TABLE 1. PCR Primers

Gene	Primer Sequence (5′–3′)	Product Size (bp)	GenBank Accession ID
Cytokeratin K12	Forward: TCCTCCTGCAGATTGACAACG Reverse: TTCCAGGGACGACTTCATGG	511	NM_010661
Pax6	Forward: AGTTCTTCGCAACCTGGCTA Reverse: TGAAGCTGCTGCTGATAGGA	500	NM_013627
Keratocan	Forward: AGGATGCCTTCATTCACGGAC Reverse: GCTCATTGTGGTGCTTATGGGG	491	NM_008438
Lumican	Forward: TGCTGTCTCGGCTTCTCTGAAAG Reverse: AACATCCCCCACATTCCCAACC	567	NM_008524
CD34	Forward: CCTTATTACACGGAGAATGGTGGAG Reverse: AAGAGGCGAGAGAGGAGAAATGGG	477	NM_133654
Vimentin	Forward: GAACGGAAAGTGGAATCCTTGC Reverse: GGTTGGCAGAGGCAGAGAAATC	591	NM_011701
Aldh	Forward: CTTCCAGCGGGTCATAAATCTG Reverse: AGCCAGCAAAACAAGTGTCAGG	528	NM_007436
Gapd	Forward: GACCACAGTCCATGCCATCAC Reverse: TCCACCACCCTGTTGCTGTAG	453	NM_008084

collagenase (Sigma-Aldrich) and 38 U/mL hyaluronidase (Sigma-Aldrich) for 30 minutes at 37°C.

Stromal cells were mechanically dissociated into single cells, and cultured in DMEM/F12 (1:1) supplemented with 20 ng/mL epidermal growth factor (EGF; Sigma-Aldrich), 10 ng/mL of fibroblast growth factor 2 (FGF2, Sigma-Aldrich), B27 supplement (Invitrogen, Carlsbad, CA), and 10^3 U/mL leukemia inhibitory factor (LIF; Chemicon International Inc., Temecula, CA) at a density of 5×10^5 cells/mL in a 37°C 5% CO_2 incubator. Initial culture was performed in 24-well plates or 35-mm dishes and then subcultured to 25-cm^2 culture flasks. The spheres were then further subcultured in 75 cm^2 culture flasks after 7 to 14 days, which was repeated every 7 to 14 days. Medium was changed every 5 to 7 days. All dishes and flasks used for sphere culture were polystyrene, noncoated vessels obtained from Asahi Techno Glass (Tokyo, Japan). Stromal sphere cells were examined by immunocytochemistry and RT-PCR. To allow cells to differentiate, cells dissociated from corneal spheres were cultured in serum-free or DMEM/F12 medium (10% FBS) supplemented with or without 2 ng/mL TGF-β (Sigma-Aldrich) for 4 days. Subcultured cells were stained by calcein-AM (Dojindo Laboratories, Tokyo, Japan), as described,[41] to visualize cell morphology. Primary stromal discs of mouse cornea were cultured in keratinocyte-serum free medium (K-SFM; Invitrogen) or DMEM/F12 with 10% FBS for 10 days (37°C, 5% CO_2), to identify any contamination by epithelial cells.

Immunocytochemistry

Immunocytochemistry was performed as described previously.[42] In brief, mouse corneal sphere cells and cells freshly isolated from mouse cornea were attached to glass slides by cytospin preparation (Auto Smear CF-120; Sakura, Tokyo, Japan) and then fixed in 4% paraformaldehyde for 15 minutes at 4°C. Cells were incubated in blocking serum for 30 minutes and then incubated with primary antibodies for 60 minutes. Primary antibodies used were anti-cytokeratin K12 (1:100, Santa Cruz Biotechnology, Santa Cruz, CA), anti-Pax6 (1:500, Chemicon International, Inc.), anti-vimentin (1:100, Santa Cruz Biotechnology), and anti-αSMA (1:200, Laboratory Vision, Fremont, CA). Immunoreactivity of primary antibodies was visualized with secondary antibodies conjugated with Cy3 or FITC (Jackson ImmunoResearch Laboratories, West Grove, PA).

Reverse Transcription–Polymerase Chain Reaction

Sphere cells and freshly dissociated corneal cells were collected and immediately frozen in liquid N_2. cDNAs were synthesized with a cDNA synthesis kit (Life Sciences, Inc., St. Petersburg, FL) from total RNA also prepared with a kit (RNeasy; Qiagen, Hilden, Germany). Gene-specific primers used for cytokeratin K12 (K12), Pax6, vimentin, keratocan, lumican, CD34, aldehyde dehydrogenase (Aldh), and Gapd are shown in Table 1. PCR was then performed (GeneAmp 9700; Applied Biosystems, Foster City, CA). The PCR products were analyzed by agarose gel electrophoresis.

Collagen Gel Contraction Assay

Collagen gel contraction assay was performed as described previously,[43-47] with some modifications. Collagen gels were prepared according to instructions provided by the manufacturer (Cellmaterix Type I-A; Nitta Gelatin, Osaka, Japan). In brief, collagen was mixed with 10-fold concentrated DMEM/F12 medium and 50 mM NaOH containing 260 mM $NaHCO_3$ and 200 mM HEPES at a proportion of 8:1:1 (vol/vol/vol) at 4°C. Then a 0.2-mL aliquot of the solution was placed in the center of each well of a six-well cell culture cluster (Corning Inc., Corning, NY) and allowed to polymerize at 37°C for 30 minutes in a cloning ring 10 mm in diameter (Asahi Techno Glass). Cells cultured in medium containing 10% FBS were harvested and suspended at 2×10^5 cells /mL. Eighty-five micrometers of the cell suspension was applied to a polymerized collagen gel and incubated overnight in a 37°C 5% CO_2 incubator. On day 1, the cloning ring was removed, and 2.5 mL of 10% FBS-containing medium was added to each well to submerge the cells. To examine TGF-β-dependent collagen gel contraction, TGF-β was added at a 0.1- or 1-ng/mL final concentration. As an inhibitor, an anti-TGF-β antibody (0.1 ng/mL) was also added in the medium for selected dishes. FBS-containing media with or without TGF-β and/or TGF-β antibody were changed on day 3. Gel thickness was measured on day 5 with an inverted phase-contrast microscope, by adjusting the plane of focus from the bottom to the top of the gel and recording the distance that the stage had been moved.

Data are expressed as the mean ± SD. Post hoc comparisons between groups was performed with the Tukey procedure. Differences were considered significant at $P < 0.01$.

Results

Sphere Formation from Stromal Cells

More than five mice were used to prepare corneal stromal cells in each experiment. From 10 corneas, $1.32 \pm 0.16 \times 10^4$ cells ($n = 3$) were isolated, and subcultured cells proliferated into spheres, to yield an average of $7.97 \pm 0.35 \times 10^7$ cells per 75 cm^2 flask ($n = 6$) after four passages (P4). Sphere cells were propagated for >12 passages through 5 months without loss of viability. To avoid contamination of epithelial and endothelial cells, stromal discs were carefully prepared as described in the Materials and Methods sections. Dissociated cells from mouse stromal discs formed spheres when cultured in serum-free

medium containing EGF and FGF2 (Fig. 1A, left). To exclude the possibility that spheres may have originated from contaminating epithelial cells, we first performed primary cultures of mouse corneal discs, with or without dispase treatment, followed by epithelium separation. K-SFM with low Ca^{2+} was used to examine epithelial expansion.[48,49] When untreated discs were cultured, migration of epithelial and stromal cells was observed in K-SFM and in DMEM/F12 containing 10% FBS, respectively (Fig. 1B, left). There were no epithelial cells migrating from dispase-treated discs in both media, whereas fibroblasts migrated from the discs in DMEM/F12 with serum (Fig. 1B, right). We further cultured dissociated epithelial cells under conditions that allowed stromal spheres to form by 14 days. As a result, no spheres were observed in the epithelial cell culture (Fig. 1A, right). To demonstrate whether the spheres were hollow or solid, confocal microscopy of 4′,6′-diamino-2-phenylindole (DAPI)-stained spheres was performed. Imaging in different focal planes showed that the inside of spheres was filled with cells, not hollow (Fig. 1C).

We then examined the expression of epithelial and stromal markers in primary and subcultured spheres (P10) by RT-PCR. Stromal markers examined were the proteoglycans, keratocan, and lumican,[13,14,50,51] as well as CD34, which was recently reported to be expressed in keratocytes.[52-54] As shown in Figures 2A and 2B, in addition to the mesenchymal intermediate filament vimentin, the expression of the genes described earlier were detected in the stromal spheres. On the contrary, K12 and Pax6, both of which are expressed in corneal epithelium,[10,32,55-58] were not detected in sphere cells (Fig. 2A).

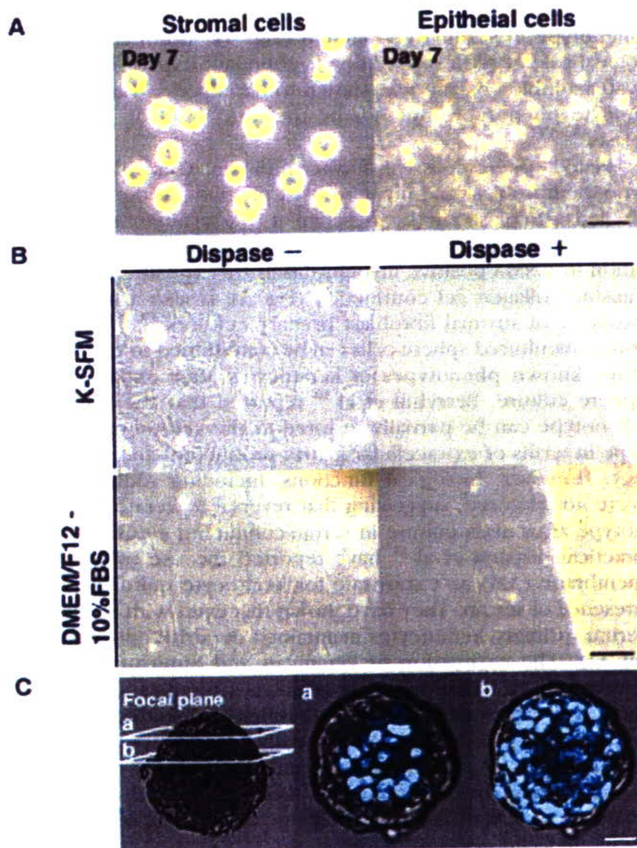

FIGURE 1. Sphere cells derived from the mouse corneal stroma. (A) Mouse corneal stroma and epithelium were separated by dispase treatment. Cells were cultured in DMEM/F12 supplemented with EGF and FGF2. After 7days' culture, spheres formed from stromal cells, but not from epithelial cells. (B) Mouse corneal discs were cultured in K-SFM or DMEM/F12 with 10% FBS. Epithelial cells migrated from intact corneal discs in K-SFM (*top left*) but not from dispase-treated (denuded) discs (*top right*). Expanding fibroblastic cells were still observed after dispase treatment. (C) Confocal images of the sphere in two different focal planes, a and b, as shown schematically (*left*). *Blue*: DAPI-stained nuclei. Scale bar: (A) 50 μm; (B) 100 μm; (C) 10 μm.

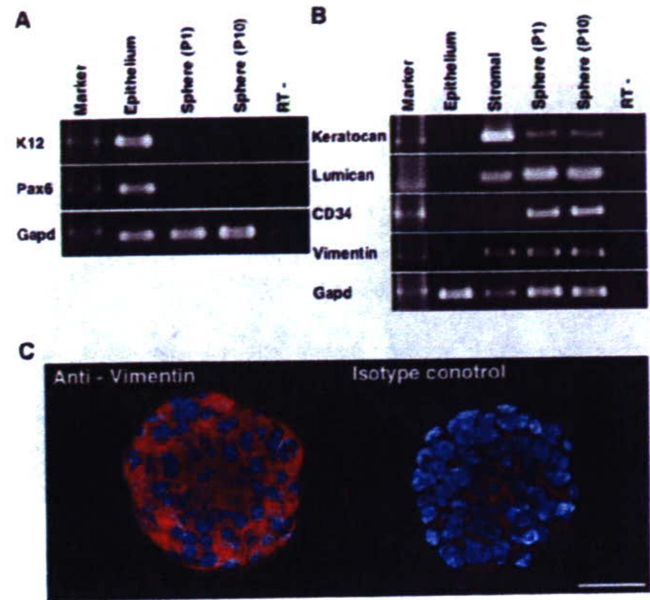

FIGURE 2. Sphere cells express keratocyte markers. (A, B) Total RNA was prepared from epithelial, stromal, and sphere cells. RT-PCR was performed with gene-specific primers. The epithelial markers K12 and Pax6 were not detected in the spheres (A). In contrast, the keratocyte markers keratocan, lumican, and CD34 were detected (B). Immunocytochemical analysis showed expression of the mesenchymal marker, vimentin, in spheres (C). *Blue*: nuclei of cells counterstained with DAPI. Scale bar, 50 μm.

Immunocytochemical analysis of spheres did not detect K12 and Pax6 expression (not shown), whereas vimentin staining was positive (Fig. 2C). These results show that sphere cells were of stromal, not epithelial, origin.

Characteristics of Sphere Cells

Sphere cells plated on collagen I-coated dishes in serum-free medium exhibited a dendritic morphology consistent with keratocytes (Fig. 3A).[3-10] RT-PCR showed that expression of keratocan and Aldh were retained under these conditions (Fig. 4). In contrast, the morphology of sphere cells subcultured in 10% serum were fibroblastic, and the expression of these genes was not detected (Fig. 4). Corneal sphere cells further differentiated to express α-SMA after exposure to TGF-β, which is consistent with the myofibroblast phenotype (Figs. 3C, 3D). Furthermore, when cells were subcultured on collagen gels in the presence of TGF-β, fibroblast-mediated gel contraction was observed (Fig. 5). Without TGF-β, contraction to 68.5% ± 1.75% of the original gel thickness was observed, whereas contraction was enhanced to 50.2% ± 3.96% or 29.4% ± 1.96% of the original thickness in the presence of 0.1 ng/mL or 1 ng of TGF-β, respectively ($P < 0.01$). TGF-β-dependent contraction was reduced to control levels when anti-TGF-β antibody was added to the medium.

Discussion

We successfully isolated and subcultured sphere-forming cells from the mouse corneal stroma, yielding a multifold increase in available cells for further experiments. Zhao et al.[10] have re-

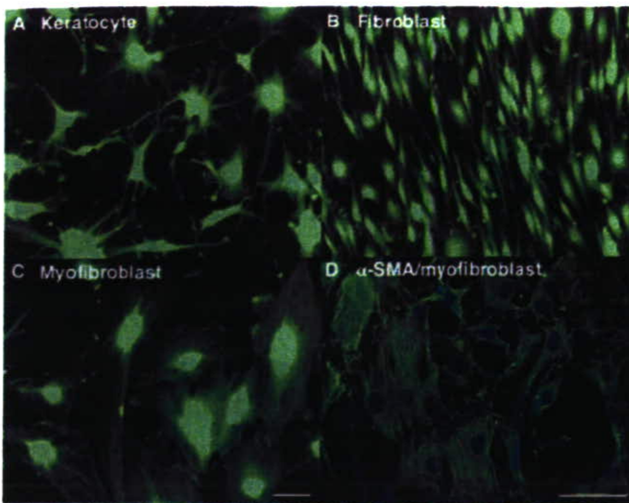

Figure 3. **Phenotype of corneal stromal sphere cells stained by calcein-AM. Dissociated sphere cells were dendritic in SFM (A) and fibroblastic in adherent culture with medium containing 10% FBS (B). In the** presence of TGF-β, morphology of adherent cells became myofibroblastic (**C**), and the cells expressed α-SMA, detected by immunocytochemistry (**D**, *green*). *Blue*: nuclei of cells counterstained with DAPI. Scale bar, 50 μm.

ported that cells present in limbus-derived spheres are derived from the limbal epithelium but not the stroma. However, the corneal sphere cells that we isolated did not express the epithelial markers K12 or Pax6 throughout the study, and furthermore, exhibited properties of corneal keratocytes when subcultured in serum-free medium. The morphology of the subcultured cells as shown in Figure 3 was similar to that of keratocytes in situ, and together with the expression of keratocan, lumican, Aldh, and CD34 in the subcultured cells, the

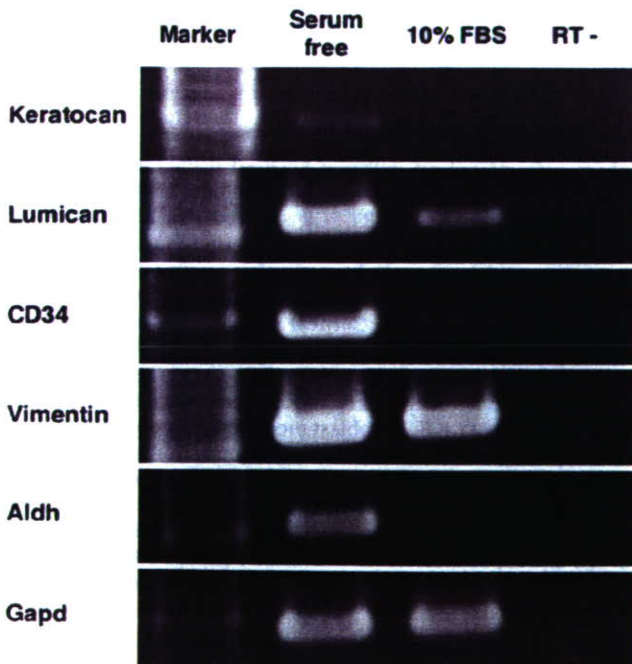

Figure 4. RT-PCR analysis of keratocyte markers expressed in sphere cells subcultured on plastic. Keratocan and Aldh were expressed only in cells in SFM, whereas lumican, CD34, and vimentin were also detected in cells cultured in the presence of 10% FBS.

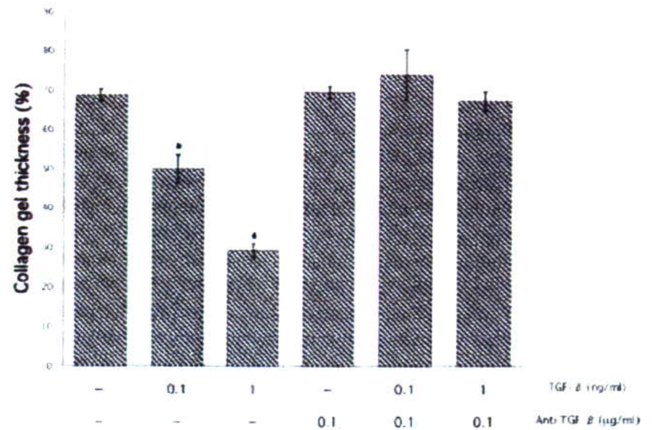

Figure 5. Collagen gel contraction assay of fibroblasts. Mouse corneal spheres were allowed to differentiate in 10% serum-containing medium. Dose-dependent TGF-β-induced collagen gel contraction was observed, which was inhibited by an anti-TGF-β antibody (*$P < 0.01$).

collective evidence shows that these cells were of keratocyte origin. Although most genes were expressed during sphere cultures and maintained after adhesion to plastic dishes, keratocan, and Aldh were exclusively expressed in the keratocyte phenotype in serum-free medium (Fig. 4). Although the biological role of Aldh is not known, abundant expression of the water-soluble enzyme was shown to be expressed in the keratocyte phenotype, but not by the fibroblasts or myofibroblasts.[32,59]

Plated sphere cells can further be induced to differentiate into the fibroblast and myofibroblast phenotypes. Sphere cells seeded onto plastic in the presence of 10% serum exhibited the morphology and properties of stromal fibroblasts.[28] The transition to α-SMA-positive myofibroblasts by exposure to TGF-β, causing collagen gel contraction (Fig. 5), is also a functional property of stromal fibroblast primary cultures.[29,33,60] Therefore, subcultured sphere cells can be conditioned to express all three known phenotypes of keratocytes after expansion by sphere culture. Berryhill et al.[36] reported that the fibroblast phenotype can be partially restored to the keratocyte phenotype in terms of extracellular matrix production and morphology. However, biological functions, including Aldh activity, were not restored, suggesting that reversal to keratocyte phenotype after mass culture in serum-containing medium is not practical. Espana et al.[41] have reported the use of amniotic membrane (AM) as a substrate for keratocyte cultures in the presence of serum. They have shown that even with the use of serum, primary keratocytes maintained dendritic morphology on AM. The expression of keratocan and lumican was also present for up to five passages, which is a significant improvement over previous reports using artificial substrates. Still, the scarcity of keratocytes in tissue usually necessitates the use of human tissue or cells from larger animals, such as cows,[28,36] rabbits,[30] and rhesus monkeys.[61] Biochemical and molecular analysis of such cells are difficult due to the lack of available antibodies and genomic information.

During subcultures of spheres, cells that failed to form spheres were found attached to the dish as nondividing, fibroblast-like cells (data not shown). Although these cells may have had low viability, there may be a selection process that allows only cells with high growth potential to propagate as spheres. Once secondary spheres are successfully initiated, subsequent passages continue to produce spheres for at least 12 passages, the longest that we observed. To our surprise, cells from later passages continued to show the keratocyte phenotype when subcultured on plastic, suggesting the possible presence of

committed progenitor cells during the sphere culture stage. Many aspects of the keratocyte are still not understood, and the availability of cells from the mouse cornea should be a powerful tool in studying the biology of these cells.

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Original Article

Individual tissue culture system in a disposable capsule with hypoxic atmosphere

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Abstract

In the present study, a novel system for the hypoxic culture of individual tissues was established for the subculturing of cell lines for research as well as for clinical culture of primary cells. To provide a hypoxic environment throughout the process of tissue handling and culture, we designed a clean bench with CO_2 gas circulation and a hypoxic culture incubator containing disposable capsules. The bench top was covered with an acrylic chamber, and an atmosphere of 5.0% CO_2-air was maintained using a sensor control. The cleanliness class of the chamber could easily be improved to 1 within 5 min of circulative filtration, even though it was found to be 10^5 before the unit was operated. Gas buffer solution (220 ml of 20 mM HEPES, 25 mM $NaHCO_3$, pH 7.4) placed in a 500-ml plastic capsule in the unit stabilized the culture environment by functioning as a heat storage and gas pool. The inflow of air that occurred by the cap of the capsule was opened was excluded by the infusion of purging gas (5.0% CO_2 and 95% N_2); the O_2 level returned to 2.0% within 4 min, after which the gas supply automatically switched to the culture gas (2.0% O_2, 5.0% CO_2, and 93% N_2). If this purging process was omitted, restoration of the O_2 level required 120 min, even though the inner volume was only 280 ml.

Keywords: individual tissue culture, gas circulation clean bench, hypoxic culture, disposable capsule

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Introduction

Tissue culture plays an important role in cell biology, and it is mainly focused towards the subculturing of established cell lines. Culture equipments such as the clean bench and CO_2 incubator have been widely employed, and they provide the appropriate settings for the handling of tissues by supplying filtered fresh air and an atmosphere with saturated humidity and 5.0% CO_2, respectively. It is well known that the oxygen tension in the periphery is substantially lower than that of fresh air. Only some mutant variants that tolerate the given culture conditions such as the atmosphere and culture medium can adapt to long-term subculturing. Although media compositions have been discussed in detail, few efforts have been channeled towards ensuring that the oxygen tension at the original growth environments remains unchanged. The development of a hypoxic culture system will enable the subculturing of many more malignant tissues than what is currently possible. Recent advances in regenerative medicine require the culturing of primary cells or stem cells, and advances with regard to transplantation in humans demand more precise duplication of the original growth environments. Recent reports have indicated the influence of hypoxic culture on some cellular functions. BeWo cells - an in vitro model of human trophoblasts - were cultured in 2% O_2, and RT-PCR conducted after the culture indicated that transcription of the organic cation transporter OCTN2 was higher than that after culture in 20% O_2[1]. Hirao et al.[2] observed that when MC3T3-E1 cells and calvariae from 4-day-old mice were cultured in conditions of 20% or 5% O_2, osteoblastic differentiation and the subsequent transformation to osteocytes were promoted by low oxygen tension.

Some structures and mechanisms of conventional culture equipments are not designed to maintain low oxygen tension in the environment when tissues are being handled and cultured. The present study established a new hypoxic tissue culture system for the culturing of cell lines and primary cells in research and clinical settings, respectively.

Materials and Methods

Measurements with regard to particles in air: The degree of air cleanliness was defined in terms of "cleanliness classes" that are specified by the number of particles of size 0.5 μm or more in 1 cubic foot of air[3]. For in-

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stance, cleanliness class 100 indicates less than 100 particles of the specified size in 1 cubic foot of air. Particle size and number were simultaneously measured using a light-scattering particle counter (KC-03A, Rion, Tokyo, Japan). When an intake air stream is passed through a high-intensity laser beam, the particles in the stream scatter light. The particle sizes were divided across 5 categories in terms of the particle diameter (0.3–0.5 µm, 0.5–1.0 µm, 1.0–2.0 µm, 2.0–5.0 µm, and >5.0 µm), and the number of particles belonging to each category was assessed.

Measurement of temperature and O_2 concentration: The temperature sensor in the device (K470; Techno-Seven, Tokyo, Japan) had a resolution of ± 0.02°C. The O_2 concentration was measured using a galvanic current sensor.

Preparation of premixed gas and accuracy control: In the present study, we used commercially supplied premixed gases for culture (2.0% O_2, 5.0% CO_2, and 93% N_2) and for purging the capsules (5.0% CO_2 and 95% N_2). Pure O_2, CO_2, and N_2 gases were mixed according to their weights of corresponding molar ratios and filled in a container; in a pre-shipment review, their composition was assessed using gas chromatography. Post-receipt accuracy control was performed as follows: when the gases were aerated in a solution containing 20 mM HEPES, 25 mM $NaHCO_3$ at 37°C, and the composition was considered accurate if the pH of the solution remained at 7.4 ± 0.05 after gas equilibration.

Results

1. Clean bench with CO_2 gas circulation

In the present study, a new clean bench with CO_2 gas circulation and a built-in microscope was developed. As shown in Fig. 1, the bench top was covered with an acrylic chamber to prevent leakage of the ambient atmosphere; it resembled an infant incubator. Pure CO_2 was infused using a gas sensor control to maintain the composition at 5.0% CO_2-air, and the temperature was maintained at 30–37°C by using a temperature control (Fig. 2). In addition, if the tissue did not allow exposure to 5.0% CO_2-air, the culture dish was isolated in a small chamber placed on the bench top, and humidified culture gas was supplied to it. While fresh air is filtered only once in the conventional clean bench, the enclosed 5.0% CO_2-air was circulated through HEPA filter every 24 s in the present system. As shown in Fig. 3, the cleanliness class of the air was found to be approximately 4×10^5 before operation of the unit was initiated; particles larger than 0.5 µm were not found, and only 10 particles of size 0.3–0.5 µm were observed in 1 cubic foot of air after 5 min of operation. Repeated filtration easily yielded cleanliness class 1. Thus far, if bench tops were contaminated by some infectious material such as body fluid, they were merely wiped clean. In the present system, however, a disposable clear film is placed as a covering shield on the bench top, and it is discarded after each operation.

2. Hypoxic culture of individual tissues in disposable capsules

We developed a disposable capsule for hypoxic tissue culture (Fig. 4). A 500-ml plastic capsule containing 220 ml of the gas buffer solution (20 mM HEPES, 25 mM $NaHCO_3$) was used; it functioned as a heat storage as well as a gas pool. The culture bath had a 16-well aluminum block for heat storage, and the block and the inner space were maintained at 37°C by using a temperature sensor (Fig. 5). First, the gas buffer solution was equilibrated by infusion of a small amount of the culture gas (10 ml/min) at least overnight. When the door was opened for 10 s, a slight temperature change of 0.1°C was observed in the inner space of the capsule, and it was restored within 7 min.

When conducting tissue culture using this capsule, the inflow of air when the cap is opened should be excluded as soon as possible; The purging gas was flushed (500 ml/min) immediately after the cap was closed, and the O_2 level was restored to 2.0% within 4 min; the gas supply automatically switched to the culture gas, which was continuously infused (10 ml/min) to maintain positive pressure (Fig. 6). If this process was omitted, the restoration of the O_2 level required 120 min, even though the inner volume was only 280 ml. In this system, gas control with a CO_2 sensor was unnecessary, and improper control due to the sensor deterioration was not required to be considered. Gas equilibration in each capsule was roughly estimated by the color of phenol red in the gas buffer solution, and the precise control of the culture environments were monitored by measuring the temperature and pH of the gas buffer solution. Although simultaneous culturing of multiple tissues in a single CO_2 incubator is usually possible, the present method that facilitates the culturing of individual tissues in disposable capsules has some advantages: the individual dish can be easily identified without any confusion, and the culture conditions are not disturbed when the door of the unit is opened.

Discussion

The cleanliness class of room air is generally 10^6–10^5. A low-dust environment with a cleanliness class of 10^2 would be provided when the conventional clean bench is operated under the optimum conditions. However, tissues are handled in fresh air, and under these conditions, O_2 may be dissolved in the medium, and the pH could change due to CO_2 removal. The present clean bench with CO_2-air circulation had overcome the above defects, and the functions had been rather similar with the conventional CO_2 incubator.

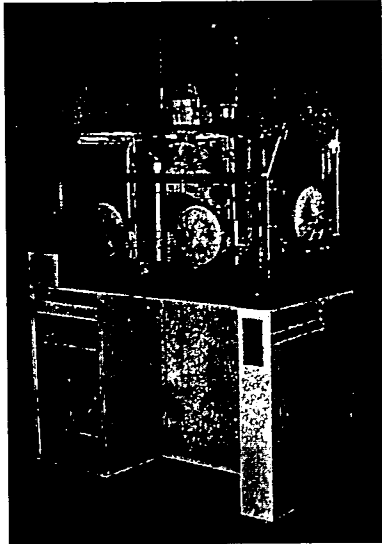

Fig. 1 Clean bench with CO_2 gas circulation

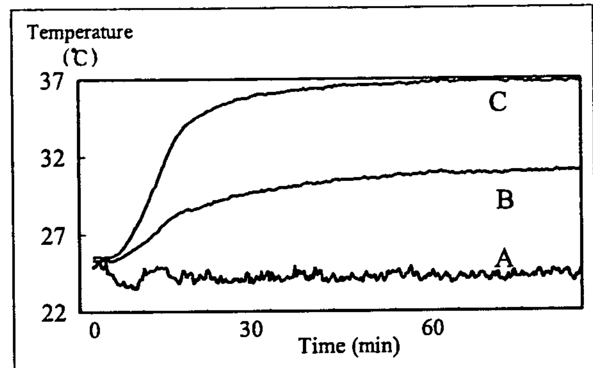

Fig. 2 Temperature of the clean bench
A: fresh air, B: ambient atmosphere, C: bench top

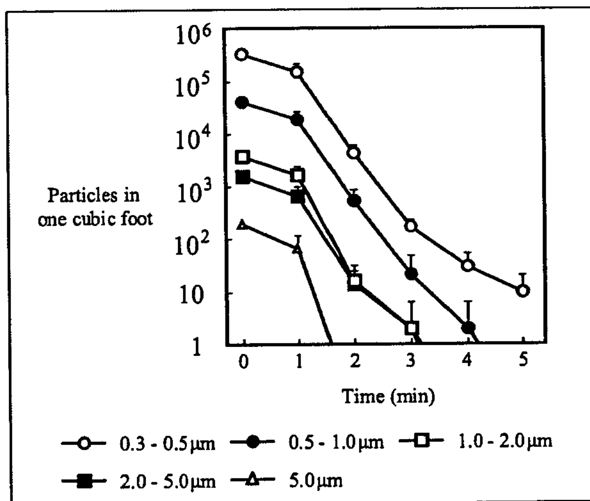

Fig. 3 Change in cleanliness class after beginning of operation
The values are represented as mean ± standard error. They were measured at 5 points on the bench.

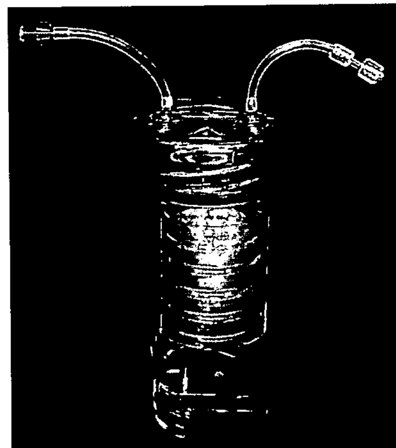

Fig. 4 Disposable capsules for hypoxic tissue culture
A maximum of 5 culture dishes (6.0-cm diameter) can be placed on the tray on the stainless steel stand. The gas buffer solution is placed at the bottom of the stand. The 2 tubes protruding from the cap are the gas inlet and outlet.

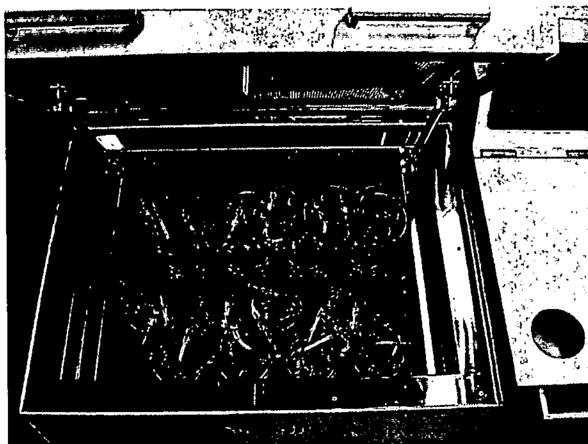

Fig. 5 Culture bath with a 16-well aluminum block

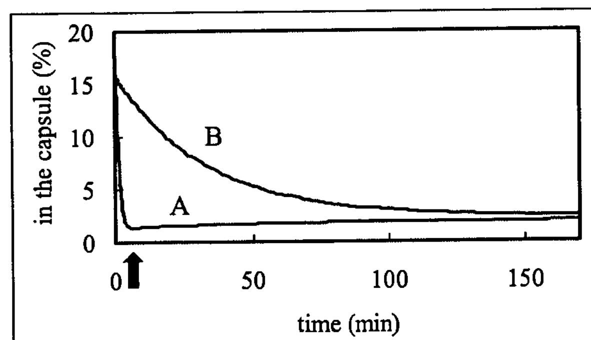

Fig. 6 Effect of gas purging on the restoration of the O_2 level after the cap is closed
A: purging with 5.0% CO_2 and 95% N_2, B: no purging, but 10 ml/min supply of 2.0% O_2, 5.0% CO_2, and 93% N_2. The arrow indicates gas switching.

The conventional CO_2 incubator poses a structural problem if hypoxic tissue culture is to be performed. The inflow of fresh air cannot be avoided when the door is opened. The concentration of CO_2 can be restored by infusing pure CO_2 gas. As shown in Fig. 6, it is very difficult to remove O_2 once it enters the chamber, and the tissues may be exposed to a high concentration of O_2. It is well known that the partial pressure of O_2 in fresh air (159 mmHg) is reduced to 100 and 25 mmHg or less, respectively, in arterial blood and at the periphery. When the saturated vapor pressure at 37°C was estimated to be 47 mmHg, those in 5.0% CO_2-air and the culture gas were calculated to be 142 and 14.3 mmHg, respectively. Numerous authors have shown that reactive oxygen species (ROS) exert various harmful effects such as lipid and protein peroxidation and membrane and DNA damage[4–7], however, only a few reports have addressed the effects of O_2 tension in culture environments[1,2,8]. Tissues and cells in body fluids are protected from O_2 and ROS by physiological antioxidants that are limited in artificial culture media, and an extremely high level of O_2 promotes ROS generation. Since the tolerance of cells to O_2 and ROS differs, the optimum O_2 concentration should be established for each cell line in order to minimize cellular damages and subsequent mutation. In the present system, by changing the composition of the premixed gas, the concentration of O_2 can easily be set, in the range of 0%–95%.

Acknowledgement

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Concise Review: Isolation and Characterization of Cells from Human Term Placenta: Outcome of the First International Workshop on Placenta Derived Stem Cells

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Key Words. Human placenta • Fetal membranes • Amnion • Chorion • Mesenchymal stromal cells • Fetal tolerance

ABSTRACT

Placental tissue draws great interest as a source of cells for regenerative medicine because of the phenotypic plasticity of many of the cell types isolated from this tissue. Furthermore, placenta, which is involved in maintaining fetal tolerance, contains cells that display immunomodulatory properties. These two features could prove useful for future cell therapy-based clinical applications. Placental tissue is readily available and easily procured without invasive procedures, and its use does not elicit ethical debate. Numerous reports describing stem cells from different parts of the placenta, using nearly as numerous isolation and characterization procedures, have been published. Considering the complexity of the placenta, an urgent need exists to define, as clearly as possible, the region of origin and methods of isolation of cells derived from this tissue. On March 23–24, 2007, the first international Workshop on Placenta Derived Stem Cells was held in Brescia, Italy. Most of the research published in this area focuses on mesenchymal stromal cells isolated from various parts of the placenta or epithelial cells isolated from amniotic membrane. The aim of this review is to summarize and provide the state of the art of research in this field, addressing aspects such as cell isolation protocols and characteristics of these cells, as well as providing preliminary indications of the possibilities for use of these cells in future clinical applications. STEM CELLS *2008;26:300–311*

Disclosure of potential conflicts of interest is found at the end of this article.

INTRODUCTION

Human placenta, besides playing a fundamental and essential role in fetal development, nutrition, and tolerance, may also represent a reserve of progenitor/stem cells. Considering the complexity of the structure of the placenta, we have focused our attention on cells isolated from the amniotic and chorionic fetal membranes and reached a consensus on the minimal criteria for definition of mesenchymal cells derived from both of these membranes. Cord blood and amniotic fluid-derived cells are not included in this discussion.

From the data presented by the participants, the following points were evident.

- Cells isolated from placental tissue should be verified to be of fetal origin (using methods sensitive enough to detect maternal contamination of 1% or less).
- Four regions of fetal placenta can be distinguished: amniotic epithelial, amniotic mesenchymal, chorionic mesenchymal, and chorionic trophoblastic. From these regions, the following cell populations are isolated: human amniotic epithelial cells (hAEC), human amniotic mesenchymal stromal cells

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Table 1. Specific surface antigen expression at passages 2–4 for amniotic mesenchymal stromal cells and human chorionic mesenchymal stromal cells

Positive (≥95%)	Negative (≤2%)
CD90	CD45
CD73	CD34
CD105	CD14
	HLA-DR

(hAMSC), human chorionic mesenchymal stromal cells (hCMSC), and human chorionic trophoblastic cells (hCTC).

- Cells from each layer demonstrate variable plasticity. Because of their plasticity, the term stem cell has been used in the literature to describe a number of cells isolated from placenta. Self renewal and "hierarchy," which are normally considered a hallmark of stem cells, have not been clearly demonstrated in the different placenta derived cell types, and therefore the term "stem cell" at this time is not always appropriate. However, it may be interesting to mention that recent reports propose an alternative stem cell concept whereby plasticity is essential to define stemness, and self renewal and hierarchy are optional characteristics [1, 2].
- According to criteria recently proposed by Dominici et al. for bone marrow-derived mesenchymal stromal cells [3], mesenchymal cells isolated from fetal membranes should be termed mesenchymal stromal cells (hAMSC and hCMSC).

Minimal criteria for defining hAMSC and hCMSC are as follows:

Adherence to plastic;
Formation of fibroblast colony-forming units;
A specific pattern of surface antigen expression (Table 1);
Differentiation potential toward one or more lineages, including osteogenic, adipogenic, chondrogenic, and vascular/endothelial; and
Fetal origin.

The participants of the workshop concluded that placenta is an important source of stem/progenitor cells, and additional useful cell types in this tissue may yet be defined. Here, we briefly describe the structure of this organ and discuss isolation, characterization, and differentiation protocols for hAEC, hAMSC, and hCMSC, as well as the immunomodulatory properties, in vitro and in vivo preclinical studies, and cell banking strategies for these cell populations. We do not include hCTC in our discussion as very little definitive information has been published on these cells to date.

The Placenta

Placental Compartments

The fetal adnexa is composed of the placenta, fetal membranes, and umbilical cord. The term placenta is discoid in shape with a diameter of 15–20 cm and a thickness of 2–3 cm. From the margins of the chorionic disc extend the fetal membranes, amnion and chorion, which enclose the fetus in the amniotic cavity, and the endometrial decidua. The chorionic plate (Fig. 1) is a multilayered structure that faces the amniotic cavity. It consists of two different structures: the amniotic membrane (composed of epithelium, compact layer, amniotic mesoderm, and spongy layer) and the chorion (composed of mesenchyme and a region of extravillous proliferating trophoblast cells interposed in varying amounts of Langhans fibrinoid, either covered or not by syncytiotrophoblast). Villi originate from the chorionic plate and anchor the placenta through the trophoblast of the basal plate and maternal endometrium. From the maternal side, protrusions of the basal plate within the chorionic villi produce the placental septa, which divide the parenchyma into irregular cotyledons (Fig. 1).

Some villi anchor the placenta to the basal plate, whereas others terminate freely in the intervillous space. Chorionic villi present with different functions and structure. In the term placenta, the stem villi show an inner core of fetal vessels with a distinct muscular wall and connective tissue consisting of fibroblasts, myofibroblasts, and dispersed tissue macrophages (Hofbauer cells). Mature intermediate villi and term villi are composed of capillary vessels and thin mesenchyme. A basement membrane separates the stromal core from an uninterrupted multinucleated layer, called syncytiotrophoblast. Between the syncytiotrophoblast and its basement membrane are single or aggregated Langhans cytotrophoblastic cells, commonly called cytotrophoblast cells.

Fetal membranes continue from the edge of the placenta and enclose the amniotic fluid and the fetus. The amnion is a thin, avascular membrane composed of an epithelial layer and an outer layer of connective tissue, and is contiguous, over the umbilical cord, with the fetal skin. The amniotic epithelium (AE) is an uninterrupted, single layer of flat, cuboidal and columnar epithelial cells in contact with amniotic fluid. It is attached to a distinct basal lamina that is, in turn, connected to the amniotic mesoderm (AM) (Fig. 2). In the amniotic mesoderm closest to the epithelium, an acellular compact layer is distinguishable, composed of collagens I and III and fibronectin. Deeper in the AM, a network of dispersed fibroblast-like mes-

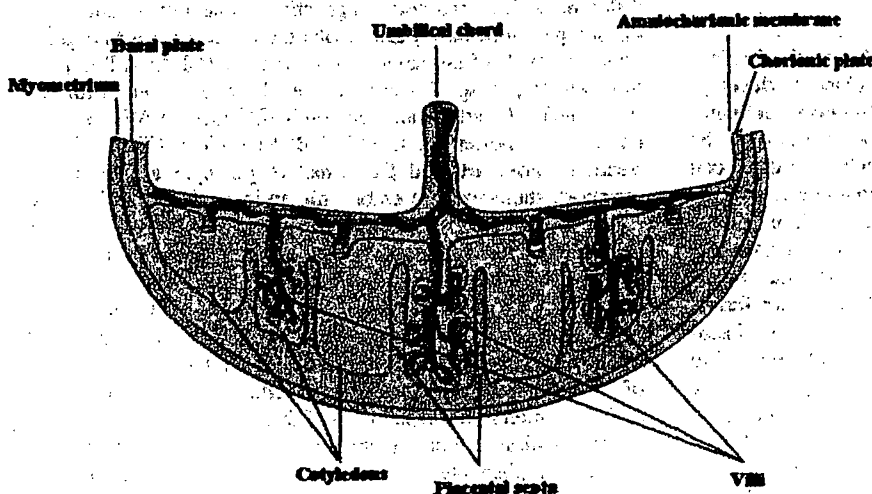

Figure 1. Schematic section of the human term placenta. During placenta formation, from the maternal side, protrusions of the basal plate within the chorionic villi produce the placental septa, which divide the parenchyma into irregular cotyledons. Each cotyledon contains several villi, which originate from the chorionic plate. Fetal blood vessels are located within the branches of the villi.

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Figure 2. Cross-sectional representation of human fetal membranes (amnion and chorion). The amnion is composed of an epithelial layer of cuboidal and columnar cells, which lie on top of a mesodermal layer consisting of an upper acellular compact layer and a deeper layer containing dispersed fibroblasts. The chorionic membrane consists of a mesodermal layer and a layer of extravillous trophoblast cells. Abbreviations: AE, amniotic epithelium; AM, amniotic mesoderm; CM, chorionic mesoderm; CT, chorionic trophoblast.

enchymal cells and rare macrophages are observed. Very recently, it has been reported that the mesenchymal layer of amnion indeed contains two subfractions, one having a mesenchymal phenotype, which is referred to throughout this review as amniotic mesenchymal stromal cells, and the second containing monocyte-like cells [4].

A spongy layer of loosely arranged collagen fibers separates the amniotic and chorionic mesoderm (Fig. 2). The chorionic membrane (chorion leave) consists of mesodermal and trophoblastic regions (Fig. 2). Chorionic and amniotic mesoderm are similar in composition. A large and incomplete basal lamina separates the chorionic mesoderm from the extravillous trophoblast cells. The latter, similar to trophoblast cells present in the basal plate, are dispersed within the fibrinoid layer and express immunohistochemical markers of proliferation. The Langhans fibrinoid layer usually increases during pregnancy and is composed of two different types: a matrix type on the inner side (more compact) and a fibrin type on the outer side (more reticulate). At the edge of the placenta and in the basal plate, the trophoblast interdigitates extensively with the deciduas [5, 6].

Embryological Development

In humans, by days 6–7 after fertilization (during the implantation window), the blastocyst implants and placenta development begins. At this stage, the blastocyst is flattened and composed of an outer wall (trophoblast) that surrounds the blastocystic cavity. A small group of larger cells, the inner cell mass, is apposed to the inner surface of the trophoblastic vesicle. The embryo, umbilical cord, and amniotic epithelium are derived from the inner cell mass. As the blastocyst adheres to the endometrium, invading trophoblasts erode the decidua, facilitating implantation of the blastocyst. By 8–9 days after fertilization, trophoblastic cells at the implanting pole of the blastocyst proliferate robustly, forming a bilayered trophoblast. The outer of the two layers becomes the syncytiotrophoblast by fusion of neighboring trophoblast cells, whereas the inner cells (cytotrophoblast) remain temporally unfused.

The proliferating cytotrophoblasts and the syncytiotrophoblasts give rise to a system of trabeculae intermingled with hematic lacunae. From these trabeculae are generated the primordial villi that are distributed over the entire periphery of the chorionic membrane. Villi in contact with the decidua basalis proliferate to form the leafy chorion or chorion frondosum, whereas villi in contact with the decidua capsularis degenerate into the chorion leave.

At day 8–9 after fertilization, the inner cell mass differentiates into two layers: the epiblast and the hypoblast. Subsequently, from the epiblast, small cells that later constitute the amniotic epithelium appear between the trophoblast and the embryonic disc and enclose a space that will become the amniotic cavity. On the opposite side, between the hypoblast and cytotrophoblast, the exocoelomic membrane and its cavity modify to form the yolk sac. The extraembryonic mesoderm arranges into a connective tissue that surrounds the yolk sac and amniotic cavity, giving rise to amniotic and chorionic mesoderm. Gastrulation, the process through which the bilaminar disc differentiates into the three germ layers (ectoderm, mesoderm, and endoderm) and develops a defined form, with a midline and cranio-caudal, right-left, and dorsal-ventral body axes, occurs during the 3rd week after fertilization [6, 7].

Fetal Placenta Tissue Cell Populations

Human Amniotic Epithelial Cells

Recent reports indicate that hAEC express stem cell markers and have the ability to differentiate toward all three germ layers. These properties, the ease of isolation of the cells, and the availability of placenta as a discard tissue, make the amnion a potentially useful and noncontroversial source of cells for transplantation and regenerative medicine. For isolation of epithelial cells, the amniotic membrane is stripped from the underlying chorion and digested with trypsin or other digestive enzymes (Table 2) [8–11]. Epithelial cells are specifically released by brief trypsin digests of 20–40 minutes each. A detailed protocol is available providing a step-by-step isolation procedure along with photos of the process [12]. Isolated cells readily attach to plastic or basement membrane-coated culture dishes. Culture is commonly established in a simple medium such as Dulbecco's modified Eagle's medium supplemented with 5%–10% serum and epidermal growth factor (EGF) (Table 2), where the cells proliferate robustly and display typical cuboidal epithelial morphology [10, 11, 13]. Normally, 2–6 passages are possible before proliferation ceases. Cells do not proliferate well at low densities. Amniotic membrane contains epithelial cells with different surface markers, suggesting some heterogeneity of phenotype. Immediately after isolation, hAEC appear to express very low levels of human leukocyte antigen (HLA)-A,B,C [10]; however, by passage 2, significant levels are observed (Table 2). Additional cell surface antigens on hAEC include ATP-binding cassette transporter G2 (ABCG2/BCRP), CD9, CD24, E-cadherin, integrins $\alpha 6$ and $\beta 1$, c-met (hepatocyte growth factor receptor), stage-specific embryonic antigens (SSEAs) 3 and 4, and tumor rejection antigens 1-60 and 1-81 [11, 14]. Surface markers thought to be absent on hAEC include SSEA-1, CD34, and CD133, whereas other markers, such as CD117 (c-kit) and CCR4 (CC chemokine receptor), are either negative or may be expressed on some cells at very low levels. Although initial cell isolates express very low levels of CD90 (Thy-1), the expression of this antigen increases rapidly in culture [11, 14]. Additional surface markers are presented in Table 2.

In addition to surface markers, hAEC express molecular markers of pluripotent stem cells, including octamer-binding protein 4 (OCT-4), SRY-related HMG-box gene 2 (SOX-2), and

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Table 2. Human amniotic epithelial cells: isolation protocols, phenotype, and in vitro differentiation

Procedure		Procedure description	References
Isolation protocols		Mechanical peeling of amnion membrane from the underlying chorion followed by digestion with the following: dispase II (2.4 U/ml) for 1 hour or trypsin-EDTA (different concentrations and incubation times). Centrifugation (200g for 10 minutes) of solution containing released cells, discarding the remaining membrane	[9, 11, 12, 22, 24, 28, 29, 31, 133]
Phenotype at passages 2–4		Mesenchymal and hematopoietic markers: CD105+, CD90+, CD73+, CD44+, CD29+, HLA-A,B,C+, CD13+, CD10+, CD166+, CD49d-, CD49e+, CD117 (+/− very weak signal), CD14−, CD34−, CD45−, HLA-DR− Embryonic cell markers: SSEA-3+, SSEA-4+, TRA 1-60+, TRA 1-81+, SSEA-1− Others: CD324 (E-cadherin)+, POU5F1+, SOX2+, CFC1+, NANOG+, DPPA3+, PROM1+, PAX6+, FOXD3−, GDF3−, CD140b+, CD349−, GCTM2+	[11, 28, 29, 133] (Bühring HJ, Treml S, Cerabona F et al., unpublished data)
Differentiation potential	Adipogenic	DMEM high glucose (or DMEM/Ham's F-12 medium), 10% FBS, 0.5 mM isobutylmethylxanthine, 1 μM dexamethasone, 10 μM insulin, 200 μM indomethacin	[28, 133]
	Chondrogenic	DMEM high glucose, 1% FBS, 6.25 μg/ml insulin, 10 ng/ml TGF-β1, 50 ng/ml fresh ascorbic acid	[133]
	Osteogenic	DMEM high glucose (or DMEM/Ham's F-12 medium), 10% FBS, 10 μM dexamethasone, 10 nM 1-α,25-dehydroxyvitamin D3, 50 μg/ml ascorbic acid, 10 mM β-glycerophosphate	[28]
		MesenCult Human Osteogenic Stimulatory Kit (StemCell Technologies, Vancouver, BC, Canada, http://www.stemcell.com)	[29]
	Skeletal myogenic	DMEM/Ham's F-12 medium (or DMEM high glucose), 10% FBS, 5% human serum (or horse serum), 50 μM hydrocortisone (0.1 μM dexamethasone)	[28]
	Cardiomyogenic	DMEM, 10% FBS, 55 μM 2-mercaptoethanol, 1 mM sodium pyruvate, 1 mM ascorbic acid 2-phosphate	[11]
		DMEM/Ham's F-12 medium, 10% FBS, 1 mM ascorbic acid	[20]
	Neurogenic	DMEM high glucose, 10% FBS, 30 μM all-trans retinoic acid	[133]
		DMEM, 10% FBS, 55 μM 2-mercaptoethanol, 1 mM sodium pyruvate, 5×10^{-7} M all-trans retinoic acid, 10 ng/ml FGF-4	[11]
		DMEM/Ham's F-12 medium, 10% FCS, 5×10^{-5} M all-trans retinoic acid, 10 ng/ml FGF4, N-2, B-27	[20]
	Pancreatic	DMEM, 10% FBS, 55 μM 2-mercaptoethanol, 1 mM sodium pyruvate, 10 mM nicotinamide on collagen I-coated plate	[11]
		DMEM (or DMEM/Ham's F-12 medium) containing N_2 supplement (Gibco, Grand Island, NY, http://www.invitrogen.com), 10 mM nicotinamide	[28]
	Hepatic	DMEM, 10% FBS, 55 μM 2-mercaptoethanol, 1 mM sodium pyruvate, dexamethasone 10^{-7} M, 0.1 μM insulin for 3 weeks, addition of 1 mM phenobarbital for the final 3 days, on collagen I-coated plate	[11]
		DMEM, 10% FBS, 20 ng/ml HGF, 10 ng/ml FGF-2, 10 ng/ml oncostatin M, 100 nM dexamethasone, 10 U/ml heparin sodium salt	[22]
		DMEM/Ham's F-12 medium, 10% FCS + 0.1 μM insulin, 1×10^{-7} M dexamethasone	[20]

Abbreviations: DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; FCS, fetal calf serum; HGF, hepatocyte growth factor; SSEA, stage-specific embryonic antigen; TGF, transforming growth factor; TRA, tumor rejection antigen.

Nanog [11]. The suggestion that hAEC may be pluripotent was supported by the report by Tamagawa et al. [15]. These investigators created a xenogeneic chimera of human amnion cells with mouse embryonic stem cells, in vitro. Chimeric aggregates were maintained, and human contribution to all three germ layers was established. Later studies indicated that differentiation of hAEC can be directed. Sakuragawa et al. identified neural and glial markers on cultured hAEC [16]. Later studies reported that cultured hAEC synthesize and release acetylcholine, catecholamines [9, 17, 18], and dopamine [19, 20]. Hepatic differentiation of hAEC was also reported. Sakuragawa et al. reported that cultured hAEC produce albumin (Alb) and α-fetoprotein (AFP) and that Alb- and AFP-positive hepatocyte-like cells could be identified integrated into hepatic parenchyma following transplantation of hAEC into the livers of severe combined immunodeficiency (SCID) mice [21]. The hepatic potential of hAEC was confirmed and extended [11, 22, 23], whereby in addition to Alb and AFP production, other hepatic functions, such as glycogen storage and expression of liver-enriched transcription factors, such as hepatocyte nuclear factor (HNF) 3γ and HNF4α, CCAAT/enhancer-binding protein (CEBP α and β), and several of the drug metabolizing

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Figure 3. Culture of placenta derived stem cell types (phase contrast micrograph pictures; magnification, ×200). (A): Primary human amniotic epithelial cell culture. (B): Human amniotic mesenchymal stromal cells, passage 4. (C): Human chorionic mesenchymal stromal cells, passage 4.

genes (cytochrome P450) could be demonstrated. The wide range of hepatic genes and functions identified in hAEC suggests that these cells may be useful for liver-directed cell therapy.

Differentiation of hAEC to another endodermal tissue, pancreas, was also reported. Wei et al. [24] cultured hAEC for 2–4 weeks in the presence of nicotinamide to induce pancreatic differentiation. Subsequent transplantation of the insulin-expressing hAEC corrected the hyperglycemia of streptozotocin-induced diabetic mice. In the same setting, hAMSC were ineffective, suggesting that hAEC, but not hAMSC, were capable of acquiring a β-cell fate.

The studies reviewed above indicate that hAEC are unique cells with many stem cell characteristics. Cell types from all three germ layers were produced in vitro. There is currently strong in vitro and in vivo evidence of neural, pancreatic, and hepatic differentiation of hAEC.

Mesenchymal Stromal Cells from Amnion and Chorion: hAMSC and hCMSC

hAMSC and hCSMC are thought to be derived from extraembryonic mesoderm [7]. Extensive phenotypical and functional characterization is available on hAMSC [25–29], whereas there are few reports of investigations on hCMSC [28, 30, 31]. The available details are summarized in Tables 3 and 4.

hAMSC and hCMSC can be isolated from first-, second-, and third-trimester mesoderm of amnion and chorion, respectively [25–29, 31]. Here, when using the terms hAMSC and hCMSC we refer to cells from term placenta unless otherwise specified. For hAMSC, isolations are usually performed with term amnion dissected from the deflected part of the fetal membranes to minimize the presence of maternal cells. Homogenous hAMSC populations can be obtained by a two-step procedure: minced amnion tissue is treated with trypsin to remove hAEC, and the remaining mesenchymal cells are then released by digestion with collagenase [32] or collagenase and DNase [33]. The yield from term amnion is about 1 million hAMSC and 10-fold more hAEC per gram of tissue [33].

hCMSC are isolated from both first- and third-trimester chorion after mechanical and enzymatic removal of the trophoblastic layer with dispase. Chorionic mesodermal tissue is then digested with collagenase [28] or collagenase plus DNase [31]. Mesenchymal cells have also been isolated from chorionic fetal villi through explant culture, although maternal contamination is more likely [30, 31, 34].

Both hAMSC and hCMSC adhere and proliferate on tissue culture plastic and can be kept until passages 5–10. Reports suggest that hAMSC proliferation slows beyond passage 2, although both first-trimester hAMSC and hCMSC proliferate better than third-trimester cells [28]. Theoretically, term amnion may yield up to 5×10^8 hAMSC [35]; however, in practice, yields are typically 4 million hAMSC per 100 cm^2 of starting material, with a fourfold expansion after 1 month (two passages) (G. Bilic, S. Zeisberger, and A.H. Zisch, unpublished data).

Phase micrographs (Fig. 3) show the distinct morphology of cultured hAMSC, hCMSC, and hAEC. Expression of CD49d (α4 integrin) on hAMSC distinguishes them from hAEC [29]. In culture, neither vimentin nor cytokeratin 18 expression is specific for hAMSC or hAEC, respectively. Transmission electron microscopy of hAMSC shows mesenchymal and epithelial characteristics. This hybrid phenotype is interpreted as a sign of multipotentiality and is not found in hCMSC, which are more primitive and metabolically quiescent. Specifically, compared with hAMSC, transmission electron microscopy of hCMSC show a simpler cytoplasmic organization. The most relevant features include the presence of stacks of rough endoplasmic reticulum cisternae, dispersed mitochondria and glycogen lakes, whereas features of higher specialization, such as presence of assembled contractile filaments, prominence of endocytotic traffic, and junctional communications, are lacking. Overall, the ultrastructural characteristics of hCMSC resemble those found in the hematopoietic progenitors and in the blue small round cell tumors (e.g., Ewing sarcomas), suggesting their position at the higher levels of the stem cell hierarchy [36]. The surface marker profile of cultured hAMSC, hCMSC, and mesenchymal stromal cells (MSC) from adult bone marrow are similar. All express typical mesenchymal markers (Table 1) but are negative for hematopoietic (CD34 and CD45) and monocytic markers (CD14) [26, 28, 29, 31]. Surface expression of SSEA-3 and SSEA-4 and RNA for OCT-4 has been reported [24, 29, 35, 37] (G. Bilic, S. Zeisberger, and A.H. Zisch, unpublished data). However, immunofluorescence staining of mesenchyme tissue did not detect SSEA-3 or SSEA-4 [39]. Both first- and third-trimester hAMSC and hCMSC express low levels of HLA-A,B,C but not HLA-DR [28, 29], indicating an immunoprivileged status.

Both hAMSC and hCMSC differentiate toward "classic" mesodermal lineages (osteogenic, chondrogenic, and adipogenic) [26, 28, 29, 31, 35], and differentiation of hAMSC to all three germ layers—ectoderm (neural), mesoderm (skeletal muscle, cardiomyocytic and endothelial), and endoderm (pancreatic)—was reported (Table 3). Questions remain as to the proportion of hAMSC that exhibit plasticity and the full maturation of progeny [39].

Human amniotic and chorionic cells successfully and persistently engraft in multiple organs and tissues in vivo. Human chimerism detection in brain, lung, bone marrow, thymus, spleen, kidney, and liver after either intraperitoneal or intravenous transplantation of human amnion and chorion cells into neonatal swine and rats was indeed indicative of an active migration consistent with the expression of adhesion and migration molecules (L-selectin, VLA-5, CD29, and P-selectin ligand 1), as well as cellular matrix proteinases (MMP-2 and MMP-9) [41].

Immunology of the Placenta

Maternal acceptance of the fetal allograft is not completely understood. The paradox of maternal tolerance of the fetus was first raised more than 50 years ago by Peter Medawar, who proposed that: (a) there is a physical separation between the

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Table 3. Human amniotic mesenchymal stromal cells: isolation protocols, phenotype, and in vitro differentiation

Procedure		Procedure description	References
Isolation protocols		Mechanical peeling of amnion membrane from the underlying chorion followed by digestion with various concentrations of collagenase (0.75–2 mg/Ml) and DNase (20–75 μg/ml) for 30–120 minutes at 37°C	[27–29, 31–33, 37, 38]
Phenotype at passages 2–4		Mesenchymal and hematopoietic markers: CD105+, CD90+, CD73+, CD44+, CD29+, HLA-A,B,C+, CD13+, CD10+, CD49c+, CD49d+, CD49e+, CD54+, CD166+, CD271^{low}, CD14−, CD34−, CD45−, CD31−, HLA-DR−, CD133−, CD3− Embryonic cell marker: Oct-4+ Others: CD349+, CD140b+, CD324 (E-cadherin)−	[26, 28, 29, 31, 35] (Bühring HJ, Treml S, Cerabona F et al., unpublished data)
Differentiation potential	Adipogenic	DMEM, 10% FCS, 0.5 mM isobutylmethylxanthine, 200 μM indomethacin, 10^{-6} M dexamethasone, 10 μg/ml insulin	[28, 35]
		Bullet Kit Adipogenic Differentiation Media (Cambrex, Walkersville, MD, http://www.cambrex.com)	[31]
	Chondrogenic	DMEM high glucose, 1% FBS, 6.25 μg/ml insulin, 10 ng/ml TGF-β1, 50 μg/ml fresh ascorbic acid	[28]
		DMEM, 6.25 μg/ml insulin, 6.25 μg/ml transferrin, 6.25 μg/ml selenous acid, 5.33 μg/ml linoleic acid, 1.25 mg/ml BSA, 0.35 mM proline, 1 mM sodium pyruvate, 10^{-7} M dexamethasone, 0.1 mM L-ascorbic acid-2-phosphate supplemented with 10 ng/ml transforming growth β-3	[35]
		DMEM, 100 nM dexamethasone, 50 μg/ml L-ascorbic acid 2-phosphate, 1 mM sodium pyruvate, 40 μg/ml proline, ITS (5 μg/ml insulin, 5 μg/ml transferrin, 5 ng/ml selenous acid), 5 ng/ml TGF-β1	[31]
	Osteogenic	DMEM high glucose, 10% FBS, 10 mM β-glycerophosphate, 50–200 μg/ml ascorbic acid, 10^{-8} M dexamethasone, 1α,25-dihydroxyvitamin-D3	[24, 26–28, 35, 37]
		Commercial media: MesenCult Human Osteogenic Stimulatory Kit (StemCell Technologies); Bullet Kit Osteogenic Differentiation Media (Cambrex)	[29, 31]
	Skeletal myogenic	DMEM high glucose, 10% FBS, 5% horse serum, 50 μM hydrocortisone, 0.1 μM dexamethasone	[133]
		DMEM, 5% FBS, 40 (Sigma) MCDB-201%, ITS-LA+BSA 100×, 10^{-8} M dexamethasone, 10^{-4} M ascorbic acid 2-phosphate, 10 ng/ml bFGF, 10 ng/ml VEGF, 10 ng/ml insulin-like growth factor 1	[35]
	Angiogenic	Matrigel adherence, 50 ng/ml VEGF, 2% FBS	[35]
	Neurogenic	DMEM high glucose, 10% FBS, 30 μM all-trans retinoic acid	[28]
		DMEM, 100 μM butylated hydroxyanisol, 10 μM forskolin, 2% DMSO, 5 U/ml heparin, 5 nM K252a, 25 mM KCl, 2 mM valproic acid, 1× N2 supplement, 10 ng/ml bFGF, 10 ng/ml PDGF-BB	[27]
	Pancreatic	DMEM containing N_2 supplement (Gibco), 10 mM nicotinamide	[24]
	Cardiomyogenic	DMEM, 10% FBS, 10 ng/ml bFGF, 50 ng/ml activin A	[37]
		Mixed ester of hyaluronan with butyric and retinoic acid treatment	[80]

Abbreviations: bFGF, basic fibroblast growth factor; BSA, bovine serum albumin; DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; FCS, fetal calf serum; ITS-LA, insulin transferrin selenous acid-linolenic acid; MCDB, molecular cellular and developmental biology; PDGF, platelet-derived growth factor; TGF, transforming growth factor; VEGF, vascular endothelial growth factor.

mother and fetus, (b) the fetus is antigenically immature, and (c) the mother possesses an immunological inertness [42]. Since the time of Medawar, several mechanisms that contribute to tolerance induction have been proposed, although how they are regulated and driven to interact with each other remain unclear.

In particular, different explanations have been proposed regarding the regulation of maternal T-cell proliferation at the fetal maternal interface. It has been shown that some cells of the syncytiotrophoblast express the tryptophan catabolizing enzyme indoleamine 2,3-dioxygenase (IDO), resulting in the depletion of tryptophan and inhibition of T-cell proliferation, which may provide protection of the fetus from maternal T-cells [43, 44]. However, in IDO knockout mice, fetus rejection was not observed [45]. Therefore, even though IDO activity has been hypothesized as a key mechanism for protecting the allogenic fetus, it is not the sole determinant since other mechanisms, perhaps redundant in normal mice, can compensate for the loss of IDO activity during gestation.

Furthermore, soluble HLA-G molecules produced by placenta induce apoptosis of activated CD8+ T-cells [46] and inhibit CD4+ T-cell proliferation [46]. Trophoblast cells expressing HLA-G may also inhibit natural killer cells that could induce fetal rejection [48]. In addition, trophoblast cells that express Fas ligand (CD95L) have been demonstrated to induce apoptosis of maternal Fas (CD95)-expressing lymphocytes [49],

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Table 4. Human chorionic mesenchymal stromal cells: isolation protocols, phenotype, and in vitro differentiation

Procedure	Procedure description		References
Isolation protocols	Mechanical removal of surrounding layers after treatment with 2.4 U/ml dispase II at 37°C, followed by 1–3 hours treatment with Worthington collagenase II (270 U/ml) or collagenase A (0.83 mg/ml)		[28, 31]
Phenotype at passages 2–4	Mesenchymal and hematopoietic markers: CD105+, CD90+, CD73+, CD44+, CD29+, HLA-A,B,C+, CD13+, CD166+, CD49e+, CD271^{low}, CD10+, CD14−, CD34−, CD45−, CD117−, CD133−, HLA-DR− Embryonic stem cell marker: SSEA-4−/+ Others: CD349+, CD140b+, CD324−		[28, 31, 134] (Bühring HJ, Treml S, Cerabona F et al., unpublished data)
Differentiation potential	Adipogenic	DMEM high glucose, 10% FBS, 0.5 mM isobutylmethylxanthine, 1 μM dexamethasone, 10 μM insulin, 200 μM indomethacin	[28]
		Bullet Kit Adipogenic Differentiation Media (Cambrex)	[31]
	Chondrogenic	DMEM high glucose, 1% FBS, 6.25 μg/ml insulin, 10 ng/ml TGF-β1, 50 ng/ml fresh ascorbic acid	[28]
		DMEM, 100 nM dexamethasone, 50 μg/ml L-ascorbic acid 2-phosphate, 1 mM sodium pyruvate, 40 μg/ml proline, ITS (5 μg/ml insulin, 5 μg/ml transferrin, 5 ng/ml selenous acid), 5 ng/ml TGF-β1	[31]
	Osteogenic	10 μM dexamethasone, 10 nM 1α, 25-dihydroxyvitamin D3, 50 μg/ml ascorbic acid, 10 mM β-glycerophosphate	[28]
		Bullet Kit Osteogenic Differentiation Media (Cambrex)	[31]
	Skeletal myogenic	DMEM high glucose, 10% FBS, 5% horse serum, 50 μM hydrocortisone, 0.1 μM dexamethasone	[28, 31]
	Neurogenic	DMEM high glucose, 10% FBS, 30 μM all-trans retinoic acid	[28]

Abbreviations: DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; TGF, transforming growth factor.

therefore representing another possible mechanism for contributing to the maintenance of fetomaternal tolerance. The mechanisms discussed above are not definitive, and it is very likely that they all play a role in the complex phenomenon of fetal maternal tolerance.

Role of Fetal Membranes in Tolerance

It has been proposed that cells from fetal membranes have immunoregulatory properties. Amniotic membrane and hAEC are used on skin wounds, burn injuries, and chronic leg ulcers and to prevent adhesions in surgical procedures [50–57]. Amniotic membrane is also used in ocular surface reconstruction to promote development of normal corneal or conjunctival epithelium [57] without immunosuppression or acute rejection. Furthermore, transplantation of allogenic amniotic membrane or hAEC without immunosuppression does not induce acute immune rejection [8, 58–61]. In vitro studies show that cells isolated from amniotic and chorionic membranes do not elicit an allogeneic or xenogeneic immune response but actively suppress lymphocyte proliferation [29, 41, 62]. Human amniotic membrane and hAEC have been shown to survive for prolonged times in immunocompetent animals, including rabbits [63], rats [64], guinea pigs [65], and bonnet monkeys [66]. In addition, long-term engraftment was observed after i.v. injection of heterogeneous human amniochorionic cells into newborn swine and rats, with human microchimerism detected in bone marrow, brain, lung, and thymus [41], suggesting active migration and integration into specific organs and indicating active tolerance of the xenogeneic cells.

Placenta as a Potent Niche Source for Hematopoietic Stem/Progenitor Cells: Insights from the Mouse Placenta

In mouse placenta the allantois, an extension of the primitive streak, fuses with the chorionic plate, giving rise to umbilical vasculatures and mesoderm of fetal placenta. Allantoic mesoderm interdigitates with trophoblasts to form the placenta labyrinth. Allantois-derived umbilical arteries are the site of hematopoietic stem cell (HSC) ontogeny. In 1961, Till and McCulloch reported that placenta harbors transplantable hematopoietic activity [67]. Melchers detected B cell progenitors in placenta prior to their appearance in fetal liver [68]. Multipotential myeloerythroid progenitors are present at a higher frequency in mouse placenta than in yolk sac or fetal liver [69]. Mouse placenta is a niche for developing HSCs without facilitating myeloid or erythroid differentiation. The distribution of HSC in placenta and the aorta-gonad-mesonephros region was compared, a dramatic expansion of placenta HSC (50 HSC-long-term repopulating units per organ) was reported at embryonic day (E) 12.5 [70], and HSC were detected in the vascular labyrinth of placenta of Ly-6A (Sca-1) green fluorescent protein transgenic mice [71]. Hematopoietic cytokines produced by human placental MSC, such as stem cell factor, Flt 3 ligand, interleukin-6, and macrophage colony-stimulating factor, functionally support a sevenfold expansion of long-term culture-initiating cells [72], and additional factors were identified and cloned [73].

Mouse placenta serves as a source and a functional niche for HSC [74]. Prefusion allantois and chorion are potent sources for hematopoietic progenitors. Allantois and chorion, isolated prior to the union between the three major circulatory systems (um-

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bilical cord, yolk sac, and cardiovascular) in the conceptus, express Runx1, a key transcriptional factor critical for HSC ontogeny, and CD41, a hallmark for initiation of definitive hematopoiesis [75], and also generate myeloid and definitive erythroid lineages following explant culture [76]. Furthermore, hemogenic potential and Runx1 expression are independent of the union of the tissues, reflecting their intrinsic hematopoietic property. The identification of critical niche components for HSCs from placenta may be useful for ex vivo HSC expansion.

Preclinical Studies in Animal Models

Hepatic Regeneration

Hepatocyte transplantation has become a novel method for treatment of liver disease [77]. However, the technique is currently limited by a shortage of suitable hepatocytes for transplant procedures. The large number of hepatic genes and functions identified in human amniotic epithelium (hAE) suggest that if effective and efficient methods were developed to induce complete hepatic differentiation, hAE could be a useful source of cells for transplant procedures. Preclinical investigations with hepatic differentiation of hAEC have been promising. In vitro, differentiation procedures have yielded cells that express a number of liver functions, including the transcription factors HNF3γ, and C/EBPα, HNF1, HNF4α, pregnane x receptor, and constitutive androstane receptor, and differentiated liver genes, including albumin, α1-antitrypsin (A1AT), glucose-6-phosphatase, carbamoyl phosphate synthase I (CPS-I), glutamine synthase, phosphoenolpyruvate carboxykinase, tyrosine aminotransferase, transthyretin, and the drug metabolizing genes CYP1A1, 1A2, 2A6, 2B6, 2C8, 2C9, 2D6 2E1, 3A4, 7, and 7A1 [11, 14, 22, 23]. In addition to quantification of RNA, drug metabolism dependent on CYP1A and CYP3A enzymatic activity has been demonstrated [11, 78]. In unpublished work, the list of hepatic genes identified in hAE include transport proteins, including P-glycoprotein, multidrug resistance protein 1, ABCG2, the bile salt export pump, uridine diphosphate glucuronosyl transferase 1A1 (UGT1A1), and ornithine transcarbamylase (OTC) (Miki T, Marongiu F, Strom S, unpublished data). Following transplantation into the liver, successful engraftment and survival of human [21, 78] or rat amniotic epithelial cells (AEC) [79] has been demonstrated. Recent unpublished work extends these observations to more than 6 months. Human A1AT could be detected by Western blot in mouse serum [14], clearly indicating that hAEC perform this important hepatic function in vivo. Human albumin was detected in the sera and peritoneal fluid of SCID mice who received peritoneal implants of human amniotic membrane [22]. At this time, there are no reports of the characterization of hepatic gene expression in long-term recipients of AE transplants or that the transplantation of AEC can support animals with acute or long-term defects in liver function. These important preclinical studies will be needed before the therapeutic potential of hAE can be fully assessed.

At least 30 genes known to be expressed in mature human liver are expressed in hAEC directed toward hepatic differentiation in culture, suggesting that transplantation of hAE-derived hepatocytes could be an effective therapy for liver disease. It is significant that among the hepatic genes expressed are A1AT, OTC, CPS-I, and UGT1A1. Mutations in these genes cause metabolic liver diseases that are currently corrected by liver transplantation. Perhaps hAEC transplants could provide a novel cell therapy for patients suffering liver or lung disease from A1AT deficiency, or those at risk for neurological effects due to the inability to metabolize and excrete ammonia (OTC and CPS-I) or bilirubin (UGT1A1).

Cardiac Repair

Myocardial infarction, ischemia, and stroke are important consequences of end-stage occlusive vascular disease. Present-day therapies are inadequate and palliative, so stem cell therapy has been investigated. Coculture experiments with neonatal rat heart explants have confirmed that hAMSC integrate into cardiac tissues and differentiate into cardiomyocyte-like cells. After transplantation into myocardial infarcts in rat hearts, hAMSC survived for 2 months and differentiated into cardiomyocyte-like cells [37].

A mixed ester of hyaluronan and butyric and retinoic acids (HBR) was reported to promote cardiogenic/vasculogenic differentiation of human amniochorionic (AC)-derived cells. HBR enhanced the expression of cardiomyogenic genes (GATA4, NKX 2.5) and proteins (sarcomeric myosin heavy chain and α-sarcomeric actinin) but not skeletal muscle myosin D (MyoD) or neurogenic features (Neurogenin). Cells treated with HBR express both cardiac and endothelial markers, such as von Willebrand factor (vWF), and enhance cardiac repair in infarcted rat heart. AC transplants increase capillary density at the infarct border, normalize left ventricular function, and decrease scar formation. Transplantation of HBR-preconditioned AC-derived cells further enhanced capillary density and the yield of human vWF-expressing cells, also decreasing the infarct size. Some engrafted, HBR-pretreated AC-derived cells were also positive for connexin 43 and cardiac troponin I. These improvements may be related to the local or paracrine secretion of angiogenic, antiapoptotic, and mitogenic factors following human MSC transplantation, in addition to the differentiation of AC to vascular cells. These "trophic" effects may provide a major contribution to the therapeutic potential of MSCs for myocardial infarction and dilated cardiomyopathy. These findings provide an innovative approach to cell therapy of heart failure, whereby AC secrete trophic factors and, under the influence of HBR, may show enhanced cardiac and vascular differentiation [35, 80].

Placenta Derived Stem Cells for Treating Neurological Disorders

Human AEC have shown particular potential for treating central nervous system disorders. Since the discovery that hAEC have stem cell properties [11], express neural and glial markers and neural-specific proteins, and also have the capacity to produce and secrete neurotransmitters [9, 17], cell therapy with these cells has been considered [9, 16]. Successful transplants of hAEC into caudate nucleus [19, 20, 81], hippocampus [82], and spinal cord [66] have been reported. Transplantation of hAEC in a rat model of Parkinson's disease reversed the condition and prevented neuronal death [19, 20]. When hAEC were transplanted into ischemic hippocampus, they differentiated into "neuron-like" cells [82]. Following transplantation into the transected spinal cord of monkeys, hAEC aided a robust regeneration of host axons and prevented death of axotomized neurons of the spinal cord [66]. Transplantation of hAEC into the lesioned areas of a contusion model of spinal cord injury in rats was performed without immunosuppression. Cells survived up to 120 days with no evidence of inflammation or rejection. Animals showed gradual functional improvement using the Basso, Beattie, and Brensnahan (BBB) locomotor rating scale and ultimately reached a score of 19, just 2 points below normal animals. Improvement was also observed in lesion control animals; however, improvement was faster during acute and subacute phases of recovery in transplant recipients. Wu et al.

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reported similar findings [83]. Early improvement in the BBB scale is thought to indicate that hAEC provide neuroprotection [84]. In narrow-beam and inclined-plane tests, the performance of hAEC-transplanted animals was significantly improved compared with lesion control animals. These tests measure corticospinal tract [85] and rubrospinal tract [86, 87] function, respectively. Improvement following hAEC transplants indicates improvement of pyramidal (corticospinal) and extrapyramidal (brain stem) systems of motor tracts controlling locomotion. hAEC secrete neurotrophic factors [88], whereas medium conditioned by hAEC has been shown to be neurotrophic for E18 rat cortical cells. Novel EGF-like neurotrophic factors were thought to mediate this effect [89]. hAEC-conditioned medium also supported survival of E10 chicken neural retinal cells, which were otherwise dependent on fibroblast growth factor-2 (FGF-2) [90, 91]. Although FGF-2 and EGF were not detected in media by immunoblotting, FGF-2 and EGF gene and protein expression was reported in cryopreserved hAEC [89, 92]. Recently, MSCs were found to produce "neuron-like" cells, but their function is yet to be proven [93].

In conclusion, hAEC transplants produce beneficial results in animal models of spinal cord injury. They were found to exhibit neuroprotection in acute phases of injury and facilitate regeneration of long tracts in long-term phases of recovery, as measured by behavioral assessment. The beneficial effects may be mediated through the secretion of novel neurotrophic factors.

Cell Tracking

In preclinical studies, tracking of transplanted cells is essential. Using cell labeling together with imaging, cells can be traced noninvasively [94–102]. Stem cells from different sources have been labeled using radionuclides, magnetic nanoparticles, or reporter genes, in both preclinical and clinical studies [95, 103, 104]. In contrast to other imaging techniques, luminescence imaging detects live cells, since the reporter gene, luciferase, generates photon emission only in the presence of ATP, luciferin, and oxygen [105]. Reporter gene transfection protocols established for adipose-derived stem cells have been successfully applied in hAMSC [102], allowing luminescence imaging of their survival, migration, and distribution in preclinical in vivo models.

CELL AND TISSUE BANKING

General Aspects of Banking for Human Use

Cell or tissue banking follows specific rules. European Community directive 2003/94/EC lays down the principles and guidelines of good manufacturing practice (GMP) for medicinal products for human use. This directive is particularized in the so-called GMP Guidelines [106]. Standards of quality and safety for the donation, procurement, testing, processing, preservation, storage, and distribution of human tissues and cells, with annotations to traceability, adverse reactions, and technical requirements concerning production and quality control, are found in directive 2004/23/EC and specified in 2006/17/EC and 2006/86/EC. Other institutions such as The European Association of Tissue Banks (ETAB) and Joint Accreditation Committee-ISCT & EBMT (JACIE) also publish standards for cell and tissue banking and interpreting laws [107–109].

Cell Banking

So far, most experience in preservation of placental tissue-derived cells has been gained with cord blood, which contains both hematopoietic and mesenchymal stem cells. When cord blood transplantation proved effective, many cord blood banks were established, offering collection and banking for public (allogeneic) or private (autologous or allogeneic) use. Cord blood is procured from natural births or caesarean sections [110]. Different methods for the reduction of red blood cells, plasma volume, and cryopreservation exist [111]. Cord blood products containing cryoprotectants (e.g., dimethyl sulfoxide [DMSO]) are frozen at a controlled-rate and can be stored in liquid nitrogen for at least 15 years without the loss of their engraftment potential in vivo [110, 112].

In contrast to cord blood [113, 114], fetal membrane stem cells are presently preserved mainly for research. However, as these cells gain interest for their regenerative and immunomodulatory properties (described above, in Immunology of the Placenta) [29, 115], future medical needs may require concomitant application of cord blood and placental cells from the same donor. After cryopreservation, hAMSC and hCMSC can be differentiated at least along the osteogenic lineage (S. Hennerbichler, unpublished data). However, no conclusive studies are so far available on the influence of cryopreservation on plasticity.

Tissue Banking

The use of amniotic membrane has history spanning almost 100 years. The first reported clinical use of amniotic membrane was in 1910, when it was applied in skin transplantation [116]. Shortly after, application was expanded to treat burned and ulcerated skin [117, 118] and conjunctival defects [119]. Since its rediscovery in 1995 [120], it has been widely applied in ophthalmology, surgery, and wound healing [121]. Besides its nearly unlimited availability, easy procurement, and low processing costs for therapeutic application, many beneficial properties of this tissue, including bacteriostatic, anti-inflammatory, analgesic, wound healing, reepithelialization, reduced scarring, and anatomical and vapor barrier properties, have been reported [56, 122–124].

Currently, freeze-dried, γ-sterilized, decellularized, glycerol-preserved, and cryopreserved amniotic membranes are used in ophthalmology and wound care [123, 125–128]. Questions remain as to how these different processing and preservation methods influence sterility, viability, and growth factor release [129, 130] (S. Hennerbichler, unpublished data).

When placentae from caesarean sections were collected, 0 of 10 tested were contaminated with aerobic or anaerobic bacteria, whereas bacteria were detected in 4 of 10 naturally born placenta tested (S. Hennerbichler, unpublished data). It may therefore be preferential to collect placentae from caesarean sections.

Membranes were further investigated for viability and growth factor release after glycerol (50% glycerol) or cryopreservation (10% DMSO). Cryopreserved membrane retained cell viability and released several angiogenic growth factors and cytokines [129, 130], whereas storage in glycerol at 4°C resulted in immediate cell death [129]. Others have confirmed that different processing methods such as irradiation influence the growth factor content of amniotic membrane [131].

CONCLUSION

In 2004, the journal Transplantation ran an editorial entitled "Amnion and chorion cells as therapeutic agents for transplantation and tissue regeneration: a field in its infancy" [132]. This editorial referred to one of the first reports showing that human fetal membranes harbor cells with progenitor/stem cell potential that were tolerated by the recipients such that they would engraft in animals even after xenotransplantation [40]. In this review,

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we have presented recent advances in this field, with the aim of defining the placenta derived cell subpopulations and their plasticity, as well as providing protocols for their isolation and differentiation. Finally, the preclinical studies reported strongly support the hypothesis that placenta holds much promise for the development of cell-based therapies for clinical applications in the near future.

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Disclosure of Potential Conflicts of Interest

S.C.S., University of Pittsburgh, has acted as a consultant and received financial compensation within the last 2 years from Cambrex, Walkersville, MD.

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