ORIGINAL PAPER

Placental stem cell differentiation into islets of Langerhans-like glucagon-secreting cells

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Abstract

Background and Purpose: For the past few years, in an attempt to find new sources of cells that may be used in cell therapy, numerous researchers have highlighted the particular properties of mesenchymal stem cells. Mesenchymal stem cells can be isolated from adult tissues such as the bone marrow or adipose tissue, but also from other organs such as the human placenta. Our study focuses adult stem cells isolated from the chorionic villi in an attempt to differentiate them into islets of Langerhans in order to study their differentiation potential, as a future background for cell therapy. *Experimental Design*: Full-term placentas were prelevated from volunteer women that have just delivered a normal pregnancy. After a mechanical fragmentation of the placenta, the chorion fragments are transferred in a dish with dispase before the enzyme is inactivated using fetal calf serum. The cell suspension is filtered in order to obtain a single-cell suspension. After the adherence of the first cells, the proliferation rate increased progressively and cell morphology is kept the same for several passages. In order to correctly differentiate placental stem cells into glucagon-secreting cells, we used a culture method on a scaffold with sequential exposure to different growth factors. The underlying substrate used contained type IV collagen, chytosan, Matrigel and laminin. Molecular biology techniques were carried out to investigate the gene expression of the stem cells. *Results*: Our results show that exendin-4 is able to induce the differentiation of placental stem cells into glucagon-secreting cells. We also notice the absence of the insulin gene, a conclusion that may be explained by the fact that our phenotype is a partial one, incomplete, closer to islet cell progenitors than to insulin-producing progenitors. *Conclusions*: The identification of the placenta as a valid source for stem cells has important practical advantages because it is easily accessible, it raises no ethical issues and cells are easily to isolate in a large enough number to use. The future knowledge and manipulation of the signaling pathways that determines the dramatic phenotype shift may provide the basis for efficient cell differentiation, with great impact on regenerative medicine and tissue engineering.

Keywords: placental mesenchymal stem cells, regenerative medicine, glucagon-secreting cells.

⊟ Introduction

For the past few years, in an attempt to find new sources of cells that may be used in cell therapy, numerous researchers have highlighted the particular properties of mesenchymal stem cells [1–4]. Several studies have shown the superior role of adult stem cells in regenerative medicine and tissue engineering because their administration, following the isolation and *in vitro* culture leads to the cell's engraftment in certain tissues and the release of anti-inflammatory cytokines [5]. Mesenchymal stem cells can be isolated from adult tissues such as the bone marrow or adipose tissue, but also from other organs such as the human placenta. The identification of the placenta as a valid source for stem cells has important practical advantages because it is easily accessible, it raises no ethical issues and cells are easily to isolate in a large enough number to use.

Besides, immunology characteristics as a low HLA level turns the placental stem cell into a interesting candidate for cell transplantation [6–8], as recently published data prove the plasticity of these cells, allowing the differentiation into cartilage, bone, muscle cells or endothelial cells [9, 10].

Our study focuses adult stem cells isolated from the chorionic villi in an attempt to differentiate them into islets of Langerhans in order to study their differentiation potential, as a future background for cell therapy.

⊟ Material and Methods

Patient selection and tissue processing

Full-term placentas were prelevated from volunteer women that have just delivered a normal pregnancy.

A written informed consent was signed prior to the study, in accordance to all international legal and ethical laws at the 2nd Gynecology Clinic in Cluj-Napoca. All tissues were tested to exclude HIV-infection, toxoplasmosis, cytomegalovirus and rubella virus infection.

All tissues were processed within three to four hours, in order not to affect cell viability, after having been evaluated by a certified pathologist.

Cell isolation and culture

After a mechanical fragmentation of the placenta, the tissue is washed with phosphate buffer solution (PBS). The chorion fragments are transferred in a 50 mL Falcon dish with 15 mL of dispase (Sigma Aldrich, St. Louis, MO, USA) for 30 minutes at 37^0C, before the enzyme is inactivated using fetal calf serum (FCS). Following centrifugation at 1200 rpm for seven minutes, the cell suspension is filtered in order to obtain a single-cell suspension. Cell viability was evaluated using the Trypan Blue staining technique. Red blood cell lysis was possible using ammonium chloride (0.8% NH_4Cl and 0.1 mM EDTA) for 10 minutes at 4^0C and 41^0C.

Cells were cultured at 37^0C, 7% CO_2 for seven days before the culture medium is first changed and a 70–80% cellular confluence obtained. The first passage is possible after three to six weeks, depending on each case and the fibroblast-like phenotype is noticed after 7–15 days in culture. After the adherence of the first cells, the proliferation rate increased progressively and cell morphology is kept the same for several passages. The culture medium contained DMEM/F12, 20% FCS, penicillin, streptomycin, 2 mM L-Glutamine, 1% non-essential amino acids, 1 mM sodium piruvate and 55 μM β-mercapto-ethanol.

RNA extraction and RT–PCR

RNA was extracted from chorionic mesenchymal cells in the culture passage 3–4 (80% confluence) and from the mononuclear fraction of umbilical cord blood stored in liquid nitrogen. Total RNA was extracted from approximately 0.5×10^7 cells using the PureYield RNA Midiprep System kit (Promega). The synthesis of cDNA corresponding to messenger RNA was carried out using the ImProm Reverse Transcription System kit (Promega).

The reaction mixture consisted of 0.5–1 μg RNA, 15 u/μg AMV reverse-transcriptase; buffer solution (10 mM Tris-HCl with pH 9.0 at 25^0C, 50 mM KCl, 0.1% Triton X-100); dNTP solution 1 mM each; $MgCl_2$ 5 mM; recombinant ribonuclease inhibitor 1 u/μL; oligo-dT 0.5 μg/μg RNA; nuclease-free ultra pure water. The described mixture was incubated at 45^0C for 45 minutes, followed by heating at 95^0C for 5 minutes and cooling at $0-5^0$C for another 5 minutes. Subsequently, the cDNA obtained was stored at -20^0C until its use in PCR amplification reactions. Amplification was performed under standard conditions, using the components of the GoTaq PCR Core System II kit (Promega). The cDNA amount was generally 5–10 ng/μL and negative controls were always prepared (ultrapure water instead of standard DNA).

The reaction also included: Taq polymerase 0.025 u/μL; buffer solution; dNTP solution 0.2 mM each; $MgCl_2$ 1.5 mM; primers and nuclease-free ultra pure water. The program used was a standard amplification scheme in which the melting temperatures of specific primers varied: denaturation 95^0C, two minutes; 35–45 cycles of 95^0C, 30 seconds – t^0C (depending on

primers), one minute – 72^0C, two minutes; 72^0C, five minutes; storage at 4^0C. The primer sequences, the size of the amplification product and the melting temperature used are indicated in Table 1.

Table 1 – *The primer sequences, the size of the amplification product and the melting temperature*

Primers	Sequence (5'→3')	Size of the product [bp]	Melting temperature [^{0}C]
Amilase	agttagctataaattatgcacaag tgcttggaagcatcaagtctgaac	411	56
Insulin	tttgtgaaccaacacctgtgc aagggctttattccatctctctc	326	56
Pancreatic lipase	ctcctatatactaatgagaaccca attcaacaaaatatgccacttctgc	286	56
Glucagon	tgaaaagcatttactttgtggct ttcttcaacaatggcgacctcttct	422	56

Immunofluorescence staining

The cells were cultured on chamber slides until confluence was reached, after which they were fixed with 4% formaldehyde solution in PBS for 20–45 minutes at room temperature. The membrane was permeabilized with a 0.1% Triton X-100 solution in PBS for 20 minutes, and for blockage various solutions corresponding to the antibody used, i.e. 1% BSA (bovine serum albumin) in PBS, normal donkey serum or 10% BSA in PBS for 45 minutes at room temperature, was used. The primary monoclonal antibodies used were Nanog, Sox-2 (R&D), Oct-3/4, alkaline phosphatase, SSEA-4, CD105 (Santa Cruz Biotechnologies) and CD29 (Bekton–Dickinson), all mouse anti-human antibodies, and the secondary goat anti-mouse antibodies IgG1 and IgM were marked with FITC, Texas Red and PE (Santa Cruz Biotechnologies). Incubation with the primary antibody was performed at 4^0C for one hour or during the night, and in the case of the secondary antibody for 45–60 minutes at 37^0C. The slides were assembled with an antifade medium containing DAPI in order to evidence the nuclei and were examined using a Zeiss Axiovert microscope by inversed phase fluorescence.

Differentiation protocol

In order to correctly differentiate placental stem cells into glucagon-secreting cells, we used a culture method on a scaffold with sequential exposure to different growth factors. The underlying substrate used contained: 15 μg/mL type IV collagen, 0.1 mg/mL chytosan, 4 mg/mL Matrigel and 2 μg/cm² laminin. The protocol includes four steps: in the first step, during four days we used Dulbecco's Modified Eagles Medium (DMEM) (1000mg glucose/L)/F12 1:1, 2% BSA (Bovine Serum Albumin), 0.5% N_2 (Gibco), 0.5% B27, 3 ng/mL transforming growth factor β (TGF-β) and 0.2 μg/mL rifampicin. The second step, for another four days, we added F12/MCDB 201 1:1, 0.5% BSA, 0.5% B27, 0.5% ITS (insulin, transferin and selenium), 2 μM retinoic acid and 10 ng/mL basic fibroblast growth factor (bFGF). The third step contains DMEM with 5000 mg glucose/L, 0.5% BSA, 1% N_2, 0.5% ITS, 50 ng/mL epidermal growth factor (EGF) for five days and the final step lasts eight days, using DMEM low glucose/F12, 0.5% BSA, 0.5% ITS, 10 ng/mL bFGF, 50 ng/mL exendin-4 and 10 ng/mL BMP4 (all from Sigma-Aldrich).

◻ Results

In the current study, our research team has successfully managed to isolate placental mesenchymal stem cells. Cell adherence was observed after 2–3 days of primary cell culture, at the beginning cells having a round shape and becomes spindled-shaped with time until finally reaching a fibroblast-like shape. Cell passage was carried out at 70–80% confluence and a part of the cells was kept at -170^0C in liquid nitrogen, for further use. We tested the expression of genes known to have an important role in stem cell biology, including embryonal stem cell biology. As shown in previously published data, one such genes present in both amniotic membrane stem cells and chorion stem cells in Oct3/4, responsible for the synthesis of a key regulatory protein for the undifferentiated state (Figure 1).

Also, the telomerase levels (hTERT) and the protein it encodes are usually highly expressed by embryonal stem cells. Nevertheless, the cells isolated by our team did not express this gene. This proves that placental stem cells have a limited proliferative potential but the

risk for the development of different tumors, both benign or malignant, after transplantation does not exist. After the genetic evaluation of the cell population, the pluripotency potential was tested by differentiation into cartilage, bone or adipose cells. These preliminary results confirm once more the plasticity of placental stem cells (data not shown).

The next step, by using a complex differentiation protocol, we have successfully managed to obtain, starting from placental stem cells, glucagon-secreting cells, a key hormone in the regulation of glucose blood levels in the human body. At the end of the culture period, cells changed their phenotype into a fibroblast-like one, lost the ability to adhere to the bottom of the culture flask and showed a cuboid-like shape, forming spheroids in suspension (Figures 2 and 3).

Confirming the results of immunocytochemistry protocols, the genotypical characterization of the differentiated cells highlights the expression of the glucagon gene, characteristic for alpha cells of the Langerhans islets (Figure 4).

(A) **(B)**
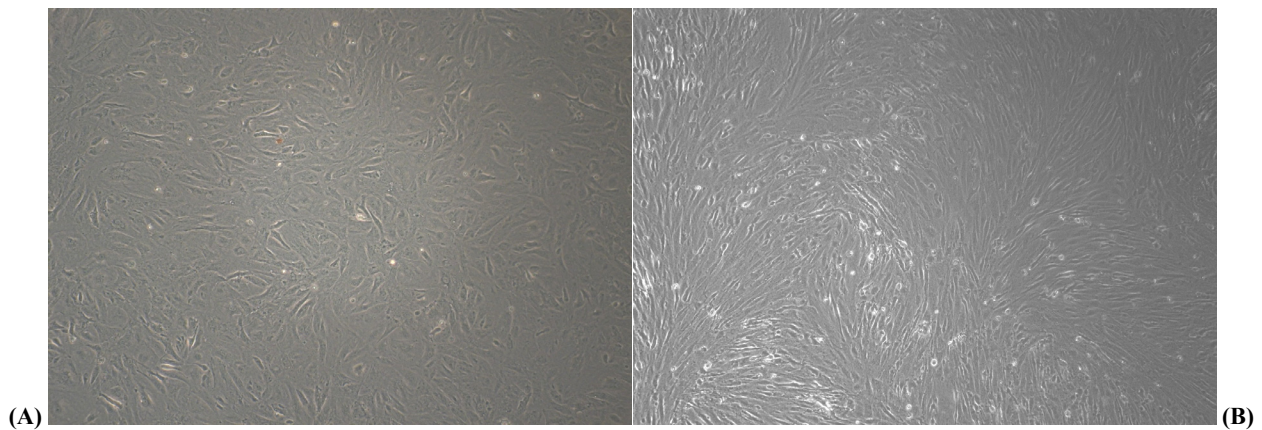
Figure 1 – *Placental mesenchymal stem cells at subconfluence (A) (day 29, passage 1) and passage 3 (B).*
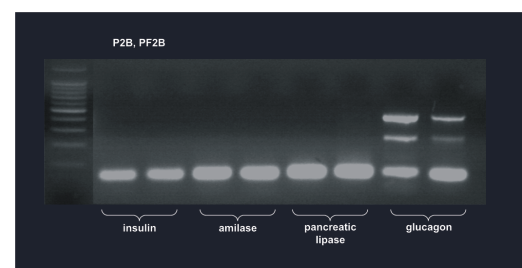

Figure 2 – *Spheroids resembling islets of Langerhans in the final step of cell differentiation.*
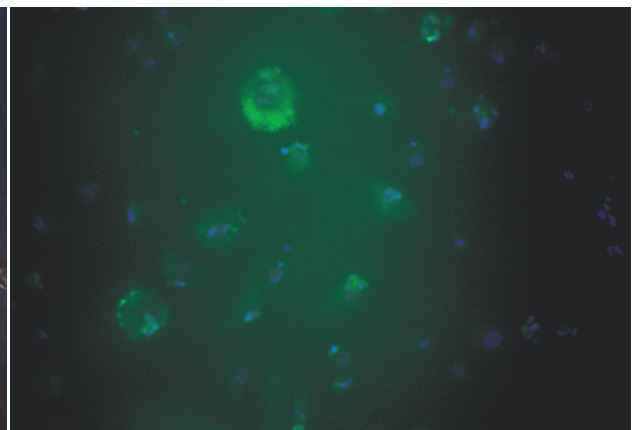
Figure 3 – *Positive immunocytochemistry staining for glucagons (chorion cells, passage 8).*

Figure 4 – *The gene expression of the final step of pancreatic differentiation. Cells express the glucagon gene.*

Discussion

Recent progress in the control of stem cell differentiation has brought new hope for the future treatment of diseases considered today as incurable. One among the sources of pluripotent cells is the human placenta [11–15] and recent knowledge shows that this organ, along with the Yolk sack and the liver, is a source of hematopoietic progenitor cells during fetal development [16–18]. The use of such cells has been proven useful in the treatment of various hematological and genetic conditions [19–21], the maintenance of the pluripotency state being closely connected to the existence of characteristic niches. The histological tissue architecture, similar to that of the bone marrow, with hematopoietic cells residing in a niche formed by mesenchymal cells with multi-lineage development potential, has brought us to the valid hypothesis that the placental villi may also be formed by a small population of cells with properties similar to that of adult stem cells [22–25]. Also, the access to these cells is a lot easier because for the past few years the hematopoietic placental cells, but also the mesenchymal stem cells, have been preserved immediately after birth [26–30].

Regarding cell isolation, it follows a easy protocol, the most suitable medium being formed by DMEM/F12, fetal calf serum, penicillin, streptomycin, L-Glutamine, non-essential amino acids, sodium piruvate and β-mercapto-ethanol, for the primary culture, but also for the secondary culture conditions with a smaller fetal calf serum concentration. In our study, the epidermal growth factor did not influence the isolation and expansion of the cells from the amiotic membrane and also, red blood cell lysis using NH_4Cl did not influence at all the primary culture. As shown in previous studies, the genetic profiling reveals the expression of pluripotency genes, as Oct 3/4, a strong argument for the use of these cells in regenerative medicine.

Most interesting are the results concerning the change of the phenotype during the differentiation process. Immediately after isolation, adherent cells had a fibroblast-like phenotype but as the differentiation process carries on, the cells begin to show a epithelial-like shapes, turning into cuboid cells. During the final steps, the volume becomes smaller, cells become round and form clusters made out of 30–40 cells in suspension. This pattern of organization may be compared to that of the islets of Langerhans, found in the adult pancreas [31–34].

The maintenance of blood sugar levels is important for the normal physiology of the body and this homeostasis is maintained using a feedback system in which insulin and glucagon have key roles. These hormones are secreted by alpha and beta cells, a close relationship unifying these two cell types. The secretion of glucagon in the adult body is controlled either directly, depending on the glucose concentration, either indirectly through the interaction between alpha and beta cells. Between the two cell types, there is a close relationship at the molecular level and the particular distribution of the small blood vessels favors this very connection [35–37].

Usually, both alpha and beta cells are very stable, have a long lifespan and when they divide alpha cells give rise of other alpha cells, with the same rule going for beta cells [38]. Some studies have shown that a common progenitor during pancreatic embryogenesis gives birth to both cell types [39], and also in the case of the loss of an important part of the beta cell population, their regeneration is possible because of the alpha cell population. This suggests that alpha cells can present less differentiated progenitors of beta ones. Responsible for this conversion are a series of transcription factors that include Pdx1, Nkx6.1 or Pax4. The mentioned factors regulate the balance between the alpha and beta phenotype, with a predisposition for activation found on alpha cell membrane [40–42]. The mentioned observations point out the fact that alpha cells may be used as a source of insulin-producing cells [43–46]. Another fact is that during transformation there is a period of time in which cells secrete both insulin and glucagon, another evidence that supports the hypothesis that alpha cells may act as progenitor cells when beta cell number drops.

Conclusions

In the presently-published study, we used a four-step differentiation protocol. In the final period, we used exendin-4, a substance known to help in the process of pancreatic islet differentiation. Exendin-4 is a synthetic analogue of GLP-1 (glucagon-like peptide), an incretin secreted postprandial by L-cells, found in the small intestine structure. This acts on cell that expresses the GLP-1R receptor on the membrane, such as beta cells, brain, lung or even heart cells. The expression by alpha cells has been reported, but remains controversial. Our results show that exendin-4 is able to induce the differentiation of placental stem cells into glucagon-secreting cells. We also notice the absence of the insulin gene, a result that may be explained by the fact that our phenotype is a partial one, incomplete, closer to islet cell progenitors than to insulin-producing progenitors.

The future knowledge and manipulation of the signaling pathways that determines the dramatic phenotype shift may provide the basis for efficient cell differentiation, with great impact on regenerative medicine and tissue engineering.

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