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ORIGINAL ARTICLE

Clinically applicable human adipose tissue-derived mesenchymal stem cells delivering therapeutic genes to brainstem gliomas

SA Choi[1,2,9], YE Lee[1,3,9], PA Kwak[1,2], JY Lee[1,4], SS Kim[3], SJ Lee[5], JH Phi[1,2], K-C Wang[1], J Song[6], SH Song[7], KM Joo[3,5,8] and S-K Kim[1,2]

Pediatric brainstem glioma is an incurable malignancy because of its inoperability. As a result of their extensive tropism toward cancer and the possibility of autologous transplantation, human adipose-derived mesenchymal stem cells (hAT-MSC) are attractive vehicles to deliver therapeutic genes to brainstem gliomas. In this study, in a good manufacturing practice (GMP) facility, we established clinically applicable hAT-MSCs expressing therapeutic genes and investigated their therapeutic efficacy against brainstem glioma in mice. For feasible clinical applications, (1) primary hAT-MSCs were cultured from human subcutaneous fat to make autologous transplantation possible, (2) hAT-MSCs were genetically engineered to express carboxyl esterase (CE) and (3) a secreted form of the tumor necrosis factor-related apoptosis-inducing ligand (sTRAIL) expression vector for synergistic effects was delivered by a gene transfer technology that did not result in genomic integration of the vector. (4) Human CE and sTRAIL sequences were utilized to avoid immunological side effects. The hAT-MSCs expressing CE ± sTRAIL showed significant therapeutic effects against brainstem gliomas *in vitro* and *in vivo*. However, the simultaneous expression of CE and sTRAIL had no synergistic effects *in vivo*. The results indicate that non-viral transient single sTRAIL gene transfer to autologous hAT-MSCs is a clinically applicable stem cell-based gene therapy for brainstem gliomas in terms of therapeutic effects and safety.

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INTRODUCTION

Brainstem glioma is one of the most malignant and aggressive brain tumors in children.[1,2] In most brainstem glioma cases, neurosurgical intervention is not possible because of the tumor being located in a vital part of the brain. Moreover, conventional anticancer therapies, including chemotherapy and radiation therapy, show minimal therapeutic benefits. The absence of effective treatments for brainstem gliomas has resulted in poor clinical outcomes; the mean overall survival period is <1 one year, and the 5-year progression-free survival is $<10\%$.[2,3] Therefore, novel therapeutic strategies targeting glioma cells in the brainstem are urgently needed.

The dynamic migratory activities of stem cells to damaged brain areas[4,5] or brain tumors[6,7] have provided new treatment tools for anticancer gene therapy. Mesenchymal stem cells (MSCs) have been considered as especially appropriate candidates for stem cell-based gene therapy.[4,8–16] Autologous MSCs can easily be obtained from various organs of patients without any ethical problems. In addition, the stemness of MSCs can be effectively maintained *in vitro* for long periods of time, which enables clinically applicable numbers of cells to be obtained and makes possible the adoption of non-viral gene transfer techniques. As the migratory capacity and therapeutic potential of human adipose tissue-derived MSCs (hAT-MSCs) was confirmed in various experimental brain tumor models,[17] the addition of therapeutic genes to MSCs could provide major treatment effects against brainstem gliomas.

Carboxyl esterase (CE) converts the blood–brain barrier-permeable chemotherapeutic agent, CPT-11 (irinotecan-7-ethyl-10-[4-(1-piperidino)-1-piperidino] carbonyloxycamptothecin),[18] into the toxic metabolite, SN-38.[19–21] In many previous studies, rabbit CE was utilized as a therapeutic gene to increase local concentrations of SN-38 in brain tumors.[22,23] However, xenoproteins can have unexpected immunological side effects clinically.

Glioma cells express the death receptors 4 and 5 (DR4 and DR5).[24,25] The receptors induce apoptosis of cancer cells in response to their membrane-bound ligand, tumor necrosis factor-related apoptosis-inducing ligand (TRAIL).[26–30] Accordingly, a secreted form of TRAIL (sTRAIL) has been considered as an alternative candidate for a therapeutic gene.[29,31,32] Moreover, the different therapeutic mechanisms of the two genes (CE and sTRAIL), when they are simultaneously present in the MSCs, could potentiate the treatment effects of MSCs against brainstem gliomas.

To obtain clinically applicable MSCs with maximal therapeutic effects against brainstem gliomas, we cultured hAT-MSCs from human adipose tissue, established hAT-MSCs expressing human

[1]Division of Pediatric Neurosurgery, Pediatric Clinical Neuroscience Center, Seoul National University Children's Hospital, Seoul National University College of Medicine, Seoul, Republic of Korea; [2]Adolescent Cancer Center, Seoul National University Cancer Hospital, Seoul, Republic of Korea; [3]Department of Health Sciences and Technology, SAIHST, Sungkyunkwan University, Seoul, Republic of Korea; [4]Department of Anatomy, Seoul National University College of Medicine, Seoul, Republic of Korea; [5]Department of Anatomy and Cell Biology, Sungkyunkwan University, School of Medicine, Suwon, Republic of Korea; [6]Department of Laboratory Medicine, Seoul National University Bundang Hospital, Seongnam, Republic of Korea; [7]Department of Laboratory Medicine, Seoul National University Hospital, Seoul, Republic of Korea and [8]Samsung Biomedical Research Institute, Samsung Medical Center, Seoul, Republic of Korea. Correspondence: Dr KM Joo, Department of Anatomy and Cell Biology, Sungkyunkwan University School of Medicine, Suwon, Republic of Korea. or Dr S-K Kim, Division of Pediatric Neurosurgery, Pediatric Clinical Neuroscience Center, Seoul National University Children's Hospital, Seoul National University College of Medicine, 101 Daehak-ro, Jongno-gu 110-744, Seoul, Republic of Korea.
E-mail: kmjoo@skku.edu or nsthomas@snu.ac.kr
[9]These authors contributed equally to this work.
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CE ± sTRAIL in a good manufacturing practice (GMP) facility and validated the therapeutic effects of the MSCs using a mouse brainstem glioma model. The results provide important data for clinical trials using MSCs with therapeutic genes against brainstem gliomas.

MATERIALS AND METHODS

Cell culture

The hAT-MSCs were isolated from the anterior chest subcutaneous fat during operations for vagus nerve stimulation insertion for intractable epilepsy. The preparation of MSCs from fat tissues was approved by the institutional review board (IRB) of the Seoul National University Hospital (IRB number: 0606-049-176). hAT-MSCs were cultured as described previously.[9,10] The cells were maintained in the GMP facility of Seoul National University Hospital and used before passage 5. The human glioma cell lines, U-87MG and T98G (ATCC, Manassas, VA, USA), were cultured in Dulbecco's modified Eagle's medium (Life Technologies, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin (P/S). The human embryonic kidney cell line, 293FT, was cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 1% penicillin/streptomycin, 1% non-essential amino acids (Sigma-Aldrich, St Louis, MO, USA), and 1% sodium pyruvate (Life Technologies).

Characterization of primary cultured hAT-MSCs

Primary cultured hAT-MSCs were labeled with fluorescein isothiocyanate- or phycoerythrin-conjugated antibodies against CD14, CD34, CD73, CD90 (BD Biosciences Pharmingen, Heidelberg, Germany), CD45 or CD105 (Chemicon, Temecula, CA, USA) and then analyzed by a FACScan cytometer (BD Biosciences Pharmingen). The hAT-MSCs were stimulated to differentiate using a mesenchymal differentiation kit (Life Technologies) for 2–3 weeks as suggested by the manufacturer. Differentiation was validated by histologic staining using Oil red O, Alizarin red S and Alcian blue (Sigma-Aldrich).

hAT-MSCs expressing human CE ± sTRAIL

A cloning package including an entry vector (pENTR/D-TOPO) and a lentiviral destination vector (pLenti7.3/V5-DEST) (Life Technologies) was utilized. The destination vector harbors a puromycin resistance gene under the control of the PGK promoter. Alternatively, the puromycin resistance gene was replaced by a green fluorescent protein (GFP) gene. The human CE complementary DNA was cloned from HepG2 cells using a Phusion high-fidelity DNA polymerase kit (New England Biolabs, Knowl Piece, Hitchin, UK) and specific primers (Supplementary Table 1). The human CE complementary DNA was inserted into the entry vector and then transferred to the lentiviral destination vector with the puromycin resistance gene. The packaging of the lentivirus was induced in 293FT cells using the lentiviral destination vector with the human CE gene, the helper vectors (VSVG and PAX2), and a calcium phosphate transfection kit following the manufacturer's protocol (Life Technologies). The supernatant was collected for 72 h. The viral titers were determined by transduction of 293FT cells with serial dilutions of the lentivirus containing the GFP gene, which was produced in parallel. Primary cultured hAT-MSCs were infected with the lentivirus containing the human CE gene using a multiplicity of infection = 1 and 6 μg ml^{-1} polybrene (Sigma-Aldrich). Infected cells were selected with 1 μg ml^{-1} puromycin for 4 days and then with 0.5 μg ml^{-1} puromycin for 4 days. When colonies formed, CE expression was confirmed by reverse transcriptase-PCR and western blotting.

A sTRAIL DNA construct consisting of the Flt3L secretion-inducing domain, the trimerization enforcing domain (the isoleucine zipper sequence) and the receptor-binding domain of the human TRAIL gene (coding for amino acids 114–281)[7] was synthesized (Bioneer, Daejeon, South Korea). The sTRAIL DNA construct was amplified by PCR with specific primers (Supplementary Table 1). The PCR product was inserted into the entry vector and then transferred to the lentiviral destination vector with the GFP gene. To introduce the lentiviral vector with the sTRAIL DNA construct into hAT-MSCs, electroporation was performed using a Neon Transfection System (Life Technologies) following the manufacturer's protocol. Briefly, 6×10^5 hAT-MSCs in a 100 mm^2 culture dish were electroporated with 20 μg of the vector at a pulse magnitude and duration of 1400 V and 20 μs in a GMP facility. Transfection efficiency was determined 48 h after transfection using a fluorescence microscope.

Reverse transcriptase-PCR

Total RNA was extracted with an RNeasy Plus Mini kit (Qiagen, Hilden, Germany). The complementary DNA was synthesized from 1 μg of total RNA using superscript III Reverse Transcriptase (Life Technologies) following the manufacturer's instructions. PCR was conducted with EF-taq DNA polymerase (Solgent, Daejeon, South Korea) with 30 amplification cycles using specific primers for CE, sTRAIL, DR4, DR5 and β-actin (Supplementary Table 1).

Western blotting

Cells were lysed in RIPA lysis buffer (15 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate, 50 mM Tris (pH 8.0)). After centrifugation at 15 000 r.p.m. for 30 min, the supernatant was harvested. The amount of protein was analyzed using a BCA protein assay kit (Thermo, Waltham, MA, USA). Protein samples (30 μg) were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, transferred to polyvinylidene difluoride membranes (Millipore, Billerica, MA, USA), and probed with an anti-CE (Abcam, Cambridge, MA, USA), DR4 (Santa Cruz Biotechnology, Santa Cruz, CA, USA), DR5 or glyceraldehyde 3-phosphate dehydrogenase (Cell Signaling, Danvers, MA, USA) antibody. The primary antibodies were reacted with appropriate horseradish peroxidase-conjugated secondary antibodies (Thermo), which were visualized with Super ECL solution (GE Healthcare, Little Chalfont, Buckinghamshire, UK) following the manufacturer's instructions.

In vitro CPT-11 conversion assay

Cells, 1×10^6 hAT-MSCs or hAT-MSCs with CE (hAT-MSC-CE), were treated with 5 μM CPT-11 (Sigma-Aldrich) for 72 h. The concentrations of SN-38 in the conditioned media were measured by high-performance liquid chromatography-tandem mass spectrometry as previously described.[9]

Enzyme-linked immunosorbent assay

To quantify the amount of sTRAIL protein, 3×10^5 hAT-MSCs were plated in 60 mm^2 dishes and maintained for 72 h. The sTRAIL protein was measured in the conditioned medium with a human TRAIL/TNFSF10 Quantikine ELISA kit (R&D systems, McKinley, MN, USA) according to the manufacturer's protocol.

Cell viability test

Cells, 3×10^3 hAT-MSCs or hAT-MSC-CE, were seeded in each well of 96-well plates and treated with various concentrations of CPT-11 (0, 2.5, 5, 10, 20, 40, 80, 160 and 320 μM) for 72 h. Cell viability was measured enzymatically by an EZ-cytox kit (Daeil Lab Survice, Seoul, South Korea).

TUNEL assay

After 1×10^4 U-87MG cells were seeded in 96-well plates, they were treated with the same concentration of the TRAIL peptide or sTRAIL from the culture medium. After 24 h, TUNEL assays were performed with an ApopTag Apoptosis Detection kit (Millipore) according to the manufacturer's instructions. To quantify apoptosis, TUNEL-positive nuclei were counted ($n = 3$ for each group). Percentages of apoptotic cells were expressed as percentage of TUNEL-positive nuclei to DAPI-stained nuclei.

Co-culture experiment

In all, 1×10^3 U-87MG cells were cultured with 3×10^3 hAT-MSCs, hAT-MSC-CE, hAT-MSC-sTRAIL or hAT-MSC-CE.sTRAIL for 24 h and treated with or without 5 μM CPT-11 for 48 h. U-87MG-specific cell viability was determined by Cytotox 96 non-radioactive cytotoxicity assay (Promega, Dane, WI, USA) according to the manufacturer's instructions.

The brainstem glioma mouse model

All animal studies were approved by the Institutional Animal Care and Use Committee (IACUC) at Seoul National University (IACUC number: 13-0290-COA2). Six-week-old female BALB/c-nude mice (Orient Bio Inc., Sungnam, South Korea) were anesthetized by intraperitoneal injection of 20 mg kg^{-1} Zoletil (Virbac, Carros, France) and 10 mg kg^{-1} xylazine (Bayer, Leverkusen, Germany). The skull was exposed, and a small burr hole was made using a 26-gauge needle. The stereotactic coordinates were 1.5 mm to the right of midline on the lambdoid suture and 5.0 mm depth from the skull surface.[33]

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Then, 4×10^4 U-87MG glioma cells in 3 µl phosphate-buffered saline (PBS) were injected using a 26-gauge Hamilton syringe at injection rate of 1 µl min^{-1}.

In vivo therapeutic efficacy of hAT-MSCs containing therapeutic genes

Three days after the tumor cell implantation, the mice were randomized into six groups and treated with PBS+saline, PBS+CPT-11, hAT-MSCs +CPT-11, hAT-MSC-CE+CPT-11, hAT-MSC-sTRAIL+CPT-11 or hAT-MSC-CE. sTRAIL+CPT-11. The hAT-MSCs were labeled with cell-stalker TM II CSR (Biterials Co., Yongsan, Seoul, South Korea) before injection. The PBS or hAT-MSCs (1.2×10^5 in 6 µl PBS) were stereotactically injected into the established tumor site using the same burr hole and stereotactic coordinates. Two days after the injection, the mice received intravenous injections of saline or CPT-11 (15 mg kg^{-1}) every day for 5 days. After a 2-day rest period, the treatment was repeated. To measure tumor volume, mice were killed at 27 days after the tumor cell implantation. In addition, the overall survival was measured. The development of symptoms requiring euthanasia was considered as mortality. Survival was followed until the mice died or for a maximum of 100 days, at which time animals were killed.

Histological analysis

At the end-point of the short-term tumor volume tests (27 days after the tumor cell transplantation), mice were perfused with 4% paraformaldehyde under deep anesthesia, and brains were harvested. The fixed brain tissues were dehydrated, embedded in an optimum cutting temperature compound (Tissue-Tek, Torrance, CA, USA) and sectioned into 10 µm sections. Sections were stained with hematoxylin and eosin, and tumor volumes were estimated as described previously.[12] For immunofluorescence staining, sectioned tissues were incubated with the primary antibodies, anti-Ki67 or active caspase-3 antibody (Abcam). The proliferating and apoptotic indices were analyzed as the percentage of positively stained cells in five randomly selected fields using a fluorescence microscope.

Statistical analysis

All values were calculated as the means ± s.d. or were expressed as a percentage of controls ± s.d. Multiple groups were analyzed by analysis of variance or Kruskal–Wallis analysis. The survival data were presented as Kaplan–Meier plots and analyzed by the log-rank test. *P*-values < 0.05 were considered statistically significant.

RESULTS

The establishment and functional characterization of hAT-MSCs containing therapeutic genes

In this study, hAT-MSCs were designed to deliver two types of therapeutic genes, human CE and sTRAIL (Figure 1a). CE converts the blood–brain barrier-permeable cytotoxic agent, CTP-11, into

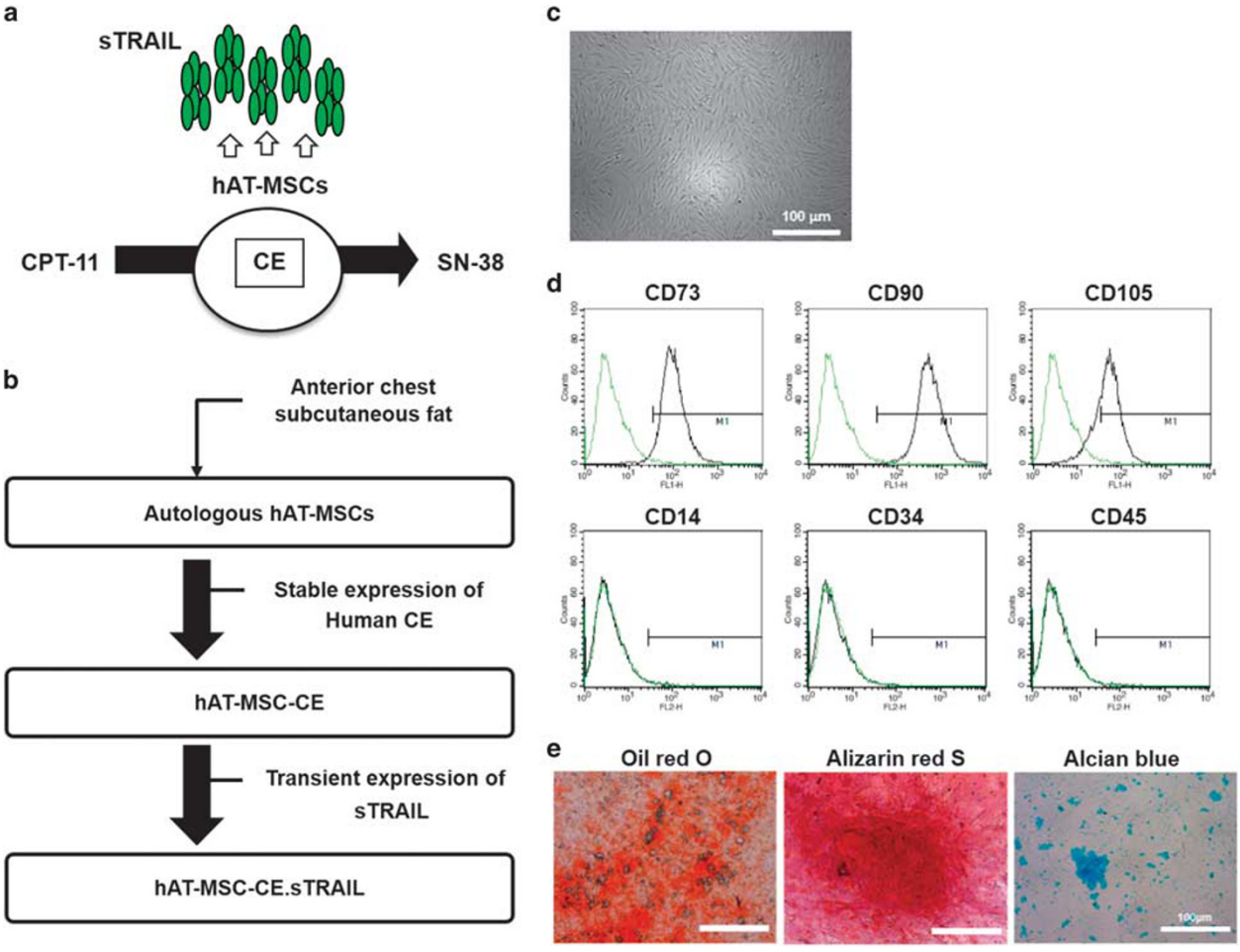

Figure 1. Human adipose-derived mesenchymal stem cells (hAT-MSCs) isolated from the anterior chest subcutaneous fat during operations for vagus nerve stimulation insertion for intractable epilepsy. (**a**) Mode of action of the two therapeutic genes, carboxyl esterase (CE) and a secreted form of tumor necrosis factor-related apoptosis-inducing ligand (sTRAIL) in hAT-MSCs. (**b**) Experimental scheme used to establish hAT-MSCs that produce CE and a sTRAIL (hAT-MSC-CE.sTRAIL). (**c**) Fibroblast-like morphologies of isolated cells. (**d**) Flow cytometric analysis of primary cultured cells. Cells were positive for CD73, CD90 and CD105 but negative for CD14, CD34 and CD45. (**e**) The differentiation potential of primary cultured cells is shown: Oil red O indicates adipocytes, Alizarin red S, osteocytes, and Alcian blue, chondrocytes.

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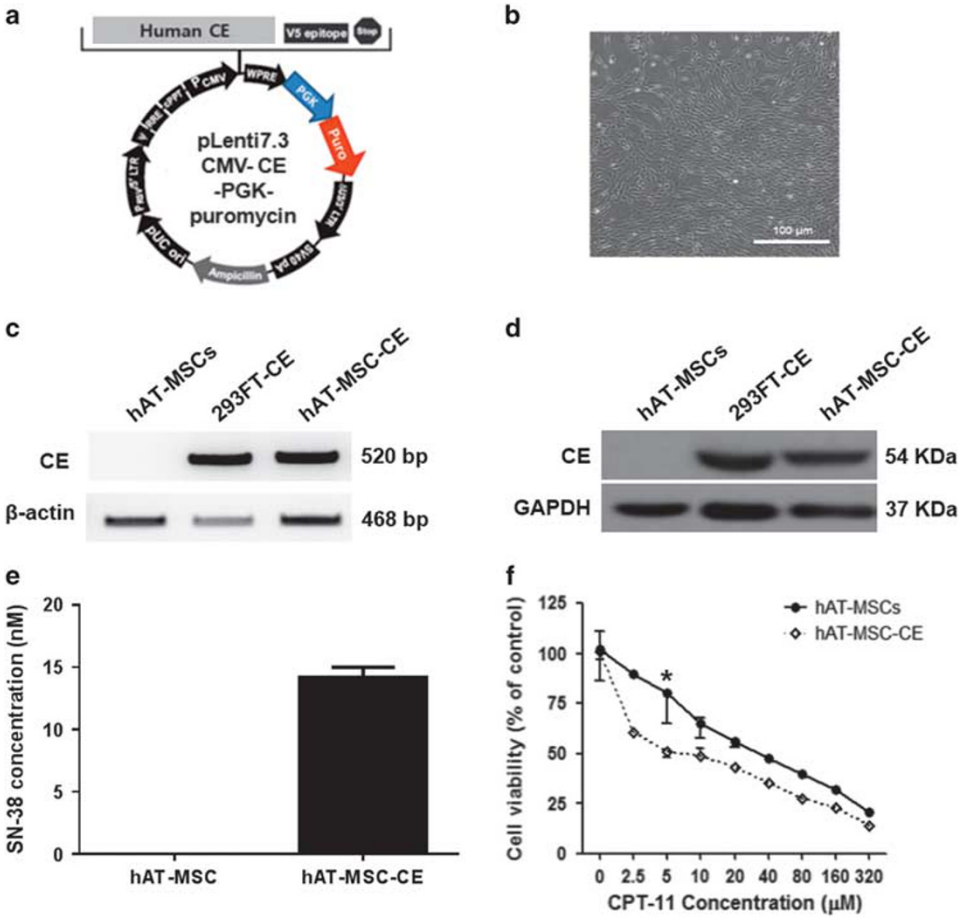

Figure 2. Establishment and characterization of human adipose-derived mesenchymal stem cells (hAT-MSCs) containing the carboxyl esterase gene (hAT-MSC-CE). (**a**) The lentiviral expression vector map for the human CE, pLenti7.3 CMV-CE-PGK-puromycin vector. (**b**) Cellular morphologies of hAT-MSCs after transfection with the lentivirus and selection with puromycin. (**c**) CE expression in hAT-MSCs is confirmed by reverse transcriptase (RT)-PCR and (**d**) western blotting. (**e**) SN-38 concentrations in the conditioned media are measured by HPLC-MS/MS. (**f**) The IC_{50} of CPT-11 against hAT-MSCs and hAT-MSC-CE is determined by measuring cell viability. $^*P < 0.05$.

the more toxic metabolite, SN-38, which would increase local concentrations of SN-38 with low systemic side effects. Moreover, sTRAIL secreted from hAT-MSCs would independently provoke apoptosis of glioma cells through the receptors, DR4 and DR5. The establishment scheme of hAT-MSCs containing the therapeutic genes is illustrated in Figure 1b.

We isolated cells from subcutaneous fat and cultured the fibroblast-like cells in MSC expansion medium (Figure 1c). At passage 3 *in vitro*, the cells were positive for the MSC markers, including CD73, CD90 and CD105, but negative for the hematopoietic markers CD14, CD34 and CD45 (Figure 1d). Moreover, they could differentiate into adipocytes, osteocytes and chondrocytes (Figure 1e). These results indicated that the primary cultured cells were hAT-MSCs.

The human CE therapeutic gene was delivered to primary cultured hAT-MSCs using a lentiviral system for stable expression (Figure 2a). Even after puromycin selection, the cellular morphologies of hAT-MSCs with CE (hAT-MSC-CE) were not significantly altered (Figure 2b). The expression of human CE was confirmed at both the mRNA and protein levels (Figures 2c and d). The conversion of CPT-11 to SN-38 was measured at 72 h after *in vitro* CPT-11 (5 μM) treatment of hAT-MSCs with or without CE. More than 14 nM of SN-38 was detected only in hAT-MSC-CE cells (Figure 2e), validating the function of the introduced human CE gene. Moreover, CPT-11 was more toxic to hAT-MSC-CE ($IC_{50} = 11.0 \pm 4.0$ μM) than hAT-MSCs ($IC_{50} = 31.9 \pm 1.2$ μM; Figure 2f). The expression of the TRAIL receptors (DR4 and DR5) in glioma cells was confirmed by reverse transcriptase-PCR (Figure 3a) and western blotting (Figure 3b). The two bands for splicing variants of DR5 were detected.[34] The DR5 expression level was higher than that of DR4 in U-87MG and T98G glioma cells. It has been reported that TRAIL-induced apoptosis in glioma cells originates from DR5 activation.[35–37] Based on the expression of the DRs in glioma cells, we transiently introduced an sTRAIL DNA construct by electroporation. As TRAIL acts as a trimeric form, a protein sequence that induces trimerization (an isoleucine zipper) was attached to the receptor-binding domain of the human TRAIL protein (amino acids 114–281) (Figure 3c). Moreover, a signal peptide that provokes protein excretion[7] (Flt3L secretion-inducing domain) was added to the TRAIL protein to facilitate its secretion from the hAT-MSCs (Figure 3c).The successful transduction of the sTRAIL DNA expression vector was validated by GFP (Figure 3d). The transduction efficiency was approximately 60%. The accumulation of sTRAIL in the culture medium was confirmed by enzyme-linked immunosorbent assay at 12, 24, 48 and 60 h after electroporation (Figure 3e). The sTRAIL collected from the culture medium induced the apoptosis of U-87MG cells with a potency equivalent to the positive control, a commercially available TRAIL peptide (Figure 3f). When the ratio of TUNEL-positive apoptotic cells of the recombinant TRAIL (22.3 ± 2.5%), hAT-MSC-sTRAIL (27.0 ± 6.6%)

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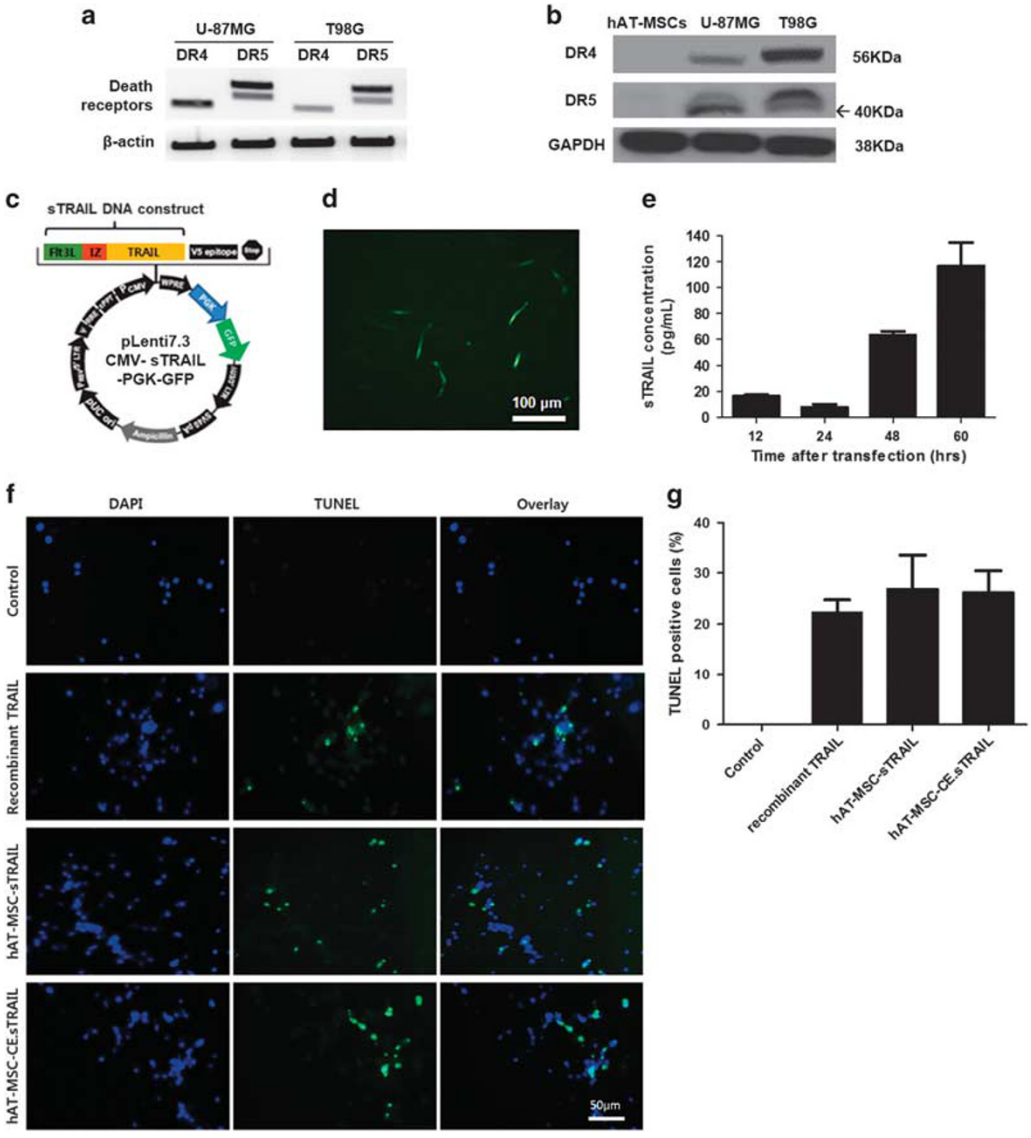

Figure 3. Establishment and characterization of human adipose-derived mesenchymal stem cells (hAT-MSCs) a secretary form of tumor necrosis factor-related apoptosis-inducing ligand (hAT-MSC-sTRAIL). (**a**, **b**) DR4 and DR5 expression in glioma cells was confirmed by reverse transcriptase (RT)-PCR (**a**) and western blotting (**b**). (**c**) The lentiviral expression vector map for the sTRAIL, pLenti7.3 CMV-sTRAIL-PGK-GFP vector. (**d**) Transfection efficiency of the sTRAIL lentiviral vector was confirmed by GFP expression using fluorescence microscopy. (**e**) Concentrations of sTRAIL from hAT-MSC-sTRAIL culture media were examined by enzyme-linked immunosorbent assay (ELISA) at 12, 24, 48 and 60 h after transfection. (**f**) The sTRAIL-induced apoptosis signal (green) in U-87MG cells was observed by TUNEL assays. DAPI (blue) = nuclei. (**g**) Apoptotic cell death was quantified by the percentage of TUNEL-positive cells in the DAPI-stained cells.

and hAT-MSC.CE.sTRAIL (26.3 ± 4.2%) group was quantified, the ratios were not significantly different (Figure 3g).

The efficacy *in vitro* of hAT-MSCs expressing therapeutic genes

To measure the treatment efficacy of hAT-MSCs with or without therapeutic genes against glioma cells *in vitro*, hAT-MSCs, hAT-MSC-CE, hAT-MSC-sTRAIL and hAT-MSC-CE.sTRAIL were established as indicated (Figure 1b). Expression of CE and/or sTRAIL was confirmed (Figure 4a). Although the sTRAIL mRNA levels were highest at 24 h after electroporation, sTRAIL accumulated continuously in the culture medium of hAT-MSC-CE.sTRAIL (Figure 4b). To confirm the therapeutic effects of hAT-MSC-CE, hAT-MSC-sTRAIL and hAT-MSC-CE.sTRAIL cells, specific viability of U-87MG cells was measured after co-culture. CPT-11 alone showed *in vitro* toxicity to U-78MG cells in the hAT-MSC+CPT-11 group (Figure 4c). Comparing with the hAT-MSCs+CPT-11 group, hAT-MSC-CE, hAT-MSC-sTRAIL and hAT-MSC-CE.sTRAIL cells reduced U-87MG cell survival significantly more after CPT-11 treatment. Furthermore, hAT-MSC-CE.sTRAIL cells showed more potent cytotoxic effects than hAT-MSC-sTRAIL cells (Figure 4c, $P < 0.05$), indicating the additive or synergistic effects of CE with sTRAIL.

The brainstem glioma mouse model

To test the effects of hAT-MSCs with therapeutic genes against brainstem glioma *in vivo*, xenograft transplantation was adopted. However, previous animal models for brainstem glioma were based on immune competent rats because of technical issues and would provoke acute immune rejection against human cells. To

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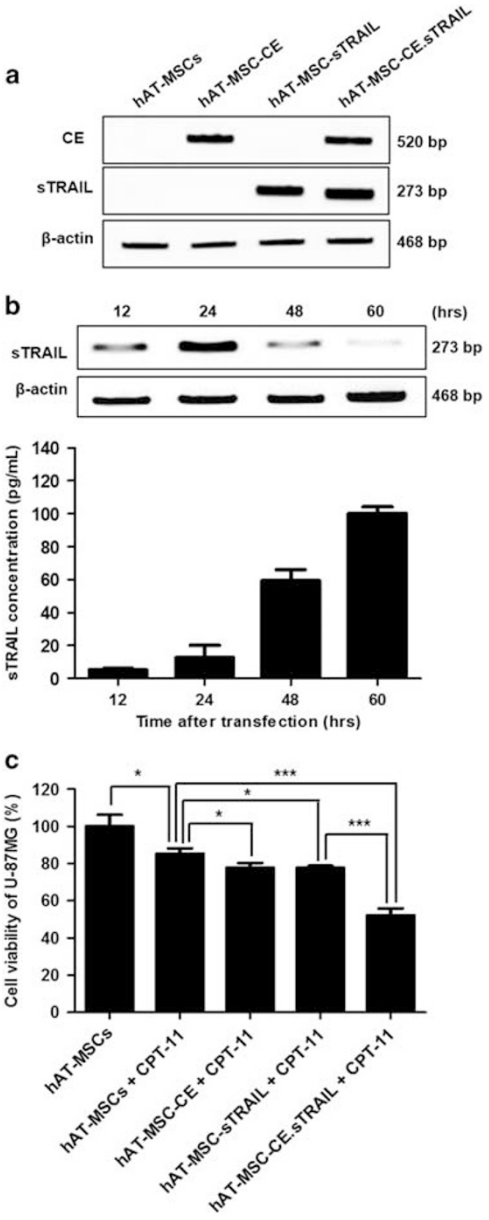

Figure 4. *In vitro* bystander effects of human adipose-derived mesenchymal stem cells (hAT-MSCs) producing carboxyl esterase (CE) and a secreted form of tumor necrosis factor-related apoptosis-inducing ligand (hAT-MSC-CE.sTRAIL). (**a**) CE and sTRAIL expression in hAT-MSCs-CE ± sTRAIL was confirmed by reverse transcriptase (RT)-PCR. (**b**) sTRAIL expression in hAT-MSC-CE.sTRAIL, and sTRAIL concentrations in the conditioned medium of hAT-MSC-CE.sTRAIL were measured by RT-PCR and enzyme-linked immunosorbent assay (ELISA), respectively, at 12, 24, 48 and 60 h after electroporation. (**c**) U-87MG cells and hAT-MSC-CE ± sTRAIL were co-cultured with 5 μM CPT-11 at 3:1 ratio. After 48 h in co-culture, U-87MG cell survival was determined. $^{*}P < 0.05$, $^{***}P < 0.001$.

overcome this limitation, we adopted the brainstem glioma animal model using immune compromised BALB/c-nude mice.[33,38] We first confirmed that stereotactic injection could target the brainstem of mice using blue ink (Figures 5a and b). Then, 4×10^4 human U-87MG glioma cells were injected to the brainstem of BALB/c-nude mice ($n = 6$). At 28 days after the implantation, brainstem gliomas were consistently formed in all mice (Figures 5c and d).

The therapeutic potential *in* vivo of hAT-MSCs expressing therapeutic genes

Using the established brainstem glioma mouse model, we evaluated the differential therapeutic efficacies of hAT-MSC, hAT-MSC-CE, hAT-MSC-sTRAIL and hAT-MSC-CE.sTRAIL cells. Six groups of mice were treated, including Group 1 = PBS+saline, Group 2 = PBS+CPT-11, Group 3 = hAT-MSC+CPT-11, Group 4 = hAT-MSC-CE+CPT-11, Group 5 = hAT-MSC-sTRAIL+CPT-11 and Group 6 = hAT-MSC-CE.sTRAIL+CPT-11 (Figure 6a; $n = 15$ for each group). The treatment schedule for the evaluation of short-term tumor volume is indicated in Figure 6b. Compared with the tumor volume of the hAT-MSC+CPT-11 group ($30.5 \pm 4.9\ mm^3$), the hAT-MSC-sTRAIL+CPT-11 ($13.9 \pm 8.1\ mm^3$, $P < 0.01$) and hAT-MSC-CE.sTRAIL+CPT-11 groups ($10.4 \pm 4.8\ mm^3$, $P < 0.001$) showed significantly smaller tumor volumes (Figures 6c and f). However, there were no significant tumor volume differences among the PBS+saline ($38.5 \pm 9.2\ mm^3$), PBS+CPT-11 ($35.4 \pm 8.4\ mm^3$), hAT-MSCs+CPT-11 and hAT-MSC-CE+CPT-11 ($32.6 \pm 4.9\ mm^3$) groups. Apoptotic cell death increased only in the hAT-MSC-sTRAIL +CPT-11 and hAT-MSC-CE.sTRAIL+CPT-11 groups compared with the hAT-MSCs+CPT-11 group (Figures 6d and g). In contrast, there were no changes in the proliferation of U-87MG cells (Figures 6e and h).

The significant treatment effects *in vivo* were confirmed by survival analysis. The establishment of brainstem animal models and treatment schedules was identical to that in the previous experiments (Figure 7a). Consistent with the previous results, significant survival benefits were observed only in the hAT-MSC-sTRAIL+CPT-11 and the hAT-MSC-CE.sTRAIL+CPT-11 groups compared with the hAT-MSCs+CPT-11 group (Figures 7b and c). However, a synergistic improvement of the treatment effects of the hAT-MSC-CE.sTRAIL+CPT-11 group compared with the hAT-MSC-sTRAIL+CPT-11 group was not observed *in vivo* ($P = 0.6159$), although there was a statistically significant difference between the hAT-MSCs+CPT-11 group and the hAT-MSC-CE.sTRAIL+CPT-11 group (Figures 7b and c).

DISCUSSION

An essential clinical limitation in brainstem glioma treatment is the absence of therapeutic modalities to target glioma cells in critical parts of the brain. Many research groups have demonstrated that stem cells can deliver therapeutic genes to cancer cells in the brain parenchyma with significant therapeutic benefits.[8–11,22,37,39–47] However, there are several issues to be resolved for their actual clinical application: (1) avoidance of possible immunological rejection of allograft stem cells, (2) immunological safety of therapeutic genes that are not expressed in human tissues, (3) genetic stability of stem cells that could be affected by the transfer of therapeutic genes to stem cells and (4) selection of adequate therapeutic genes for the maximal treatment effects.

In this study, we aimed to establish clinically applicable stem cells expressing therapeutic genes for the treatment of brainstem gliomas. To address the previous issues, several strategies were adopted in this study: (1) autologous MSCs were cultured from human subcutaneous fat in a GMP facility, (2) CE, a therapeutic gene, was cloned from human cells to avoid using non-human proteins by virus infection, (3) the integration of sTRAIL therapeutic gene into the genomic DNA of the stem cells was prevented by the use of a non-viral transfection method, that is, electroporation and (4) stem cells were engineered to express two therapeutic genes, CE and sTRAIL, which have independent mechanisms of action and should have synergistic therapeutic effects.

The hAT-MSCs from the subcutaneous fat showed *in vitro* proliferation and differentiation potentials consistent with previous reports.[4,5] These results indicated that autologous hAT-MSCs

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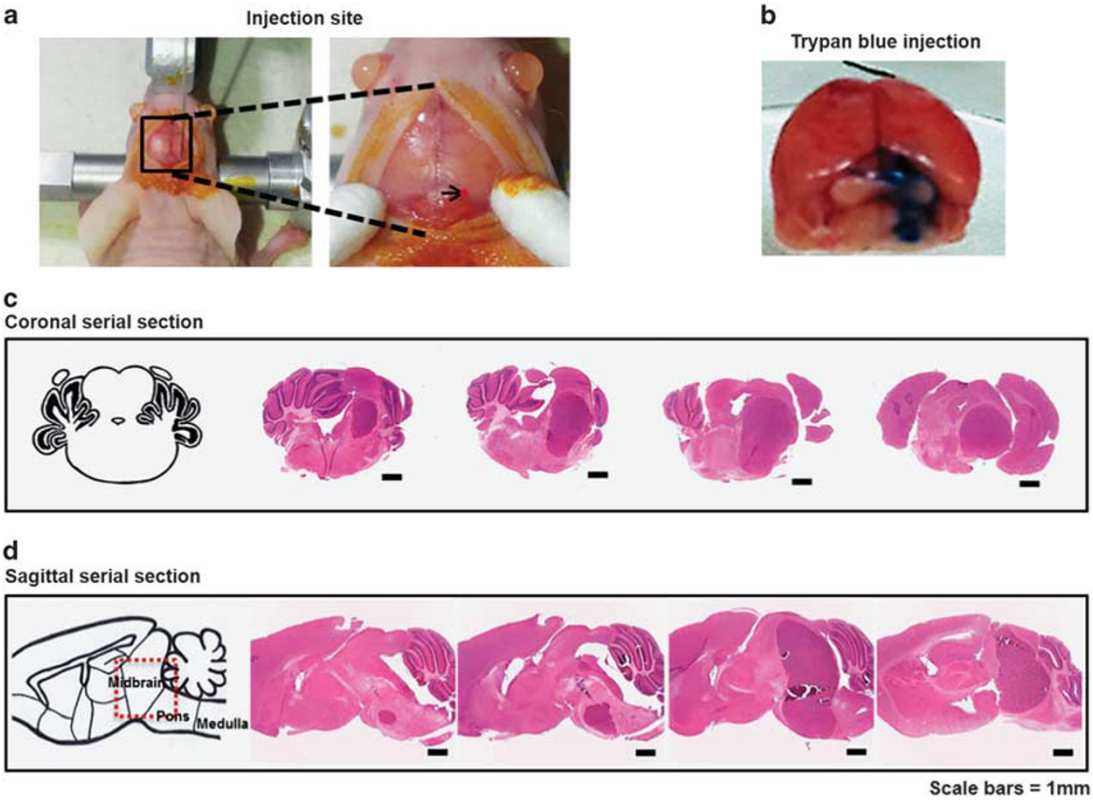

Figure 5. Xenograft brainstem glioma mouse model. (**a**) Immune-deficient BALB/c-nude mice were mounted on the stereotactic device for injection. The stereotactic coordinates show the site for drilling (red dot, arrow) in relation to the mouse skull landmarks. (**b**) Stereotactic injection targeted the pons of the mice. (**c**, **d**) Hematoxylin and eosin (H&E) staining of coronal (**c**) and sagittal (**d**) sections demonstrate the establishment of the brainstem gliomas in the mouse brains.

could be prepared in the clinic during minimal surgical interventions. As the hAT-MSCs proliferated continuously *in vitro*, the therapeutic genes could be transferred to the hAT-MSCs by a physical method, electroporation. Compared with chemical gene transfer technologies, electroporation does not involve any possibly toxic chemicals, a property that could be beneficial in producing clinically applicable stem cells expressing therapeutic genes. Nevertheless, several limitations, including low transfection efficiency and restricted duration of sTRAIL expression, need to be considered.

To increase the therapeutic effects of hAT-MSCs, we established hAT-MSCs that express two therapeutic genes, CE and sTRAIL, simultaneously. Although rabbit CE has been commonly utilized as a therapeutic gene,[48] we transferred human CE to hAT-MSCs to avoid using proteins of non-human origin. Although synergic effects were observed *in vitro*, hAT-MSC-CE.sTRAIL and hAT-MSC-sTRAIL showed similar treatment outcomes in two independent experiments *in vivo*. These unexpected results could be because of the utilization of human CE. Although the activity of human CE in converting CPT-11 to SN-38 was directly validated by measuring SN-38 concentrations in the conditioned medium, it has been reported that rabbit CE is 100- to 1000-fold more efficient at converting CPT-11 to SN-38 than human CE. The level of SN-38 (14.28 nM) in this study is the concentration that could reduce the cell viability *in vitro,* but it may not be enough *in vivo*.[49–51] The low efficacy of the CE gene *in vivo* may be caused by the low activity of the human enzyme. In contrast to CE, the therapeutic gene, sTRAIL, was successfully expressed in hAT-MSCs, and the protein was secreted into conditioned media *in vitro*. sTRAIL mRNA was maintained only transiently after physical gene transfer. The cytotoxic activities of sTRAIL against human glioma cells were validated *in vitro* and *in vivo*. In the case of delivering a suicide gene, including CE, early drug treatment would cause cell death before producing the therapeutic efficacy. Although sTRAIL combined with CE showed significant *in vitro* and *in vivo* tumor cell killing effects, the hAT-MSC-CE.sTRAIL cells could be destroyed by early injection of CPT-11. Actually, hAT-MSC-sTRAIL+CPT-11 group showed longest survival day albeit it is not statistically significant. In subsequent studies, proper doses and times should be considered.

Other studies of combined therapeutic gene therapy using stem cells have been conducted. The preclinical study of neural stem cells expressing two therapeutic genes (HSV-TK and cytosine deaminase) showed effective inhibition of glioma development.[40,46,47] In addition, another investigation showed that human amniotic fluid-derived MSCs delivering endostatin and CE2 exhibit antitumor effects in a mouse glioma xenograft model.[52] Those previous studies indicated that the selection of adequate therapeutic gene combinations could increase the treatment efficacy of stem cells against brainstem gliomas, although sTRAIL +CE showed little synergistic effect in this study.

In this study, we have adopted a mouse brainstem glioma model using intracranial injection of human U-87MG glioma cells into the pontine region of BALB/c-nude immune deficient mice.[33] This mouse brainstem glioma model has several benefits. (1) The pathology of the tumors closely resembles that of the intrinsic pontine gliomas. (2) BALB/c-nude mice are free from immunological problems in xenograft model systems. (3) The mouse model entails less expense in buying, housing, handling, and imaging the animal models and smaller amounts of drugs in therapeutic tests compared with previous rat models.[32,38]

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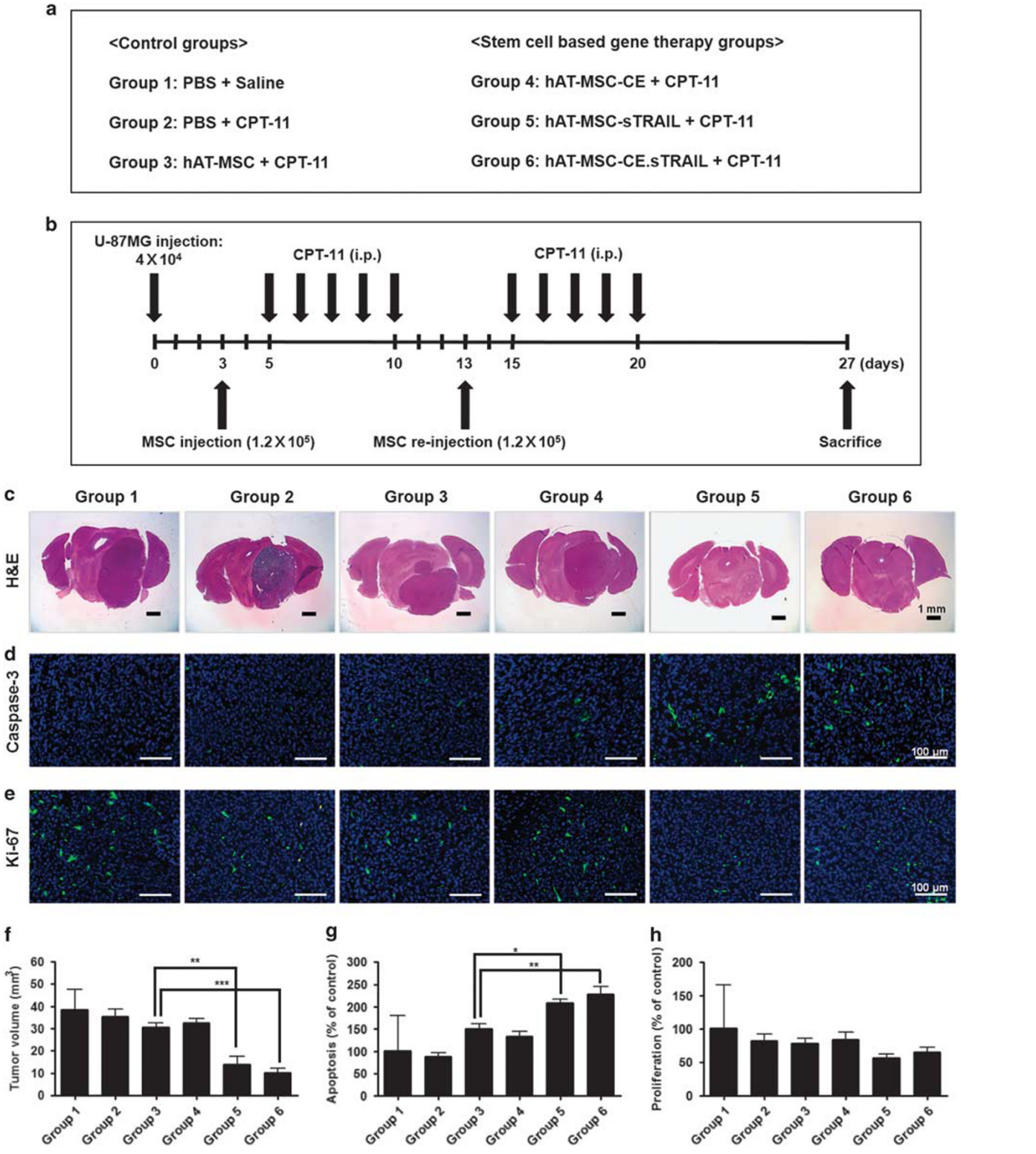

Figure 6. The therapeutic effects *in* vivo of hAT-MSCs containing therapeutic genes against brain gliomas. (**a**) Mice were randomly divided into six groups ($n = 15$ for each group). (**b**) Schematic plot of the study design for short-term therapeutic efficacy. (**c**-**h**) Twenty-seven days after the tumor cell transplantation, tumor volume (**c**, **f**), apoptotic cell death (**d**, **e**; green = active caspase-3) and proliferation potential of U-87MG glioma cells (**e**, **h**; green = ki-67-positive proliferating cells) were compared. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$.

Based on the results of this study, we hypothesize that autologous hAT-MSCs could produce significant treatment benefits against brainstem gliomas when the sTRAIL gene is transferred alone into the cells transiently. This protocol would not provoke immunological side effects nor require genomic DNA integration of the therapeutic genes, which would maintain the genomic stability of autologous hAT-MSCs. We anticipate that the design of the present study could serve as a prototype of stem cell-based

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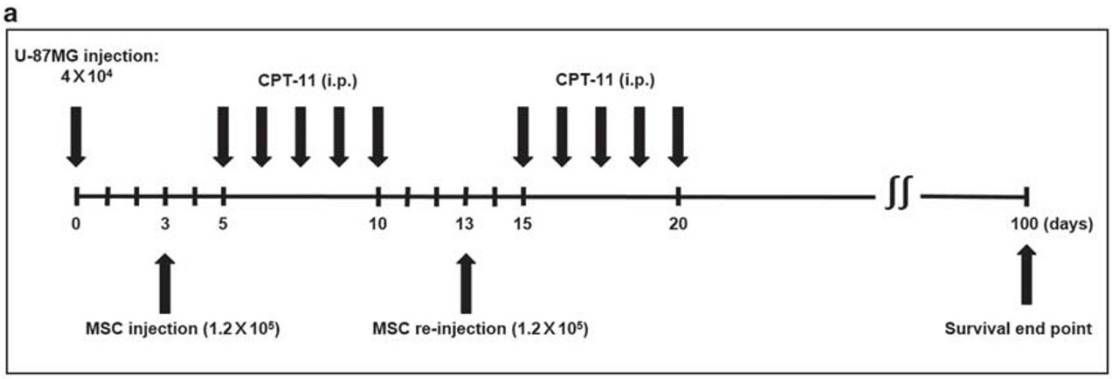

b

	Group 1	Group 2	Group 3	Group 4	Group 5	Group 6
Median survival	31	29.5	30.5	30.5	36	36

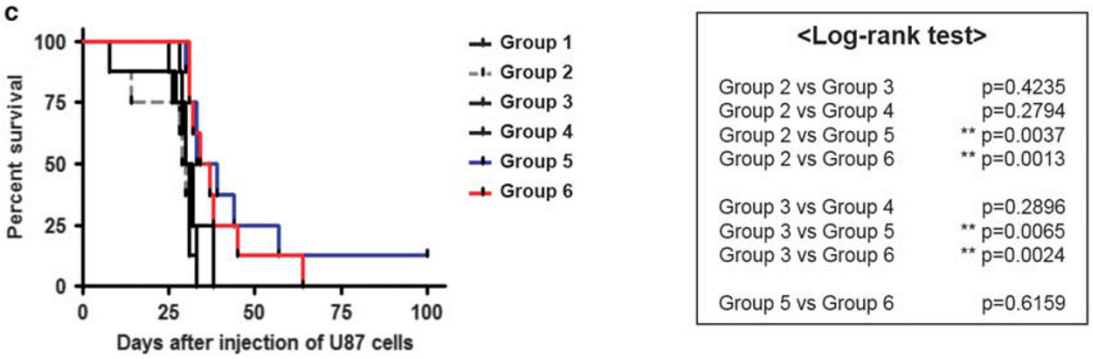

Figure 7. The effects of human adipose-derived mesenchymal stem cells (hAT-MSCs) containing therapeutic genes on the long-term survival of brainstem glioma-bearing mice. (**a**) Schematic plot of the study design for long-term therapeutic efficacy. (**b**) Median survival time of the groups (days). (**c**) Kaplan–Meier survival curves and results of a long-rank test.

gene therapy for brainstem gliomas, although further technical advances could potentiate the therapeutic efficacy.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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