Isolation and characterization of stem cells from the placenta and the umbilical cord

CARMEN MIHAELA MIHU1), D. MIHU2), N. COSTIN2), D. RUS CIUCĂ3), S. ŞUŞMAN1), R. CIORTEA2)

1) Department of Histology
2) Department of Obstetrics–Gynecology II
3) Department of Pathological Anatomy
"Iuliu Hațegianu" University of Medicine and Pharmacy, Cluj-Napoca

Abstract
In addition to its essential role in the development, nutrition and immunological tolerance of the product of conception, human placenta is an important source of stem cells. Over the past years, scientific research has been aimed at isolating and characterizing mesenchymal cells and amniocytes, which show a high plasticity and are found in the chorionic villi and the membranes. At the level of the umbilical cord, two types of stem cells can be found: hematopoietic and mesenchymal. The blood of the umbilical cord is already in the focus of attention of researchers, as an important source of hematopoietic stem cells that can be used for transplantation.

Keywords: stem cells, placenta, amniocytes, mesenchymal cells.

Stem cells
The notion of stem cells is a developing concept [1, 2]. Stem cells are undifferentiated cells, capable of self-renewal and differentiation into specific lineage cells. Self-renewal can be maintained along many generations, increasing the number of cells. Through differentiation, stem cells give birth to daughter cells, also called progenitor or precursor cells. The latter are capable of further differentiation, but not of self-renewal [3].

Over the past years, the following terms related to the proliferation and differentiation capacity of stem cells have been delimited [4]:

1. Totipotent stem cells, which have the genetic potential to differentiate into any type of cell in the organism, including placental cells and extra-embryonic tissues, having in this way all the properties necessary for the obtaining of an entire human fetus (these cells are present until the eight cell zygote stage). These are non-specialized cells, characterized by a normal karyotype (diploid cells), self-renewal capacity, symmetric and disymmetric division, specific phenotypic and molecular markers: Oct-4, SSEA-1, SSEA-3/-4 clonogenicity (they generate a colony of genetically identical cells), absence of the G1 checkpoint (they do not require external stimuli for the initiation of DNA replication), extensive proliferation capacity, the capacity to produce embryoid bodies and teratomas that contain all the three main germ differentiation lines [5, 6].

2. Pluripotent stem cells, which can be isolated from the internal blastocyst mass and are capable of forming tissues originating in the three embryonic layers (endoderm, mesoderm and ectoderm), except for placental cells and extra-embryonic tissues.

3. Pluripotent stem cells, cells that can generate a limited number of cell types or tissues, restricted to one embryonic layer, which can be identified in developing fetuses and adult organisms.

4. Unipotent stem cells, which can generate a single cell type.

Stem cells can also be classified according to the developing stage of the organism in which they are obtained [7].

Embryonic stem cells, derived from the internal cell mass of the preimplantation blastocyst

They are pluripotent stem cells. Embryonic stem cells are capable of self-renewal and differentiation into all cell lineages and show an exceptional capacity of maintaining this non-differentiation state in long-term in vitro cultures (1–2 years, with a cell division period at every 36–48 hours) [8]. This is an important characteristic of embryonic stem cells, which differentiates them from somatic cells and even from mesenchymal stem cells, which have a finite replicative power, a capacity directly related to telomerase activity. The role of telomerase consists of adding repetitive telomeric sequences at the ends of chromosomes, maintaining in this way the length of chromosomes and the initiation of cell division. Telomerase activity is characteristically increased in embryonic cells, moderate in hematopoietic stem cells and variable and even absent in somatic cells. Their use is limited for
ethical reasons (involving the destruction of the embryo), as well as by the fact that they are incriminated in the occurrence of tumors at a high rate after their transplantation.

Fetal stem cells

Fetal stem cells, for example cells taken from the amniotic fluid, are pluripotent and express the specific SSEA-4 (Stage Specific Embryo Antigen-4) and the stem cell marker Oct-4, but they do not express SSEA-1/-3, CD34, CD133, BMP-4 (Bone Morphogenic Protein-4), or alkaline phosphatase [8]. Consequently, stem cells isolated from the amniotic fluid express several key markers of embryonic stem cells, but not all of them, suggesting the fact that they are not as primitive as embryonic cells, but they have a higher potential than adult cells. These cells may form embryonic bodies in vitro like embryonic cells, but they cannot generate teratomas when implanted in immunodeficient mice [9].

Adult stem cells

Adult stem cells are obtained postnatally from organisms, from tissues of endodermal, mesodermal and ectodermal origin. These cells are pluripotent, they do not express specific totipotent cell markers and do not form teratomas. The most studied are hematopoietic stem cells of mesodermal origin, which are the basis of bone marrow transplantation. They are considered to have a more limited potential than embryonic cells.

Embryonic germ stem cells

Embryonic germ stem cells are obtained from fetal gonads. They show all the features of stem cells.

Stem cells’ therapy

Cell therapy involves the manipulation of live cells, which are complex systems with an unpredictable behavior under transplantation conditions. The implementation of stem cells’ therapy in human clinics requires extensive studies on the biology of stem cells [10–12].

The manipulation of stem cells poses many ethical and technical problems and the translation from preclinical to clinical research requires the fulfillment of some essential conditions: obtaining of a sufficient number of cells, non-invasive isolation and purification methods, increased expansion (proliferation) capacity, control of cell differentiation, immunological characterization, molecular and functional characterization, reproducibility of experiments, no damage to the host organism [13].

The current sources of stem cells are embryonic stem cells and adult type stem cells. Both sources have advantages and disadvantages.

Embryonic stem cells are pluripotent cells that may generate all the specialized cell types in the organism. The obtaining method is non-invasive, leading to the destruction of the embryo. The obtaining of an increased number of embryonic stem cells is limited by the induction of the spontaneous differentiation phenomenon, as well as by the limited possibilities to control differentiation aimed at a certain cell type.

Another disadvantage of the culture of human embryonic stem cells is the extremely low rate of stabilization of cell lines. In 2005, about 100 cell lines were stabilized worldwide, without a sufficiently rigorous characterization. Another obstacle to the use of embryonic stem cells is represented by the risk of induction of teratomas.

Adult type stem cells do not pose ethical problems, because they are isolated from adult organisms. These cells are in very small numbers, but sufficient to ensure tissue regeneration. They can be mobilized for the repair of lesions in an organ or a tissue. Under these conditions, stem cells proliferate and differentiate to the cells of the damaged tissue. After the exhaustion of the endogeneous stem cell stock, a recruitment of non-hematopoietic stem cells from peripheral blood and bone marrow occurs. This entire process requires the release of cytokines and other growth factors. The classic adult stem cell is the hematopoietic stem cell (HSC), which is capable to generate all hematopoietic cell lineages. Bone marrow has been used since the 70’s as a source of hematopoietic stem cells in bone marrow transplantation. HSCs were isolated from the mouse spleen and were termed spleen-colony forming units. For 20 years, these spleen-colony forming cells were identified as hematopoietic cell progenitors, but transplantation studies on lethally irradiated animals have shown the heterogeneity of these cells, suggesting the presence of other cells, with stem cell features, but with an enlarged potential. In 1982, these new pluripotent adult stem cells were described for the first time, under the name of CFU-f (Colony-Forming-Unit fibroblasts) [14].

These were subsequently termed mesenchymal stem cells (MSC). Mesenchymal stem cells are classically obtained from bone marrow, but can be isolated from any tissue in the organism (adipose tissue, muscle, myocardium, and pancreas). The major disadvantage of adult stem cells is that they have a much lower differentiation potential compared to embryonic stem cells. Mesenchymal stem cells may differentiate into a variety of cell types: osteoblasts, chondrocytes, myocytes, adipocytes, neuronal cells [15, 16].

Over the past decade, an unexpected evolution in the study of the biology of the stem cell has been found due to the accumulation of evidence demonstrating a much greater plasticity of adult stem cells than the criteria established by the definition imposed by embryonic stem cells [17]. At the same time, efforts are made in order to understand the molecular mechanisms that dictate the plasticity of these cells, as well as for the development of modalities of use in cell therapy [18].

In human clinics, the most common source of MSC is adult bone marrow. The percentage of MSC in bone marrow is quite low (0.001–0.01%) and it decreases even more with age. Another disadvantage of this source is the fact that bone marrow harvesting is an invasive procedure.

The features of fetal stem cells are intermediate between embryonic and adult features. Compared to adult stem cells, fetal stem cells show an increased telomerase activity. Compared to embryonic stem cells,
they have a lower rate of DNA lesions occurring due to cell divisions or mutagenic agents, an extremely favorable aspect for tissue engineering [19].

**The placenta is the most important source of stem cells**

The placenta is one of the most important sources of stem cells, and has been studied extensively over the past period. The placenta fulfills two main desiderata of cell therapy: obtaining of an as high as possible number of cells and use of non-invasive methods for their harvesting [20, 21].

From the placenta, amniotic cells and mesenchymal stem cells may be isolated. An important source of stem cells is the umbilical cord [22–24].

**Amniotic cells**

Many clinical and experimental studies have demonstrated that the transplantation of the amniotic membrane promotes reepithelization, reduces inflammation and fibrosis and modulates angiogenesis. It is supposed that human amniotic epithelial cells (EA) express stem cell markers have important immunological capacities and can differentiate into all three embryonic layers. These properties, along with the easy isolation of cells and the availability of the placenta, make the amnion be a source of cells to be used for transplantation and regenerative medicine [25–27].

For the isolation of epithelial cells, the amniotic membrane is easily removed mechanically from the underlying chorion and is digested with trypsin or other digestive enzymes (usually a short reaction duration of 20–40 minutes is sufficient). The isolated cells are attached to the plastic surfaces of the culture plates, lined or not with substances that compose the basal membranes. The culture is performed in simple media, such as DMEM supplemented with 5–10% serum and possibly epidermal growth factor (EGF), in which cells proliferate quite well (in particular at higher densities) and show a typical cuboidal epithelial morphology [28].

Two to six passages are normally possible. Immediately after isolation, these express very low HLA-A, -B, -C levels, but after two passages these levels increase [29].

The surface antigens present are ABCG/BCRP, CD9, CD24, E-cadherin, integrins α6 and β1, HGF, SSEA-3/4, Tra-1-60 and Tra-1-81. In addition, these cells are characterized by the absence of certain markers such as SSEA-1, CD34, and CD133. Other markers such as CD117 and CCR4 are also negative or are expressed in very low amounts.

Amniotic cells, which are complementary to the surface markers, express pluripotential markers such as OCT-4, SOX-2 and Nanog.

Recent studies have evidenced the fact that these cells can be differentiated. Neural and glial markers have been successfully identified. Subsequently, it has been demonstrated that amniocytes can synthesize and release acetylcholine, catecholamines and dopamine [25, 30].

Hepatic differentiation has also been reported. In culture, the cells differentiated into hepatocytes produce albumin and alpha-fetoprotein; these cells can be subsequently shown to be integrated in the hepatic parenchyma, after transplantation to SCID mice. This potential for hepatic differentiation has been confirmed by many authors, who have also demonstrated other functions specific for hepatocytes, such as the glycogen storing capacity, as well as the expression of some transcription factors specific for hepatic cells such as: HNF-4 alpha, HNF-3 gamma and CEBP-alpha and -beta. The expression of the cytochrome P450 gene has also been evidenced, this cytochrome having the capacity to metabolize drugs, a liver specific function. All these results cause amniocytes to be considered an important source in cell therapy in the case of hepatic diseases [10].

The differentiation of amniocytes into other endodermis-derived tissue structures has also been reported. Encouraging results have been obtained in the differentiation into pancreatic cells. It was successfully differentiated amniocytes into pancreatic cells, after a culture in the presence of nicotinamide for 2–4 weeks. Then it was transplantation in mice with experimentally induced diabetes resulted in the correction of glycemia levels. It should be mentioned that the same result was not obtained by the use of placenta-derived mesenchymal cells [28, 31].

The studies performed indicate the fact that amniocytes are cells with stem cell characteristics. Cells that are normally derived from the ectodermis, the mesodermis and the endodermis have been obtained by the differentiation of amniocytes in vitro [28, 29, 32].

**Mesenchymal stem cells**

Mesenchymal stem cells (MSC) were initially identified in the bone marrow of adult subjects [33, 34]. Subsequently, they were also evidenced in fat tissue, bone and the placenta. It is accepted that these cells have the capacity to regenerate mesenchymal tissues and blood cells. A number of studies have shown that these cells are not immunogenic and have immunomodulating capacities. The mechanism of this is not clear; it is only known that MSC can inhibit the action of T-lymphocytes as well as the differentiation and proliferation of monocytes. These properties make them extremely interesting in transplantation therapy, where they can improve the results of bone marrow transplantsations and diminish the body reaction to transplanted tissues [35, 36].

The most important source of mesenchymal stem cells is currently bone marrow. However, the harvesting of MSC at this level raises many problems: it is difficult to access and it involves the use of invasive techniques. With age, the differentiation capacity of these cells decreases, but the chances for the donor to be the carrier of a viral infection increase. This is why alternative sources from where MSC could be isolated have been sought. An important source is human placenta. MSC can be isolated from the amniotic membranes and the placental villi [37, 38].
Amniotic and chorionic mesenchymal cells (AM and CM) are probably derived from the extraembryonic mesodermis. Chorionic mesenchymal cells are much less investigated than amniotic cells. AM are preferably isolated from the amnion at term, from the reflected portion of the membranes, in order to minimize the presence of maternal cells. Chorionic cells are isolated from the membranes and from the fetal portion of the placenta, after the mechanical and enzymatic removal of the trophoblast layer using dispase. Mesenchymal cells can be isolated by explant type cultures, from the choriocytic plate (from the area situated in the close proximity of the amniotic membrane) and the chorionic villi, although in this latter case the risk of maternal contamination is higher. Both AM and CM have a good adhesion and proliferate on the plastic surface of culture containers for a limited number of passages [34, 39].

AM and CM differ not only morphologically (culture aspect), but also phenotypically, functionally and even ultrastructurally. The examination by transmission electron microscopy (TEM) of AM has demonstrated the presence of mesenchymal and epithelial characteristics. This hybrid phenotype is interpreted as a sign of pluripotentiality and is not seen in CM, which are more primitive cells, with latent metabolic functions. Under TEM, CM has a simpler cytoplasmic organization than AM. The most important elements include the presence of groups of cisternae of the endoplasmic reticulum, dispersed mitochondria and glycogen pools, while more specialized elements (such as contractile filaments, prominent endocytic traffic, junctional communications) are completely absent. The following are positive for embryonic stem cell markers: SSEA-4, Tra-1-61, Tra-1-80 and Oct-4. In addition, these cells could be differentiated into osteocytes, chondrocytes, adipose cells, neuronal and neuroglial cells [37, 38]. Recent studies have evidenced the fact that these cells can generate cardiomyocytes, unlike amniocytes, which could not be differentiated into these cell types. Moreover, their injection in mouse models with myocardial infarction has resulted in an increase of survival. All these findings cause mesenchymal stem cells from the placenta to be an important source in cell therapy [9, 40, 41].

Umbilical cord cells

At the level of the umbilical cord, the two types of stem cells can be found: hematopoietic (UC–HS) and mesenchymal, which in their turn can be found in the blood (UC–MS) or in Wharton’s jelly (UC–MM) [42].

The blood of the umbilical cord has long been in the focus of attention of researchers as an important source of stem cells, used for transplantation, for several reasons:

- It contains a higher number of primitive hematopoietic stem cells (HSC) per volume unit, which proliferate more rapidly than bone marrow HSC;
- There is a lower risk of rejection after transplantation;
- Transplantation does not require a perfect HLA antigen match (unlike in the case of bone marrow);
- The UC blood has already been successfully used in the treatment of inborn metabolic errors;
- Methods for the collection, storage and refrigeration of human blood were developed as early as 1940, so there is no need for a new technology for the mononuclear cells from UC blood.

Interestingly, blood MSC (UC–MS) can produce cytokines, which facilitate grafting in the donor and in vitro HSC survival, being more efficient from this point of view compared to bone marrow MSC [34].

UC vessels and the surrounding mesenchyma (including the connective tissue known as Wharton’s jelly) derive from the embryonic and/or extraembryonic mesodermis. Thus, these tissues, as well as the primitive germ cells, are differentiated from the proximal epiblast, at the time of formation of the primitive line of the embryo, containing MSC and even some cells with pluripotential potential. It is speculated that the UC matrix material is derived from a primitive mesenchyma, which is in a transition state towards the adult bone marrow mesenchyma.

The blood from the placenta and the umbilical cord is relatively easy to collect in usual blood donation bags, which contain anticoagulant substances. Mononuclear cells are separated by centrifugation on Ficoll gradient, from which the two stem cell populations will be separated [43, 44]:

- HSC which express certain characteristic markers (CD34, CD133);
- MSC that adhere to the culture surface under certain conditions (e.g. modified McCoy medium and lining of vessels with FBS or FCS).

The MSC from the umbilical cord matrix (UC–MM) are obtained by different culture methods depending on the source of cells: connective matrix, subendothelial cells from the umbilical vein or even whole umbilical cord explant. They are generally well cultured in DMEM medium, supplemented with various nutritional and growth factors, in certain cases the prior treatment of vessels with hyaluronic acid being beneficial [45].

Placental stem cells are the object of study for many researchers. A common result of these studies is that, although placental stem cells do not show the plasticity and the self-regeneration capacity of embryonic stem cells, they are superior to adult type stem cells.

Many laboratories have succeeded in differentiating them into cell types belonging to the three germinal layers: adipocytes, osteoblasts, chondrocytes, myocytes, neurons, hepatocytes.

The morphological, phenotypic and genetic characterization of placental stem cells is also aimed at evidencing the differences between the various placental compartments (amniotic membrane, amniotic and chorionic mesenchymal cells, cells isolated from the umbilical cord). Their high immunological tolerance supports their use as an adequate source in cell therapy.

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Corresponding author
Carmen Mihaela Mihu, Associate Professor, MD, PhD, Department of Histology, “Iuliu Hațieganu” University of Medicine and Pharmacy, 6 Louis Pasteur Street, 400 349 Cluj-Napoca, Romania; Phone +40264–595 433, +40727–305 085, E-mail: carmenmihu2004@yahoo.com

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