Human Amniotic Epithelial Cells Cultured in Substitute Serum Medium Maintain Their Stem Cell Characteristics for Up to Four Passages

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Background and Objectives: The common applied culture medium in which human amniotic epithelial cells (hAECs) maintain their stem cell characteristics contains fetal calf serum (FCS) and thus is not compatible with possible future clinical applications due to the danger of animal derived pathogens. To overcome this problem, we replaced FCS with serum substitute supplement, a serum substitute used in the in vitro fertilization for embryo development, in the common applied culture medium and cultured hAECs in this substitute serum medium (SSM).

Methods and Results: Purity validation and characterization of freshly isolated and cultured hAECs was assessed through the expression of stem cell specific markers by RT-PCR (gene expression), by immunofluorescence staining and FACS (protein expression). Furthermore, karyotype was performed at passage four in order to exclude possible chromosome anomalies in hAECs cultured in SSM. The differentiation potential of hAECs into the cardiomyogenic lineage was tested through cardiac Troponin T expression by immunohistochemistry. hAECs cultured in SSM maintained expression of all the major pluripotent genes Sox-2, Oct-4 and Nanog as well as the expression of the embryonic stem cell specific surface antigens SSEA-4, SSEA-3 and TRA-1-60 over four passages. Using cardiac differentiation medium containing 10% serum substitute supplement, hAECs differentiated into cardiac troponin T expressing cells.

Conclusions: We can conclude that, hAECs maintain their stem cell characteristics when cultured in SSM for up to 4 passages. This makes possible future clinical applications of these cells more feasible.

Keywords: Amniotic epithelial cells, Serum free, Stem cells, In vitro culture

Introduction

In previous studies, human amniotic epithelial cells (hAECs) were documented as having the potential of in-vitro differentiation to all three germ layers: ectodermal neural cells (1, 2), mesodermal cardiomyocytes (1, 2) and endodermal hepatocytes and pancreatic cells (3-5). The fact that these cells possess low antigenic potential (1), an-

ti-inflammatory properties (6) and low risk to form in-vivo teratomas (2) makes them attractive for potential stem cell based therapies.

Co-culturing of embryonic stem cells with animal derived feeder cells has been shown to present a risk of contamination with animal derived pathogens that could be transmitted to the patient (7, 8). Much effort has been done in the development of feeder-free and serum free culture systems for human embryonic stem cells (9-11). In such serum free culture systems, fetal calf serum (FCS) is often replaced by knockout Serum Replacement (SR). Although SR contains fewer components than FCS, it is still undefined and is a proprietary formulation, largely composed of bovine serum albumin and still carries the risk of contamination with animal derived pathogens (12).

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In high density cultures, hAECs form spheroid structures which retain stem cell characteristics and, therefore, do not require other cell derived feeder layers to express stem cell specific markers (2). In this study, we cultured hAECs with serum substitute supplement routinely used as culture media for protein supplements in gamete and embryo manipulation for assisted reproductive procedures (13).

Materials and Methods

Tissue collection

Placentas were obtained from uncomplicated vaginal deliveries or elective cesarean sections from healthy mothers who signed informed consent. The amnion layer was mechanically peeled off from the chorion and transferred to the laboratory in PBS supplemented with antibiotics. In the laboratory, the amnion was meticulously washed with PBS supplemented with antibiotics.

Isolation and characterization of human amniotic epithelial cells (hAECs)

Our isolation protocol was based on previous publications (2, 14). Briefly, in order to release hAECs, the amnion membrane was first placed in a 50 ml centrifugation tube (BD Falcon, Franklin Lakes, NJ, USA) containing 10 ml 0.25% trypsin/EDTA (Kibbutz Beit-Ha'Emek, Israel) and was shaken for 30 seconds at room temperature. The amnion membrane was then transferred into two new 50 ml centrifugation tubes (Falcon), each containing 15 ml 0.25% trypsin/EDTA (Beit-Ha'Emek) and was shaken again in a Comfort shaker (Comfort. Heto Master Shake, Heto-Holten A/S Type: SBD50-1, Paris, France) at 200 rpm (12×g) at 37°C for 10 minutes. The cells from the first 10 minutes of digestion were discarded, in order to exclude debris. The amnion membrane was then transferred into two new 50 ml centrifugation tubes (Falcon), each containing 15 ml 0.25% trypsin/EDTA (Beit-Ha'Emek) and shaken at 37°C for 30 minutes in a Comfort shaker (Comfort. Heto Master Shake). Following an additional 30 minutes of incubation, the amnion membrane was discarded. 10 ml of standard medium was added to the digests, which were then centrifuged at 1,300 rpm, in order to remove trypsin. Cells were pooled, filtered through a 100 µm cell strainer and counted in a hemocytometer (14). Purity validation of freshly isolated hAECs was assessed with CD34 to exclude possible contamination with hematopoietic stem cells such as umbilical cord blood or embryonic fibroblasts and by the expression of cytokeratin together with the expression of the three embryonic stem cell specific surface markers (SSEA-4,

SSEA-3 and TRA-1-60) in order to distinguish between amniotic epithelial cells and amniotic mesenchymal stem cells (15).

Cell cultures

The hAECs were plated on 60 mm or 100 mm diameter plastic petri dishes (Falcon) in standard culture medium (StM) or in substitute serum medium (SSM) at a density of 12.7×10^4 cells per cm^2 . The StM was Dulbecco's modified Eagle's medium (Beit-Ha'Emek) supplemented with 20% FCS, 2 mM L-glutamine, 1% nonessential amino acids, 1 mM sodium pyruvate, 1% antibiotic-antimycotic (all from Beit-Ha'Emek), 55 µM 2-mercaptoethanol (Sigma-Aldrich, St. Louis, MO, USA) and 10 ng/ml EGF (Sigma-Aldrich). In SSM, FCS was replaced by 20% serum substitute supplement (Irvine Scientific, Santa Ana, CA, USA). Serum substitute supplement (Irvine Scientific) consisted of 6% total protein (weight/volume) in a normal saline. The protein component contained 84% human serum albumin from a therapeutic grade source material and 16% alpha and beta globulins.

Cell cultures were maintained in a humidified atmosphere containing 5% CO_2 at 37°C. Upon reaching confluence, the cells were harvested from tissue culture dishes by trypsin/EDTA (Beit-Ha'Emek), centrifuged and were then re-cultured. For cardiomyocyte differentiation, hAECs were cultured in StM containing 10% FCS without EGF, or in SSM, containing 10% serum substitute supplement (Irvine Scientific) without EGF, supplemented with 1 mM ascorbic acid 2-phosphate (Sigma-Aldrich) on microscope slides (Superfrost, Menzel GmbH, Braunschweig, Germany) under mineral oil for immunohistochemistry at a density of 1×10^4 cells per cm^2 for 2~3 weeks.

Immunohistochemistry

Immunohistochemistry was performed using the Histo-stain-plus kit (Zymed laboratories Inc., USA). Isolated amniotic epithelial cells grown on microscope slides (Superfrost, Menzel GmbH) were fixed with ice cold acetone for 10 minutes and quenched with 3% hydrogen peroxide in methanol to eliminate endogenous peroxidase activity. The slides were then washed, blocked and incubated at room temperature with primary antibodies: mouse anti-human cytokeratin 22-cocktail (Biomedica, Foster City, CA, USA), mouse monoclonal anti-human cardiac troponin T, sc-20025 (Santa Cruz Biotechnology, Inc., CA, USA) and mouse anti-human CD34 (Signet, Princeton, New Jersey, USA). Secondary antibodies used: Histo-stain-Plus broad-spectrum biotinylated second antibody (Zymed laboratories Inc.). The slides were then de-

veloped with a substrate-chromagen solution of amino-ethyl carbazole (Zymed Laboratories Inc.). The slides were photographed through an upright light microscope (Olympus Europa GmbH, Hamburg, Germany) using a Nikon DS Fi1 digital camera (Nikon Instruments Inc., Melville, NY, USA).

Fluorescence activated cell sorter analysis (FACS)

Freshly isolated and cultured hAECs were examined for surface antigens commonly found on embryonic stem cells (16, 17). The following specific primary monoclonal antibodies ($2 \mu\text{g/ml}$ each) were used to detect surface antigen expression: mouse monoclonal anti-human SSEA-4 sc-21704, rat monoclonal anti-human SSEA-3 sc-73066 and mouse monoclonal anti-human TRA-1-60 sc-21705 (all from Santa Cruz Biotechnology). Secondary goat anti-mouse IgG, DyLight488 (Thermo Scientific, Rockford, IL, USA) was used for SSEA-4 and TRA-1-60; and secondary goat anti-rat IgG conjugated with AlexaFluor-488 (Invitrogen Molecular Probes, Eugene, Oregon, USA) was used for SSEA-3. Negative controls were run in parallel without the primary antibody, with the secondary fluorescein-conjugated antibody only, in order to verify specificity of secondary antibody binding. Cells (1×10^6 cells/ml) were washed in PBS (Beit-Ha'Emek) containing 5% fetal calf serum (Beit-Ha'Emek) at 1,250 rpm, 4°C for 10 minutes and then incubated with the primary antibody for 45 minutes in ice. After three washings in PBS containing 5% fetal calf serum at 1,250 rpm, 4°C for 10 minutes each time, the cells were incubated with the secondary, fluorescein-conjugated antibody at room temperature for 30 minutes in the dark. After three washings in PBS containing 5% fetal calf serum at 1,250 rpm, 4°C for 10 minutes each time, 500 μl PBS containing 1% paraformaldehyde (Electron Microscope Sciences, Belgar) was added to the pellet and the cell solution was stored at 4°C in the dark until analysis. Cells were analyzed on a flow cytometer (FACS Calibur, CA, USA).

Immunofluorescence staining

For immunofluorescence analysis, hAECs were cultured in StM or in SSM on cover glasses (Menzel GmbH, Braunschweig, Germany) at a density of 12.7×10^4 cells per cm^2 . The cells were washed 3 times with PBS and fixed with 4% paraformaldehyde (Electron Microscope Sciences, Belgar) in PBS for 10 minutes at 4°C , then washed twice with PBS and permeabilized for 5 minutes at 4°C with 0.2% Triton (Sigma-Aldrich) in PBS. After a PBS wash, the cells were incubated for 30 minutes with blocking buffer (PBS supplemented with 3% BSA), then washed twice

with PBS and were then incubated for 30 minutes at room temperature with primary antibodies (mouse monoclonal anti-human Oct-3/4 sc-5279, mouse monoclonal anti-human SSEA-4 sc-21704, rat monoclonal anti-human SSEA-3 sc-73066 and mouse monoclonal anti-human TRA-1-60 sc-21705, all from Santa Cruz Biotechnology) $1 \mu\text{g}$ per cover glass in 700 μl PBS supplemented with 1.5% BSA. After five washings with PBS, the cells were incubated for 30 minutes in the dark, at room temperature, with secondary fluorescein-labeled antibodies (for Oct-3/4, SSEA-4 and TRA-1-60: goat anti-mouse IgG, DyLight 488 (Thermo Scientific); and for SSEA-3: goat anti-rat IgG conjugated with AlexaFluor-488 (Invitrogen Molecular Probes), 0.5 μg per cover glass in 700 μl PBS supplemented with 1.5% BSA. Following two washings with PBS, the cells were incubated with $1 \mu\text{g/ml}$ DAPI (GX12369, Inno-Train Diagnostik GmbH, Kronberg/Taunus, Germany) for 1~2 minutes at room temperature in the dark, washed twice with PBS and were then mounted with DPX (Sigma-Aldrich) onto microscope slides (Superfrost, Menzel GmbH). Negative controls were run in parallel by replacing the primary antibody with mouse and respectively rat IgG isotype control antibody (Santa Cruz Biotech) at the same concentration as the primary antibody ($1 \mu\text{g}$ pr slide in 700 μl PBS supplemented with 1.5% BSA). No specific immuno-reactivity was detected in these negative control specimens. Stained cells were photographed at a magnification of $\times 20$, using an upright fluorescence microscope Axioscop 2 (Carl Zeiss GmbH, Hamburg, Germany) and a CCD camera hooked to the system (Roper Scientific Camera, Roper Industries Inc., Sarasota, Florida, USA).

RNA isolation for RT-PCR

For reverse transcription-polymerase chain reaction (RT-PCR), hAECs were plated on 100 mm or 60 mm diameter plastic petri dishes (Falcon) at a density of 12.7×10^4 cells per cm^2 and cultured in StM or in SSM. RNA was isolated from freshly isolated hAECs and from cultured hAECs at the end of each passage, using the EZ-RNA Total RNA isolation kit (Beit-Ha'Emek), in accordance with the manufacturer's instructions. RNA concentration was determined spectrophotometrically.

First strand cDNA synthesis for RT-PCR

To obtain the cDNA, 5 μg RNA was denatured at 70°C for 10 min and then reverse transcribed in the presence of 25 ng/ μl random primer (Promega, Mannheim, Germany), 2.5 mM MgCl_2 , 0.5 mM deoxy-NTPs, 10 mM dithiothreitol, and 10 U ribonuclease H- reverse transcriptase (SuperScript II RT, Life Technologies, Inc Invitrogen,

Dorset, UK.) for 60 min at 42°C, and 5 min at 95°C. Subsequently, 10 µl of the resulting cDNA was used as a template for PCR.

Reverse transcription-polymerase chain reaction (RT-PCR)

The 1st strand cDNA product, 3 µl, was subsequently amplified in a total volume of 30 µl PCR assay, containing 6 µl Red load Taq master (LAROVA GmbH, Teltow, Germany), 0.5 µM of each primer and was filled up to a total volume of 30 µl with sterile PCR grade water. Primers for Beta actin, GAPDH, Oct-4, Sox-2 and Nanog were used. The PCR amplification for all primers was performed for 45 cycles in an automated thermocycler profile. Each primer was normalized for sub-saturation condition. We used a series of dilutions of a bulk RNA preparation, in order to determine sub-saturation conditions for the PCR products using GAPDH. The amplification parameters were denaturation at 94°C for 5 minutes, followed by 45 cycles as follows: denaturation at 94°C for 30 seconds, annealing at 57°C for 1 minute, elongation at 72°C for 90 seconds and a 72°C extension for 10 minutes at the end of the program. RT-PCR products were analyzed by a 2.5% agarose ethidium bromide gel electrophoresis. Images were captured with Polaroid film (Hertfordshire, UK) under UV light. Amplification of the housekeeping gene GAPDH transcripts was performed simultaneously, in order to confirm RNA integrity and efficiency and for quantification of cDNA. Negative control reactions containing samples without cDNA or Taq enzyme were used.

Primer design

The sequences of appropriate primers were obtained from the gene bank and prepared at IDT Inc., Hy-Labs, Rehovot, Israel.

BETA ACTIN

BETA ACTIN FWD 5'-AGGCATCCTCACCTGAAGTA-3';

BETA ACTIN REV 5'-CACACGCAGCTCATTGTAGA-3';

105 bp product

GAPDH

GAPDH FWD 5'-TGATGACATCAAGAAGGTGGTGAAG-3';

GAPDH REV 5'-TCCTTGGAGGCCATGTGGGCCAT-3';

230 bp product

OCT-4

OCT-4 FWD 5'-GAGGAGTCCCAGGACATGAA-3';

OCT-4 REV 5'-GTGGTCTGGCTGAACACCTT-3';

151 bp product

SOX-2

SOX-2 FWD 5'-GCCGAGTGGAACCTTTTGTC-3';

SOX-2 REV 5'-GTTCATGTGCGCGTAACTGT-3';

264 bp product

NANOG

NANOG FWD 5'-CTGTGATTTGTGGGCCTGAA-3';

NANOG REV 5'-TGTTTGCCCTTTGGGACTGGT-3';

153 bp product

Karyotype

hAECs were cultured for three passages in StM and respectively in SSM and were then released from the tissue culture flasks by trypsin/EDTA (Beit-Ha'Emek), centrifuged and seeded onto cover glasses (Menzel GmbH) at a density of 12.7×10^4 cells per cm^2 . 24 hours later, the cells were treated with 0.15 µg/ml colcemid (Invitrogen GIBCO Carlsbad, California, USA) for 30 minutes and were then exposed to 0.0375 M KCL hypotonic solution for 10 minutes. Cells were fixed with 3 : 1 methanol:acetic acid. G-banding was performed with 4 : 1 Gurr's/Leishmann's stain (Sigma-Aldrich).

Differentiation into cardiac troponin T expressing cells

The differentiation potential of hAECs into cardiomyogenic lineage was tested through cardiac Troponin T expression by immunohistochemistry. For this, hAECs were cultured in a differentiation medium containing 10% FCS (Beit-Ha'Emek), or respectively, a 10% serum substitute supplement (Irvine Scientific) without EGF, supplemented with 1mM ascorbic acid 2-phosphate (Sigma-Aldrich) on microscope slides (Superfrost, Menzel GmbH) at a density of 1×10^4 cells per cm^2 for 2~3 weeks.

Statistical analysis

Unless stated differently, results were expressed as mean $\pm$ SD. Student's t-test was used when appropriate. $p < 0.05$ was considered significant.

Results

Characterization of freshly isolated hAECs

Purity validation and expression of stem cell specific markers: Purity of freshly isolated hAECs was clarified with positive staining against cytokeratin and negative for

Fig. 1. Immunohistochemical staining, FACS analysis, immunofluorescence staining and RT-PCR of freshly isolated hAECs. Immunohistochemical staining for cytokeratin cocktail (A) and CD34 (B); scale bar=20 μ m in A and B. (C) FACS analysis: black bar, SSEA-4; white bar, SSEA-3; dashed bar, TRA-1-60. (D) Immunofluorescence staining, Oct-4, TRA-1-60, SSEA-4 and SSEA-3 are in green color. Blue - DAPI nuclear staining; scale bar=20 μ m. (E) RT-PCR with photographs of representative gene products bands. Beta actin, 105 bp product; Oct-4, 151 bp product; Sox-2, 264 bp product and Nanog, 153 bp product. FACS: flow activated cell sorter; hAECs: human amniotic epithelial cell; bp: base pairs.

CD34. About 98% of all freshly isolated hAECs were cytokeratin positive (Fig. 1A), while no CD34 staining was observed, thus excluding contamination with hematopoietic stem cells such as umbilical cord blood or embryonic fibroblasts (Fig. 1B).

The percentage of freshly isolated hAECs demonstrating stem cell similarity was assessed by FACS using three embryonic stem cell specific surface markers. Freshly isolated hAECs from 5 different placentas were analyzed. Results are depicted as mean $\pm$ SD of % of cells expressing embryonic stem cell specific surface marker in Fig. 1C. Among freshly isolated hAECs, 16.54 $\pm$ 14.58% of cells expressed SSEA-4, 26.36 $\pm$ 11.17% of cells expressed SSEA-3 and 20.8 $\pm$ 16.5% of cells expressed TRA-1-60.

Protein expression of stem cell specific markers in freshly isolated hAECs, tested by immunofluorescence staining against SSEA-4, SSEA-3, TRA-1-60 and Oct-4 is

presented in Fig. 1D.

The protein expression of the three embryonic stem cell specific surface markers (SSEA-4, SSEA-3 and TRA-1-60) validate that these cells are in fact amniotic epithelial cells and not amniotic mesenchymal cells, as it has been shown that the latter do not express the three embryonic stem cell specific surface markers at the protein level (15).

The results of the RT-PCR are shown as photographs of representative bands of the gene products in a 2.5% agarose ethidium bromide gel from five independent experiments in Fig. 1E. Freshly isolated hAECs were found to express all three stem cell specific transcription factors.

Maintenance of hAECs stem cell characteristics cultured in substitute serum medium (SSM): FACS was used to assess the percent of hAECs expressing the three embryonic stem cell specific surface markers cultured in substitute serum medium (SSM) and in standard culture

Fig. 2. Expression of stem cell specific markers in hAECs cultured in SSM and in StM up to passage 5. (A) Flow activated cell sorter analysis of hAECs cultured in SSM and respectively in StM over two passages. Results are expressed as mean $\pm$ SD of % of cells expressing the specific surface marker. White bar, StM; black bar, SSM. $p < 0.05$ ([†]versus StM, ^{††}versus passage 1) (B) RT-PCR of Oct-4, Sox-2 and Nanog gene expression in hAECs cultured in SSM and in StM over 5 passages. Photographs of representative bands of the gene products in a 2.5% agarose gel are shown. GAPDH, 230 bp product; Oct-4, 151 bp product; Sox-2, 264 bp product and Nanog, 153 bp product. FACS: flow activated cell sorter; hAECs: human amniotic epithelial cells; StM: standard culture medium; SSM: substitute serum medium; SD: standard deviation; bp: base pairs.

medium (StM) over the first 2 passages (Fig. 2A). At passage 1, a significantly higher percentage of hAECs cultured in SSM, expressed SSEA-4, SSEA-3, and TRA-1-60 compared to cells cultured in StM (SSEA-4, $59.26 \pm 0.44\%$ in SSM versus $31.14 \pm 2.35\%$ in StM, $p = 0.0036$; SSEA-3, $57.8 \pm 1.4\%$ in SSM versus $31.03 \pm 2.2\%$ in StM, $p = 0.005$; and for TRA-1-60, $42.54 \pm 2.5\%$ in SSM versus $15.83 \pm 5.63\%$ in StM, $p = 0.026$). However, at passage 2, no significant difference in the percentage of hAECs expressing embryonic stem cell specific surface marker was observed in cells cultured in SSM compared to those cultured in StM. In hAECs cultured either in StM, or SSM the percentage of cells expressing SSEA-4 decreased significantly at passage 2 compared to passage 1 ($14.53 \pm 3.6\%$ versus $31.14 \pm 2.35\%$ $p = 0.03$ and $20.58 \pm 0.86\%$ versus $59.26 \pm 0.44\%$, $p = 0.0003$ respectively).

The maintenance of stem cell similarity of hAECs cultured in SSM and in StM up to 5 passages was tested by the gene expression of transcription factors involved in the maintenance of pluripotency and self renewal by RT-PCR

(Fig. 2B). The results are shown as photographs of representative bands of the gene products in a 2.5% agarose ethidium bromide gel from 8 independent experiments in Fig. 2B. hAECs cells cultured in SSM and in StM maintained Oct-4 and Nanog expression up to 5 passages. Sox-2 expression was maintained until passage 4 in hAECs cultured in SSM, and disappeared already at passage 2 in StM.

The protein expression of SSEA-4, SSEA-3, TRA-1-60 and Oct-4 was tested by immunofluorescence staining at passage 1, 3 and 5. Expression of all the tested markers was maintained in hAECs cultured in SSM and in StM up to 5 passages. Representative pictures from five independent experiments are shown in Fig. 3.

Karyotype of hAECs cultured in SSM and respectively in StM at passage 4: In order to exclude chromosome anomalies, a karyotype analysis of hAECs cultured in SSM and in StM was performed at passage 4 and was found normal (Fig. 4A).

Differentiation potential of hAECs into cardiac tropo-

Fig. 3. Immunofluorescence staining of hAECs cultured in SSM and in StM up to passage 5. Immunofluorescence staining of hAECs at passage 1 (A), 3 (B) and 5 (C), (A-1), (B-1) and (C-1) in StM, (A-2), (B-2) and (C-2) in SSM. Green color, Oct-4, TRA-1-60, SSEA-4 and SSEA-3; Blue - DAPI nuclear staining of the corresponding cells; scale bar=20 μ m. hAECs: human amniotic epithelial cells; StM: standard culture medium; SSM: substitute serum medium.

nin T expressing cells: In order to test the differentiation potential of hAECs into cardiac troponin T expressing cells, freshly isolated hAECs were cultured on microscope slides for 13~17 days in cardiac differentiation medium and respectively in StM (control) or in SSM (control). Representative pictures of five independent experiments are shown in Figs. 4B and C. Troponin T expression was observed in hAECs cultured in differentiation medium starting from day 13 with serum substitute supplement (Fig. 4B-1) and in day 17 with FCS (Fig. 4C-1). No expression of Troponin T was observed in the cells cultured in the control (self-renewal) medium (Fig. 4B-2, 4C-2).

Discussion

Substantial effort is devoted in developing culture systems for stem cells, free of animal derived ingredients (11, 12, 18). Consequently, fetal calf serum (FCS) has been replaced by knockout serum replacement (19-21). Still, the latter is largely composed of bovine serum albumin and therefore has the risk of contamination with animal derived pathogens, jeopardizing possible use in future clinical applications. In the present study, we reported on the use of substitute serum culture, applied commonly in assisted reproductive procedures, in which hAECs can be

Fig. 4. Karyotype of hAECs cultured in SSM and StM and differentiation of hAECs into cardiac troponin T expressing cells. (A) Karyotype of hAECs cultured in StM (A-1) and respectively in SSM (A-2) at passage 4. (B, C) Representative pictures of the immunohistochemistry of cardiac Troponin T expression. (B-1) hAECs cultured in differentiation medium containing substitute serum supplement (day 13); scale bar=20 μ m. (C-1) hAECs cultured in differentiation medium containing FCS (day 17); scale bar=20 μ m. (B-2) and (C-2) hAECs cultured in self renewal medium (days 13 and 17 respectively); scale bar=20 μ m in B-2 and C-2. hAECs: human amniotic epithelial cells; StM: standard culture medium; SSM: substitute serum medium; FCS: fetal calf serum.

cultured up to four passages retaining stem cell like characteristics. Purity validation of freshly isolated hAECs was exerted by positive staining to cytokeratin and negative for CD34, thus excluding contamination with hematopoietic stem cells such as umbilical cord blood or embryonic fibroblasts. Furthermore, the protein expression of the three embryonic stem cell specific surface markers (SSEA-4, SSEA-3 and TRA-1-60) validate that the freshly isolated hAECs are in fact amniotic epithelial cells and not amniotic mesenchymal cells, as it has been shown that the latter do not express the three embryonic stem cell specific surface markers at the protein level (15).

Stem cell similarity of freshly isolated hAECs was confirmed by the expression of stem cell specific genes and surface markers, as reported by others (1, 2).

hAECs cultured in SSM were found to maintain the expression of embryonic stem cell specific surface markers for up to 4 passages and were presented with an intact karyotype at passage four. hAECs lack telomerase activity and thus have limited proliferation capacity (2). This might explain the limitation in maintaining stem cell

characteristics for only 4 passages. However, the absence of telomerase in these cells reduces the risk for hAECs to form in-vivo teratomas and brings about an advantage for possible clinical application. In our study, in the hAECs cultured in StM containing 20% FCS, Sox-2 gene expression disappeared already at the second passage, but was maintained up to passage four in hAECs cultured in SSM. The transcription factors Oct-4, Sox-2 and Nanog are the three core transcription factors involved in cellular pluripotency (22-24). Sox-2 can heterodimerize with Oct-4 and up-regulate the expression of Oct-4, Sox-2 and Nanog (25-27). Overexpression of Sox-2 along with Oct-4, c-Myc and Klf4 has been described to be sufficient to reprogram mouse fibroblast cells to the pluripotent state (28-30). Sox-2 is necessary for regulating multiple transcription factors that affect Oct-4 expression. Thus, the essential function of Sox-2 is to stabilize stem cells in a pluripotent state by maintaining the requisite level of Oct-4 expression (31). Furthermore Sox-2 is expressed in cells that retain their ability to proliferate, whereas it is down-regulated in cells that become post-mitotic (32, 33).

hAECs can be cultured in serum free medium maintaining their stem cell characteristics for up to four passages. The serum free culture medium increases the safety of hAECs for future clinical applications.

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Potential conflict of interest

The authors have no conflicting financial interest.

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