

Placental-derived stem cells: Culture, differentiation and challenges

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Abstract

Stem cell therapy is a promising approach to clinical healing in several diseases. A great variety of tissues (bone marrow, adipose tissue, and placenta) are

potentially sources of stem cells. Placenta-derived stem cells (p-SCs) are in between embryonic and mesenchymal stem cells, sharing characteristics with both, such as non-carcinogenic status and property to differentiate in all embryonic germ layers. Moreover, their use is not ethically restricted as fetal membranes are considered medical waste after birth. In this context, the present review will be focused on the biological properties, culture and potential cell therapy uses of placental-derived stem cells. Immunophenotype characterization, mainly for surface marker expression, and basic principles of p-SC isolation and culture (mechanical separation or enzymatic digestion of the tissues, the most used culture media, cell plating conditions) will be presented. In addition, some preclinical studies that were performed in different medical areas will be cited, focusing on neurological, liver, pancreatic, heart, muscle, pulmonary, and bone diseases and also in tissue engineering field. Finally, some challenges for stem cell therapy applications will be highlighted. The understanding of the mechanisms involved in the p-SCs differentiation and the achievement of pure cell populations (after differentiation) are key points that must be clarified before bringing the preclinical studies, performed at the bench, to the medical practice.

Key words: Fetal membrane; Placenta; Embryonic stem cells; Mesenchymal stem cells; Cell therapy

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Core tip: Fetal membranes are a source of stem cells, namely placenta-derived stem cells (p-SCs), which are in between embryonic and mesenchymal stem cells sharing characteristics with both, such as the capacity to differentiate in all germ layers and the dearth of tumorigenicity. Many preclinical studies have been investigated the potential of p-SC use in different medical areas, such as neurology, cardiology,

orthopedics, gastroenterology, and tissue engineering, showing promising results, but also some drawbacks. The present review will focus on different aspects of biological properties and potential clinical uses of p-SCs.

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INTRODUCTION

Stem cell therapy is a promising approach to clinical healing in several diseases. Neurology^[1], orthopedics^[2] and cardiology^[3] are just a few medical branches in which the benefits of stem cell therapy have been mentioned. The prolific capacity of stem cells (either embryonic or mesenchymal), taken together with the ability to generate different cell lineages and its self-renewal property are the major features of these cells that allow their use and manipulation in biology and medicine^[4,5]. A great variety of tissues (bone marrow, adipose tissue, and endometrium) are potentially sources of stem cells and among them the placenta is a transient organ from which it is possible to obtain mesenchymal stem cells^[5].

The term placenta constitutes a very reliable rich source of fetal mesenchymal stem cells^[6-9]. These cells are capable of differentiating into multiple different cell types and have immunological properties that suggest their use in an allogenic transplantation setting^[10-12].

In this respect, it is important to remember that placenta has a fundamental role in maintaining fetomaternal tolerance and, therefore, the immunomodulatory properties of these cells have been investigated with the aim of exploring their applicability in cell therapy-based treatments^[11-13]. Their recovery does not involve any invasive procedures for the donor and their use does not create any ethical issue. In addition, the fact that placenta is generally discarded after birth as medical waste and is available in large supplies, makes placental-derived stem cells (p-SCs) excellent candidates for cell therapy. The current review is focused on the biological properties, culture and potential cell therapy applications of p-SCs.

P-SC

Biological properties

Animal development is initiated by fertilization of the egg with sperm, which is immediately followed by mitotic cell divisions, or cleavages, to generate blastomeres. For eutherians, such as the mouse and human, the first cell differentiation event is the establishment of two distinct cell lineages: the trophoblast (TE) and the inner cell mass (ICM)^[14].

TE engages in implantation by directly interacting with the mother's uterus, and gives rise to tissues in the placenta. It is only after implantation that the three germ layers form from the ICM, which ultimately generates all the tissues in the animal body^[14]. Pluripotency is a characteristic of the ICM and is related to the expression of octamer binding transcription factor 4 (Oct4). Oct4 is essential to prevent ICM from diverting towards the TE lineage. Another important transcription factor, caudal type homeobox 2 (Cdx2), is specifically expressed in TE. It was demonstrated that Cdx2 is necessary to repress the expression of Oct4 in TE^[15]. Moreover, Cdx2 null embryos exhibit high incidence of apoptosis^[16].

Embryonic stem cells (ESCs), which are ICM-derived cell lines, despite of showing pluripotency and self-renewal capacity, have ethical concerns restraints^[4], because of their tumorigenic potential. On the other hand, mesenchymal stem cells (MSCs) are not subject of these ethical restraints, but show multipotency, that means a lower capacity to multiply and originate different cell lineages^[5]. Surrogate systems to study early placental development in the human species have been tried using human and mouse embryonic stem cells under bone morphogenetic protein (BMP) 2/4 stimulus to trophoblast differentiation and spontaneous formation of embryoid bodies^[17,18]. MSCs are found in a lot of tissues, like the dentary tissue, adipocytes and bone marrow which culture provide inadequate cell numbers and have limitations like donor site morbidity and the need of aspiration to collect them, besides being a well-established culture. Attempts to overcome these problems were tried with alternative sources of MSCs and cells isolated from reproductive organs showed to be very useful. Thus, ovaries, uterus (endometrium) and specially the placenta^[19-21], because of its discarded as medical waste after delivery, are potential sources of MSCs that can be used without ethical restrictions.

The p-SCs, which are TE-derived cell lines, gather some features from adult and embryo cells: possible differentiation to originate each of germinative cells and absence of carcinogenicity^[22-27]. In the First International Workshop on p-SCs terminologies standardization have been made^[28], and according to the cell origin we should have amniotic epithelial or mesenchymal stromal cells, chorionic mesenchymal stromal or trophoblastic cells. In the present review it will be considered p-SCs in general, referring to any kind of them.

Cellular characterization

Immunophenotype characterization is the most common procedure used to distinguish different cell clusters, by means of surface marker expression, and some essays employed are immunolabeling^[29] and flow cytometry^[30]. The p-SCs show profiles merged with those found in embryonic and mesenchymal stem cells^[26]. None of these three cell lineages (embryonic,

mesenchymal, and p-SC as well) express the clusters of differentiation (CD) 11b, 34, and 45. Regarding CD expression, p-SCs are positive for 29, 73 and 166, and negative for 11b, 31, 34, 45. In addition, they are positive for human leucocyte antigen (HLA) A, B, C and negative for HLA-DR, DP, DQ^[26,31,32].

The p-SCs fulfill the essential characteristics to categorize MSC^[33] and are capable to originate ectodermal, endodermal, and mesodermal (adipocytes, osteocytes, and chondrocytes) lineages *in vitro*^[25,31]. Moreover, p-SCs express surface cell markers only expressed by embryo-derived cells, such as Oct-4, SRY (sex determining region Y)-box 2 (Sox 2), Nanog, stage-specific embryonic antigen (SSEA)-1, SSEA-4, germ cell tumor marker (GCTM)-2, Tra-1-60, and Tra-1-80^[22-24]. High levels of Oct-4, Sox 2 and Nanog were detected in p-SCs using q-PCR method^[25]. Musashi-1, vimentin, polysialylated-neural cell adhesion molecule, and Nestin were observed in cells from amniotic membrane, unlike other MSCs, when evaluated by immunofluorescence technique^[34].

Cell culture

Many different protocols for isolation of fetal membranes-derived stem cells are found in literature. For human beings and large animals, a separation of amniotic epithelial cells^[23,35], amniotic mesenchymal cells^[7] and chorionic mesenchymal cells^[8] are possible, meanwhile for small animals, such as rodents, such characterization is not possible^[31].

Fetal membranes are submitted to enzymatic disaggregation following different protocols, which differ among themselves particularly regarding enzymatic digestion and duration^[7,8,23,31,35]. In every protocol, the cells are incubated with trypsin-EDTA or other enzymes, at 37 °C, and then, are harvested through physical methods. Then cells are plated onto noncoated tissue culture dishes or flasks. Serum, like fetal bovine serum (FBS) ranging from 10% to 20%, must be added to the media. The most commonly used media are Roswell Park Memorial Institute (RPMI)-1640, Dulbecco's modified eagle medium (DMEM), Minimum essential medium (EMEM), and DMEM/F12. Also, media should be enriched with nonessential amino acids, L-glutamine, β -mercaptoethanol, sodium pyruvate, and antibiotics. Cells are raised at controlled conditions of air atmosphere and temperature. For maintenance of the cell culture, p-SCs must have their medium (such as DMEM or RPMI) changed every other day. To passage the cells, it should be used trypsin, and the cells are generally plated into T75 tissue culture flasks. Such culturing conditions are very similar with MSCs. On the other hand, ESCs need to be plated into mouse embryonic fibroblasts - MEFs - feeder layer or Matrigel™ and the medium (such as DMEM-F12 or mTeSR™-1) must be changed every single day. To passage the cells, it should be used collagenase type IV or dispase, and the cells are generally plated into 6-well

plates.

Finally, p-SCs may be cryopreserved using different media containing various concentrations of dimethyl sulfoxide, FBS and DMEM. Viability and cell numbers must be evaluated after thawing.

CELL THERAPY USING P-SC

Basic considerations

Three characteristics are important for the clinical application of p-SCs: the lack of ethical restrictions, pluripotency and their low immunogenicity^[12]. An explanation for this is the low expression of MHC class II molecules, allowing these cells to be effectively employed in transplants^[26].

Regarding to cellular therapy we have to consider the existence of cell banking due to the large amount of cells needed. Thus, those cells must have essential properties like to keep stable in subcultures, availability, great prolificity, freezing resistance, and high viability after thawing. The p-SCs fulfill most of these requirements. It has been mentioned that p-SCs even at subculture 30 showed high cellular division rates keeping a steady karyotype^[25]. Different from p-SCs and ESCs, all other MSCs do not neither replicate nor exhibit high levels of cellular survival around subcultures 8 to 10.

Considering the freezing process, p-SCs require ordinary media (like MSCs, unlike ESCs), show intermediate resistance to low temperature (in between ESCs and MSCs) and show great viability after frozen-thawing (like ESCs, unlike MSCs).

Some of the p-SC uses in research are listed in Table 1, at the end of this section.

Neurological diseases

Human p-SC demonstrated neuroprotective effects after stroke in rats. Treatment of stroke with p-SCs (*via* intravenous administration) significantly increased vascular endothelial growth factor (VEGF), hepatocyte growth factor (HGF) and brain derived neurotrophic factor (BDNF) levels in the ischemic brain compared to controls (dextran vehicle or phosphate buffer saline) after middle cerebral artery occlusion^[36].

In Alzheimer's disease, β -amyloid peptide is considered to be its root cause. Also, the neuroinflammatory process mediated by β -amyloid plaque-induced microglial cells and astrocytes contributes to Alzheimer's disease pathogenesis. Thus, it was demonstrated that p-SCs transplanted into an Alzheimer's disease mouse model modulated the properties of microglial cells toward a β -amyloid peptide plaque-reducing anti-inflammatory response. Moreover, p-SCs injected mice, compared to phosphate-buffered saline controls, had higher levels of β -amyloid degrading enzymes, reduced levels of proinflammatory cytokines, increased levels of anti-inflammatory cytokines (TGF- β and IL-10), slower progression of Alzheimer's pathology, and

Table 1 Preclinical studies using placental-derived stem cells for different disease models

Disease	Animal model	Cells	Ref.
Stroke	Rats	p-SC	[36]
Alzheimer	Mouse	p-SC	[37]
Injured liver model	Rat	p-SC	[39]
Myocardial infarction	Rat	amniotic membrane fragment	[43]
Myocardial infarction	Pig	p-SC	[44]
Lung fibrosis model	Mouse	p-SC	[47]
Idiopathic pulmonary fibrosis	Human (trial study)	p-SC	[48]
Osteogenesis imperfecta	Murine	p-SC	[49]
Bone lytic lesions	Mice	p-SC	[50]
Bone disease	rabbit	p-SC + scaffold	[51]

p-SC: Placenta-derived stem cell.

improved memory function^[37].

Liver disease

Cell therapy for liver diseases has also been investigated. Among other sources of MSCs, p-SCs showed the greatest potential for hepatogenic differentiation and proliferation *in vitro*. The p-SCs, mainly those from chorionic plate, expressed higher levels of hepatocyte growth factor after differentiation^[38].

In another study, chorionic-plate derived mesenchymal stem cells isolated from placenta could trigger autophagy to enhance regeneration in carbon tetrachloride injured rat liver model. After p-SC transplantation they observed reduction in apoptosis (caspase activity) and increasing levels for autophagy, survival and regeneration in liver cells^[39].

Pancreatic disease

Human placenta-derived mesenchymal stem cells (p-SCs) have the potential to differentiate into insulin producing cells^[40,41]. Also, p-SCs can form islet-like cell clusters which are capable of restoring normoglycemia when transplanted into streptozotocin-induced diabetic Balb/C mice^[42].

Heart disease

In order to evaluate whether human amniotic membrane could limit postischemic cardiac injury, a fragment was applied onto the left ventricle of rats that had undergone ischemia through left anterior descending coronary artery ligation. The authors observed that the amniotic membrane fragment onto ischemic rat hearts could significantly reduce postischemic cardiac dysfunction once the rats showed higher values of left ventricle ejection fraction, fractional shortening and wall thickening on echocardiographic examinations^[43].

In another study of myocardial infarction, pre-treated (with a hyaluronan mixed ester of butyric and retinoic acid - HBR) p-SCs were intramyocardial injected in a pig model. Treated animals showed

smaller infarct scar size and a significant improvement of the end-systolic wall thickening and circumferential shortening of the infarct border zone evaluated by magnetic resonance imaging^[44].

Muscle disease

First-trimester chorionic-villi-derived cells were evaluated for potential therapeutic use in Duchenne muscular dystrophy, which is a X-linked disorder characterized by the absence of dystrophin at the sarcolemma of muscle fibers. The p-SCs efficiently differentiated into myotubes after myogenic induction, at which point Nanog and Sox2 were down-regulated, whereas MyoD, myogenin, desmin and dystrophin were up-regulated. The p-SCs could be efficiently directed to differentiate *in vitro* into skeletal muscle cells that express dystrophin as the last stage marker of myogenic differentiation^[45].

The myogenic differentiation of fetal stem cells (p-SC, amniotic fluid stem cells, cord blood and wharton's jelly cells), were already reviewed and considerations about limitations and perspectives for therapeutically use were done^[46].

Pulmonary disease

On a mouse model of bleomycin-induced lung fibrosis, cellular therapy using p-SCs promoted a decrease in neutrophil infiltration and a significant reduction in the severity of fibrosis, compared to control^[47].

On a single centre, non-randomized, dose escalation phase 1b trial study, human patients with moderately to severe idiopathic pulmonary fibrosis received p-SCs *via* peripheral vein and they were evaluated follow up 6 mo. The variables analyzed were lung function (forced vital capacity and diffusing capacity for carbon monoxide), 6-min walk distance, gas exchange (assessed by resting PaO₂), and lung fibrosis score (assessed by high-resolution computed tomography chest). Compared to baseline, at 6 mo all parameters were unchanged, with no evidence of worsening fibrosis and no side effects reported in the patients who had received p-SC injections^[48].

Bone disease

Human fetal early chorionic stem cells (p-SCs) treatment was studied in a murine osteogenesis imperfecta model. It was demonstrated that intraperitoneal injection of p-SCs in osteogenesis imperfect neonates reduced fractures, increased bone ductility and bone volume, increased the numbers of hypertrophic chondrocytes, and upregulated endogenous genes involved in endochondral and intramembranous ossification^[49].

Regenerative medicine using p-SC for bone disease (large lytic lesions) secondary to multiple myeloma was investigated. The authors founded p-SCs inhibitory effects on myeloma bone disease and tumor growth were dose-dependent and intrabone engraftment of p-SCs inhibited bone disease and tumor growth in mice severe combined immunodeficiency-rab model.

The p-SCs also promoted apoptosis in osteoclast precursors and inhibited their differentiation, indicating a promising therapeutic approach for myeloma osteolysis using cytotherapy^[50].

Tissue engineering

The utility of p-SCs for bone tissue engineering has been investigated using different biomaterials and showing promising results. Transplantation of human p-SCs grown in a silk fibroin/hydroxyapatite scaffold into injured radius segmental bone in rabbits enhance tissue repair^[51]. A combination of p-SCs and polylactide/poly(ϵ -caprolactone)-poly(ethylene glycol)-poly(ϵ -caprolactone) fibrous scaffolds promoted good cellular response and excellent osteogenic potential *in vitro*^[52].

An elastic scaffold, obtained combining a poly-(ether)urethane-polydimethylsiloxane (PEtU-PDMS) semi-interpenetrating polymeric network (s-IPN) with fibrin, was used as a substrate for *in vitro* studies of human p-SC growth and differentiation. The s-IPN PEtU-PDMS/fibrin combined scaffold allowed a better proliferation and metabolic activity of p-SC cultured up to 14 d, compared to the ones grown on plastic dishes and also sustained the beginning of p-SCs differentiation process towards a cardiomyogenic lineage^[53].

As an alternative to corneal transplantation, researchers evaluated a biomaterial derived from fetal membranes to promote corneal repair. The pericellular matrix of decidua-derived mesenchymal cells was demonstrated to provide a xeno-free substrate for efficient culture of human corneal endothelial cells. The matrix enhanced corneal cell attachment, promoted cell proliferation, and suppressed apoptosis, offering a viable *in vitro* expansion protocol for human corneal endothelial cells^[54].

Cell-based therapy, using p-SC, combined with bioactive materials improved bone regeneration prior to dental implant. Amniotic epithelial cells, loaded on a calcium-phosphate synthetic bone substitute, displayed a reduced fibrotic reaction, a limited inflammatory response and an accelerated process of angiogenesis. In addition, the presence of p-SCs significantly stimulated osteogenesis by enhancing bone deposition, as suggested by the presence of p-SCs entrapped within the newly deposited osteoid matrix and by their ability to switch-on the expression of osteocalcin when transplanted into host tissues^[2].

CHALLENGES

A routine medical practice using stem cells is an exciting promise. And for this reason, any preclinical study regarding cell therapy must be considered cautiously. Besides the rapid evolution in differentiating stem cells into many different cell types, all protocols result in a mix cell preparations and how to get specialized cells in sufficient amount and purity is still a challenge. So, the really understanding of all the

mechanisms from culturing stem cells to transplanting differentiated ones into a patient is of great importance in order to let the dream comes true.

CONCLUSION

In summary, either the fetal or the maternal components of the placenta may be considered potential sources of stem cells once they share features from both cells of embryonic and mesenchymal origin. Phenotypically, these cells show common characteristics of embryo-derived and adult stem cells and do not express hematopoietic cell markers CD11b, CD 34 and CD 45. Different media to culture and stimuli to onward differentiation in p-SCs are under investigation and they have been used on research trials of cell therapy with promising results. Furthermore, it is important to get enough purity cells and to understand the mechanism of differentiation and engraftment of p-SC to improve cell-based therapy.

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