

Placenta as a Source of Stem Cells and as a Key Organ for Fetomaternal Tolerance

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The placenta encloses the very beginnings of the mystery of life, but discloses an ever-increasing amount of information toward our understanding not only of cell development, maturation, and differentiation, but to an even greater extent, the fundamental mechanisms of immunological tolerance.

For many years, the human placenta has attracted the attention of scientists because of the essential role it plays in development of the growing embryo by facilitating gas and nutrient exchange between the mother and fetus, while this tissue has intrigued researchers for an even longer time because of its role in maintaining fetomaternal tolerance. More recently, this tissue has also been investigated as a potential source of stem cells for application in regenerative medicine.

2.1 Placenta Structure

The human term placenta is round or oval in shape with a diameter of 15–20 cm and a thickness of 2–3 cm. The decidua constitutes the maternal portion of the placenta and is derived from the maternal endometrium. The portion of the decidua at which implantation takes place is called the decidua basalis, while the portion adjacent to the chorion leave is termed the decidua capsularis. The decidua parietalis covers the remainder of the endometrium.

The fetal portion of the placenta is composed of the placental disk and the amniotic and chorionic membranes.

The placental disk is composed of the chorionic plate and the basal plate, which form a base and cover, respectively, to enclose the intervillous space. The multilayered chorionic plate faces the amniotic cavity and is composed of a spongy layer, followed by the chorionic mesodermal layer, and a Langans' fibrinoid layer interposed with highly variable amounts of proliferating extravillous cytotrophoblast cells. The amnion covers the face of the chorionic plate, which is closest to the amniotic cavity, while chorionic villi project from the other side of the chorionic plate and either terminate freely in the intervillous space where maternal blood flows, or anchor the placenta through the trophoblast of the basal plate to the endometrium.

Despite the fact that there are different types of villi with different functional specializations, all villi exhibit the same basic structure, consisting of an inner stromal core containing fetal vessels and connective tissue, in which mesenchymal cells, fibroblasts, myofibroblasts, and fetal tissue macrophages (Hofbauer cells) are dispersed. A basement membrane separates the stromal core from an uninterrupted multinucleated outer layer, called syncytiotrophoblast, with single or aggregated cytotrophoblast cells found between the syncytiotrophoblast and its basement membrane. The ramifications of the villous trees differ in their caliber, vessel structure, stromal arrangement, and position within the villous tree itself, and can be distinguished as stem villi, which mechanically support the structure of the villous tree, immature intermediate villi, which act as growth zones and produce new sprouts, and mature intermediate villi and terminal villi, both of which represent the main exchange area in the third trimester placenta. Fetal blood is carried to the villi

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via the branches of the umbilical arteries. After circulating through the capillaries of the villi, the fetal blood absorbs oxygen and nutritional materials from, and transfers waste products to the maternal blood through the villous walls. The purified and nourished fetal blood is then carried back to the fetus via the umbilical vein.

The basal plate is the most intimate and important contact zone between maternal and fetal tissue. It is composed of a superficial stria of Rohr's fibrinoid, which faces the intervillous space, followed by a layer of extravillous cytotrophoblast and connective tissue, and another fibrinoid layer (Nitabuch's fibrinoid layer), which is located next to the compact decidual layer. In term placenta, the basal plate is usually of variable thickness owing to the fact that it loses its typical layering as gestation progresses. Protrusions extending from the basal plate into the intervillous space produce the placental septa, which divide the fetal part of the placenta into the irregular cotyledons.

At the regions of placenta that are in contact with the decidua capsularis during gestation, the intervillous space is obliterated so that the chorionic plate and the basal plate fuse with each other forming the chorionic membrane (commonly called the chorion leave), which consists of a chorionic mesodermal (CM) and chorionic trophoblastic (CT) region. The chorionic mesoderm consists of a network of collagen bundles intermingled with finer fibrils in which fibroblasts and macrophages are usually observed. A basal lamina separates the chorionic mesoderm from the highly variable layer of extravillous trophoblast cells that represent the only residue of the former villi of the chorion frondosum (see section on Embryological Development of the Placenta) intermingled with trophoblastic residues of the primary chorionic plate and basal plate.

The amnion is an uninterrupted membrane, which is in contact with the amniotic fluid on its inner surface, while on the other side it is in contact with the chorion leave, the chorionic plate, and the umbilical cord. The amnion is contiguous over the umbilical cord with the fetal skin.

Structurally, the amniotic membrane is a thin avascular sheet composed of an epithelial layer and connective tissue. The amniotic epithelium (AE), which is in contact with the amniotic fluid, is a single layer of flat, cuboidal to columnar epithelial cells, which is attached firmly to a distinct basal lamina that is in turn

connected to the amniotic mesoderm (AM). In the amniotic mesoderm, an acellular compact layer of interstitial collagens I, III, and fibronectin, and a deeper network of widely dispersed fibroblast-like mesenchymal cells and rare macrophages are distinguishable.

The amniotic mesoderm and chorionic mesoderm are loosely connected via a spongy or intermediate layer, which is a reticular zone composed of loosely arranged collagen fibers that results from the incomplete fusion of amniotic and chorionic mesoderm during early pregnancy. Both layers contribute to the mechanical stability of the membranes, but it is the fibers of the compact layer of the AM which confer most of the tensile strength to the fetal membranes.^{1,2}

2.2 Embryological Development of the Placenta

Development of the placenta begins as soon as the blastocyst implants in the maternal endometrium (6–7 days after fertilization). At this stage, the blastocyst is a flattened vesicle in which most of the cells form an outer wall (trophoblast), which surrounds the blastocystic cavity (blastocoel). A small group of larger cells, known as the inner cell mass, is apposed to the inner surface of the trophoblastic vesicle. The trophoblast eventually gives rise to the chorion, whereas the embryo, the umbilical cord, and the amnion are derived from the inner cell mass.

As the blastocyst adheres to the endometrial epithelium, the invading trophoblast erodes the deciduas, allowing the embedding of the blastocyst. During implantation, the trophoblastic cells of the implanting pole of the blastocyst show increased proliferation, resulting in a bilayered trophoblast, made up of a multinucleated outer syncytiotrophoblast, which originates from fusion of neighboring trophoblast cells, and an inner, mononucleated cytotrophoblast layer.

By day 8, small intrasyncytial vacuoles appear in the syncytiotrophoblast mass at the implantation pole. These vacuoles grow rapidly and become confluent, forming a system of hematic lacunae separated by lamellae and pillars of syncytiotrophoblast (trabeculae). Primary villi can be observed after invasion of the cytotrophoblast into the trabeculae, while the lacunae form the intervillous space where maternal blood flows.

In early pregnancy, the entire chorionic membrane is covered by villi, which are almost uniform in size, but which soon begin to develop unequally. At the anti-implantation pole, villous degeneration and fibrinoid deposition in the intervillous space give rise to the smooth chorion or chorion leave, while at the implantation pole, villous proliferation forms the leafy chorion or chorion frondosum.

At day 8–9 after fertilization, morphological changes occur in the inner cell mass, which differentiates into two layers, the epiblast and the hypoblast, that together form the bilaminar embryonic disk. From the epiblast, some small cells, that will later constitute the amniotic epithelium, appear between the trophoblast and the embryonic disk and enclose a space that will become the amniotic cavity. The three germ layers of the embryo (endoderm, mesoderm, ectoderm) will also originate from the epiblast.

Once the lining of the amnion has developed, the amniotic cavity surrounds the embryo from all sides and amniotic fluid begins to accumulate within the amniotic cavity. The accumulation of amniotic fluid within the amniotic cavity causes the amnion to expand and ultimately to adhere to the inner surface of the trophoblast (chorion).

From the other side of the bilaminar disk, some cells from the hypoblast migrate along the inner wall of the blastocoel giving rise to the exocoelomic membrane. The exocoelomic membrane and the blastocoel modify to form the yolk sac, while cells of the exocoelomic membrane and the adjacent trophoblast form the extraembryonic reticulum. Some hypoblast cells then migrate along the outer edges of extraembryonic reticulum to form a connective tissue known as the extraembryonic mesoderm, which surrounds the yolk sac and amniotic cavity, and later forms the amniotic mesoderm (AM) and chorionic mesoderm (CM). The amniotic mesoderm and chorionic mesoderm are separated by a cavity called the exocoel, which is compressed during amniotic cavity expansion.^{1,3}

All these events occur before gastrulation (third week after fertilization), the process through which the bilaminar disk differentiates into the three germ layers (ectoderm, mesoderm, and endoderm), which leads to the hypothesis that placental tissues themselves may harbor cells that display the potential to differentiate toward different lineages.

2.3 Immunology of the Placenta

Immune evasion by the allogeneic fetus has intrigued immunologists since the beginning of the twentieth century with the observation of Little (1924) that the mother must in some way be able to tolerate the presence and growth of the fetus, leading him to propose that the embryo might have “no definite physiological characteristics which are individual enough to be recognized as foreign by the mother.” In 1932, Witebsky and Reich suggested that human trophoblast may be nonantigenic and could be capable of acting as a barrier between the mother and the fetus. However, it was Medawar who identified the truly paradoxical nature of the immunological relationship between the mother and the fetus in 1953, declaring that “the immunological problem of pregnancy may be formulated thus: how does the pregnant mother contrive to nourish within itself, for many weeks or month, a fetus who is an antigenically foreign body?”⁴ In what eventually became well known as Medawar’s paradox, Medawar proposed that the lack of fetal rejection by the mother might be explained by three mechanisms: (a) that there is an anatomical barrier between the fetus and the mother; (b) that the fetus is antigenically immature; (c) that the maternal immune system might be immunologically inert.⁵

Since the time of Medawar, it has become evident that these mechanisms cannot fully explain why the fetus is not rejected by the mother, and other site-specific immune suppression mechanisms must therefore be considered.

For many years, in accordance with the first mechanism of Medawar’s paradox, the trophoblast was considered an impenetrable barrier, which prevents exposure of the fetus to the maternal immune system. More recently, however, bidirectional transfer of fetal and maternal cells through this tissue has been reported by numerous investigators. Fetal cell microchimerism was originally demonstrated in female mice,⁶ and long-term persistence of fetal cells in the bone marrow of these animals postpartum has been observed. During human pregnancy, fetal cells enter the maternal circulation from as early as 6 weeks into gestation⁷ and can persist in maternal blood and tissues for decades after pregnancy⁸ without any signs of graft-versus-host reaction or graft rejection. Data concerning the health consequences of persistent fetal cells in maternal tissues are contradictory. Initially, fetal cells were thought to

be implicated in autoimmune diseases, based on the observation that increased levels of fetal microchimerism were detected in women affected by autoimmune diseases.⁹⁻¹² However, to date there is no concrete evidence to prove that fetal cells cause autoimmune disease,¹³ and increasing scientific evidence now suggests that these cells may actually help to combat disease. Support for this hypothesis comes from studies which show that fetal microchimerism is commonly detected in the peripheral blood of healthy women,^{8,14} while the multilineage differentiation potential of fetal cells, which have been transferred to the mother, has also been demonstrated,¹⁵ suggesting that these cells may play a role in tissue regeneration. Furthermore, fetal cell microchimerism may also confer a beneficial effect by performing immune surveillance for malignant cells, as supported by the observation that fetal cell chimerism is reduced in women with breast cancer compared to healthy women.¹⁵⁻¹⁷

With regard to the second mechanism of Medawar's paradox, it has been shown that fetal cells do in fact express MHC I and MHC II, which are antigenically mature and detectable in maternal circulation.¹⁸ The lack of expression of the classical MHC class I and MHC class II molecules by the trophoblast cells, which are in contact with maternal circulation, was long considered to be a mechanism for evading detection and destruction by maternal cells. However, it was later shown that interstitial trophoblast populations, which are in contact with maternal decidua, do in fact express the MHC class I molecule.^{19,20} Furthermore, studies by Shomer and Rogers using transgenic technology showed that expression of allogeneic MHC class I molecules on various trophoblast populations does not increase fetal loss, even in the presence of defects in the Fas/FasL pathway.^{21,22}

Finally, concerning the third point of Medawar's paradox, it is clear that the maternal immune system is not inert during pregnancy, and is instead able to recognize fetal cells, as proven by the observation that fetal tissues are rejected when transplanted into pregnant rats.²³ Moreover, it has also been shown that the maternal immune system is able to attack the pre-implantation blastocyst when the zona pellucida is removed.²⁴ Although maternal T cells respond to fetal antigens during normal pregnancy, the nature of the immune response appears to change during gestation, as demonstrated by conflicting data regarding expansion and deletion of maternal T cell subsets at different

time points during gestation.²⁵⁻²⁸ The production of alloantibodies by maternal B cells to paternally inherited antigens has also been reported, and while alloantibody production increases with subsequent pregnancies, it does not affect the outcome of the pregnancy.^{29,30}

2.4 Possible Mechanisms Controlling Fetomaternal Tolerance

Many local mechanisms that contribute to protection of the fetus from the maternal immune system have been identified at the fetomaternal interface, although it is not yet clear how these mechanisms interact with each other.

The most well-known of these mechanisms have been summarized in several reviews³¹⁻³⁶ and those which have been most commonly described include: (a) expression of nonclassical MHC molecules by trophoblastic cells; (b) expression of the IDO enzyme by placental cells, resulting in tryptophan depletion and kyurenine production; (c) FasL expression by trophoblastic cells; (d) expression of complement regulator proteins by trophoblastic and decidual cells.

Regarding the first of the mechanisms listed here, it has been shown that trophoblastic cells express the nonclassical HLA molecules HLA-E, HLA-F, and HLA-G. While the function of HLA-F is unknown, protection of the fetus from allogeneic T-cell responses and NK cell-mediated damage have been attributed to HLA-G,³⁷ which is supported by the observation that T-cell proliferation is inhibited when these cells are cultured in mixed lymphocyte reactions with HLA-G-transfected cells.³⁸ In vitro studies have shown that HLA-G can also induce apoptosis of lymphocytes which have been previously activated through the Fas/FasL pathway.³⁹ Meanwhile, it has been hypothesized that the effect of HLA-G on NK cell activity is not induced directly, but rather, that it requires the expression of HLA-E on trophoblastic cells. It is thought that HLA-G promotes and stabilizes the expression of HLA-E at the cell surface, allowing it to bind the CD94–NKG2 inhibitory receptor on NK cells, which leads to inhibition of NK activity.^{40,41} In addition, the interaction of HLA-G with dendritic cells through KIR-related leukocyte Ig-like receptors may have an indirect effect on the immune response by tolerizing

dendritic cells and facilitating the generation of regulatory T cells.^{42,43}

Regarding the role of Indoleamine 2,3-dioxygenase (IDO) in promoting fetomaternal tolerance, evidence from a study by Munn and Mellor suggests that synthesis of this tryptophan-catabolizing enzyme by placental cells could provide protection of the fetus from maternal T-cells, with the observation that inhibition of this enzyme during murine pregnancy resulted in fetal allograft rejection.⁴⁴ IDO is expressed by trophoblast giant cells in mice,⁴⁵ and is thought to prevent immune responses to the fetus by inhibiting maternal T cell activation either by depriving T cells of tryptophan⁴⁶ or by producing catabolites of tryptophan (kynurenines), which prevent activation and proliferation of T cells, B cells, and NK cells in vitro.⁴⁷ However, subsequent studies have shown that IDO-knockout in mice still results in normal litters,⁴⁸ suggesting that other mechanisms, such as the presence of another enzyme, tryptophan 2,3-dioxygenase, which also promotes tryptophan catabolism,⁴⁹ can compensate for the loss of IDO activity during gestation. It has been reported that IDO may also have indirect effects on immune responses by affecting the function of IDO-expressing dendritic cells, thereby preventing T cell regulation.⁵⁰ While tryptophan catabolism appears to be essential in murine pregnancy, its role in human pregnancy is less clear.³² Although it is known that IDO is expressed by extravillous and villous trophoblast cells in humans, and that its expression increases during the first week of pregnancy and diminishes during the second trimester,⁵¹ IDO deficiency has not been reported as a cause of pathology during human pregnancy.

Support for the hypothesis that apoptosis may be an important determinant in fetomaternal tolerance comes from studies which suggest that maternal tolerance of the fetus may be mediated by the Fas/FasL system, which plays a critical role in promoting apoptosis, and was also identified some years ago as an important pathway for controlling maternal immune responses at the fetomaternal interface.⁵²⁻⁵⁴ The maternal decidua and fetal tissues express FasL on their cell surface and cause apoptosis of activated maternal Fas-expressing lymphocytes,^{52,55} with apoptosis detectable at the maternal-fetal interface throughout gestation.^{56,57} However, recent studies implicate a more complex role of FasL in fetomaternal tolerance, with the demonstration that this molecule may promote allograft rejection

rather than survival.^{58,59} Although some mechanisms to explain this have been proposed from studies that report the presence of FasL in trophoblast microvesicles, which can promote fetal rejection,^{60,61} a more complete understanding of the role of Fas in fetomaternal tolerance is still required.

A role for the complement system has also been hypothesized in the control of fetomaternal tolerance. This system is a component of natural immunity that can be activated by pathogens, and also after transplantation of allogeneic or xenogeneic cells, resulting in induction of inflammatory cell chemotaxis, enhanced phagocytosis, and promotion of cell lysis by the membrane attack complex. Therefore, the complement system must be tightly regulated in order to protect tissues from damage associated with the inflammatory process, and in the context of fetomaternal tolerance, it has been shown that complement regulatory molecules play an important role in allowing the fetus to regulate maternal processes that would otherwise result in fetal tissue damage. In mice, expression of the complement regulator protein *Crry* prevents deposition of the C3 and C4 complement components, thereby preventing activation of the complement cascade at the fetomaternal interface.^{62,63} The role of *Crry* in contributing to fetomaternal tolerance in mice is confirmed by the observation that a deficiency in this protein results in gestational failure.⁶⁴ Unlike mice, humans express multiple types of complement regulatory molecules at the fetomaternal interface, such as DAF, MCP, and CD59, and a role for these molecules in regulation of the complement cascade at the C3 level has also been demonstrated.^{65,66} The expression of complement regulatory molecules by invading fetal trophoblast cells could be the result of a response to sublytic levels of complement activity, which may be encountered by these cells as they invade the uterine decidua, via a mechanism analogous to that observed during organ transplantation in which increasing levels of antibody and complement activation have been shown to result in increased resistance of the graft to complement-mediated injury.⁶⁷

In trying to understand the mechanisms of fetomaternal tolerance, the possible role of specific leukocyte subtypes that are present at the fetomaternal interface, and which very likely play different and important roles in this process, should also be considered.^{31,68} For further reading in this area, we refer readers to comprehensive reviews that have been published describing

the characteristics of different leukocyte types which have been identified at the fetomaternal border either at the trophoblastic or decidual level, including NK cells,⁶⁹⁻⁷¹ regulatory T cells,^{33,72,73} dendritic cells, and macrophages.^{74,75} Here, we will focus instead on results which have recently been obtained from studies exploring the immunomodulatory features of cells derived from the amniotic fetal membrane, and their possible roles in fetomaternal tolerance.

Support for the hypothesis that cells derived from the fetal membranes may contribute to fetomaternal tolerance comes from studies which demonstrate that cells isolated from amniotic and chorionic membranes do not induce allogeneic or xenogeneic T-cell responses, and actively suppress T-cell proliferation.^{76,77} Furthermore, both human amniotic membrane and human amniotic epithelial cells have been shown to survive for prolonged periods of time after xenogeneic transplantation into immuno-competent animals, including rabbits,⁷⁸ rats,⁷⁹ guinea pigs,⁸⁰ and bonnet monkeys.⁸¹ Additionally, long-term engraftment has been observed after intravenous injection of human amniotic and chorionic cells into newborn swine and rats, with human microchimerism detected in several organs,⁷⁶ suggestive of active migration and tolerogenic potential of the xenogeneic cells. In addition, long-term survival of rat amnion-derived cells, with no evidence of immunological rejection or tumor formation, has been observed after allogeneic in utero transplantation of these cells into the developing rodent brain.⁸²

Recently, in the stromal layer of the amniotic membrane, two subpopulations have been identified, which differ in their expression of HLA-DR, CD45, CD14, CD86, CD11b, and which possess either T-cell suppressive or stimulatory properties.⁸³ Even though the roles of these two populations in the amniotic membrane are not yet known, it is tempting to speculate that they may both play a role in controlling fetomaternal tolerance.

In summary, although many mechanisms have been postulated in order to explain maternal acceptance of the fetus, the cause of this phenomenon remains to be clarified and many questions still remain: Is there an initiating mechanism for fetomaternal tolerance, or does it result from the cumulative effect of several mechanisms that interact with each other? If the latter is true, how then are these mechanisms integrated? In any case, it is clear that further studies are needed to gain a complete understanding of the mechanisms of

fetomaternal tolerance, which will constitute a fundamental tool for developing strategies of tolerance induction for organ transplantation, cell therapy, and tissue engineering in the future.

2.5 Placenta as a Source of Hematopoietic Stem Cells

Studies performed in mice have proven that in the embryo, hematopoiesis takes place in several anatomical locations, including the yolk sac, the aorta-gonad-mesonephros (AGM), the fetal liver, and the placenta.⁸⁴ However, the exact involvement of each of these regions in the processes of emergence, maturation, and expansion of hematopoietic stem cells has not yet been defined.

The mouse placenta is comprised of trophoblast and two mesodermal components: the chorionic mesoderm, which forms a continuum with the yolk sac (a bilayered organ composed of extraembryonic mesoderm and visceral endoderm) and the allantoic mesoderm, an appendage arising from the posterior primitive streak. The allantois fuses with the chorionic plate and gives rise to the umbilical vasculature and the mesodermal components of the fetal placenta. Interdigitations of the allantoic mesoderm with the trophoblast result in formation of the placental labyrinth, which is the site of oxygen and nutrient exchange between maternal and fetal blood.⁸⁴

The yolk sac, which was long considered to be the only site capable of producing hematopoietic stem cells (HSCs), is the first hematopoietic site to appear in mammals, producing the first primitive blood cells that terminally differentiate after circulating to the fetal liver.⁸⁵ The intra-embryonic AGM region, which is composed of the dorsal aorta, its underlying mesenchyme, and the adjacent vitelline and umbilical arteries, can also generate HSCs *de novo*. Furthermore, a recent study has shown that this region harbors precursors that display high proliferative potential, and the capacity for hematopoietic self-renewal and endothelial cell differentiation.⁸⁶

The fetal liver is the main site of hematopoietic expansion and differentiation during gestation, but unlike the yolk sac and AGM region, it is a site of hematopoietic colonization and not an intrinsic source of hematopoietic cells.⁸⁷

Appreciation of placental contribution to mammalian fetal hematopoiesis was gained after the discovery that the avian allantois retains cells with hematopoietic and endothelial potential.⁸⁸ Subsequent studies in mice revealed that the placenta contains multipotential clonogenic progenitors, which are present before liver colonization commences. These cells have the capacity to self-renew and to repopulate the hematopoietic system in irradiated adult hosts.^{89,90} It has also been reported that prior to fusion, the allantois and chorion are both potent sources of hematopoietic progenitors, as revealed by their expression of a key transcriptional factor for hematopoiesis (Runx1).⁹¹⁻⁹⁴ A recent study has provided further strong evidence that the mouse fetal placenta functions as a hematopoietic organ, with the demonstration that placenta-derived hematopoietic cells are capable of producing both myelo-erythroid and B and T lymphoid progeny, therefore confirming the multipotentiality of HSCs derived from placenta. Interestingly, it has also been demonstrated that HSCs emerge in large vessels within the placenta, leading to the proposal that the small vessels that constitute the placental labyrinth may serve as a niche where HSCs convene for maturation and expansion prior to colonization of the fetal liver.⁹⁵

2.6 Placenta as a Source of Nonhematopoietic Multipotent Stem and/or Progenitor Cells: In Vitro and In Vivo Studies

In addition to playing an essential role in fetal development, nutrition and maintenance of fetal tolerance, and acting as a source of hematopoietic stem cells, placental tissue also draws great interest as a source of other types of progenitor/stem cells, including mesenchymal stem cells.

Since 2002, numerous studies have demonstrated the presence of progenitor cells from different regions of the placenta through in vitro characterization and differentiation experiments. As summarized in recent reviews, various approaches have been reported for isolating cells, which display progenitor cell characteristics from different regions of placental tissues, namely, the mesodermal areas of the amniotic and chorionic fetal membranes, and the amniotic epithelium. Studies exploring the differentiation potential of these cells

have yielded promising results indicating that they display plasticity and are capable of in vitro differentiation toward lineages of the three germ layers: ectoderm, mesoderm, and endoderm.^{96,97} Here, we will use the nomenclature reported in a recent review when referring to cells derived from the different placental regions: hAEC for human amniotic epithelial cells, hAMSC for human amniotic mesenchymal stromal cells, and hCMSC for human chorionic mesenchymal stromal cells.⁹⁶ The review above also sets out a general consensus which has been established regarding the main features of mesenchymal stromal cells from human fetal membranes (hAMSC and hCMSC). Specifically, the minimum criteria for identifying hAMSC and hCMSC include: adherence to plastic; formation of fibroblast colony-forming units; a specific pattern of surface antigen expression whereby mesenchymal markers (CD90, CD73, CD105) are expressed (as shown by greater than 95% positivity for these markers), while hematopoietic markers (CD45, CD34, CD14, HLA-DR) are not expressed (as shown by positivity of less than 2%); fetal origin of the cells and differentiation potential toward one or more lineages including osteogenic, adipogenic, chondrogenic, and vascular/endothelial.⁹⁶

In support of the hypothesis that hAMSC may display some degree of pluripotency, gene expression of octamer binding protein-4 (OCT-4),^{77,98-101} SRY-related HMG-box gene 2 (SOX-2), reduced expressin-1 (Rex-1), and Nanog¹⁰¹ have been reported in these cells, while positivity for the stage-specific embryonic antigens SSEA-3 or SSEA-4 on hAMSC is still debated.^{96,102}

A possible association between hAMSC and the neuronal lineage has been demonstrated by studies that show that when freshly isolated, these cells express neuronal (Nestin, Musashi1, neuron-specific enolase, neurofilament medium, MAP2) and glial markers (glial fibrillary acidic protein), with increased expression of some of these observed after differentiation in specific neural induction media.^{101,103,104}

The potential of hAMSC to differentiate into hepatocytes was studied by Tamagawa and colleagues, who have shown that these cells express hepatocytic markers such as albumin, α -fetoprotein (α -FP), cytokeratin 18 (CK18), α_1 -antitrypsin (α_1 -AT), and hepatocyte nuclear factor-4 α (HNF-4 α). Furthermore, after hepatic induction of these cells, increased expression of the above-mentioned genes was observed, together with production of albumin and α -fetoprotein and storage of glycogen.¹⁰⁵

Investigation of the cardiomyogenic potential of hAMSC has shown that these cells express the cardiac-specific transcription factor GATA4 and cardiac-specific genes such as atrial myosin light chain (MLC)-2a, ventricular myosin light chain MLC-2v, and the cardiac troponins cTnI and cTnT. hAMSC have also been shown to integrate into cardiac tissue and differentiate into cardiomyocyte-like cells after transplantation into myocardial infarcts in rat hearts.⁹⁹

Enhancement of the cardiomyogenic and vasculogenic differentiation of human amniochorionic-derived cells has been observed after exposure of these cells to a mixed ester of hyaluronan, butyric, and retinoic acid (HBR). In particular, increased expression of cardiomyogenic (GATA4, NKX2.5) and endothelial genes (VEGF, vWF), as well as enhanced expression of the cardiac markers sarcomeric myosin heavy chain, α -sarcomeric actinin and connexin 43, has been observed in HBR-treated amniochorionic cells compared to untreated cells. Meanwhile, injection of both HBR-pretreated and non-pretreated cells into infarcted rat hearts has been shown to result in recovery to essentially normal indices of cardiac function.¹⁰⁶

In experiments investigating the angiogenic potential of amniotic membrane-derived cells, basal expression of endothelial-specific markers (FLT-1, KDR) and spontaneous differentiation into endothelial cells have been observed, while both of these have been shown to be enhanced by exposure of the cells to vascular endothelial growth factor (VEGF).¹⁰⁰

Not only do the stromal regions of placenta seem to contain progenitor/stem cells, but interesting data have also been obtained through studies of hAEC. Expression of embryonic stem cell markers such as the stage-specific embryonic antigen SSEA-4, TRA-1-60, and TRA-1-81 has been reported for these cells,^{102,107} and in addition, they have also been demonstrated to express molecular markers of pluripotent stem cells, including octamer-binding-protein-4 (OCT-4), SRY-related HMG-box gene 2 (SOX-2), and Nanog.^{107,108} The pluripotency of hAEC is further supported by a study of Tamagawa et al., whereby a xenogeneic chimeric embryo was created by mixing amniotic cells with mouse embryonic stem cells, with demonstration that amnion-derived cells were then able to contribute to the formation of all three germ layers.¹⁰⁹

Interestingly, the mesenchymal marker vimentin, although absent on freshly isolated hAEC, has also been shown appear during culture.^{110,111} The significance

of the expression of both epithelial and mesenchymal markers by hAEC remains to be elucidated, although it could be due to the spontaneous commencement of differentiation during culture, or perhaps to the so-called epithelial to mesenchymal transition in the amnion, as also suggested by Sakuragawa and co-workers.¹⁰⁴

To date, numerous studies have been undertaken to explore the differentiation capacity of hAEC, yielding results that confirm the plasticity of these cells.⁹⁶

A neuronal predisposition of hAEC has been demonstrated through pioneeristic studies by Sakuragawa and colleagues, who showed that these cells express neuronal and glial markers,¹¹² and also perform neuronal functions such as synthesis and release of acetylcholine, catecholamines, neurotrophic factors (brain-derived neurotrophic factor and neurotrophin-3), activin, and noggin.^{104,113-117} Furthermore, hAEC conditioned medium has been shown to have neurotrophic effects on rat cortical neurons¹¹⁶ and can support the survival of chicken neural retinal cells,¹¹⁸ while more recently, it has also been shown that human amniotic membrane promotes the growth of chicken dorsal root ganglia neurons in the absence of neurotrophic factors.¹¹⁹

Preclinical studies in animal models demonstrate that hAEC may be useful for central nervous system regeneration by exhibiting neuroprotective and neuroregenerative effects during acute phases of injury. For example, Sankar and coworkers observed robust regeneration of host axons and enhanced survival of axotomized spinal cord neurons after transplantation of hAEC into lesioned areas of a contusion model of spinal cord injury in monkeys.⁸¹ Improved performance in locomotor tests in cell-treated animals compared to lesion control animals was also observed.⁸¹ Meanwhile, in a rat model of Parkinson's disease, restoration of striatal dopamine levels and behavioral improvement have been observed after transplantation of hAEC,¹²⁰⁻¹²² while transplantation of these cells into the brains of rats which had undergone middle cerebral artery occlusion resulted in improvement of behavioral dysfunction and reduced infarct volume.¹²³

Hepatocyte-like features of hAEC have also been observed *in vitro* by several groups. These cells have been shown to express liver-enriched transcription factors including hepatocyte nuclear factor (HNF) 3 γ and HNF4 α , CCAAT/enhancer-binding protein (CEBP) α and β) and CYP450 enzymes, as well as hepatocyte-related

genes including α 1-antitrypsin (α 1AT), cytokeratin 18 (CK18), glutamine synthetase (GS), carbamoyl phosphate synthetase-1 (CPS-1), phosphoenolpyruvate carboxykinase (PEPCK), and drug metabolism-related genes, CYP2D6 and CYP3A4.^{107,124} In vitro expression of human serum albumin and α -fetoprotein (AFP) has also been reported for hAEC, as well as typical hepatic functions such as albumin synthesis and production and storage of glycogen.^{125,126} Studies in mice suggest that hAEC may also be able to perform hepatic functions in vivo, with human albumin detected in the sera and peritoneal fluid of SCID mice which had received peritoneal implants of human amniotic membrane.¹²⁵ Furthermore, a study in which hAEC were transplanted into SCID mice has demonstrated that human α 1-antitrypsin could subsequently be detected by Western blot in the sera of these animals,¹¹⁰ while another study has shown that integrated AFP- and Alb-positive hepatocyte-like cells could be identified in hepatic parenchyma of SCID mice two weeks after hAEC transplantation.¹²⁶ Interestingly, the authors of this latter study also showed that hAEC which had been genetically modified to express the *LacZ* gene were able to integrate in liver parenchyma, suggesting that these cells could also be useful as gene carriers for patients with congenital liver disorders.

The ability of hAEC to differentiate toward the pancreatic lineage has also been reported, whereby these cells were induced to produce insulin through culture in the presence of nicotinamide. The insulin-producing hAEC were then able to normalize blood glucose levels after transplantation into streptozotocin-induced diabetic mice.⁹⁸ Ultrastructural features characteristic of beta pancreatic cells, as well as expression of the pancreatic marker amylase alpha 2B (AMYB2) and glucagon production have also been observed after culture of hAEC in pancreatic differentiation media.¹⁰⁸

2.7 Conclusion

To conclude on the possibilities for the future of placenta-derived cells in the clinical setting, it is clear that these cells hold great promise for the reasons that have been discussed in this chapter. The presence of different sources of stem cells in the placenta, from the pluripotent amniotic ectoderm-derived cells to the mesenchymal and hematopoietic stem cells, as well as the plasticity of these

cells, which has been shown through in vitro studies, and finally, their promising immunological properties, lead us to hypothesize with confidence that placenta-derived cells, or at least some of their subpopulations, could be applied for the development of new therapeutic strategies. Furthermore, the fact that placental tissue can be procured in nearly unlimited supply without harming the mother or the fetus, as well as the fact that its use raises ethical support rather than objection, and finally, the possibility of collecting and banking these cells at birth, together constitute strong evidence that the placenta indeed represents a potential oasis in the search for new and viable stem cell sources.

Although holding much promise for future applications in regenerative medicine, many questions remain open in the field of placenta-derived stem cell research. Given our current understanding of the cells from placental tissues, perhaps the most important of these is whether it is the plasticity or immunomodulatory properties of placental cells that will make them most useful in clinical applications in the future. Current knowledge leaves open both possibilities, although it appears that ever-increasing attention is being turned toward the effect that these cells have on the surrounding environment. Literature published to date appears to lend stronger support to the hypothesis that placental cells exert the bulk of their actions in vivo by secreting factors which support the growth, survival, or differentiation of other cells, rather than themselves undergoing differentiation to regenerate damaged or diseased tissue. In any case, it is clear that the human placenta still harbors many clues to understanding the processes of tissue development and tolerance, which will no doubt open new doors for the development of therapeutic treatments which can overcome current shortcomings in the field of regenerative medicine.

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