

# Differentiation of human amniotic epithelial cells into Schwann-like cells via indirect co-culture with Schwann cells *in vitro*

SHUANG ZHU<sup>1\*</sup>, JIACHUN LI<sup>1\*</sup>, QINGTANG ZHU<sup>1</sup>, TING DAI<sup>2</sup>, BO HE<sup>1</sup>,  
XIANG ZHOU<sup>1</sup>, JIANPING XIANG<sup>1</sup> and XIAOLIN LIU<sup>1</sup>

<sup>1</sup>Department of Microsurgery and Hand Surgery,  
The First Affiliated Hospital of Sun Yat-sen University; <sup>2</sup>Department of Biotechnology,  
School of Basic Science, Guangzhou Medical University,  
Guangzhou, Guangdong 510080, P.R. China

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**Abstract.** Human amniotic epithelial cells (hAECs) exhibit multi-lineage differentiation ability. The present study investigated the possibility that hAECs possess the potential to differentiate into Schwann-like cells using an *in vitro* indirect co-culture approach. hAECs were isolated via enzymatic digestion, and immunocytochemistry and flow cytometry were performed to identify the hAECs. The hAECs were co-cultured with Schwann cells (SCs) to differentiate the hAECs into Schwann-like cells via induced proximity. The expression of typical S-100 SC markers in the co-cultured hAECs was determined via immunocytochemistry. For the functional experiments, reverse transcription quantitative polymerase chain reaction (RT-qPCR) was performed to measure the expression levels of nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF) and glial cell-derived neurotrophic factor (GDNF) mRNA. In addition, neurite outgrowth was measured in PC12 cells following co-culture with the differentiated hAECs. Subsequent to co-culture with SCs for 21 days, the hAECs exhibited spindle-like morphology. The immunocytochemistry results revealed that the co-cultured hAECs expressed S-100, indicating differentiation into Schwann-like cells. RT-qPCR revealed that NGF, BDNF and GDNF expression was upregulated upon differentiation. The average axon length of the PC12 cells increased from

21.32±5.45 to 51.32±8.56  $\mu$ m subsequent to co-culture with the differentiated hAECs. These results demonstrate that this indirect co-culture microenvironment induced the hAECs to differentiate into Schwann-like cells that exhibited the morphological, phenotypic and functional characteristics of SCs. Therefore, the use of differentiated hAECs that exhibit the characteristics of SCs provides a promising alternative to the present techniques used for peripheral nerve regeneration.

## Introduction

Annually, >3,500,000 patients worldwide suffer from peripheral nerve injuries, accounting for 3% of all trauma patients. Among the various types of peripheral nerve injury, transection injuries, in which the nerve trunk is completely separated, particularly those that result in large defects, may produce a marked impact on patients' quality of life, and in these cases, reconstructive surgery is required as a therapeutic strategy to achieve nerve regeneration. Several studies have attempted to accelerate the regeneration of traumatized nerves via the application of microsurgical techniques (1-6). However, despite advancements in these techniques, complete recovery is rarely achieved (7,8).

Schwann cells (SCs) are considered to be the primary structural and functional cells of the peripheral nervous system, and these cells exert a critical supportive role in peripheral nerve regeneration (9,10). The transplantation of cultured SCs into various peripheral nerve grafts scaffolds has been demonstrated to promote axonal regeneration across nerve gaps (11). However, the clinical application of SCs remains controversial as obtaining sufficiently large numbers of SCs is difficult. In the past few decades, bone marrow stromal cells (BMSCs) and adipose-derived stem cells (ADSCs) have been considered as alternative cell sources for tissue regeneration following peripheral nerve injury, as these cells are capable of selectively differentiating into SCs and enhancing axonal regeneration (12,13). Each of these cell types has advantages; however, numerous problems have been associated with the use of each of the currently available cell sources, including

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*Correspondence to:* Professor Jianping Xiang or Professor Xiaolin Liu, Department of Microsurgery and Hand Surgery, The First Affiliated Hospital of Sun Yat-sen University, 58 Zhongshan 2nd Road, Guangzhou, Guangdong 510080, P.R. China  
E-mail: 328438430@qq.com; gzjianpingxiang@qq.com  
E-mail: gzxiaolinliu@hotmail.com

\*Contributed equally

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complications regarding cell survival, immune rejection, tumorigenesis and ethical issues. Therefore, the development of an ideal cell source is important to address these problems. Recently, human amniotic epithelial cells (hAECs) have been reported to exhibit stem cell characteristics, and these cells have been shown to differentiate into liver cells, fat cells, heart cells and nerve cells *in vitro* (14-19). hAECs are easily accessible and highly abundant since the amnion is normally discarded following birth. Furthermore, hAECs exhibit low immunogenicity and have the potential to differentiate into various types of functional cell (20). These characteristics render hAECs an alternative source of adult stem cells for SC transplantation for peripheral nerve regeneration.

In the present study, whether hAECs possess the potential to differentiate into Schwann-like cells was investigated via indirect co-culture of hAECs with SCs *in vitro*. Immunocytochemical staining was performed to determine the biological features of the differentiated cells. Co-culture of the differentiated hAECs with PC12 cells was conducted to assess the effect of differentiated hAECs on axon regeneration.

## Materials and methods

**Isolation and culture of hAECs.** In brief, discarded placentas from uncomplicated cesarean resections at 37-40 weeks gestational age were obtained from The First Affiliated Hospital of Sun Yat-sen University (Guangzhou, China) with the approval of the University of Sun Yat-sen Institutional Review Board. The amnion layer was mechanically peeled away from the chorion and washed multiple times using phosphate-buffered saline (PBS; Invitrogen, Carlsbad, CA, USA) to remove the blood. The amnion layer was then cut into 2x2 cm sections and processed according to previously described methods (21). To release the hAECs, the amnion sections were incubated at 37°C in 0.05% trypsin containing 0.2 g/l ethylenediaminetetraacetic acid (Invitrogen/Gibco-BRL; Carlsbad, CA, USA). Subsequently, the trypsin was inactivated by adding 10 ml Dulbecco's modified Eagle's medium/F-12 Ham nutrient mixture (DMEM/F-12; 1:1; Gibco-BRL) supplemented with 10% heat-inactivated fetal bovine serum (FBS; Invitrogen Life Technologies). The cells from the initial 10 min of digestion were discarded to remove any debris. The cells from the second and third 30-min digestions were pooled and washed three times with PBS. The hAECs were then plated on six-well dishes at a seeding density of 20,000 cells/cm<sup>2</sup> in DMEM/F-12 (1:1) culture medium supplemented with 10% FBS, 1% penicillin/streptomycin (Invitrogen), 1% GlutaMAX™ (Invitrogen) and 10 ng/ml epidermal growth factor (EGF; PeproTech, Rocky Hill, NJ, USA). The hAECs from the initial culture were subcultured to passage 1 using a split ratio of 1:3. The above-mentioned culture medium was replaced every three days.

**Isolation and culture of SCs.** Rat SCs were harvested as previously described, with minor modifications (22). Briefly, for collection of the sciatic nerves, the male Sprague-Dawley rats (1-3 days old, weighing 8-15 g; Laboratory Animal Center of Sun Yat-sen University, Guangzhou, China) were anesthetized by cooling on ice one at a time. Once the animal had stopped moving it was decapitated, and the head placed into

a collection bag. The body was spayed with 70% ethanol. The sciatic nerves were exposed, dissected out under a binocular microscope (Leica S6E, Wetzlar, Germany) and placed in a sterile culture dish (3-cm diameter; Costar, Corning Inc., Corning, NY, USA) containing ice-cold PBS with 1% penicillin/streptomycin, where they were maintained until the nerves had been harvested from all of the rat. Then, the sciatic nerves were enzymatically dissociated via sequential incubation at 37°C in 0.3% collagenase type II (Sigma-Aldrich, St. Louis, Missouri, USA) and 0.125% trypsin (Invitrogen/Gibco-BRL) for 30 and 10 min, respectively. The mixture was triturated, centrifuged and resuspended in DMEM/F-12 supplemented with 10% FBS and 1% penicillin/streptomycin. On the following day, 10 μM cytosine arabinoside (Sigma-Aldrich) was added, and the mixture was allowed to incubate for an additional 48 h to remove any fibroblasts. Subsequently, the culture was maintained in DMEM/F-12 supplemented with 10% FBS, 1% penicillin/streptomycin and 2 ng/ml heregulin (Sigma-Aldrich, St. Louis, MO, USA) to stimulate SC proliferation. All media and supplements were purchased from Invitrogen/Gibco-BRL. All experiments were performed in accordance with the guidelines for the use of experimental animals from the National Institutes of Health and ethical approval for this study was obtained from the Ethics Committee of Sun Yat-sen University.

**Viability and proliferation assays for hAECs.** The hAECs were harvested during the logarithmic growth phase and digested using trypsin. To examine cell proliferation and viability, the hAECs (5x10<sup>3</sup> cells/well) were plated in 96-well dishes and incubated in DMEM/F-12 supplemented with 10% FBS. Plates were incubated at 37°C in 5% CO<sub>2</sub> for 24 or 48 hours. Four hours prior to the end of the incubation time, 20 μl 3-(4,5-dimethylthiazol-2-yl) 2,5-diphenyltetrazolium bromide solution (MTT, Sigma-Aldrich) was added to each well. Following a 4 h of incubation, plates were centrifuged and culture medium was removed. Precipitated formazan crystals were dissolved by adding 150 μL DMSO to each well. The microplates were shaken at room temperature for 10 min. A microplate reader (OPTImax; Molecular Dynamics, Sunnyvale, CA, USA) was used to measure the absorbance (A) of each well at 540 nm, and the average values were obtained. Experiments were repeated ≥3 times, and the data are presented as the means ± standard deviation (SD).

**Flow cytometric analysis.** Isolated hAECs were harvested and washed using 0.5% bovine serum albumin (BSA)/0.01 M PBS solution. Following fixation using 4% paraformaldehyde (Invitrogen), the cells were resuspended in 0.5% BSA/PBS solution and incubated for 1 h at room temperature with fluorescein isothiocyanate (FITC)-conjugated primary mouse monoclonal antibody against CD34 (MA1-12103; Thermo Fisher Scientific, Waltham, MA, USA; 1:200). Negative controls were produced using mouse immunoglobulin G 1-isotyped antibodies conjugated to FITC (Beckman Coulter, Marseille, France). The samples were analyzed using a BD FACSCanto™ II system (BD Biosciences, San Jose, CA, USA). A total of 3x10<sup>4</sup> events were captured using the BD FACSDiva™ software (BD Biosciences) histogram feature. The data were further analyzed using CellQuest software (BD Biosciences)

and WinMDI software version 2.9 (Scripps Research Institute; La Jolla, CA, USA).

**Differentiation of hAECs via co-culture with SCs.** hAECs were co-cultured with SCs in a double-chamber system with SCs on the upper level and hAECs on the lower level of the culture plate. The cultures were suspended in DMEM/F-12 culture medium at a density of  $2 \times 10^4$  cells/well in a 6-well dish (Costar; Corning Inc., Corning, NY, USA). Culture inserts (Costar; Corning Inc.), each containing  $8 \times 10^4$  SCs, were placed in four of the six wells using one insert/well. An insert membrane with a pore size of  $0.4 \mu\text{m}$  diameter was used to permit only the transmission of soluble factors and to prevent direct interaction between the hAECs and the SCs. Half the volume of the co-culture medium was replaced every three days. hAECs under identical conditions but without co-culture with SCs served as negative controls and SCs alone served as positive controls.

**Immunocytochemistry.** The characteristics of differentiated hAECs at the protein expression level were assessed via immunocytochemistry. The cells were fixed at room temperature with 4% paraformaldehyde for 10 min. Subsequently, the cells were washed with PBS, followed by permeabilization for 30 min using 0.1% Triton X-100. Non-specific binding sites were blocked by incubation with 5% goat serum for 1 h. The following primary antibodies were employed for immunocytochemistry: monoclonal mouse against Pan-cytokeratin (4545; 1:200) and monoclonal mouse against S-100 (5529; 1:200) obtained from Cell Signaling Technology (Danvers, MA, USA). The cells were incubated in the primary antibodies overnight at  $4^\circ\text{C}$ . The cells were washed three times with PBS and incubated with the corresponding goat secondary antibodies (4408; 1:500) for 2 h at room temperature. Subsequent to washing twice with PBS, the cells were incubated in 4',6'-diamidino-2-phenylindole dihydrochloride (1:1,000; Sigma-Aldrich) for 5 min at room temperature to counterstain the cell nuclei. The specimens were washed three times with PBS, each for 5 min, and were then mounted using an antifade solution. Immunolabeled cells were examined via fluorescence microscopy (Zeiss Axiovision Z1, Oberkochen, Germany). Images were digitally recorded and processed using Image-Pro Plus (Media Cybernetics, Rockville, MD, USA).

**RNA isolation.** To collect total RNA,  $2 \times 10^6$  cells were seeded on 10 cm diameter dishes in 8 ml growth medium. The cultures were maintained at  $37^\circ\text{C}$  in a humidified atmosphere of 5%  $\text{CO}_2/95\%$  air. The media were replaced, including the drug of interest. After six days, total RNA was isolated using an Isogen kit (Nippon Gene, Tokyo, Japan) according to the manufacturer's instructions. RNA quantity and purity were determined via spectrophotometry (DU-65; Beckman Coulter, Fullerton, CA, USA).

**Reverse transcription quantitative polymerase chain reaction (RT-qPCR).** RT-qPCR was performed using an RNA PCR kit [avian myeloblastosis virus (AMV)] version 2.1 (Takara Bio, Inc., Shiga, Japan) according to the manufacturer's instructions and with a PCR Thermal Cycler (Takara Bio, Inc.). First-strand cDNA was synthesized from total RNA (1 mg)

using AMV reverse transcriptase XL primed using 50 pmol random 9 mers (Takara Bio, Inc.). The first-strand reaction was conducted as follows:  $30^\circ\text{C}$  for 10 min,  $50^\circ\text{C}$  for 30 min,  $99^\circ\text{C}$  for 5 min and  $58^\circ\text{C}$  for 5 min. The reverse-transcription reaction products (10 ml) were utilized as templates for PCR using 0.2 mM of each primer. Primers specific to rat nerve growth factor (NGF), glial cell-derived neurotrophic factor (GDNF), brain-derived neurotrophic factor (BDNF) and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) were designed using Primer Express 2.0 software (Applied Biosystems, Foster City, CA, USA) and were obtained from Shanghai HuiRui Biotechnology Co., Ltd. (Shanghai, China). The PCR products were separated via precast 2% agarose gel (Daiichi Pure Chemicals Co., Ltd., Tokyo, Japan) electrophoresis and visualized via SYBR-Green 2 (FMC Bioproducts, Rockland, ME, USA) staining using a ultraviolet trans-illuminator (UVP Inc., San Gabriel, CA, USA). The signal intensity of the PCR products was determined using Image J software. The expression levels of the GDNF, BDNF and NGF PCR products were quantified as determined by the intensity of the PCR products compared with that of the corresponding GAPDH reactions.

**Functional co-culture assay with PC12 cells.** PC12 cells were plated in six-well chamber slides pre-coated with collagen (Sigma-Aldrich) at a density of 1,000 cells/well. The PC12 cells were incubated for 24 h in DMEM supplemented with 5% horse serum, 10% FBS, 100 U/ml penicillin and 100  $\mu\text{g}/\text{ml}$  streptomycin. At 24 h prior to plating the PC12 cells, 293T cells and hAECs differentiated towards a SC phenotype were seeded on  $0.4 \mu\text{m}$  pore size culture inserts at a seeding density of  $2 \times 10^4$  cells/insert and incubated at  $37^\circ\text{C}$ , 95% humidity and 5%  $\text{CO}_2$ . After 48 h, the inserts were checked for cell adherence and were added to the PC12 chamber slides such that molecules secreted by the cells were able to permeate to the PC12 cells without direct cell contact. Neuron-like outgrowth was measured using an Olympus IX81 microscope (Olympus Corporation, Tokyo, Japan) and a U-CMAD 3 camera (Olympus Corporation). Cell body size and axon length were analyzed using SIS image analysis software version 3.2 (Olympus Soft Imaging Solutions GmbH, Münster, Germany). PC12 cells with axons longer than the average cell diameter were used for data analysis. Cell axon length was analyzed using image-pro-plus (SIS, Münster, Germany).

**Statistical analysis.** All data were analyzed using SPSS 11.0 statistical software (SPSS, Inc., Chicago, IL, USA). The experimental data are expressed as the mean  $\pm$  SD. The Student's t-test was performed to compare the values.  $P < 0.05$  was considered to indicate a statistically significant difference.

## Results

**Identification of hAECs.** The viability of the isolated cells was  $>95\%$ , as determined via trypan blue staining. In the presence of EGF, hAECs proliferated robustly and formed a confluent monolayer of cobblestone-shaped epithelial cells (Fig. 1A). Approximately all isolated cells reacted with antibodies against pan-cytokeratin, which revealed the epithelial nature of the cells and the lack of contamination with human amniotic mesenchymal stromal cells (Fig. 1B). The absence of

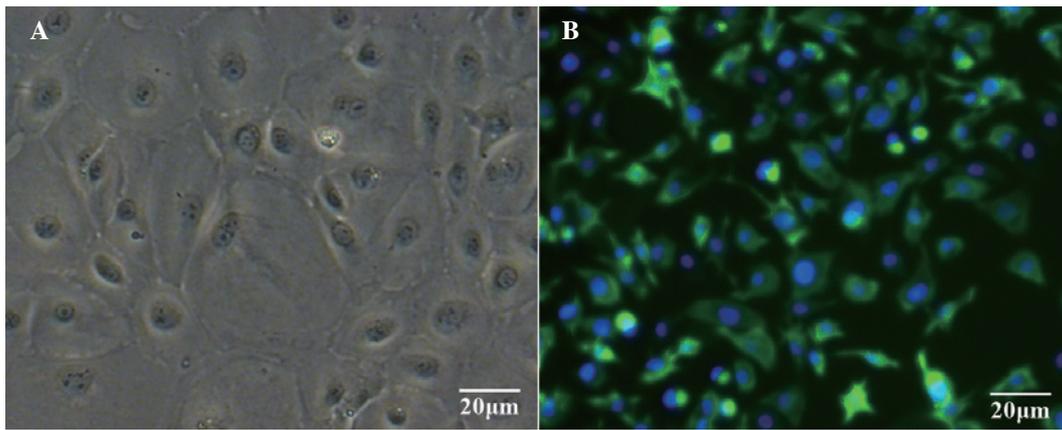


Figure 1. Identification of human amniotic epithelial cells. (A) Microscopic examination of human amniotic epithelial cells seven days after culture (magnification, x40). (B) Immunostaining against pan-cytokeratin (magnification, x20).

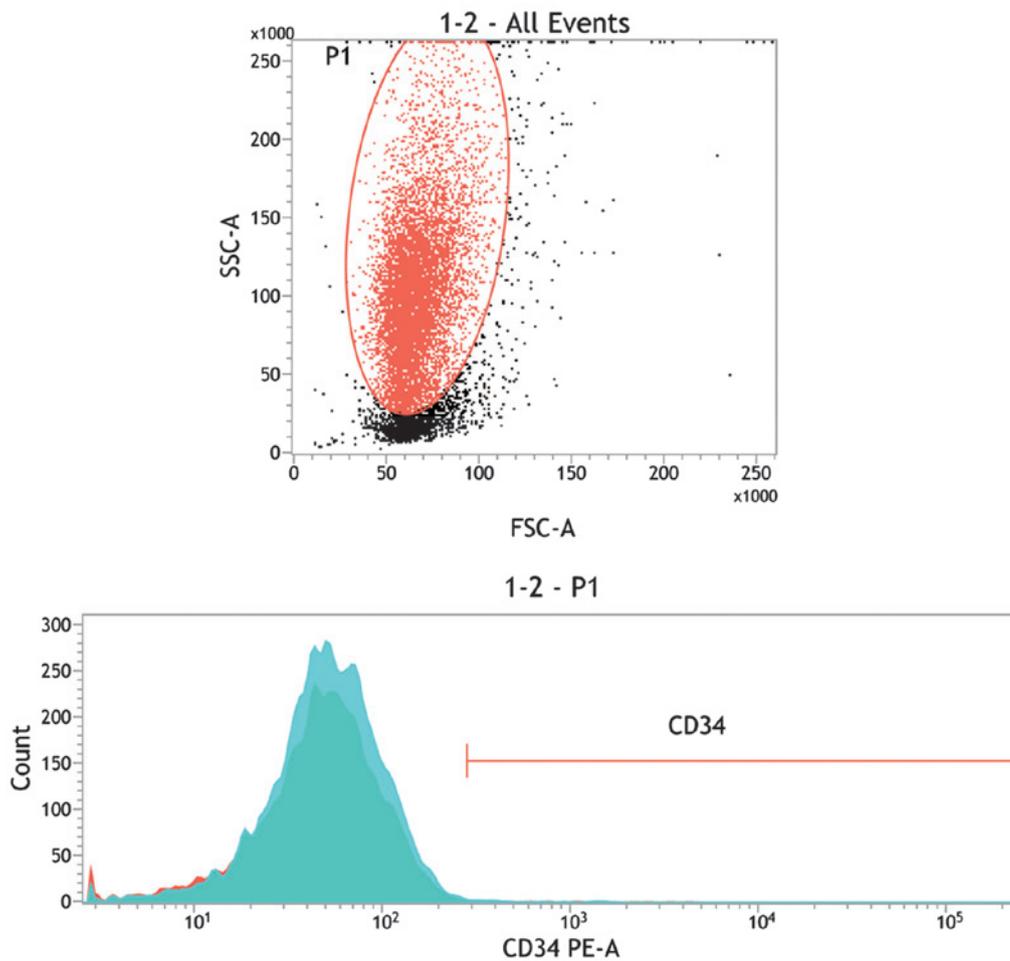


Figure 2. Flow cytometry analysis of human amniotic epithelial cells. SSC, side light scatter; FSC, forward light scatter; CD, cluster of differentiation; PE, phycoerythrin.

CD34-positive cells in this population indicates that the isolates were not contaminated with hematopoietic stem cells, such as umbilical cord blood cells or embryonic fibroblasts (Fig. 2).

*Identification of differentiated hAECs.* hAECs cultured alone appeared as a monolayer of cobblestone-shaped epithelial cells (Fig. 3A). Following co-culture of the cells with SCs for

21 days, certain hAECs exhibited a typical bipolar or tripolar morphology (Fig. 3B). As compared with undifferentiated hAECs (Fig. 3C), differentiated hAECs exhibited positive expression of S-100 (Fig. 3D).

*Expression levels of NGF, BDNF and GDNF mRNA following differentiation.* To examine the biological function

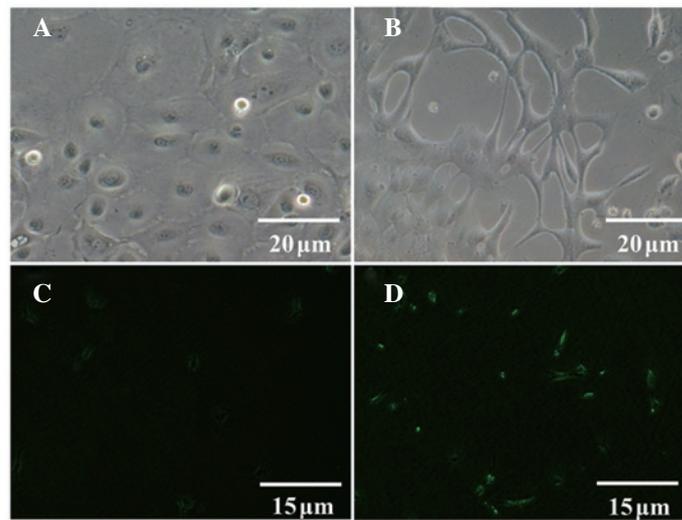


Figure 3. Differentiation of hAECs. (A) Microscopy of primary hAECs on day 7 revealed a spindle-like morphology. (B) Following differentiation, hAECs exhibited atypical bipolar or tripolar morphology. (C) Immunostaining of undifferentiated hAECs against S100 revealed negative expression. (D) However, differentiated hAECs exhibited positive S100 expression. hAECs, human amniotic epithelial cells.

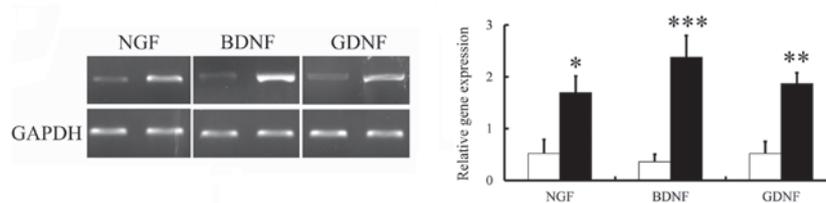


Figure 4. mRNA expression levels of neurotrophic factors in hAECs. The results of reverse transcription quantitative polymerase chain reaction analysis of NGF, BDNF and GDNF mRNA expression levels. Representative results are shown, and the data are presented as the mean  $\pm$  standard deviation (n=6). A two-tailed Mann-Whitney U test was performed for statistical analysis of differential expression levels. \*P<0.05, \*\*P<0.01 and \*\*\*P<0.001. hAECs, human amniotic epithelial cells; NGF, nerve growth factor; BDNF, brain-derived neurotrophic factor; GDNF, glial cell-derived neurotrophic factor.

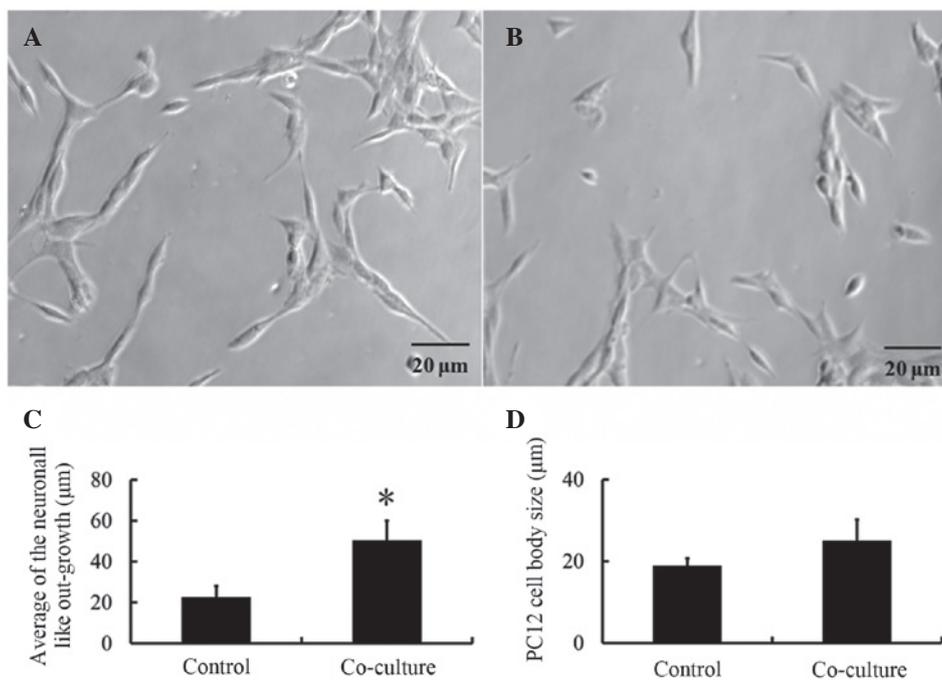


Figure 5. Effect of co-culture with differentiated hAECs on the axonal sprouting of PC12 cells. Microscopic images of (A) PC12 cells co-cultured with 293T cells as a control or (B) with differentiated hAECs. Comparison of (C) the average axon length of PC12 cells and (D) the average diameter of the PC12 cell bodies subsequent to co-culture for 10 days. The data are presented as the means  $\pm$  standard deviation of PC12 cell cultures in media (n=6). A two-tailed Mann-Whitney U test was performed for statistical analysis. \*P<0.05 compared with the control group. hAECs, human amniotic epithelial cells.

of the differentiated cells, the expression levels of the NGF, BDNF and GDNF neurotrophic factors in the cells were measured via RT-qPCR using specific primers, as described above. Neurotrophic factors are polypeptides or proteins that are released by neurons or non-neuronal cells that exert marked effects on the central and peripheral nervous systems (23). Exogenous administration of NGF has been reported to completely prevent the death of axotomized sensory neurons (24,25). Subsequent to co-culture of the hAECs with SCs, the expression levels of NGF mRNA in the hAECs increased 1.55-fold as compared with treatment with saline (Fig. 4). Subsequently, whether co-culture results in a sustained increase in BDNF and GDNF mRNA expression levels was determined. The BDNF and GDNF mRNA levels were also significantly increased following co-culture (1.5-fold,  $P < 0.05$ ; and 1.8-fold,  $P < 0.05$ , respectively).

**PC12 cell morphology.** To determine the effect of differentiated hAECs on PC12 cell axon regeneration subsequent to co-culture of PC12 cells with differentiated hAECs, the number of PC12 cells that exhibited axonogenesis, the cell body sizes and the overall length of the outgrown fibers were quantified. Fig. 5 clearly presents the biological activity of the differentiated cells. Statistical analysis of the neuron-like outgrowth of the PC12 cells revealed significant induction of axonogenesis following co-culture ( $P < 0.05$ ;  $51.32 \pm 8.56 \mu\text{m}$ ) as compared with the control group ( $21.32 \pm 5.45 \mu\text{m}$ ). In addition, the average cell body size of the PC12 cells was determined during the culture period since alterations in cell morphology and size indicate higher metabolic activity induced by external stimuli. The cell body sizes were measured for each group. The average PC12 cell body size increased following incubation in the co-culture. However, these marginal changes were not statistically significant.

## Discussion

In the present study, hAECs were differentiated into SCs using a co-culture method. The results revealed that, following differentiation, the hAECs exhibited a spindle-like morphology. The hAECs were immunopositive for the S-100 SC markers. Subsequently, the function of the differentiated hAECs was analyzed. The RT-qPCR results revealed that NGF, BDNF and GDNF expression was upregulated as compared with the control group. In addition, following the co-culture of differentiated hAECs with PC12 cells, the differentiated cells significantly increased the average axon length of the PC12 cells.

In recent years, numerous strategies have been developed to prepare nerve conduits involving biomaterials that incorporate growth factors, cells and natural microarchitectural structures (26,27). To more effectively repair nerve defects, the construction of nerve grafts that integrate these factors is a promising alternative to the employment of autologous nerves. SCs are a good candidate for use as seed cells. However, treatment with primary SCs as a cell therapeutic remains limited by the associated donor site morbidity (28). In addition, for clinical applications, the time period required to generate a sufficient quantity of cells is not always feasible (29). Therefore, various studies have attempted to identify an alternative method. hAECs, which exhibit characteristics of embryonic and

pluripotent stem cells, may be utilized for cell therapy without legal or ethical problems (30). The differentiation of hAECs has been widely investigated. Using  $\beta$ -mercaptoethanol, Banerjee *et al.* (31) found that human amniotic membranes were differentiated towards the SC lineage *in vitro*. Following differentiation, the cells expressed BDNF and GDNF, a finding concurrent with that of the present study.

Contrary to BMSCs or ADSCs, hAECs are not stem cells. However, hAECs do possess the multi-lineage differentiation potential required to develop into tissues of three germ layers (ectodermal, mesodermal and endodermal lineages), a finding which has been demonstrated by multiple studies (32-38). In addition, the demonstration of hAEC expression of pluripotent and multipotent markers, such as octamer-binding protein (Oct)-3/4, SRY-related HMG-box 2, Nanog, fibroblast growth factor 4, reduced expression 1, frizzled class receptor 9, bone marrow stromal cell antigen 1 and ATP-binding cassette sub-family G member 2, provides further support for the multi-lineage differentiation potential of hAECs (32,39,40).

In the present study, the overall proportion of differentiated hAECs remained low. Two factors may account for this phenomenon. On one hand, the quantity of co-cultured hAECs was large. Incubation of an excessive number of cells together may not only restrict cell growth and differentiation but also limit the relative effect of the proteins secreted by the SCs. Conversely, Miki *et al.* (32) found that hAECs grow as three layers when cultured in dishes: A layer of cells in the supernatant, an intermediate layer and a layer of cells attached to the culture dish. Furthermore, the expression levels of the pluripotent markers Oct-4 and Nanog were elevated in the cells that were present above the basal layer of hAECs, which indicates that the cells above the basal layer exhibited greater differentiation ability. This phenomenon may partially explain why particular hAECs differentiate more quickly and easily. In the present study, due to the only partial differentiation of hAECs into SCs observed, the function of the cells following co-culture was subsequently examined.

PC12 cells are a well-defined and widely used model for the analysis of axon regeneration (41). In the present study, following co-culturing, the average axon length was observed to increase from  $21.32 \pm 5.45$  to  $51.32 \pm 8.56 \mu\text{m}$ . The PC12 cell body size may also have increased, as alterations in cell morphology and size indicated a higher metabolic activity induced by external stimuli. A marginal, but not significant, increase in the average cell body size was demonstrated. This result implied that unlike average axon length, cell body size may not provide an appropriate marker of regeneration.

In conclusion, in general, using a co-culture technique, hAECs were demonstrated in the present study to differentiate towards SCs *in vitro*. The differentiated hAECs exhibited certain characteristics of SCs and greatly increased the axon length of PC12 cells. These promising results indicate that this method may provide a good strategy for the treatment of nerve injuries in humans.

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