2 days, U251MG cells were stained with PE-annexin-V and 7-AAD to detect the dead cell populations. The dead cell population was the sum of annexin-V-positive/7-AAD-negative, annexin-V-positive/7-AAD-positive, and annexin-V-negative/7-AAD-positive cells. Data are shown as the mean±SD from three independent experiments. *p≤0.05; **p≤0.01.*

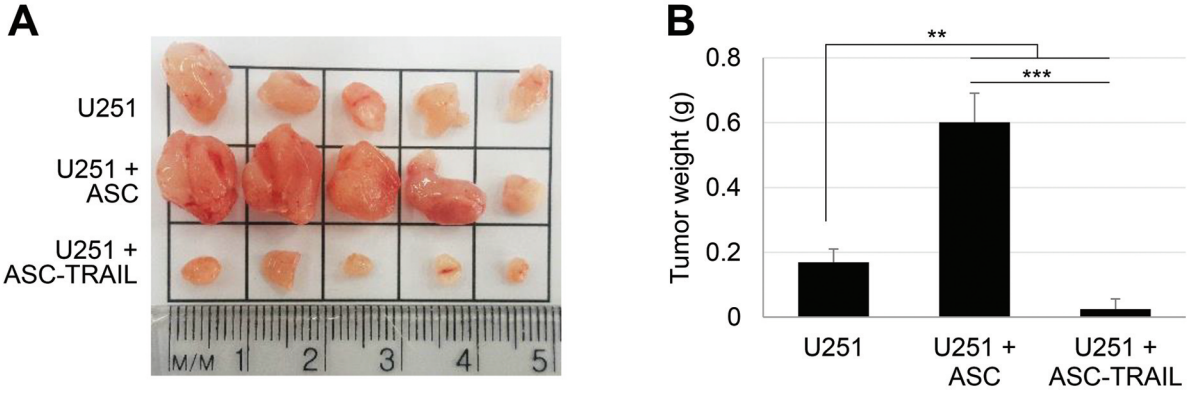

Figure 4. *Anti-tumor effects of TRAIL-overexpressing adipose tissue-derived mesenchymal stem cells (ASCs) in xenograft animal models. (A) Morphology of tumor mass. At 3 weeks after injection of U251MG and/or ASCs, mice (n=5) were euthanized in a CO_2 chamber and the tumor mass was separated and photographed. (B) Tumor weight at 3 weeks after injection with ASCs. Tumor weight was measured using an electronic balance. Data are shown as the mean±SE. **p≤0.01; ***p≤0.001 (n=5).*

nanoparticles and the pentapeptide Ku70 have been shown to enhance cellular uptake and expression of the target gene with transfection efficiency of more than 80% (30).

We observed that with CPP as an enhancer, the transfection efficiency of X-tremeGENE increased up to 53.9%, TRAIL expression increased by 3 folds, and ASC-TRAIL could inhibit tumor growth *in vitro* and *in vitro*. Transfection efficiency of our system was about 25% lower than that of Ho and Murpski's method (29, 30). However, compared to X-tremeGENE alone, CPP with X-tremeGENE increased TRAIL expression in ASCs by more than 3 times and TRAIL secretion by about 37.6%.

The genetic engineering methods of MSCs using a non-viral vector must not only have high transfection efficiency but also not cause toxicity to MSCs. Electroporation, a method of relatively high MSC transfection efficiency (40%) of non-viral vectors, greatly reduces the viability of MSCs (<50% viable) (31). Compared to cancer cell lines, MSCs proliferate very slowly. Therefore, the transfection method should not affect MSC viability. We confirmed that when CPP was used together with lipofectamine 2000 (Invitrogen, Eugene, OR, USA), PolyMag magnetofection reagent (OZ Biosciences, Marseille, France), and CombiMag transfection reagent (OZ Biosciences), the expression of the target gene could be increased dramatically. More importantly, when CPP was used together with the above transfection reagents, cytotoxicity in MSCs was rarely observed (Data not shown). In the U251MG xenograft model, ASC-TRAIL reduced tumor mass, but ASC increased tumor mass significantly. In conclusion, CPP can be used as an enhancer for non-viral genetic manipulation of ASCs that can be applied to clinical studies for cancer treatment.

Conflicts of Interest

The Authors declare no conflicts of interest regarding this study.

Authors' Contributions

Study concepts and study design, data analysis and interpretation, quality control of data and algorithms: Jaesik Shin, Yongdae Yoon, Soon Koo Baik, Kum Whang and Young Woo Eom. Data acquisition: Yongdae Yoon, Soonjae Hwang, Joon Hyung Sohn, Minjeong Jo and Woo-Seung Kim. Investigation: Jaesik Shin, Yongdae Yoon and Young Woo Eom. Statistical analysis, visualization of the results: Soon Koo Baik, Soonjae Hwang and Ki-Jong Rhee. Manuscript preparation: Young Woo Eom. Manuscript editing and review: Jaesik Shin, Ki-Jong Rhee, Kum Whang and Young Woo Eom. All Authors approved the manuscript.

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