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Placental Structure and Biological Aspects of Fetal Membranes Cultured *in vitro*

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1. Introduction

The placenta is a transient organ responsible for maternal fetus nutrient and oxygen exchange. It is important to the modulation of the maternal immune response to antigens of paternal and fetal origin and a source of a great variety of hormones that ensure the maintenance of gestation. Pathological conditions of pregnancy, fetal growth and delivery, abnormal placentation and surgical problems always attracted the medical sciences attention. The diversity of placental types among eutherian mammals has often raised important questions about phylogeny. *In vitro* culture of placental cells is an approach that allows the investigation of hormonal production and metabolic process as well as pathological disorders of common occurrence in human beings and animals. This chapter aims to show the most important features on culturing placenta derived cells and cell populations with properties of progenitor/stem cells: identification of the structures, isolation of the cells, maintenance of the cell culture, and applicability in research trends.

2. The placenta

Retention and development of the fertilized egg within the maternal body (histotrophic and hemotrophic viviparity) are observed in most mammals, reptiles and a few lower organisms. The growing embryo is nourished by the mother, usually through a placenta or similar structure. Vertebrate viviparity with the development of a placenta in the uterus evolved 150-100 million years ago and is responsible for the adaptability and dispersion of eutherian mammals all over the diverse habitats.

The Mammalian class is divided into two subclasses, Prototheria and Theria, which includes the two major groups of viviparous mammals, the marsupials and placentals.

Four superorders of eutherian mammals were identified by techniques of molecular phylogenetics. The two oldest superorders Afrotheria (elephants and others) and Xenarthra

(armadillos, anteaters and sloths) have either endothelial or hemochorial placentas. Members of the superorder Euarchontoglires exhibit hemochorial placentas (Glires – rodents and lagomorphs) and endotheliochorial placentation (Euarchonta – tree shrews). Epitheliochorial placentas are observed in the last superorder to arise, the Laurasiatheria (horses, ruminants and various other species). It seems that the likely path of evolution in Afrotheria was from endotheliochorial to hemochorial placentation and the same condition apparently occurs with the Xenarthra and the bats. It is possible that endotheliochorial placentation was the more primitive and that epitheliochorial placenta evolved twice, once in the Laurasiatheria and once in Euarchontoglires. Mammalian phylogeny has been extensively studied (1–4).

The placenta should be considered as an apposition of the fetal membranes to the uterine tissue for physiological exchange, and in mammals the definitive placentation is of the chorio-allantoic type, in which mesodermal blood vessels, from the allantois external layer, form a vascular bridge between the embryo and the chorion, allowing a broad vascularization, and increasing efficiency in the mother-fetus exchange (5). In this situation, the maternal-fetal interface is enlarged, because chorionic villous fills the uterine crypts. In the chorion-vitelline placenta, there is a fusion between the yolk sac and its blood vessels network with the chorion. Here the allantois is never in contact with the chorion and it is observed in some non-mammalian vertebrates. In mammalian species, however, it occurs temporarily, while the allantois is developing from the intestine, to constitute the chorio-allantoic placenta.

Despite of all the work to develop systems of classification and to use placental structure to aid in the investigation of phylogenetic relationships among species of different orders, there is a lack of morphological and electron microscopic studies of the placenta in a great number of organisms, limiting the efforts to generalizations. The classical systems of classification of the placenta, based on structure, are of limited significance. Considering that the trophoblast could be apposed to uterine epithelium, to the endothelium of maternal vessels or directly to maternal blood, the placenta is classified as epitheliochorial, endotheliochorial or hemochorial, respectively. However, electron microscopy studies have been shown that a continuous fetal endothelium and chorion always persist, although very thin in some tissue extensions, reducing the diffusion distance between fetal and maternal circulation, even in epitheliochorial placentas.

It is now well recognized that the number of layers between fetal and maternal circulation do not have any relationship to the placenta's ability to supply oxygen to the fetus. Interhaemal area is reduced in many ways in the different types of placentation. In the ruminant's placenta (epitheliochorial and synepitheliochorial) the indentation of trophoblast and uterine epithelium by blood vessels decreases the interhaemal distance; in the endotheliochorial placentae this reduction is achieved by the indentation of trophoblast by fetal capillaries within the labyrinth. In the haemochorial placentation several features occur to reduce the thickness of the trophoblast, like alternation of thick and thin regions in the rabbit placenta (See (6) for detailed information).

The placenta developed as an organ to nutrient and oxygen exchange between the mother and fetus and also to avoid immunological rejection by the maternal immune system. Cells from the mother and the fetus intermingle throughout gestation and the extension of trophoblast invasion apparently is related to the modulation of maternal immune response against the allogenic conceptus (7). In the epithelialchorion placentation there are minimal changes in the uterine mucosa during gestation, whereas in the haemochorial placentation the endometrium is differentiated into decidua. These changes allow the establishment and maintenance of pregnancy and fetal survival with great diversity among different species.

2.1. Organogenesis

At the beginning of the mammalian development, the conceptus differentiates into an inner cell mass and an outer layer of cells, the trophoblast, which solely contributes to extra-embryonic membranes formation (4,8). The tissue on the maternal component of the placenta usually is of epithelial or connective tissue origin of the ovary, oviduct or uterus. The fetal component is a derivative of ectodermal epithelium, the trophoblast or trophoctoderm.

Diverse populations of trophoblasts, all derived from the embryonic trophoctoderm, have morphological, functional and molecular diversity within and across species. The extra-embryonic membranes of the vertebrate conceptus form from the three germ layers of the embryo - the endoderm, mesoderm and ectoderm. These germ layers form the yolk sac, initially a single layer of ectoderm that is progressively transformed in a bilaminar and trilaminar structure, after the migration of a layer of endoderm internal to the ectoderm, and a growth of mesoderm between these two first layers. In mammals, the growth of mesoderm transforms only the superior part of the bilaminar yolk sac into the trilaminar vascularized structure, which is the basis for development of the chorion, amnion and allantois. Fusion of mesoderm and endoderm vascularizes promptly, whereas mesoderm and trophoctoderm rarely form blood vessels. The vascularized yolk sac fused with the ectoderm form the fetal component of the choriovitelline placenta, and the vascularized allantois plus chorion forms the fetal part of the chorioallantoic placenta.

The trophoctoderm and the inner cell mass become separate during early gestation and non interconvertible cell lineages. In mouse, for instance, *Cdx2* is expressed predominantly in the trophoblast and *Oct3/4* only in the inner cell mass (6). In the human placenta (9), the cell types that constitute placental villi are different populations of trophoblasts, the syncytiotrophoblasts and cytotrophoblasts that cover the surface of villi and bathe in maternal blood within intervillous space. Trophoblasts are formed during the first stage of pregnancy and are the first cells to differentiate from the fertilized egg. They have two cell populations, the undifferentiated cytotrophoblasts and fully differentiated syncytiotrophoblasts. The syncytiotrophoblasts are a continuous, specialized layer of epithelial cells.

Mesenchymal cells, Hofbauer cells (mesenchymal derived macrophages) and fibroblasts are found between fetal vessels and trophoblasts in human species. The last group of cells is fetal vascular cells (vascular smooth muscle cells, endothelial and perivascular cells

(pericytes). Hofbauer cells are the macrophages in the placenta villous stroma. These cells are of mesenchymal origin and expand during the first and second trimesters in placental villous tissues mesenchymal stem cells are differentiated by vasculogenesis and angiogenesis, transform into hemangioblastic cell cords, which are believed to be the precursors of capillary endothelial cells and hematopoietic stem cells. Mesenchymal stem cells can also differentiate into perivascular cells, which are considered predecessors of capillary endothelial cells. Pericytes (perivascular cells with dendritic processes) are found surrounding capillary endothelium and venules. They support endothelial cells and are important for maintaining vessel stability and microvascular integrity.

In mouse, the trophoblast differentiates in two pathways: rapid proliferation of polar trophoblastic cells originate the extraembryonic ectoderm and the ectoplacental cone; and the mural trophoblastic cells that originate the primary giant trophoblastic cells by endoreplicating their DNA but not dividing, like in other rodent giant cells. These cells, however, are not analogous to that observed in the human placenta, despite bearing the same name.

Chorionic binucleate cells are observed in the ruminant and horse placentas (6). In the mare, these are migratory transient cells and never fuse with maternal cells. In the cow and ewe, nevertheless, this population of fetal cells migrates through the chorionic tight junction to fuse with uterine epithelial cells throughout gestation. These cells are derived from uninucleate trophoblast cells by consecutive nuclear divisions, the second without subsequent cytokinesis. The chorionic binucleate cells comprise 15 to 20 per cent of the trophoblast epithelial cells and produce a great variety of hormones. This is a unique feature of the ruminant placenta.

2.2. Structure and classification

Placenta has been described in the early studies (5) as more variable in structure than any other mammalian organ, and consequently it is classified in different ways, but other classification (10), which distinguishes the layers between fetal trophoblast and maternal endometrial surface, is considered the most useful, despite its limitations regarding biological and evolutionary aspects, as it was mentioned before.

Morphologically, the placenta is classified according to the configuration of the maternal-fetal interface. The area of fetomaternal exchange is increased by the formation of placental folds (villi) which is characteristic among families. Four types of placenta are described: in the diffuse placenta (horse and pig) the allantochorion is involved in the formation of the organ and it is almost entirely in contact with the endometrium; the cotyledonary placenta, observed in ruminants, shows multiple and discrete areas of attachment to the endometrium. The fetal portion is called cotyledon and the maternal contact sites are the caruncles. The hemotrophic structures formed by this fusion are called placentomes.

The placenta seen in carnivores like dogs and cats is called zonary, and the chorionic villi occupy the equatorial region of the chorionic sac, where they attach to the endometrium;

finally, a discoid placenta is present in rodents and primates, in which an area of the chorion (discoid in shape) adhere to the endometrial stroma.

Regarding to its internal structure, the placenta is classified in villous and labyrinthine; in the former, chorionic villi are present and they penetrate into uterine crypts; fetal blood is transported in vessels through these structures. In the labyrinthine placenta, maternal blood circulates through channels within the fetal syncytiotrophoblast.

Histologically, the placenta is classified according to the layers between fetal and maternal blood, examined by light microscopy. The fetal components are endothelium, mesenchyma and trophoblast; maternal tissues are uterine epithelium, connective tissue and endothelium. When the uterine epithelium is in contact with the chorion (pig and horse) the placenta is called epitheliochorial; in ruminants, the uterine epithelium is removed and the maternal connective tissue is in contact with the chorion (Syndesmochorial placenta); uterine epithelium and connective tissue could be absent, and so the maternal endothelial basement membrane is in contact with the chorion, like in the carnivores placenta, and finally, in the haemochorial placenta, all the maternal tissue layers are removed, the chorion is in direct contact with the maternal circulation and such situation is observed in anthropoids and rodents.

Some other criteria of classification have been proposed, like capillary position, regional specialization, tissue lost at parturition and accessory placental structures. For the classical criteria see (11) and for updates, see (6).

2.3. Physiology

The placenta is related to the production and metabolism of gonadotropins, steroids and prostaglandins hormones that are responsible for the pregnancy establishment and maintenance, placental maturation and parturition. Throughout gestation uterine environment shows great plasticity, which is necessary for fetal growth, being mediated by hormonal production of the placenta. This organ produces a great variety of hormones, including steroid hormones, peptides and insulin-like growth factors. In the human species, these factors are related to proliferation, invasion and differentiation of the trophoblast. Progesterone (P4) secretion in mammals is necessary to prepare the uterine environment to receive the developing conceptus. Embryonic signals, in the absence of P4, are inefficient to rescue the corpus luteum from the uterus luteolytic mechanisms, like in the ungulates, or to extend the luteal lifespan, as it occurs in the human species. In the ewe, hypophysectomy before day 50 of gestation causes corpus luteum regression and abortion. After this, the placenta produces P4 in sufficient concentrations to maintain pregnancy, even in the absence of ovaries and hypophysis.

In ruminants, binucleate cells (BNC) are present in the trophoblast since the pre-implantation period until parturition and these cells are found in the endometrium by day 22 of pregnancy. BNC produce placental lactogen, P4, and prostaglandins E-2 and I-2. In the mare these cells produce the equine chorionic gonadotropin during their migration by the

uterine connective tissue. In the cow steroid production are altered throughout gestation. Two to three days before parturition, P4 levels decrease, and estradiol, in contrast, increase, and high levels are observed in the last week of gestation.

BNC produce prostaglandins and it is possible that they are related to the maternal recognition of pregnancy in some species, like swine. It has been suggested that estrogens and prostaglandins have a physiological role in normal expulsion of the placenta in cattle.

In the ruminant placenta, the trophoblast produces interferons that are responsible by the maternal recognition of pregnancy. Interferon tau has been recognized, in ungulates, as an inhibitor of luteolysis. Atypical interferons are produce by the swine blastocyst and its relationship with the maternal recognition of pregnancy is less clear. Type I interferons are produced by the human placenta and it is accepted that they are hormones related to the establishment of gestation in this species. For details see (12,13).

3. *In vitro* culture of placental tissues

3.1. Primary culture

Basically the source of tissues to primary culture is human term placentas or animal placentas obtained in abattoirs. Cells can be obtained by mechanical disaggregation of placental tissues (amniochorion and allantochorion membranes, cotyledons, caruncles and the endometrium itself). Usually tissues are rinsed in Hank's balanced salt solution (HBSS) or in phosphate-buffered saline (PBS) enriched with antibiotics (gentamicin, penicillin, streptomycin), to avoid bacterial contamination, and they are progressively dispersed with surgical scissors in at least six baths. After rinsed, tissues are cultured in suspension in Minimum Essential Medium (EMEM) with Earle's salts. Disaggregate tissues contain different cell types and it may be necessary to go through a separation process. Amniocytes may be aspirated directly from placental fetal fluid and cultured in specialized medium such as Amniomax. These cells have adherent properties and form small aggregates in culture plastic flasks, being ideal for cariotyping. Basic culture conditions are 37^o C and 5% CO₂ atmosphere. Briefly, a protocol of cell separation is described. First, remove fat and dead tissues. Wash it with PBS twice. Slice placenta in trophoblast regions and transfer to a sterilized conical tube with cold PBS (10%) on ice. Release trophoblasts from chorionic villi by trypsin digestion. Plate the cells. Deplete macrophages by adherence to plastic. Freshly isolated cells are predominantly trophoblast villous that can be identified by verifying the expression of epidermal growth factor receptor (EGFR). Tissues and cells cultured primarily can be induced, by different ways, to produce proteins, hormones and other metabolites. The cell genome can be altered under experimental conditions in order to achieve research purposes, and different systems of tissue culture and cell manipulation could be assembly to diagnostic techniques and intracellular information flow investigation.

Primary cultures are obtained by disaggregation of a tissue sample, either mechanically or enzymatically. The resulting suspension contains a proportion of cells capable of attaching to a surface, as in tissue culture flasks and dishes, forming a monolayer. Another type of cell

culture, the primary explants, consists of cells that migrate out from small fragments of tissue and adhere to the growth surface. Tissue disaggregation is capable of generating larger cultures in short periods of time, but explants culture may still be preferable where only small fragments of tissue are available or the fragility of the cells precludes survival after disaggregation.

Generally, from human beings and large animals, placentae are obtained at term of normal pregnancies and after caesarean section or vaginal delivery. Amnion and chorion membranes are manually separated from each other. The membranes and other placental tissues as well, are washed extensively in PBS or HBSS containing 100 U/ml penicillin and 100 µg/ml streptomycin to avoid bacterial contamination. Blood clots present in the cotyledons may be mechanically removed.

In small animals like rats and mouse (*Rattus norvegicus* and *Mus musculus*), it is a common practice to excise the uteri from pregnant animals, transfer them to Petri dishes with cold PBS, and then, collect and pool the placentae in a new Petri dish. Harvested placentae are washed thoroughly with PBS containing antibiotics. Rat amnion may become distinguished as almost transparent, and may be peeled off from chorion and dissected (14). Otherwise, both membranes are processed together in order to obtain the fetal membrane-derived cells.

Samples are cut into small fragments (3 × 3 or 5 × 5 cm) and may be stored in 50 ml vials filled with serum-free and phenol red-free Dulbecco's Modified Eagle Medium (DMEM) in sterile conditions. The number of cells and their viability, either when stored at room temperature and processed within 24 hours or when stored at 4°C and processed within 28 days, are the same as in fresh membranes, as previously reported (15,16). Those fragments may be either used as primary explants or submitted to enzymatic disaggregation.

Small fragments of amniotic membrane may be directly engrafted to a site of injury, as focal ischemic regions in the heart, lung fibrosis, skin wounds, chronic leg ulcers, and liver fibrosis. The benefits of such therapeutic approaches as the ability to promote re-epithelialization have been shown to reduce inflammation and fibrosis and to modulate angiogenesis (16–19).

Explants culture may also be done using fetal placental villi. Bundles of chorionic anchoring (stem) villi are separated from the chorionic plate and spread out using forceps to separate individual villi from each other. The villi are washed multiple times in PBS, cut into small pieces (1 × 1 mm) and cultured intact. When outgrowing cells reach 80% to 90% confluence, the villi pieces remaining intact are carefully removed, transferred into, and subsequently cultured on new plates to get a sufficient amount of cells for the experimental procedures. Recently it was reported that abundant vasculature present in the human placenta can serve as a source of myogenic cells able to migrate within dystrophic muscle and regenerate myofibers (20).

Instead of using an entire fragment, fetal membranes may be subjected to enzymatic disaggregation, as reported in literature by various different protocols, which differ from

each other mainly regarding the enzyme employed and the digestion length. However, all of them include an incubation step at 37°C with some enzyme (dispase, trypsin/EDTA, collagenase) followed by collection of the cells through filtration and centrifugation. Different strategies employing diverse enzymes will be presented. Moreover, it is important to have in mind the kind of cells going to be isolated and the possibility of separating the fetal membranes. Chorion, cotyledons, and especially the amnion are rich sources of mesenchymal stromal/stem cells. The amnion is also comprised of an epithelial layer from which amnion epithelial cells are obtained.

In order to isolate the amniotic mesenchymal stromal cells, first incubate membrane fragments with dispase (2.4 U/ml in PBS) for 9 minutes, followed by collagenase I (0.75 mg/ml) + DNase (20 µg/ml) solution in PBS for 150 minutes. An alternative option is only one digestion step using 1 mg/ml collagenase I for 120 minutes. Chorionic mesenchymal stromal cells may be isolated by performing two 9-minute dispase digestions, separated by one 9-minute wash step in RPMI-1640 containing 100 U/ml penicillin, 100 µg/ml streptomycin, and 10% heat-inactivated fetal bovine serum (FBS). Another way is to replace the dispase of the first digestion step by collagenase IV. After gentle centrifugation (150 × *g* for 3 minutes), filtrate each supernatant containing amniotic or chorionic mesenchymal stromal cells in a 100 µm cell strainer. However, mesenchymal stromal cells may be isolated from the placenta without previously separation of its components. In such situation, mince pieces from fetal membranes and incubate for 10 minutes in DMEM with 0.25% trypsin/EDTA, 10 U/ml DNase I, and 0.1% collagenase. Tissues should be pipetted vigorously up and down, avoiding foam, for 5 minutes. Allow large pieces of tissue to settle under gravity for 5 minutes. Transfer the supernatant to a fresh tube, neutralize with FBS, and then spin at 1500 rpm for 10 minutes.

Amniotic undigested pieces (when amnion is incubated either with dispase and collagenase I or collagenase I only, as previously described) may be submitted to another digestion step with trypsin (0.25% in PBS) for 2 minutes, in order to obtain epithelial cells.

Moreover, epithelial cells may be harvested from amniotic membrane fragments after digestion only with trypsin/EDTA, at 37°C. One way is to use the enzyme at 0.125% and incubate three times at 20 minutes each. Alternatively, two digestion steps of 30 minutes each, shaking (0.20% trypsin/EDTA) or two steps of 20 minutes (0.25% trypsin/EDTA) are considered. Trypsin should be inactivated by adding FBS. Collect the cells after centrifugation (1000 rpm for 5 minutes). It is important to highlight that, in all situations, the amniotic epithelial cell layer must be scraped out to remove the underlying tissues, such as the spongy and fibroblast layers, to obtain a pure epithelial layer.

In addition to all disaggregation protocols for amniotic membrane, to achieve different populations of cells (amniotic mesenchymal stromal and amniotic epithelial), the whole membrane may be digested giving a mix of cells, known as amniotic derived cells. Membrane fragments are digested with 0.25% trypsin/EDTA for 30 minutes at 37°C. Cells are collected after filtration in 100 µm cell strainer and three wash steps in cold PBS.

Another protocol requires three digestion steps (1x15 minutes; 2x30 minutes) in 0.05% trypsin at 37°C. Wash cells three times in PBS and collect after centrifugation (1000 rpm for 5 minutes).

Another placental tissue used for cell isolation is cotyledon. Minced cotyledon is digested with 0.25% trypsin for 60 minutes at 37°C. Incubate the undigested fragments with 12.5 U/ml collagenase I for 60 minutes at 37°C. Collect cells by filtration (100 µm cell strainer) and centrifugation (300 × g for 10 minutes).

Finally, when fetal membranes are difficult to distinguish (i.e. small animals as mouse) incubate the placenta with dispase (2.4 U/ml in PBS) for 5 minutes at 37°C, followed by collagenase (0.75 mg/ml) + DNase (20 µg/ml) in PBS for 90 minutes at 37°C. After filtration (100 µm cell strainer) and centrifugation (300 × g for 5 minutes) a mix of amniotic and chorionic cells are obtained. Alternatively, digest placenta fragments with 300 U/ml collagenase II for 1 hour at 37°C in water shaker. Neutralize enzyme activity with α -minimal essential medium (α -MEM) containing 10% FBS. Collect the cells after filtration and centrifugation.

Cells obtained after any of the procedures mentioned above must be plate onto noncoated tissue culture dishes or flasks. Cells may be seeded at $7 \times 10^3/\text{cm}^2$ (amniotic mesenchymal stromal cells) and $14\text{--}21 \times 10^3/\text{cm}^2$ (amniotic epithelial cells). The medium should be supplemented with serum, mainly FBS. There is a common practice, in some research centers, to use 20% of serum for the primary culture, and then, replace it for 10% in further passages. However, such cells are perfectly capable to survive and proliferate when incubate with 10% serum from the first passage. The most commonly used media are Roswell Park Memorial Institute (RPMI)-1640, DMEM, α -MEM, and DMEM/F12. It is also important to supplement the medium with antibiotics (100 U/ml penicillin and 100 µg/ml streptomycin or 1% antibiotic-antimycotic solution), L-glutamine (2 mM), nonessential amino acids (1%), β -mercaptoethanol (55 µM), and sodium pyruvate (1 mM). Cells are incubated at 37°C in a humid atmosphere (5% CO₂).

Morphological characterization of the cells, by light microscopy, indicates that amniotic epithelial cells show ground or oval shapes, cluster formation, relatively big nucleus (some can reach half of the cell diameter) and many fat drops in the cytoplasm. Both amniotic and chorionic mesenchymal stromal cells show the fibroblast-like appearance and very high proliferation rate, reaching 70-80% confluence after one to three weeks from harvesting.

Cells may either be used in experimental procedures just right after culturing or be frozen in 10% dimethyl sulfoxide (DMSO) and 90% FBS. An alternative freezing media is 10% DMSO, 40% DMEM, 50% FBS. Prior to using these cells, it is recommended to analyze the viability, which may be determined by use of trypan blue dye, and cell number in a hemocytometer.

In addition, it is important to highlight that all experimental procedures must have ethics committee's approval and, regarding human placental tissues, the previous donor consent.

3.2. Established (transformed) cell lines

There are a lot of cell lines derived from placenta. These cells are cultured with subtle differences, for research purposes and some of them are able to secrete hormones and other molecules. Some examples will be present.

BeWo (ATCC® number CCL-98™) is a human epithelial cell line, derived from choriocarcinoma, that produces progesterone, human chorionic gonadotropin (hCG), placental lactogen, estrogen and other reproductive hormones. BeWo is cultured in F-12K medium enriched with 10% FBS. This cell is sensitive to human poliovirus 3 and the vesicular stomatitis virus. Other human epithelial cell lines derived from choriocarcinoma are JEG-3 (ATCC® number HTB-36™) and JAR (ATCC® number HTB-144™). JEG-3 produces hCG, progesterone and placental lactogen. The cells are able to transform steroid precursors to estrone and estradiol. The line is cultured with EMEM supplemented with 10% FBS. JAR cells produce estrogen, progesterone, hCG, and placental lactogen and are cultured in RPMI 1640 supplemented with 10% FBS.

The *M. musculus* mast cells 10P2 (ATCC® number CRL-2034™) and 10P12 (ATCC® number CRL-2036™) were established by transformation of placental cells from a 10 day mouse embryo, and are cultured in suspension with RPMI 1640 medium supplemented with 0.05 mM 2-mercaptoethanol and 10% FBS. These cells possess receptors for IgE. Both lines represent a transformed stage of a very early hematopoietic precursor and share surface antigens with multipotent stem cells. The 11P0-1 (ATCC® number CRL-2037™) cell line is similar with the others except for being derived from a 11 day mouse embryo.

The FC-47 (ATCC® number CRL-6094™) is a fibroblast derived from the cat (*Felis catus*) normal placenta, with adherent properties and cultured in DMEM supplemented with 10% FBS.

The 3A-(tPA-30-1) (ATCC® number CRL-1583™) is a human SV40 transformed cell line, derived from placenta, showing an epithelial morphology. When incubated at 40°C, these cells are able to synthesize hCG and alkaline phosphatase. The cells express the transformed phenotype at the permissive temperature (33°C) and the non-transformed phenotype at the non-permissive temperature (40°C). The line has a limited life expectancy of 15 to 18 passages before entering the crisis stage. Medium required for incubation is α -MEM supplemented with 10% FBS.

ChaGo-K-1 (ATCC® number HTB-168™) is a human epithelial cell line, derived from bronchogenic carcinoma, that produces hCG alpha subunit only, estradiol, progesterone, and mucin (apomucin, MUC-1, MUC-2). The cells are cultured in RPMI 1640 supplemented with 10% FBS.

There are several cell lines derived from normal human placenta, which are available for research purposes, such as Hs 726.PI (ATCC® number CRL-7460™), Hs 730.PI (ATCC® number CRL-7464™), Hs 795.PI (ATCC® number CRL-7526™), Hs 798.PI (ATCC® number

CRL-7529™), Hs 799.PI (ATCC® number CRL-7530™), Hs 815.PI (ATCC® number CRL-7548™). All of them are cultured in DMEM supplemented with 10% FBS, at 37°C, in humidity atmosphere (5% CO₂).

3.3. Placenta-derived stem/progenitor cells

Various undifferentiated stem cell sources have been proposed for regenerative medicine, each having their advantages and drawbacks. Embryonic stem cells (ESCs) are characterized by pluripotency and an unlimited self-renewal capacity, but they may present high tumorigenic potential and their use is associated with major ethical concerns. In contrast, mesenchymal stem cells (MSCs) are not ethically restricted, but show limited capacity to proliferate and differentiate into different cell lineages (multipotency). MSCs reside within bone marrow, adipose tissue, dental pulp, and many other tissues. Although bone marrow MSCs are the most studied and the best established, there are some limitations of their use due to invasive nature of bone marrow aspiration, donor site morbidity, inadequate cell numbers, and the limited capacity of proliferation and differentiation. Thus, researchers started looking for alternative sources of MSCs that can be obtained noninvasively and in sufficient quantity. Studies have shown that MSCs may be isolated from placenta (either from maternal or fetal components, especially from the amnion), which are generally discarded as medical waste after delivery, and are therefore without ethical concerns associated with their use. Placenta-derived MSCs have been described to combine characteristics from both embryonic and mesenchymal stem cells: the ability to differentiate into all three germ layers and lack of tumorigenicity. Although specific fetal membrane components have already been considered in the above section “primary culture” of this chapter, here aspects of placenta MSCs will be discussed, without distinction between any fetal membranes as a specific source of cells, even though the amniotic MSCs are the most studied. It should be highlighted that efforts toward standardization of the terminologies used have been made (21) in the First International Workshop on placenta-derived stem cells, who proposed the following nomenclature: amniotic epithelial cells, amniotic mesenchymal stromal cells, chorionic mesenchymal stromal cells, and chorionic trophoblastic cells.

One way to identify a population of cells is by performing its immunophenotype characterization, mainly for surface marker expression. Current methodologies employed are immunocytochemistry, immunofluorescence, and flow cytometry. Placenta MSCs reveal profiles in between embryonic and adult stem cells, as summarized in Table 1 where it is shown some of the most used surface markers, as cluster of differentiation (CD), for categorizing stem cell populations. It is important to note that neither embryonic nor mesenchymal cells (and placenta-derived MSC as well) express the hematopoietic stem cell markers CD11b, CD34, and CD45. Placenta MSCs show a positive expression profile for CD29, CD73, CD166, and major histocompatibility complex (MHC) class I molecules (human leucocyte antigen (HLA) -A, -B, -C) and a negative expression profile

for CD11b, CD31 (endothelial marker), CD34, CD45, and MHC class II molecules (HLA-DR, -DP, -DQ).

Although there are common properties between placenta and bone marrow derived MSCs, flow cytometric analyses performed by different research groups report that placenta MSCs do not express CD271 (22) and show different patterns for CD90 expression: either low [4.7% (14)], moderate [22.5% (23)], or high expression [> 95% (22)] and even the presence of two distinct subpopulations of placenta MSCs positive for CD90 (24).

Taken together, placenta MSCs satisfy the minimal criteria for identifying multipotent mesenchymal stromal cells (27). Furthermore placenta MSCs, different from other mesenchymal cells, are able to differentiate into ectodermal (neural and retinal cells), endodermal (pancreatic beta cells), and mesodermal (adipocytes, osteocytes, and chondrocytes along with myotubule formation and endothelial cells) lineages *in vitro* (24,25).

Moreover, placenta MSCs are positive for surface markers that are expressed by embryonic stem cells but not by mesenchymal cells. Among them are octamer binding protein 4 (Oct-4), Sox2, Nanog, stage-specific embryonic antigen (SSEA)-1, SSEA-4, GCTM2, Tra-1-60, and Tra-1-80 which are routinely evaluated by immunocytochemistry or flow cytometry (14,23,26). Even when analyzing such expression using more sensitive assays, as in real time polymerase chain reaction (q-PCR), it is observed that placenta MSCs significantly express high levels of Oct-4, Nanog and Sox2 (25). In addition, it was detected by immunofluorescence analysis that human amniotic stem cells, different from other MSCs, are positive for the neural stem cell markers Nestin, Vimentin, Musashi-1, and PSA-NCAM (28). However, all of these data should be analyzed carefully. Some studies indicate variable percentages of positive cells for different pluripotent markers, indicating that more studies are need.

Besides the pluripotency potential of placenta MSCs and the lack of ethical concerns, an important characteristic that lead placenta MSCs to a great importance for clinical application is their low immunogenicity. This property is partially explained by the fact that placenta MSCs do not express MHC class II molecules (HLA-DR, -DP, -DQ) and co-stimulatory molecules *in vitro*, as reported by different research groups and using different methodologies (flow cytometry, qPCR, western blotting), indicating that such cells can be effectively used for both autologous and allogenic transplantations. It was recently demonstrated that fetal membrane MSCs are capable of suppressing proliferation in a mix lymphocyte reaction, decreasing interferon (IFN)- γ and interleukin (IL)-17 production, stimulating IL-10 secretion, and increasing levels of adhesion markers CD54, CD29, and CD49d (29). In addition, placenta MSCs are able to engraft and survive long-term in various organs and tissues without evidence of inflammation or rejection after transplantation into neonatal animals and after *in utero* transplantation into pregnant rats (30,31). Therefore, placenta MSCs are viable candidates for cell-based therapeutic approaches.

Surface marker	Cell types			References
	ESC	MSC	Placenta – MSC	
CD11b	-	-	-	(24)
CD34	-	-	-	(14,22–27)
CD45	-	-	-	(14,22–24,26,27)
CD29	-	+	+	(14,22,24–27)
CD73	-	+	+	(22–26)
CD90	-	+	ND	(14,22–25,27)
CD105	-	+	-	(23,25,27)
CD166	-	+	+	(23,26)
CD271	-	+	-	(22)
Nanog	+	-	+	(25,26)
Oct-4	+	-	+	(14,23,25,26)
Sox-2	+	-	+	(23,25,26)
SSEA-1	+	-	+	(14)
SSEA-4	+	-	+	(23,26)
GCTM-2	+	-	+	(23,26)
Tra-1-60	+	-	+	(26)
Tra-1-80	+	-	+	(26)

(+): presence; (-): absence; ND: not defined

Table 1. Surface marker expression from embryonic stem cells (ESC), adult mesenchymal stem cells (MSC), and placenta derived mesenchymal stem cells (placenta-MSC).

When considering cellular therapy it is important to keep in mind that a great number of cells will be needed and cell banking may be required. As a result, it is imperative that cells possess some important characteristics: abundant source, high proliferation rate, stability over further passages, efficient freezing, and high viability after thawing. Placenta MSCs have been demonstrated to fulfill such requirements. It was recently reported that placenta MSCs at passage 30 are still able to proliferate in normal rates and keep a stable karyotype (25). Such characteristics are observed in ESCs but not in other types of MSCs, such as those derived from bone marrow or adipose tissue, which don't proliferate well and go to senescence around passages 8-10. Compared to ESCs, MSCs are more resistant to cryopreservation. Whilst ESCs require complex and expansive reagents to be frozen and great number of cells die, placenta MSCs are successfully frozen in 40% DMEM, 50% FBS, 10% DMSO, as well as bone marrow and adipose tissue MSCs, with high recovery rates after thawing. Among many different assays to evaluate cellular viability, the measurement of mitochondrial metabolic rate using MTT (3-[4, 5-dimethyl-2-thiazolyl]-2, 5-diphenyl-2H-tetrazolium bromide) to indirectly reflect viable cell numbers has been widely applied. However, if the purpose is to evaluate the viability and the number of cells immediately

before their transplantation to human or animals, trypan blue dye exclusion assay is preferable because only the unavailable cells are dyed, while the desired cells are maintained viable, ready to use.

Before considering using placenta MSCs for clinical purposes, preclinical studies may be performed and the results must be carefully interpreted in order to assure safety for the patient and success in the therapy. Many preclinical studies on placenta-derived cells and amniotic membrane were reviewed (32), considering a wide range of diseases as neurological, pancreatic, muscle, vascular, cardiac, and pulmonary disorders along with liver fibrosis and applications for tissue engineering. Overall the results are promising and, although differentiation of placenta MSCs to specific lineages has been considered the first necessity for therapeutic applications *in vivo*, it seems that the beneficial effects reside on paracrine effects. However, both mechanisms (differentiation or paracrine) are not mutually exclusive and can account for the promising results reported in the literature.

As placenta MSCs share with ESCs some common characteristics such as surface marker expression and plasticity *in vitro*, it was assumed that placenta cells could regenerate damaged tissues due to pluripotency potential. To date, however, preclinical studies have failed to demonstrate the differentiation of engrafted cells. Most of the time the success of the cell transplantation may more likely be due to the secretion of bioactive molecules that could act on other cells and on the microenvironment which they occupy thereby promoting endogenous tissue repair or eliciting other beneficial effects (anti-inflammatory, anti-scarring, angiogenic effects) through paracrine actions.

The abundance of pluripotent cells, high yield, rapid proliferation rates, stable karyotype, plasticity and immunomodulatory properties make placenta MSCs an ideal choice for clinical and tissue engineering applications. Nevertheless, further studies are needed to demonstrate the pluripotency ability of placenta MSCs *in vivo* and to elucidate the mechanisms by which these cells promote physiological and clinical improvements.

4. Conclusion

Eutherian mammalian fetus growth is characterized by the early development of fetal membranes, specially the trophoblast, which produces hormones for the establishment, maintenance and end of pregnancy. The fetomaternal interface varies among species and is a basis for placental structure classification. Primary cultures from placenta are obtained in an easy and not consuming fashion allowing a great number of research applications. Various undifferentiated stem cell sources have been proposed for regenerative medicine. In this setting placenta-derived cells show some advantages as potential to differentiate into the three germ layers, lack of tumorigenicity, low immunogenicity, and more importantly that they are isolated in high yields from sources generally considered medical waste, avoiding ethical concerns.

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