HUMAN PLACENTA- DERIVED STEM CELLS - RECENT FINDINGS BASED ON THE MOLECULAR SCIENCE

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Abstract
The human placenta is a complex, multifunctional transient fetomaternal organ. The placenta is composed of the maternal decidua basalis and its fetal part, consisting of the mesenchymal and trophoblast cell lineages. Both the placenta and the amniotic membranes are abundant in readily available placenta-derived mesenchymal stem cells (PD-MSCs). The clinical application of the PD-MSCs opens new perspectives for regenerative medicine and the treatment of various degenerative disorders. Their properties depend on their paracrine activity – the secretion of the anti-inflammatory cytokines and specific exosomes. In contrast to the PD-MSCs, the trophoblast stem cells (TSCs) are much more elusive. They can only be isolated from the blastocyst-stage embryos or the first-trimester placental tissue, making that procedure quite demanding. Also, other cultures require specific, strictly controlled conditions. TSCs may be potentially used as an in vitro model of various placental pathologies, facilitating the elucidation of their mysterious pathogenesis and creating the environment for testing the new drug efficiency. Nonetheless, it is unlikely that they could be ever implemented as a part of novel cellular therapeutic strategies in humans.

Running title: Current knowledge on the placental stem cells

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**Introduction**

The placenta is a complex fetomaternal organ build of maternal decidua basalis and its fetal part, which is composed of trophoblast cells, as well as the blood vessels and fetal connective tissue. Trophoblast cells are divided into three major cellular populations syncytiotrophoblast (ST), cytotrophoblast (CT), and extravillous cytotrophoblast cells (EVT) [1]. The placenta regulates multiple physiological processes in human pregnancy. Its activity depends on the function of various cellular subpopulations, which have different characteristic properties. ST and CT cells form the structure of placental villi. They are responsible for the transfer of respiratory gases and energy substrates across the placental barrier and the elimination of the metabolic wastes from the fetal circulation. Moreover, from the middle part of the pregnancy, ST cells serve as a substantial origin of gestational hormones such as progesterone and human chorionic gonadotropin [2]. Abnormal placentation leads to the development of numerous gestational complications. EVT cells are mainly involved in the regulation of the invasiveness of the placenta. They penetrate the maternal decidua basalis and promote the invasion and remodeling of uterine spiral arteries to provide the optimal fetomaternal blood flow [3].

The term placenta and amniotic membranes are a rich source of multipotent mesenchymal stem cells (MSCs) that are capable of differentiating into multiple cell lines that have a potential to form numerous types of connective tissue (e.g., bone, cartilage, tendon, ligament, muscle, or the adipose tissue) [4,5]. In general, the placental tissue after the delivery is treated as common medical waste. Considering that, the potential source of the placenta-derived mesenchymal stem cells (PD-MSCs) is relatively easy to obtain. Due to the advances in laboratory techniques that have been made in recent years, both their isolation and the subsequent culture protocols are relatively simple [6]. The allogeneic PD-MSCs are thought to be a promising tool for clinicians, especially in regenerative medicine and the treatment of degenerative disorders. PD-MSCs and other MSCs could directly differentiate into specific cells in damaged tissues and facilitate their regeneration through the secretion of various molecules such as growth factors and cytokines [7].

Besides the mesenchymal stem cells, the early developing placental tissue is the origin of the population of trophoblast stem cells (TSCs) [8]. TSCs are defined as self-renewing, multipotent cells that give rise to all trophoblast cell lineages (ST, CT, and EVT) that form the fetal part of the developing placenta. In contrast to animal models, human TSCs are quite elusive. Their identification and isolation are much more elaborate, as they are highly restricted to the blastocysts obtained during the in vitro fertilization and first-trimester placental tissues [8]. The establishment of efficient TSCs culture requirements may enable creation of novel cell models that could be used as a readily available tool to explain the pathophysiology of the numerous pregnancy complications and as a safe in vitro environment for the testing of new drug efficacy.

**Human embryonic development**

Embryogenesis is initiated by the fusion of oocyte and sperm, which creates a zygote that subsequently divides, achieving the morula stage [9]. Then the blastocyst forms on approximately 4-5th day post-fertilization and consists of inner cell mass (ICM), which give rise to all three germ layers that afterward create embryo and trophoderm (TE), which is the precursor of trophoblast cells. The endometrium is receptive, allowing embryo implantation for only about 3-5 days of the menstrual cycle [10]. The first stage of human placenta development occurs after implantation that takes place 6-7 days post-conception when polar TE, the part of TE that is contiguous with ICM, attaches to the mucosa inside the uterine cavity [9,11,12]. After implantation, TE divides into two lineages: mononuclear cytotrophoblast and multinucleated syncytiotium [12,13]. In the following days, primitive syncytiotium penetrates the maternal endometrium, which is known as decidua [14]. This leads to the creation of fluid-filled lacunar spaces that shortly merge into blood sinusoids as the maternal capillaries are damaged [11,12]. Simultaneously cytotrophoblast starts to invade syncytiotium forming the first villous-like structures. Subsequently, secondary villi are formed as the core is penetrated by extraembryonic mesenchymal cells. Around 17-18th day post-fertilization, tertiary villi develop as the first fetal blood vessels appear. About the day 32nd post-fertilization, fetal circulation connects with the vessels formed inside the placenta via the umbilical cord. [12–15].

At this time, all types of trophoblast cells, such as syncytiotrophoblast, villous cytotrophoblast, and extravillous trophoblast, are present [13,16]. First-trimester CT cells may differentate and give rise to other trophoblast cells - ST and EVT [8,16]. Okae et al. suggest that preimplantation blastocyst-derived TE cells and postimplantation CT cells are the sources of human TSCs [8]. Research in mice shows that the homeobox protein CDX-2 (CDX2) is essential to TE differentiation. Expression of CDX2 was also observed in human placenta development. However, it cannot be considered a specific marker of human TSCs because human placenta formation varies from any other species [8,13,16].

ST is covered with microvilli that increase its surface area, which is directly contacted with maternal decidua, participating in gases and nutrients exchange between the fetus and the mother. ST has a crucial role in impeding pathogens penetration into the uterus and partakes in transferring mater-
Intrauterine growth restriction (IUGR) is one of the most severe gestational complications. The pathogenesis of that condition is not clear but is usually associated with placental insufficiency. The IUGR increases the risk of stillbirth, multiple congenital malformations, and even the incidence of type 2 diabetes and cardiovascular diseases in adulthood [12,23]. The results of a porcine study indicate that IUGR-MSCs show increased adipogenic and fibrogenic capacity. Their osteogenic and chondrogenic properties were significantly decreased compared to normal MSCs. The central hypothesis that comes from that study about the PD-MSCs’ potential role in the pathophysiology of that condition is quite surprising and should be tested in humans [23].

The role of human placenta-derived mesenchymal stem cells is not well-studied in pathological pregnancies. Nonetheless, it is known that these cells can secrete many molecules, such as growth factors, cytokines, chemokines, and extracellular vesicles (EVs) [24,25]. We can distinguish amniotic mesenchymal stromal cells and chorionic mesenchymal stromal cells, but their secretory functions are nearly the same [26]. Because the collection and isolation of PD-MSCs, during the pregnancy are somewhat challenging and raise ethical issues, we cannot assess their role in the pregnancy-related pathologies in prospective clinical trials.

Human placental stem cells – characteristic, methods of isolation, molecular research, and potential clinical applications

The broad definition of placenta-derived stem cells includes several types of stem cells that originate from different parts of the human placenta and amniotic membranes. The participants of the “First International Workshop on Placenta-derived Stem Cells,” which was held in Brescia, Italy, in 2007, reached a consensus on the nomenclature of the placenta-derived stem cells. They concluded that there are two stem cell populations that derive from the amniotic membranes (human amniotic epithelial cells (hAEC) and human amniotic mesenchymal stromal cells (hAMSC), as well as two subgroups that derive from placenta per se, the population of human chorionic mesenchymal stromal cells (hCMSC) and human chorionic trophoblastic cells (hCTC) [27]. This review focuses mostly on the populations of the mesenchymal and trophoblast stem cell lineages.

Placenta-derived mesenchymal stem cells

The same workshop experts established the hAMSC and hCMSC defining criteria that included the confirmation of their fetal origin, adherence to standard plastic flasks, a capability to form the fibroblast colony-forming units and differentiate into one or more cell lines form the following list – osteogenic, adipogenic, chondrogenic, and vascular/endothelial. Moreover, both stem cell populations have to exhibit the pronounced expression of the following antigens – CD90, CD73, CD105, and do not manifest the expression of the hematopoietic markers – CD45, CD34, CD14, and HLA-DR [27].

It has been established that cell populations classified as hCMSC could be effectively isolated from the various regions of term placentas such as cho-
rionic plate and villous chorion [24]. Samples could be obtained from either the patients who underwent the cesarean section procedure or delivered vaginally [24,28,29]. The fragments of chorionic tissue are surgically dissected from the placentas. After the collection, the placental tissue samples should be soaked in a mixture of cold phosphate-buffered saline (PBS) and antibiotics. The specimens should be transferred to the laboratory immediately after the collection, where the samples are repeatedly washed in PBS, manually separated from the amniotic membranes, and cut into pieces smaller than 1 mm. The above-mentioned procedures are usually similar in various isolation protocols. There are numerous possible approaches for further cell preparations. We will shortly describe one of them. Afterward, the prepared material is suspended for 60 minutes in the culture media mixed with the digestive enzymes – collagenase and dispase. Then, the suspension is centrifuged, and the supernatant is discarded. In the next step, the cell pellet is lysed in the ammonium-chloride-potassium (ACK) buffer for 5 minutes and once again centrifuged to separate the pellet from the supernatant. The collected pellet is suspended in the serum-free Dulbecco's modified Eagle's medium (DMEM) or the minimum essential medium Eagle – alpha modification (αMEM) with antibiotics and cultured in the culture flasks in the stable, controlled conditions at 37°C in 5% CO2/95% air [24,30,31]. Cells adhere to the plastic plates after about three days of cultivation. Cells could be harvested when the cultures reach about 80% confluence or passed to new culture flasks with fresh growth media [24]. Similarly, to hMSC, the hAMSC can be isolated from the amniotic membranes' mesenchymal layer [6]. The amnion is manually separated from the chorion, minced into small fragments, and digested by dispase. The fragments are suspended in RPMI complete culture media and treated with collagenase and DNase I. After the centrifugation, the cells are finally suspended and cultured in the Chang medium C with the glutamine at 37°C in 5% CO2 [28].

It was established that PD-MSCs and MSCs derived from the other tissues possess similar growth properties in the in vitro cultures [31]. Nonetheless, the same authors noted that the relative expression of the several stemness marker genes was the lowest in the population of PD-MSCs in comparison to those measured in MSCs derived from the bone marrow, umbilical cord blood, and the adipose tissue. Only the expression of the NANOG, Krüppel-like factor 4 (KLF4), and Inhibin beta A (INHBA) was detected in the PD-MSCs population. They did not detect the expression of the octamer-binding transcription factor 4 (OCT4), sex-determining region Y-box 2 (SOX2), MYC, LIN28, and REX1 genes [31]. Heo et al. reported that PD-MSCs had the potential to differentiate into adipocytes and chondrocytes but did not acquire specific features of osteogenic differentiation [31]. Some authors examined the osteogenic potential of PD-MSCs obtained from various parts of the placenta. They reported that stem cells isolated from the amniotic membranes have higher osteogenic differentiation potency than the chorionic ones [4,5]. In contrast to those results, the hCMSC had a higher potential to differentiate into the adipocytes [4].

Multiple studies revealed that PD-MSCs themselves produce several anti-inflammatory cytokines and excrete the nanovesicles, called the exosomes, which may possess extraordinary regenerative properties [32]. The collection and isolation of the PD-MSCs' exosomes are quite complex, multi-stage procedures. Briefly, in normal conditions, the exosomes are released continuously out of the cells into the culture media. After 48 hours of incubation, the conditioned culture media is collected and sequentially centrifuged following the previously described isolation and purification protocols [30,33–35]. The isolated PD-MSCs' exosomes are identified by the membrane presence of CD63 antigen [30]. It was reported that the treatment of myoblasts with the purified PD-MSCs' exosomes or conditioned media increased their differentiation and decreased the expression of fibrogenic genes. Moreover, PD-MSCs transplantation significantly reduced the level of inflammation and fibrosis in mdx mice – a mouse model for studying Duchenne muscular dystrophy. It was speculated that those activities could be evoked by the mi-RNA molecules [32]. Furthermore, conditioned medium from the PD-MSCs and purified PD-MSCs' exosomes have an ability to stimulate the secretion of several proangiogenic cytokines, and as a result, to increase the viability and support the migration of the endothelial cells as well as enhance endothelial tube formation in vitro [24,36].

There have been few studies that analyzed the properties of these cells in the treatment of common human diseases. Most studies are based on the use of animal models. Abumaree et al. showed that PD-MSCs have an impact on lymphocytes. To be exact, they have immunosuppressive properties, which could be used in the autoimmune diseases’ treatment protocols [37]. PD-MSCs also reduce the blood concentrations of interleukin-5, which could have a significant impact on the treatment of asthma [29]. Other studies proved that PD-MSCs and their exosomes demonstrate proangiogenic activity so that they might be a new treatment method for ischemic diseases or dermal wounds [24,38–40]. Moreover, PD-MSCs with NK4 molecule expression have antitumor activity, and they are ideal vectors to target the glioblastoma multiforme, which is the most common CNS-tumor with an abysmal prognosis [41]. There are several reports that PD-MSCs could also be used to cure neurological diseases like…
Duchenne muscular dystrophy, multiple sclerosis, spina bifida, or autoimmune encephalomyelitis. Their EVs contain proteins, which provide neuronal survival and development. That promotes myelin regeneration by inducing oligodendrocyte precursor cells to differentiate into mature myelinating oligodendrocytes [25,32,35,42,43]. Furthermore, dementias reportedly can be treated with PD-MSCs because of their convalescent effect on cognitive behavior, connected with the recovery of the choliner- gic neuronal populations [44]. Park et al. used murine PD-MSCs to treat diabetic gastropathy in mice, giving positive results [45]. All these studies show that PD-MSCs have immense potential in almost every field of medicine.

Trophoblast stem cells
Numerous animal studies focused on TSCs isolation and their other cellular characteristics [46–50]. Human studies are less accessible mainly due to the possible ethical dilemmas that may arise when the laboratory procedures are performed on the human tissues obtained during early pregnancy. After the years of ongoing investigations, it has been announced that the human trophoblast stem cells can be efficiently directly isolated from the blastocyst-stage embryos and first-trimester placental tissues, obtained from the planned terminations, and then long-term cultured under the strictly regulated conditions [8]. The optimal culture medium should include CHIR99021 (a Wingless/Inte-grated (WNT) activator), epidermal growth factor (EGF), A83-01 and SB431542 (transforming growth factor-beta inhibitors), valproic acid (a histone deacetylase inhibitor), and Y27632 (a Rho-associated protein kinase inhibitor) [8]. More studies analyzed the population of TSCs that derived from the induced pluripotent stem cells (iPSCs) [51–54]. However, TSCs isolated from the fetal tissues are treated as medical waste after delivery, their limited plasticity, it is unlikely that TSCs could be implemented in the regenerative medicine cell treatment protocols. However, the influence of their secretory profile on the other somatic cell lineages has not been studied yet.

Conclusions
Placenta-derived stem cells definition includes a heterogeneous group of stem cells with various plasticity and self-renewal capacities. Mesenchymal stem cells could be relatively easily isolated from term placentas and amniotic membranes. Their isolation does not raise ethical issues because those tissues are treated as medical waste after delivery, making them readily available. Moreover, PD-MSCs have well described and optimized culture requirements. Both mesenchymal cell lines could be applied in future cell treatment protocols. Molecular analyses revealed that their regenerative properties are connected with their paracrine activity – secretion of numerous cytokines and exosomes. Even though the first groundbreaking reports on the successful outcomes of the human trophoblast stem cells isolation and culture were published, those cells remain elusive and do not cease to be intriguing.

Ethical approval
The conducted research is not related to either human or animal use.

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The authors declare they have no conflict of interest.

References:


