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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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

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.
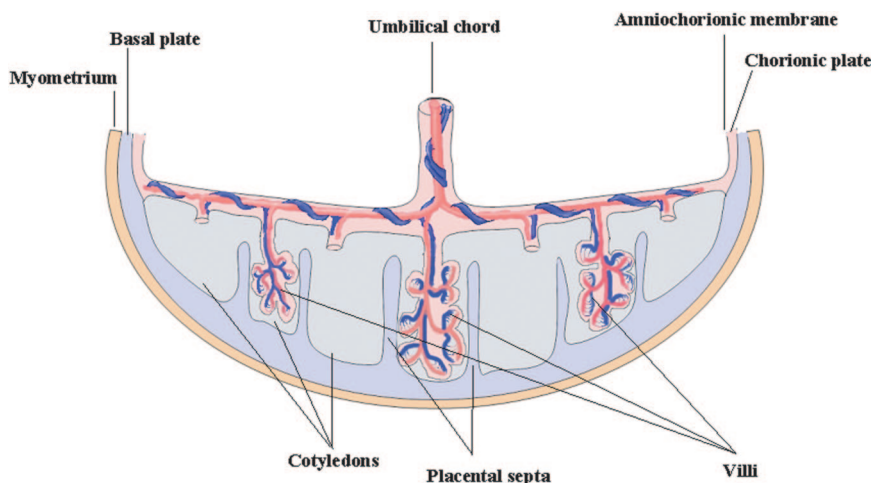
THE PLACENTA

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.
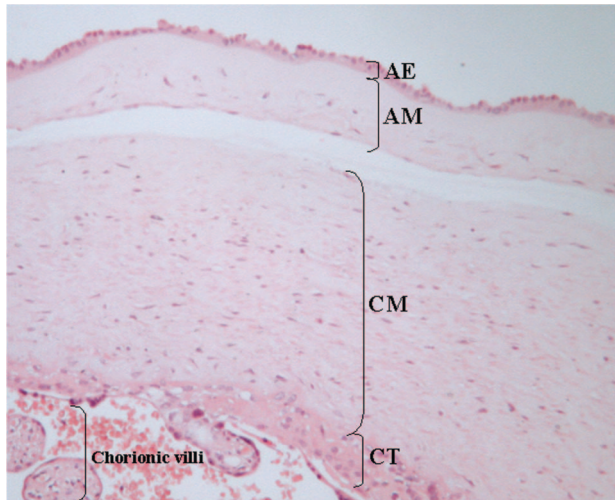
Figure 2. Cross-sectional representation of human fetal membranes (amnion and chorion). The amnion is composed of an epithelial layer of cuboidal and columnar cells, which lie on top of a mesodermal layer consisting of an upper acellular compact layer and a deeper layer containing dispersed fibroblasts. 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 α6 and β1, 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
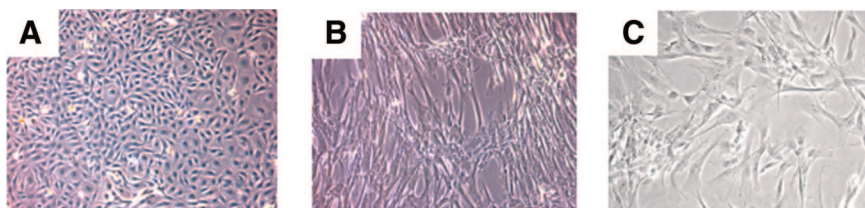
Table 2. Human amniotic epithelial cells: isolation protocols, phenotype, and in vitro differentiation

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^{-5} 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 oncostastin 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

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

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 ng/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 linolenic 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, 1,α25-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^{-8} 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],

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-

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 Bresnahan (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.

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,

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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