Chapter 3
Human Amniotic Membrane: A Potential Tissue and Cell Source for Cell Therapy and Regenerative Medicine

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Abstract The human amniotic membrane (HAM) is the innermost membrane surrounding the fetus. HAM is a highly abundant and readily available tissue that is becoming appreciated as an alternative to adult bone marrow mesenchymal stem cells (BM-MSCs) useful for cell therapy and regenerative medicine. This tissue provides high efficiency in noninvasive and safe MSC recovery with no intrusive procedures. HAM contains two cell types from different embryological origins: human amnion epithelial cells (hAECs), derived from the embryonic ectoderm, and human amnion mesenchymal stromal cells (hAMSCs), derived from the embryonic mesoderm. hAMSCs and hAECs are immune-privileged cells that can be isolated without the sacrifice of human embryos, avoiding immunological rejection problems and the ethical conflict of using human embryonic stem cells (hESCs). Regarding their immunophenotype, both cell types demonstrate the expression of the common well-defined human mesenchymal and embryonic stem cell markers and the absence of hematopoietic markers. Moreover, both cell populations have similar multipotential for in vitro differentiation into all three germ layers: ectoderm,

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mesoderm, and endoderm lineages. Indeed, the potential application of amnion-derived cells in a variety of diseases, in particular those associated with degenerative processes, is under clinical or preclinical investigation. The HAM has other biological properties important for tissue engineering, including anti-fibrosis, anti-inflammatory, anti-scarring, antimicrobial, as well as adequate mechanical properties and low immunogenicity. Therefore, amnion allografts are widely applied in ophthalmology, plastic surgery, dermatology, and gynecology. In this chapter, the localization, isolation, characterization, and differentiation potential of amnion-derived cells are discussed. Moreover, the potential clinical applications of either amnion-derived cells or the whole HAM are also reviewed.

Keywords Human amniotic membrane • Adult bone marrow mesenchymal stem cells • Human amnion mesenchymal stromal cells • Cell therapy • Regenerative medicine

3.1 Mesenchymal Stem Cell Concept

Mesenchymal stem cells (MSCs) are multipotent non-hematopoietic progenitors located within the stroma of the bone marrow and other organs that are phenotypically characterized by the expression of several markers (e.g., CD73, CD90, and CD105) and the lack of expression of CD14 or CD11b, CD19 or CD79α, CD34, CD45, and HLA-DR surface molecules [1, 2]. According to a proposal of the International Society for Cellular Therapy [3], three criteria define all types of stem cells: self-renewal, multipotency, and the ability to reconstitute a tissue in vivo. Since there are no specific markers for MSCs, the main criteria for their identification are adherence to the plastic of the tissue culture flask, fibroblast-like morphology, prolonged capacity for proliferation, and the capacity to differentiate into cells of mesodermal lineage in vitro. MSCs are classified, according to the developmental stage from which they are obtained, into embryonic, fetal, or adult stem cells. hESCs are pluripotent and could give rise to all specialized cell types of the organism. However, the tumorigenicity of these cells and technical and ethical considerations limit their availability. In contrast, adult stem cells are rare cells thought to be present in all tissues and responsible for maintaining the homeostasis of the specific tissue [4]. These cells, previously thought to be limited in potential, have been shown to differentiate into multiple mesoderm-type lineages, including chondrocytes, osteoblasts, adipocytes, tenocytes, myotubes, astrocytes, and hematopoietic-supporting stroma [5–7], and also into cell types of ectodermal (e.g., neurons) and endodermal (e.g., hepatocytes) origin [8].

These cells have been isolated from several tissues such as bone marrow [2, 9], articular cartilage [10], synovial membrane [11, 12], perichondrium [13], periosteum [14], connective tissue of dermis and skeletal muscle [15], adipose tissue [16, 17], peripheral blood [18–20], liver [21], lung [22], placenta [5, 23–25], umbilical cord [26–28], umbilical cord blood [29], amniotic fluid [23, 25, 30], and amniotic

membrane [31–33]. Moreover, the list of tissues with the potential for tissue engineering is increasing because of recent progress in stem cell biology [34].

Cell therapy using MSCs is a new clinical approach for the treatment of a large number of genetic and degenerative human diseases, including hematopoietic and immune system disorders, diabetes, heart failures, chronic liver injuries, and neurodegenerative disorders. The recent use of autologous or allogenic stem cells has been suggested as an alternative therapeutic approach for cartilage treatment [35, 36]. Human MSCs are probably responsible for normal tissue renewal as well as for response to injury [37–39]. Stem cell transplantation uses cells isolated from small tissue samples, proliferated in culture, to obtain the appropriate number for clinical applications. The use of autologous MSCs avoids immunological rejection problems and the ethical conflict of using hESCs. For these reasons, MSCs are a promising cell resource for tissue engineering and cell-based therapies [38]. The interest in MSCs and their possible application in cell therapy have resulted in a better understanding of the basic biology of these cells. Due to the low number of MSCs that can be isolated from a tissue sample, culture expansion is necessary to obtain adequate cell numbers for clinical purposes and for the analysis of molecular mechanisms.

The bone marrow is the traditional tissue source used for obtaining adult MSCs, but it has a number of disadvantages. The most important limitations are the accessibility and that the procedure required for obtaining this type of tissue is invasive, painful, and associated with morbidity. In addition, the number of cells obtained is low and the potential for proliferation and differentiation declines with donor age [40, 41]. Therefore, the identification of alternative sources of MSCs for both therapeutic and research purposes would be beneficial.

The HAM or amnion has recently emerged as another novel and alternative source of stem cell populations. The HAM is the innermost membrane surrounding the fetus. Because it arises from embryonic epiblast cells prior to gastrulation, it has been suggested that it may retain a reservoir of stem cells throughout pregnancy [42].

3.2 Human Amniotic Membrane or Amnion

The placenta is a structure of fetal-maternal origin with a round shape, 15–20 cm in diameter, and 2–3 cm in thickness [43]. The thickness of the full-term amnion varies between humans and depends on the location of the sample. HAM functions as a filter and preventive shock absorber that protects against infections, traumas, and toxins. This organ is involved in the maintaining fetal tolerance and allows nutrient uptake and gas exchange with the mother but also contains a high number of progenitor cells or stem cells. Moreover, the volume of term placenta makes it an attractive source of stem cells, since as an average human term placenta weighs more than 590 g [44]. HAM develops from extraembryonic tissue and consists of both a fetal component (the chorionic plate) and a maternal component (the deciduas) that are comprised of an epithelial monolayer, a thick basement membrane,

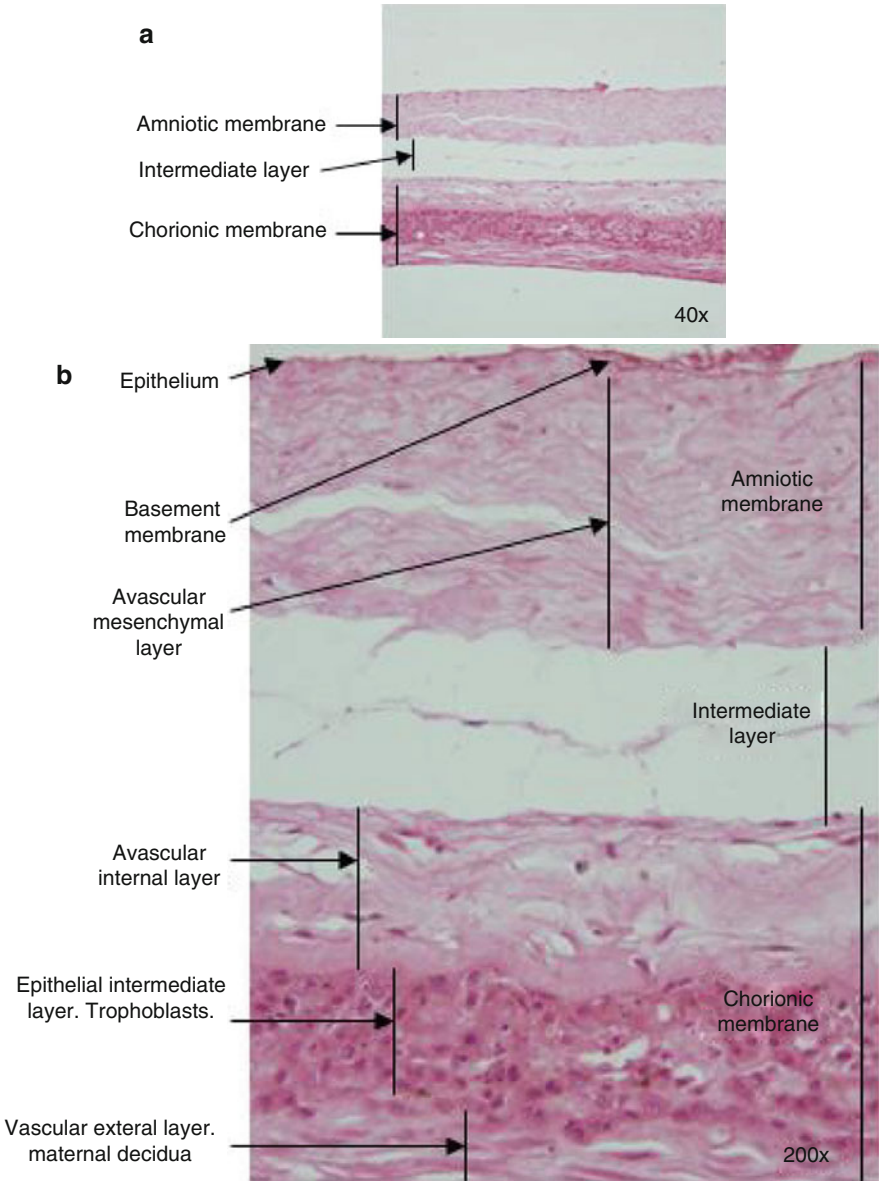

Fig. 3.1 Structure of the fetal membrane at term stained with hematoxylin and eosin (HE). Original magnification: 40× (**a**) and 200× (**b**)

and an avascular stroma [45, 46] (Fig. 3.1). The amnion is a thin (up to 2 mm), avascular, strong, elastic, translucent, and semipermeable fetal membrane attached to the chorionic membrane. Both the amnion and chorion form the amniotic sac

filled with amniotic fluid, providing and protecting the fetal environment. The outer layer, the chorion, consists of trophoblastic chorionic and mesenchymal tissues. The inner layer, the amnion, consists of a single layer of ectodermally derived epithelium uniformly arranged on the basement membrane, which is one of the thickest membranes found in any human tissue, and a collagen-rich mesenchymal layer [47]. This mesenchymal layer can be subdivided into the compact layer, forming the main fibrous skeleton of the HAM, the fibroblast layer, and an intermediate layer, which is also called the spongy layer or *zona spongiosa* [45]. Resistance to rupture of HAM is provided by the collagen present in the basement membrane of the amnion. Spontaneous premature rupture of the fetal membranes complicates 1–4 % of the pregnancies. This is due to multiple factors such as infection and genetic predisposition. These premature ruptures are associated with elevated expression levels of relaxins, low expression levels of extracellular matrix (ECM) proteins synthesized by the fetal membranes, or to degradation of these proteins by induced matrix metalloproteinases (MMPs) and subsequent ECM remodeling [48].

The two layers of the amniotic membrane originate at day 8–9 after fertilization, when implantation of the blastocyst has occurred. The inner cell mass of the blastocyst differentiates into two layers, the epiblast and the hypoblast; both layers form the bilaminar embryonic disc. The epiblast gives rise to the three germ layers (ectoderm, mesoderm, and endoderm) and the amniotic epithelium [49].

3.3 Localization of Human Amniotic Membrane-Derived Cells

The localization of HAM-derived cells was examined by our group [31, 32]. We assessed the co-localization of different stem cell markers in histological sections of amniotic membrane by means of immunofluorescence assays. In particular, we studied the co-localization of the CD44, CD90, CD105, and CD271 markers.

Our group did not observe any cells in which co-localization of three and/or four stem cell markers occurred. However, we frequently observed co-localization of double markers, for example, we found CD105 co-located with CD90, CD44 co-located with CD90, and CD271 co-localized with CD44 (Fig. 3.2). Most cells labeled with the different stem cell markers were hAMSCs from the thick basement membrane, although in some membranes we observed hAECs, derived from the embryonic ectoderm, that were labeled only for the CD105 marker. hAMSCs are derived from embryonic mesoderm [50] and are sparsely distributed in the stroma underlying the amnion epithelium [51]. On the other hand, hAECs form a continuous monolayer of embryonic ectodermally derived epithelium uniformly arranged on the basement membrane in contact with the amniotic fluid.

The immunofluorescence results of our group [31, 32] indicated that the HAM contains at least two different cell types having stem cell characteristics and that

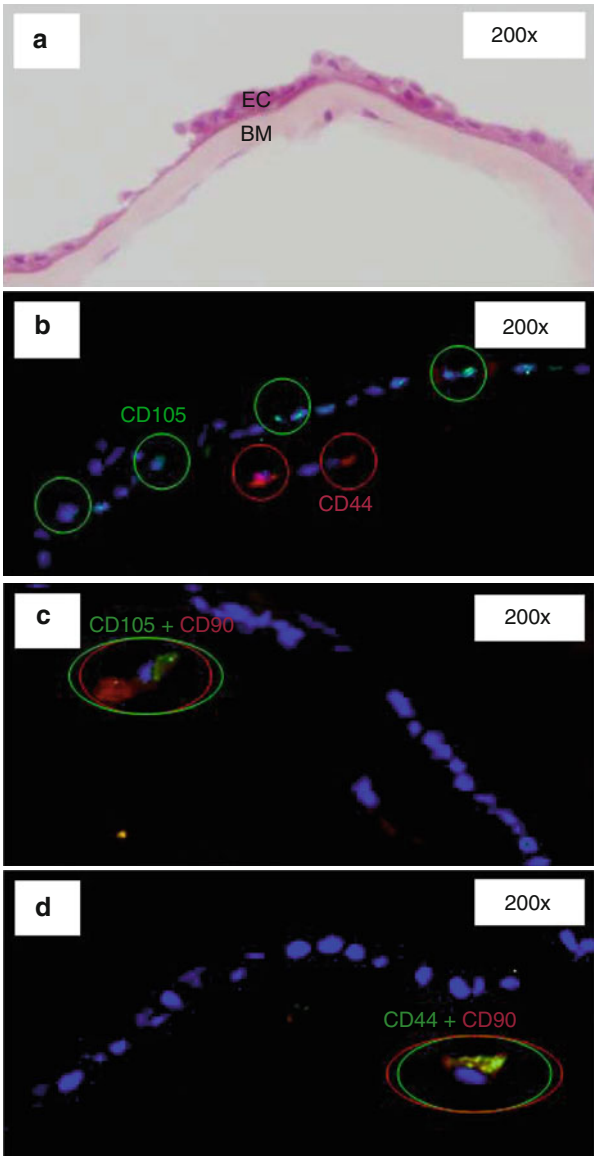

Fig. 3.2 Localization of HAM-derived cells, hAMSCs and hAECs, in healthy HAMs. Representative section of the HAM stained with HE (**a**), indicating the epithelial cells from the extraembryonic ectoderm (*EC*) and the thick basement membrane (*BM*). Immunofluorescence analysis of stem cell marker expression of human amnion cells, nuclei were counterstained with 4′,6-diamidino-2-phenylindole (DAPI) (**b**–**d**). Representative images of hAECs, positive for CD105, and hAMSCs, positive for CD44 (**b**). Representative photos of hAMSCs positive for CD105-CD90 (**c**) and CD44-90 (**d**). Original magnifications: 200× (Images taken from Díaz-Prado et al. [32])

these cells are located in the basement membrane and in the single layer of ectodermally derived epithelium. These common and well-defined human MSCs markers were previously described for bone marrow MSCs. Moreover, we showed that hAECs are positive for the epithelial marker cytokeratin 7, which confirms its epithelial nature (Fig. 3.3).

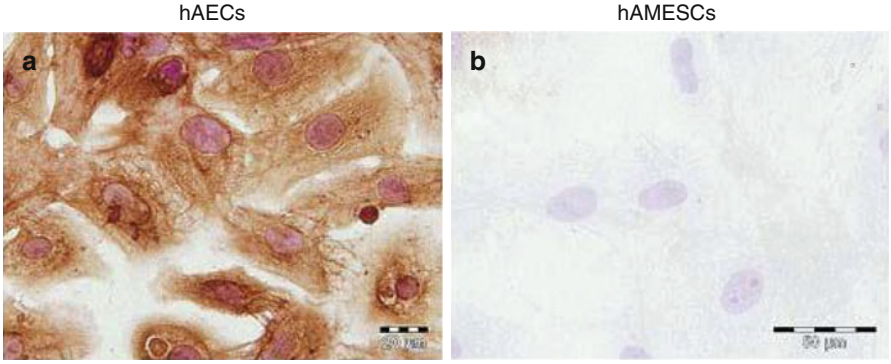

Fig. 3.3 hAECs (**a**) and hAMSCs (**b**) were stained with CK7 antibody by means of immunohistochemistry

3.4 Human Amniotic Membrane as a Source of Stem Cells

HAM expresses only moderate levels of major histocompatibility complex (MHC) class I antigens and MHC class II antigens on its surface. HAM-isolated cells have anti-inflammatory properties. Moreover, there was no evidence of tumorigenicity when isolated human amniotic cells were transplanted into human volunteers or into patients in an attempt to correct lysosomal storage diseases [52–54]. Therefore, hAECs and hAMSCs seem to be immune-privileged cells and suitable for allotransplantation and regenerative medicine [40, 55].

Because fetal tissues are routinely discarded postpartum, HAMs have proved to be abundant, inexpensive, and easily obtained with a virtually limitless availability [45, 47, 56–58]. Therefore, the HAM represents a very useful source of progenitor cells for a variety of applications. Because human embryos are not sacrificed for the isolation of progenitor cells from HAMs, the current controversies associated with the use of hESCs can be avoided [43, 55, 56, 58]. Given the minimal ethical and legal issues associated with HAM cell usage, further investigation into their functional potentials in vivo is warranted.

HAM is becoming appreciated as an alternative to bone marrow for adult MSCs for regenerative medicine. This tissue provides high efficiency in MSC recovery with no intrusive procedures [33]. Moreover, harvesting cells from the HAM is noninvasive and safe. A major advantage of cells isolated from the HAM is that they are harvested after birth and can be cryogenically stored to be available in a timely manner for patient therapy after being thawed and expanded for use in tissue engineering, cell transplantation, and gene therapy.

MSCs from first-, second- and third-trimester placental compartments, including the amnion, chorion, decidua parietalis, and decidua basalis, were isolated and represent less than 1 % of the cells present in the human placenta [22, 33, 59, 60].

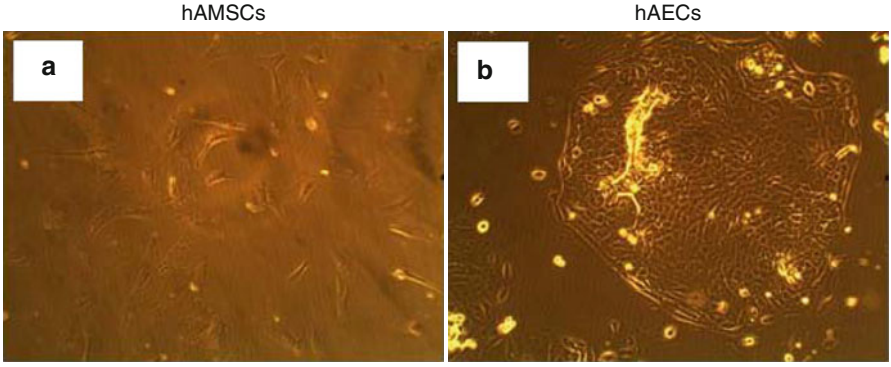

Fig. 3.4 Morphology of cultured hAMSCs (**a**) and hAECs (**b**) isolated from healthy HAM. Original magnifications: 100×

HAM contains two different cell types from different embryological origin [33, 61]: hAECs, derived from embryonic ectoderm [50] which form a continuous monolayer that contacts the amniotic fluid, and hAMSCs, derived from embryonic mesoderm [50] which are sparsely distributed in the stroma that underlies the amnion epithelium [51]. Both hAECs and hAMSCs secrete various antiangiogenic and anti-inflammatory proteins such as interleukin (IL)-1 receptor antagonist; activin A; tissue inhibitors of metalloproteinases (TIMP-1, TIMP-2, TIMP-3, and TIMP-4); and IL-10 which are deposited within the amniotic membrane stroma [62]. Hyaluronic acid may act as a ligand for CD44 and may entrap inflammatory cells in the stroma.

Some papers reported the isolation of HAM-derived cells, from the mesenchymal and epithelial regions of the amnion, from the full-term amnion after its manual separation from the chorion. Bailo et al. [63] isolated and characterized amnion and chorion cells from human term placenta suggesting that both kinds of cells may represent an advantageous source of progenitor cells with potential applications in a variety of cell therapy and transplantation procedures. For this purpose, different methods to isolate HAM-derived cells have been published [33, 59, 63–67].

All these protocols start with a mechanical separation of the amniotic membrane from the underlying chorion through the spongy layer [43]. This step is followed by a digestion with trypsin, dispase, or other digestive enzymes, in different concentrations and for different periods of time, to release the hAECs from the basal membrane. hAMSCs can be subsequently released through subsequent digestion with collagenase [49], alone or combined with DNAase [23].

Regardless of the morphological features of human amnion-derived cells, hAMSCs show plastic adherence and fibroblast-like growth usually observed with MSCs from bone marrow (Fig. 3.4). After 3–4 weeks of hAMSCs culture, it is possible to obtain a population of adherent mesenchymal cells morphologically identical to MSCs isolated from bone marrow. These stromal cells are easy to expand in vitro for at least 9 passages without morphological changes. Furthermore, their immunophenotypic characterization demonstrates the presence of common well-defined

human MSC markers previously described for bone marrow (CD90, CD44, CD73, CD166, CD105, CD29) with the absence of the hematopoietic markers CD34 and CD45 and the concomitant lack of fibroblast marker [68, 69]. The absence of hematopoietic or monocytic marker gene expression excludes the possibility that the observed plasticity of these cells is due to contamination with stem cells from fetal or cord blood or with embryonic fibroblasts. This antigen expression pattern is consistent with the data previously published in cells isolated from the amnion and other regions of the term placenta [5, 51, 63]. hAMSCs are also positive for pluripotency markers such as Oct4 (octamer-binding protein 4), NANOG, SOX2 (SRY-related HMG-box gene 2), and REX-1 [49], but positivity for embryonic stem cell markers, SSEA-3 or SSEA-4, remains debated [49]. hAMSCs may be considered as superior to adult MSCs in their differentiation and proliferation capacity due to their higher OCT4 mRNA levels [33]. Moreover, hAMSCs also express low levels of HLA-A, HLA-B, and HLA-C, but do not express HLA-DR, indicating that these stromal cells may be useful in clinical transplantation procedures [49].

On the other hand, isolated hAECs are small-size cells that are easy to expand in vitro for at least three passages without morphological changes; they display epithelial morphologies and grow into a tightly packed, cobblestone monolayer in culture [70] (Fig. 3.4). These cells generally have a central or eccentric nucleus, one or two nucleoli, and abundant cytoplasm, usually vacuolated [66]. hAECs are positive for desmin and vimentin [58]. These epithelial cells also reveal an antigen expression profile characteristic of culture-expanded MSCs [51], since they are positive for the same markers as for hAMSCs. Primary hAECs seem to contain class IA and class II HLAs, consistent with a low risk of tissue rejection [42]. They do not express HLA-A, HLA-B, and HLA-C belonging to class I of the MHC and HLA-DR and HLA-DQ belonging to the class II MHC [45, 65]. When these cells follow pancreatic or hepatic differentiation, but not cardiogenic differentiation, express a significant percentage of class IA but not class II HLAs [71]. In addition, hAECs secrete a number of immunosuppressive factors that target the innate and adaptive immune systems, which may support survival following transplantation [70]. Evidence for long-term self-renewal is not still available for hAECs, probably may be due to the absence of telomerase that limits their ability to divide in culture.

Phenotypes of the two cell populations (Fig. 3.5), hAMSCs and hAECs, are maintained from passage 0 to passage 9 [32]. It is important to notice that although both populations show similar signature regarding cell surface receptor expression pattern, they show many differences with regard to cell shape and cell arrangement [32, 51]. These same findings were previously described by Bilic et al. [51]. These investigators isolated these two populations and concluded that hAECs and hAMSCs in culture exhibited and maintained a similar marker profile of mesenchymal progenitors. hAECs also express surface markers of undifferentiation normally present on embryonic stem and germ cells such as SSEA-4 and STRO-1. Both embryonic stem cell markers are present in more quantity in hAECs than in hAMSCs [32, 42, 51], possibly indicating that hAECs could be at a more early state of undifferentiation. In this regard, Ilancheran et al. [42] also showed that hAECs

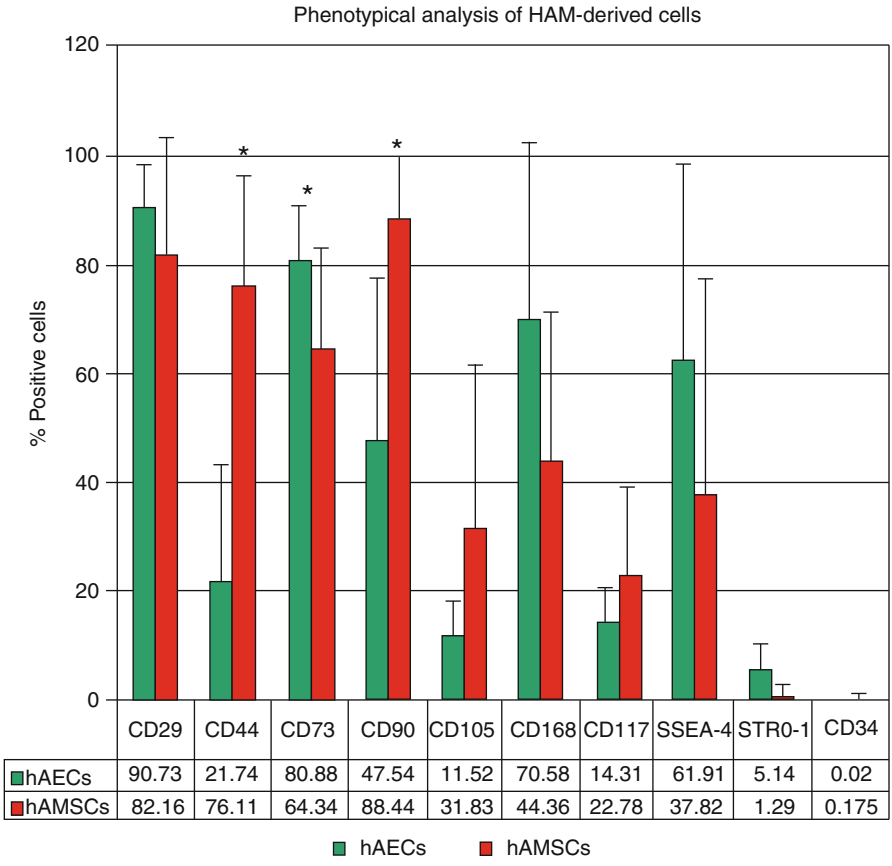

Fig. 3.5 Analysis of hematopoietic and standard adult stem cell markers on hAECs and hAMSCs. *Means *P* value <0.05 (Mann–Whitney *U*-test)

expressed SSEA-3 (stage-specific embryonic antigen 3), SSEA-4, TRA-1–60 (tumor rejection antigen) and TRA-1–81, and other antigens such as the ABCG 2/BCRP (a member of the ATP-binding cassette superfamily), CD9, CD24, CD90, CD117, E-cadherin, integrin α6 and β1, and c-met (receptor growth factor of the hepatocyte) [43, 45]. It has to be noted that initially isolated, hAECs are not homogenously positive for all these antibodies. Some surface markers such as CCR4- and CD117-positive cells are very rare, while others such as CD9 and integrin α6 and β1 are expressed on virtually 100 % of the cells, indicating that hAECs are a heterogeneous cell population with respect to cell surface profiling [67]. These epithelial cells also express Oct4, NANOG, SOX2, REX-1, FGF4, Lefty-A, and TDGF-1gene products associated with pluripotent embryonic stem cells [49, 67, 72, 73]. When hAECs are cultured as an adherent monolayer for several weeks, small spheroids are evidenced over the cobblestone pavement of epithelial cells. These cell clusters express SSEA-3, SSEA-4, TRA 1–60, and TRA 1–80 stem cell-specific cell surface

antigens. Moreover, the stem cell molecular marker genes Oct4 and NANOG are also expressed in the small cell clusters, suggesting that hAECs form embryonic body-like structures that maintain their stem cell nature in culture [73].

hAECs and hAMSCs can be grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 20 % fetal bovine serum (FBS) and 1 % penicillin-streptomycin (P/E) and seeded into culture flasks. Moreover, hAECs could be cultured with or without the addition of growth factors such as epidermal growth factor (EGF) or basic fibroblast growth factor (bFGF) and mostly in the absence of leukemia inhibitor factor (LIF) [48]. Both populations should be expanded in a humidified 5 % CO_2 atm at 37 °C. After the isolation of both cell types, it is advisable to perform immunohistochemical stainings (e.g., for cytokeratin 7, CK7) to demonstrate the purity of both populations. In this regard, only hAECs may be positive for this or other epithelial markers. In monolayer cultures, these hAECs are positive for low molecular weight cytokeratins, confirming their epithelial nature. Moreover, although initially they are vimentin-negative, hAECs become vimentin-positive during cell culture. Vimentin-positive hAECs remain positive for cytokeratins, indicating that in vitro culture may induce dedifferentiation of these epithelial cells [67].

Recently, Parolini et al. [49] published a comparison of key features of HAM-derived cells and human BM-MSCs. These authors postulated that BM-MSCs have a higher cell doubling time than hAECs, while for the hAMSC this time was not reported yet. Regarding the maximum number of passages, it ranges from 5 to 10 for hAMSCs, 10–20 for BM-MSCs, and 30 for hAECs. But there is a contradiction with the passage number at which HAM-derived cells stop proliferation. Based on the literature, proliferation slows down with every passage and cells settle into senescence until proliferation ceases. For example, Miki et al. [66] and Parolini et al. [65] state that hAECs grow normally for 2–6 passages before proliferation ceases. On the contrary, Bilic et al. [51] confirmed that hAECs and hAMSCs proliferation almost stops beyond passage 5, whereas Toda et al. [58] postulated that hAECs senescence is reached at lower passages, P3 or P4. However, Alviano et al. [33] and Soncini et al. [59] indicated that hAMSCs are easily expanded in vitro for at least 15 passages without any visible morphological alterations, but they used cells not exceeding P4 for cell characterization and multilineage differentiation potential studies.

Another comparison between placental cells and BM-MSCs was the aim of the paper published by Barlow et al. [5]. These authors compared human placenta-derived MSCs (the placental tissue included amnion, chorion, and decidua) and human bone marrow-derived MSC in terms of cell characteristics, optimal growth conditions, mesodermal lineage differentiation, and in vivo safety specifically to determine if human placenta-derived MSCs could represent a source of human MSC for clinical trials. They demonstrated that both populations were similar in terms of growth condition requirements and in terms of subsequent biological characterization. However, both populations differed with respect to their proliferation capabilities at different seeding densities. In this regard, human bone marrow-derived MSCs proliferated more slowly than human placenta-derived MSCs in every experiment.

Also the latter had greater long-term growth ability than the former. Moreover, MSCs from both sources exhibited similar morphology, size, and cell surface phenotype, and mesodermal differentiation ability with the exception that human placenta-derived MSC consistently appeared less able to differentiate to the adipogenic lineage. In line with the results obtained, these authors suggested that human placenta is an acceptable alternative source for human MSC.

All published protocols to obtain HAM-derived cells yield hAMSCs, but no studies have compared their efficacy in the isolation. Our group [31] compared two protocols, described in the literature by Alviano et al. [33] and Soncini et al. [59], for the isolation of hAMSCs from the HAM. Alviano's protocol involved three digestions (one mechanical and two enzymatic), whereas Soncini's protocol used only two enzymatic digestions. This study included the comparison of hAMSCs, isolated using both methodologies, in terms of their phenotypic characterization and their in vitro potential for differentiation toward osteogenic, adipogenic, and chondrogenic mesodermal lineages. Both protocols allowed the successful isolation and culture of cells attached to the culture flask with fibroblast-like cell morphology from full-term placenta. These cells showed similar immunophenotype but with differences in cell yield and in the in vitro differentiation potential into the main mesodermal lineages. In particular, quantitative studies showed that Soncini's protocol typically showed an increase in the hAMSCs isolation yield of almost tenfold with regard to Alviano's protocol. Also, the former protocol allowed the isolation and expansion of a larger number of cells in a very short time period. This ready and rapid availability of cells is one criterion required of a source of MSCs for it to be considered for cell transplantation. Therefore, the differences found using both protocols should be taken into account when using these cells for cell therapy.

3.5 Differentiation Potential of Human Amniotic Membrane-Derived Cells

Placental MSCs have been shown to differentiate into chondrogenic, osteogenic, endothelial, hepatocytic, myogenic, and neurogenic lineages, with some differences among cell types depending on the placental tissue sources [8, 33, 40, 43, 50, 56, 68, 69, 74, 75] (Figs. 3.6 and 3.7).

hAMSCs differentiation to neuronal lineage has been demonstrated by the fact that these cells express neuronal markers (nestin, Musashi 1, neuron-specific enolase, neurofilament medium, microtubule-associated protein [MAP]-2 and Neu-N) and glial (GFAP) markers, after their culture in specific neural-induction media [50, 60, 75, 76].

Tamagawa et al. [74] showed that hAMSCs were able to differentiate into cells with characteristics of hepatocytes. In this regard, native cells expressed typical hepatocytic mRNA such as albumin, CK (cytokeratin) 18, α-fetoprotein, α1-antitrypsin, and HNF-4α, but only glucose-6-phosphatase and ornithine transcarbamylase expression and glycogen storage were observed after in vitro hepatic induction.

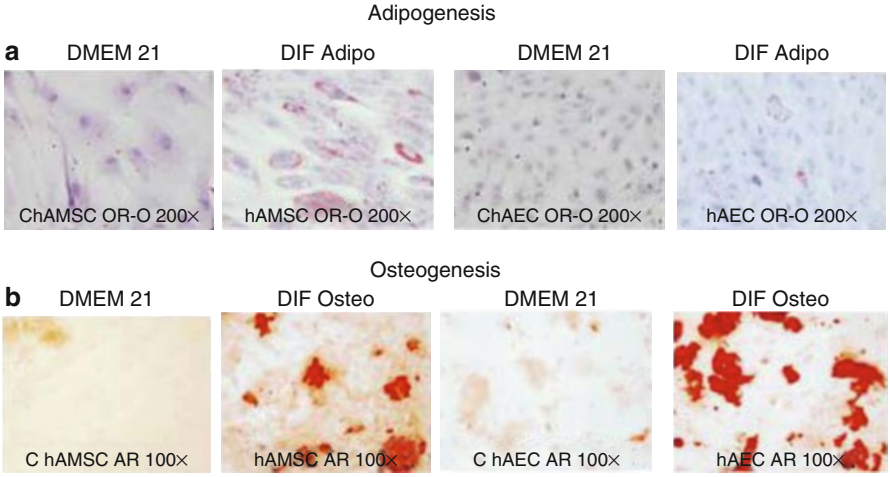

Fig. 3.6 Adipogenic (*DIF Adipo*) and osteogenic (*DIF Osteo*) differentiation of human amnion mesenchymal stromal cells (*hAMSCs*) and human amnion epithelial cells (*hAECs*) with their respective controls (*C hAMSC* and *C hAEC*) grown for 21 days in Dulbecco's Modified Eagle Medium (*DMEM 21*). The presence of adipocytes was assessed by detection of lipid drops using Oil Red O (*OR-O*) stain (**a**). The presence of the calcium deposits characteristic of osteoblasts was detected using Alizarin Red (*AR*) stain (**b**). Original magnifications: 200× (**a**) and 100× (**b**) (Images taken from Díaz-Prado et al. [32])

Regarding hAMSC differentiation toward mesodermal lineage, In't Anker et al. [22] demonstrated the potential of hAMSCs to differentiate into osteogenic and adipogenic cells. After osteogenic differentiation, hAMSCs suffered morphologic changes and showed calcium deposits when they were stained with von Kossa's dye. On the other hand, and after adipogenic differentiation, hAMSCs become multi-vacuolated cells that were stained with Oil Red O stain. Later, Portmann-Lanz et al. [60] showed the capacity of these stromal cells for differentiation to chondrogenic and myogenic lineages. Chondrogenic differentiation of these cells was demonstrated by the presence of abundant collagen in the ECM by means of Alcino's blue dye. Myogenic differentiation of hAMSCs has been determined by RT-PCR since Portmann-Lanz et al. [60] demonstrated the mRNA expression of myogenic transcription factors such as MyoD and myogenin and the protein expression of desmin in hAMSCs cultured in differentiation media. Alviano et al. [33] confirmed these results and also were the first to demonstrate the angiogenic differentiation potential of these cells. This latter study revealed that hAMSCs, after culture in induction media with VEGF, expressed endothelial-specific markers such as the receptors of the vascular endothelial growth factor 1 and 2 (FLT-1, KDR), ICAM-1, as well as the appearance of CD34 and von Willebrand Factor (vWF)-positive cells.

Regarding cardiomyogenic potential, it has been demonstrated that hAMSCs expressed cardiac-specific genes such as GATA4, MLC-2a (myosin light chain), MLC-2v, cTnI, and cTnT [77, 78] after cardiomyogenic induction. Zhao et al. [77]

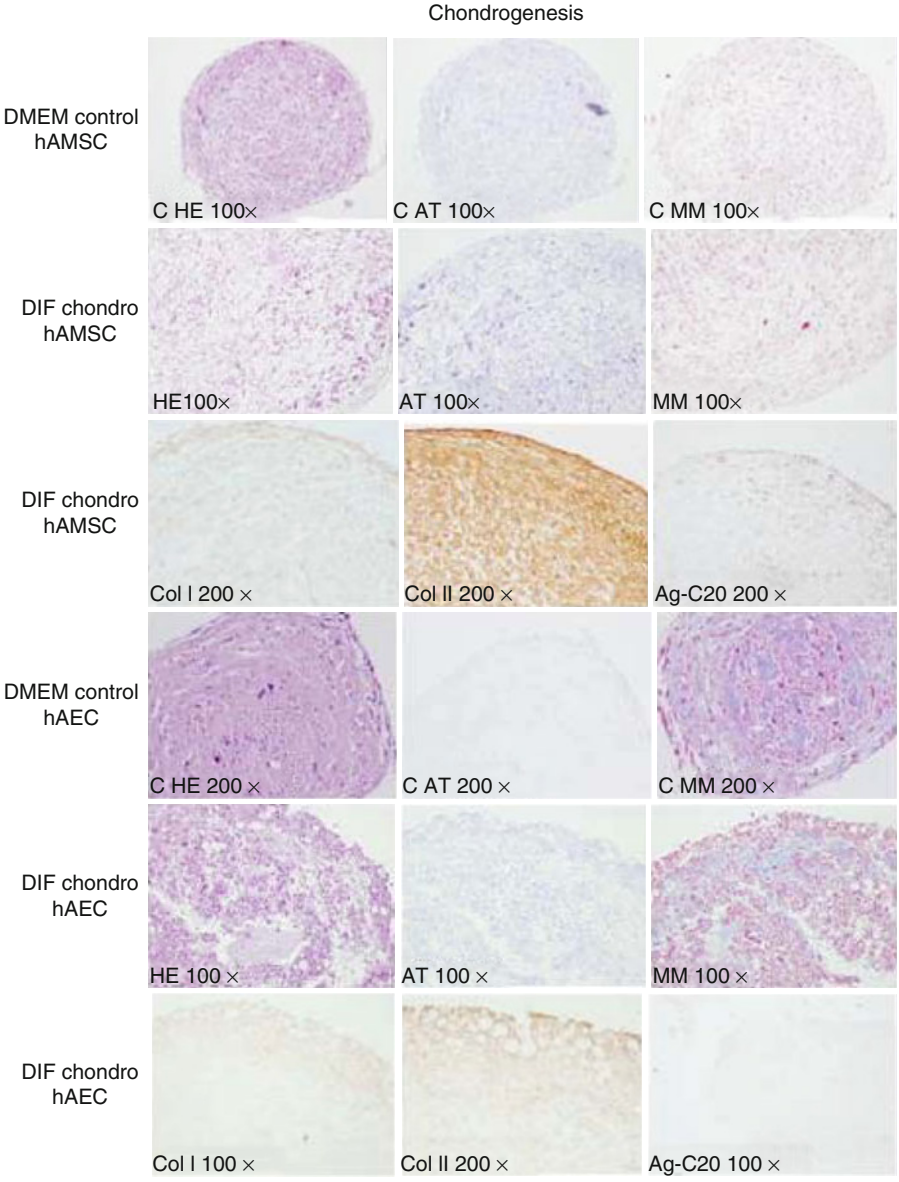

Fig. 3.7 Chondrogenic differentiation (*DIF Chondro*) of human amnion mesenchymal stromal cells (*hAMSCs*) and human amnion epithelial cells (*hAECs*) and their respective controls (*C hAMSC* and *hAEC*) grown for 21 days in Dulbecco's Modified Eagle Medium (*DMEM*). Micropellets were stained with HE, Masson's trichrome (*MM*), and toluidine blue (*AT*) for proteoglycans. Immunodetection of Agg (*Ag-C20*) and collagen type II (*Col II*) was performed to detect molecules characteristic of hyaline cartilage. Immunodetection for collagen type I (*Col I*) was also assessed. Original magnifications: 100× and 200× (Images taken from Díaz-Prado et al. [32])

showed that after hAMSCs transplantation into the myocardial infarcts in rat hearts, these cells survived in the scar tissue for at least 2 months and differentiated into cardiomyocyte-like cells. On the other hand, spontaneous differentiation of hAMSCs toward myofibroblasts has also been observed after their culture in standard medium (DMEM/FBS) within 2 passages [79].

The ability of hAECs to differentiate into cardiomyocytic, myocytic, osteocytic, adipocytic (mesodermal), pancreatic, hepatic (endodermal), neural, and astrocytic (neuroectodermal) cells in vitro has been established [42, 43, 73, 80]. However, in contrast with embryonic stem cells, hAECs did not form tumors up to 7 months posttransplantation in SCID/Beige mice [42, 73]. The capacity of hAECs to differentiate into cell types from all three germ layers may be associated with the fact that the hAECs are directly derived from the epiblast and thus may retain the plasticity of pregastrulation embryonic stem cells.

The pluripotency of hAECs was supported by the study of Tamagawa et al. [81]. The ultimate approach to determine the pluripotency of amniotic epithelium-derived stem cells is to generate chimeric animals by injecting the single stem cell into a blastocyst. If the stem cell contributes all germ layer cells in the chimeric animal, pluripotency will be confirmed [67]. Tamagawa et al. [81] created a xenogeneic chimera with hAECs and mouse embryonic stem cells in vitro. This chimera gives rise to cells of all germ layers, confirming the in vitro pluripotency of hAECs. Later studies have corroborated the ability of hAECs to in vitro differentiate into cells from the three germ layers [42, 43, 73, 80].

hAECs have characteristics of neural progenitor cells since freshly epithelial cells constitutively express a number of neural genes, including neuron-specific enolase (NSE), NF-M, and myelin basic protein (MBP), perhaps suggesting a predilection for neural differentiation [70]. Exposure of hAECs to all-trans-retinoic acid and FGF4 resulted in adoption of an elongated, neural morphology and enhanced expression of some differentiation markers for neural stem such as nestin and GAD (glutamate decarboxylase). Differentiation to astrocyte-like and oligodendrocyte-like cells was also evidenced by expression of glial fibrillary acidic protein (GFAP) and cyclic nucleotide phosphodiesterase (CNP), respectively [73]. Kakishita et al. [82] and Elwan and Sakuragawa [83] demonstrated the differentiation of the epithelial cells to neural cells (ectodermal lineage) with capacity to synthesize and release acetylcholine, catecholamines, neurotrophic factors, activin, noggin, and dopamine, suggesting their possible utility in the treatment of neural degenerative diseases. In this regard, several studies have already been published showing promising results in animal models with Parkinson's disease and mucopolysaccaridosis type VII. Studies of intracerebral grafting of hAECs for the treatment of a mouse model of Parkinson's disease showed that these epithelial cells can synthesize and release catecholamine and neurotrophic factors such as nerve growth factor, neurotrophin-3, and brain-derived neurotrophic factor [82, 84, 85]. Kosuga et al. [86] suggested that transplantation of hAECs transduced with adenoviral vectors can be employed for the treatment of congenital lysosomal storage disorders.

Hepatic differentiation (endodermal lineage) of hAECs was reported by Sakuragawa et al. [87]. They demonstrated albumin and α-fetoprotein production from cultured hAECs, and when LacZ-labeled human hAECs were transplanted into the liver of an immunodeficient mouse, the transplanted cells were found to integrate into the hepatic plate. Some reports demonstrated that these epithelial cells also displayed other functional properties associated with hepatocytes, such as glycogen storage and expression liver-enriched transcription factors, such as hepatocyte nuclear factor (HNF)-3γ and HNF4α, CCAAT/enhancer-binding protein (C/EBP-α and C/EBP-β), and several of the drug-metabolizing genes (cytochrome P450) [73, 88, 89]. Some papers showed albumin production and induction of early markers of hepatic differentiation of hAECs after the addition of specific growth factors to the culture media such as FGF-2, hepatocyte growth factor, oncostatin M, and heparin sodium salt [88]. These findings suggest the potential utility of hAECs to restore hepatic tissues that have been diseased or injured.

Differentiation of hAECs to another endodermal lineage, pancreatic, was reported. Wei et al. [90] cultured these epithelial cells in the presence of nicotinamide to induce pancreatic differentiation, and they observed that the treated cells initiated the expression of multiple pancreatic genes, including the transcription factor Pax-6 and the hormones glucagon and insulin. Subsequent transplantation of these insulin-expressing cells in the spleen of diabetic SCID mice normalized the levels of serum glucose for several months after the transplant, indicating the therapeutic potential of hAECs to treat diabetes mellitus type I. Later, Miki et al. [73] showed by RT-PCR analysis that, after pancreatic differentiation, hAECs express pancreatic α- and β-cell markers such as the transcription factors PDX-1 (pancreatic duodenum homeobox 1), PAX-6 (paired box homeotic gene 6), and NKX2.2 (NK2 transcription factor-related locus 2) and the mature hormones insulin and glucagon.

The differentiation of hAECs to cardiac cells (mesodermal lineage) was first evaluated by Miki et al. [73]. They demonstrated by RT-PCR that cardiac-specific genes atrial and ventricular myosin light chain 2 (MLC-2A and MLC-2V, respectively) and the transcription factors GATA-4 and Nkx 2.5 are expressed or induced in hAECs cultured in media supplemented with ascorbic acid 2-phosphate for 14 days. The immunohistochemical analysis of alpha-actinin expression showed a staining pattern very similar to the one reported for hESC-derived cardiomyocytes.

Differentiation of hAECs to another mesodermal lineages was reported by Ilancheran et al. [42], who showed that native hAECs can differentiate into cells with a phenotype and marker characteristic of mesodermal-derived myocytes, osteocytes, and adipocytes.

3.6 Preclinical Studies of Amnion-Derived Cells Applications

There are a limited number of studies showing results of preclinical investigations using amnion-derived cells [91]. New research focusing on alternative therapeutic applications is currently in progress.

Some reports suggested the beneficial effects of primary hECs in lung fibrosis when they were transplanted into a mouse model. After the transplantation, these cells expressed surfactant proteins and displayed lamellar bodies indicating their differentiation into type II pneumocytes in vivo. hECS transplantation reduced collagen deposition, induced its degradation, and overall reduced fibrosis in the injured lungs [92]. Regarding liver fibrosis, hECs transplantation also showed a significant reduction in the number of hepatic cells producing collagen [93].

Cell therapy using hAECs was assessed for the treatment of pancreatic diseases. In rat models of insulin-dependent diabetes mellitus, transplanted hAECs were able to normalize blood glucose level, since they were able to differentiate into pancreatic β-cells in vivo [90]. On the other hand, and for the treatment of muscle diseases, when hAMSCs were transplanted into a mouse model of Duchenne muscular dystrophy, they underwent myogenic differentiation or fusion with host muscle cells [94].

Moreover, the differentiation potential of hAECs into neurons and glial cells was investigated by several groups for the treatment of neurological disorders which affect both the spinal cord and the brain. For example, for the treatment of Parkinson's disease, hAECs transplanted into an immunosuppressed rat model of Parkinson's disease produced dopaminergic and other diffusible molecules with trophic and beneficial activities on dopaminergic neurons [82, 84]. In case of ischemic stroke, hAECs transplanted into ischemic rats resulted in an improvement of behavioral dysfunction and reduction of infarct volume. These beneficial effects probably could be due to the hECs differentiation toward neurogenic lineage in vivo and to the paracrine actions of the neurotrophic factors secreted by these amnion epithelial cells [95]. Moreover, hAECs have been investigated to treat spinal cord injury. When these cells were transplanted into a monkey or rat models, hAECs prevented degeneration of axotomized neurons and exerted neurotrophic effects, in part due to the release of neurotrophic factors by hAECs [96].

3.7 Clinical Application of Human Amniotic Membrane as Scaffold

HAM has been reported for the first time as a biological dressing to heal skin wounds a century ago [97]. Davis was the first to report the use of fetal membranes as surgical materials in skin transplantations. Later, other surgery applications for HAM have been reported, such as its use as a biological dressing for skin wound treatment, chronic leg ulcers, and burn injuries. Since the 1940s, the use of de-epithelialized HAM has been well documented in ophthalmology for the treatment of Stevens-Johnson syndrome, cicatricial pemphigoid, acute thermal and alkali burns, pterygium surgery, and limbal stem cell transplantation among others [98–102]. HAMs have also been used as biologic dressings for plastic surgery, dermatology, and gynecology procedures [103–107]. In management of open wounds, HAM provides a clean and closed wound in the shortest time possible; it avoids fluid, nutrient, and heat loss; prevents wound infection and pain; and reduces mobility. The amnion adheres firmly

to an exposed surface. Moreover, HAM can provide a healthy new substrate suitable for reepithelization and epithelial healing [47]. These properties enable surgeons to apply the graft on various tissue surfaces without need for suturing or application of secondary dressings. Immediately after grafting, the process of biodegradation begins and the membrane self-dissolves over a period of time from days to 3–4 weeks depending on the characteristics of the wound, the presence or absence of coexisting pathogens, the polarization of the applied graft, and the type of graft applied.

Importantly, full-term placentas are evaluated after the birth of the baby and are discarded at the hospital as medical waste. Therefore, HAMs are inexpensive and easily obtained with an availability that is virtually limitless, negating the need for mass tissue banking [45, 47, 57, 58]. The HAM possesses clinical considerable advantages to make it potentially attractive as a biomaterial. It is antimicrobial, antifibrosis, antiangiogenic, and antitumorigenic and has acceptable mechanical properties. It also reduces pain and inflammation, inhibits scarring, enhances wound healing and epithelialization, has analgesic properties, acts as an anatomical and vapor barrier, and modulates angiogenesis, all important requirements for tissue engineering [45]. Several growth factors, such as TGF-β, βFGF, EGF, TGF-α, keratinocyte growth factor, and hepatocyte growth factor, produced from amniotic membrane, are involved in some of these processes [4]. All these characteristics are not shared by other natural or synthetic polymers, highlighting the clinical advantages of HAM as a scaffold compared to other biocompatible products. Also, amnion shows little or no immunogenicity, and the immune response against the graft, if there is, is slight and ineffective, so it does not represent transplantation risks. On the contrary, chorion shows high immunogenicity, and for this reason, it is not used as biomaterial for transplantation purposes. It is important to note that HAM has been approved as a medical material by the Food and Drug Administration [67].

Nowadays, HAMs are used as allograft in general surgery for reconstructions, as an autograft in neonatal reconstruction surgery and as a scaffold in tissue engineering research [48]. The low cost of amnion graft preparation and the very good clinical results in multipurpose applications have made it a viable alternative to other natural (i.e., preserved human skin) and synthetic wound dressings [108]. Moreover, for all the clinical applications, HAM is usually preserved and stored using different methods such as cryopreservation, irradiation, air drying, lyophilization, or glycerol preservation.

3.8 Summary

The HAM, an abundant, inexpensive, and readily obtained tissue that is discarded postpartum, represents a valuable cell and tissue source of great interest in the field of cell therapy and regenerative medicine. Both cell populations isolated from HAM, hAMSCs and hAECs, show an antigen expression profile characteristic of culture-expanded MSCs and differentiation potential into ectodermal, mesodermal, and endodermal lineages. hAMSCs, hAECs, and HAM fragments were used in

preclinical studies to treat pancreatic, muscle, vascular, lung, and liver diseases. However, more studies are needed to demonstrate the potential effects of either amnion-derived cells or amnion allografts in animal models of different diseases in the hope of increasing their future clinical applications.

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References

1. Mrugala D, Dossat N, Ringe J, Delorme B, Coffy A, Bony C, Charbord P, Häupl T, Daures J-P, Noël D, Jorgensen C (2009) Gene expression profile of multipotent mesenchymal stromal cells: identification of pathways common to TGFβ3/BMP2-induced chondrogenesis. Cloning Stem Cells 11:61–76
2. Kastrinaki M-C, Andreakou I, Charbord P, Papadaki HA (2008) Isolation of human bone marrow mesenchymal stem cells using different membrane markers: comparison of colony/cloning efficiency, differentiation potential, and molecular profile. Tissue Eng Part C Methods 14:333–339
3. Dominici M, Le Blanc K, Mueller I, Slaper-Cortenbach I, Marini F, Krause D, Deans R, Keating A, Prockop DJ, Horwitz E (2006) Minimal criteria for defining multipotent mesenchymal stromal cells. The International Society for Cellular Therapy position statement. Cytotherapy 8:315–317
4. Yu SJ, Soncini M, Kaneko Y, Hess DC, Parolini O, Borlongan CV (2009) Amnion: a potent graft source for cell therapy in stroke. Cell Transplant 18:111–118
5. Barlow S, Brooke G, Chatterjee K, Price G, Pelekanos R, Rossetti T, Doody M, Venter D, Pain S, Gilshenan K, Atkinson K (2008) Comparison of human placenta- and bone marrow-derived multipotent mesenchymal stem cells. Stem Cells Dev 17:1095–1108
6. Minguell JJ, Conget P, Erices A (2000) Biology and clinical utilization of mesenchymal progenitor cells. Braz J Med Biol Res 33:881–887
7. Caplan AI (1991) Mesenchymal stem cells. J Orthop Res 9:641–650
8. Pasquinelli G, Tazzari P, Ricci F, Vaselli C, Buzzi M, Conte R (2007) Ultrastructural characteristics of human mesenchymal stromal (stem) cells derived from bone marrow and term placenta. Ultrastruct Pathol 31:23–31
9. Yoo JU, Barthel TS, Nishimura K, Solchaga L, Caplan AI, Goldberg VM, Johnstone B (1998) The chondrogenic potential of human bone-marrow-derived mesenchymal progenitor cells. J Bone Joint Surg Am 80:1745–1757
10. Alsalameh S, Amin R, Gemba T, Lotz M (2004) Identification of mesenchymal progenitor cells in normal and osteoarthritic human articular cartilage. Arthritis Rheum 50:1522–1532
11. Fickert S, Fiedler J, Brenner RE (2003) Identification, quantification and isolation of mesenchymal progenitor cells from osteoarthritic synovium by fluorescence automated cell sorting. Osteoarthritis Cartilage 11:790–800
12. De Bari C, Dell'Acio F, Tylzanowski P, Luyten FP (2001) Multipotent mesenchymal stem cells from adult human synovial membrane. Arthritis Rheum 44:1928–1942
13. Dounchis JS, Goomer RS, Harwood FL, Khatod M, Coutts RD, Amiel D (1997) Chondrogenic phenotype of perichondrium-derived chondroprogenitor cells is influenced by transforming growth factor-beta 1. J Orthop Res 15:803–807

14. Nakahara H, Bruder SP, Haynesworth SE, Holecek JJ, Baber MA, Goldberg VM, Caplan AI (1990) Bone and cartilage formation in diffusion chambers by subcultured cells derived from the periosteum. Bone 11:181–188
15. Young HE, Steele TA, Bray RA, Hudson J, Floyd JA, Hawkins K, Thomas K, Austin T, Edwards C, Cuzzourt J, Duenzl M, Lucas PA, Black AC Jr (2001) Human reserve pluripotent mesenchymal stem cells are present in the connective tissues of skeletal muscle and dermis derived from fetal, adult, and geriatric donors. Anat Rec 264:51–62
16. Zuk PA, Zhu M, Ashjian P, De Ugarte DA, Huang JI, Mizuno H, Alfonso ZC, Fraser JK, Benhaim P, Hedrick MH (2002) Human adipose tissue is a source of multipotent stem cells. Mol Biol Cell 13:4279–4295
17. Zuk PA, Zhu M, Mizuno H, Huang J, Futrell JW, Katz AJ, Benhaim P, Lorenz HP, Hedrik MH (2001) Multilineage cells from human adipose tissue: implications for cell-based therapies. Tissue Eng 7:211–228
18. Villaron EM, Almeida J, Lopez-Holgado N, Alcoceba M, Sánchez-Abarca LI, Sanchez-Guijo FM, Alberca M, Pérez-Simon JA, San Miguel JF, Del Cañizo MC (2004) Mesenchymal stem cells are present in peripheral blood and can engraft after allogenic haematopoietic stem cell transplantation. Haematologica 89:1421–1427
19. Kuznetsov SA, Mankani MH, Gronthos S, Satomura K, Bianco P, Robey PG (2001) Circulating skeletal stem cells. J Cell Biol 153:1133–1140
20. Zvaifler NJ, Marinova-Mutafchieva L, Adams G, Edwards CJ, Moss J, Burger JA, Maini RN (2000) Mesenchymal precursor cells in the blood of normal individuals. Arthritis Res 2:477–488
21. Le Blanc K, Götherström C, Ringdén O, Hassan M, McMahon R, Horwitz E, Anneren G, Axelsson O, Nunn J, Ewald U, Nordén Lindeberg S, Jansson M, Dalton A, Aström E, Westgren M (2005) Fetal mesenchymal stem-cell engraftment in bone after in utero transplantation in a patient with severe osteogenesis imperfecta. Transplantation 79:1607–1614
22. In't Anker PS, Scherjon SA, Kleijburg-van der Keur C, de Groot-Swings GM, Claas FH, Fibbe WE, Kanhai HH (2004) Isolation of mesenchymal stem cells of fetal or maternal origin from human placenta. Stem Cells 22:1338–1345
23. Steigman SA, Fauza DO (2007) Isolation of mesenchymal stem cells from amniotic fluid and placenta. Curr Protoc Stem Cell Biol Chapter 1:Unit 1E.2
24. Matikainen T, Laine J (2005) Placenta-an alternative source of stem cells. Toxicol Appl Pharmacol 207(2 Suppl):544–549
25. Fauza D (2004) Amniotic fluid and placental stem cells. Best Pract Res Clin Obstet Gynaecol 18:877–891
26. Samuel GN, Kerridge IH, O'Brien TA (2008) Umbilical cord blood banking: public good or private benefit? Med J Aust 188:533–535
27. Baksh D, Yao R, Tuan RS (2007) Comparison of proliferative and multilineage differentiation potential of human mesenchymal stem cells derived from umbilical cord and bone marrow. Stem Cells 25:1384–1392
28. McGuckin CP, Forraz N, Baradez MO, Navran S, Zhao J, Urban R, Tilton R, Denner L (2005) Production of stem cells with embryonic characteristics from human umbilical cord blood. Cell Prolif 38:245–255
29. Mareschi K, Biasin E, Piacibello W, Aglietta M, Madon E, Fagioli F (2001) Isolation of human mesenchymal stem cells: bone marrow versus umbilical cord blood. Haematologica 86:1099–1100
30. You Q, Cai L, Zheng J, Tong X, Zhang D, Zhang Y (2008) Isolation of human mesenchymal stem cells from third-trimester amniotic fluid. Int J Gynaecol Obstet 103:149–152
31. Díaz-Prado S, Muíños-López E, Hermida-Gómez T, Rendal-Vázquez ME, Fuentes-Boquete I, de Toro FJ, Blanco FJ (2011) Isolation and characterization of mesenchymal stem cells from human amniotic membrane. Tissue Eng Part C Methods 17:49–59
32. Díaz-Prado S, Muíños-López E, Hermida-Gómez T, Rendal-Vázquez ME, Fuentes-Boquete I, de Toro FJ, Blanco FJ (2010) Multilineage differentiation potential of cells isolated from the human amniotic membrane. J Cell Biochem 111:846–857

33. Alviano F, Fossati V, Marchionni C, Arpinati M, Bonsi L, Franchina M, Lanzoni G, Cantoni S, Cavallini C, Bianchi F, Tazzari PL, Pasquinelli G, Foroni L, Ventura C, Grossi A, Bagnara GP (2007) Term amniotic membrane is a high throughput source for multipotent mesenchymal stem cells with ability to differentiate into endothelial cells in vitro. BMC Dev Biol 7:11
34. Bianco P, Robey PG (2001) Stem cells in tissue engineering. Nature 414:118–121
35. Jung DI, Ha J, Kang BT, Kim JW, Quan FS, Lee JH, Woo EJ, Park HM (2009) A comparison of autologous and allogenic bone marrow-derived mesenchymal stem cell transplantation in canine spinal cord injury. J Neurol Sci 285:67–77
36. Koga H, Shimaya M, Muneta T, Nimura A, Morito T, Hayashi M, Suzuki S, Ju YJ, Mochizuki T, Sekiya I (2008) Local adherent technique for transplanting mesenchymal stem cells as a potential treatment of cartilage defect. Arthritis Res Ther 10:R84
37. Hombach-Klonisch S, Panigrahi S, Rashedi I, Seifert A, Alberti E, Pocar P, Kurpisz M, Schulze-Osthoff K, Mackiewicz A, Los M (2008) Adult stem cells and their trans-differentiation potential–perspectives and therapeutic applications. J Mol Med 86:1301–1314
38. Pittenger MF (2008) Mesenchymal stem cells from adult bone marrow. Methods Mol Biol 449:27–44
39. Tsai MS, Hwang SM, Chen KD, Lee YS, Hsu LW, Chang YJ, Wang CN, Peng HH, Chang YL, Chao AS, Chang SD, Lee KD, Wang TH, Wang HS, Soong YK (2007) Functional network analysis on the transcriptomes of mesenchymal stem cells derived from amniotic fluid, amniotic membrane, cord blood, and bone marrow. Stem Cells 25:2511–2523
40. Wei JP, Nawata M, Wakitani S, Kametani K, Ota M, Toda A, Konishi I, Ebara S, Nikaido T (2009) Human amniotic mesenchymal cells differentiate into chondrocytes. Cloning Stem Cells 11:19–25
41. Ilancheran S, Moodley Y, Manuelpillai U (2009) Human fetal membranes: a source of stem cells for tissue regeneration and repair? Placenta 30:2–10
42. Ilancheran S, Michalska A, Peh G, Wallace EM, Pera M, Manuelpillai U (2007) Stem cells derived from human fetal membranes display multilineage differentiation potential. Biol Reprod 77:577–588
43. Insausti CL, Blanquer M, Bleda P, Iniesta P, Majado MJ, Castellanos G, Moraleda JM (2010) The amniotic membrane as a source of stem cells. Histol Histopathol 25:91–98
44. Abdulrazzak H, Moschidou D, Jones G, Guillot PV (2010) Biological characteristics of stem cells from foetal, cord blood and extraembryonic tissues. J R Soc Interface 7:S689–S706
45. Niknejad H, Peirovi H, Jorjani M, Ahmadiani A, Ghanavi J, Seifalian AM (2008) Properties of the amniotic membrane for potential use in tissue engineering. Eur Cell Mater 15:88–99
46. Jin CZ, Park SR, Choi BH, Lee KY, Kang CK, Min BH (2007) Human amniotic membrane as a delivery matrix for articular cartilage repair. Tissue Eng 13:693–702
47. Wilshaw SP, Kearney JN, Fisher J, Ingham E (2006) Production of an acellular amniotic membrane matrix for use in tissue engineering. Tissue Eng 12:2117–2129
48. Dovebra MP, Pereira PNG, Deprest J, Zwijsen A (2010) On the origin of amniotic stem cells: of mice and men. Int J Dev Biol 54:761–777
49. Parolini O, Soncini M, Evangelista M, Schmidt D (2009) Amniotic membrane and amniotic fluid-derived cells: potential tools for regenerative medicine? Regen Med 4:275–291
50. Tamagawa T, Ishiwata I, Ishikawa H, Nakamura Y (2008) Induced in vitro differentiation of neural-like cells from human amnion-derived fibroblast-like cells. Hum Cell 21:38–45
51. Bilic G, Zeisberger SM, Mallik AS, Zimmermann R, Zisch AH (2008) Comparative characterization of cultured human term amnion epithelial and mesenchymal stromal cells for application in cell therapy. Cell Transplant 17:955–968
52. Sakuragawa N, Yoshikawa H, Sasaki M (1992) Amniotic tissue transplantation: clinical and biochemical evaluations for some lysosomal storage diseases. Brain Dev 14:7–11
53. Scaggiante B, Pineschi A, Sustersich M, Andolina M, Agosti E, Romeo D (1987) Successful therapy of Niemann–Pick disease by implantation of human amniotic membrane. Transplantation 44:59–61
54. Akle CA, Adinolfi M, Welsh KI, Leibowitz S, McColl I (1981) Immunogenicity of human amniotic epithelial cells after transplantation into volunteers. Lancet 2:1003–1005

55. Kim SS, Song CK, Shon SK, Lee KY, Kim CH, Lee MJ, Wang L (2009) Effects of human amniotic membrane grafts combined with marrow mesenchymal stem cells on healing of full-thickness skin defects in rabbits. Cell Tissue Res 336:59–66
56. Chang Y-J, Hwang S-M, Tseng C-P, Cheng F-C, Huang S-H, Hsu L-F, Hsu L-W, Tsai M-S (2010) Isolation of mesenchymal stem cells with neurogenic potential from the mesoderm of the amniotic membrane. Cells Tissues Organs 192:93–105
57. Hennerbichler S, Reichl B, Pleiner D, Gabriel C, Eibl J, Redl H (2007) The influence of various storage conditions on cell viability in amniotic membrane. Cell Tissue Bank 8:1–8
58. Toda A, Okabe M, Yoshida T, Nikaido T (2007) The potential of amniotic membrane/amnion-derived cells for regeneration of various tissues. J Pharmacol Sci 105:215–228
59. Soncini M, Vertua E, Gibelli L, Zorzi F, Denegri M, Albertini A, Wengler GS, Parolini O (2007) Isolation and characterization of mesenchymal cells from human fetal membranes. J Tissue Eng Regen Med 1:296–305
60. Portmann-Lanz CB, Schoeberlein A, Huber A, Sager R, Malek A, Holzgreve W, Surbek DV (2006) Placental mesenchymal stem cells as potential autologous graft for pre- and perinatal neuroregeneration. Am J Obstet Gynecol 194:664–673
61. Wolbank S, Peterbauer A, Fahrner M, Hennerbichler S, van Griensven M, Stadler G, Redl H, Gabriel C (2007) Dose-dependent immunomodulatory effect of human stem cells from amniotic membrane: a comparison with human mesenchymal stem cells from adipose tissue. Tissue Eng 13:1173–1183
62. Hao Y, Ma DH, Hwang DG, Kim WS, Zhang F (2000) Identification of antiangiogenic and antiinflammatory proteins in human amniotic membrane. Cornea 19:348–352
63. Bailo M, Soncini M, Vertua E, Signoroni PB, Sanzone S, Lombardi G, Arienti D, Calamani F, Zatti D, Paul P, Albertini A, Zorzi F, Cavagnini A, Candotti F, Wengler GS, Parolini O (2004) Engraftment potential of human amnion and chorion cells derived from term placenta. Transplantation 78:1439–1448
64. Dazzi F, Marelli-Berg F (2008) Mesenchymal stem cells for graft-versus-host disease: close encounters with T cells. Eur J Immunol 38:1479–1482
65. Parolini O, Alviano F, Bagnara GP, Bilic G, Bühring HJ, Evangelista M, Hennerbichler S, Liu B, Magatti M, Mao N, Miki T, Marongiu F, Nakajima H, Nikaido T, Portmann-Lanz CB, Sankar V, Soncini M, Stadler G, Surbek D, Takahashi TA, Redl H, Sakuragawa N, Wolbank S, Zeisberger S, Zisch A, Strom SC (2008) Concise review: isolation and characterization of cells from human term placenta: outcome of the first international workshop on placenta derived stem cells. Stem Cells 26:300–311
66. Miki T, Marongiu F, Ellis E, Strom S (2007) Isolation of amniotic epithelial stem cells. Curr Protoc Stem Cell Biol 3:1E.3.1–1E.3.9
67. Miki T, Strom SC (2006) Amnion-derived pluripotent/multipotent stem cells. Stem Cell Rev 2:133–142
68. Mihu CM, Rus Ciuc D, Sorit u O, Su man S, Mihu D (2009) Isolation and characterization of mesenchymal stem cells from the amniotic membrane. Rom J Morphol Embryol 50: 73–77
69. Kobayashi M, Yakuwa T, Sasaki K, Sato K, Kikuchi A, Kamo I, Yokoyama Y, Sakuragawa N (2008) Multilineage potential of side population cells from human amnion mesenchymal layer. Cell Transplant 17:291–301
70. Marcus AJ, Woodbury D (2008) Fetal stem cells from extra-embryonic tissues: do not discard. J Cell Mol Med 12:730–742
71. Pappa KI, Anagnou NP (2009) Novel sources of fetal stem cells: where do they fit on the developmental continuum? Regen Med 4:423–433
72. Miki T, Mitamura K, Ross MA, Stolz DB, Strom SC (2007) Identification of stem cell marker-positive cells by immunofluorescence in term human amnion. J Reprod Immunol 75:91–96
73. Miki T, Lehmann T, Cai H, Stolz DB, Strom SC (2005) Stem cell characteristics of amniotic epithelial cells. Stem Cells 23:1549–1559

74. Tamagawa T, Oi S, Ishiwata I, Ishikawa H, Nakamura Y (2007) Differentiation of mesenchymal cells derived from human amniotic membranes into hepatocyte-like cells in vitro. Hum Cell 20:77–84
75. Sakuragawa N, Kakinuma K, Kikuchi A, Okano H, Uchida S, Kamo I, Kobayashi M, Yokoyama Y (2004) Human amnion mesenchyme cells express phenotypes of neuroglial progenitor cells. J Neurosci Res 78:208–214. Erratum in: J Neurosci Res 2005; 79:725
76. Kim J, Kang HM, Kim H, Kim MR, Kwon HC, Gye MC, Kang SG, Yang HS, You J (2007) Ex vivo characteristics of human amniotic membrane-derived stem cells. Cloning Stem Cells 9:581–594
77. Zhao P, Ise H, Hongo M, Ota M, Konishi I, Nikaido T (2005) Human amniotic mesenchymal cells have some characteristics of cardiomyocytes. Transplantation 79:528–535
78. Tanaka M, Chen Z, Bartunkova S, Yamasaki N, Izumo S (1999) The cardiac homeobox gene Csx/Nkx2.5 lies genetically upstream of multiple genes essential for heart development. Development 126:1269–1280
79. Li W, He H, Chen YT, Hayashida Y, Tseng SC (2008) Reversal of myofibroblasts by amniotic membrane stromal extract. J Cell Physiol 215:657–664
80. Miki T, Marongiu F, Ellis EC, Dorko K, Mitamura K, Ranade A, Gramignoli R, Davila J, Strom SC (2009) Production of hepatocyte-like cells from human amnion. Methods Mol Biol 481:155–168
81. Tamagawa T, Ishiwata I, Saito S (2004) Establishment and characterization of a pluripotent stem cell line derived from human amniotic membranes and initiation of germ layers in vitro. Hum Cell 17:125–130
82. Kakishita K, Elwan MA, Nakao N, Itakura T, Sakuragawa N (2000) Human amniotic epithelial cells produce dopamine and survive after implantation into the striatum of a rat model of Parkinson's disease: a potential source of donor for transplantation therapy. Exp Neurol 165:27–34
83. Elwan MA, Sakuragawa N (1997) Evidence for synthesis and release of catecholamines by human amniotic epithelial cells. Neuroreport 8:3435–3438
84. Kakishita K, Nakao N, Sakuragawa N, Itakura T (2003) Implantation of human amniotic epithelial cells prevents the degeneration of nigral dopamine neurons in rats with 6-hydroxydopamine lesions. Brain Res 980:48–56
85. Uchida S, Inanaga Y, Kobayashi M, Hurukawa S, Araie M, Sakuragawa N (2000) Neurotrophic function of conditioned medium from human amniotic epithelial cells. J Neurosci Res 62:585–590
86. Kosuga M, Takahashi S, Sasaki K, Enosawa S, Li XK, Okuyama S, Fujino M, Suzuki S, Yamada M, Matsuo N, Sakuragawa N, Okuyama T (2000) Phenotype correction in murine mucopolysaccharidosis type VII by transplantation of human amniotic epithelial cells after adenovirus-mediated gene transfer. Cell Transplant 9:687–692
87. Sakuragawa N, Enosawa S, Ishii T, Thangavel R, Tashiro T, Okuyama T, Suzuki S (2000) Human amniotic epithelial cells are promising transgene carriers for allogenic cell transplantation into liver. J Hum Genet 45:171–176
88. Takashima S, Ise H, Zhao P, Akaike T, Nikaido T (2004) Human amniotic epithelial cells possess hepatocyte-like characteristics and functions. Cell Struct Funct 29:73–84
89. Davila JC, Cezar GG, Thiede M, Strom S, Miki T, Trosko J (2004) Use and application of stem cells in toxicology. Toxicol Sci 79:214–223
90. Wei JP, Zhang TS, Kawa S, Aizawa T, Ota M, Akaike T, Kato K, Konishi I, Nikaido T (2003) Human amnion-isolated cells normalize blood glucose in streptozotocin-induced diabetic mice. Cell Transplant 12:545–552
91. Parolini O, Caruso M (2011) Review: preclinical studies on placenta-derived cells and amniotic membrane: an update. Placenta 32(Suppl 2):S186–S195
92. Moodley Y, Ilancheran S, Samuel C, Vaghjiani V, Atienza D, Williams ED, Jenkin G, Wallace E, Trounson A, Manuelpillai U (2010) Human amnion epithelial cell transplantation abrogates lung fibrosis and augments repair. Am J Respir Crit Care Med 182:643–651

93. Manuelpillai U, Tchongue J, Lourensz D, Vaghjiani V, Samuel CS, Liu A, Williams ED, Sievert W (2010) Transplantation of human amnion epithelial cells reduces hepatic fibrosis in immunocompetent CCl4-treated mice. Cell Transplant 19:1157–1168
94. Kawamichi Y, Cui CH, Toyoda M, Makino H, Horie A, Takahashi Y, Matsumoto K, Saito H, Ohta H, Saito K, Umezawa A (2010) Cells of extraembryonic mesodermal origin confer human dystrophin in the mdx model of Duchenne muscular dystrophy. J Cell Physiol 223:695–702
95. Liu T, Wu J, Huang Q, Hou Y, Jiang Z, Zang S, Guo L (2008) Human amniotic epithelial cells ameliorate behavioural dysfunction and reduce infarct size in the rat middle cerebral artery occlusion model. Shock 29:603–611
96. Wu ZY, Hui GZ, Lu Y, Wu X, Guo LH (2006) Transplantation of human amniotic epithelial cells improves hindlimb function in rats with spinal cord injury. Chin Med J (Engl) 119:2101–2107
97. Davis JW (1910) Skin transplantation with a review of 550 cases at the John Hopkins Hospital. Johns Hopkins Med J 15:307
98. Tsai RJ, Tsai RY (2010) Ex vivo expansion of corneal stem cells on amniotic membrane and their outcome. Eye Contact Lens 36:305–309
99. Sangwan VS, Burman S, Tejwani S, Mahesh SP, Murthy R (2007) Amniotic membrane transplantation: a review of current indications in the management of ophthalmic disorders. Indian J Ophthalmol 55:251–260
100. Gomes JA, Romano A, Santos MS, Dua HS (2005) Amniotic membrane use in ophthalmology. Curr Opin Ophthalmol 16:233–240
101. Dua HS, Gomes JA, King AJ, Maharajan VS (2004) The amniotic membrane in ophthalmology. Surv Ophthalmol 49:51–77
102. Grueterich M, Espana EM, Tseng SC (2003) Ex vivo expansion of limbal epithelial stem cells: amniotic membrane serving as a stem cell niche. Surv Ophthalmol 48:631–646
103. Tejwani S, Kolari RS, Sangwan VS, Rao GN (2007) Role of amniotic membrane graft for ocular chemical and thermal injuries. Cornea 26:21–26
104. Rinastiti M, Harijadi, Santoso AL, Sosroseno W (2006) Histological evaluation of rabbit gingival wound healing transplanted with human amniotic membrane. Int J Oral Maxillofac Surg 35:247–251
105. Santos MS, Gomes JAP, Hofling-Lima AL, Rizzo LV, Romano AC, Belfort R Jr (2005) Survival analysis of conjunctival limbal grafts and amniotic membrane transplantation in eyes with total limbal stem cell deficiency. Am J Ophthalmol 140:223–230
106. Meller D, Pires RT, Mack RJ, Figueiredo F, Heiligenhaus A, Park WC, Prabhasawat P, John T, McLeod SD, Steuhl KP, Tseng SC (2000) Amniotic membrane transplantation for acute chemical or thermal burns. Ophthalmology 107:980–989
107. Morton KE, Dewhurst CJ (1986) Human amnion in the treatment of vaginal malformations. Br J Obstet Gynaecol 93:50–54
108. Díaz-Prado S, Rendal-Vázquez ME, Muíños López E, Hermida-Gómez T, Rodríguez-Cabarcos M, Fuentes-Boquete I, de Toro FJ, Blanco FJ (2010) Potential use of the human amniotic membrane as a scaffold in human articular cartilage repair. Cell Tissue Bank 11:183–195