

## Research Article

# Enhanced inhibition of tumor growth using TRAIL-overexpressing adipose-derived stem cells in combination with the chemotherapeutic agent CPT-11 in castration-resistant prostate cancer

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## ABSTRACT

**Background:** This study investigated the inhibition of tumor growth in castrate-resistant prostate cancer (CRPC)-bearing mice by tumor necrosis factor–related apoptosis-inducing ligand (TRAIL)-overexpressing adipose-derived stem cells (ADSCs) (hTERT-ADSC.sTRAIL), which was enhanced by combined treatment with CPT-11.

**Materials and methods:** An hTERT-ADSC.sTRAIL cell line was established by transfection with a lentiviral vector (CLV-Ubic) encoding the human sTRAIL gene. Quantitative polymerase chain reaction and Western blots were performed to confirm gene overexpression. An invasion study for the selective migration ability toward PC3 cells was performed. In the *in vivo* study, the tumor volume in mice treated with ADSC.sTRAIL and CPT-11 was measured.

**Results:** Carboxylesterase was generated from hTERT-ADSCs. The gene expression of sTRAIL from hTERT-ADSC.sTRAIL was shown. The directional migration of ADSC.sTRAIL cells toward PC3 cells was significantly stimulated by PC3 cells *in vitro* ( $P < 0.05$ ). In the *in vitro* study, the viability of PC3 cells significantly decreased in the presence of ADSC.sTRAIL ( $62.7 \pm 2.0\%$ ) and CPT-11 compared with that of CPT-11 alone ( $83.0 \pm 1.0\%$ ) at a cell ratio as low as 0.05 (PC3: ADSC.sTRAIL) ( $P < 0.05$ ). The proportion of apoptotic PC3 cells significantly increased in the presence of ADSC.sTRAIL ( $37.2 \pm 2.1\%$ ) and CPT-11 compared with that of CPT-11 alone ( $16.5 \pm 1.0\%$ ) ( $P < 0.05$ ). In the *in vivo* study, the inhibition of tumor growth in CRPC-bearing mice by TRAIL-overexpressing adipose stem cells was enhanced by combined treatment with the chemotherapeutic agent CPT-11 compared with that in the treatment with CPT-11 alone. Immunohistochemical staining of the removed tumors showed anti-TRAIL–positive cells and apoptotic bodies after hTERT-ADSC.sTRAIL treatment or combined treatment with hTERT-ADSC.sTRAIL and CPT-11.

**Conclusions:** Therapeutic stem cells expressing sTRAIL genes combined with CPT-11 can provide a new strategy for treating CRPC in clinical trials using the patients' own ADSCs.

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## 1. Introduction

Prostate cancer (PC) is one of the most common cancers in men and is the second most common cause of death among all cancers(1). The current limitations in treating PC depend on the

treatment of castrate-resistant prostate cancer (CRPC). Tumor necrosis factor–related apoptosis-inducing ligand (TRAIL) induces apoptosis selectively in a variety of tumor cells with minimal effects on normal cells and tissues, suggesting it as an attractive cancer therapeutic candidate(2, 3). TRAIL and its death receptors have been introduced as targets for the development of tumoricidal agents since the 1990s(4).

However, most localized cancers, including PC and CRPC cell lines, PC3 and DU145(5), are resistant to TRAIL-induced apoptosis. To overcome this limitation of TRAIL-induced apoptosis, research has focused on developing compounds or combinations to improve

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TRAIL susceptibility(6). Recently, cell therapy has been used in many studies, especially stem cells in combination with TRAIL(7).

Because stem cells (SCs) migrate toward cancer, the introduction of genes into SCs is an attractive cell-based delivery strategy(8, 9). The homing and migration of SCs into tumors is well-known and has been demonstrated by many experimental studies(10). Our previous study also showed the efficacy of SCs as an effective PC treatment(11).

Adipose-derived SCs (ADSCs) have the potential for expanded applications because they are easy to obtain without ethical issues(12). The patient's own ADSCs can be obtained easily in their undifferentiated state without inducing an immune response, and using the patient's own ADSCs prevents immune rejection. Human ADSCs can be transduced with therapeutic genes with high efficiency and rapidly expanded to numbers required for therapeutic applications(13).

Recently, the chemotherapeutic agent CPT-11 (irinotecan), a derivative of the natural alkaloid camptothecin, was shown to enhance TRAIL-induced apoptosis in several cancer cell lines, including prostate, breast, and colon(4, 14). Combined treatment with TRAIL and CPT-11 showed enhanced apoptosis in PC cells by regulating BCL-2 family proteins and activating caspases(14). CPT-11 is activated by human liver carboxylesterase to generate SN-38 (7-ethyl-10-hydroxy-camptothecin), a topoisomerase I inhibitor that is 100- to 1000-fold more potent than irinotecan *in vitro* and *in vivo* (15).

To overcome the previously known limitation of TRAIL resistance in PC treatment, this study innovated the strategy of treating with both ADSCs and CPT-11. This study was performed to investigate the inhibition of tumor growth in CRPC-bearing mice by TRAIL-overexpressing ADSCs in combination with the chemotherapeutic agent CPT-11. The cytotoxicity of the combined treatment was also investigated.

## 2. Materials and Methods

The general study design and methods were executed similar to our previous study protocol(11).

### 2.1. Cell culture

An ASC52TELO, hTERT-ADSC (ATCC® SCRC-4000™, ATCC, Manassas, VA, USA) cell line was purchased and cultured in Dulbecco's modified Eagle medium (DMEM) (GibcoBRL, Grand Island, NY, USA) supplemented with 2 mM L-glutamine, 100 U/mL penicillin, 100 µg/mL streptomycin, and 10% heat-inactivated fetal bovine serum (FBS, GibcoBRL).

The PC3 PC mouse cell line (Korean Cell Line Bank, Seoul, Korea) was cultured in DMEM supplemented with 2 mM L-glutamine, 100 U/mL penicillin, 100 µg/mL streptomycin, and 10% heat-inactivated FBS (GibcoBRL). All of the cells were propagated in 5% CO<sub>2</sub> and 95% air at 37 °C in a humidified incubator and routinely passaged via trypsinization.

### 2.2. Generation of hTERT-ADSC.TRAIL lines

Recombinant lentivirus was generated from CLV-Ubic/sTRAIL containing the entire coding sequence of the rabbit CE gene. Briefly, transfection was completed via calcium phosphate coprecipitation. The medium was exchanged on the next day, and the supernatant was harvested 16 to 20 h later, which served as the recombinant lentivirus stock. The cells were infected with the aforementioned retroviruses at 37 °C for 4 to 6 h in the medium containing 8 µg/mL of polybrene (Sigma-Aldrich, St. Louis, MO, USA). The medium was replaced with fresh virus-free medium, and the cells were

incubated for two days at 37 °C. The infected cells were selected by the addition of puromycin (Sigma) to a final concentration of 3 µg/mL for two weeks, and the puromycin-resistant cells were expanded for subsequent experiments. The hTERT-ADSC.sTRAIL cell line encoding the human TRAIL gene was established.

### 2.3. Analysis of hTERT-ADSC.CE

#### 2.3.1. Reverse transcription-polymerase chain reaction -PCR

Total RNA was extracted from the selected cell clones using the TRIzol reagent (GIBCO). Reverse transcription (RT) was performed using the total RNA and random primers. RT-polymerase chain reaction (PCR) was performed using the following primers: forward: 5'- ATGGTGATTGTCATAGTGCTCC -3'; reverse: 5'- GCAAG-CAGGGTCTGTCAAGA-3'. GAPDH controls were used to confirm equal protein loading. The amplification program was as follows: a denaturation step of 3 min, followed by 35 cycles at 95 °C for 1 min, 63 °C for 1 min, and 72 °C for 1 min. The amplification products were examined by electrophoresis in 1.2% agarose 1X TAE gels with ethidium bromide staining.

#### 2.3.2. Western blot analysis

The total proteins were extracted from the cell lysates using RIPA lysis buffer (Thermo Fisher Scientific, Waltham, MA, USA), followed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis to isolate the proteins. The isolated proteins were then transferred to polyvinylidene fluoride membranes (Millipore, Billerica, MA, USA) for Western blotting. Five percent skim milk was used to block the membranes, followed by incubation with specific primary antibodies against TRAIL (Abcam, Cambridge, UK) and β-actin (Santa Cruz Biotechnology, Dallas, TX, USA). Later, peroxidase-conjugated secondary antibodies (Santa Cruz Biotechnology) were used to target the proteins on the membrane. The bands were analyzed following their visualization using enhanced chemiluminescence reagent (Amersham Biosciences, Little Chalfont, Buckinghamshire, UK).

#### 2.3.3. Flow cytometry analysis

A Cyflow Cube 8 FACS instrument (SysmexPartec, Görlitz, Germany) was used for flow cytometry analysis, and the data were analyzed using standard FSC Express software (De Novo Software, Los Angeles, CA, USA). The hTERT-ADSC and hTERT-ADSC cells were subjected to flow cytometry analysis using anti-CD29 (OriGene Technologies, Rockville, MD, USA), CD 34 (Invitrogen, Carlsbad, CA, USA), CD 45 (LDBIO, Seattle, WA, USA), CD 90 (OriGene Technologies, Rockville, MD, USA), and CD 105 (OriGene Technologies, Rockville, MD, USA) antibodies. A two-color flow cytometry system (BD FACS Canto II; Becton-Dickinson, Franklin Lakes, NJ, USA) was used to analyze the immunostained cells. The percentage of stained cells was calculated by comparing the results to the corresponding negative controls.

### 2.4. Cell invasion assays of hTERT-ADSCs.TRAIL in PC

Matrigel-coated transwell cell culture chambers (8-µm pore size; Merck Millipore, Billerica, MA, USA) were used to assess the tropism of hTERT-ADSC.sTRAIL for tumor cells. Medium or 1.5 x 10<sup>4</sup> WPMY-1, human prostatic myofibroblasts (ATCC, Manassas, VA, USA), were loaded in the lower well of 24-well plates 24 h before the start of the experiment. The hTERT-ADSCs and hTERT-ADSC.sTRAIL (10<sup>5</sup>) cells in serum-free medium were placed on the 8-µm pore-size inserts coated with ECMMatrix. The lower wells containing the cells were washed with phosphate-buffered saline (PBS), filled with a serum-free medium, assembled for the migration assay, and incubated for 48 h. The noninvasive cells and

ECMatrix were wiped away from the inside of the insert, and the cells were stained for 20 min. The invading cells were photographed under a microscope. The results were evaluated by directly counting the number of migrated cells in five fields and calculating the mean.

## 2.5. *In vivo* migration toward prostate cancer

All procedures were conducted in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals (2001) and approved by the Institutional Animal Care and Use Committee of the hospital. Adult male nude mice, each weighing 20 – 25 g were purchased (OrientBio, Seongnam, Korea). The animals were housed in a temperature-controlled environment with a 12:12-h day-night cycle. All mice ate regular chow and had free access to water. The mice were anesthetized with isoflurane (BKPharm, Goyang, Korea), and  $1.0 \times 10^6$  PC3 cells (in 100  $\mu$ l of PBS) were injected subcutaneously into the flank using a 30-gauge Hamilton syringe. The mice were anesthetized with isoflurane (BKPharm, Goyang, Korea) two weeks after PC3 inoculation. PBS or  $1 \times 10^6$  hTERT.ADSC or hTERT.ADSC.sTRAIL cells mixed with 100  $\mu$ l of saline were injected into the left ventricle using a 100-micrometer syringe with a 26-gauge needle. After two weeks, the mice were euthanized, and the prostate cancer tumor induced at the flank was removed.

To confirm the *in vivo* migration toward prostate cancer, RT-PCR for green fluorescent protein (GFP), sTRAIL, and  $\beta$ -actin was performed on the tumors induced at the flank by hTERT-ADSC.sTRAIL via intracardiac injection to determine whether the tumors demonstrated the presence of GFP and rabbit CE.

Quantitative RT-PCR (qRT-PCR) was performed using Maxima SYBR Green/ROX qPCR Master Mix (Thermo Fisher Scientific). The qRT-PCR reactions were run on a PikoReal 96 (Thermo Fisher Scientific) under cycling conditions of 95 °C for 15 seconds (denaturation), 55 °C for 30 seconds (annealing), and 72 °C for 60 seconds (extension) for 45 cycles. The gene expression level, normalized to  $\beta$ -actin, was then calculated using the  $2^{-\Delta\Delta}$  formula with reference to the hTERT-ADSC.sTRAIL. The primers are shown in Table 1.

## 2.6. Confirmation of chemoattractant ligands and receptors

RT-PCR was used to determine the transcription of the ligand, stem cell factor, stromal cell-derived factor 1, and vascular endothelial growth factor (VEGF) in the PC3 prostate cancer cells and their corresponding receptors, c-kit, chemokine receptor 4 (CXCR4), VEGF receptor (VEGFR)-1, VEGFR2, and VEGFR3 in the hTERT-ADSC cells.

## 2.7. *In vitro* experiments

### 2.7.1. Antitumor effect of hTERT-ADSC.sTRAIL

Apoptosis and the viability of PC3 cells exposed to hTERT-ADSC.sTRAIL and CPT-11 were assessed. The hTERT-ADSC.sTRAIL cells ( $5 \times 10^3$  cells/well) were plated in 96-well plates (Falcon, Becton-Dickinson Co.). After 24 h, the cells were treated with 5  $\mu$ M CPT-11 (Sigma-Aldrich). The cells were incubated at 37 °C for three days, and the cell viability was quantified. All experiments were conducted in quadruplicate. The cell viability was determined using a modified version of the MTT assay (Promega, Madison, WI, USA), which was based on the conversion of the tetrazolium salt of MTT to a formazan product by mitochondrial dehydrogenase. A total of 10  $\mu$ l MTT solution was added to each well and incubated for 4 h at 37 °C. The color was extracted with dimethyl sulfoxide (Sigma-Aldrich) at 37 °C for 20 min. The formazan product was quantified by measuring the absorbance of the reaction at 570 nm using a microplate reader (Infinite F50; Tecan, Männedorf, Switzerland). The cell viability was expressed as the mean  $\pm$  standard error (SE) percent of the control viability, which was set to 100.

### 2.7.2. Cytotoxicity of hTERT-ADSC.sTRAIL to PC3 cells

The hTERT-ADSC.sTRAIL and PC3 prostate cancer cells ( $1 \times 10^4$  cells) were plated. The ratios of hTERT-ADSC.sTRAIL and PC3 prostate cancer cells were 0, 0.1, and 0.01 in 96-well cell culture plates (Falcon, Becton-Dickinson Co.) in DMEM plus 3% FBS and incubated at 37 °C. After 24 h, the PC3 prostate cancer cells cocultured with hTERT-ADSC.sTRAIL cells were treated with 5  $\mu$ M CPT-11. The microplates were incubated at 37 °C in a humidified atmosphere of 5% CO<sub>2</sub> and 95% air for three days, and the cytotoxicity was measured by a colorimetric assay.

**Table 1**  
PCR primer sequences.

Gene	Sequence	Size (bp)
CE	Sense: 5'-TGCTGGGCTATCCACTCTCT-3'	237
	Antisense: 5'-CTCCAGCATCTCTGTGTTGA-3'	
SCF	Sense: 5'-ACTTGGATTCTCACTTGCATT-3'	505
	Antisense: 5'-CTTCTCAGGACTTAATGTTGAAG-3'	
c-kit	Sense: 5'-GCCACAATAGATTGGTATTT-3'	332
	Antisense: 5'-AGCATCTTTACAGCGACAGTC-3'	
SDF-1	Sense: 5'-ATGAACGCCAAGGTCGTGGTC-3'	200
	Antisense: 5'-GGCTGTTGTGCTTACTTGTTT-3'	
CXCR4	Sense: 5'-CTCTCCAAGGAAAGCGAGGTGGACAT-3'	733
	Antisense: 5'-AGACTGTACTGTAGGTGCTGAAATCA-3'	
VEGF	Sense: 5'-AAGCCATCCTGTGTGCCCTGATG-3'	541
	Antisense: 5'-GCTCCTTCTCCTGCCCGCTCAC-3'	
VEGFR1	Sense: 5'-GCAAGGTGTGACTTTTGTTC-3'	512
	Antisense: 5'-AGGATTCTTCCCTGTGTA-3'	
VEGFR2	Sense: 5'-ACGCTGACATGTACGGTCTAT-3'	438
	Antisense: 5'-GCCAAGCTTGTACCATGTGCG-3'	
VEGFR3	Sense: 5'-AGCCATTCATCAACAAGCCT-3'	298
	Antisense: 5'-GGCAACAGCTGGATGCATA-3'	
$\beta$ -actin	Sense: 5'-GCA CCA CAC CTT CTA CAA TG -3	619
	Antisense: 5'-TGC TTG CTG ATC CAC ATC TG -3	

Carboxylesterase (CE); stem cell factor (SCF); stromal cell-derived factor 1 (SDF-1); C-X-C Motif Chemokine Receptor 4 (CXCR4); Vascular endothelial growth factor (VEGF); Vascular endothelial growth factor receptor (VEGFR).

### 2.7.3. Propidium iodide/annexin V flow cytometry analysis

The proportion of apoptotic cells was determined by flow cytometry analysis. The hTERT-ADSC.sTRAIL cells were stained with annexin V–FITC and propidium iodide (FITC Annexin V Apoptosis Detection Kit; BD Pharmingen, Franklin Lakes, NJ, USA), and evaluated using a Cyflow Cube 8 FACS instrument (Sysmex Partec, Görlitz, Germany). The data were analyzed using standard FSC Express software (De Novo Software, Los Angeles, CA, USA).

## 2.8. In vivo experiments

The mice were anesthetized with isoflurane (BKPharm, Goyang, Korea). PC3 cells ( $1.0 \times 10^6$  in 100  $\mu$ l PBS) were injected subcutaneously into the flank using a 30-gauge Hamilton syringe. One and two weeks after intracardiac injection of hTERT-ADSC.sTRAIL, the animals were treated with 1.7 mg/kg/day of CPT-11 diluted in 100  $\mu$ l of PBS i.p. in two rounds of five consecutive days with a break of two days. At days 0 and 14 days after the injection, the tumor volumes were measured using calipers, and the volume was calculated based on the formula, volume = length  $\times$  width<sup>2</sup>/2. To evaluate the effect of a high concentration compared with that of a low concentration of CPT-11, at weeks one and two after the intracardiac injection of hTERT-ADSC.sTRAIL, the animals were treated with 13.5 mg/kg/day of CPT-11 diluted in 100  $\mu$ l of PBS intraperitoneal injection (i.p.) in two rounds of five consecutive days with a break of two days. On days 0 and 14 after the injection, the tumor volumes were measured. The results are expressed as the mean tumor volume  $\pm$  SE. Fourteen days after the injection, the mice were euthanized, and the induced tumors were removed.

## 2.9. Immunohistochemistry

The removed tumors were analyzed immunohistopathologically for TRAIL to demonstrate the presence of the delivered TRAIL and its involvement in anticancer activity. We used a TRAIL antibody (1:100 ADI-AAP-470, Enzo Life Sciences INC, Farmingdale, NY, USA) and a DAB Detection IHC Kit (Abcam, Cambridge, UK). The TRAIL assay was performed on tumor tissues that were removed after two weeks of treatment with hTERT-ADSC.sTRAIL or the combination of hTERT-ADSC.sTRAIL and CPT-11. The stained sections were visualized using a microscope (Olympus, Tokyo, Japan). Low-power (X100) or high-power (X400) fields were examined.

To detect apoptosis, a terminal deoxynucleotidyl transferase–mediated dUTP nick end-labeling (TUNEL) assay was performed. We used a TdT apoptosis detection kit (Takara Bio Inc, Mountain View, CA, USA) and a DAB Detection IHC Kit (Abcam). The TUNEL assay was performed on tumor tissues that were removed after two weeks of treatment with hTERT-ADSC.sTRAIL or the combination hTERT-ADSC.sTRAIL and CPT-11. The stained sections were visualized using a microscope (Olympus, Tokyo, Japan). Low-power (X100) or high-power (X400) fields were examined.

## 2.10. Statistics

Two-way ANOVA and a posthoc Tukey test were used to determine the significant differences in cell viability and tumor volume. The data are presented as means  $\pm$  SE. A *P* value of  $< 0.05$  was considered statistically significant.

## 3. Results

### 3.1. Generation of hTERT-ADSC

The ASC52TELO, hTERT-ADSC (ATCC® SCRC-4000™) is an immortalized human adipose mesenchymal SC line containing

hTERT. The cells were shown to express mesenchymal SC markers CD29, CD90, and CD105, but not CD34 or CD45 markers for hematopoietic SCs. The telomerase assay [telomerase repeat amplification protocol (TRAP)] showed that the cells expressed markers for telomerase activity (Product sheet from ATCC® SCRC-4000™, ATCC, Manassas, VA, USA).

### 3.2. Gene expression of TRAIL from hTERT-ADSC.sTRAIL

hTERT-ADSC.sTRAIL cells were produced by lentiviral transduction of the sTRAIL gene using a CLV-Ubic puromycin/sTRAIL vector. The expression of sTRAIL transcripts from hTERT-ADSC.sTRAIL was confirmed by RT-PCR. The expression of sTRAIL protein from hTERT-ADSC.sTRAIL was confirmed by Western blotting. sTRAIL transcripts and protein were demonstrated only in hTERT-ADSC.sTRAIL, but not in hTERT-ADSC cells (Fig. 1).

### 3.3. hTERT-ADSC.sTRAIL phenotype

hTERT-ADSC and hTERT-ADSC.sTRAIL cells expressed cell type–specific markers for mesenchymal stem cell markers CD29, CD90, and CD105, but did not express CD34 or CD45 cell type markers for hematopoietic stem cells (Fig. 2).

### 3.4. Migration study

#### 3.4.1. Invasion study

Whereas only a few hTERT-ADSC and hTERT-ADSC.sTRAIL cells migrated toward media or the WPMY-1 cells, the directional migration of hTERT-ADSC and hTERT-ADSC.sTRAIL cells toward PC3 cells was significantly stimulated by PC3 cells *in vitro* ( $P < 0.05$ ). The migration capacity was not affected by sTRAIL transgene expression. The directional migration of hTERT-ADSC and hTERT-ADSC.sTRAIL cells toward PC3 cells was also significantly stimulated by PC3 cells treated with 5  $\mu$ M CPT-11 *in vitro* ( $P < 0.05$ ). The migration capacity of hTERT-ADSC.sTRAIL cells was not affected by treatment with 5  $\mu$ M CPT-11 for 72 hours ( $P < 0.05$ ) (Fig. 3A and B).

#### 3.4.2. In vivo migration study

RT-PCR demonstrated the presence of GFP and human sTRAIL from the induced prostate cancer tissue removed from the mice. GFP (fold) mRNA was not detectable in PBS. However, it was seen in the ADSCs ( $1.0 \pm 0.1$ ). The mRNA level of sTRAIL in the treated mice ( $2.7 \pm 0.7$ ) was higher than that in the ADSC-treated group ( $1.0 \pm 0.1$ ,  $P < 0.05$ ) (Fig. 3C). Because neither GFP nor human sTRAIL exists in mouse tissue, the presence of GFP or human sTRAIL demonstrates the selective migration of hTERT-hADSC.sTRAIL toward the prostate cancer induced in the mice. The intracardiac delivery of hTERT-hADSC.sTRAIL toward targeted prostate cancer was confirmed.

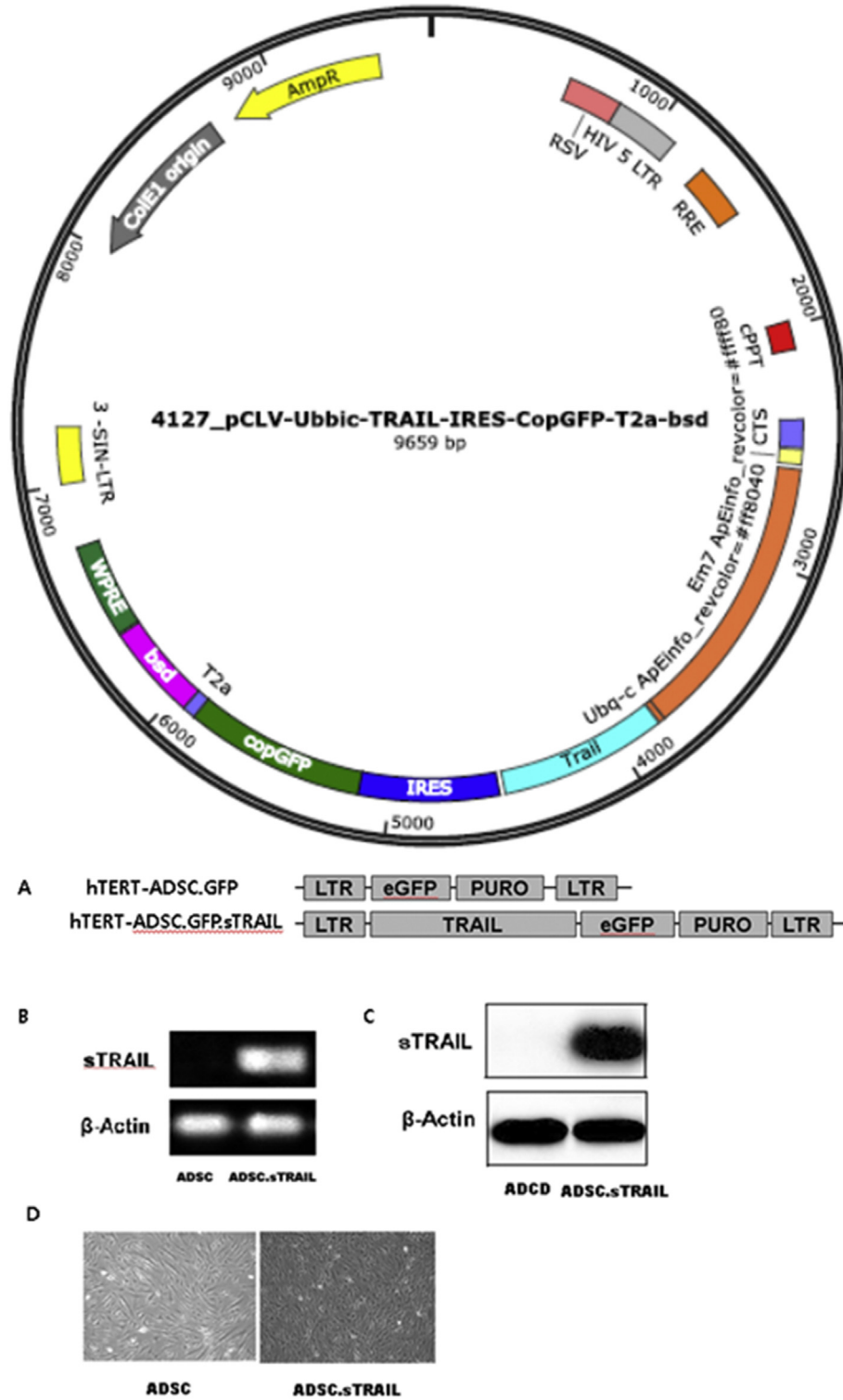
#### 3.4.3. Confirmation of chemoattractant ligands and receptors

The presence of ligands, stem cell factor, stromal cell–derived factor 1, and VEGF in PC3 prostate cancer cells and their corresponding receptors, c-kit, CXCR4, VEGFR1, VEGFR2, and VEGFR3 was confirmed in the hTERT-ADSC cells by RT-PCR (Fig. 3D).

### 3.5. In vitro antitumor effects of hTERT-ADSC.sTRAIL

#### 3.5.1. In vitro suicide effect of hTERT-ADSC.sTRAIL

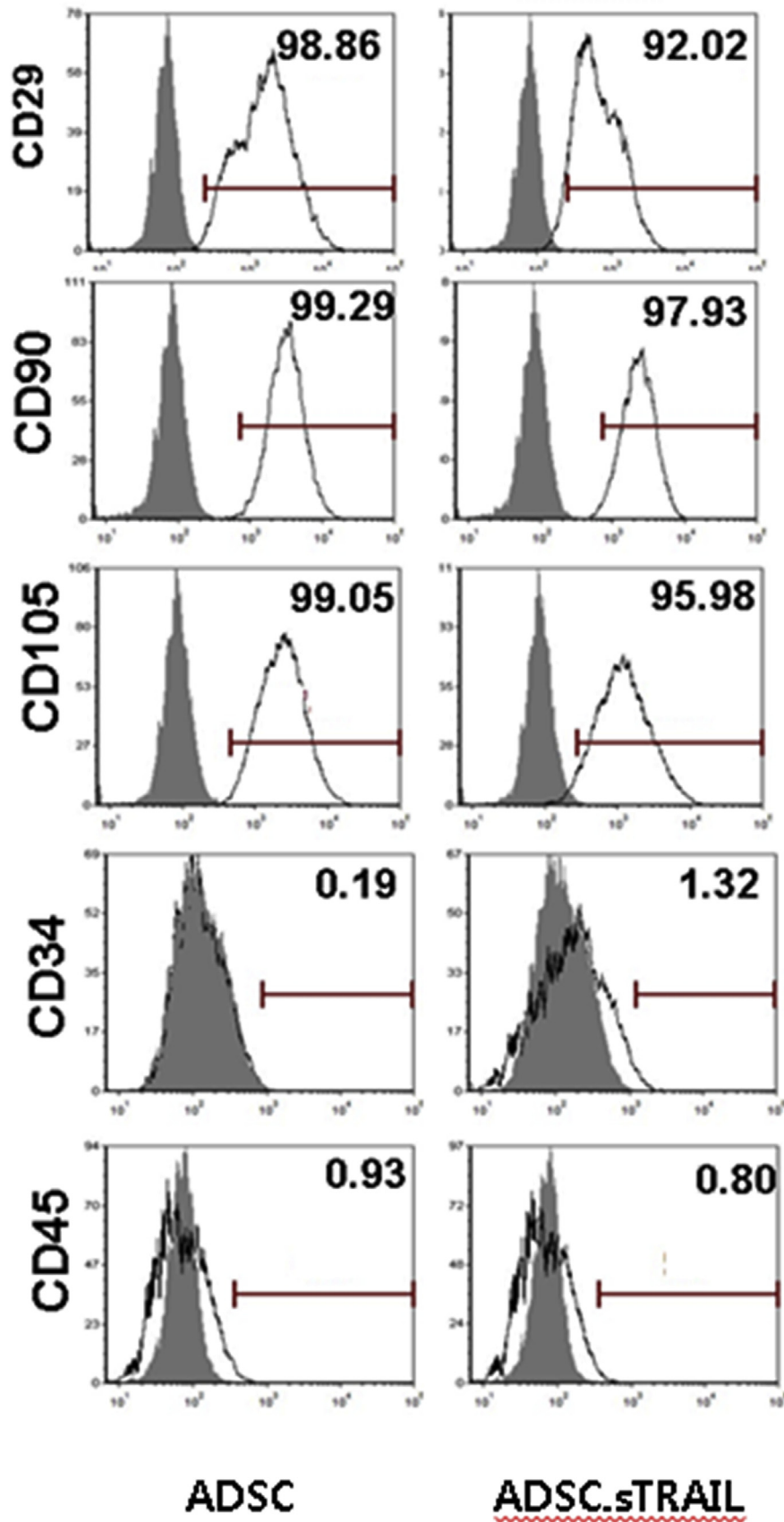
To analyze the *in vitro* cytotoxicity of CPT-11 on hTERT-ADSC.sTRAIL cells, we used a cell viability assay. hTERT-hADSC.sTRAIL cells were cultured under varying concentrations of CPT-11 (0.01 – 5  $\mu$ M) for 72 h. The cell viability was not changed



**Fig. 1.** The sTRAIL-overexpressing hTERT immortalized human adipose stem cell line (hTERT-ADSC.sTRAIL). (A) hTERT-ADSC and hTERT-ADSC.sTRAIL were generated by the lentiviral transduction of GFP and the sTRAIL gene using the CLV-Ubic vector. (B), (C): The expression of the sTRAIL transcript or protein was confirmed by RT-PCR or Western blots, respectively. (D) Phase-contrast microscopy of the hTERT-ADSC.GFP line (left) and hTERT-hADSC.GFP.sTRAIL (right) line. ADSC = hTERT-hADSC, sTRAIL = secretable trimeric forms of tumor necrosis factor (TNF)-related apoptosis-inducing ligand, ADSC = adipose-derived stem cells, ADSC.sTRAIL = sTRAIL-overexpressing ADSC, RT-PCR = reverse transcription-polymerase chain reaction.

by treatment with CPT-11 from 0.01 to 0.5 μM. At 1 μM CPT-11, the viability of hTERT-hADSC cells (92.5 ± 1.8%) and hTERT-hADSC.sTRAIL cells (94.3 ± 2.4%) was lower than that of hTERT-hADSC (100.0 ± 3.5%) and hTERT-hADSC.sTRAIL (100.0 ± 1.1%) at

0 μM CPT-11. At a final concentration of 5 μM cpt-11, the hTERT-ADSC (80.3 ± 2.0%) and hTERT-ADSC.sTRAIL cell lines (84.4 ± 1.2%) showed lower cell viability at 72 hours than treatment with 0.1 μM cpt-11 alone (100.9 ± 1.1%, *P* < 0.05). A concentration of



**Fig. 2.** Cell markers of hTERT-ADSC.GFP and hTERT-ADSC.GFP.sTRAIL cells. hTERT-ADSC cells express cell type-specific markers for mesenchymal stem cell markers CD29, CD90, and CD105, but do not express CD34 or CD45 cell type markers for hematopoietic stem cells. ADSC = hTERT immortalized human adipose-derived stem cells, ADSC.sTRAIL = sTRAIL-overexpressing ADSCs.

CPT-11 greater than 5  $\mu$ M was required to achieve an effective *in vitro* suicide effect of hTERT-hADSC.sTRAIL by cpt-11 treatment (Fig. 4A).

3.5.2. *In vitro* cytotoxicity of hTERT-ADSC.sTRAIL

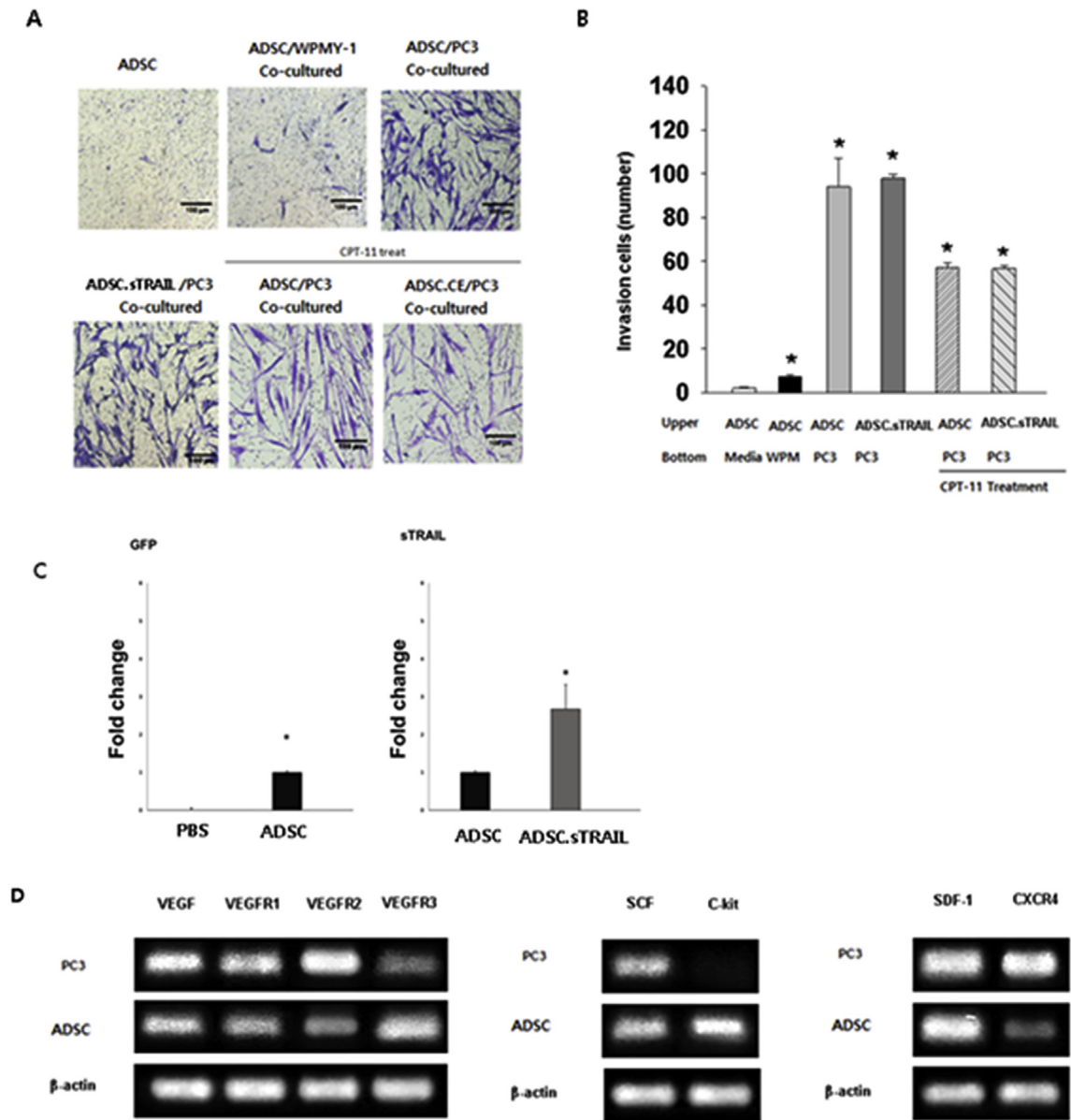
The selective cytotoxicity toward PC3 prostate cancer cells was mediated by hTERT-ADSC.sTRAIL in the presence of 5  $\mu$ M CPT-11 *in vitro* for 72 hours. The viability of the PC3 cells was significantly decreased by combined treatment with ADSC.sTRAIL and CPT-11 ( $62.7 \pm 2.0\%$ ) compared with treatment with CPT-11 alone ( $83.0 \pm 1.0\%$ ) at a cell ratio of 0.05 (PC3: ADSC.sTRAIL) ( $P < 0.05$ ). The percentage of viable hTERT-ADSC.sTRAIL cells was significantly decreased compared with CPT-11 treatment alone (Fig. 4B).

3.5.3. *In vitro* apoptotic effect of ADSC.sTRAIL

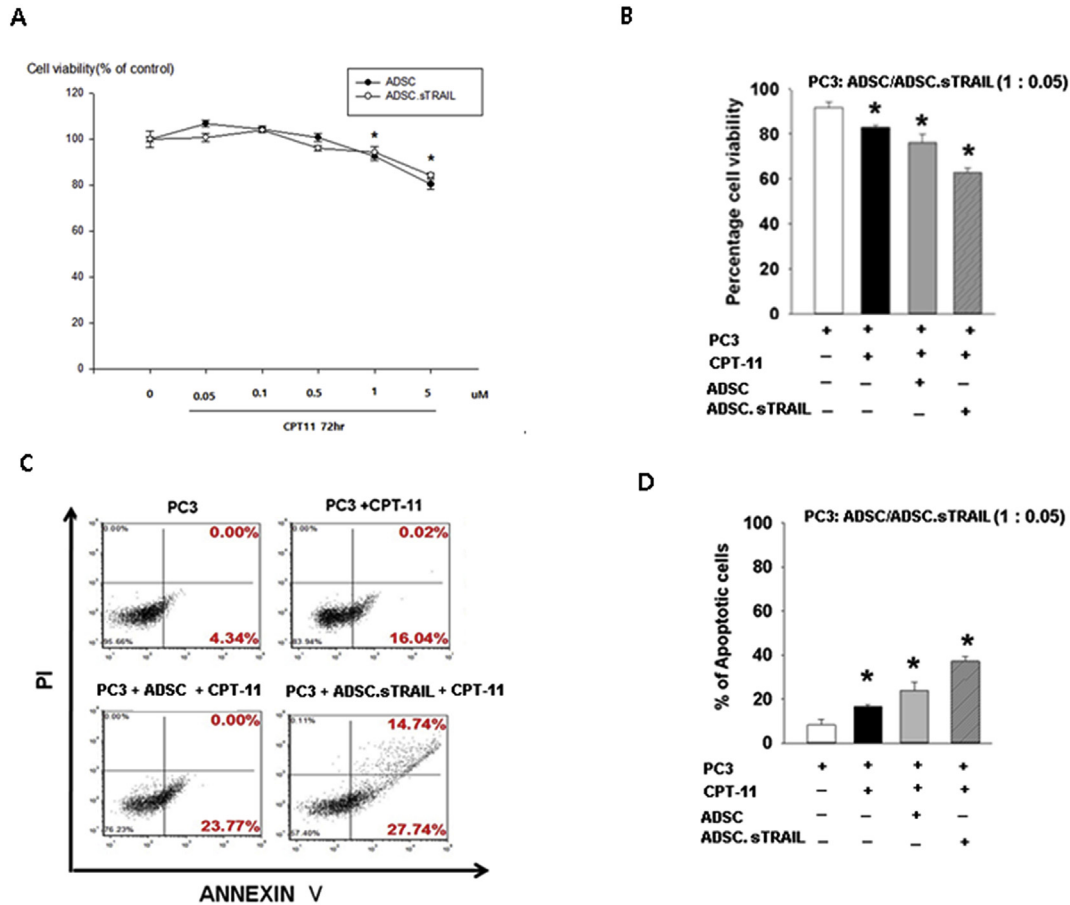
Flow cytometry for annexin V/propidium iodide staining showed that the percentage of annexin V–positive apoptotic PC3 cells was increased in the ADSC.sTRAIL (42.5%) and the 5  $\mu$ M CPT-11–treated group compared with the group treated with CPT-11 alone (16.06%) (Fig. 4C). The percentage of apoptotic PC3 cells significantly increased in the hTERT-ADSC.sTRAIL and CPT-11–treated group ( $37.2 \pm 2.1\%$ ) compared with the group treated with CPT-11 alone ( $16.5 \pm 1.0\%$ ,  $P < 0.05$ ) (Fig. 4D).

3.6. Tumor growth inhibition by the systemic administration of hTERT-ADSC.sTRAIL *in vivo*

Prostate cancer was induced in mice (Fig. 5A and B). The systemic administration of hTERT-ADSC.sTRAIL in combination with



**Fig. 3.** hTERT-ADSC.sTRAIL line targets prostate cancer. (A), (B) The invasion study shows the migration of hTERT-ADSC or hTERT-ADSC.sTRAIL to prostate cancer. (C) Intracardiac delivery of hTERT-ADSC targeting prostate cancer. qPCR demonstrated GFP and human sTRAIL from experimentally induced prostate cancer tissues removed from the mice. (D) Expression of chemoattractant factors. The presence of the ligands, SCF, SDF-1, and VEGF in PC3 cells and their corresponding receptors c-kit, CXCR4, VEGFR1, VEGFR2, and VEGFR3 in hTERT-ADSC cells. ADSC = hTERT-ADSC, stem cell factor, SCF = stromal cell–derived factor, SDF = vascular endothelial growth factor, VEGF = vascular endothelial growth factor, VEGFR = vascular endothelial growth factor receptor, ADSC = hTERT immortalized human adipose stem cell line, ADSC.sTRAIL = sTRAIL-overexpressing ADSCs. \* $P < 0.05$ .



**Fig. 4.** *In vitro* effect of combined treatment with hTERT-ADSC.sTRAIL and cpt-11. (A) The suicide effect. At greater than 1 μM CPT-11, the viability of ADSC.sTRAIL cells was lower than at more than 0 μM CPT-11 ( $P < 0.05$ ). (B) The cytotoxicity of ADSC.sTRAIL and 5 μM CPT-11 toward PC3 at cocultured ratios of 0.05. The cell viability of PC3 decreased significantly by treatment with both CPT-11 and ADSC.sTRAIL compared with CPT-11 treatment alone. (D) Flow cytometry for propidium iodide/annexin V staining showed that the percentage of annexin V-positive apoptotic PC3 cells increased in the presence of ADSC.sTRAIL and CPT-11 compared to CPT-11 treatment alone. (E) The percentage of apoptotic cells significantly increased in the ADSC.sTRAIL-treated group compared with those treated with cpt-11 alone ( $P < 0.05$ ). ADSC = hTERT immortalized human adipose-derived stem cells, ADSC.sTRAIL = sTRAIL-overexpressing hTERT immortalized human adipose stem cells. \* $P < 0.05$ .

CPT-11 reduced prostate cancer volume. Two weeks after CPT-11 treatment, the volume of the prostate cancer tumors was  $2089.0 \pm 264.9\%$  (control group),  $968.8 \pm 118.6\%$  (hTERT-ADSC.sTRAIL group),  $830.1 \pm 161.9\%$  (CPT-11 group),  $901.1 \pm 158.4\%$  (hTERT-ADSC + CPT-11 group), and  $476.4 \pm 59.5\%$  (hTERT-ADSC.sTRAIL + CPT-11 group) of the baseline prostate cancer volume 0 weeks after CPT-11 treatment (Fig. 5C).

After the systemic administration of hTERT-ADSC.sTRAIL combined with CPT-11, prostate cancer tumor growth was inhibited compared with the control group ( $P < 0.05$ ). After systemic administration of hTERT-ADSC.sTRAIL in combination with CPT-11, prostate tumor growth was inhibited compared with the hTERT-ADSC.sTRAIL or CPT-11 monotherapy groups ( $P < 0.05$ ). The inhibitory effects of ADSC.sTRAIL and CPT-11 were greater than those of CPT-11 alone.

Two weeks after the systemic administration of hTERT-ADSC.sTRAIL in combination with 13.5 mg/Kg/day CPT-11, the prostate cancer volume decreased up to  $86.7 \pm 4.2\%$  (hTERT-ADSC.sTRAIL + 13.5 mg/Kg/day CPT-11) and  $140.2 \pm 15.6\%$  (13.5 mg/Kg/day CPT-11 monotherapy), compared with  $100 \pm 17.5\%$  (baseline prostate cancer volume at 0 weeks) ( $P < 0.05$ ) (Fig. 5D). The inhibitory effects of hTERT-ADSC.sTRAIL combined with 13.5 mg/Kg/day CPT-11 were better than those of 13.5 mg/Kg/day CPT-11

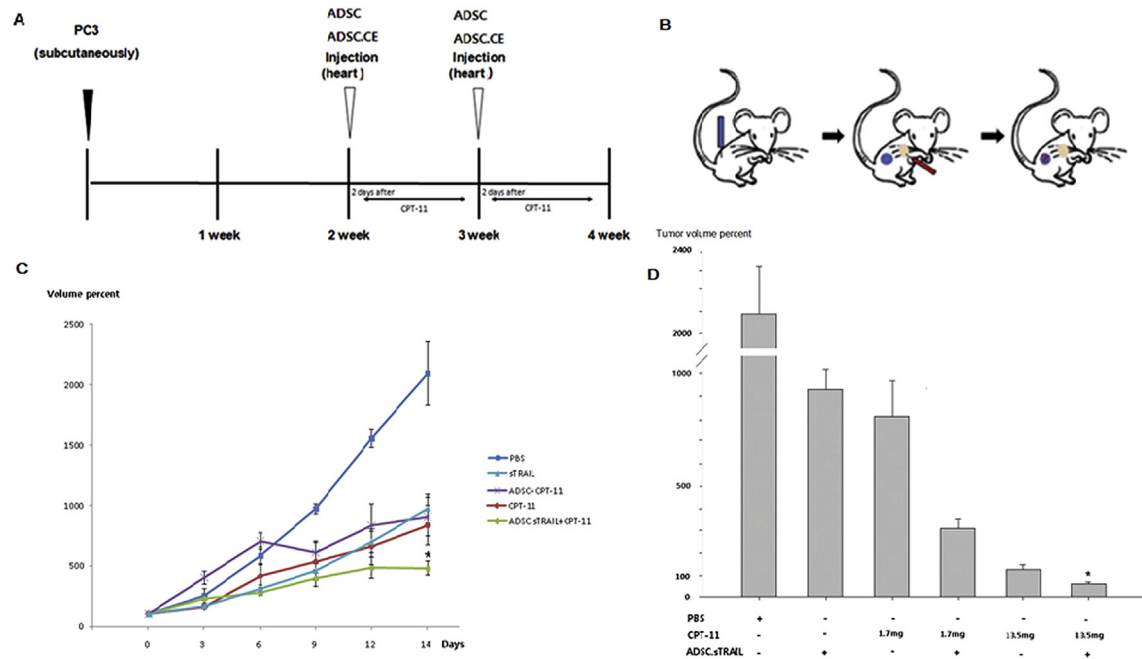
monotherapy and decreased the prostate cancer volume up to 61.8%.

Hematoxylin and eosin staining of the transplanted PC3 cell tumor showed the induction of prostate cancer two weeks after the injection of PC3 prostate cancer cells into the flanks of the mice (Fig. 6A). The tumor was well-demarcated from dermal tissue and composed of closely packed cancer cells. The neoplastic cells remaining in the peripheral lesion were shrunken and had distinct borders. Geographic necrosis was found (Fig. 6A–D). Immunohistochemical staining showed anti-TRAIL-positive cells in the removed tumor by treatment with hTERT-ADSC.sTRAIL or the combination of hTERT-ADSC.sTRAIL and CPT-11 (Fig. 6E and F), and hyperchromatic nuclei were centrally located with condensed eosinophilic cytoplasm, which has apoptotic bodies (Fig. 6G and H).

#### 4. Discussion

Although initial clinical trials using the death ligand TRAIL have shown disappointing results, TRAIL is still an attractive cancer therapeutic agent because it can induce remarkable apoptosis in tumors with minimal toxicity to normal tissues. However, several cancers, including PC and CRPC, are resistant to TRAIL-induced apoptosis. To overcome the known limitation of





**Fig. 5.** Treatment with hTERT-hADSC.sTRAIL cells and CPT-11 has a significant therapeutic effect *in vivo*. (A) The schematic summary of the treatment. (B) Illustration of the induction of prostate cancer using PC3 cells, the systemic injection of hTERT-ADSC.sTRAIL cells, and the migration of gene-modified stem cells toward prostate cancer. Blue: PC3; red: hTERT-ADSC.sTRAIL cells. (C) Administration of hTERT-hADSC.sTRAIL in combination with 1.7 mg/kg/day CPT-11 reduced prostate cancer tumor volumes in mice. The inhibitory effects of hTERT-ADSC.sTRAIL combined with CPT-11 were higher than those of hTERT-ADSC.sTRAIL or CPT-11 alone. (D) The inhibitory effects of hTERT-ADSC.sTRAIL combined with a high CPT-11 concentration (13.5 mg/kg/day) were better than those at a low CPT-11 concentration (1.7 mg/kg/day). ADSC = hTERT immortalized human adipose stem cell line, hTERT-ADSC.sTRAIL = sTRAIL-overexpressing hTERT immortalized human adipose stem cells, H&E = hematoxylin & eosin. \* $P < 0.05$ .

TRAIL resistance in PC treatment, we added several combinations to classic TRAIL, including secreted trimeric forms of TRAIL, ADSC, and CPT-11.

TRAIL induces apoptosis selectively in a variety of tumor cells, but not in normal cells or tissues, suggesting it as an excellent therapeutic candidate for cancer with reduced toxicity (2, 3). Because of low toxicity in normal tissue or cells and apoptosis by extrinsic and intrinsic pathways, TRAIL and anti-TRAIL antibodies could be promising antitumor agents (16). In this study, the sTRAIL transcript and protein were detected only in hTERT-ADSC.sTRAIL, but not in hTERT-ADSC cells (Fig. 1), indicating that gene modification was successfully accomplished.

Gene therapy has several advantages compared with conventional drug therapies. First, the high-dose and long-term expression of a drug can be achieved relatively easily. The drug may be delivered locally and specifically to the target tissues. ADSCs can be used as delivery vehicles for therapeutic genes.

ASC52TELO, hTERT-ADSC (ATCC® SCRC-4000™) is an immortalized human adipose mesenchymal SC line containing hTERT. The cells express markers specific for mesenchymal SCs CD29, CD90, and CD105, but not hematopoietic SC markers CD34 or CD45. The telomerase assay showed the expression of the marker for telomerase activity. Therefore, the modification of ADSCs with hTERT did not alter the SC properties.

The major advantage of using SCs to treat cancer is their ability to migrate and infiltrate tumors. Moreover, this tumor-specific tropism may be exploited to target minor distant metastases and infiltrate malignant satellites after the complete removal of the main tumor. In this study, the directional migration of hTERT-ADSC.sTRAIL cells toward PC3 cells was significantly stimulated by PC3 cells *in vitro*. The migration ability was not affected by sTRAIL transgene expression or by treatment with 5  $\mu$ M CPT-11 for 72 hours. In this study, the directional migration of hTERT-

ADSC.sTRAIL cells toward PC3 cells was significantly stimulated by PC3 cells *in vitro* (Fig. 3). The migration capacity was not affected by sTRAIL transgene expression or treatment with 5  $\mu$ M CPT-11 for 72 hours (Fig. 3). Neither gene modification using lentiviral transduction nor transgene expression affected SC differentiation, migration properties, or pluripotency transcription factor expression (10, 16-18). RT-PCR demonstrated the presence of GFP and human sTRAIL in the removed prostate cancer tissues induced by the injection of PC3 cells into the flanks of mice (Fig. 3C). Because artificially transfected human sTRAIL or GFP does not exist in normal mouse tissues, the presence of human sTRAIL or GFP in the experimentally induced prostate cancer tissues demonstrated the selective migration of hTERT-hADSC.sTRAIL cells toward induced prostate cancer in mice. The hTERT-ADSC.sTRAIL cells migrated to the implanted tumor sites and reduced the tumor volume in mice exposed to CPT-11.

The combined treatment of TRAIL and CPT-11 potentiated the antitumor effect without the need to increase the concentration of CPT-11 and with reduced toxicity. The generation of TRAIL-overexpressing SCs triggered the persistent release of TRAIL at high concentrations from the SCs. CPT-11 is known to augment the TRAIL-induced apoptosis of cancer (14). In this study, the *in vitro* suicide effect of ADSC.sTRAIL was shown at a concentration of CPT-11 greater than 5  $\mu$ M (Fig. 4A). A concentration of CPT-11 greater than 5  $\mu$ M was required for the effective *in vitro* suicide effect of hTERT-hADSC.sTRAIL cells by treatment with CPT-11. Cytotoxicity toward PC3 prostate cancer cells was mediated by hTERT-ADSC.sTRAIL cells in the presence of 5  $\mu$ M CPT-11 *in vitro* for 72 hours (Fig. 4B). hTERT-ADSC.sTRAIL cells exerted a strong selective cytotoxic effect on PC3 prostate cancer cells, even at a ratio of hTERT-ADSC.sTRAIL to PC3 cells of 0.05 to 1. In this study, hTERT-ADSC.sTRAIL cells induced apoptosis *in vitro* in the combined treatment with 5  $\mu$ M CPT-11 for 72 hours (Fig. 4C). The

percentage of apoptotic cells was significantly increased in the hTERT-ADSC.sTRAIL cells compared with the hTERT-ADSC cells (Fig. 4 D).

In the *in vivo* study, the systemic administration of hTERT-ADSC.sTRAIL in combination with 1.7 mg/Kg/day CPT-11 reduced the prostate cancer tumor volume. After the systemic administration of hTERT-ADSC.sTRAIL in combination with CPT-11, the PC tumor growth was significantly inhibited compared with that of CPT-11 treatment alone (Fig. 5D). The inhibition of castration-resistant prostate cancer tumor growth using TRAIL-overexpressing adipose stem cells was enhanced by the addition of the chemotherapeutic agent CPT-11. This suggests that the antitumor effect of the prolonged production of higher local concentrations of TRAIL released from hTERT-ADSC.sTRAIL and the potentiation by CPT-11 increased the therapeutic efficiency.

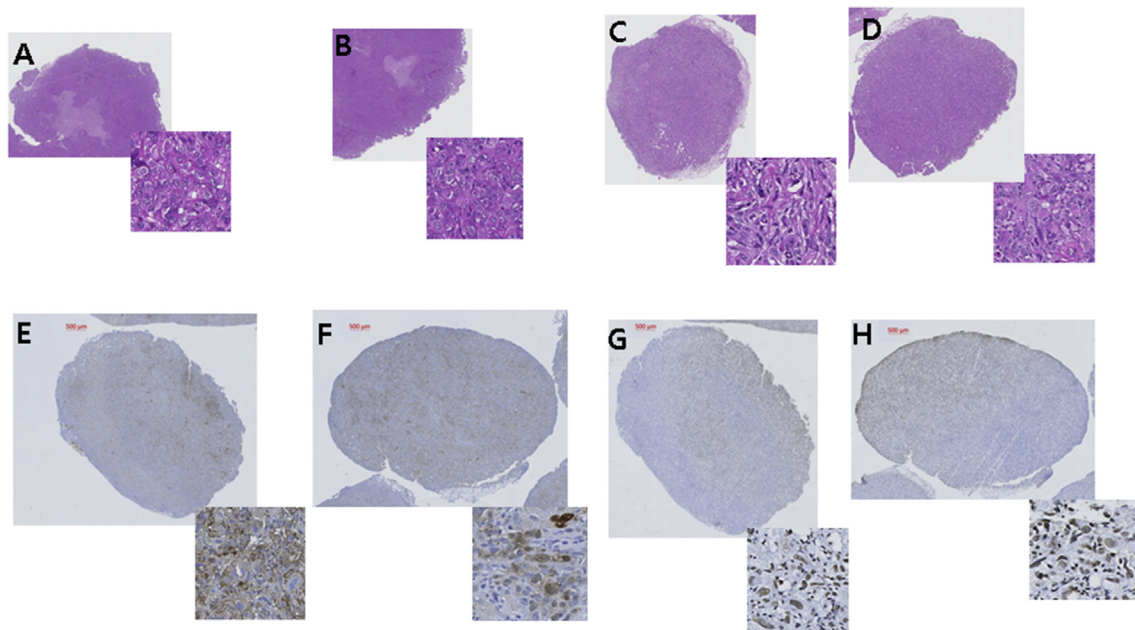
The inhibitory effects of hTERT-ADSC.CE combined with 13.5 mg/Kg/day CPT-11 were better than those of hTERT-ADSC.sTRAIL combined with 1.7 mg/Kg/day CPT-11 (Fig. 5E). The inhibitory effects of hTERT-ADSC.sTRAIL combined with 13.5 mg/Kg/day CPT-11 were better than those of 13.5 mg/Kg/day CPT-11 treatment alone and decreased the volume of the prostate cancer tumors up to 61.8% compared with 13.5 mg/Kg/day CPT-11 monotherapy (Fig. 5E). Because the treatment of hTERT-ADSC.sTRAIL cells in combination with CPT-11 decreased the effective concentration of CPT-11, enhanced efficacy of CPT-11 with reduced toxicity can be expected.

Immunohistochemical staining of the removed tumor showed anti-TRAIL-positive cells after hTERT-ADSC.sTRAIL or hTERT-ADSC.sTRAIL treatment combined with CPT-11 (Fig. 6E and F) and

hyperchromatic nuclei were centrally located with condensed eosinophilic cytoplasm, which had apoptotic bodies (Fig. 6G and H). Immunohistopathological examination of the removed tumors for TRAIL demonstrated that the delivered TRAIL was present and involved in anticancer activity. This suggests that TRAIL-induced apoptosis in the prostate cancer and CPT-11 augmented TRAIL-induced apoptosis in prostate cancer.

Recently, several studies have tried to overcome the limitation of resistance to classical TRAIL-induced apoptosis in PC. Mohr et al.(7) showed that the combination of small-molecule drugs with SC.sTRAIL enhanced the sensitization of PC cells to TRAIL and reduced cytokine production related to the side effects of TRAIL. Chen et al.(19) showed that a novel combination of cyproterone acetate and TRAIL caused apoptosis in CRPC cell lines, including PC3 and DU145.

Although our study demonstrated remarkable findings using the novel combination of sTRAIL, ADSCs, and CPT-11, there were several limitations. First, our study did not include any results using full-length TRAIL. There are conflicting results on the efficacy differences between full-length TRAIL and sTRAIL. A second limitation was the lack of molecular analyses to define the crucial mechanism of hTERT-ADSC.sTRAIL in PC. To date, several detailed mechanisms of TRAIL in cancer have been reported. Besides the classical mechanism of DR5 upregulation, the sensitization of tumors or cancer SCs to SC-TRAIL-induced apoptosis; increases in anti-apoptotic molecules; the activation of caspase-8, which induces caspase-3 via cleavage (extrinsic pathway) or caspase-3 activation (intrinsic pathway); and unfolded protein response induced by endoplasmic reticulum (ER) stress need to be elucidated in PC.



**Fig. 6.** Immunohistochemistry of removed prostate cancer tumors. (A)–(D) H&E staining of induced prostate cancer two weeks after the implantation of PC3 cells into the flanks of the mice. (A) Tumors in PC3-transplanted mice (X100), Inset: (X400). The tumor is well-demarcated from the dermal tissue and composed of closely packed cancer cells. (B) Tumors in PC3-transplanted mice after 13.5 mg/kg/day CPT-11 systemic treatment (X100). (C) Tumors in PC3-transplanted mice after hTERT-ADSC.sTRAIL systemic treatment (X100). (D) Tumors in PC3-transplanted mice after hTERT-ADSC.sTRAIL combined with 13.5 mg/kg/day CPT-11 systemic treatment (X100). Geographic necrosis was seen. The neoplastic cells remaining in the peripheral lesion were shrunken and had distinct borders. (E), (F) TRAIL immunohistochemical staining of tumors after treatment shows anti-TRAIL-positive cells. (E) Tumors in the PC3-transplanted mice after hTERT-ADSC.sTRAIL systemic treatment (X100), Inset: (X400). (F) Tumors in PC3-transplanted mice after hTERT-ADSC.sTRAIL combined with 13.5 mg/kg/day CPT-11 systemic treatment (X100), Inset: (X400) (G), (H) TUNEL immunohistochemical staining of tumors after treatment shows TUNEL-positive cells. (G) Tumors in PC3-transplanted mice after hTERT-ADSC.sTRAIL systemic treatment (X100), Inset: (X400). (H) Tumors in PC3-transplanted mice after hTERT-ADSC.sTRAIL combined with 13.5 mg/kg/day CPT-11 systemic treatment (X100), Inset: (X400). Hyperchromatic nuclei are centrally located with condensed eosinophilic cytoplasm, which have apoptotic bodies. Inset: Apoptotic cells with apoptotic bodies. Bar. 500 μm. H&E = hematoxylin & eosin, TUNEL = terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling.

Further characterization of the precise mechanisms by which the combined treatments enhanced the susceptibility to TRAIL-induced apoptosis is warranted. Third, in the *in vitro* and *in vivo* studies, our study did not demonstrate the apoptotic effect of hTERT-ADSC.sTRAIL or hTERT-ADSC on PC3 cells without CPT-11 and did not show an *in vivo* treatment effect of hTERT-ADSC.sTRAIL or hTERT-ADSC on PC3 cells without CPT-11. Lastly, other CRPC cells, such as 22RV1 or VCaP, should be included to demonstrate the universality of the combined treatment effect on CRPC.

## 5. Conclusions

Human ADSCs in combination with the sTRAIL transgene migrated to PC, potentiated cytotoxic effects against tumor cells *in vitro* and exerted a robust inhibition of tumor growth *in vivo* in combination with the prodrug CPT-11. These results indicate that therapeutic sTRAIL-overexpressing ADSCs combined with CPT-11 represents a new strategy for clinical trials for CRPC treatment using the patients' own ADSCs.

## Author contributions

SHL and YSS conceived and designed the experiments. HYL, JHK, SHD, WJY, EUO, JYH, and YSH performed the experiments. JHK, SHL, and YSS analyzed the data. SHL and YSS wrote the paper.

## Conflicts of interest

There is no conflict of interest.

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## Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.pnrl.2020.07.002>.

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