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Human placenta – stem cell source for obtaining pancreatic progenitors

SERGIU ŞUŞMAN[1)*], DAN RUS-CIUCĂ[1)*], OLGA SORIŢĂU[2)], RĂZVAN CIORTEA[3)], MIHAI GÎRLOVANU[1)], DAN MIHU[3)], CARMEN MIHAELA MIHU[1)]

[1)]*Department of Histology, "Iuliu Haţieganu" University of Medicine and Pharmacy, Cluj-Napoca, Romania*
[2)]*Laboratory of Radiobiology and Tumor Cell Biology, "Prof. Dr. Ion Chiricuţă" Oncology Institute, Cluj-Napoca, Romania*
[3)]*Department of Obstetrics–Gynecology II, "Iuliu Haţieganu" University of Medicine and Pharmacy, Cluj-Napoca, Romania*

*These authors contributed equally to this work.

Abstract

Objectives: The apparition of sugar diabetes is produced by the decrease of the number and capacity of beta cells to secrete insulin. Cell mass recovery through cell therapy might be one of the solutions for treating this disease. The use of various cell sources of different differentiation grades has been tried over the last years. Decoding the molecular mechanisms of the pancreatic morphogenesis is essential for obtaining cells having a phenotype, which would be very similar to the mature cells located in the pancreatic endocrine component. In this study, in order to obtain pancreatic progenitors, we used stem cells harvested from the mesenchymal component of the amniotic membrane, cells with particular immunological properties, which are effective in transplant. *Materials and Methods*: Isolated cells from the placenta (amniotic membrane) have undergone a three-stage differentiation protocol. The modulation of glucose concentration, the type of substrate (collagen + laminin) and the use of nicotinamide and exedin-4 were the main selective conditions of differentiation micro-environment. The differentiated cells were analyzed from the point of view of proteins (immunofluorescence – IF), gene expression (real-time polymerase chain reaction – RT-PCR) and morphological changes. *Results*: Isolated cells from the placenta membrane induced for pancreatic differentiation expressed transcription factors, which are characteristic for pancreatic progenitors (Pdx1 and PAX4). During the experiment, the cells modified their morphology by forming islet-like clusters. They were positively for dithizone staining and expressed insulin as shown by immunocytochemistry. *Conclusions*: The isolated cells from the placenta can be differentiated towards pancreatic progenitors by using specific protocols.

Keywords: placenta, stem cells, pancreatic progenitors.

Introduction

Type 1 diabetes is produced by the destruction of insulin secreting beta cells resulting in a defective use of glucose in the body [1–3]. Current treatments use the administration of insulin in order to improve the glycemic level, but this kind of treatment is not the best protocol with long-term consequences over the entire body [4–6]. Destroyed beta cells replacement is the best solution in the treatment of type 1 diabetes, and this disease is considered the perfect model for cell therapy protocols [7–9]. Therefore, numerous studies aim decrypting the molecular mechanisms involved in the pancreas development [10–12]. Several cell types were involved for differentiation in order to be used in cell therapy [13–17]. Among them, the mesenchymal stem cells are important due to their special immunological properties, which make them extremely useful in type 1 diabetes cell treatment [18–21].

In the present study, we focused upon mesenchymal placental stem cells taken from the amniotic membrane. Besides the immunological properties specific to the placental stem cells, they lack any ethical issues and they can be obtained in sufficient amounts. They can also be stored at birth in order to be used throughout the lifetime of the individual.

Materials and Methods

Placenta selection

Placentas were taken from pregnant women included in the study after signing an agreement according to the laws and international ethical stipulations. All patients had a normal pregnancy evolution and normal delivery. All placental samplings were tested in order to exclude the ones with HIV (human immunodeficiency virus), toxoplasmosis, cytomegalovirus and rubella infection. The processing was made in the first four hours following their harvesting to avoid decreasing of cell viability.

Isolation and culture of cells

After the mechanical fragmentation of the placentas, the placental tissue was washed with phosphate-buffered saline (PBS). The decollated fragments of the membranes had been transferred into a 50 mL Falcon tube with 15 mL of 0.25% trypsin (Sigma-Aldrich, St. Louis, MO, USA) and exposed for 5 minutes at 37^0C on a rotary agitator. A vigorous pipetting of tissue fragments was followed by inactivation of trypsin with complete medium containing 10% fetal calf serum (FCS) and centrifugation. After discarding the supernatant, an enzymatic cocktail with 0.25% trypsin and 0.1% collagenase type IV (Gibco) was

added on membrane fragments and digested for another five minutes. Then, the enzymes were inactivated using medium containing 10% FCS. This process was followed by a 7-minute centrifugation at 1200 rpm and the resuspended cells in complete medium were filtered with Filcon's meshes (70 μm pore) to get a single cell suspension. Cell viability was evaluated by Trypan Blue staining. Erythrocyte lysis was performed by using an ammonium chloride solution buffer (0.8% NH_4Cl and 0.1 mM EDTA – ethylenediamine-tetraacetic acid) with exposure of primary cell suspensions for 10 minutes at 41^0C. Remaining nucleated cells were seeded in Cole flasks (25 cm^2) (Nunc) in 5 mL of complete medium and cultivated at 37^0C, 7% CO_2 during seven days. The cell culture media was first changed after 6–7 days. We performed the first passage after a variable interval of 1–2 weeks when we obtained the characteristic fibroblastoid phenotype and the cells monolayer reached a confluence of 70–80%. At the following passages, the cell proliferation rate gradually grew keeping the characteristic mesenchymal cell phenotype during several passages. The culture media for the primary cultures was as it follows: DMEM (Dulbecco's modified Eagle's medium) high glucose/F12 medium (ratio 1:1), 20% FCS, penicillin, streptomycin, 2 mM L-glutamine, 1% non-essential amino acids (NEA), 1 mM sodium piruvate and 55 μM, β-mercapto-ethanol (all reagents from Sigma-Aldrich). For further passages, the FCS concentration was 15%. At the 5th passage, the isolated cells were characterized for stemness markers expression by immunocytochemical staining and real-time polymerase chain reaction (RT-PCR). Cells expressed pluripotency markers Oct3/4, SOX-2, Nanog, SSEA-4, Rex-1 and mesenchymal stem cells antigens CD29 and CD105 [22].

Differentiation protocol

The substrate of collagen and laminin, as an important element in extracellular signaling, was created by a layer-by-layer method in 24-well dishes. The first layer was composed of 0.5 mL sterile solution of 15 μg/mL (in PBS) of collagen IV from human placenta applied in each well and left to dry in a laminar flow hood. The dried collagen substrate sustained the second laminin layer obtained using the same method with a laminin solution of 15 μg/mL (in PBS) (all reagents were purchased from Sigma-Aldrich). This procedure was repeated three times in order to obtain a stratified substrate [23]. The differentiation protocol started with the exposure of the stem cells monolayer to a pre-differentiation medium for obtaining the common hepato-pancreatic progenitors, consisting of DMEM low glucose/MCDB201 medium, 1% bovine serum albumin (BSA), 4.7 μg/mL linoleic acid, 1% insulin–transferring–selenium (ITS), 10 ng/mL platelet-derived growth factor (PDGF), 10 ng/mL epidermal growth factor (EGF), 10 nM dexamethasone, 10 mM ascorbic acid, 1% L-glutamine, 1% antibiotics and 1% NEA (Sigma-Aldrich). After 24 hours, the growth factors were replaced with 20 ng/mL hepatocyte growth factor (HGF) and 20 ng/mL Oncostatin M (Sigma-Aldrich) and the cells were exposed to this medium for another three days. The pancreatic differentiation protocol consisted of three main stages. After three days of culture, some of the cells detached and this cell suspension was transferred into plates prepared with collagen-laminin substrate, after a short centrifugation at 1200 rpm for four minutes and the cell pellet was resuspended. For the first step, we used P1 medium containing DMEM high glucose (concentration of glucose 6.5 g/L)/MCDB201 medium (ratio 1:1), 1% BSA, 1% N1 supplement, 0.5% B27 supplement (from Gibco), 10 ng/mL EGF, 10 ng/ml basic fibroblast growth factor (bFGF), 4 ng/mL HGF, 20 UI/mL heparin, 1% ITS, 1% L-glutamine, 1% penicillin–streptomycin, 1% NEA. The cells were inoculated on the collagen + laminin plates. A 7-day culture period was performed. The cells remained in suspension but their morphology changed, the cell aggregates becoming more dense and condensed having exhibiting a spherical shape of a larger dimension.

In the second stage, after seven days, the cell suspension was centrifuged and resuspended in P2 medium and seeded on other plates with collagen + laminin substrate. The culture in P2 medium took another seven days. P2 medium consisted of DMEM low glucose at 1 g/mL/MCDB201, 10% FCS, 10 mM nicotinamide, 1% L-glutamine, 1% penicillin–streptomycin, 1% NEA.

In the third and last stage, the cultivated cell suspension was removed after seven days, centrifuged and resuspended in P3 medium and reinoculated on other plates with substrate. This stage of culture lasted seven days. P3 medium consisted of DMEM low glucose/ MCDB201, 10% FCS, 10 mM exendin-4, 1% L-glutamine, 1% penicillin–streptomycin, 1% NEA. After seven days of culture in P3 medium, 10 ng/mL glucagon and 3 ng/mL of transforming growth factor β1 (TGF-β1) were added into the media for at least three more days (Figure 1).

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 the umbilical cord blood stored in liquid nitrogen. The 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; 5 mM $MgCl_2$; 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 five minutes and cooling at 0–50^0C for another five minutes. Subsequently, the obtained 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^{0}C, two minutes; 35–45 cycles of 95^{0}C, 30 seconds – t^{0}C (depending on primers), one minute – 72^{0}C, two minutes; 72^{0}C, five minutes; storage at 4^{0}C.

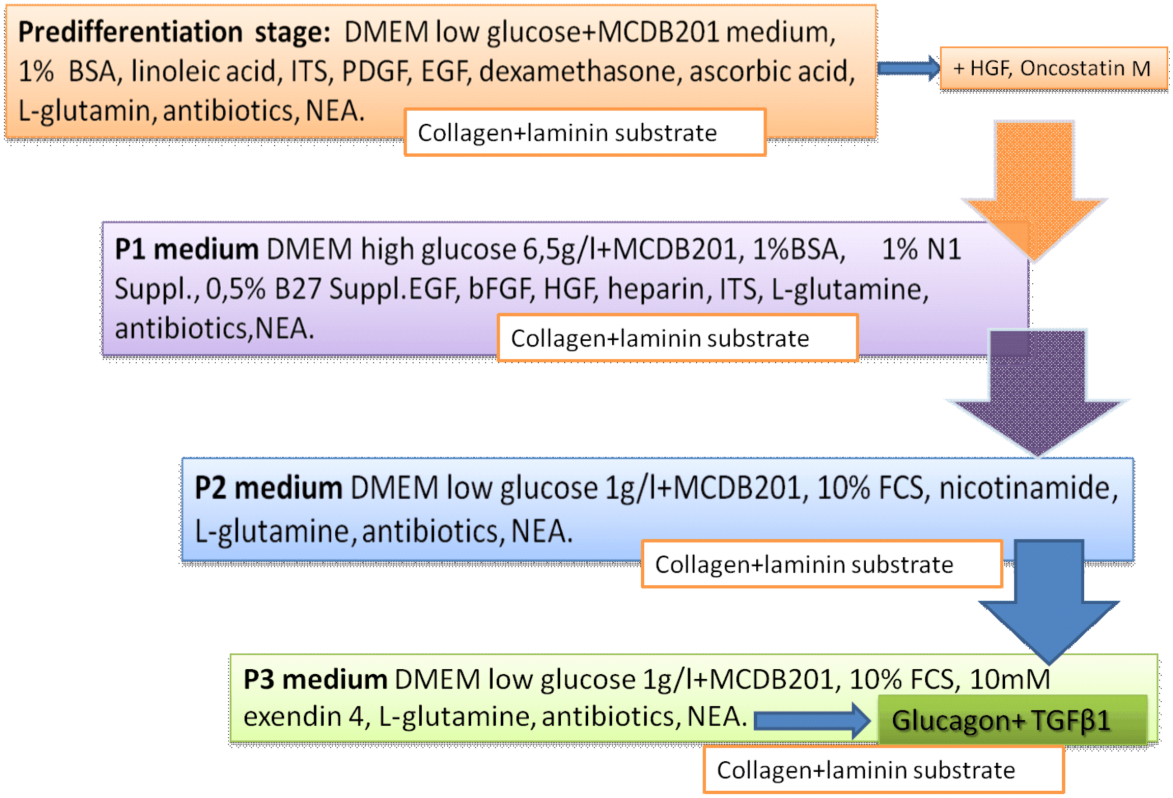

Figure 1 – ***A concise diagram of differentiation protocol applied for obtaining pancreatic cells.***

Immunocytochemical staining

At the end of the third phase of differentiation protocol, cell suspensions were centrifuged (centrifuged) at 600 rpm, washed three times with PBS and fixed in 4% paraformaldehyde solution in PBS for 45 minutes at room temperature. The membranes were permeabilized with 0.1% Triton X-100 solution in PBS for 30 minutes and for blockage of unspecific antibody binding, were used 10% BSA in PBS for 45 minutes at room temperature. The anti-insulin primary antibody (Sigma-Aldrich) was a mouse anti-human type and the secondary antibodies were goat anti-mouse IgG1 marked with Texas red (Santa Cruz Biotechnologies, Dallas, Texas, USA). Incubation with the primary antibody was made at 4^{0}C overnight and for the secondary antibody, incubation was made for 45 minutes at 37^{0}C. All staining steps were followed by three washes with PBS by centrifugation. The slides were assembled with an antifade medium containing DAPI (4',6-diamidino-2-phenylindole) in order to evidence the nuclei and were examined using a Zeiss Axiovert microscope by reversed phase fluorescence with 546 nm and 346 nm filters.

Dithizone staining

At the end of the pancreatic differentiation protocol, the cell clusters were stained for 30 minutes by adding in each well 20 µL in of 10 mg/mL dithizone solution (dissolved in dimethyl sulfoxide – DMSO) in 1.5 mL culture medium. The incubation was performed in incubator at 37^{0}C. The visualization of stained cells was done with a reverse phase Zeiss Axiovert microscope, and image acquisition with a MRM Axiocam camera.

Results

Isolated cells from the amniotic membrane were processed using repeated trypsinization steps in order to be isolated. After adhesion, these cells characteristically showed the fibroblastoid-type morphology. The first passage was undertaken after 1–2 weeks when they reached the 70–80% confluency. After the first passage, the proliferation rate of the cells gradually grew, keeping the phenotype during several passages. The isolated cells behavior in terms of adhesion, proliferation and their genetic profile were characteristic to mesenchymal stem cells as shown before [22].

The applied three steps differentiation protocol of stem cells into pancreatic progenitors applied combined with culture on a collagen–laminin substrate induced dramatic changes on cells adhesion and morphology. Some of the cells detached from the surface of the plates after three days of culture in P1 medium and formed floating clusters in the medium (Figure 2A). Cell suspensions were transferred on other substrate treated plates. Regarding the change of cell morphology, a very interesting phenomenon was the loss of adhesion to the collagen–laminin substrate and the formation of cell aggregates in suspension (Figure 2B) with a more defined spherical shape as the cultures evolved in presence of P3 medium, resembling with the Langerhans islets (Figure 2C). The aggregates survived for another seven days and cell den-

sity grew by adding TGF-β and glucagon (Figure 2D). They were composed of approximately 20–30 cells, of small size and spherical shape.

Before the immunohistochemical coloration the differentiation towards the pancreatic phenotype was emphasized by staining with a zinc chelate (dithizone), used in order to establish the purity of the isolated beta cells for the transplant patients with type 1 diabetes. Beta cells having a high Zn^{2+} concentration, an important metal for secreting insulin, will color in red due to its capture. Some of the cell aggregates obtained after the differentiation were positive for staining with dithizone (Figure 3).

Immunocytochemical staining with anti-insulin antibodies (primary antibody) conjugated with secondary antibodies marked with Texas red, with a DAPI as counter staining of the nucleus, showed positivity for insulin of obtained cell aggregates by using this protocol (Figure 4).

RT-PCR analysis revealed that differentiated cells expressed two of the most important transcription factors that are essential to the pancreatic differentiation: Pdx1 and PAX4. The expression of a characteristic marker associated to the mature pancreatic phenotype can also be noticed (nestin). The common embryological origin with that of the liver is shown by the presence of a hepatic cell-specific enzyme, glutathione synthetase (GS) (Figure 5).

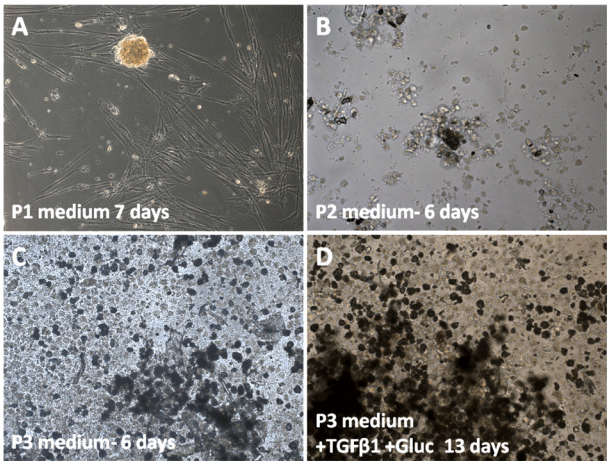

Figure 2 – ***Phase contrast images of stadial pancreatic differentiation on collagen–laminin substrate. (A) Mesenchymal cells harvested from the amniotic membrane changed their morphology forming cell aggregates in suspension in the first seven days of culture with P1 medium. (B) Morphological aspect after six days of cells cultivated in P2 medium. (C) Increased number of cell aggregates with a more defined spherical shape after six days of culture with P3 medium. (D) Cell density grew by adding TGF-β and glucagon (×100).***

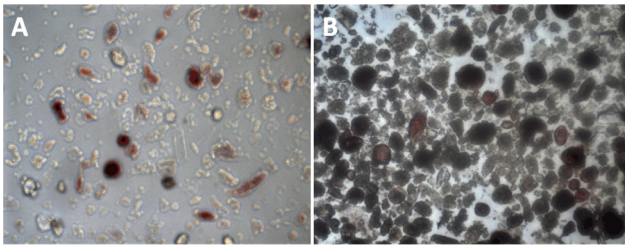

Figure 3 – ***Contrast phase images of cell aggregates in suspension stained with dithizone at the end of the culture phase with media P3 (A) and after adding TGF-β and glucagon (B) (×200).***

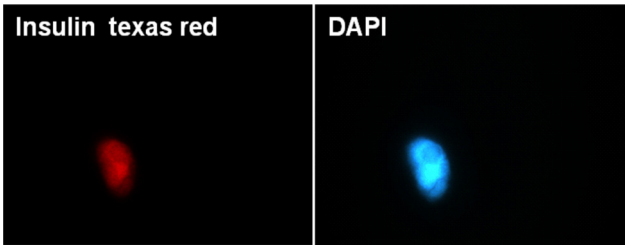

Figure 4 – ***Immunohistochemical staining for insulin of the cell aggregates.***

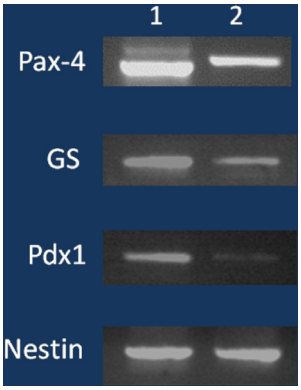

Figure 5 – ***Gene expression in cells induced to differentiate into pancreatic progenitors: strong expression for PAX4 and nestin, and in a lesser extent for Pdx1 and glutathione-synthetase.***

Discussion

The ultimate purpose regarding the diabetes type I treatment is maintaining an optimal level of sugar in blood, avoiding hypoglycemic episodes. Experimental transplants of beta cells carried on diabetic patients have partly proven that this kind of treatment might be a good solution. Since the pancreas provides a limited source of beta cells through isolation, new sources of stem cells meant to undergo differentiation into insulin secreting cells and new protocols are necessary. Using embryonic and adult stem cells in this matter has shown promising results. However, more work has to be carried out in this direction in order to improve the outcome. Concerning the embryonic stem cells several protocols of differentiation have been applied leading to variable results [24, 25]. It should be mentioned, though, that in this case, as well as in all the other cases that make use of embryonic stem cells, the latter come not only with a series of advantages but also with disadvantages. Their remarkable plasticity makes them difficult to control when trying to obtain a certain type of specialized cell. In order to overcome this challenge, all the intracellular signaling pathways must be carefully handled, which is currently impossible. This explains the controversial results obtained using embryonic stem cells, as well as the discrepancy between their theoretical and practical potential regarding their therapeutic use [26]. Concerning the placenta as a source of insulin secreting cells, there are few studies in this direction. Mainly, two of them are representative, both of them carried out by Chinese researchers. In the first one, Sun & Ji [27] uses a simple protocol. The medium is classic, the Dulbecco's modified Eagle's medium consisting in a high concentration of glucose and containing 10% fetal bovine serum (FBS), 1% NEA, beta-mercaptoethanol

and glutamine to which is added bFGF, the basic fibroblast growth factor. The cells are passaged after 2–3 days/ every 2–3 days and studied using the phase microscope on a daily basis. Even though the protocol is simple, the researchers managed to record Pdx1 gene expression, insulin 1, and insulin 2 at/after 7 and 14 days. Chang *et al.* uses a more complex protocol on cells isolated from the placenta: the medium is the Dulbecco's modified Eagle's medium/F12 1:1 medium containing ITS, 0.6% glucose, 25 g/mL insulin, 100 g/mL transferrin, 20 nM progesterone, putrescine, 30 nM selenium chloride, 2 mM glutamine, 3 mM sodium bicarbonate, 20 ng/mL bFGF, and 20 ng/mL hepatocyte growth factor [28].

Starting with the existing data in literature in the current study, we tried to obtain pancreatic endocrine component progenitors beginning with isolated pluripotent cells at the level of the human amniotic membrane of the placenta. Isolated cells express at both the level of gene expression and the level of proteins pluripotential markers that are necessary for the application of differentiation protocols. Regarding cell isolation, this is made easily with a protocol used by us and already mentioned previously [22]. At the moment, there are studies which tried using different sources of cells [29–34]. Furthermore, the protocols used are quite heterogeneous, the obtained results being variable. At the moment, presently there is no general protocol accepted [35–38].

In our study, we accomplished the differentiation towards the pancreatic endocrine progenitors during three stages. In the first stage, the two parameters, the duration and the substrate that we used, were important. A period of seven days was the optimal time. Furthermore, we have got the best results by using a combination of collagen and laminin as a culture substrate.

Three types of medium were used with modulation of glucose levels depending on the differentiation stage that progressively was reached by the cells. During the first stage, we cultivated the cells in a medium with high glucose concentration (6.5 g/L) containing a combination of growth factors and specific supplements for neuronal differentiation such as N1 and B27.

The second stage took seven days, during which the concentration of glucose in the DMEM/MCDB201 medium was lowered down to 1 g/L. Nicotinamide, which is an important phenotype inductor, was used in the P2 medium. In the last stage, the low concentration of glucose was maintained and exedin-4 was added and then glucagon, for another three days, were introduced in the medium. This protocol allowed us to modify the phenotype of isolated mesenchymal cells. During the first stage, these cells became non-adherent, they detached and formed clusters in suspension of approximately 30–40 cells, similar to the Langerhans islets. These cell clusters proved to be dithizone-positive, a zinc chelator used to measure the purity of the Langerhans islets in the transplant. As for the gene expression, two essential transcription factors for the development of the pancreas, Pdx1 and PAX4, were brought forward. Their presence proves that the obtained cells are progenitors of the endocrine pancreatic component. Until now, the differentiation protocols used in order to differentiate stem cells succeeded in obtaining only partial phenotypes, incomplete in comparison with adult cells that have a sophisticated differentiation program. We recently saw that in the matter of differentiation of stem cells towards beta cells they look like a fetal phenotype rather than an adult phenotype [39]. In our study, we obtained positivity both for PAX4, which is characteristic for fetal beta phenotype and for Pdx1 expressed in adult beta cells. The phenotype we obtained can be an intermediate one. As for the proteic expression, the cells we obtained were positive for insulin in immunofluorescence.

Conclusions

On gene and proteic level, isolated mesenchymal cells from the placenta expressed pluripotential markers and can be used to obtain pancreatic progenitors. Although at this point there is not a consensus regarding the protocol to be used, it seems that the substrate used and the glucose concentration are important factors. Furthermore, exendin-4 and nicotinamide are essential in the final stages of differentiation protocol. The resulting phenotype is incomplete and immature although marker expression characteristic of beta cells is present. Future studies are imperative for the improvement of protocols used in order to obtain a functional phenotype similar to normal beta cells.

Conflict of interests

The authors declare that they have no conflict of interests.

Acknowledgments

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Corresponding author
Sergiu Şuşman, Lecturer, MD, PhD, Department of Histology, "Iuliu Haţieganu" University of Medicine and Pharmacy, 6 Pasteur Street, 400349 Cluj-Napoca, Romania; Phone +40747–074 018, e-mail: serman_s@yahoo.com

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