

Isolation and characterization of chorionic mesenchymal stem cells from the placenta

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Abstract

The aim of the study was the isolation and characterization of mesenchymal stem cells from the placental chorion from a genotypical and phenotypical point of view. The placentas included in the study were derived from term pregnancies with a normal evolution. Along with the placentas, umbilical cord blood, maternal and newborn peripheral blood samples were taken. The isolation and culture of chorionic and, incidentally, trophoblastic cells was followed by the determination of markers of the former cells. They expressed proteins and genes characteristic of stem cells. Immunofluorescence and evaluation of gene expression evidenced the pluripotential properties of these cells and also their higher position on the differentiation pathway. HLA expression provides information that might help explain the immunological mechanisms of tolerance between the maternal organism and fetal structures.

Keywords: mesenchymal stem cells, placenta, chorion, chorionic plate.

Introduction

Placenta is one of the most feasible stem cell sources and much study is dedicated nowadays towards unraveling its mysteries. It fulfills the two important requirements of cell therapy, namely a high number of cells and an easy non-invasive way to harvest them. Still, placenta stem cells have not received the same attention as others, like embryonic stem cells (ESCs), bone marrow or umbilical cord blood. The fact that is emerging at present is that even if placenta cells do not have the plasticity and self-renewing capacity of ESCs, they are still superior to adult type stem cells and may have properties which are somewhere in between these two categories, just like the placenta as an organ. This study is focused on the isolation and characterization of chorionic mesenchymal stem cells.

Materials and Methods

Patient selection

Term placentas from pregnancies with a normal evolution were taken after the obtaining of the informed consent of the pregnant women (Clinic of Gynecology II, Cluj-Napoca). The group of pregnant women included in the study was selected on a volunteer basis, after a previous examination of the general medical history and of the pregnancy evolution. The patients with obstetric diseases or other associated systemic diseases were excluded from the study.

Harvesting and processing

All placentas were harvested immediately after a term natural delivery in a sterile environment and were transported in cold PBS (phosphate buffered saline) solution in a thermally insulated container on ice. They were processed within 3–24 hours, the latter being kept on ice in a mixture of PBS and antibiotics (penicillin + streptomycin), similar with the one used in culture. Along with the placentas, umbilical cord blood, maternal peripheral blood and, in one case, the peripheral blood of the newborn, were taken. They were all processed as soon as possible (usually within an hour) by separating the mononuclear fraction using Ficoll density gradient centrifugation and storing it in liquid nitrogen. The placentas were also assessed macro- and microscopically. Hematoxylin–Eosin (HE) stained histological preparations were completed.

Isolation and culture

After thoroughly washing the placenta and detaching the fetal membranes, a small fragment from the chorion (approximately 2–3 cm³) was dissected from the chorionic plate, as far away from the maternal side as possible. The cells were isolated using several combinations of mechanical and enzymatic treatment with dispase and/or collagenase IV. The cell suspension was filtered and the cells were seeded in Cole flasks in culture medium and incubated at 37°C in a 5% CO₂ atmosphere.

Immunofluorescence

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. 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 were used. The primary monoclonal antibodies used were Nanog, Sox-2 (R&D), 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°C for one hour or during the night, and in the case of the secondary antibody for 45–60 minutes at 37°C. The slides were mounted with an antifade medium containing DAPI in order to evidence the nuclei and were examined using a Zeiss Axiovert microscope by reversed phase fluorescence.

RNA extraction and RT-PCR

Total RNA was isolated from chorionic mesenchymal cells in culture passage 3–4 (80% confluence) and from the mononuclear fraction of umbilical cord and peripheral blood stored in liquid nitrogen. RNA extraction was performed using TRIzol Reagent (Invitrogen) according to the manufacturer's instructions. Only mRNA was transformed into cDNA by using oligo-dT primers in the reaction mixture. This mixture was incubated at 45°C for 45 minutes, followed by heating at 95°C for 5 minutes and cooling at 0–5°C for another 5 minutes. Subsequently, the cDNA obtained was stored at -20°C until its use in PCR amplification reactions. Amplification was performed under standard conditions. The program used was a standard amplification scheme in which the melting temperatures of specific primers varied: denaturation 95°C, 2 minutes; 35–45 cycles of 95°C, 30 seconds – t°C (depending on primers), 1 minute – 72°C, 2 minutes; 72°C, 5 minutes; storage at 4°C. The primer sequences and the size of the amplification product are indicated in the table below (Table 1). The PCR products were then separated by electrophoresis on 2% agarose gel and photographed with a UV transilluminator.

As a loading control for RNA, we used three ubiquitous genes (β_2 -microglobulin, CBF β and GAPDH).

Table 1 – The genes, primer sequences and the size of the amplification product

Genes	Primer sequences (5'→3') forward and reverse	Size of the product [bp]
Oct-4	aggagtcctcaggacatcaaag; tcgtttggctgaataccttc	146
Rex-1	atggctatgtgtgctatgagc; ctcaacttcttagtgcatcc	447
TERT	catcatcaaacccagaacac; caaacagctgttctccatgt	371
Nanog	attataaatctagagactccag; tcctgaataagcagatccatg	407
Sox-2	aagcgctttttgatcctgattc; accacaccatgaaggcattcatg	363

Genes	Primer sequences (5'→3') forward and reverse	Size of the product [bp]
SCF	tatttaatcctctcgtcaaaac; agaattcttcaggagtaaagag	369
β_2 - microglobulin	actccaaagattcaggtttactc; catgatgctgcttacatgtc	302
HLA-DR α	ttgaagaatttgacgactgtg; aaactcccagtgcttgagaagag	407
Vimentin	ttcagagagaggaagccgaaaac; tttaagggcacaccctcacag	422
c-kit (CD117)	ttcagcgagagttaatgattctg; tgtattcacataaacattaaatg	400
Thy-1 (CD90)	aaagaagcacgtgctcttggc; actcagagaagtaggatctctg	379
GAPDH	acaactttggatcgtggaa; aaattcgtgtcataccagg	458
CBF β	aacgaggagtttttaggaagc; attcagaatcatggagccttc	273
Tie-2	tctgtactgctgtatgaacaatg; tttaccactgtttacttctatag	405
GFAP	tatagacaggaagcagatgaag; agactccaggctcgcaggtaag	381
HLA-ABC	accaggacacggagctcgtggagacca; cacactttacaagctgtgagagacaca	354

HLA typing

Two HLA types, class I (HLA-A) and class II (HLA-DQB1) were assessed in DNA samples from chorionic (Ch) and amniotic membrane (AM) mesenchymal stem cells, umbilical cord (UCB) blood, and from maternal (MB) and newborn (NB) peripheral blood. Like in the case of RT-PCR, DNA was extracted from culture cells for chorion and from the cryopreserved mononuclear fraction for UCB and peripheral blood. The DNA was stored at -20°C in a Tris-HCl and EDTA solution.

HLA typing was performed using the Dynal RELI SSO HLA Typing kit in combination with Dynal RELI SSO Strip Detection Reagent kit (both from Dynal, Invitrogen), based on three important stages: (1) PCR amplification of HLA sequences from genomic DNA; (2) hybridization of the amplified products on the surface of strips on which sequence-specific oligonucleotide probes (SSO) were immobilized; (3) detection of the amplified products bound to the probes by a color reaction.

Results

In the experiments performed, we succeeded in isolating and culturing mesenchymal cells from the placental chorion. Their *in vitro* behavior was different depending on the isolation method. The ones which were isolated only using mechanical methods or enzymatic digestion with collagenase IV alone had a strange and unexpected epithelioid morphology (Figure 1A), with a high degree of adherence, removal with trypsin being difficult and requiring higher concentrations than usual. The cells also had a low proliferation rate and a low propensity for secondary culture development. After closer study of their morphology, comparing them with similar cells in the HE stained placenta (Figure 1D), we hypothesized that these cells are actually intermediate trophoblastic cells (Figure 1C).

The cells obtained after digestion with collagenase IV and dispase (Figure 1B) could form a primary culture quite rapidly (even after one week), had the typical fibroblast-like appearance that was expected and a very high proliferation rate.

These isolated cells readily initiated adhesion and proliferation, reaching a 70–80% confluence after one to three weeks from harvesting. At the following passages, the cells had a higher growth rate, reaching a 90–100% confluence after four days. The most adequate medium proved to be a 1:1 DMEM/F12 mixture, 20% fetal serum, penicillin-streptomycin, 2 mM L-glutamine, 1% NEA, 1 mM sodium pyruvate, 55 μ M β -mercapto-

ethanol for the primary culture and the same medium with a 15% fetal serum concentration in secondary cultures. Erythrocyte lysis with ammonium chloride had no favorable effect on the primary culture; on the contrary, the cells grew better in an erythrocyte-enriched environment.

The immunofluorescent staining showed that our cells express various amounts of markers that are consistent with mesenchymal stem cells (Figure 2). The pluripotency marker Oct-3/4 had an intensely positive expression, while Sox-2 and Nanog a weakly positive expression. Mesenchymal stem cell markers: CD29 and CD105 had a moderate expression.

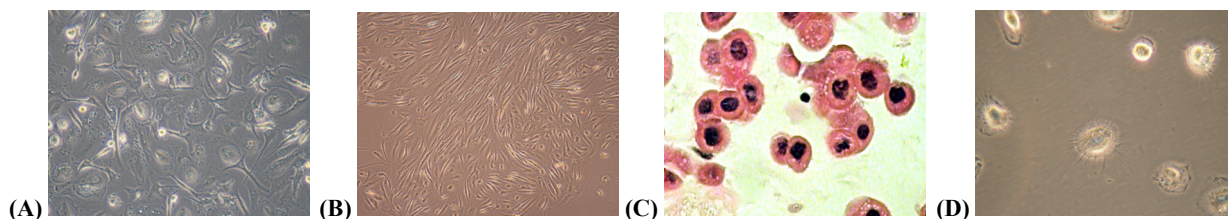


Figure 1 – Mesenchymal stem cells from the chorion: (A) mechanical treatment; (B) collagenase IV + dispase; trophoblastic cells in culture (C) and in HE staining (D).

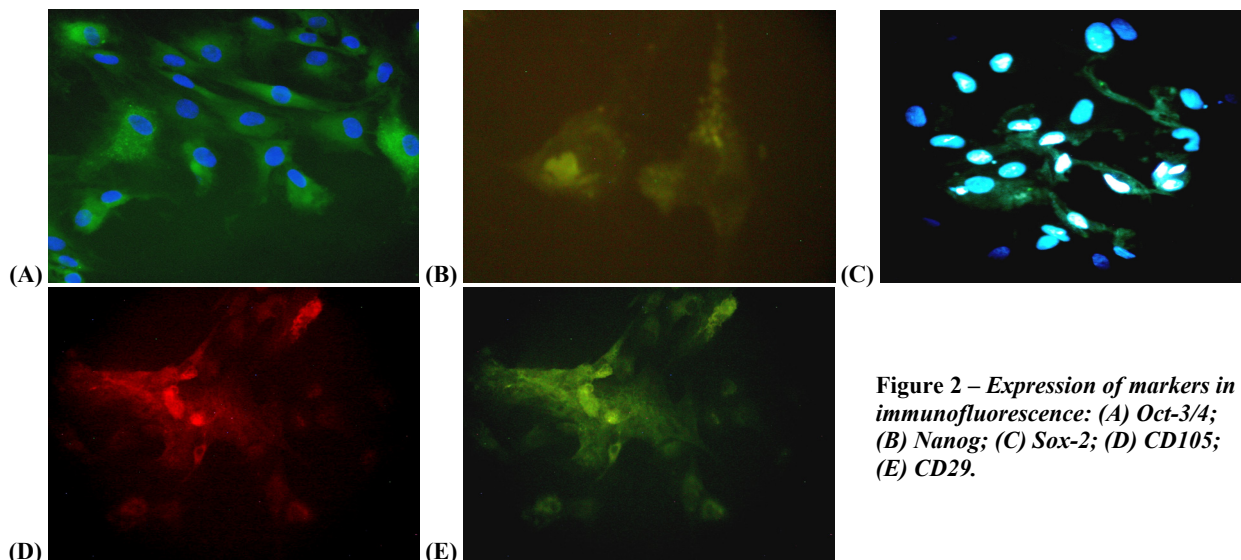


Figure 2 – Expression of markers in immunofluorescence: (A) Oct-3/4; (B) Nanog; (C) Sox-2; (D) CD105; (E) CD29.

The gene expression levels are shown in Figure 3 and it is obvious the expression profiles of stem cells from the amniotic membrane and from the chorion are very similar, almost identical. A relatively high expression level of Oct-3/4, the gene involved in the maintenance of the regeneration capacity of stem cells in general and of the undifferentiated state in embryonic stem cells in particular, is noted. Rex-1, another gene involved in the functioning of embryonic stem cells, whose role in these placental mesenchymal cells is not clear, is expressed at a lower, but still significant level. Nanog, a cell-fate regulatory molecule known to be important for embryonic stem cell self-renewal, which also plays a novel role in tumor development, is variably expressed. This may be due to the numerous pseudogenes it contains, which cannot be amplified by one pair of primers.

The levels of telomerase reverse transcriptase (TERT) and of the protein encoded by it are usually

very high in embryonic stem cells, but in the case of our cells, they were obviously undetectable. A somewhat surprising fact, contrary to the literature data, is the absence of a gene frequently found in stem cells, namely Sox-2, which, together with Oct-3/4 and Nanog, controls the proliferation and fate of ESCs. In our experiments, it seems that its protein levels assessed by immunofluorescence are also extremely low, being present in a very small number of cells. Vimentin is an important marker of mesenchymal cells in general; therefore, its presence comes as no surprise.

An extremely important finding, with crucial practical implications in the future transplantation of these placental cells in particular, is the absence of the HLA-DR α transcript from chorionic and also amniotic mesenchymal cells in all the tested cases, while HLA-ABC is still highly expressed.

The expression profile also showed us that that our stem cells express genes found in other types of more

differentiated cells, like Tie-2, Thy-1 (endothelial cells), c-kit and its ligand SCF (hematopoietic cells), GFAP (glial cells).

The HLA-A and HLA-DQB1 phenotype determined for chorionic cells compared to that of amniotic membrane stem cells, UCB, maternal and newborn blood is shown in Figure 4. It can be noted that the phenotype of the analyzed placenta cells is identical to that of the mother's peripheral blood.

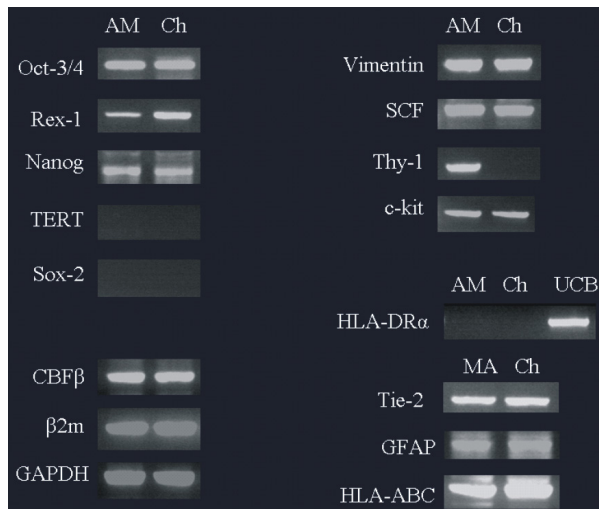


Figure 3 – Gene expression levels in chorionic (Ch) and amniotic membrane (AM) stem cells (UCB – umbilical cord blood).

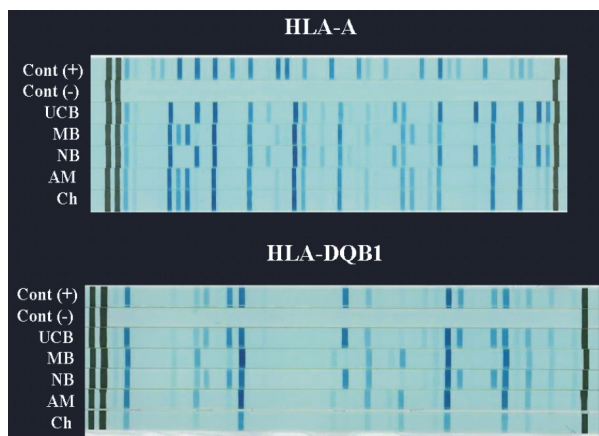


Figure 4 – Expression of HLA-A and HLA-DQB1 phenotype in the studied cells: Cont (+) positive control; Cont (-) negative control; chorionic (Ch) and amniotic membrane (AM) mesenchymal stem cells; umbilical cord (UCB) blood; maternal (MB) and newborn (NB) peripheral blood.

Discussion

The mesenchymal cells of the chorion can represent an important source of cells with pluripotential characteristics [1–6]. In this study, we succeeded in isolating and culturing these cells in the attempt to characterize them phenotypically and genotypically, and later to differentiate them into as many lineages as possible. For an optimal isolation, the use of a double enzymatic digestion, with both collagenase IV and dispase, seems to be essential in order to get rid of the

trophoblastic cells and culture only fibroblast-like chorion cells. However, our observations showed that even if trophoblasts and the sought-after stem cells were cultured together, in some cases the mesenchymal cells finally gained an advantage over the others because of their higher rate of proliferation and the reduced viability of epithelioid cells in the basic culture conditions that were employed. Moreover the Brescia Convention [4] on the types of stem cells in the placenta clearly states the stem properties of the so-called “chorion trophoblastic cells”.

As in the case of amniotic stem cells, the presence of erythrocytes in culture seems to have a favorable effect, compared to their lysis using ammonium chloride. Moreover, growth factors like EGF or bFGF did not stimulate the proliferation of cells, but bFGF seemed to have a beneficial effect on their in vitro lifespan, increasing the number of passages the cells could be kept in culture in a stem-like state (data not shown). It appears that the stem cells from all the components of the placenta are capable of losing their stem properties and of spontaneous differentiation (usually towards an osteoblast-like lineage) after a high number of passages (30 or more). This phenomenon can sometimes be stopped or even reversed using bFGF or higher amounts of FBS.

The main characteristic of stem cells is their pluripotency and their self-regeneration capacity, properties conferred by the presence of some cell transcription factors, of which the most studied are Oct-3/4 and Nanog. Oct-3/4 and Nanog are expressed in embryonic stem cells, tumor germ cells and adult stem cells. They are essential in the formation of the internal cell mass and are necessary for the self-regeneration function [1, 7]. Moreover, Nanog and Sox-2 are involved in early embryonic development and the maintenance of the non-differentiation state of stem cells and expressed by non-differentiated cells and teratocarcinoma cells [8–11, 14].

Adult stem cells characteristically express other markers such as CD29, CD105, CD73, CD90, and CD166 [12, 13, 15, 16]. Some surface markers expressed by the cells isolated from the amniotic membrane and the chorion are pluripotentiality markers. All the three key ESC transcription factors, namely Oct-3/4, Nanog and Sox-2 were strongly or weakly positive, together with some specific proteins for mesenchymal stem cells such as CD29 and CD105 [17, 18, 20].

The levels of telomerase reverse transcriptase (TERT) and of the protein encoded by it are usually extremely high in embryonic stem cells, but in the case of our cells, they were obviously undetectable. This aspect has two sides: placental cells do not have such a long life in culture, but on the other hand, they do not have the risk of generating various benign or malignant tumors (teratoma, teratocarcinoma) after transplantation [19, 20].

An important finding is the absence of the HLA-DRα transcript from chorionic mesenchymal cells in all the tested cases, while class I HLA molecules, evaluated

using the HLA-ABC cluster, are highly expressed. It is already known that one of the mechanisms, which ensures the immunological tolerance of the maternal organism towards the fetal organism, is the reduction of the expression of HLA molecules (especially of class II) on the surface of fetal cells and of some placental cells [7, 10, 21]. Unfortunately, we could not test the presence of HLA-G molecules which play a crucial part in this tolerance mechanism.

The RT-PCR study also proved that the placenta stem cells do not fit precisely the “classical” definition for a stem cell, being somewhere further along the differentiation pathway. They can be placed between multipotent and adult stem cells, with genetic and behavioral characteristics of both, making them a unique research tool in the stem cell world. As a confirmation of our findings, the Brescia Symposium has decided to call this type of cells from the placenta as “chorionic stromal mesenchymal cells”, not stem cells [4].

The HLA typing of our cells in comparison with the mother and newborn led us to the same conclusion as for amniotic stem cells, namely that they have a maternal phenotype. In the beginning of placenta stem cell studies [6], they were considered to have either a maternal or a fetal origin, but more recent articles confirm their maternal phenotype [7]. It is already known that one of the mechanisms, which ensure the immunological tolerance of the maternal organism towards the fetal organism, is the reduction of the expression of HLA molecules on the surface of fetal cells and of some placental cells [7, 10, 21].

These findings have a much deeper meaning because of their possible use in transplantation, dealing with an adaptive change in HLA phenotype in order to avoid immune rejection.

✉ Conclusions

In this study, we succeeded in isolating and culturing mesenchymal cells from the chorionic plate.

The fibroblast morphology of these cells in culture is characteristic to mesenchymal cells.

Immunofluorescence and the assessment of the gene expression showed that these cells possess some pluripotent features reminiscent of ESCs, but also certain traits from more differentiated cells, which are also mesenchymal in nature.

The HLA expression provides information that might help explain the immunological mechanisms of tolerance between the maternal organism and fetal structures.

Note

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