Human amniotic membrane-derived epithelial stem cells display anticancer activity in BALB/c female nude mice bearing disseminated breast cancer xenografts

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Abstract. Breast cancer is one of the most common malignant tumors and the leading cause of mortality among women. In this study, we propose a human stem cell transplantation strategy, an important method for treating various cancers, as a potential breast cancer therapy. To this end, we used human amniotic membrane-derived epithelial stem cells (hAECs) as a cell source for performing human stem cell transplantation. hAECs have multipotent differentiation abilities and possess high proliferative potential. We transplanted hAECs into female BALB/c nude mice bearing tumors originating from MDA-MB-231 breast cancer cells. Co-culturred hAECs and MDA-MB-231 cells at a ratio of 1:4 or 1:8 (tumor cells to stem cells) inhibited breast cancer cell growth by 67.29 and 67.33%, respectively. In the xenograft mouse model, tumor volumes were significantly decreased by 5-flurouracil (5-FU) treatment and two different ratios of hAECs (1:4 and 1:8) by 84.33, 73.88 and 56.89%, respectively. Treatment of nude mice with hAECs (1:4) produced remarkable antitumor effects without any side-effects (e.g., weight loss, death and bruising) compared to the mice that received only 5-FU treatment. Tumor progression was significantly reduced by hAEC treatment compared to the xenograft model. On the other hand, breast tissues (e.g., the epidermis, dermis and reticular layer) appeared to be well-maintained following treatment with hAECs. Taken together, these results provide strong evidence that hAECs can be used as a safe and effective cancer-targeting cytotherapy for treating breast cancer.

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Introduction

Breast cancer is the most common form of cancer affecting women worldwide and each year its incidence rate tends to increase (1). Furthermore, breast cancer is the leading cause of mortality in women due to its malignancy resulting from excess proliferation, invasion and metastasis (2). Chemotherapy is the main treatment for patients with advanced metastatic disease, such as breast cancer (3). 5-Fluorouracil (5-FU), irinotecan, oxaliplatin, chlorambucil, taxol and vincristine are widely used as conventional chemotherapeutic drugs, but these compounds are sometimes associated with disease resistance, toxicity and other undesirable side-effects (2). Consequently, there are substantial needs for novel and effective therapies with low toxicity for treating breast cancer.

Stem cell therapy has drawn attention as an alternative therapeutic tool for regenerative medicine and a treatment for various diseases including cancer (4-6). In this study, we propose a new breast cancer therapeutic strategy using human stem cell transplantation (5-10). A number of previous studies have suggested that various human stem cells exert antitumor effects. For example, mesenchymal stem cells (MSCs) have been shown to inhibit the growth of rat colon carcinoma when co-injected with tumor cells. MSCs derived from human fetal skin, have also been shown to inhibit the growth of MCF-7 breast cancer cells *in vitro*. Finally, human umbilical cord matrix stem cells have been shown to significantly attenuate the growth of MDA-MB-231 human breast carcinoma cells *in vitro* and in a mouse xenograft model (5,6,8,11).

In the present study, we explored the potential of human amniotic membrane-derived epithelial stem cells (hAECs) to serve as a stem cell transplantation therapy that targets breast cancer. hAECs are derived from the human amnion, which is a membrane that forms the amniotic sac which surrounds and protects the embryo. hAECs are easily isolated from amnion or amniotic fluid readily available during gestation and at the time of birth (12-14). Thus, hAECs are not associated with any moral/ethical issues unlike human embryonic stem cells and therefore are advantageous for stem cell research

and clinical use (5). A number of studies have reported that hAECs possess a multipotent differentiation ability and high proliferation potential by expressing molecular markers such as *OCT4*, *SOX2*, *LEFTY2*, *FGF-4*, *PEX1* and *CRLPTO* which are required for self-renewal and pluripotency (12,13).

In our previous studies, genetically engineered stem cells expressing a suicide gene and cytokine showed antitumor effects in diverse human cancers (15,16). In this study, to evaluate the effectiveness of hAEC transplantation therapy against breast cancer, we first investigated the growth attenuation potential of hAECs against MDA-MB-231 human breast carcinoma cells in an *in vitro* cell culture study and a female BALB/c nude mouse xenograft model. Subsequently, the tumor targeting capacity of hAECs in the mouse xenograft model was observed by a fluorescence staining assay. We also examined the safety of hAEC transplantation therapy by histologically analyzing breast tissues and evaluating the survival rate compared to treatment with 5-FU in an animal experiment. Based on the results from our study, we suggest that hAECs are capable of effectively suppressing the growth of human breast cancer cells and can be a safe tumor-targeting tool for anticancer therapy.

Materials and methods

Cell culture. The human breast carcinoma cell line, MDA-MB-231 (Korean Cell Line Bank, Seoul, South Korea), was cultured in RPMI (PAA Laboratories, Linz, Austria) supplemented with 10% fetal bovine serum (FBS; Hyclone Laboratories, Inc., Logan, UT, USA), 1% penicillin G and streptomycin (Cellgro Mediatech, Inc., Manassas, VA, USA), 1% antifungal HEPES (Invitrogen Life Technologies, Carlsbad, CA, USA) and 0.1% antimycoplasmal plasmocin (Invivogen, San Diego, CA, USA) at 37˚C in a humidified atmosphere of 5% CO_2-95% air. The bovine fibroblast (bovine FB) cell line (obtained from Chungbuk National University, Cheongju, South Korea), was cultured in Dulbecco's modified Eagle's medium (DMEM; Hyclone Laboratories, Inc., Logan, UT), supplemented with 10% FBS, 1% penicillin G and streptomycin, 1% HEPES and 0.1% plasmocin at 37˚C in a humidified atmosphere of 5% CO_2-95% air. Cells were detached with 0.05% trypsin/0.02% EDTA (PAA Laboratories) in Mg^{2+}/Ca^{2+}-free Hank's balanced salt solution (HBSS).

Human amniotic tissue was obtained from Guro Korea Medical Hospital (Seoul, Korea), under an informed consent and isolation and culture procedures were performed with the approval of the Seoul National University Institutional Review Board (IRB no. 0611/001-002). The amniotic tissue was washed several times with PBS to remove blood and incubated with 0.05% trypsin-EDTA (Invitrogen) for 1 h. The amniotic epithelial cells were collected and suspended in standard culture medium consisting of Keratinocyte-SFM (K-SFM) supplemented with 0.031 mg/ml human recombinant epithelial growth factor (EGF), 12.4 mg/ml bovine pituitary extract (all from Invitrogen) and 10% FBS (Hyclone Laboratories).

Cell growth assay to evaluate the effects of hAECs or 5-FU treatment. To investigate the effect of hAECs on the growth of breast cancer cells, MDA-MB-231 and bovine FB cells (control) were seeded at a concentration of 4,000 cells/well in 96-well plates and cultured in 0.1 ml of an indicated medium supplemented with 5% FBS. After 24 h, hAECs were added at densities of 16,000 and 32,000 cells/well and co-cultured for 5 days. The number of viable cells was assessed by 3-(4,5-dimethylthiazol-2-yl)-2,5-dyphenyltetrazolium bromide (MTT) assay. MTT (Sigma-Aldrich Corp., St. Louis, MO, USA) solution (5 mg/ml) was added to each well (10 μl per well) and the plates were incubated for 4 h at 37˚C. Supernatants were removed and 100 μl of 99% dimethyl sulfoxide (DMSO) Junsei Chemical Co., Tokyo, Japan) were added to each well to dissolve the resultant formazan crystals. Optical densities of the wells were measured at 540 nm using an ELISA plate reader (VERSA man; Molecular Devices, Sunnyvale, CA, USA).

We then investigated the effect of 5-FU on MDA-MB-231 cells to determine its appropriate injection concentration *in vivo*. MDA-MB-231 cells were seeded at a density of 4,000 cells/well in 96-well plates and cultured in 0.1 ml RPMI medium with 5% FBS. After 24 h, fresh RPMI (supplemented with 5% FBS) was added and the cells incubated for a further 24 h before being treated with 5-FU. Finally, cells were treated with 5-FU (Sigma-Aldrich Corp.) at concentrations of 100, 200, 300, 400, 500 and 600 μg/ml for 4 days. An MTT assay was then performed as described above.

Cytokine array. We performed a cytokine array to detect the expression of multiple cytokines in hAECs. Samples of hAECs culture media, were collected from hAEC cultures at passage 2. Fresh medium was used as the negative control. The cytokine array was performed according to the RayBio® Human Cytokine Antibody Array kit protocol (RayBiotech, Inc., Norcross, GA, USA). The intensities of signals were quantified by densitometry. The positive control was used to normalize the results from different membranes.

MDA-MB-231 cell xenograft model. Healthy female BALB/c nude mice were purchased from the Central Lab Animal, Inc. (Seoul, South Korea). Five-week-old female BALB/c nude mice were housed in a specific pathogen-free (SPF) facility, at the Laboratory Animal Research Center in Chungbuk National University. Mice were allowed to acclimate for 1 week after arrival. MDA-MB-231 cells ($1.0x10^6$) suspended in 100 μl PBS were mixed with 50% Matrigel Matrix (BD Co., Bedford, MA, USA) and injected subcutaneously into the right mammary fat pads of 6-week-old female BALB/c nude mice, as described previously (17-20). After cell transplantation, tumors were measured every week with a vernier caliper (Mitutoyo Co., Tokyo, Japan) and tumor volume was calculated using the following formula: (π/6) x length x width x height (17).

Therapeutic effect of hAECs. Seven weeks after tumor implantation (all tumors were at least 250-300 mm^3 in volume), the animals were randomly divided into four groups. Mice in group 1 (control, n=9) were treated with a circumtumoral injection of PBS (100 μl). Mice in group 2 (5-FU, n=6) were treated with an intraperitoneal injection of 5-FU (50 mg/kg/d) once a day for 12 days. Mice in group 3 [hAECs (1:4), n=6] were treated with a circumtumoral injection of $4.0x10^6$ chloromethyl-benzamido-1,1'-dioctadecyl-3,3,3',3'-tetramethylindo carbocyanime perchlorate (CM-DiI)-labeled hAECs in 100 μl of PBS. Mice in group 4 [hAECs (1:8), n=6] were treated with a circumtumoral

injection of $8.0x10^6$ CM-DiI-labeled hAECs in 100 μl of PBS. The ratio of 1:4 or 1:8 represents the ratio of cancer cells to stem cells.

In this study, CM-DiI (Sigma-Aldrich Corp.) was used to label the hAECs, as it is non-diffusible and binds covalently to cellular thiols. This dye also persists for at least 10 weeks *in vivo*. hAECs were stained with CM-DiI before injection according to the manufacturer's instructions (21).

Fluorescence staining analysis. Fluorescence staining was performed to detect the presence of hAECs at the breast tumor locus. We injected CM-DiI-labeled hAECs into tumor-bearing animals and the breast tumors were then subjected to fluorescence microscopy analysis. Tumor specimens were fixed in a 10% formalin solution (Junsei Chemical Co., Ltd.) and embedded in paraffin. Sections (8-μm thick) were cut from the paraffin block. Breast tumor slides were fixed in a 10% formalin (OCI Co., Ltd., Ulsan, South Korea) solution for 5 min and then rinsed twice with PBS. The nuclei of all cells were stained with 4',6-diamidino-2-phenylindole (DAPI; Sigma-Aldrich Corp.) for 10 min. After DAPI staining, tumor cells were detected by fluorescence microscopy (1X71 U-LH100HG inverted microscope; Olympus, Tokyo, Japan) using a WU emission filter; hAECs labeled with CM-DiI were observed using a WIG emission filter.

Histopathological analysis. A histological analysis of breast and tumor specimens from each group of xenografted mice was performed. Breast and tumor tissue specimens were fixed in a 10% formalin solution and embedded in paraffin. Sections (5-μm and 9-μm thick) were cut from the paraffin blocks and then stained with hematoxylin and eosin (Sigma-Aldrich Corp.), using the standard methods of the manufacturer. Breast and tumor tissue structures were observed using a light microscope (BX51 U-LH100HGWIG; Olympus).

Statistical analysis. All data were analyzed with GraphPad Prism software (GraphPad Software Inc., San Diego, CA, USA). Data from the *in vitro* and *in vivo* experiments are presented as the means ± SD and the means ± SEM, respectively. Statistical analysis was performed using one-way ANOVA followed by Dunnett's multiple comparison tests. A P-value >0.05 was considered to indicate a statistically significant difference. Analysis of Kaplan-Meier plots compared the survival of six mice per group, using the log-rank (Mantel-Haenszel) test.

Results

hAECs inhibit the growth of MDA-MB-231 cells in vitro. To determine whether hAECs inhibit the *in vitro* growth of MDA-MB-231 breast cancer cells, a co-culture study was carried out. When co-cultured with hAECs, significant growth inhibition was induced in the MDA-MB-231 cells, but not in the bovine FB cells. hAECs attenuated the growth of MDA-MB-231 cells by 67.29 and 67.33%, when the treatment ratios of tumor cells to stem cells were 1:4 and 1:8, respectively, as shown in Fig. 1A. There was no significant difference in MDA-MB-231 cell growth according to the ratio of stem cells to tumor cells within this range. To determine the appropriate concentration of 5-FU for the mouse xenograft model as the positive control, we also carried out a cytotoxicity test to evaluate the effects of 5-FU on MDA-MB-231 cells. MDA-MB-231 cell viability was significantly decreased with 100 μg/ml of 5-FU (Fig. 1B). Based on this result, we determined that the appropriate 5-FU concentration *in vivo* was 50 mg/kg/d.

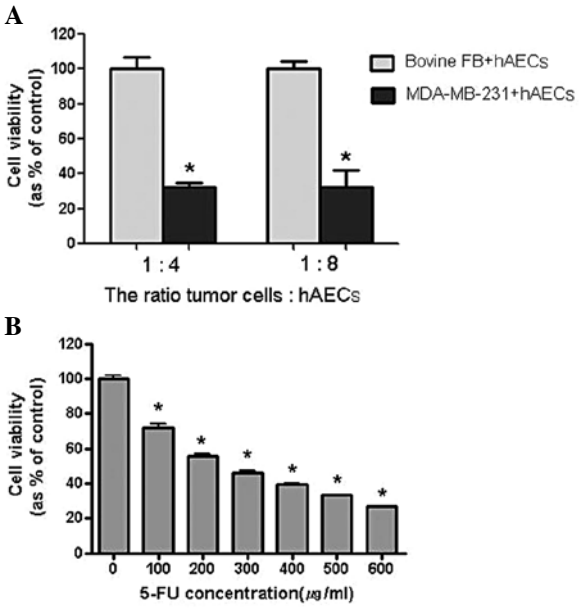

Figure 1. The effects of hAECs or 5-FU on the viability of breast cancer cells *in vitro*. (A) MDA-MB-231 or bovine FB cells were seeded ($4.0x10^3$) and treated with increasing numbers of hAECs ($1.6x10^4$ and $3.2x10^4$). Cell viability was measured using an MTT (5 mg/ml) assay. (B) MDA-MB-231 cells ($4.0x10^3$) were seeded and treated with 5-FU at indicated concentrations. MDA-MB-231 cell viability was significantly decreased (from 74 to 28.11%) according to the 5-FU concentration (100 to 600 μg/ml). Values represent the means ± SD. *P<0.05 was considered to indicate a statistically significant difference.

hAECs express various cytokines. The results from the cytokine array showed that hAECs express multiple cytokines such as TNF-α, TNF-β, TGF-β, IFN-γ, IL-2, IL-3, IL-4, M-CSF and IL-8 (Fig. 2A). Although the levels of anticancer-associated cytokines appeared to be modest as demonstrated in Fig. 2B, we concluded that the antitumor effects of the hAECs may be associated with the expression and release of these cytokines.

hAECs inhibit the growth of breast tumors in female BALB/c nude mouse. To examine the antitumor effect of hAECs on breast cancer cells *in vivo*, an MDA-MB-231 cell xenograft study was carried out in female BALB/C nude mice. The subcutaneous injection of MDA-MB-231 cells into the mammary fat pad, led to the development of breast tumors in the nude mice within 7 weeks. Two circumtumoral injections of hAECs administered 30 days after MDA-MB-231 cell transplantation reduced the tumor burden compared to the control group as observed by measuring the tumor volume (Fig. 3A). When we measured the tumor volume of the four groups [(control, 5-FU, hAECs (1:4) and hAECs (1:8)] after 30 days, the tumor volumes decreased by 84.33, 73.88 and 56.89% in the 5-FU, hAECs (1:4) and hAECs (1:8) groups, respectively, compared to the control group (Fig. 3B).

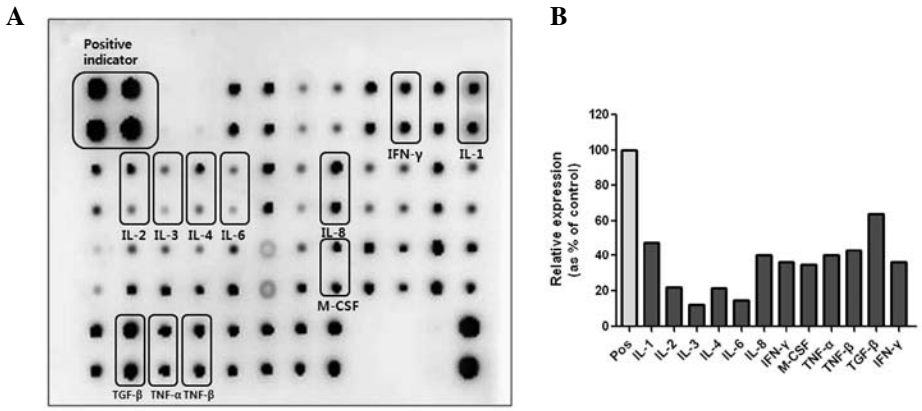

Figure 2. Expression of multiple cytokines by hAECs. (A) The Human Cytokine Antibody Array 3 Map. Cytokine arrays were performed according to the RayBio® Human Cytokine Antibody Array protocol. (B) The intensities of the signals were quantified by densitometry. The cytokine array demonstrated that hAECs express multiple cytokines, such as TNF-α, TNF-β, TGF-β, IFN-γ, M-CSF, IL-2, IL-3, IL-4 and IL-8.

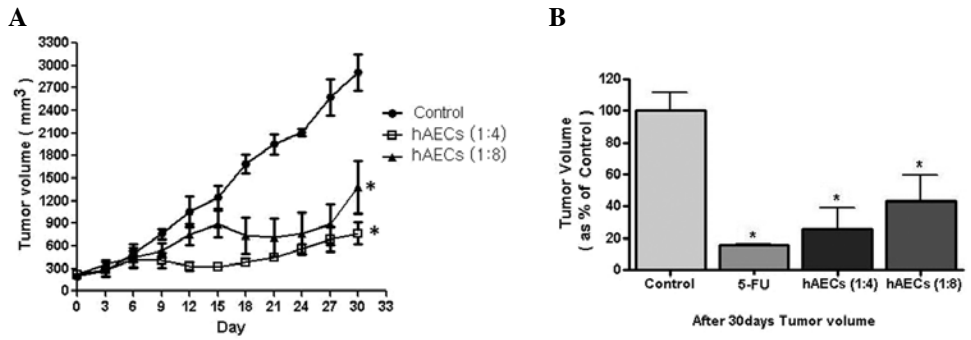

Figure 3. Antitumor activity of hAECs in the mouse xenografts. (A) Once subcutaneous tumors were established, hAECs ($4.0x10^6$ and $8.0x10^6$) were injected circumtumorally. Tumor volumes were measured every 3 days during the treatment. (B) The tumor volumes were measured at the end of the experiment (30 days). Two circumtumoral injections of hAECs administered 30 days after MDA-MB-231 cell transplantation reduced the tumor burden compared to the control group as observed by measuring the tumor volume. Values represent the means ± SEM. *P<0.05 compared to the control.

hAECs are localized in the breast tumor locus. In the stained breast tumor tissues, DAPI-stained tumor cells appeared blue and CM-DiI-labeled hAECs were red in the fluorescence micrographs. Localization of the hAECs at the breast tumor locus was clearly observed when the two images were merged as shown in Fig. 4. CM-DiI-labeled hAECs were found within the tumor site. The results of the fluorescence staining showed that hAECs are intratumorally localized in the breast tumors.

hAECs help maintain the original breast tissue structure and tumor targeting. Breast and tumor tissue sections stained with hematoxylin and eosin showed the histological characteristics of each group (Fig. 5A). Breast tissues of the control group clearly showed high density of tumor cells due to invasiveness, which is a general characteristic of breast tumors (19,21). The breast tissues of the control group did not maintain their original structures, such as the dermis and reticular layers, apart from the epidermis. However, breast tissues of the hAEC group maintained a distinct epidermis, dermis and reticular layers. In the 5-FU group, breast tissues were almost destroyed due to the toxic effect of 5-FU. Tumor tissues of the control group clearly showed a high density of MDA-MB-231 cells as demonstrated in Fig. 5B. However, in the hAEC treatment groups, the destruction of tumor tissues was observed in part. These results indicate that hAEC treatment may suppress breast cancer infiltration in the breast by inhibiting tumor growth and thus may help to maintain the original structure of breast tissues.

hAECs prolong the survival of mice with xenografts. The mice in each treatment group were monitored for survival. After 30 days, mice in the hAEC (1:4) and hAEC (1:8) group, exhibited a higher survival rate (100%) than those in the control (83.33%) and 5-FU (75%) groups (Fig. 6). In Fig. 6, the xenografted mice treated with hAECs (1:4) or hAECs (1:8), showed an increased survival following treatment at the end of the 30-day experimental period.

Discussion

Previous studies have suggested that human stem cell therapy can be an important therapeutic tool for treating various types of cancer (4-6,11,22). Stem cells have a self-renewal ability and powerful differentiation capacity. Furthermore, stem cells are considered to be an ideal carrier for anticancer gene delivery due to their capacity of specific tumor-oriented

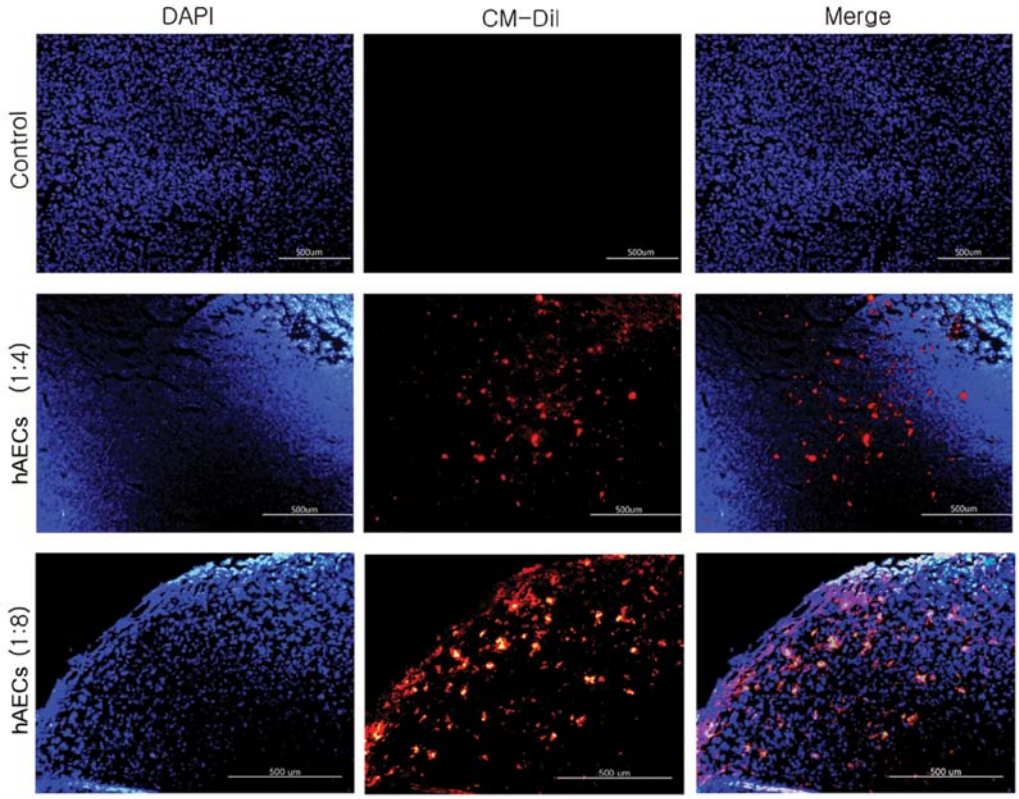

Figure 4. Fluorescent images of hAECs in the breast tumor locus. hAECs were labeled with CM-DiI and injected into tumor-bearing animals. The breast tumors were stained with DAPI and subjected to fluorescent microscopy analysis. The DAPI-stained tumor cells appeared blue and CM-DiI-labeled hAECs appeared red in fluorescent micrographs. Localization of the hAECs in the breast tumor locus was clearly observed when the two images were merged.

migration (4,21,23) and are thus anticipated to be an effective tumor-targeting anticancer agent. Indeed, multiple stem cells engineered to express anticancer genes at a specific tumor locus, have been reported to effectively suppress tumors (1,4,11,14,17,22-27). However, certain reports have shown that non-engineered native stem cells have an inherent ability to inhibit the growth of several types of cancer cells (28,29). However, the use of engineered stem cells can help solve unexpected problems associated with transfected genes, such as mutation, inappropriate insertion into genomic DNA and viral vector virulence.

In this study, we examined the anticancer effects of human stem cell transplantation targeting breast cancer, using hAECs that are naïve stem cells and not genetically engineered. hAECs are derived from the human amnion and are readily isolated from the amnion or amniotic fluid that is usually discarded after birth. Therefore, these cells are much easier to obtain and are not associated with any controversial issues unlike human embryonic stem cells. Additionally, hAECs have a powerful potential to differentiate into all three germ layers *in vitro*. Due to these advantages, hAECs are considered to be suitable replacements for human embryonic stem cells.

In the present study, we first evaluated the intrinsic *in vitro* and *in vivo* therapeutic effect of hAECs on the human MDA-MB-231 breast cancer cell line. In the *in vitro* co-culture study, hAECs effectively inhibited the growth of MDA-MB-231 cells, but did not affect normal bovine FB cells. In the female BALB/c nude mouse xenograft model, circumtumorally-administered hAECs significantly attenuated tumor growth in breast tissues and increased mouse survival rate. However, following treatment with a greater number of hAECs, there was no increase in tumor suppressing capacity, but rather a decrease, as shown in the mouse xenograft model. Fluorescence staining of hAECs in the breast tumor was performed to observe their existence within the tumor tissue. In this analysis, hAECs appeared to localize precisely in the breast tumor locus, which is an important step in hAEC-induced tumor suppression.

The anticancer effect of hAECs may be associated with growth inhibitors produced by hAECs themselves along with their tumor targeting capacity. It is well known that diverse types of factors, such as TGF-β, IFN-γ and TNF-α secreted by stem cells, can inhibit the cell cycle and stimulate apoptosis of cancer cells (5). In the cytokine assay, we confirmed that hAECs express not only cytotoxic cytokines, such as M-CSF, TNF-α, TNF-β, IFN-γ and TGF-β, but also various interleukins such as IL-1, IL-2, IL-3, IL-4, IL-6 and IL-8. Some interleukins, such as IL-2, IL-4 and IL-3, are known to enhance the cytotoxicity of NK cells which can attack cancer cells and restrict tumor formation (28). Further studies are required to investigate the more precise mechanism by which hAECs exert their anticancer effect on breast tumors.

We also examined the safety of hAEC transplantation by comparing this procedure to 5-FU treatment. 5-FU is a chemotherapeutic agent and widely used for treating various types of cancer such as breast, gastric, colorectal and liver. However, 5-FU produces typical side-effects such as myelo-suppression, nausea, vomiting, diarrhea and stomatitis (2). In the present

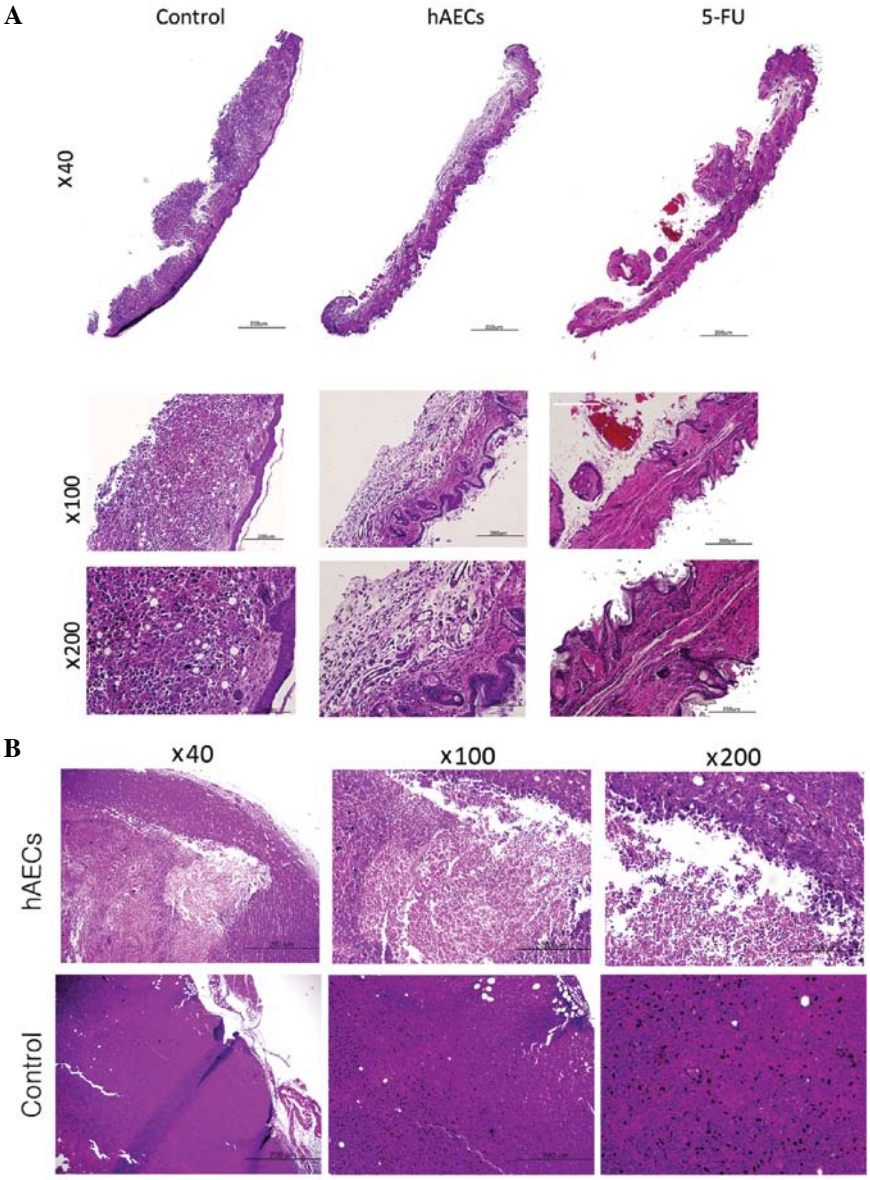

Figure 5. Histopathological images of breast tissue in MDA-MB-231 cell xenografted mice. Breast and tumor tissue specimens were fixed in 10% formalin solution and embedded in paraffin. Sections (5 and 9 μm thick) were cut from the paraffin blocks and stained with hematoxylin and eosin using standard methods. (A) Breast and tumor tissue structures were detected using light microscopy (x40, x100 and x200 magnifications). (B) Breast tissues of the control group clearly showed high density of tumor cells, while breast tissues of the hAEC groups maintained a distinct epidermis, dermis and reticular layers. In the 5-FU group, breast tissues were almost destroyed due to the toxic effect of 5-FU.

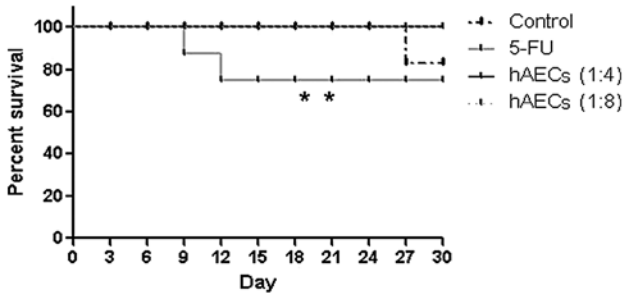

Figure 6. Survival of mice with MDA-MB-231 cell xenografts. Mice in each treatment group were monitored for survival during the 30 days experimental period. Analysis of Kaplan-Meier plots compared the survival of six mice per group using the log-rank (Mantel-Haenszel) test. hAECs (1:4) and hAECs (1:8) groups exhibited a higher survival rate (100%) than the control (83.33%) and 5-FU (75%) group. Values represent the means ± SEM. **P<0.05 was considered to indicate a statistically significant difference.

study, 5-FU was found to be quite toxic, although this compound had outstanding anticancer effects on breast tumor *in vivo* and *in vitro*. In the histological analysis, breast tissues of the 5-FU group were found to be almost completely destroyed, while the original breast tissue structures including the epidermis, dermis and reticular layer, were relatively well maintained in the hAEC groups. Likewise, mice in the 5-FU group showed serious side-effects, such as weight loss, bruising and a 75% survival rate during the treatment period. In contrast, mice in the hAEC groups showed no adverse effects and maintained 100% viability.

In this study, using an increased number of hAECs did not enhance their antitumor effect *in vivo*. CM-DiI-labeled hAECs intratumorally localized in the breast tumors were detected by fluorescent images. Following treatment with hAECs at the

ratio of 1:8 (ratio of cancer cells to stem cells), CM-DiI-labeled hAECs were localized mainly in the tumor sites than after treatment with hAECs at the ratio of 1:4. However, a stronger antitumor effect was observed following treatment with hAECs at the ratio of 1:4 than the ratio of 1:8. Further studies are required in order to clarify the distinct antitumor effects of hAECs. In conclusion, the present study is the first to demonstrate that non-engineered naïve hAECs significantly reduce the viability of breast cancer cells both *in vivo* and *in vitro*. Our findings clearly demonstrate that hAEC transplantation can be a safe cancer-targeting cytotherapy for treating breast cancer.

Acknowledgements

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