

Amnion-Derived Pluripotent/Multipotent Stem Cells

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Abstract

Amniotic epithelium is derived from the epiblast by approx 8 d after fertilization. Other parts of the placenta are derived from extraembryonic tissue. In addition to this developmental difference, amniotic epithelial (AE) cells are known to have unique characteristics, such as low level expression of major histocompatibility complex antigens, and a less restricted differentiation potential. The differentiation of the AE cells to the neural lineage is well documented. Recently, we reported that AE cells from term placenta express several stem cell surface markers that are commonly found on pluripotent stem cells such as embryonic stem cells, and that in culture, AE cells differentiate into cell types from all three germ layers. In this review, we describe the unique characteristics of the AE stem cells and summarize previous work concerning the stem cell nature of cells from amnion.

Index Entries: Amnion; pluripotent; stem cell; Oct-4; placenta.

Introduction

There is an increasing evidence that human placenta contains pluripotent and/or multipotent stem cells. Specific types of stem cells, such as trophoblastic, hematopoietic, and mesenchymal stem cells, will be discussed in other articles in this issue. Here, we will review stem cells derived from amnion of human placenta, specifically amniotic epithelial (AE) cells. Unlike other parts of placenta, amniotic epithelium is derived from pluripotent epiblast at day 8, far before gastrulation (days 15-17), which is a "tipping point" at which cell fate is specified. The other components of placenta such as chorion differentiate from extraembryonic trophoblast. Because the amnion differentiates from the epiblast at a time when it retains pluripotency, it is reasonable to speculate that AE cells may have escaped the specification that accompanies gastrulation and that AE cells may preserve some or all of the characteristics of the epiblast such as pluripotency. Previously, we reported that some AE cells express cell surface antigens characteristic of pluripotent

embryonic stem (ES) cells such as SSEA-3, SSEA-4, TRA 1-60, and TRA 1-81. In addition, AE cells express molecular markers of pluripotency, *Oct-4* and *Nanog*, and differentiation into all three germ layers was demonstrated in vitro. These data suggest that, like ES cells, AE cells may be a useful stem cell for cell transplantation and regenerative medicine.

Placenta is discarded after a live birth. Therefore, the use of placenta as a stem cell source would obviate most, if not all, ethical, religious, or political concerns. If it were definitively demonstrated that each of us arrived at birth with "spare parts" that could be derived from placenta, these stem cells would be a unique recycling biotechnology. In theory, placental stem cells could be isolated from every newborn baby. In the United States, more than 4 million live births occur per year (e.g., 4,115,590 births in 2004). Once technology is established to develop pluripotent stem cell lines from each placenta, it would be easy to complete banks with all major histocompatibility complex (MHC) immunotypes for further clinical

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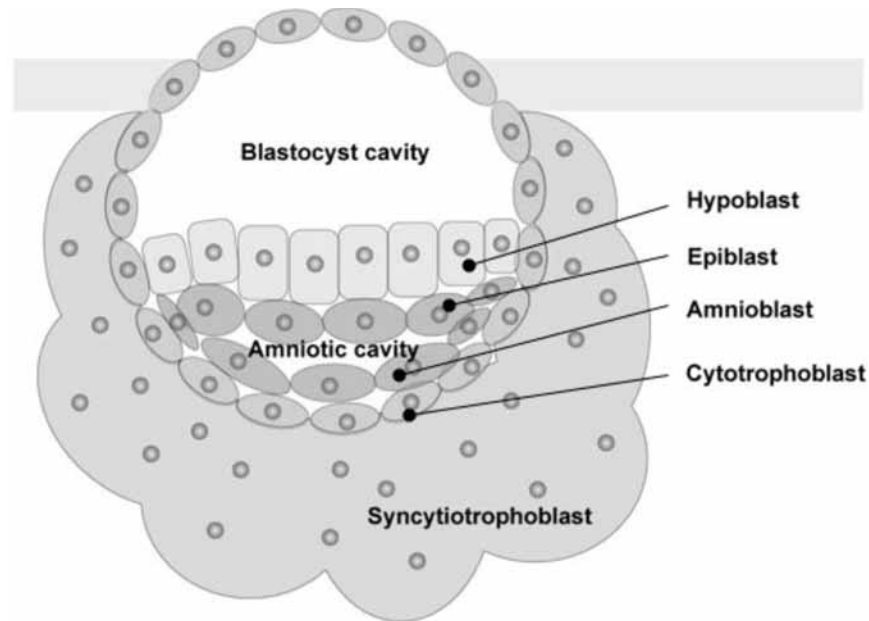


Fig. 1. Illustration of embryo at day 8. At 8 d after fertilization, inner cell mass differentiates into epiblast and hypoblast. An amniotic cavity appears in the middle of the epiblast. The epiblasts adjacent to the cavity, termed amnioblasts, eventually form the amniotic epithelial layer.

applications. These potential advantages lead us to investigate the possible stem cell nature of AE cells.

Development of the Amnion

About the 8 d after fertilization, the human blastocyst is partially embedded in the endometrial stroma. The outer cells (trophoblast) of the blastocyst differentiate into two layers and migrate into the stroma. The inner cell mass or embryoblast also differentiates into two layers, the hypoblast and epiblast. Epiblast is the source of all three germ layers and eventually forms the developing embryo. At the same time, a small cavity (amniotic cavity) appears within the epiblast. Epiblast cells adjacent to the cytotrophoblast are called amnioblasts (Fig. 1). Amnioblasts bordering the amniotic cavity differentiate to amniotic epithelia and the cavity enlarges.

Concomitantly, a layer of extraembryonic mesenchyme around the AE cell layer develops in to the amniotic connective tissue. At 31–35 d of gestation, the amnion is made up of three layers. The innermost layer toward the amniotic cavity is the amniotic epithelium. Outside of this is a layer of connective tissue cells. The connective tissue consists of very fine argyrophil fibers at early stages, which are replaced with several types of collagens produced by fibroblasts. The outermost layer separating the amnion from embryonic coelom consists of a solid, single layer of strongly flattened cells with marked cell borders. This mesothelium-like coating disappears when the amnion and chorion start to unite (at the end of 3 mo). At full term, the placenta is a discoid with a diameter of 15–20 cm, is approx 3 cm thick, and weighs about 500–600 g. The fetal surface of the placenta is covered entirely by the amnion, which has a surface area is approx 700–1200 cm².

It is a common misconception that the amnion is derived from the extraembryonic endoderm or extraembryonic trophoderm such as the yolk sac or chorion. However, the

amnion is derived from the epiblasts, the pluripotent cells, which eventually give rise to all of the cell types of the embryo.

Isolation and Characterization of Amniotic Epithelial Cells

Amnion is easily stripped from the underlying chorion and deciduas at the coarse intermediate layer of connective tissue (Fig. 2; arrow). The thin, nearly transparent amnion membrane, which contains AE cells and mesenchymal fibroblasts (AMF), is obtained after several washing steps to remove blood. For isolation of AE cells, amniotic membrane is trypsinized, which releases the AE cells from the supporting connective tissue and the AMF. In our laboratory, membrane is incubated with 0.05% trypsin with 0.53 mM EDTA for 10 min to dissociate debris from the surface of the membrane, followed by two sequential 40-min trypsinizations. The “after” photo (Fig. 2) shows that most of the epithelial cells are removed from the amniotic membrane by trypsinization and that the connective tissue (AMF) layer remains intact. With this simple protocol, 200–300 million AE cells can be isolated from one placenta.

The number of AE cells increases in the course of development (1). The rapid surface growth of the amniotic epithelium is caused by mitotic division. The mitosis rate peaks at around day 20 and decreases thereafter. By 180 d of development, mitosis is rarely observed (2). However, isolated human AE cells showed robust proliferation in culture with numerous mitotic events (3). Despite a lack of robust growth in vivo, these data indicate that, some or all AE cells preserve their proliferation potential. The proliferation of AE cells in vivo may be repressed during late gestation in response to interleukin (IL)-6 superfamily ligands (3).

In the presence of epidermal growth factor (EGF) or transforming growth factor- α , AE cells proliferate robustly and form a confluent monolayer of cobblestone-shaped epithelial cells.

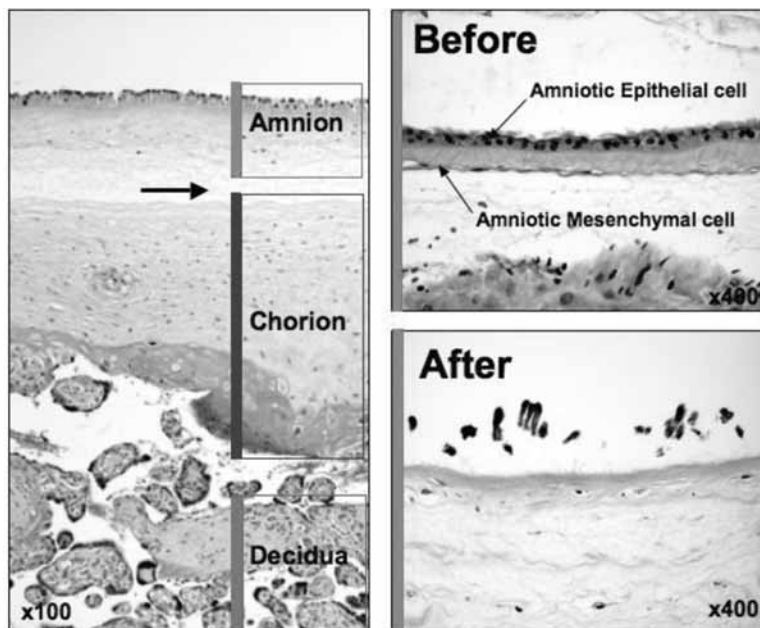


Fig. 2. Histology of amnion. Normal human placenta sample as sectioned and stained with hematoxylin and eosin. In the left panel, the three major components, amnion, chorion, and decidua, were shown. The arrow indicates the coarse intermediate layer of connective tissue. The right panel shows photos of membrane “before” and “after” trypsinization. Only amniotic epithelial cells were dissociated by trypsinization. Although not shown, subsequent collagenase digestion would release the amniotic mesenchymal cells from the connective tissue.

Proliferation ceases in the absence of EGF and giant palm-shaped multinucleated cells form, which are reminiscent of the description of trophoblast differentiation of ES cells (4). It is known that the amount of EGF in amniotic fluid increases during pregnancy. It has been suggested that a balance of EGF and IL-6 superfamily ligand signaling regulate AE cell proliferation *in vivo*.

Characterization of Amniotic Epithelial Cells

Cytokeratins and vimentin are used as markers to identify cells of different lineages. Vimentin is normally expressed by mesenchymal cells such as fibroblasts or myocytes as well as vascular endothelial cells. Recent reports suggest that vimentin may be a marker of stem/progenitor cells, especially neural stem cells (5,6).

In monolayer cultures of AE cells, virtually 100% of the cells react with antibodies to low-molecular weight cytokeratins, confirming their epithelial nature. Interestingly, although initially vimentin-negative, AE cells become vimentin-positive during cell culture. Vimentin-positive AE cells remain positive for cytokeratins. These data indicate that *in vitro* culture or environmental influences may induce dedifferentiation of AE cells.

Cell Surface Antigens

In culture, AE cells proliferate to form a population of epithelial cells with uniform morphology. However, even morphologically homogenous monolayer cultures may contain cell types with different growth or differentiation potential. Following isolation, AE cells express cell surface antigens known to be expressed on other stem cells (Table 1). In addition to the typical stem cell markers such as SSEA-3, SSEA-4, TRA 1-60, and TRA 1-81, AE cells share many cell surface

antigens with human ES cells. It has to be noted that initially isolated, naive AE cells are not homogeneously positive for all of these antibodies. Some surface markers such as CCR4- and CD117-positive cells are very rare, while others such as CD9, integrin α_6 , and integrin β_1 are expressed on virtually 100% of the cells. These data suggest that naive AE cells are a heterogeneous cell population with respect to cell surface profiling. The profile of cultured AE cells is similar to that of ES cells at a time where they start differentiating within an embryonic body. Undifferentiated ES cells are not a completely homogeneous population (7). A fluorescence-activated cell sorting analysis of a typical ES culture will show that up to 20% of the cells are positive for SSEA1 and 50–80% of the cells will react with antibodies to SSEA-3, SSEA-4, TRA 1-60, or TRA 1-81. As with the AE cultures, even clonally expanded ES cells may contain cells at different stages of differentiation and with a different profile of surface markers. Clearly, more research will be needed to determine if the culture of AE cells can be optimized to maintain their stem cell characteristics and a homogenous profile of surface markers.

ABCG2 Expression and Side Population Cells

One of the cell surface antigens identified on AE cells is ABCG2, a member of the ATP-binding cassette superfamily. Expression of this protein by AE cells suggests that they might have similar properties to the side population cells identified in bone marrow. The ABCG2 gene product is a multidrug resistance transport protein which effluxes, among other things, the Hoechst dye, 33342, from cells (8). In 1996, Goodell et al. (9) reported a method for isolating hematopoietic stem cells in a single step based on the retention of this dye. A small subpopulation of cells termed the side population (SP) cells

Table 1
Summary of Cell Surface Marker Expression

<i>Cell surface antigens</i>	<i>Am S</i>	<i>Em S</i>	<i>Neu S</i>	<i>Mes S</i>	<i>Hem S</i>
ABCG2	+	+	+		+
Angiotensin converting enzyme					+
BMP-Rs				+	
CCR4 (CC chemokine receptor)	±	+			
CD9	+	+	+		
CD24	+	+			
CD34	–				+
CD117 (c-kit)	±			+	
CD133	–	+	+		+
CXCR4 (CXC chemokine receptor)			+		+
E-cad	+	+			–
Endoglin (CD105)				+	
EphB4					+
FABP7			+		
FGF-R4			+		
Frizzled-9 (Wnt ligand binding receptor)			+		
Glut1			+		
Integrin a1				+	
Integrin a6	+	+			
Integrin b1	+	+			
PODXL (podocalyxin-like)		+			+
SSEA-1	–	±			
SSEA-3	+	+			
SSEA-4	+	+			
STRO-1				+	
TRA 1-60	+	+			
TRA 1-81	+	+			
VCAM-1 (CD106)				+	
VEGF R2 (FLK-1)					+

Am S, amnion-derived stem cells; Em S, embryonic stem cells; Neu S, neural stem cells; Mes S, mesenchymal stem cells; Hem S, hematopoietic stem cells.

Stem cell and progenitor cell surface marker expression were summarized and compared with the expression of other stem cells. Marker expression on AE cells was analyzed in our laboratory and data from other stem cells were obtained from published literature. Blank cells

showed rapid efflux of the dye which was dependent on the expression of ABCG2. In subsequent experiments, it was determined that hematopoietic stem cells were contained within the SP. Later, the SP phenotype was observed in a wide variety of stem cells including neural stem cells (10) and muscle-derived stem cells (11). Recently, SP cells were identified in both the AE and AMF fractions from amnion (12). The significance of these observations is not known.

Molecular Markers of Stem Cells

Along with the stem cell-specific surface markers, AE cells express molecular markers of stem cells. There is consensus agreement that pluripotent human ES cell lines express Oct-4, SOX-2, Lefty-A, fibroblast growth factor (FGF)-4, rex-1, and TDGF-1 (cripto, 57; 13), and nanog (14). When examined by reverse-transcription (RT)-PCR, all stem cell marker genes were found to be expressed in the freshly isolated AE cells and/or in cultured cells.

Oct-4 Expression

Among those molecular stem cell markers, Oct-4 is known as one of the transcription factors that play a critical role in

maintaining pluripotency and self-renewal. Oct-4 belongs to the Pit-Oct-Unc (POU) family of transcriptional regulators (15–17) and regulates the pluripotency of human and mouse ES cells (18). In the early mouse embryo, Oct-4 is only expressed in pluripotent cell types such as cleavage stage blastomeres, the inner cell mass of the blastocyst, and the epiblast of the egg cylinder. At gastrulation, Oct-4 expression is downregulated in somatic cells and was thought to be maintained mainly in the primordial germ cells. During the postnatal period, Oct-4 expression is detected in growing oocytes and spermatogonia (18). Pluripotent stem cells in culture, ES cells, embryonal carcinoma cells, and embryonic germ (EG) cells all express Oct-4. This pattern of expression reflects the key role played by Oct-4 in the maintenance of pluripotency (19). The expression of Oct-4 is controlled epigenetically by hypermethylation of the enhancer/promoter region (20).

Epiblasts, which redirect developmental origin of AE cells, express Oct-4 as long as they remain undifferentiated (17). The expression of Oct-4 in AE cells suggests that they maintain the pluripotency of the undifferentiated epiblast. Oct-4 expression is observed in most AE cells. Although some display nuclear-localized Oct-4, in the majority of AE cells, the expression is

cytoplasmic. EG cells also show cytosol-positive Oct-4 staining, indicating that AE cells may be at a developmental stage similar to that of EG cells.

Further investigation will be required in order to determine whether the stem cell marker-positive cells are remnants of the pluripotent cells from the fetus or if amniotic cells maintain their stem cell nature for a separate, specific function which has yet to be determined. If placental stem cells are maintained throughout the pregnancy, the mechanism and the functional implications of this will be the basis of future exploration.

Multipotent adult progenitor cells (MAPCs) were identified from adult bone marrow mesenchymal stem cells of human, rat, and mouse. This rare cell population has pluripotent differentiation ability *in vitro* and *in vivo* and the cells express Oct-4. However, the expression of Oct-4 in MAPCs is 1000-fold lower than that of ES cells (21). In contrast, monkey (*Macaca fascicularis*) AE cells expressed Oct-4 mRNA in almost the same manner as ES cells (unpublished observations). These data suggest that AE cells may be more like ES cells than adult stem cells.

Pluripotency of Amniotic Epithelium-Derived Stem Cells

A wide variety of investigations performed since 1980 have provided evidence of the existence of pluripotent stem cells in amniotic fluid. 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. Of course, it would be unethical to perform this experiment with human blastocyst. Tamagawa et al. (22) established cell lines from cells isolated from whole amnion, which contain AE cells and AMFs. The cell line HAM-1 was mixed with mouse early embryonic stem cells (EES-7) to form an aggregation chimera. The xenogeneic chimera embryo was maintained until all three primordial germ layers were formed. The contribution of amnion-derived stem cells to all three germ layers of the xenogeneic aggregation chimera was demonstrated. Tamagawa's report is a remarkable demonstration of pluripotency *in vitro*.

Spheroid Formation

When AE cells were cultured as an adherent monolayer for several weeks, small clusters or "spheroids" or cell clusters were noticed over the cobblestone pavement of epithelial cells. These spheroids are similar in structure to embryoid bodies (EB) described cultures of ES cells. Stem cell markers were examined by immunohistochemistry in the spheroid structures from long-term (24 d) cultures. Immunohistofluorescent staining revealed that the rim of cells on the outer edge of each EB-like spheroid structure expressed stem cell-specific cell surface antigens SSEA-3, SSEA-4, TRA 1-60, and TRA 1-81 (23). Data presented in Fig. 3 shows surface staining with antibodies to SSEA4 and cytoplasmic activity for alkaline phosphatase. Expression of the cell surface stem cell markers was mainly restricted to the spheroid-like structures, whereas more differentiated cells in the epithelial monolayer surrounding the EB-like structures did not react with these antibodies. The stem cell molecular marker genes, *Oct-4* and *Nanog*, are also predominantly expressed in the cells of the spheroids. These data

indicate that AE cells form EB-like structures over the monolayer of attached AE cells which better support and maintain their stem cell nature in culture. The spheroid structures growing over the monolayer of other cells is reminiscent of ES cells growing over a feeder layer of stromal cells. Miyamoto et al. (24) reported a feeder layer function of AE cells. Primate ES cells (Cynomolgus monkey CMk6 cell line) were cultured on human AE cells, and the ES cells were maintained in an undifferentiated state as was shown by *Oct-4* expression and formation of teratomas after injection into immunodeficient mice. These data are completely consistent with our findings. Unique cell-to-cell interaction between stem cells and a feeder-like monolayer of AE cells may be useful to maintain the "stemness" of AE stem cells.

Localization of Stem Cells in Human Placenta

Recently, we succeeded in the localization of the stem cell marker-positive cells in a whole mount of the amnion membrane (25). The stem cell marker-positive cells were uniformly scattered over the membrane. Interestingly, the stem cells were not found in clusters, but rather were surrounded by stem cell marker negative cells. This observation stimulates our imagination of the existence of a "stem cell niche." Most tissue stem cells have their own "niche," i.e., a specific microenvironment which helps to maintain the stem cells in an undifferentiated state. Much like the feeder layer experiments described previously, the stem cell marker-negative AE cells may be playing the role of a "niche" for the stem cell marker-positive cells. The specific cell-to-cell contact and signaling pathways involved in this process are currently under investigation. Other explanations are also possible. During development, when the amnioblasts first differentiate from the pluripotent epiblast, the cells stray from the organized development of the embryo. Without restrictions or other external regulatory signals, some of the cells of the amnion may randomly differentiate, whereas others preserve their stem cell characteristics.

Differentiation Potential of Amniotic Epithelial Cells

We have previously shown the potential of AE cells to differentiate into ectoderm, endoderm, and mesoderm lineage cells *in vitro* (23). Although our report showed for the first time that AE cells from one placenta can differentiate into all three germ layer cells, it has also been demonstrated by other groups that AE cells have the potential to differentiate into various types of cells. Sakuragawa and coworkers, (26) the leading investigators in this field, demonstrated that cultured AE cells express markers of glial and neuronal progenitor cells. Further experiments succeeded to induce the differentiation AE cells into the cells that synthesize and release acetylcholine (27) and catecholamines (28). Furthermore, dopamine-producing cells were produced from AE cells (29) and transplanted into the brain of animals with a model of Parkinson's disease (30). Tyrosine hydroxylase-positive cells were immunohistochemically confirmed at the transplanted site of recipients and a therapeutic effect was observed for about 1 mo.

Because the amnioblast is sometimes also referred to as the amniotic ectoderm perhaps there is a propensity for the AE cells to differentiate toward the neural lineage (ectodermal). It is an interesting corollary that ES cells have the same

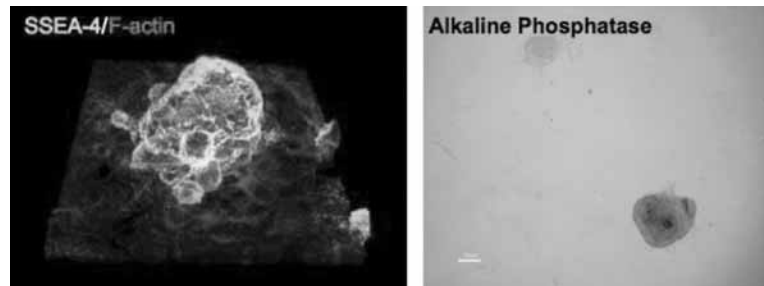


Fig. 3. Characterization of spheroid. Amniotic epithelial (AE) cells form spheroid-like structure on the monolayer AE cells. The cells of the spheroid express stem cell marker cell surface antigens, such as SSEA-4. Monolayer AE cells were visualized with F-actin counterstaining (rhodamine-phalloidin). Right, a phase-contrast image shows spheroid-specific alkaline phosphatase expression. Alkaline phosphatase activity was detected only in the spheroid cells.

propensity to differentiate along a neural lineage. Our RT-PCR data indicate that some of the neuronal progenitor marker genes are expressed in the naive AE cells (23).

As with ectoderm differentiation, the potential for AE cells to differentiate to endoderm was also reported. In 2000, Sakuragawa et al. (31) showed albumin secretion from cultured human AE cells and when LacZ-labeled human AE cells were transplanted into the liver of an immunodeficient mouse, the transplanted cells were found to integrate into the hepatic plate. Our group has also performed human AE cell transplants into immunodeficient mice, and observed cells with the morphology of hepatocytes, which expressed human albumin or α -1 antitrypsin. Furthermore, we detected human α -1 antitrypsin circulating in the serum of recipient mice, which confirmed the engrafted human AE cells function as hepatocytes in mouse liver. Although these are encouraging preliminary data, a more complete study of the fate of AE cells following transplantation into the liver is needed and is currently underway. Nakajima et al. (32) established a method to isolate rat amniotic cells. The transplanted rat AE cells survived in the liver following allogeneic transplantation for at least 30 d. There are reports of the induction of early markers of hepatic differentiation of AE cells following the addition of specific growth factors to culture media. Takashima et al. (33) used hepatocyte growth factor, FGF-2, heparin sodium salt, and oncostatin M to induce albumin-producing hepatocyte-like cells. The same group also reported that they could induce AE cells to produce insulin and could normalize blood glucose in diabetic model animals following transplantation of the AE cells into the spleen (34).

There is no report of mesoderm lineage differentiation except by our group (23). We applied culture conditions that were used to induce cardiac differentiation of ES cells. Although functional assays were not performed, the amniotic epithelium-derived cardiomyocytes expressed cardiac-specific genes and immunostaining of α -actinin protein was demonstrated. The α -actinin expression pattern was identical to that reported for ES-derived cardiomyocytes (35).

The experiments summarized so far demonstrate that differentiation of AE cells can be induced and somewhat directed by exposure to exogenous growth factors or chemicals. The plasticity of AE cells is further shown by growth factor-induced differences in gene and protein expression and also by changes

in cell morphology. The effect of a number of growth factors on AE cells is summarized in (Fig. 4).

Amniotic Mesenchymal Fibroblasts

At the hypoblast stage, a primary yolk sac is formed. Extraembryonic mesoderm cells derived from the hypoblast form a new cell layer beneath the amnioblast and parietal endoderm. The extraembryonic mesoderm cells eventually form the amniotic mesenchymal layer (36). Therefore, the amnion consists of epiblast-derived AE cells and hypoblast-derived AMF. Mesenchymal stem cells can differentiate into multiple mesenchymal lineages, such as adipocytes, osteoblasts, chondrocytes, and myocytes. Human MSCs are mainly isolated from bone marrow, but many other sources are proposed. AMF also express MSC cell surface markers and differentiation capability. A variety of protocols are used to isolate AMFs from placenta. Most reports use collagenase digestion. However, as shown in Fig. 2, it is difficult to obtain a pure AMF population without contamination of AE cells. Although trypsinization releases only AE cells from the connective tissue, subsequent collagenase digestion will release all remaining cells. Aside from the reports on the mesenchymal stem cell characteristics of AMFs, some reports suggest that AMF may be pluripotent, with the capability to differentiate into cells beyond the mesenchymal lineage such as pancreatic cells, cardiomyocytes, or neural cells. Wei et al. (34) induced pancreatic differentiation of AE cells and AMF cells with similar culture conditions. The results showed AMF-derived insulin-producing cells achieved one-third the efficacy of AE cell-derived insulin-producing cells upon transplantation. Because the starting material was a mix of AE cells and AMF, these data may be explained by the presence of pluripotent AE cell contamination in the AMF.

Senescent Nature of Amniotic Epithelial Cells

Continuous growth of human AE cells in culture leads to replicative senescence. Under the culture conditions used to date, after 6–10 passages, AE cells fall into a terminally non-dividing state. If AE cells are cultured at very low densities, senescence occurs even earlier. This discrepancy indicates that the senescence mechanism is not simply controlled by telomere length. Integrin-dependent epidermal growth factor receptor (EGFR) activation is one of the signaling mechanisms involved in cell proliferation (37). AE cells express a number

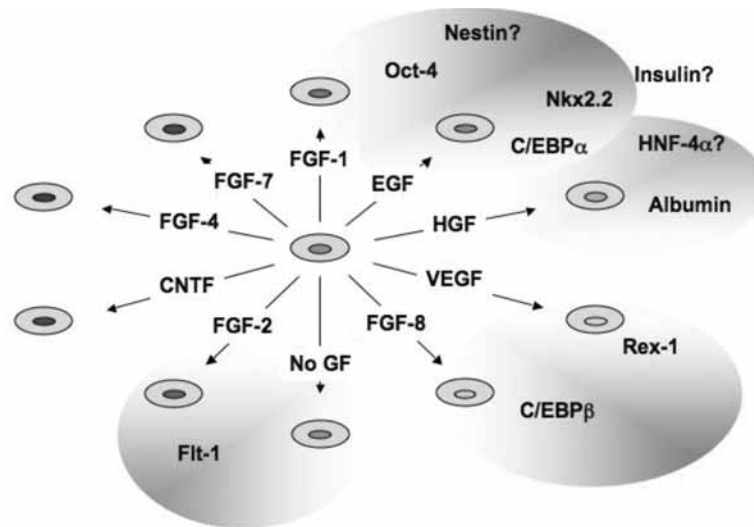


Fig. 4. Summary of gene induced by exogenous growth factors. Naive AE cells were cultured with several growth factors for 1–2 wk. The expression of key genes was evaluated by real-time quantitative reverse-transcription PCR and compared with the gene expression of starting (naive) AE cells.

of integrins. Although it remains to be proven, AE cell proliferation may depend on the integrin and EGFR-associated molecular complex and cell-to-cell interactions, which are facilitated by higher density culture (38). In our hands, human telomerase reverse transcriptase mRNA expression was not detected by RT-PCR in naive AE cells. This might suggest that AE cells are sufficiently quiescent to preserve the characteristics of differentiated somatic cells. To address this question, the length of AE telomeres was investigated. Mosquera et al. (39) performed precise evaluation of telomeres length and telomerase activity on amniotic fluid cells. They described that telomerase activity was 34–48% of that of a HeLa cell line at 22 d after the initiation of culture, and decreased thereafter. According to Southern blotting of terminal restriction fragment length, the telomere length initially observed progressively decreased after each round of DNA replication. Amniotic fluid cells are a heterogeneous cell population; however, many are cytokeratin-positive cells like the AE cells that we describe (40). If the data on amniotic fluid cells are representative of AE cells, it may be useful to release the telomerase constraints with specific factors. In fact, exposure of AE cells to trichostatin A, a histone deacetylase inhibitor, temporarily and reversibly reactivated human telomerase reverse transcriptase expression on AE cells (unpublished observations). This suggests that optimized culture conditions might be created which may allow establishing continuously growing lines of pluripotent AE cells.

Cytogenetic Stability and Tumorigenicity

Telomerase activity is detectable in human ES cells, MAPCs, human germ cells, and 80–90% of human tumor samples. Telomerase-active stem cells, including ES cells and MAPCs, sometimes become aneuploid. Aneuploidy is seen more frequently once mouse MAPCs have been expanded for greater than 60–70 population doublings and following repeated freeze–thaw episodes. Furthermore, telomerase-active

ES cells form teratomas when the stem cells are injected into the muscles of severe combined immunodeficient-beige mice. By contrast, when from half a million to 2 million human AE cells were injected into more than 50 individual mice and observed for an average of 60 d (max. 516 d), no AE cell transplants led to the development of tumors by any route of administration in severe combined immunodeficient-beige mice or Rag-2 knockout mice. No cytogenetic abnormalities were observed in cultured AE cells by simple analysis of the karyotype. The safety of AE cells upon transplantation has been shown in a clinical setting. Some physicians focused on the storage of glycogen by AE cells and transplanted minced amnion or rolled tissue into glycogen-deficient patients in an attempt to correct the metabolic defect. It is well known that amnion does not express HLA class II antigens and only expresses class I antigens at low levels. These observations lead researchers to speculate that AE cells would not be recognized by the immune system and that allogeneic transplants of AE cells would survive indefinitely. Researchers transplanted AE cells into the forearm of volunteers to determine whether an immunological reaction would occur. No immunological reaction was noted, and importantly, in neither case was there a report of tumorigenicity of the AE cells (41–43).

Drug Discovery and Toxicology

Stem cells are expected to become an important new tool for drug development (44). Amnion-derived stem cells may be an ideal cell source for these *in vitro* assays. The successful differentiation of AE to cells with mature cardiac or hepatic functions would be critical. Current preclinical models of cardiotoxicity for drug candidates are difficult because of problems with appropriate cell models and the availability of the most useful cell types. Primary human cardiomyocytes are excellent tools with which to screen new chemical entities for efficacy and safety. However, the availability of healthy human cardiomyocytes is limited. AE cells have the potential to

differentiate into cardiomyocytes. Another essential cell type for drug development is the hepatocyte because of its high level expression of drug-metabolizing enzymes. AE cells have been shown to differentiate into hepatocyte-like cells that demonstrate hepatic gene expression as well as some drug-metabolizing functions. In addition to the expression of basal levels of all of the hepatic genes examined to date, amniotic epithelium-derived hepatocyte-like cells express regulated, inducible cytochrome P450 genes. Importantly, the specific drug-metabolizing enzymes are regulated and induced by prototypical inducing agents in a manner similar, if not identical, to authentic human hepatocytes. Once culture conditions are optimized to produce mature cardiomyocytes or hepatocytes from AE cells, this may be a nearly unlimited source of cells for drug metabolism and toxicology purposes.

Clinical Applications

The use of de-epithelialized human amniotic membrane has been well documented in ophthalmic surgery for the treatment of Stevens-Johnson syndrome, ocular cicatricial pemphigoid, acute thermal and alkali burns, pterygium surgery, and limbal stem cell transplantation (45,46). For this application, only the basement membrane of amnion is needed to serve as a biological scaffold for epithelial cell migration as well as for anti-inflammatory properties. An important point is that human amnion has been approved as a medical material by the Food and Drug Administration.

The low antigenicity of amnion may be an advantage for amniotic epithelium-derived stem cell transplantation or cell replacement therapy. Human MHC antigens are expressed very weakly (class I) or not at all (class II) on AE cells (47,48). This potential advantage was utilized during AE cell transplantation to correct lysosomal storage disease (42). More than 50 cases of AE cell/tissue transplantation had been performed in various institutes. Although the therapeutic effect for the correction of lysosomal storage disease was varied and was transient at best, there were no clear evidences of immunological reaction to, or rejection of, the transplanted cells. Finally from a safety standpoint, as we have described in the section on tumorigenicity, AE cells are clearly nontumorigenic when transplanted into immunodeficient animals and no tumor formation has been reported when human AE cells or tissues were transplanted into patients.

Ethical Considerations

The basis of most of the current ethical concerns with ES cells is dependent on the need to derive the cell type from developing blastocysts. Because the blastocyst could potentially develop into an embryo if it were transferred to a suitably prepared recipient, the argument can be made that the derivation of ES cells interrupts normal development, which might have produced a human life. Derivation of ES-like stem cells from placenta would not be expected to raise the same objections. Currently normal full-term placenta is evaluated after the birth of the baby and is discarded at the hospital as medical waste. Because the tissue is only made available following a live birth and is not used for any other purpose, the isolation and use of stem cells from a discarded placenta would not be expected to elicit any ethical concerns. No potential

human life is interrupted to derive the stem cells; in fact, the cells only become available following a normal live birth. Once technology is established to isolate and/or propagate pluripotent stem cells from the placenta, this medical waste may turn into valuable property. The precedent and current use of umbilical cord blood stem cells has helped establish protocols for the possible future use of amnion-derived stem cells. It has been established that the placenta belongs to the baby and that parents execute control over the use of the placental tissues/cells on behalf of the baby. Therefore, the use of human placenta-derived cells for research purposes and clinical applications would not be expected to elicit current or future ethical or legal conflicts.

Summary

Here, we review the stem cell characteristics of amnion cells, especially AE cells. During development, amnion differentiates early from the pluripotent epiblast prior to cell type specification, which occurs at gastrulation. Additionally, the amnion remains separate from the developmental signals, which regulate differentiation of the epiblast to form all of the cell types and organs of the developing embryo. At least some of the AE cells seem to have preserved the pluripotent characteristics of the epiblast. Cell surface marker expression suggests that approx 10% of the AE cells express markers which are commonly found on ES cells.

Eight years from the first report of the establishment of human ES cells, the classification of stem cells is now generally held only for cells which fulfill the dual requirements of self-renewal and pluripotency. However, even this definition does not enjoy complete agreement (49). Plasticity or differentiation potential is an essential characteristic of stem cells, which could be used for further clinical applications. On the other hand, "unlimited self-renewal" may not be an essential requirement. Cells in adult tissues do not have unlimited growth potential and most organs function normally without clearly defined stem cell compartments. Extended growth potential and self-renewal are only required when the initial stem cell source is limited and not able to generate sufficient numbers of cells for therapeutic use without expansion in culture. If abundant pluripotent cells could be obtained from the starting material for differentiation protocols or for cell transplantation and regenerative medicine applications, the requirement for extended self-renewal capacity becomes less important. Furthermore, extended culture carries its own risks. Long-term culture of cells is frequently accompanied by genetic changes in the karyotype and tumorigenicity.

Placenta, the source of AE cells, is abundantly available as a discarded tissue and is free of the ethical concerns of other stem cells. It could easily be imagined that banks of AE cells could be established which contained a precise MHC match for every possible transplant recipient without the need for extensive technical manipulations such as somatic nuclear transfer. So far, no stem cells are able to differentiate into therapeutically useful cell types *in vitro* or their differentiation is not well controlled. As with the other stem cells, further investigations will be required to induce AE cells to differentiate to therapeutically useful cells. However, because they are abundantly available without ethical concerns and because of the

advantages of pluripotency, low immunogenicity, and lack of tumorigenicity, amniotic epithelium-derived stem cells may be an extremely useful cell source for transplantation and organ and tissue regeneration.

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