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Original Articles

Adipose tissue-derived mesenchymal stem cells cultured at high density express IFN-β and TRAIL and suppress the growth of H460 human lung cancer cells

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ABSTRACT

Although mesenchymal stem cells (MSCs) have been reported to inhibit tumor growth, the mechanism controlling this tumor suppression function is unclear. Here, we report that high-density (40,000 cells/cm^2) cultured adipose tissue-derived MSCs (40K-ASCs) expressed interferon (IFN)-β and tumor necrosis factor-related apoptosis-inducing ligand (TRAIL); we also found that serum deprivation during cell culture induced the expression of IFN-β and TRAIL. In addition, the mRNA expression of *IFN-β*, but not *TRAIL*, was increased during the washing step required for the transplantation of normal-density (5000 cells/cm^2) cultured ASCs (5K-ASCs). When the human lung cancer cell line H460 was co-cultured with 40K-ASCs, necrotic cell death was dramatically increased *in vitro*. When ASCs were injected after four washes, both 5K-ASCs and 40K-ASCs substantially reduced tumor weight in H460-derived cancer animal models. These results suggest that serum deprivation during the culture of 40K-ASCs or during the washing step of 5K-ASCs can induce IFN-β and/or TRAIL expression, ultimately leading to the tumor suppression capability of ASCs.

1. Introduction

Although mesenchymal stem cells (MSCs) are utilized in regenerative medicine, the development of tumor tropism and promotion of tumorigenesis in pre-existing tumors limit their applicability in cancer patients [1,2]. MSCs support tumor growth through differentiation to tumor-associated fibroblasts [3–6]; suppression of immune responses [7]; promotion of angiogenesis [4,8,9]; stimulation of epithelial-mesenchymal transition [10,11]; interaction with cancer stem cells [12,13]; promotion of tumor metastasis [1,14–17]; and inhibition of tumor cell apoptosis [18–20]. MSCs have also been reported to prevent tumor growth through apoptosis induction, cell cycle and cellular signaling regulation, and immune cell infiltration induction [21].

Interferons (IFNs) are pleiotropic cytokines that regulate the innate and acquired immunity, activate host defenses against viral and bacterial infections, and function in tumor surveillance. IFNs comprise two main families: type I (IFN-α, -β, -ε, -κ, and -ω) and type II (IFN-γ) [22]. IFN-α and IFN-β are secreted by many cell types, including lymphocytes (natural killer [NK] cells, B-cells, and T-cells), macrophages, fibroblasts, endothelial cells, and osteoblasts [23]. They stimulate both

Abbreviations: 40K-ASC-CM, CM recovered from 40K-ASCs; 7-AAD, 7-aminoactinomycin D; APC, allophycocyanin; ASCs, adipose tissue-derived MSCs; Cas, caspase; CFSE, carboxyfluorescein diacetate succinimidyl ester; CM, condition medium; DMEM, Dulbecco's modified Eagle's medium; ELISA, enzyme-linked immunosorbent assay; FBS, fetal bovine serum; H460*, CFSE-labeled H460; HBSS, Hank's Balanced Salt solution; IFNs, interferons; mbTRAIL, membrane-bound TRAIL; MSCs, mesenchymal stem cells; MTT, methylthiazolyldiphenyl-tetrazolium bromide; PBS, phosphate-buffered saline; RT-PCR, reverse transcription-polymerase chain reaction; SD, standard deviation; sTRAIL, secreted TRAIL; TRAIL, tumor necrosis factor-related apoptosis-inducing ligand

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macrophages and NK cells to elicit potent anti-tumor, anti-viral, and immunomodulatory functions [24,25]. Moreover, MSCs that are primed or genetically modified with type I and II IFNs exert antitumor effects via nuclear factor-κB (NF-κB), signal transducer and activator of transcription (Stat), or tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) pathways [26–30].

Previously, we reported that adipose tissue-derived MSCs (ASCs) cultured at a high density expressed type I IFNs and suppressed tumor growth in MCF-7 breast cancer cells [31]. Herein, we investigated TRAIL expression in ASCs cultured at a high density, analyzed the mechanisms controlling IFN-β and TRAIL expression, and evaluated the tumor suppression potential of ASCs against H460 lung cancer cells *in vitro* and *in vivo*.

2. Materials and methods

2.1. Cell culture

In accordance with the procedures approved by the Institutional Review Board of the Yonsei University Wonju College of Medicine, human adipose tissues from three healthy donors (24–38 years of age) were obtained through elective liposuction procedures under anesthesia at the Wonju Severance Christian Hospital (Wonju, Korea). Informed consent was obtained from all donors. Mononuclear cells were isolated using a modified protocol as described by Zuk et al. [32]. Briefly, lipoaspirates were thoroughly washed with phosphate-buffered saline (PBS) to remove contaminated blood cells and local anesthetics. Mononuclear cells were then obtained by digestion with 0.075% type IA collagenase (Sigma-Aldrich, St. Louis, MO, USA) in PBS, centrifugation at 1200 × *g* for 5 min, and resuspension in low-glucose Dulbecco's modified Eagle's medium (DMEM; Gibco, Rockville, MD, USA) containing 10% fetal bovine serum (FBS) and penicillin/streptomycin. Debris was removed by filtration through a 100-μm mesh filter (Cell Strainer; Becton Dickinson, Franklin Lakes, NJ, USA). Mononuclear cells (5×10^6) were seeded in a 100-mm culture dish with low-glucose DMEM containing 10% FBS and penicillin/streptomycin. After 2 days, the medium was replaced to remove non-adherent cells. Thereafter, the cell culture medium was changed twice weekly, and the cells were passaged with 0.25% trypsin/0.1% EDTA (Gibco) until they reached 90% confluence. For experiments, ASCs were seeded at 5,000, 10,000, 20,000, or 40,000 cells/cm^2 (5K-ASCs, 10K-ASCs, 20K-ASCs, and 40K-ASCs, respectively) and cultured for the indicated times (1–5 days).

The human lung cancer cell line H460 was purchased from the Korean Cell Line Bank (Seoul, Korea). H460 cells were maintained in low-glucose DMEM (Gibco) supplemented with 10% FBS and penicillin/streptomycin. H460 and ASCs were indirectly co-cultured using a Transwell plate. 5K-ASCs or 40K-ASCs were cultured for 3 days (upper chamber) and then co-cultured with H460 cells (lower chamber) for a further 2 days to observe changes in cell morphology or for 1 day to analyze apoptosis rates through annexin-V staining. For direct co-culture, H460 cells were labeled with carboxyfluorescein diacetate succinimidyl ester (CFSE, Sigma-Aldrich). Briefly, H460 cells were suspended in PBS (1×10^7 cells/ml) and incubated with 5 μM CFSE in the dark at room temperature for 15 min. An equal volume of FBS was added, incubated for an additional 5 min at room temperature in the dark, and washed three times with cold PBS. The same number of 40K-ASCs cultured for 3 days before co-culture with CFSE-labeled H460 (H460*) cells was seeded and then cultured for an additional 2 days.

2.2. Reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA was extracted from 1×10^5 cells using TRIzol reagent according to the manufacturer's instructions (Gibco). Total RNA (2 μg) was reverse-transcribed with MMLV reverse transcriptase (Bioneer, Daejeon, Korea) for 1 h at 42 °C with oligo-dT primers. PCR was performed using Taq DNA polymerase (Bioneer). The specific primers used for the RT-PCR assays were 5′-TTCGAAGCCTTTGCTCTGGCAC-3′ (sense), 5′-AGATGGTCAATGCGGCGTCC-3′ (antisense) for *IFN-β*; 5′-AAGGGCTTCAGTGACCGGCT-3′ (sense), 5′-GGAGTCTTTCTAACGAGCTGACGG-3′ (antisense) for *TRAIL*; and 5′-CAAGGCTGAGAACGGGAAGC-3′ (sense), 5′-AGGGGGCAGAGATGATGACC-3′ (antisense) for *GAPDH*. Amplified products were subjected to 2% agarose gel electrophoresis and photographed using the FluorChem FC2 system (Alpha Innotech, Santa Clara, CA, USA). All results were normalized against *GAPDH*.

2.3. Immunoblotting

To detect TRAIL protein expression, cells were lysed in sample buffer (62.5 mM Tris-HCl [pH 6.8], 1% sodium dodecyl sulfate, 10% glycerol, and 5% β-mercaptoethanol), boiled for 5 min, subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and transferred to an Immobilon 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 TRAIL (1:1000; R&D Systems, Minneapolis, MN, USA); caspase (Cas)-3, cleaved Cas-3, and Cas-8 (1:1000; Cell Signaling Technology, Danvers, MA, USA); and GAPDH (1:1000; Santa Cruz Biotechnology, Dallas, TX, USA). The membranes were then incubated with horseradish peroxidase-conjugated secondary antibodies (1:2,000, Santa Cruz Biotechnology), treated with EZ-Western Lumi Pico (Dogen, Seoul, Korea), and visualized using the FluorChem FC2 system (Alpha Innotech).

2.4. Membrane-bound antigen analysis

To confirm whether ASCs express membrane-bound TRAIL (mbTRAIL) or DR5, a TRAIL receptor, ASCs were stained with antibodies conjugated with Fluor 488 or allophycocyanin (APC) against mbTRAIL (*i.e.*, mbTRAIL-Fluor 488 antibody) or DR5 (*i.e.*, DR5-APC antibody), respectively (BD Biosciences, San Jose, CA, USA). A total of 5×10^5 cells were resuspended in 0.2 ml PBS and incubated with mbTRAIL-Fluor 488 or DR5-APC antibodies for 20 min at room temperature. Fluor 488- or APC-conjugated mouse IgGs were used as the control isotype at the same concentration as the specific primary antibodies. The fluorescence intensity of the cells was evaluated by flow cytometry (BD FACSAria III; BD Biosciences), and the data were analyzed using BD FACSDiva software (BD Biosciences).

2.5. Enzyme-linked immunosorbent assay (ELISA)

To determine the concentration of secreted TRAIL (sTRAIL), ASCs were cultured at different seeding densities (5K–40K). After 5 days of culture, conditioned medium (CM) was recovered by centrifugation and then stored at −80 °C until analysis. Secreted TRAIL concentrations were measured using a human TRAIL Quantikine ELISA kit (R&D Systems) according to the manufacturer's instructions.

2.6. MTT assay

H460 cells were plated at 1×10^4 cells/cm^2 in 96-well plates and cultured for 1 day. Since the CM of 40K-ASCs may be deficient in glucose and amino acids [31] and this nutritional deficiency may influence H460 cell death, the CM obtained after a 5-day culture of 40K-ASCs was concentrated using centrifugal filter units (Millipore). The H460 cells were then treated with recombinant TRAIL (rTRAIL; R&D Systems) and concentrated- or normal CM from 40K-ASCs cultured for 1–5 days and further cultured for 1 day. Methylthiazolyldiphenyl-tetrazolium bromide (MTT; Sigma-Aldrich) dissolved in PBS was added to each well (final concentration: 5 mg/ml) and incubated at 37 °C for 2 h. MTT formazan was dissolved in 100 μl dimethyl sulfoxide and

incubated for a further 15 min with shaking before measuring the optical density at 570 nm of each well on a microplate reader (BioTek Instruments, Winooski, VT, USA).

2.7. Annexin-V/7-AAD staining

The PE-Annexin-V apoptosis detection kit I (BD Biosciences) was used according to the manufacturer's instructions. Cells were harvested, washed twice with cold PBS, and re-suspended in binding buffer. Cells were stained with PE-Annexin-V and 7-aminoactinomycin D (7-AAD) for 15 min at room temperature in the dark. Cells were then analyzed without washing on a flow cytometer (BD FACSAria III) within 1 h.

2.8. Cell cycle analysis

The cellular DNA content of live cells was analyzed by Vybrant DyeCycle Ruby staining (Molecular Probes, Eugene, OR, USA) according to the manufacturer's instructions. Briefly, H460* cells were directly co-cultured with 40K-ASCs for the indicated time periods, trypsinized, and resuspended in 0.5 ml DMEM. Cells were incubated with 5 μM ruby stain at 37 °C for 20 min in the dark. DNA content was evaluated on a flow cytometer (BD FACSAria III), and then the cell cycle stage of H460* was analyzed.

2.9. Animal studies

All animal experiments were performed according to institutional guidelines and approved by the Institutional Animal Care and Use Committee at Yonsei University Wonju College of Medicine. Five-week-old athymic nude mice were purchased from Central Lab Animal Inc. (Seoul, Korea). To completely remove FBS from cells, H460 or ASCs were washed with PBS three times, once with Hank's Balanced Salt solution (HBSS; Sigma-Aldrich), and then resuspended in HBSS for transplantation into nude mice. H460 and ASCs were mixed just prior to co-injection. Cells were suspended in 100 μl HBSS and subcutaneously injected into the flanks of nude mice. Mice were examined three times weekly, and the tumor volume ($L \times W \times H \times 0.52\ mm^3$) was measured weekly using Vernier calipers for 3 weeks. Mice were sacrificed by cervical dislocation; thereafter, tumors were excised and photographed, and tumor weights were measured using an electronic balance.

2.10. Statistical analysis

Data are expressed as the mean ± standard deviation (SD). For multiple comparisons of the mean between different groups, one-way analysis of variance was used, followed by the Scheffé post-hoc test against the control sample. Significance was set at $P \leq 0.05$.

3. Results

3.1. TRAIL expression in 40K-ASCs

Previously, we reported that ASCs cultured at high density expressed type I IFNs and induced cell death in MCF-7 breast cancer cells [31]. Since type I IFNs are known to induce the pro-apoptotic factor TRAIL [33,34], we investigated TRAIL expression after culturing ASCs at different cell densities (5K-, 10K-, 20K-, and 40K-ASCs) for 5 days. IFN-β and TRAIL were expressed only in 20K- and 40K-ASCs, according to RT-PCR and immunoblotting (Fig. 1A and B). In 40K-ASCs, *IFN-β* and *TRAIL* mRNAs were expressed from day 1 and 2, respectively (Fig. 1C). However, TRAIL protein expression was detected from day 4 (Fig. 1D). Additionally, TRAIL expression in 5K-ASCs was confirmed by exogenous IFN-β treatment in a dose-dependent manner (Fig. 1E). TRAIL is a type II transmembrane protein [35], and its extracellular carboxyl-terminal region can be proteolytically cleaved from the cell surface in a vesicle-associated or secreted form [36,37]. Therefore, we analyzed sTRAIL and mbTRAIL expression in 5K-ASCs and 40K-ASCs after 5 days of culture. The mean fluorescence intensity of mbTRAIL increased by approximately 5-fold in 40K-ASCs on day 5 compared to that in 5K-ASCs on day 1. The mean fluorescence intensity of DR5 slightly increased in 5K-ASCs at day 5 and 40K-ASCs at both day 1 and 5, but there was no significant difference between the three groups (Fig. 1F). Furthermore, sTRAIL was detected in 20K-ASCs and 40K-ASCs in a cell dose-dependent manner (Fig. 1G). Taken together, these results indicate that when ASCs were cultured at a density > 20K, IFN-β was expressed, which, in turn, induced TRAIL expression.

3.2. Cell death of H460 by 40K-ASCs

Next, we investigated whether 40K-ASCs expressing IFN-β and TRAIL would induce cell death in the TRAIL-sensitive H460 lung cancer cells. H460 cells were incubated with concentrated- or normal CM recovered from 40K-ASCs cultured for 1–5 days (40K-ASC-CM) or indirectly co-cultured with 40K-ASCs for the same time period using a Transwell system. H460* cells were directly co-cultured with 40K-ASCs. When the H460 cells were treated with concentrated- or normal 40K-ASC-CM, their viability was lowest in the 5-day 40K-ASC-CM group, but the viability in the concentrated CM group was also lower similar to that in the 5 ng/ml rTRAIL group (Fig. 2A). When the H460 cells were co-cultured with 40K-ASCs, the cellular contents, indicative of necrosis, leaked out of the cells, leading to the presence of floating cytosolic particles in the culture medium (Fig. 2B). To determine whether this leakage of cellular contents was related to the necrotic death of H460 cells co-cultured with 40K-ASCs, annexin-V-PE/7-AAD staining was performed. H460 cells co-cultured with 40K-ASCs were annexin-V-negative/7-AAD-positive, suggesting necrotic cell death (Fig. 2C). In addition, when H460* cells were cultured alone, the G0/G1 population was high at day 2, but dead-sub-G0 populations were rarely observed (Fig. 2D). However, approximately 30% of H460* cells directly co-cultured with 40K-ASCs showed sub-G0 cell death from day 1, and more than 60% sub-G0 cell death was observed on day 2 (Fig. 2D). These results suggest that 40K-ASCs could induce necrotic cell death in H460 cells.

3.3. IFN-β and TRAIL stimulate necrotic cell death in H460 cells

Since 40K-ASCs expressed both IFN-β and TRAIL and induced necrotic cell death in H460 cells, we next determined whether both IFN-β and TRAIL were required to induce necrotic cell death. Compared to control H460 cells, H460 cells cultured with either IFN-β or TRAIL for 3 days showed substantially increased apoptotic cell death, confirmed by morphological microscopy and annexin-V/7-AAD staining (Fig. 3A) and the cleavage of Cas-3 and Cas-8 (Fig. 3B). IFN-β and TRAIL induced the apoptotic body, early apoptotic population (annexin-V-positive/7-AAD-negative, 17.3 ± 2.4% for IFN-β and 25.0 ± 3.2% for TRAIL), and the expression of cleaved-cas-3 and -8 in H460 cells (Fig. 3A and B). However, when H460 cells were indirectly co-cultured with 5K-ASCs, IFN-β induced necrotic cell death in H460 cells. The dying cells, which were still attached to the bottom of the culture plate, were identical to the necrotic H460 cells observed at the beginning of the co-culture with 40K-ASCs, and the late apoptotic (annexin-V-positive/7-AAD-positive, 19.9 ± 0.8%) and necrotic population (annexin-V-negative/7-AAD-positive, 24.5 ± 2.7%) was observed, but not early apoptotic population (annexin-V-positive/7-AAD-negative) (Fig. 3A). Additionally, both Cas-3 and cleaved Cas-3 levels were reduced compared to those in IFN-β-treated H460 cells, and cleaved Cas-8 was not observed in H460 cells co-cultured with 5K-ASCs under IFN-β treatment (Fig. 3B). Incubation with 40K-ASC-CM induced cell death in H460 cells, but their viability was recovered by neutralization with anti-IFN-β and anti-TRAIL antibodies. Moreover, cell viability was recovered synergistically at low concentrations of anti-IFN-β (0.05 μg/ml) and anti-TRAIL (0.1 μg/ml) antibody treatment (Fig. 3C). As previously reported, glucose deficiency

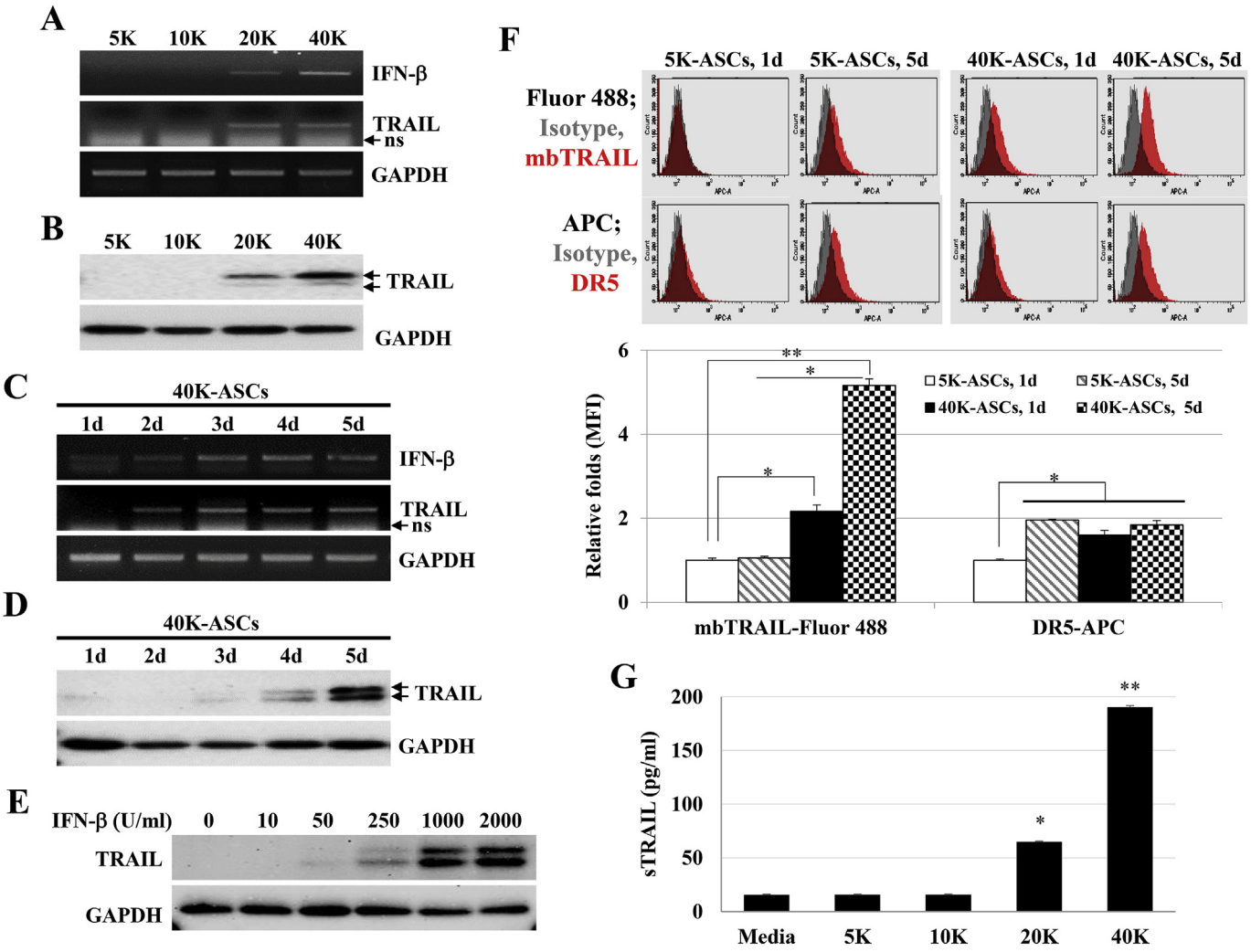

Fig. 1. TRAIL expression in ASCs cultured at high density. A–B) Expression of IFN-β and TRAIL in ASCs at different cell seeding densities. ASCs were cultured at doses of 5000 (5K), 10,000 (10K), 20,000 (20K), or 40,000 (40K) cells/cm^2 for 5 days, and the expression of IFN-β and TRAIL was evaluated by RT-PCR (A) and immunoblotting (B). C–D) TRAIL expression in 40K-ASCs after culture for the indicated time periods using RT-PCR (C) and immunoblotting (D). E) TRAIL expression in 5K-ASCs treated with IFN-β. After treatment with IFN-β for 48 h, TRAIL expression was analyzed by immunoblotting. F) Expression of membrane-bound TRAIL (mbTRAIL) in ASCs. 5K-ASCs or 40K-ASCs were cultured for 1 or 5 days, and the mean fluorescence intensity (MFI) of mbTRAIL was analyzed by flow cytometry after staining with Fluor 488-conjugated mbTRAIL antibody and APC-conjugated DR5 antibody. G) Expression of secreted TRAIL (sTRAIL). ASCs were cultured for 5 days, and conditioned media were harvested by centrifugation. Data represent the mean ± SD from three independent experiments. *$P \leq 0.05$; **$P \leq 0.01$. ns: non-specific band.

was observed in media when ASCs were cultured for 5 days at a cell concentration of > 10K or when cultured at 40K for more than 3 days [31]. Indeed, in the absence of both serum and glucose, TRAIL induced necrotic cell death in H460 cells, but not apoptosis (Fig. 3A). These results suggest that both IFN-β and TRAIL played key roles in inducing necrotic cell death in H460 cells, which depended on nutrient depletion (*i.e.*, serum and glucose) during co-culture with 40K-ASCs.

3.4. Tumor suppression by ASCs

40K-ASCs induced H460 cell death *in vitro* by expressing IFN-β and TRAIL. Therefore, we investigated whether 40K-ASCs could control tumor growth in an *in vivo* tumor model. H460 cells were subcutaneously injected with or without 5K-ASCs or 40K-ASCs into nude mice. When H460 cells were co-injected with 5K-ASCs or 40K-ASCs, tumor volumes were 2.5-fold and 1.5-fold greater than those in the control group after 1 week, respectively. However, from week 2, the tumor volumes of mice in the control group were greater than those of mice in the ASC co-injected groups (Fig. 4A and B). Furthermore, tumor weight was reduced by 15% and 23% at the end of 3 weeks in the 5K-ASC and 40K-ASC co-injected groups, respectively (Fig. 4C). These results show that 5K-ASCs and 40K-ASCs could suppress tumor growth *in vivo*, suggesting that IFN-β and TRAIL expression in 40K-ASCs may more effectively inhibit tumor growth.

3.5. Mechanism of TRAIL expression in ASCs

Since serum depletion is known to induce the expression of type I IFNs in macrophages [38], we investigated whether serum depletion would induce IFN-β and TRAIL expression in ASCs. Interestingly, when 40K-ASCs were cultured in serum-free medium, IFN-β expression was undetectable at 3, 24, and 72 h but was detectable at 6, 12, 96, and 120 h. *TRAIL* mRNA expression was detectable from 3 h and was highly expressed from 24 h (Fig. 5C). TRAIL protein expression in 40K-ASCs was detectable from 48 h and increased gradually in a time-dependent manner (Fig. 5D). However, 5K-ASCs cultured in serum-free media did not express IFN-β or TRAIL (Fig. 5A and B). Moreover, when 40K-ASCs expressing IFN-β and TRAIL were re-seeded at a low density (5K), *IFN-β*

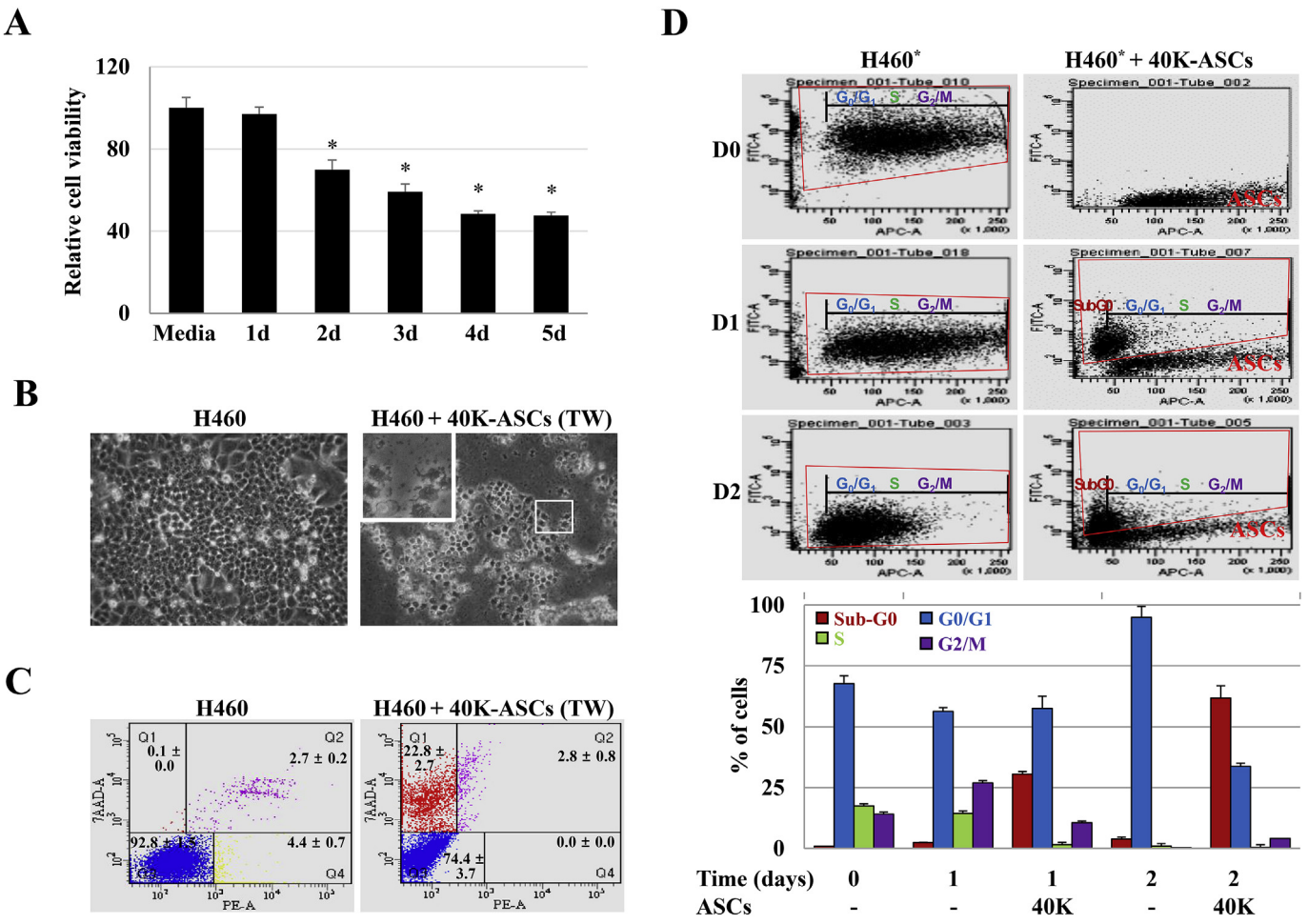

Fig. 2. Cell death of H460 cells after treatment with 40K-ASCs. A) Growth inhibition of H460 cells in conditioned media obtained from cultured 40K-ASCs (40K-ASC-CM) after 1–5 days. H460 cells were treated with 40K-ASC-CM for 24 h, and their viability was assessed using an MTT assay. Error bars represent the mean ± SD of triplicate wells. Data are from one of three independent experiments. *$P \leq 0.05$. B) Morphology of H460 cells indirectly co-cultured with 40K-ASCs. H460 cells were co-cultured with 40K-ASCs for 2 days, and then the morphology of H460 cells was observed using a light microscope (100 ×). C) Annexin-V/7-AAD staining of H460 cells indirectly co-cultured with 40K-ASCs for 2 days. Data represent the mean ± SD of three independent experiments. D) Growth suppression of CFSE-labeled H460 cells (H460*) directly co-cultured with 40K-ASCs. Cell cycle analysis of H460* cells co-cultured with 40K-ASCs or alone for the indicated time points by Vybrant DyeCycle Ruby staining. Approximate H460* cell populations are marked with red squares (upper panel); only the cell cycle stages of H460* cells were analyzed, represented by a bar graph (lower panel). Error bars represent the mean ± SD from triplicate analyses. Data were obtained from one of three independent experiments. TW: Transwell. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

and *TRAIL* mRNA was expressed during sub-culturing, but TRAIL protein expression was not observed (Fig. 5E and F). These results suggest that serum-free high-density culture of stem cells may induce IFN-β and TRAIL expression.

Although nude mice co-injected with H460 tumor cells and 40K-ASCs showed reduced tumor weight in our xenograft tumor model, 5K-ASCs also significantly reduced tumor weight (Fig. 4). We therefore investigated whether inducing IFN-β and TRAIL expression before transplanting 5K-ASCs into experimental animals would increase their anti-tumor activity. First, we examined whether IFN-β and TRAIL could be expressed in 5K-ASCs after four washes with PBS and HBSS—a routine procedure to completely remove serum components before transplantation. 5K-ASCs cultured for 5 days were treated with trypsin, washed three times with PBS and once with HBSS, resuspended in HBSS, and incubated at room temperature for the indicated times. *IFN-β* mRNA expression was observed in the washed 5K-ASCs; it gradually increased until 4 h and decreased thereafter (Fig. 6A). However, *TRAIL* mRNA and protein expression was not detected (Fig. 6A and B). Next, we investigated whether IFN-β and TRAIL expression could be detected in 5K-ASCs at any of the five washing steps, and whether the expression of these cytokines would be altered when ASCs were washed with PBS or HBSS supplemented with serum. Interestingly, *IFN-β* mRNA expression was observed only in ASCs after serum-free washing (Fig. 6C–F). Moreover, when 5K-ASCs were washed with serum-free PBS, *IFN-β* mRNA expression was observed after the second wash (Fig. 6C). Taken together, these results indicate that when ASCs were exposed to stresses such as serum starvation at a high cell density, IFN-β was expressed, leading to the induction of TRAIL expression in normal media. However, during washing with PBS or HBSS, TRAIL was not expressed even if *IFN-β* mRNA was expressed. It is possible that when ASCs are cultured at a high concentration, the serum concentration gradually decreases, causing cellular stress that may lead to the expression of type I IFNs and TRAIL.

4. Discussion

Despite our increasing knowledge of the potential role of MSCs in medical treatments, the reported roles in promotion or suppression of tumor growth conflict. Until now, the promotion of tumor growth was thought to be regulated by various trophic factors secreted by MSCs. However, only a few reports have analyzed the factors playing crucial roles in tumor growth suppression. In this study, 40K-ASCs expressed

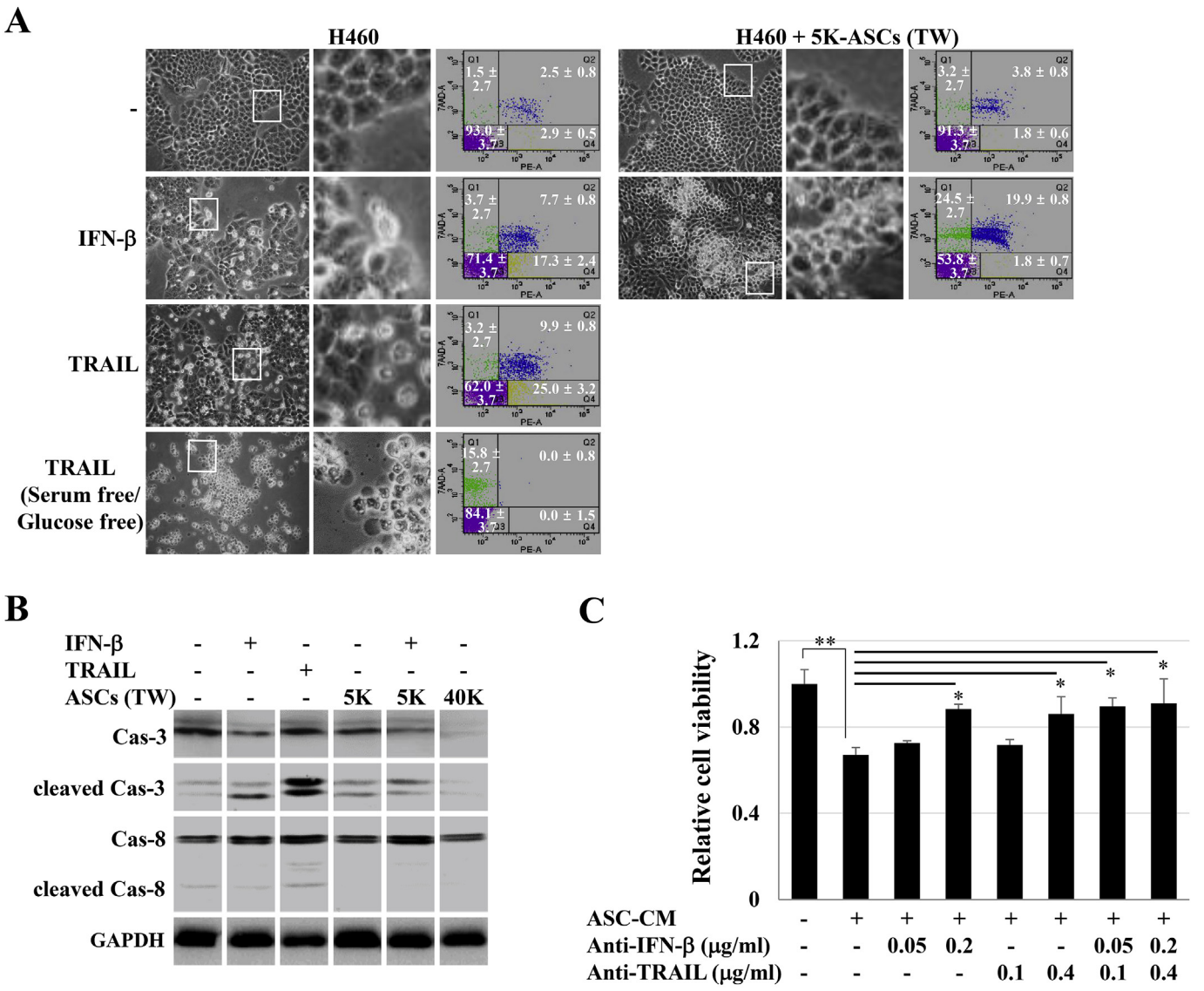

Fig. 3. Effects of IFN-β and TRAIL on cell death in H460 cells. A) Morphological changes in H460 cells with 5K-ASCs treated with IFN-β or TRAIL for 3 days (100 ×) and necrotic cell death in H460 cells by 5 ng/ml TRAIL for 1 day in serum- and glucose-deficient medium. The morphology of H460 cells was observed using a light microscope (100 ×) and cell death mode was analyzed by flow cytometer after annexin-V-PE/7-AAD staining. B) Immunoblot of caspase (Cas)-3 and Cas-8 in H460 cells with 5K-ASCs treated with IFN-β or TRAIL for 3 days. C) Viability of H460 cells treated with 40K-ASC-CM after the neutralization of IFN-β and/or TRAIL. H460 cells were cultured with 40K-ASC-CM and treated with anti-IFN-β and/or anti-TRAIL neutralizing antibodies for 24 h. Cell viability was assessed by an MTT assay. Error bars represent the mean ± SD of triplicate wells. Data were obtained from one of three independent experiments. $^{*}P \leq 0.05$ and $^{**}P \leq 0.01$.

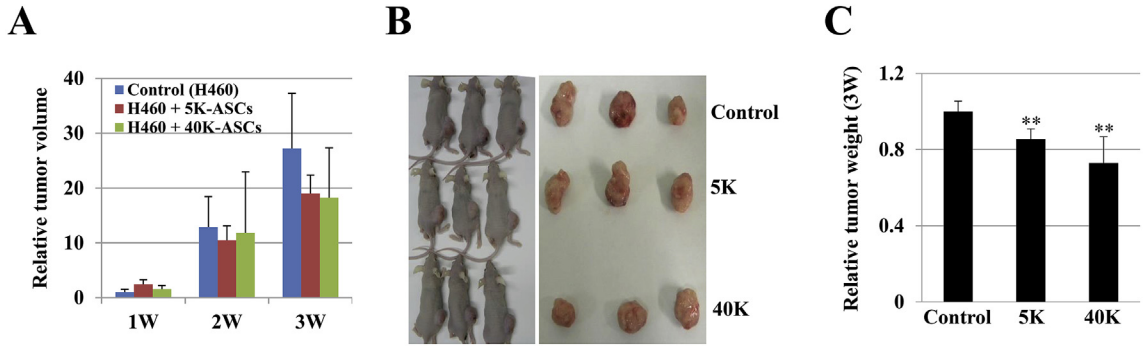

Fig. 4. Anti-proliferative effect of ASCs in H460 xenograft tumor models. A) Changes in tumor volume 3 weeks after ASC injection. For 3 weeks after co-injection of H460 and ASCs cultured at 5K or 40K, the tumor volume was measured weekly using Vernier calipers. B) Morphology of xenograft mice and tumor mass. At 3 weeks after injection of ASCs, mice were sacrificed by cervical dislocation (Control, n = 3; 5K, n = 5; 40K, n = 6), and the tumor mass was separated and photographed. Representative images from three mice are shown. C) Tumor weight at 3 weeks after injection with ASCs. Tumor weight was measured using an electronic balance. Error bars represent the mean ± SD of experimental mice. $^{**}P \leq 0.01$.

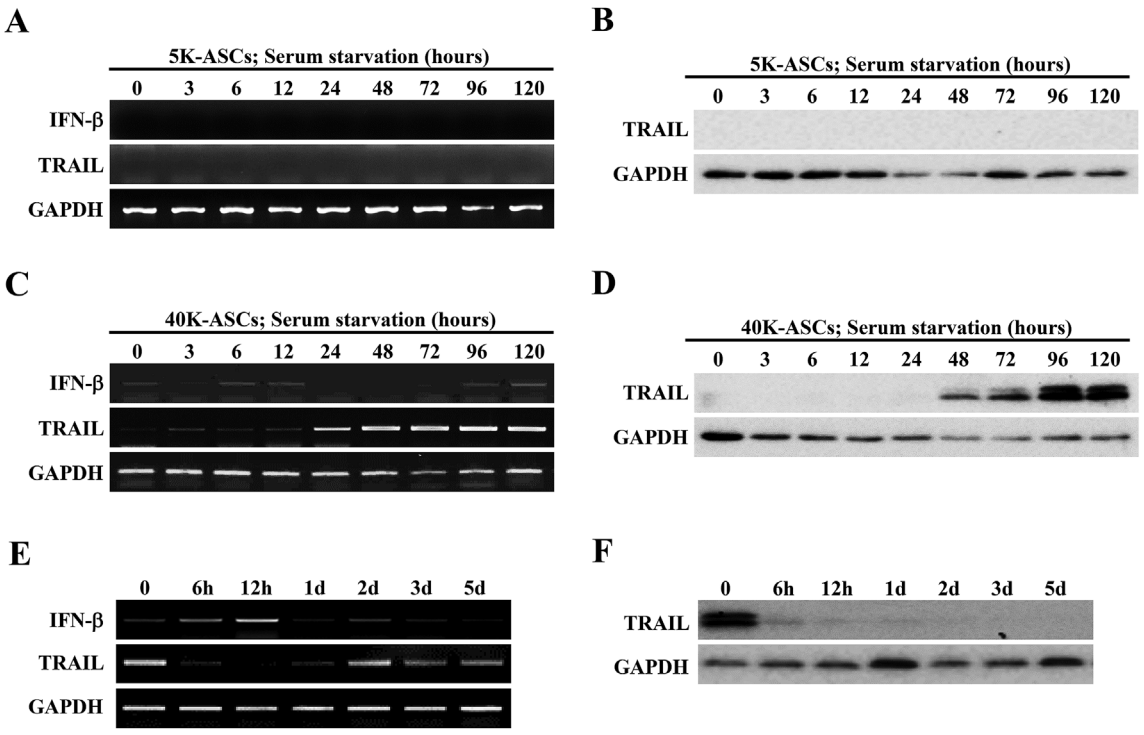

Fig. 5. IFN-β and TRAIL expression after serum starvation in 40K-ASCs or sub-culture of 5-day-cultured 40K-ASCs into 5K. A, C) mRNA expression of *IFN-β* and *TRAIL* in 5K- and 40K-ASCs after serum starvation. 5K- and 40K-ASCs were cultured in serum-free medium for the indicated time points, and then mRNA expression was evaluated by RT-PCR. B, D) TRAIL expression in 5K- and 40K-ASCs cultured with serum-free media for the indicated time points. TRAIL expression was detected by immunoblotting. E–F) IFN-β and TRAIL expression in 5K-ASCs seeded from 5-day-cultured 40K-ASCs. 40K-ASCs were cultured for 5 days and sub-cultured at 5K for the indicated time points. Total RNA and proteins were isolated from the ASCs, and the expression of IFN-β and TRAIL was analyzed by RT-PCR (C) and immunoblotting (D).

IFN-β and TRAIL because of stresses such as serum depletion, which resulted in necrotic cell death in H460 cells and suppressed tumor growth in a murine model. Interestingly, type I *IFN* mRNA, particularly *IFN-β*, was expressed after the second wash step of ASCs prior to transplantation, while *TRAIL* was not expressed after any wash. When the ASCs were washed with PBS and HBSS supplemented with 10% FBS, IFN-β was not expressed. These results suggest that IFN-β expression may be regulated by serum depletion in high-density cell culture or during the washing step. IFN-β may act in an autocrine fashion to induce TRAIL expression, but only during cell culture. In macrophages, serum deprivation was found to induce apoptosis through the autocrine secretion of type I IFNs [38]. Type I/II IFNs are well-known stimulators that can induce TRAIL expression in various cells [30,33,34,39–41]. In our system, IFN-β was expressed in ASCs cultured at high density (40K) or in serum-free conditions as well as during the washing step prior to ASC transplantation. However, TRAIL mRNA and protein expression was observed only in ASCs cultured at high density (40K) or in serum-free media. These results suggest that the autocrine effects of IFN-β combined with sufficient cellular energy sources (*e.g.*, ATP) and/or building blocks (*e.g.*, glucose, amino acids) are required for TRAIL expression. However, since the washing solution lacked ATP and the building blocks for IFN-β protein synthesis, it was hypothesized that TRAIL would not be expressed even if *IFN-β* mRNA was expressed.

IFN-β and TRAIL induced apoptotic cell death in H460 cells. However, when H460 cells were indirectly co-cultured with 5K-ASCs, IFN-β induced necrotic cell death in H460 cells. These dying cells, which were still attached to the bottom of the culture plate, were almost identical to the necrotic H460 cells observed at the beginning of co-culture with 40K-ASCs. Rapidly growing tumors may easily undergo hypoxia and nutrient (*e.g.*, glucose) deficiency due to a lack of blood supply; these metabolic stresses have been reported to mainly cause necrosis during carcinogenesis [42,43]. Indeed, treatment of H460 cells with TRAIL in the absence of both serum and glucose induced necrotic cell death, as indicated by cell morphology. Taken together, we concluded that serum deficiency causes ASCs to express IFN-β, leading to increased TRAIL expression. TRAIL then induces necroptosis in lung cancer H460 cells in an environment lacking serum and glucose.

Nevertheless, although 40K-ASCs induced the death of H460 cells very efficiently *in vitro*, the tumor weight reduction in our murine xenograft model was statistically significant, but not dramatic. These results suggest that ASCs may act very differently depending on the microenvironment. In fact, when 40K-ASCs expressing IFN-β and TRAIL were re-seeded at a low density (5K), *IFN-β* and *TRAIL* mRNA was expressed during sub-culturing, but not TRAIL protein. Thus, we hypothesized that TRAIL protein expression is regulated by the nutritional status (*i.e.*, serum and glucose) of the microenvironment. In addition to tumor growth inhibition, TRAIL plays an important role in regulating immune reactions [44–46]. Therefore, ASCs expressing TRAIL may be useful for treatment of cancer as well as various inflammatory diseases. In the future, the regulation of TRAIL expression by the microenvironment is expected to enhance the therapeutic effects of stem cell-based treatments for inflammatory disease and cancer.

Declarations of interest

None.

Conflicts of interest statement

The authors declare to have no conflicting interests.

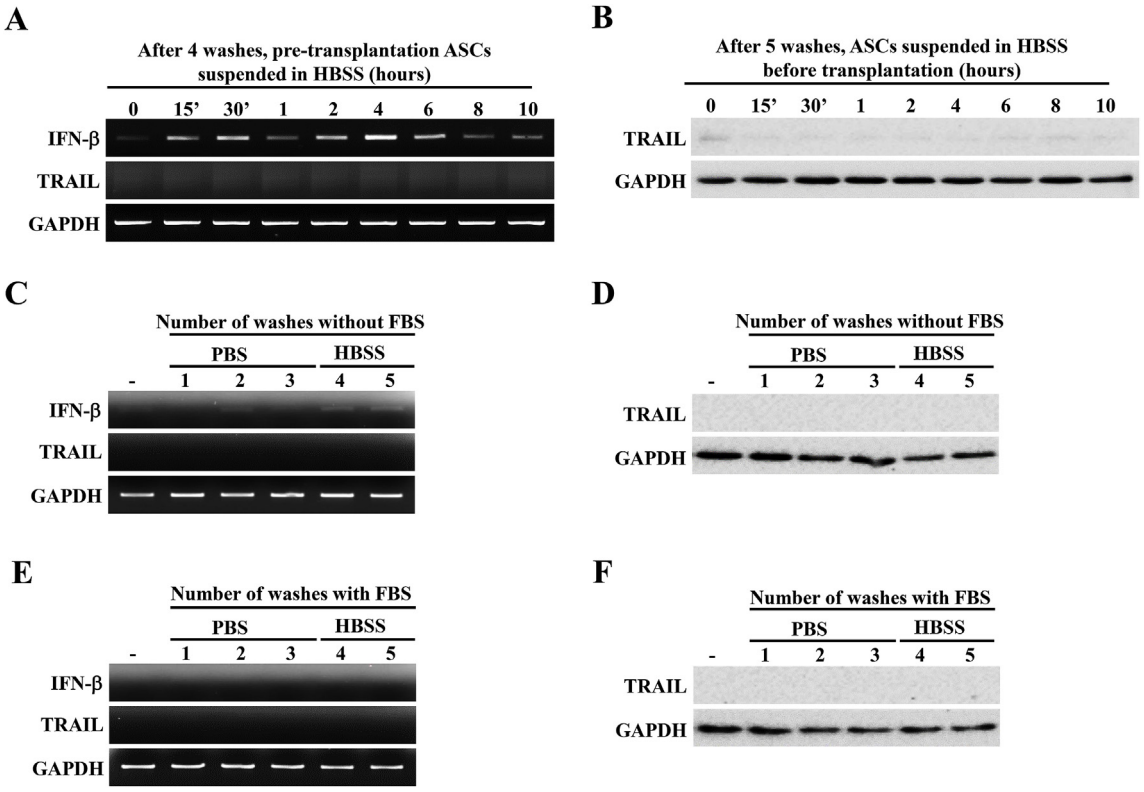

Fig. 6. IFN-β and TRAIL expression after or during the washing step in ASCs cultured at 5K. A–B) Expression of IFN-β and TRAIL in ASCs after five washes (three with PBS and two with HBSS). After four washes (three with PBS and one with HBSS), ASCs were suspended in HBSS, aliquoted, and left at room temperature for the indicated time points. Total RNA and proteins were isolated, and IFN-β and TRAIL expression was analyzed by RT-PCR (A) or immunoblotting (B). C–D) Expression of IFN-β and TRAIL in ASCs during washing with serum-free PBS and HBSS. ASCs were washed three times with PBS and twice with HBSS. Total RNA and proteins were isolated from ASCs at each washing step, and the expression of IFN-β or TRAIL was analyzed by RT-PCR (C) or immunoblotting (D). E–F) Expression of IFN-β and TRAIL in ASCs after washing with PBS and HBSS supplemented with 10% FBS. ASCs were washed three times with PBS containing 10% FBS and twice with HBSS supplemented with 10% FBS. Total RNA and proteins were isolated from ASCs at each washing step, and the expression of IFN-β and TRAIL was analyzed by RT-PCR (E) and immunoblotting (F).

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References

[1] W.T. Xu, Z.Y. Bian, Q.M. Fan, G. Li, T.T. Tang, Human mesenchymal stem cells (hMSCs) target osteosarcoma and promote its growth and pulmonary metastasis, Cancer Lett. 281 (2009) 32–41.
[2] W. Zhu, W. Xu, R. Jiang, H. Qian, M. Chen, J. Hu, W. Cao, C. Han, Y. Chen, Mesenchymal stem cells derived from bone marrow favor tumor cell growth in vivo, Exp. Mol. Pathol. 80 (2006) 267–274.
[3] C. Jotzu, E. Alt, G. Welte, J. Li, B.T. Hennessy, E. Devarajan, S. Krishnappa, S. Pinilla, L. Droll, Y.H. Song, Adipose tissue-derived stem cells differentiate into carcinoma-associated fibroblast-like cells under the influence of tumor-derived factors, Anal. Cell Pathol. 33 (2010) 61–79.
[4] E.L. Spaeth, J.L. Dembinski, A.K. Sasser, K. Watson, A. Klopp, B. Hall, M. Andreeff, F. Marini, Mesenchymal stem cell transition to tumor-associated fibroblasts contributes to fibrovascular network expansion and tumor progression, PloS One 4 (2009) e4992.
[5] P.J. Mishra, P.J. Mishra, R. Humeniuk, D.J. Medina, G. Alexe, J.P. Mesirov, S. Ganesan, J.W. Glod, D. Banerjee, Carcinoma-associated fibroblast-like differentiation of human mesenchymal stem cells, Cancer Res. 68 (2008) 4331–4339.
[6] M. Quante, S.P. Tu, H. Tomita, T. Gonda, S.S. Wang, S. Takashi, G.H. Baik, W. Shibata, B. Diprete, K.S. Betz, R. Friedman, A. Varro, B. Tycko, T.C. Wang, Bone marrow-derived myofibroblasts contribute to the mesenchymal stem cell niche and promote tumor growth, Cancer Cell 19 (2011) 257–272.
[7] M. Najar, G. Raicevic, H. Fayyad-Kazan, C. De Bruyn, D. Bron, M. Toungouz, L. Lagneaux, Immune-related antigens, surface molecules and regulatory factors in human-derived mesenchymal stromal cells: the expression and impact of inflammatory priming, Stem Cell Rev. 8 (2012) 1188–1198.
[8] I. Rajantie, M. Ilmonen, A. Alminaite, U. Ozerdem, K. Alitalo, P. Salven, Adult bone marrow-derived cells recruited during angiogenesis comprise precursors for perivascular mural cells, Blood 104 (2004) 2084–2086.
[9] D. Bexell, S. Gunnarsson, A. Tormin, A. Darabi, D. Gisselsson, L. Roybon, S. Scheding, J. Bengzon, Bone marrow multipotent mesenchymal stroma cells act as pericyte-like migratory vehicles in experimental gliomas, Mol. Ther. 17 (2009) 183–190.
[10] A.I. Caplan, J.E. Dennis, Mesenchymal stem cells as trophic mediators, J. Cell. Biochem. 98 (2006) 1076–1084.
[11] F.T. Martin, R.M. Dwyer, J. Kelly, S. Khan, J.M. Murphy, C. Curran, N. Miller, E. Hennessy, P. Dockery, F.P. Barry, T. O'Brien, M.J. Kerin, Potential role of mesenchymal stem cells (MSCs) in the breast tumour microenvironment: stimulation of epithelial to mesenchymal transition (EMT), Breast Canc. Res. Treat. 124 (2010) 317–326.
[12] S. Liu, C. Ginestier, S.J. Ou, S.G. Clouthier, S.H. Patel, F. Monville, H. Korkaya, A. Heath, J. Dutcher, C.G. Kleer, Y. Jung, G. Dontu, R. Taichman, M.S. Wicha, Breast cancer stem cells are regulated by mesenchymal stem cells through cytokine networks, Cancer Res. 71 (2011) 614–624.
[13] K. McLean, Y. Gong, Y. Choi, N. Deng, K. Yang, S. Bai, L. Cabrera, E. Keller, L. McCauley, K.R. Cho, R.J. Buckanovich, Human ovarian carcinoma-associated mesenchymal stem cells regulate cancer stem cells and tumorigenesis via altered BMP production, J. Clin. Invest. 121 (2011) 3206–3219.
[14] A.E. Karnoub, A.B. Dash, A.P. Vo, A. Sullivan, M.W. Brooks, G.W. Bell, A.L. Richardson, K. Polyak, R. Tubo, R.A. Weinberg, Mesenchymal stem cells within tumour stroma promote breast cancer metastasis, Nature 449 (2007) 557–563.
[15] S. Tsukamoto, K. Honoki, H. Fujii, Y. Tohma, A. Kido, T. Mori, T. Tsujiuchi, Y. Tanaka, Mesenchymal stem cells promote tumor engraftment and metastatic colonization in rat osteosarcoma model, Int. J. Oncol. 40 (2012) 163–169.
[16] K. Shinagawa, Y. Kitadai, M. Tanaka, T. Sumida, M. Kodama, Y. Higashi, S. Tanaka,

W. Yasui, K. Chayama, Mesenchymal stem cells enhance growth and metastasis of colon cancer, Int. J. Canc. 127 (2010) 2323–2333.
[17] S. Pinilla, E. Alt, F.J. Abdul Khalek, C. Jotzu, F. Muehlberg, C. Beckmann, Y.H. Song, Tissue resident stem cells produce CCL5 under the influence of cancer cells and thereby promote breast cancer cell invasion, Cancer Lett. 284 (2009) 80–85.
[18] S. Dias, M. Choy, K. Alitalo, S. Rafii, Vascular endothelial growth factor (VEGF)-C signaling through FLT-4 (VEGFR-3) mediates leukemic cell proliferation, survival, and resistance to chemotherapy, Blood 99 (2002) 2179–2184.
[19] S. Dias, S.V. Shmelkov, G. Lam, S. Rafii, VEGF(165) promotes survival of leukemic cells by Hsp90-mediated induction of Bcl-2 expression and apoptosis inhibition, Blood 99 (2002) 2532–2540.
[20] A. Konig, T. Menzel, S. Lynen, L. Wrazel, A. Rosen, A. Al-Katib, E. Raveche, J.L. Gabrilove, Basic fibroblast growth factor (bFGF) upregulates the expression of bcl-2 in B cell chronic lymphocytic leukemia cell lines resulting in delaying apoptosis, Leukemia 11 (1997) 258–265.
[21] K.J. Rhee, J.I. Lee, Y.W. Eom, Mesenchymal stem cell-mediated effects of tumor support or suppression, Int. J. Mol. Sci. 16 (2015) 30015–30033.
[22] G.R. Stark, I.M. Kerr, B.R. Williams, R.H. Silverman, R.D. Schreiber, How cells respond to interferons, Annu. Rev. Biochem. 67 (1998) 227–264.
[23] S. Akira, K. Takeda, Toll-like receptor signalling, Nat. Rev. Immunol. 4 (2004) 499–511.
[24] J.E. Durbin, R. Hackenmiller, M.C. Simon, D.E. Levy, Targeted disruption of the mouse Stat1 gene results in compromised innate immunity to viral disease, Cell 84 (1996) 443–450.
[25] U. Muller, U. Steinhoff, L.F. Reis, S. Hemmi, J. Pavlovic, R.M. Zinkernagel, M. Aguet, Functional role of type I and type II interferons in antiviral defense, Science 264 (1994) 1918–1921.
[26] D. Saleiro, S.G. Radecki, L.C. Platanias, Mesenchymal stromal cells and Interferon alpha (IFNalpha) in cancer immunotherapy, Transl. Cancer Res. 5 (2016) S1039–S1043.
[27] X. Ling, F. Marini, M. Konopleva, W. Schober, Y. Shi, J. Burks, K. Clise-Dwyer, R.Y. Wang, W. Zhang, X. Yuan, H. Lu, L. Caldwell, M. Andreeff, Mesenchymal stem cells overexpressing IFN-beta inhibit breast cancer growth and metastases through Stat3 signaling in a syngeneic tumor model, Cancer Microenviron 3 (2010) 83–95.
[28] L. Wang, Y. Zhao, Y. Liu, K. Akiyama, C. Chen, C. Qu, Y. Jin, S. Shi, IFN-gamma and TNF-alpha synergistically induce mesenchymal stem cell impairment and tumorigenesis via NFkappaB signaling, Stem Cell. 31 (2013) 1383–1395.
[29] X. Yang, J. Du, X. Xu, C. Xu, W. Song, IFN-gamma-secreting-mesenchymal stem cells exert an antitumor effect in vivo via the TRAIL pathway, J Immunol Res (2014) (2014) 318098.
[30] J. Du, L. Zhou, X. Chen, S. Yan, M. Ke, X. Lu, Z. Wang, W. Yu, A.P. Xiang, IFN-gamma-primed human bone marrow mesenchymal stem cells induce tumor cell apoptosis in vitro via tumor necrosis factor-related apoptosis-inducing ligand, Int. J. Biochem. Cell Biol. 44 (2012) 1305–1314.
[31] H. Ryu, J.E. Oh, K.J. Rhee, S.K. Baik, J. Kim, S.J. Kang, J.H. Sohn, E. Choi, H.C. Shin, Y.M. Kim, H.S. Kim, K.S. Bae, Y.W. Eom, Adipose tissue-derived mesenchymal stem cells cultured at high density express IFN-beta and suppress the growth of MCF-7 human breast cancer cells, Cancer Lett. 352 (2014) 220–227.
[32] P.A. Zuk, M. Zhu, H. Mizuno, J. Huang, J.W. Futrell, A.J. Katz, P. Benhaim, H.P. Lorenz, M.H. Hedrick, Multilineage cells from human adipose tissue: implications for cell-based therapies, Tissue Eng. 7 (2001) 211–228.
[33] N. Kayagaki, N. Yamaguchi, M. Nakayama, H. Eto, K. Okumura, H. Yagita, Type I interferons (IFNs) regulate tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) expression on human T cells: a novel mechanism for the antitumor effects of type I IFNs, J. Exp. Med. 189 (1999) 1451–1460.
[34] L.M. Sedger, D.M. Shows, R.A. Blanton, J.J. Peschon, R.G. Goodwin, D. Cosman, S.R. Wiley, IFN-gamma mediates a novel antiviral activity through dynamic modulation of TRAIL and TRAIL receptor expression, J. Immunol. 163 (1999) 920–926.
[35] S.R. Wiley, K. Schooley, P.J. Smolak, W.S. Din, C.P. Huang, J.K. Nicholl, G.R. Sutherland, T.D. Smith, C. Rauch, C.A. Smith, et al., Identification and characterization of a new member of the TNF family that induces apoptosis, Immunity 3 (1995) 673–682.
[36] S.M. Mariani, P.H. Krammer, Surface expression of TRAIL/Apo-2 ligand in activated mouse T and B cells, Eur. J. Immunol. 28 (1998) 1492–1498.
[37] I. Monleon, M.J. Martinez-Lorenzo, L. Monteagudo, P. Lasierra, M. Taules, M. Iturralde, A. Pineiro, L. Larrad, M.A. Alava, J. Naval, A. Anel, Differential secretion of Fas ligand- or APO2 ligand/TNF-related apoptosis-inducing ligand-carrying microvesicles during activation-induced death of human T cells, J. Immunol. 167 (2001) 6736–6744.
[38] J. Wei, Z. Sun, Q. Chen, J. Gu, Serum deprivation induced apoptosis in macrophage is mediated by autocrine secretion of type I IFNs, Apoptosis 11 (2006) 545–554.
[39] Y. Miura, T. Tsujioka, Y. Nishimura, H. Sakaguchi, M. Maeda, H. Hayashi, M. Dong, F. Hyodoh, K. Yata, H. Wada, T. Sugihara, T. Otsuki, TRAIL expression up-regulated by interferon-gamma via phosphorylation of STAT1 induces myeloma cell death, Anticancer Res. 26 (2006) 4115–4124.
[40] A. Papageorgiou, C.P. Dinney, D.J. McConkey, Interferon-alpha induces TRAIL expression and cell death via an IRF-1-dependent mechanism in human bladder cancer cells, Cancer Biol. Ther. 6 (2007) 872–879.
[41] K.P. Wandinger, J.D. Lunemann, O. Wengert, J. Bellmann-Strobl, O. Aktas, A. Weber, E. Grundstrom, S. Ehrlich, K.D. Wernecke, H.D. Volk, F. Zipp, TNF-related apoptosis inducing ligand (TRAIL) as a potential response marker for interferon-beta treatment in multiple sclerosis, Lancet 361 (2003) 2036–2043.
[42] B. Muz, P. de la Puente, F. Azab, A.K. Azab, The role of hypoxia in cancer progression, angiogenesis, metastasis, and resistance to therapy, Hypoxia 3 (2015) 83–92.
[43] C.H. Kim, H.M. Jeon, S.Y. Lee, M.K. Ju, J.Y. Moon, H.G. Park, M.A. Yoo, B.T. Choi, J.I. Yook, S.C. Lim, S.I. Han, H.S. Kang, Implication of snail in metabolic stress-induced necrosis, PloS One 6 (2011) e18000.
[44] K. Cziupka, A. Busemann, L.I. Partecke, C. Potschke, M. Rath, T. Traeger, P. Koerner, W. von Bernstorff, W. Kessler, S. Diedrich, F.U. Weiss, S. Maier, B.M. Broker, C.D. Heidecke, Tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) improves the innate immune response and enhances survival in murine polymicrobial sepsis, Crit. Care Med. 38 (2010) 2169–2174.
[45] N. Finnberg, A.J. Klein-Szanto, W.S. El-Deiry, TRAIL-R deficiency in mice promotes susceptibility to chronic inflammation and tumorigenesis, J. Clin. Invest. 118 (2008) 111–123.
[46] O. Hoffmann, J. Priller, T. Prozorovski, U. Schulze-Topphoff, N. Baeva, J.D. Lunemann, O. Aktas, C. Mahrhofer, S. Stricker, F. Zipp, J.R. Weber, TRAIL limits excessive host immune responses in bacterial meningitis, J. Clin. Invest. 117 (2007) 2004–2013.