UNIVERSITA' DEGLI STUDI DI PARMA

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Cycle XXIV

EXPLORING TISSUE REGENERATION POTENTIAL OF HUMAN PLACENTA-DERIVED MESENCHYMAL MULTIPOTENT CELLS

MIMICKING HYPERGLYCAEMIA IN VITRO AFFECTS PROLIFERATION AND DIFFERENTIATION PROPERTIES

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Introduction

Stem cells

Between fundamental properties of certain tissues of a multicellular organism resides the ability to adapt and remodel itself following physiological and environmental stimuli. This peculiar property is due to the presence and specific features of stem cells that tissues are endowed with.

A stem cell is an undifferentiated cell whose mitotic division can generate both a "daughter" cell displaying the same stemness characteristics, and a partly committed cell designed to differentiate to a defined mature cellular lineage. In the first case we speak about symmetrical division, a process well-known as self-renewal, while the second is the case of asymmetrical division. It is the balance between symmetrical and asymmetrical divisions that determines the appropriate number of stem cells and differentiated daughters. Asymmetric cell divisions are controlled by intrinsic and extrinsic mechanisms. Intrinsic mechanisms rely on the asymmetric partitioning of cell components, such as cell polarity factors or cell fate determinants. In the extrinsic mechanism, the two daughter cells are positioned asymmetrically in their environment and receive different external signals[1].

Self-renew property is commonly included together with multipotency in the general concept of stemness. Multipotency is the ability of a stem cell to differentiate towards several different mature cell types through the acquisition of their genotypic and phenotypic peculiar traits.

The starting lack of expression of specific markers of differentiation determines the number and nature of different mature cell types towards which stem cells can commit. On these basis, we distinguish between totipotent, pluripotent, multipotent and unipotent stem cells. Totipotent cells have the ability to generate an entire organism. The fertilized oocyte is considered the principal totipotent cell. Progressive mitotic divisions of zygote during embryonal development give rise to cells which lose part of their differentiation potential in behalf of acquisition of more specialized functions and phenotype (cell determination). These cells originate from the inner mass of blastocyst and are considered pluripotent because of their ability to differentiate to cells belonging to all three germ layers, including germ cells. When these cells are isolated and expanded in culture they are commonly defined embryonic stem cells (ES).

Mouse ES cells were the first to be isolated[2, 3]. The next major breakthrough was in 1998, when Thomson et al.[4] isolated ES cells from human embryos.

Human embryonic stem cells might be an ideal cell source for Tissue Engineering and Regenerative Medicine, because of their indefinite proliferation capacity and pluripotency. Unfortunately, embryonic stem cell research is controversial because cell isolation leads to embryo destruction, causing ethical and religious debate.

With the proceeding of embryonal development, two distinct cell types can be distinguished: stem cells of germ lineage (GSC) and somatic stem cells designed for organogenesis. In both cases, they are partially committed cells endowed with pluripotency and self-renewal. During foetal life, different organs own their specific populations of stem cells, which sustain normal foetal growth and evolve in adult stem cells after birth. The latter are considered unipotent or tissue-specific if their differentiation program is restricted to their own mature lineage, or multipotent if they can commit towards several types of terminally differentiated cells. Multipotency concept is often associated to that of plasticity: it defines cells that are not only able to achieve different phenotypes, but also to trans-differentiate towards mature cells that originate from other lineages[5]. A significant feature of adult stem populations is a high capacity for self-renewal in culture. Their ability to expand may be less than that of ES cells, but in some cases these cells have been shown to express telomerase so that they may not be subjected to replicative senescence. Given these intriguing properties, adult stem cells could represent an effective alternative to embryonic stem cells employment in Regenerative Medicine approaches.

Anyway, these cells play a pivotal role in the maintenance of tissue homeostasis, given that they take part in regeneration processes by replacing cellular loss caused by apoptotic death or external damage.

Adult stem cells reside in an anatomically-confined microenvironment called “niche” whose nature and specific localization change with regard to tissue type[6]. The idea of stem cells niche was first introduced in 1978 by Schofield and colleagues[7]. The simple union of several stem cells to form a group it’s not sufficient to define a niche, given that it represents a functional unit comprising stem cells, “supporting” cells and all signalling molecules that they secrete. The niche provides a sheltering environment for stem cells, in which the balance between quiescence and functional activity is maintained. Stem cells reside in the niche for an indefinite period of time during which they both self-renew and generate a committed progeny.

Interactions between stem cells and supporting cells, extracellular matrix components and secreted factors are strictly balanced in order to ensure the maintenance of the stem phenotype and to guide differentiating daughters cells to the tissue that constitute their final destination[8].

The first adult stem cells were discovered almost 50 years ago. At the beginning the common thought was that adult stem cells could only be found in limited types of tissues and organs, such as blood, epidermis, intestine, testis and respiratory system, or in such anatomical districts characterized by a high cellular turnover.

Gradually, researchers discovered that it was possible to isolate adult stem cells from a wide variety of organs, if not from all. However, to date well-recognized sources of stem cells are bone marrow, connective tissue, bone and peripheral blood. From each of mentioned tissues it is possible to distinguish between stem cells of hematopoietic origin (HSC) or non-hematopoietic origin, commonly defined mesenchymal or stromal stem cells (MSC).

Hematopoietic stem cells give rise to all red and white blood cells and also platelets. They are principally purified from bone marrow, peripheral blood after mobilisation from the bone marrow compartment with cytokines and growth factors or from umbilical cord blood. They are multipotent cells endowed with self-renewal ability and able to support regeneration of both blood and immune system cells (hemato-lymphopoiesis).

Hematopoietic stem cells were the first to be isolated and characterized. The relatively easy procedures adopted for their isolation together with their pronounced differentiation potential contributed to make hematopoietic stem cells suitable and ideal candidates for Regenerative Medicine applications, for degenerative diseases cure and for cellular therapy. In fact, their employment in the treatment of some pathologies is nowadays a validate approach (bone marrow transplantation).

During last decades however, researchers tried to expand the horizons of alternative therapies through isolation and characterization of stem cells of different origin or isolated from non-common sources, in order to increase their possible therapeutic applications and fields of exploitation.

Mesenchymal stem cells

In the development of stem cell-based therapeutic platforms for tissue regeneration, the selection of which type of stem cell to use will be enormously important. Adult mesenchymal stem cells (MSC) are considered one of the most promising tools for cell and cell-based gene therapy and an alternative to hematopoietic stem cells.

This cell type was discovered more than 40 years ago by Friedenstein, who isolated from rats bone marrow cells that could be distinguish from hematopoietic precursors thanks to their ability to adhere to plastic surfaces[9]. These cells behaved like fibroblasts precursors given that they formed bone- and cartilage-like colonies, so that they were defined colony forming unit-fibroblasts (CFU-F). Almost 20 years later, Caplan gave these cells the name they have today, Mesenchymal Stem Cells[10].

During last decades the biological features of multipotent mesenchymal/stromal cells were progressively compared first with hematopoietic stem cells and, more recently, with embryonic stem cells. Although these comparisons have been crucial in helping to clarify their nature, there is now a robust amount of data indicating that MSC in vitro represent an independent and heterogeneous group of progenitors with distinct self-renewal properties and established differentiation potential.

Self-renewal and differentiation potential are two criteria that define MSC as real stem cells. However, these characteristics have only been proved after in vitro manipulation and there is no clear description of the characteristics displayed by un-manipulated cells in vivo[11].

Mesenchymal stem cells represent only 0.001-0.01% of nucleated bone marrow cells[12], anyway they could be easily expanded using classical culture systems. Once cultured in vitro, they appear as a population of cells with marked adhesion to plastic and a spindle-shape morphology very similar to that of fibroblasts. They also express a wide range of antigens which include adhesion molecules and integrins (α1, α2, α3, α5, α6, αv, β1, β3, β4), growth factors receptors (FGFR1, FGFR2, EGFR, MET, PDGFRA, PDGFRB) and other molecules such as ICAM1, ICAM2, VCAM1, CD58 and CD73[13].

Given their multipotency characteristics, mesenchymal stem cells are found to be able to differentiate in vitro into cells of osteogenic, chondrogenic, adipogenic and myogenic lineage, while some authors report a possible commitment towards endothelial and neuronal cells.

Our limited knowledge of mesenchymal stem cells is due to the fact that they lack the expression of a unique marker for identification, in contrast to other stem cells such as HSC which are defined by the expression of CD34 surface protein.

From this point of view, it is necessary to find out molecular markers and to develop universal criteria to allow quality control of isolated cells, as well as comparison between study outcomes. Thus, the Mesenchymal and Tissue Stem Cell Committee of the International Society for Cellular Therapy proposes minimal criteria to define human MSC[14], according to which mesenchymal stem cells must express markers such as CD73, CD90 and CD105 and lack the contemporary expression of hematopoietic stem cells antigens such as CD11b, CD14, CD19, CD34, CD45, CD79alpha and HLA-DR.

To date, MSC have already found their way into a vast number of clinical studies addressing a broad variety of diseases. Even though there is no convincing evidence that MSC are involved in the process of tissue repair by trans-differentiation, they probably contribute to tissue regeneration thanks to immunomodulatory effects and interaction with other cell types.

One striking feature of MSC therapy is the cumulative data on the tolerance shown by the host to allogeneic transplantation of these cells. The mechanisms through which this immunotolerance occur are complex and haven't been completely identified yet. It has been shown that there is a low expression of alloantigens by mesenchymal stem cells, given that they express only HLA-ABC antigens of type 1 major complex of histocompatibility (MHC-I) and completely lack the expression of HLA-DR (MHC-II). Moreover, immune system cells such as dendritic and T-cells have been shown to be affected by the presence of MSC in mixed lymphocyte cultures[15].

Mesenchymal stem cells from bone marrow are still the most exploited by alternative therapies because they are well-characterized, easily available and can be subjected to standard culture protocols. Anyway, they still give rise to many doubts due to their difficult employment in the case of treatment of inherited pathologies and because their differentiation potential is strictly age donor-related.

Thus, in the last years researchers focused on the possibility to isolate cells endowed with the same stemness and plasticity features from sources unlike bone marrow. In this case, tissues and organs such as placenta, foetal annexes, amniotic fluid and umbilical cord blood have aroused great interest.

Placenta as a source of stem cells

All types of cells that form human placenta originate from proliferation and differentiation of cells of blastocyst inner mass. Given that the latter are pluripotent cells which divide asymmetrically, it is correct to hypothesize that in term placenta reside cellular progenitors able to self-renew and endowed with multipotency and plasticity. Placenta is a highly specialized organ which supports normal foetal growth and development during pregnancy together with foetal annexes and amniotic fluid. Alterations of its function or of placental differentiation have important consequences on foetus health and on his capacity of interaction with surrounding intrauterine environment. For these reasons, implant and generation of placenta are highly coordinated processes that require the direct interaction between maternal and embryonal cells, given that this organ is composed by neo-formed foetal tissues and maternal tissues of endometrial origin.

In the last few years several studies focused on the presence of stem cells with various differentiation and proliferative potentials in the amniotic fluid and placenta.

Preliminary studies have been published describing very simple protocols for the isolation of a non-specific population of cells with "mesenchymal" characteristics from amniotic fluid and placenta[16, 17]. These cells were able to proliferate in vitro, could be engineered in three-dimensional structures and used in vivo to repair tissue defects[18]. A few years later In't Anker et al. proved for the first time that both amniotic fluid and placenta were abundant sources of foetal mesenchymal stem cells that exhibited a phenotype and multilineage differentiation potential similar to that of postnatal bone marrow-derived MSC[19].

Different groups have claimed that mesenchymal cells from placenta and amniotic fluid could have more plasticity than what initially thought. Phenotypic and gene expression studies indicated mesenchymal stem cell-like profiles for both amnion and chorion cells that were positive for neuronal, pulmonary, adhesion, and migration markers.

Amniotic epithelial cells isolated from human term placenta express surface markers normally present on ES and germ cells. In addition, they express the pluripotent stem cell-specific transcription factors octamer-binding protein 4 (OCT4) and nanog. Under certain culture conditions, amniotic epithelial cells form spheroid structures that retain stem cell characteristics. Amniotic epithelial cells don't require other cell-derived feeder

layers to maintain OCT4 expression, don't express telomerase and are non-tumorigenic upon transplantation.

Because of their plasticity, the term stem cell has been used in the literature to describe a number of cells isolated from placenta. Self-renewal and "hierarchy", which are normally considered hallmarks of stem cells, have not been clearly demonstrated in the different placenta-derived cell types and therefore the term "stem cell" is not always appropriate. However, it may be interesting to mention that recent reports propose an alternative stem cell concept whereby plasticity is essential to define stemness, while self-renewal and hierarchy are optional characteristics.

The whole process through which placenta originates gives rise to a portion of organ of maternal origin (decidua basalis and parietalis) and to a region of foetal origin (amnios and chorion). The maternal side faces utero walls and presents as an opaque and spongious tissue subdivided in several lobes or cotyledons. In contrast, the foetal side is covered by the amniotic epithelial layer which gives it a shining, glossy aspect. A more detailed explanation of placentation process and organ anatomy will be supplied in following sections of this thesis (*see "Results and Discussion, Part 1."*). The direct consequence of this division is that two different kind of immature progenitors can be isolated from placenta, both of maternal and foetal derivation.

Although no significant differences have been pointed out between kinetics of growth and plasticity potential of cells of maternal and foetal origin, in present study we will only focus on isolation and characterization of placental mesenchymal foetal cells. This is the case of cells that display all peculiar features that have been described relatively to bone marrow-derived cells. These features comprise growth characteristics in culture, expression of specific surface antigens and differentiation ability[20].

Cells that originate from foetal side of placenta can constitute a more promising tool for Regenerative Medicine applications if compared to their adult counterparts. They show a significantly higher proliferative potential during standard culture protocols, while their reduced immunogenicity make them suitable for the employment in alternative medical approaches such as allotransplantation. Moreover, their isolation doesn't require the use of invasive of detrimental procedures, so that they don't arouse ethical and moral debate as in the cases of embryonic stem cells or of cells from living donor.

Aim of doctoral thesis

During planning of this doctoral study we aimed to characterize placenta as a stem compartment in which presence of cells endowed with self-renewal and plasticity could be easily detected. For this purpose, we established a protocol for the isolation of a pure population of cells with stemness features from the foetal region of this organ and defined best culture conditions in order to guarantee long-term maintenance of cells peculiar characteristics and viability. Placentas from both healthy donors and diabetic patients were collected and cells were isolated with the aim to outline eventual effects of a systemic pathology on biology of cells with stemness properties. The separation protocol was set to specifically isolate cells of chorionic derivation and belonging to mesenchymal lineage which could be defined as human Chorionic Mesenchymal Stromal Cells (hCMSC) according to literature. To test these features, both cells from healthy and diabetic donors were subjected to molecular and antigenic characterization to define their origin and nature. Their behaviour in *in vitro* culture systems was determined to evaluate the proliferation rate. Multilineage differentiation potential was also assessed through induction of adipogenic, osteogenic and chondrogenic commitment.

After having defined basic biology and behavioural features of these cells, we focused on the study of hyperglycaemia effects on their proliferative potential and differentiation properties.

We aimed to deepen current knowledge about multipotent cells responses to sustained hyperglycaemic treatment in order to give a suitable model for the study of high glucose effects on cells defined by insulin-independent glucose-metabolism. Moreover, we wanted to define optimal culture conditions which could prompt placenta-derived cells to a possible future employment in Regenerative Medicine. In particular, we decided to test cells differentiation potential towards endothelial lineage with the final purpose to provide an alternative source of cells suitable to support neo-vascularization of engineered tissues and organs. In this case, we tested hyperglycaemia effects on endothelial commitment to define its possible role as differentiating agent or as element supporting differentiation microenvironment.

Materials and Methods

Cell culture procedures

All experiments reported in this doctoral thesis were performed on tissues and cells isolated from term human placentas or its annexes (third trimester, 34-40 weeks of gestational age) collected after caesarean sections in Hospital of Parma. Isolation procedures were assessed only after written informed consent and all experiments were approved by the local institutional Ethical Committee. Donors were pregnant women aged between 30-40 with uncomplicated pregnancies, 23 placentas came from healthy patients, 18 from women affected by Type I Diabetes. In all cases, it was about placentas from male newborn children, in order to facilitate distinction between cells of foetal and maternal origin.

Chorionic tissue sampling and placenta-derived cells isolation – Establishment of a purified primary culture

After delivery, placentas were preserved at 4°C for a time period not exceeding four hours before being processed. The whole procedure took place in sterile conditions under a tissue culture hood. First of all, amniotic membrane was carefully detached and discarded to allow exposition of underlying tissue. Transversal sections from both maternal and foetal portions were collected to be addressed to morpho-histological and immunophenotypical characterization as described in the following sections (further below). Decidua's leaf and chorionic plate were then dissected to perfectly reveal foetal portion, whose parenchyma represented the starting material for a following enzymatic digestion. Several pieces of tissue were then cut off from this region and extensively washed with Phosphate Buffered Saline (PBS, 137mM NaCl, 2.7mM KCl, 8mM Na_2HPO_4, 1.46mM KH_2PO_4, pH 7.4) in order to remove as many red blood cells as possible. Samples were gradually minced in pieces not bigger than $1mm^3$ and digested in an enzyme solution containing Collagenase II 0,2% (Gibco® Life Technologies™) and Dispase II 0,2% (Roche Diagnostics, GmbH, Mannheim, Germany) in PBS. Digestion lasted two hours at 37°C in continuous agitation. At the end, not completely digested pieces of tissue were removed by filtration with a 30µm mesh filter, while the cell suspension was pelleted through 10min, centrifugation at 200x g.

To select only mononucleated cells from heterogeneous isolated population, the latter was resuspended in PBS and subjected to centrifugation on a density gradient of Lympholyte solution (1.077g/ml, Cedarlane Laboratories). Carefully, one volume of Lympholyte was poured slowly into a sterile 15ml tube, then two volumes of cell suspension were layered on it and centrifuged at 800x g for 20min. with brakes off. The density gradient centrifugation caused separation of the cells of interest from the other contaminating cell types: red blood cells and platelets were pelleted, while the mononucleated fraction containing placenta-derived cells with potential stems characteristics accumulated in a white ring (Buffy coat) at the interface between the two solutions. The ring was collected and cells washed twice by resuspending in PBS and centrifuging. At the end of procedure, isolated cells were seeded onto uncoated plastic dishes (BD Falcon™) and allowed to recover in the complete maintenance medium (see "*Cell culture conditions and media*") for at least three days.

Once cells recovered and attached to the plate surface, non-adherent fraction, including cellular debris, micro-vessels, death and red blood cells, was removed by replacing medium. At this stage, primary culture of placental cells appeared as a population of adherent cells composed by small, spindle-shaped cells of "unknown" nature and big, rounded cells belonging to macrophage lineage. The latter was eliminated from a culture *via* selective trypsinization: after medium removal, cells were washed with PBS and incubated with a 0.05% trypsin-EDTA solution for few minutes. This procedure couldn't cause detachment of macrophages, which remained strongly attached to the plastic surface, so that a pure fraction of small, spindle-shaped cells was selected.
Within 20-30 days from isolation, a pure population of placenta-derived cells could be expanded in maintenance medium or used in downstream experiments, otherwise cryopreserved following standard protocols (see "*Cell culture conditions and media*").

Human umbilical vein endothelial cell isolation

Human umbilical vein endothelial cells (HUVECs) were isolated thanks to an adaptation of Jaffe et al[21] protocol. Umbilical cords (*n*=6) were obtained from normal term pregnancies, separated from placenta soon after birth and placed in a sterile container filled with physiologic solution (0.9% NaCl). A sterile technique was used during the several steps of the manipulation of cords. The storage time until processing averaged about 1 h. Cords were inspected and all areas with clamp marks were cut off. Umbilical vein was cannulated at both ends and perfused three times with 20-30ml of warm sterile PBS solution to remove red blood cells and clots. Vein was then perfused again with a Collagenase II 0,2% (Gibco® Life Technologies™) plus Dispase II 0,2% (Roche Diagnostics, GmbH, Mannheim, Germany) solution and both ends were clamped. For enzymatic digestion, each cord was incubated in sterile medium 199 (M199) at 37°C in water bath for 30min. At the end of incubation, the collagenase/dispase solution containing isolated endothelial cells was flushed from the vein by 50ml of sterile M199 and cell suspension was centrifuged at 200x g for 10min. After discarding the supernatant, the cell pellet was resuspended in 10ml of complete growth medium (see "*Cell culture conditions and media*") and seeded on 10cm diameter plastic dish coated with 2µg/cm^2 calf skin collagen (Sigma Aldrich, Italy). To test the homogeneity of isolated cellular population, an immunocytochemical staining was carried out to detect positivity for von Willebrand factor (1:100, DAKO S.p.a., Milan, Italy) and PECAM-1 (1:50, Santa Cruz Biotechnology Inc., USA). More than 95% of the cells were positive for both antigens. Furthermore, before all experiments involving HUVEC, cell monolayers were screened for typical endothelial morphology by phase-contrast microscopy.

Cell culture conditions and media

Placenta-derived cells were cultured in a complete maintenance medium since their isolation. This medium was specifically formulated to assure cell recovery after enzymatic digestion and preservation of stemness properties during long term culture[22-24]. Cells were kept in humidified incubator at 37°C and 5% CO_2 in a medium composed of: Dulbecco's Modified Eagle's Medium supplemented with 1g/L glucose (DMEM LOW, 5.5mM glucose), 10% foetal bovine serum (FBS), 2mM L-glutamine, ITS-X (10mg/L Insulin, 5.5mg/L Transferrin, 6.7µg/L Sodium Selenite, 2mg/L Ethanolamine, Gibco® Life Technologies™), 5ng/ml FGF2, 15U/ml heparin (Sigma Aldrich, Italy) and antibiotics (100U/ml penicillin and 100mg/L streptomycin, Celbio Euroclone). During long term culture, cells were allowed to reach subconfluence, before being split 1:2. Medium was changed every 3-4 days.

Experiments in the presence of high glucose concentration were performed using complete medium as usual, but DMEM LOW was replaced with Dulbecco's Modified Eagle's Medium supplemented with 4.5g/L glucose (DMEM HIGH, 25mM glucose).

For experiments involving galactose and mannitol, media with a final 25mM concentration of osmolytes were prepared. In detail, galactose and mannitol were dissolved in DMEM LOW to reach 19.5mM concentration, so that final concentration of complete medium was equal to 25mM (5.5mM glucose + 19.5mM galactose or mannitol).

HUVEC cells were routinely grown on collagen-coated 10cm diameter dishes in Medium 199 (M199), with L-glutamine concentration corresponding to 2mM. Culture medium was supplemented with 20% foetal bovine serum (FBS), 37.5µg/ml endothelial cell growth supplement (ECGS, Millipore), 75U/ml heparin (Sigma Aldrich, Italy) and antibiotics (100U/ml penicillin and 100mg/L streptomycin, Celbio Euroclone). Cells were kept in a humidified incubator at 37°C in 5% CO_2 and split twice a week 1:2 using 0.05% trypsin–EDTA solution. Cells between passages 2 to 5 were used in experimental section of this thesis.

It is noteworthy that glucose concentration of M199 is equal to the DMEM low glucose one (1g/L, 5.5mM). In all experiments where HUVEC were treated with high glucose concentration, M199 was supplemented with a sterile 3.5g/L glucose solution to reach the final concentration of DMEM high glucose (4.5g/L, 25mM). For experiments involving galactose and mannitol, medium with a final 25mM concentration of osmolyte was prepared. In detail, galactose and mannitol were dissolved in M199 to reach 19.5mM concentration, so that final concentration of complete medium was equal to 25mM (5.5mM glucose + 19.5mM galactose or mannitol).

Cryopreservation

Several scientific studies demonstrated that cells with stemness characteristics aren't damaged by standard cryopreservation protocols. In particular, proliferation and differentiation properties are not altered by subsequent freezing-thawing cycles, as well as cell viability, specific antigens expression and kariotype[25, 26]. Between several types of cryoprotectants, Dimethyl sulfoxide (DMSO) was considered the best[27]. For these reasons, for long term storage of placenta-derived cells and HUVEC, we adopted a standardized cryopreservation procedure based on a complete medium formulated as follows: 10% DMSO, 20% FBS, DMEM low glucose or M199 respectively. After trypsinization, cells were pelleted to remove maintenance medium and resuspended in cryopreservation medium at a cell density not exceeding $1x10^6$ cells/ml. Before storage in liquid nitrogen, cells were slowly frozen to avoid any damage through a gradual drop in temperature.

Differentiation protocols

Placenta-derived cells were subjected to adipogenic, osteogenic and chondrogenic differentiation protocols in order to test their plasticity and multipotency properties. Cells that underwent these experiments came from the whole population of isolated cells, between 2 to 4 passages in maintenance culture or after freezing-thawing procedures.

On the contrary, differentiation experiments towards endothelial lineage were performed after isolated the population was subjected to immunomagnetic depletion (see "*Magnetic activated cell sorting*"), so that only the $CD34^{neg}$ cellular fraction underwent endothelial induction. As for previous differentiation procedures, cells between 2 to 4 passages were used.

Formulations of adopted differentiation media were inspired by scientific literature[28, 29] and conveniently adjusted to face cells needs and to optimize results.

Adipogenic differentiation

Adipogenic medium (AM) was a chemically defined medium composed of DMEM:Ham's F-12, 5% FBS, 2mM L-Glutamine, 1µM Dexamethasone, 10µg/ml Insulin, 100µM Indomethacin, 500µM Isobutylmethylxanthine, 17µM Pantotenic acid, 33µM Biotin and antibiotics (100U/ml penicillin and 100mg/L streptomycin). For adipogenic differentiation cells were seeded on a 6-well plastic dish (BD Falcon™) at the starting density of $1x10^5$ cells/well and were allowed to reach confluence before changing medium with induction one[30]. Fresh differentiation medium was added every 3-4 days, while maintenance medium was adopted as control medium (CM).

After 28 days induction, differentiation was assessed by detection of lipid droplets deposition through staining with Oil-Red-O (0.3% in 60% isopropanol, 10m at RT) and by gene expression analysis with Real-Time PCR. Differentiation experiment was repeated twice, obtaining comparable results.

Osteogenic differentiation

To induce differentiation towards osteogenic lineage, placenta-derived cells were cultured in a chemically defined medium[31] (OstM), whose formulation was as follows: αMEM, 10% FBS, 2mM L-Glutamine, 100nM Dexamethasone, 100µM L-Ascorbic acid 2-phosphate, 40µg/ml L-Proline, 10mM β-glycerophosphate, 1nM 1-25 vitamin D_3 and antibiotics (100U/ml penicillin and 100mg/L streptomycin). Osteogenic differentiation was assessed with gene expression analysis and quantitative evaluation of mineralization. In the first case, cells were seeded onto 6-well plate at the starting number of $3.5x10^4$ cells/well. On the other hand, cells addressed to histological analysis were cultured in 24-well plate and seeded at the density of $1.5x10^4$ cells/well. In both cases, induction lasted 28 days during which medium was changed every 3-4 days. Differentiation experiment was repeated twice with comparable results.

Neo-deposition of calcium salts was revealed by adding calcein orange dye (1µg/ml) to culture medium during last week of induction. At the end of experiment, quantitative evaluation of mineralization was performed staining cells with Alizarin Red assay according to Gregory et al[32] method. Alizarin Red S was dissolved in 10% acetic acid and incubated with cells in continuous agitation for 30min at RT. After trypsinization, cell suspension was subsequently collected to microcentrifuge tube, vortexed for 1min, heated to 85°C for 10min, cooled on ice for 5min and finally pelleted at 12,000x g for 15min. An aliquot of supernatant (300µl) was mixed with 120µl of 10% ammonium hydroxide and transferred to an opaque-walled Terasaki plate (Fisher Scientific) to be read at 405nm wavelength using a fluorometer (Wallac 1420 Victor2 multilabel counter, Perkin-Elmer).

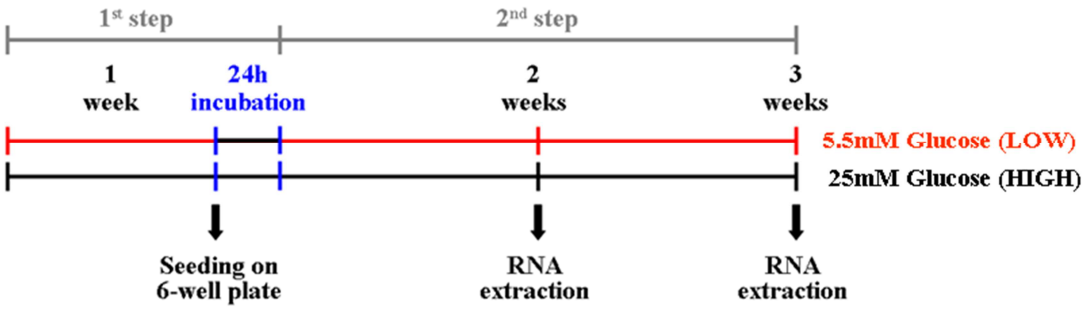
Chondrogenic differentiation

A culture system that facilitates chondrogenic differentiation of placenta-derived cells was developed starting from Johnstone et al[33] protocol. Cells were first grown in monolayer culture, then transferred into sterile tubes and allowed to form three-dimensional aggregates[34] in a chemically defined medium[35-37]. Cells between passages 2 to 4 were used. Adherent colonies were trypsinized, automatically counted and $2x10^5$ cell aliquots were spun down at 500x g in 15ml polypropylene conical tubes (BD Falcon™). The maintenance medium (also used as control medium, CM) was then replaced with a defined chondrogenic medium (ChM) consisting of DMEM high glucose, 0,1% FBS, 2mM L-Glutamine, 10nM Dexamethasone, 200mM L-Ascorbic acid 2-phosphate, 40µg/ml L-Proline, 100µg/ml Sodium Pyruvate, 10ng/ml TGF-β3, 10ng/ml IGF-1, ITS+1 (1mg/ml Insulin, 0.55mg/ml Transferrin, 0.5µg/ml Sodium Selenite, 50mg/ml bovine serum albumin and 470µg/ml linoleic acid, Sigma Aldrich, Italy) and antibiotics (100U/ml penicillin and 100mg/L streptomycin). Conical tubes were incubated at 37°C with closed caps in order to reproduce hypoxic conditions. Within 24h of incubation, cells formed an essentially spherical aggregate that did not adhere to the walls of tubes. Medium changes were carried out at 3- to 4-day intervals. Cells were harvested after 28 days of differentiation to extract RNA for gene expression analysis: aggregates were minced up in lysis buffer with an electric homogenizer and total RNA was then extracted following a standardized protocol (see "*RNA extraction*"). Pellets were also embedded in paraffin and cut into 5µm sections to be stained for both Alcian Blue dye (1% w/v solution, pH 2.5) and Masson's trichromic. Differentiation experiment was repeated twice, obtaining comparable results.

Endothelial differentiation

As previously mentioned, endothelial differentiation was assessed only on placenta-derived cell fraction depleted of CD34 positive cells.

For the purpose of maximize cellular response to treatments, a two-steps endothelial differentiation protocol was established. The first step was aimed to increase the number of cells expressing VEGF receptor 2 (KDR), in order to make cells more responsive to subsequent treatment with complete differentiation medium. As a matter of fact, in the second part of endothelial differentiation protocol cells underwent a long incubation in presence of a specific induction medium. The whole procedure is resumed in time line shown below:



Cells previously grown in low glucose medium (2 to 4 passages) were separated in two aliquots and seeded on 10cm Ø plastic dish, where they were expanded to subconfluence in presence of maintenance medium with 5.5mM (LOW) or 25mM (HIGH) glucose for one week. After this period, cells were detached and $5x10^4$ cells/well were seeded on 6-well plate to begin differentiation induction.

For the first step of protocol, cells were incubated for 24h in a medium composed of: DMEM HIGH, 2% FBS, 2mM L-Glutamine, 10ng/ml FGF2, 10ng/ml EGF, 15U/ml heparin (Sigma Aldrich, Italy) and antibiotics (100U/ml penicillin and 100mg/L streptomycin). The second step of the procedure began when medium was replaced with a chemically defined endothelial differentiation medium consisting of: DMEM LOW or HIGH glucose, 2% FBS, 2mM L-Glutamine, 10ng/ml FGF2, 50ng/ml VEGF, 15U/ml heparin (Sigma Aldrich, Italy) and antibiotics (100U/ml penicillin and 100mg/L streptomycin).

Negative controls were realized incubating cells in maintenance medium with addition of 2% FBS and, respectively, 5.5mM (LOW) or 25mM (HIGH) glucose.
During period of induction, which lasted three weeks, mRNA was extracted at two different time points, respectively after the 2nd and the 3rd week. It was then used in Real-Time PCR procedure to analyse endothelial genes expression. Differentiation experiment was repeated twice, obtaining comparable results.
To verify the degree of differentiation reached by cells at the end of induction, we performed a capillary formation assay with Matrigel™ matrix (BD Biosciences). Cells differentiated in both low and high glucose media were detached from plastic surface, seeded in a three-dimensional model on Matrigel and incubated with the same differentiation media. The ability to form vessel-like structures was monitored daily during seven days of incubation.

FACS - Cytofluorimetric analysis

Culture-expanded placenta-derived cells were phenotypically characterized by flow cytometry (FACSScan, Becton, Dickinson) in order to determine a specific pattern of antigens expression and to evaluate their stemness or immunological properties and presence of contaminating cells (hematic cells). Fluorescein isothiocyanate (FITC)- or phycoerythrin (PE)-conjugated antibodies against CD14, CD33, CD34, CD43, CD44, CD45, CD73, CD90, CD105, CD117, CD133, SSEA4, HLA-ABC and HLA- DR were used. Cells were rapidly trypsinized with 0.05% trypsin–EDTA solution and resuspended in sterile PBS containing 1% FBS and a dilution of antibodies listed above. For each of them, $2x10^4$ events were acquired and analysed with CellQuest software (Becton Dickinson Bioscence). Positive cells were identified by comparison with isotypic negative controls (FITC- and PE-conjugated IgG1, IgG2a, or IgG2b).
After depleting isolated population for CD34 positive cells (see "*Magnetic activated cell sorting*"), cytofluorimetric analysis was repeated in order to finally define phenotypic features and mesenchymal nature of placenta-derived cells designed for further experiments. In this case 34^{neg} cells were tested for positivity to CD34, CD44, CD73, CD90, CD105, CD117, CD133, HLA-ABC and HLA-DR.

Magnetic activated cell sorting

Magnetic activated cell sorting was performed on the whole population of placenta-derived cells in order to completely eliminate cells positive for CD34 antigen. The procedure was accomplished using CD34-MicroBead kit (Miltenyi Biotech), according to manufacturer's protocol. Cells grown in 75cm^2-flask (BD Falcon™) were carefully trypsinized, resuspended in PBS and collected by centrifugation. Pellet obtained was resuspended in 300µl of a specific separation buffer, so composed: 2mM EDTA, 0.5% BSA in PBS (pH 7.2). To avoid any kind of unspecific binding, cellular suspension was also mixed with 100µl of FcR Blocking Reagent. Finally, 100µl of anti-CD34 paramagnetic beads solution was added and cells were incubated for 30m at 4°C in continuous agitation. At the end of incubation, cells were washed by adding 20 volumes of separation buffer and pelleting at 300x g for 10m. Depletion of CD34^{pos} cells took place thanks to a specific separation column set on a magnetic support, which selectively retained CD34 magnetic-beads bound cells. Before separation, column and cell pellet was respectively rinsed and resuspended, both with 500µl of separation buffer. Cell suspension was then allowed to pass through the column to be collected into a 15ml tube. This collected part of cell suspension contained CD34^{neg} fraction of cells. Column was then washed four times with 500µl of separation buffer, dislodged from the magnetic support and placed into a collection tube. Detaching of retained magnetic-labelled cells was assessed by loading column with 1ml of separation buffer, which was forced to pass though the column with a plunger. After magnetic-activated cell sorting, CD34 negative cells were collected and allowed to recover by plating onto plastic dish in complete maintenance medium. Phenotype of negatively sorted cell population was characterized by FACS analysis.

Important specifications

It is important to underline that analysis of cell proliferation in different culture conditions with Resazurin, LHD and reactive oxygen species (ROS) measurement, as well as endothelial differentiation and western blot analysis (further in this section) were all performed on $CD34^{neg}$ fraction of cells, for reasons that will be later deepened in Results and Discussion section.

In following sections of this thesis, we will refer to $CD34^{neg}$ cells as **PLN17** and **PLD09**. The first acronym identifies **PL**acenta-derived cells isolated from healthy (**N**ormal) patient, the second one refers to **PL**acenta-derived cells isolated from patient affected by Type I Diabetes (**D**iabetic).

Histological analysis / Immunohistochemistry

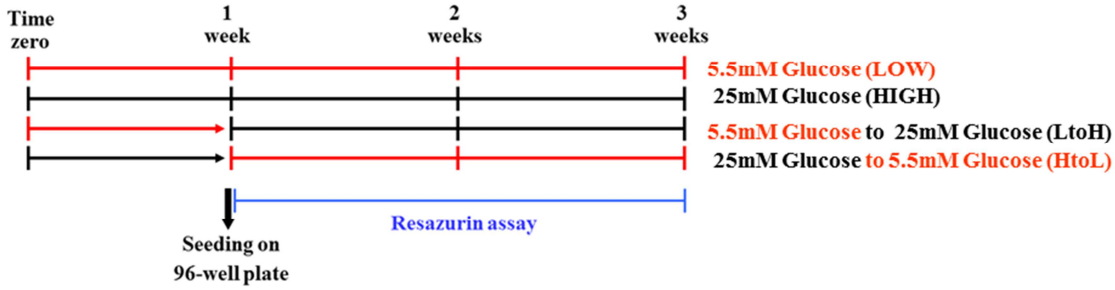
Transversal sections from both maternal and foetal portions of placenta were obtained to be addressed to morpho-histological and immunophenotypical characterization. These procedures took place in the Section of Pathological Anatomy of the Hospital of Parma. Collected samples were fixed in formalin, embedded in paraffin and 5µm sections were stained with Hematoxylin/Eosin or Alkaline phosphatase and anti-CD34 primary antibody. On the other hand, immunohistochemistry was performed following a standard protocol that planned a first step of antigens retrieval during which sections were incubated in 750-W microwave oven in 10mmol/L citrate buffer pH 6.0 (5m, 3 cycles). After that, slides were incubated overnight at 4°C in humidified chamber with following primary antibodies: 1:100 anti-CK18 (DAKO S.p.a., Milan, Italy) and 1:20 anti-c-KIT (Santa Cruz Biotechnology). Nuclei were stained with DAPI (4',6-diamidine-2-phenyndole, Invitrogen Milan, Italy), while positivity for considered antigens was revealed thanks to FITC and TRITC conjugated secondary antibodies (1:300, Sigma Aldrich, Italy).

Immunocytochemistry

Before immunomagnetic separation for CD34 antigen, immunostaining was performed on a fraction of isolated cells to reveal expression of stemness and multipotency markers. Cells were grown at low confluence on four-well glass chamber slides (Falcon Becton Dickinson Labware, Milano, Italy). Before fixation (10m in 4% paraformaldehyde, pH 7.4), cells were washed twice with PBS to completely remove medium and cell debris. Non-specific antibodies binding was blocked by incubation for 1h at RT in blocking solution containing 3% bovine serum albumin (BSA) and 5% animal serum in PBS. In detail, the latter was the serum of the animal in which secondary antibody was produced. Cells were then incubated overnight at 4 C with following antibodies diluted in blocking solution: 1:100 anti-ABCG2 (Santa Cruz Biotechnology), 1:100 anti-CD34 (IC0115, Cell Signaling), 1:200 anti-CD44 (156-3C11, Cell Signaling), 1:20 anti-C-Kit (Santa Cruz Biotechnology), 1:200 anti-Oct3-4 (C3013, Cell signaling), 1:200 anti-SSEA4 (MC813, Cell signaling), 1:200 anti-TRA-1-60 (S) (Cell signaling), 1:200 anti-TRA-1-60 (S) (Cell signaling). After incubation, cells were washed three times in PBS and incubated again (1h at RT) with FITC or TRITC conjugated secondary antibodies in blocking solution (1:300 goat anti-mouse antibody, 1:300 goat anti-rabbit antibody, Invitrogen™ Molecular Probes®). Nuclei were counterstained with DAPI (4’,6-diamidine-2-phenyndole) and cover slips mounted with Fluorescent Mounting Medium (DAKO). Cells were finally examined with Nikon eclipse TE 300 inverted microscope.

Cell growth analysis – Resazurin assay

Evaluation of multipotent placental cells (PLN17 and PLD09) and HUVEC proliferation in different culture conditions was performed in order to analyse effects of mimicking hyperglycemia in vitro. This purpose was reached through realization of several growth curves. Cell number at defined time points was determined using Resazurin assay. These studies were performed following the time line shown below.



Each cell type was subjected to four culture conditions, differing in glucose concentration or in period of incubation. Chronic culture conditions were realized by growing cells respectively in presence of 5.5mM (LOW) or 25mM (HIGH) glucose for three weeks. Chronic growth in low glucose was assumed to be control condition. The other treatments consisted of a first week of conditioning in low or high glucose medium, at the end of which glucose concentration was switched respectively to high (5.5mM glucose to 25mM glucose, LtoH) or low (25mM glucose to 5.5mM glucose, HtoL). For all treatments, after thawing procedure (time zero) cells at 3 to 5 passages were allowed to expand in 10cm Ø disposable culture dish (BD Falcon™) for one week, then they were seeded on 96-well plate (BD Falcon™) at the starting cell number of 1000 cells/well. During subsequent two weeks, Resazurin assay was performed at different time points and cell proliferation was determined. The test was assessed replacing culture media with a solution of Resazurin (110µg/ml in sterile PBS) in serum-free medium (1:10). After one hour at 37°C in humidified incubator, fluorescence at 572 nm was measured with a fluorometer (Wallac 1420 Victor2 multilabel counter, Perkin-Elmer). For all treatments at all time points the assay was carried out in quadruplicate.

Once experiments ended, growth curves were analysed with nonlinear regression and fitted to Boltzman sigmoidal curve. Statistical analysis was calculated comparing curves with one-way analysis of variance (ANOVA) followed by Bonferroni post test, assuming that distribution of data approximates normal/Gaussian one. A $P < 0.05$ was considered significant.

The same experimental protocol was followed to create growth curves of cells cultured in presence of galactose or mannitol. Experiments were assessed following the same time line shown before. In these cases, culture condition referred to as HIGH identifies cells grown in medium containing a final concentration of 25mM, made up of 5.5mM glucose and 19.5mM respectively galactose or mannitol (25mM glucose + galactose/mannitol). On the other hand, "switch" conditions have been indicated as 5.5mM glucose to 25mM glucose + galactose/mannitol (LtoH) or 25mM glucose + galactose/mannitol to 5.5mM glucose (HtoL). Growth in low glucose was assumed to be control condition also for these experiments.

Validation of Resazurin assay

The employment of Resazurin assay as method to determine cell growth in different culture conditions was validated through construction of standard curves. After thawing procedure, PLN17, PLD09 and HUVEC were divided in four aliquots and immediately incubated respectively with 5.5mM glucose, 25mM glucose, 25mM glucose + galactose (5.5mM glucose + 19.5mM galactose) and 25mM glucose + mannitol (5.5 glucose + 19.5mM mannitol). After one week of incubation, all cell types were seeded in 96-well plates at different known numbers of cells, in a range varying from $1x10^3$ to $4x10^4$ cells/well. Cells were incubated maintaining previous type and concentration of energetic substrate. Twenty-four hours after seeding, medium was changed with a solution of Resazurin (110µg/ml in sterile PBS) in serum-free medium (1:10). After one hour at 37°C in humidified incubator, fluorescence at 572 nm was measured with a fluorometer (Wallac 1420 Victor2 multilabel counter, Perkin-Elmer). For all treatments and all cell numbers the assay was carried out in quadruplicate.

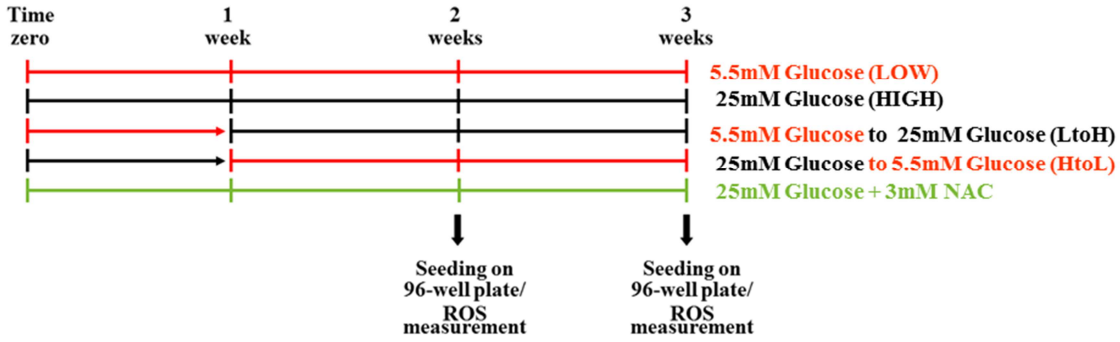
Cell death analysis – LDH assay

Analysis of multipotent placental cells death (both PLN17 and PLD09) was performed to verify an eventual cytotoxic effect of prolonged culture in presence of high glucose concentration. The test was based on detection in culture medium of Lactate Dehydrogenase (LDH) released from dying cells and was assessed with the commercial CytoTox 96well Non-Radioactive Cytotoxicity kit (Promega, Milano, Italy).

During thawing procedure, cells previously grown in low glucose medium (3 to 5 passages) were separated in two aliquots and seeded on 10cm Ø plastic dish, where they were expanded to subconfluence in presence of 5.5mM (LOW) or 25mM (HIGH) glucose for one week. After this conditioning period, cells were detached and 1000 cells/well were seeded on 96-well plate. LDH measurement was performed in quadruplicate for both culture conditions and repeated twice, respectively one week and two weeks after seeding. The total length of incubation in low or high glucose medium equals the one of growth curves experiments (three weeks). The amount of LDH released was expressed as percentage of control (5.5mM glucose). Statistical analysis was calculated with two-way analysis of variance (ANOVA) followed by Bonferroni post test.

Oxidative stress measurement

Experimental protocol performed to evaluate reactive oxygen species (ROS) production was based on the detection of CM-H_2DCFDA fluorescent probe (5-(and-6)-chloromethyl-2′,7′-dichlorodihydrofluorescein diacetate, acetyl ester) and was adapted from Ihnat et al[38]. This test was assessed in order to point out a probable increase in ROS formation due to prolonged cell exposition to high glucose concentration.
Multipotent cells from human placenta (PLN17 and PLD09) were cultured as described by following time line.



PLN17 and PLD09 underwent the same treatments described previously in the case of growth curve analysis. Briefly, after thawing procedure (time zero) cells at 3 to 5 passages were seeded on 10cm Ø culture dish and cultured in high glucose medium (25mM) for three weeks or, after a first week of incubation in low or high glucose medium, they were subjected to the “switch” of glucose concentration (LtoH and HtoL). To evaluate the effect of an anti-oxidant, cells were also treated in high glucose medium with the addition of 3mM N-Acetyl Cysteine (NAC) for three weeks. Chronic growth in low glucose (5.5mM) was assumed to be control condition.
Measurement of ROS production was performed twice, respectively after two and three weeks of culture. The day before ROS detection, cells were plated at the density of $1x10^4$ cells/well onto 96-well plate. Twenty hours after seeding, medium was removed and 2µg/ml of the cell-permeable CM-H_2DCFDA probe (Molecular Probes-Invitrogen, Eugene, OR, USA) in Hank’s Balanced Salt Solution was added to four wells per treatment and incubated at 37°C for 45 min. Fluorescence intensity was then measured

using a plate reader (Wallac 1420 Victor2 multilabel counter, Perkin-Elmer) with 488 nm excitation and 530 nm emission filters.

For all treatments, detected CM-H_2DCFDA fluorescence was expressed as percentage of control (5.5mM glucose). Statistical analysis was calculated with two-way analysis of variance (ANOVA) followed by Bonferroni post test. A P <0.05 was considered significant.

Evaluation of ROS production by HUVEC was performed on cells subjected to three treatments. For the first two, cells were chronically (three weeks) grown in high glucose medium and in high glucose with the addition of 3mM NAC. For the third treatment, cells underwent one week of pre-conditioning in high glucose medium, at the end of which glucose concentration was switched to low (25mM glucose to 5.5mM glucose, HtoL). Furthermore, measurement of ROS amount was assessed at a unique time point, at the end of culture period. In this case, treatments data were expressed as percentage of control (5.5mM glucose), while statistical analysis was based on one-way analysis of variance (ANOVA) followed by Bonferroni post test. A P <0.05 was considered significant.

Molecular Biology techniques

RNA extraction and quantification – Reverse transcription

Extraction of total RNA to address to gene expression analysis was assessed both on adherent cells and cells grown in three-dimensional aggregates using GenElute™ Mammalian Total RNA miniprep kit (Sigma Aldrich, Italy). In both cases, after washing with PBS, cells were lysed with 250µl of lysis buffer. Viscosity was reduced loading cellular lysate on a specific filtration column and centrifuging at 12,000x g for 2m. A volume of 70% ethanol was added to the homogenized lysate and mixed well, then the solution was transferred to a retention column placed in a 2 ml collection tube and centrifuged at 12,000x g for 30sec. Follow-through column was discarded, while RNA bound to column was washed with 500µl of Wash Buffer1 and centrifuged again at 12,000x g for 30sec. The column was then washed twice using 500µl of Wash Buffer2 in order to eliminate any possible carryover of contaminants and genomic DNA. The column was finally transferred to a collection tube for RNA elution: for such purpose a volume of 30-50µl of Elution Buffer was added and the column was centrifuged at 12,000x g for 1min.

After extraction, total RNA concentration was determined. Measurements were performed in double for each sample, reading an aliquot of diluted sample at 260 nm wavelength with a spectrophotometer (Biorad). Purity grade of isolated RNA was assessed by checking the 260/280nm ratio, which was considered good if greater than 1.8.

RNA was then reverse transcribed with ImProm-II™ reverse transcriptase (Promega). About 1µg of total RNA was mixed with 250ng of random primers and brought to a volume of 10.6µl with RNAse-free water. Inside a thermocycler (ICycler, Biorad), RNA and primers solution were heated at 65°C for 5min and cooled at 4°C. To complete reaction, a mix containing 1U/µl of Improm-II enzyme, 4µl of reaction buffer, 1.25µl of a 10mM mix of dNTP, 1U/µl of RNase inhibitor and 2.4µl of $MgCl_2$ solution was added. Following time and temperature steps were 5min at 25°C to allow annealing of primers and 1h at 42°C, during which reverse transcriptase catalysed cDNA synthesis. Finally, reaction was inactivated by denaturing enzyme at 70°C for 15min. To remove contaminating RNA, RNA-DNA duplex was treated with 5 units of RNase H (US Biochemicals, Cleveland, OH) at 37°C for 20 minutes.

Real-Time PCR

For Real-Time PCR, 25ng of cDNA were amplified with GoTaq® qPCR Master Mix (Promega), along with 5pmol of forward and reverse primers listed in **Table 1**. The primer set was designed according to the known sequences reported in GenBank with the help of Beacon Designer 7 software (Premier Biosoft). A final volume of 25µl composed of cDNA, primers and fluorescent reporter Sybr Green was amplified in a 36 well Rotor Gene™ 3000 (Corbett Research, Mortlake, Australia). For all probands, each cycle consisted of a denaturation step at 95°C for 15sec, followed by 30sec at the temperature specific to cause each primer pair annealing and a final polymerization step (30sec, 72°C). Fluorescence of Sybr Green reporter molecule was monitored at the end of every polymerization step. A no-template, no-reverse transcriptase control was included in each experiment. At the end of amplification, a melting curve analysis was added to verify amplicons specificity.

Samples were run in duplicate and analysis of data was made according to the Relative Standard Curve Method[39]. Data were calculated as the ratio between proband cDNA and GAPDH or RPL15 housekeeping genes cDNA. For all experiments, data of treated samples were expressed as fold changes relatively to control samples using ΔΔCt method[40]. When number of biological replicates was sufficient, statistical analysis was performed with two-way analysis of variance (ANOVA) followed by Bonferroni post test[41].

Table 1. Real-Time PCR primer pairs

Gene	Protein	Forward primer	Reverse primer	Ta °C	Amplicon size bp
Housekeeping genes					
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase	TCTCTGCTCCTCCTGTTC	GCCCAATACGACCAAATCC	55	120
RPL15	Ribosomal protein L15	GCAGCCATCAGGTAAGCCAAG	AGCGGACCCTCAGAAGAAAGC	55	100
Adipogenic differentiation markers					
FABP4	Fatty acid binding protein 4, adipocyte	GCAGAAATGGGATGGAAAA	TGCTTGCTAAATCAGGGAAAA	53	219
LEP	Leptin	GAACCCTGTGCGGATTCTTGT	GGAGGAGACTGACTGCGTGT	57	149
LPL	Lipoprotein lipase	ACAAGAGAGAACCAGACTCCAA	AGGGTAGTTAAACTCCTCCTCC	58	149
SLC2A4	Facilitated glucose transporter, member 4	TCGGGCTTCCAACAGATAGG	AGCCACGTCTCATTGTAGCTC	57	176
Osteogenic differentiation markers					
ALPL	Alkaline phosphatase	TGATGTGGAGTATGAGAGTGAC	TGAAGTGGGAGTGCTTGTATC	55	110
BGLAP	Osteocalcin	TCACACTCCTCGCCCTAT	GTCAGCCAACTCGTCACA	60	245
COL1A1	Collagen type I, alpha 1	GTCGAGGGCCAAGACGAAG	CAGATCACGTCATCGCACAAC	57	143

Gene	Protein	Forward primer	Reverse primer	Ta °C	Amplicon size bp
RUNX2	Runt-related transcription factor 2	CCAGGCAGGCACAGTCTTC	GTCAGAGGTGGCAGTGTCATC	59	182
SPARC	Osteonectin	ACAGAGGTGGTGGAAGAAAC	GAAGTGGCAGGAAGAGTCG	294	57
SPP1	Secreted phosphoprotein 1/Osteopontin	CAGCCTTCTCAGCCAAAC	CCTCAGAACTTCCAGAATCAG	127	59
VDR	Vitamin D receptor	CTGGCAGAAGTCGGAGTAGG	CTGGCAGAAGTCGGAGTAGG	58	258
Chondrogenic differentiation markers					
ACAN	Aggrecan	CCATCAACAGAGACCTAC	CGTAGCATTGTGAGATTC	58	129
BGN	Biglycan	CTCCCTCTCTCCACAAAC	GCTTCCTCATCGTTCATC	65	271
COL2A1	Collagen type II, alpha 1	CCTGGTGAACCTGGTGAAC	GCCTGGATAACCTCTGTGAC	58	204
DCN	Decorin	TGGCTACAATACAAGAGACA	GCAGAAGGAGGATGATAGT	59	312
HES1	transcription factor HES-1	AGCACAGAAAGTCATCAAAGC	TTCCAGAATGTCCGCCTTC	53	149
SOX9	transcription factor SOX-9	CTGGGAGCCAAATGAAGAAA	CCGTGGTGTGGTTGAAATG	57	115
Endothelial differentiation markers					
ANGPT1	Angiopoietin 1	CTCGCTGCCATTCTGACTCAC	GACAGTTGCCATCGTGTTCTG	57	136

Gene	Protein	Forward primer	Reverse primer	Ta °C	Amplicon size bp
ANGPT2	Angiopoietin 2	CAGGAGGCTGGTGGTTTG	CTCAGGTGGACTGGGATGTT	57	186
CD34	Hematopoietic progenitor cell antigen	GCGCTTTGCTTGCTGAGTTT	GCCATGTTGAGACACAGGGT	57	218
CDH5	Vascular endothelium cadherin 5, type 2	TACCAGCCCAAAGTGTGTGAG	GTGTTATCGTGATTATCCGTGAGG	54	152
DLL4	Delta-like protein 4	CCCTCTCCAACTGCCCTTC	GCGATCTTGCTGATGAGTGC	57	129
EDN1	Endothelin 1	ACCCACAACCGAGCACATT	CCAGTCAGGAACCAGCAGAG	57	141
ETS1	protein C-ets-1	ACCTCGGATTACTTCATTAGC	GACGACTTCTTGTTTGATAGC	55	231
GATA2	endothelial transcription factor GATA-2	CAAGATGAATGGGCAGAAC	ATAAGGTGGTGGTTGTCG	54	110
KDR	VEGF receptor 2	TCTGCCTACCTCACCTGTTTC	CGGCTCTTTCGCTTACTGTT	56	117
PECAM1	Platelet/endothelial cell adhesion molecule 1	GCTGACCCTTCTGCTCTGTT	ATCTGGTGCTGAGGCTTGAC	59	171
VCAM1	Vascular cell adhesion molecule 1	GCTGCTCAGATTGGAGACTCA	CGCTCAGAGGGCTGTCTATC	56	100
VEGF	Vascular endothelial growth factor	GCCAGCACATAGGAGAGATGAG	GCAGCGTGGTTTCTGTATCG	55	201
VWF	Von Willebrand factor	TGGAGGGAGGAGAGATTGAG	CCCAGCAGCAGAATGATGTA	56	115
TIE1	Tyrosine-protein kinase receptor Tie-1	CACGACCATGACGGCGAAT	ACTGGAGTCGGCAATCAGC	56	244

Gene	Protein	Forward primer	Reverse primer	Ta °C	Amplicon size bp
TIE2	Tyrosine-protein kinase receptor Tie-2	CAGCCTTTCCCATCCTAATC	TCTCCATCCAGTTTCCACAA	56	225
Stemness markers					
OCT4	POU domain, class 5, transcription factor 1	GAGGGCGAAGCAGGAGTC	CGGCAGATGGTCGTTTGG	59	272
SOX2	transcription factor SOX-2	GCCGAGTGGAAACTTTTGTCG	GCAGCGTGTACTTATCCTTCTT	57	154
Marker of oxidative stress					
HMOX1	Heme oxygenase 1	CAGTGCCACCAAGTTCAAGC	GTTGAGCAGGAACGCAGTCTT	57	116

Western blot

Western blot analysis was performed on multipotent cells from human placenta (PLN17 and PLD09) to examine expression of cell cycle arrest related proteins. For this reason, cells always maintained in low glucose medium (3 to 5 passages) were separated into two aliquots and seeded on 6cm Ø disposable plastic dish (BD Falcon™), where they were cultured to subconfluence in presence of 5.5mM (LOW) or 25mM (HIGH) glucose. Media were changed every 3-4 days and protein extraction was assessed after two and three weeks of chronic incubation. Total cellular protein extracts were prepared by rinsing cultures twice with cold PBS, then scraping cells directly into Laemmli sample buffer (0.625M Tris–HCl, pH 6.8, 2% SDS, 10% glycerol, 0.1M DTT and 0.002% bromphenol blue). Lysates were then transferred to Eppendorf tubes, sonicated twice for 30sec to release nuclear proteins and briefly spun in a microcentrifuge. After quantification with a modified micro Lowry protein assay, aliquots of 30mg of total proteins were mixed with Laemmli buffer, warmed at 95°C for 5min and loaded on a 12% gel for SDS-PAGE. After electrophoresis, proteins were transferred to PVDF membranes (Immobilon-P, Millipore, Millipore Corporation, MA, USA). Non-specific binding sites were blocked by incubating membranes in a commercial blocking solution (Roche Diagnostics, GmbH, Mannheim, Germany) diluted 1:10 in TBS-Tween buffer with addition of 5% bovine serum albumin (BSA). The incubation lasted two hours at room temperature. Blots were then exposed at 4°C overnight to the following antibodies diluted in a 5% BSA-TBS-Tween solution: anti-p21 (mouse monoclonal, 1:1,000, Cell Signaling Technology); anti-p15 (mouse monoclonal, 1:1,000, Cell Signaling Technology); β-tubulin (mouse monoclonal, 1:2,000, Sigma Aldrich). After washing, blots were exposed for 1h at room temperature to HRP-conjugated anti-mouse secondary antibody (Cell Signaling Technology), diluted 1:10,000 in blocking solution. Immunoreactivity was visualized with Immobilon Western Chemiluminescent HRP Substrate (Millipore). To quantify protein expression, plates were scanned with SI Densitometer (Molecular Dynamics) and analyzed with ImageQuant software. Data of treated samples were expressed as fold changes relatively to control samples and statistical analysis was calculated with two-way analysis of variance (ANOVA) followed by Bonferroni post test.

Statistical analysis

Means and standard error of the mean (SEM) were calculated with GraphPad Prism 5.0 and statistically significant differences were determined using a test specific for each experiment (see Materials and Methods). Unless otherwise specified, *n* refers to sample size. Statistical significance was considered relevant at $P < 0.05$.

Materials

Endotoxin-free FBS was purchased from BioWhittaker/Lonza, while culture media (DMEM LOW and HIGH glucose, M199, DMEM:Ham's F-12, α-MEM) were purchased from Euroclone. All growth factors were obtained from ReliaTech GmbH. Becton Dickinson was the source of antibodies used in FACS analysis. Sigma-Aldrich Italy was the source of all other chemicals not otherwise specifically indicated.

Results and Discussion

Part 1. Is placenta a source of stem cells?

Introduction and aim of the study

All cell types homing in human placenta come from proliferation and differentiation of cells from inner mass of blastocyst. Since these cells self-renew and have pluripotency properties, it is correct to suppose that cell progenitors endowed with plasticity and multiple differentiation characteristics lie in term placenta. Placental tissue draws great interest as a source of cells for regenerative medicine because is readily available and easily procured without invasive procedures and its use does not elicit ethical debate. Preliminary studies published few years ago described protocols for the isolation of a non-specific population of cells with "mesenchymal" features from human placenta[17, 18, 42, 43]. Till nowadays, numerous reports describing cells with multipotency properties from different parts of placenta, using several and different isolation and characterization procedures, have been published. This fact creates confusion, slowing down the possible employment of placenta-derived cells in cell therapy because it makes impossible the comparison of works from different groups. Considering the complexity of placenta as organ and great expectations towards its exploitation from Regenerative Medicine, an urgent need exists to define the region of origin and methods of isolation of cells derived from this tissue. For these reasons, the first part of this thesis concerned establishment of a standardized and reproducible procedure to optimize isolation and culture expansion of placenta-derived cells, as well as definition of a unique pattern of antigen expression for cells identification and characterization. Once verified stemness properties and best culture conditions of isolated placenta cells, they will be used in the second part of this thesis to analyse high glucose effects on their proliferation and differentiation potential.

Analysis of placental tissue and isolated cells - Establishment of culture conditions and antigenic characterization

Term placenta is discoid in shape with a diameter of 15-20cm and a thickness of 2-3cm. From the margins of the chorionic disc extend the foetal membranes, amnion and chorion, which enclose the foetus in the amniotic cavity. The chorionic plate is a multilayered structure that faces the amniotic cavity. It consists of two different structures: the amniotic membrane (composed of epithelium, compact layer, amniotic mesoderm and spongy layer) and the chorion (composed of mesenchyme and a region of extravillous proliferating trophoblast cells interposed in varying amounts of Langhans fibrinoid, either covered or not by syncytiotrophoblast).

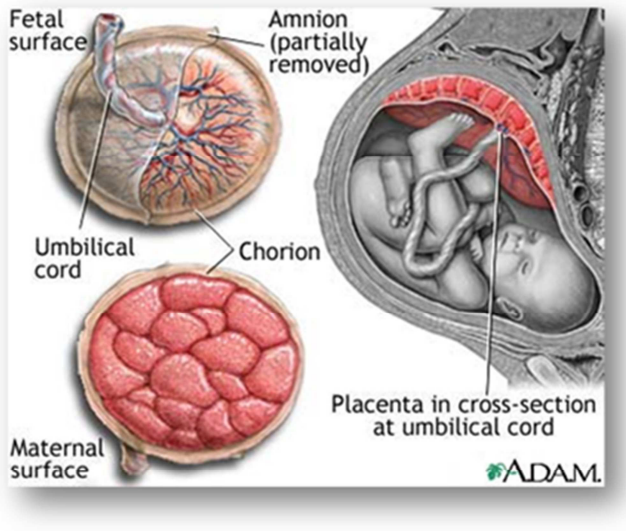


Figure 1.
Schematic representation of human term placenta
(taken from A.D.A.M. Education solutions Inc.)

The particular mechanism through which placenta originates creates a maternal (decidua basalis and decidua parietalis) and a foetal region (amnios and chorion), so that we can distinguish between isolated cells of maternal and foetal origin (**Fig. 1**). The latter appear during foetal life and display higher in vitro proliferative potential and reduced immunogenicity than their adult counterparts, so that they are generally considered the best starting material for Regenerative Medicine applications[44]. For these reasons, an isolation protocol specifically directed to avoid contamination with cells of maternal origin was established, and isolated foetal cells were employed in all experiments described in this thesis.

Villi originate from the chorionic plate and anchor the placenta through the trophoblast of the basal plate and maternal endometrium (**Fig. 2**). From the maternal side, protrusions of the basal plate within the chorionic villi produce the placental septa, which divide the parenchyma into irregular cotyledons. Some villi anchor the placenta to the basal plate, whereas others terminate freely in the intervillous space. Chorionic villi have different functions and structure. In term placenta, the stem villi show an inner core of foetal vessels with a distinct muscular wall and connective tissue consisting of fibroblasts, myofibroblasts, and dispersed tissue macrophages (Hofbauer cells). Mature intermediate villi and term villi are composed of capillary vessels and thin mesenchyme. A basement membrane separates the stromal core from an uninterrupted multinucleated layer, called syncytiotrophoblast. Between the syncytiotrophoblast and its basement membrane are single or aggregated Langhans cytotrophoblastic cells, commonly called cytotrophoblast cells (**Fig. 3**).

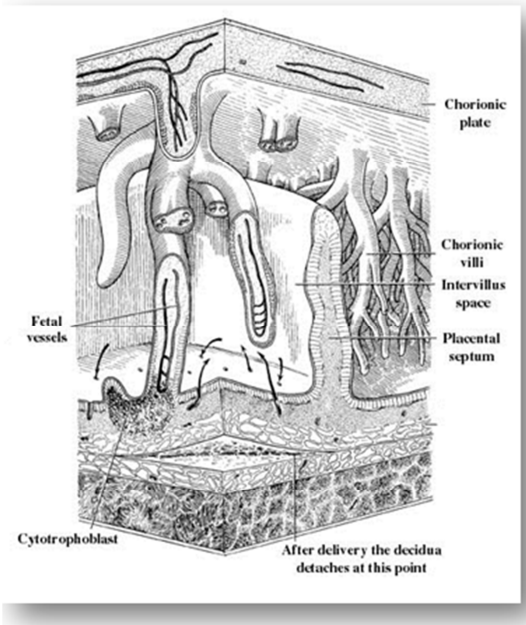


Figure 2.
Representative drawing of whole placenta in longitudinal section
(taken from Boyd JD, Hamilton WJ. The human placenta. Cambridge7 W.Heffer & Sons; 1970)

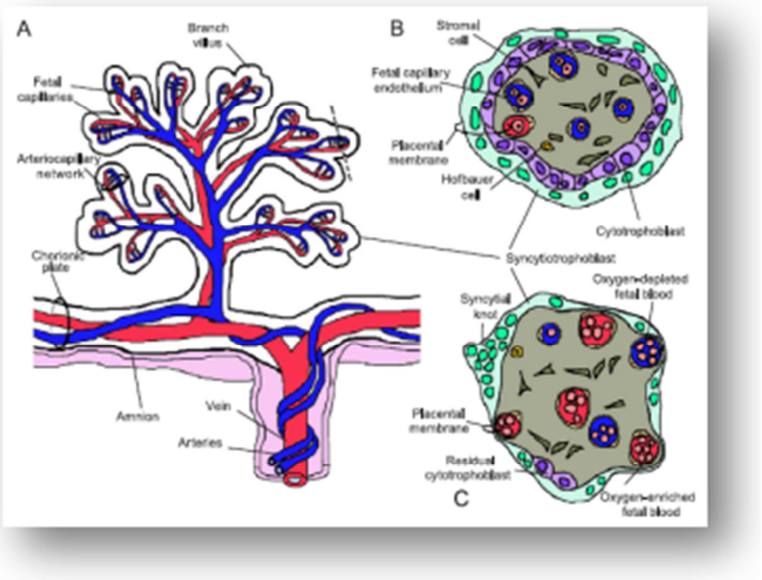
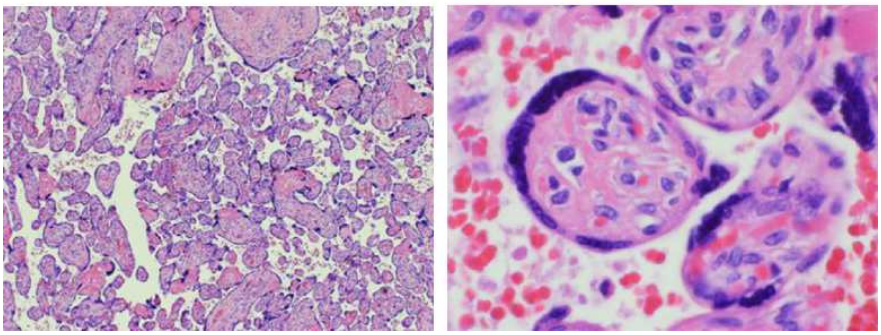


Figure 3.
A representative drawing of foetal placental circulation (**A**). Drawing of a section through a chorionic villus at approximately 10 weeks of gestational age is reproduced (**B**). A section through the chorionic villus at full term is also shown (**C**).
(Adapted from Gude et al.[45])

As described in Materials and Methods, samples of placental tissue from both maternal and foetal regions were sampled for histological and immunohistochemical analysis. **Figure 4** shows Hematoxylin/Eosin staining of transverse sections of placental villi collected from foetal region.

Figure 4.
Histological analysis of placental tissue (foetal region)
Hematoxylin/Eosin staining of placental villi, phase contrast images of representative microscopic fields, 4x (left) and 40x (right).



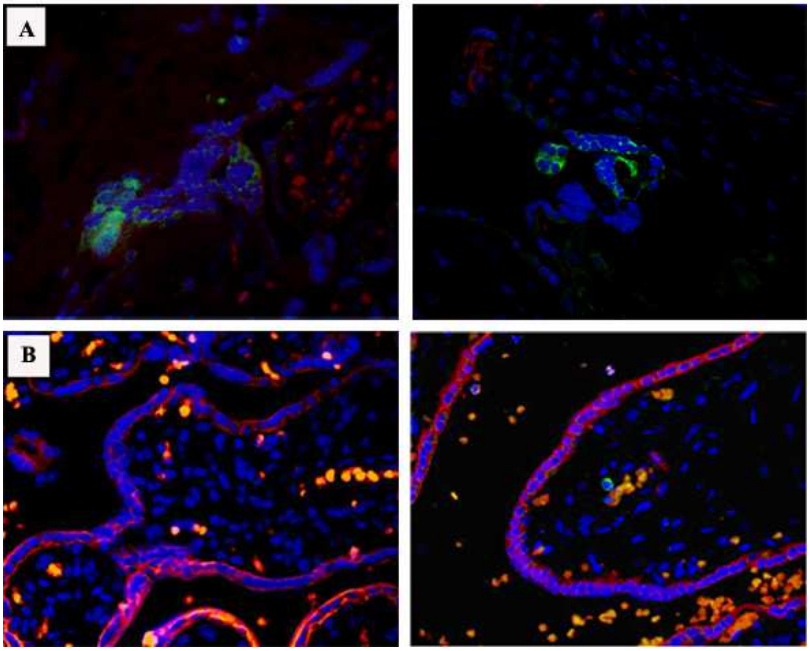
Little magnification points out typical spongy structure of placenta parenchyma, with villi of different dimensions alternated to empty spaces (intervillous space). Higher magnification shows structure of single villi. The multinucleated layer of syncytiotrophoblast is evident, enclosing stromal core of connective tissue and small vessels. Cytotrophoblastic cells underlying external layer are no more evident.

Immunohistochemical staining (**Fig. 5**) of placental tissue showed positivity for markers of undifferentiated state.

C-KIT (CD117) gene codifies for a tyrosin-kinase protein with receptor function, which is particularly expressed by embryonic stem cells and multipotent progenitors of erithroid and linfoid lineage. Binding between c-KIT receptor and its ligand SCF (Stem Cell Factor) plays a pivotal role for viability, proliferation and differentiation of multipotent cells and is considered to be the most important stimulus for hematopoiesis induction.

CK-18 is cytoskeletric protein and a specific antigen of cytotrophoblastic and epithelial lineage, whose expression was recently detected in mesenchymal cells with fibroblast-like shape isolated from first and third trimester placenta[42]. It is actually considered a marker of cells able to undergo epithelial commitment.

Figure 5.
Immunohistochemical analysis of placental tissue (foetal region)
A, Villi in transverse section, cytotrophoblastic cells, fluorescence images of representative microscopic fields, staining c-KIT (green), 40x, nuclear stain with DAPI (blue); ***B,*** Villi in transverse section, cytotrophoblastic cells, fluorescence images of representative microscopic fields, staining CK-18 (red), 40x, nuclear stain with DAPI (blue).



Images pointed out that both c-KIT and CK-18 were mostly expressed by cells of syncytio- and cytotrophoblast (where present) layers. Among cells of inner core, their presence was only detectable in blood filling villous microvessels. More interestingly, c-KIT positive cells were not uniformly widespread but formed sort of clusters or niches.

These were the first indications of presence in term placenta of cells with low grade of differentiation expressing antigens of stemness lineage.

Before antigenic characterization with cytofluorimetric analysis and immunocytochemistry, placenta-derived isolated cells were expanded in culture to assess best in vitro conditions, which could guarantee cells viability and multipotency properties maintenance. It is noteworthy that the two defining features of cells with stemness characteristics (self-renewal and multipotency) become limited when they are cultured in vitro, as they progressively senesce[46, 47], losing their proliferation and differentiation potential[48]. Having this in mind, formulation of growth medium was established starting from literature data, since several reports suggested that some cytokines can help cells to keep self-renew and multilineage commitment abilities [49, 50 23]. Since last decades, FGF2 was considered to be a potent mitogen for some connective tissue cells including osteoblasts and chondrocytes[51]. More recently, exposure of stem cells to FGF2 during mitotic expansion was demonstrated to increase cell yield and to shorten expansion time in culture[52]. Furthermore, FGF2 is nowadays a fundamental component of stem cells maintenance medium because of its ability to retain multilineage differentiation potential through suppression of senescence in culture[22, 53]. Heparin, a soluble derivative of heparan sulfate and a well known cofactor for FGF2, was also added to maintenance medium in order to optimize FGF2 function and to reduce its requirement. FGF2 was added to the final concentration of 5ng/ml because higher concentrations were demonstrated to be deleterious in the presence of heparin[54]. Finally, we decided to use a basal defined medium (DMEM) supplemented with low glucose concentration (1mg/ml). Although high glucose-containing medium is generally accepted for culture maintenance of embryonic stem cells, several observations show that it is not required for proliferation and development of cells of foetal or adult origin, so that low glucose medium is preferable[55].

After enzymatic digestion, isolated placenta derived-cells were immediately seeded on uncoated plastic surface and incubated in maintenance medium as previously described. During subsequent 20-30 days in culture, cells were accurately monitored to verify their evolution in size, shape and nutritional needs. Results are shown in **Figure 6**.

Twenty-four hours after isolation, a heterogeneous population of plastic adherent and non-adherent cells was detectable (**A**). Floating fraction was composed of cellular debris, microvessels, death cells and live cells not yet adherent. Cells attached to culture dish surface were small in size and long in shape. Seven days after enzymatic digestion, not adherent cells and other contaminants were removed through medium change. Primary culture obtained appeared as a mixed population of adherent, small spindle-shaped cells (**B**) and bigger, strongly attached to plastic surface, rounded cells with macrophage or epithelial morphology (**C**). This latter fraction of cells was discarded *via* selective trypsinization as described in Materials and Methods. About 10 days after isolation, a pure population of fibroblast-like cells was obtained (**D**). These cells could be expanded in culture without evident changes in size, shape and proliferation rate or alternatively frozen in liquid nitrogen for long term storage. When seeded at clonal density, these low passage cells (1 to 3) formed typical aggregates of small, rounded, highly proliferating cells from which single fibroblast-like cells separated (**E**).

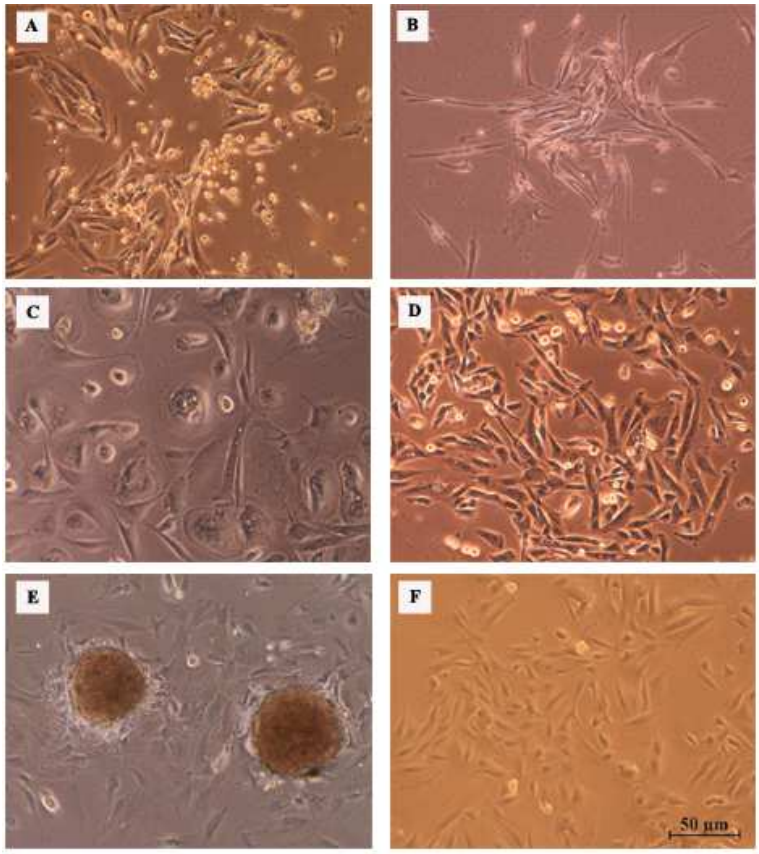


Figure 6.
Isolated human placenta-derived cells after enzymatic digestion
Phase contrast images of representative microscopic fields, 100x (all)
A, Isolated cells 24 hours after enzymatic digestion, seeded on plastic surface;
B, Primary culture: mixed population 7 days after enzymatic digestion, spindle-like cells;
C, Primary culture: mixed population 7 days after enzymatic digestion, rounded, macrophages-like cells;
D, Purified population after selective trypsinization: fibroblast-like cells 10 days after isolation;
E, Purified population after selective trypsinization: mesenchymal clusters of rounded cells with surrounding radiating fibroblast-like cells;
F, Fibroblast-like cells in culture (3-5 passages) maintain their shape and size even after repeated freezing-thawing procedures, mitosis are still detectable.

We defined them “mesenchymal clusters” or “fibroblast colony-forming units” (CFU-F) according to literature[9]. Mesenchymal clusters gradually disappeared during long term culture as passages number grew up, so that they were no more detectable at about fourth or fifth passage in culture. At this stage, isolated cells kept their fibroblast-like shape and dimensions, mitosis were still detectable so that no apparent change in proliferation potential became evident (**F**).

Proliferation rate of isolated placenta-derived cells was assesses by counting cells with an automated cell counter (Beckman Z1 Cell Counter) at defined time points (**Fig. 7**). For this calculation, cells between 3 to 5 passages were seeded on 96-well plate at the density of 1000 cells/well. Cells showed a typical growth rate with sigmoidal trend. We could distinguish a first lag phase during which proliferation of cells seeded at low density was extremely reduced. This phase generally lasted for 6 to 9 days, depending on the initial number of cells seeded. The lag phase was followed by an exponential growth phase during which cells were actively proliferating, so that we could determine a doubling time of about 23 ± 2.8 hours (data not shown). Approximately 13 to 16 days in culture were sufficient to cells to reach complete confluence (plateau phase).

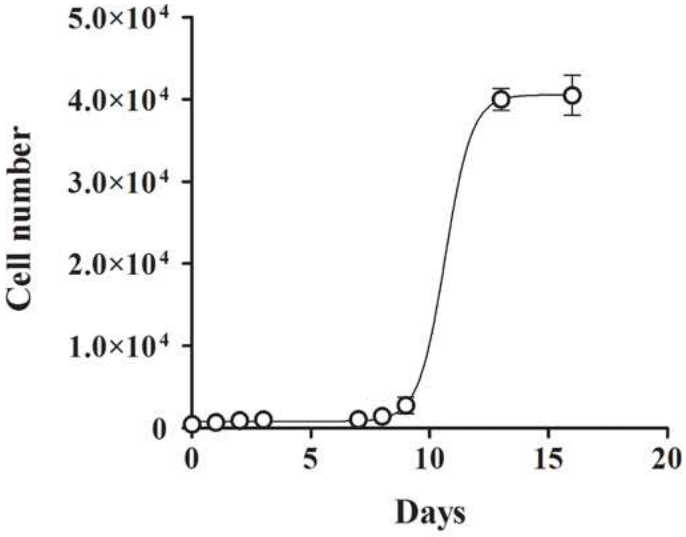


Figure 7. Proliferation rate of isolated human placenta-derived cells

After establishing optimal culture conditions and having observed isolated cells behaviour, pure population of fibroblast-like cells (2 to 4 passages) was subjected to antigenic characterization respectively with immunocytochemistry and Fluorescence Activated Cell Sorting analysis (FACS).

During immunocytochemistry, isolated cells were stained for antigens of undifferentiated state and of both hematopoietic and mesenchymal lineage (**Fig. 8**). Our purpose was to reveal the presence of cell subpopulations endowed with plasticity and multilineage differentiation potential and to define the specific germ layer from which they originated.

Oct3-4 is a transcriptional factor of germinal line and embryonic pluripotent cells. It is generally regarded as a marker of early progenitors given that it plays a pivotal role in maintenance of undifferentiated state[56].

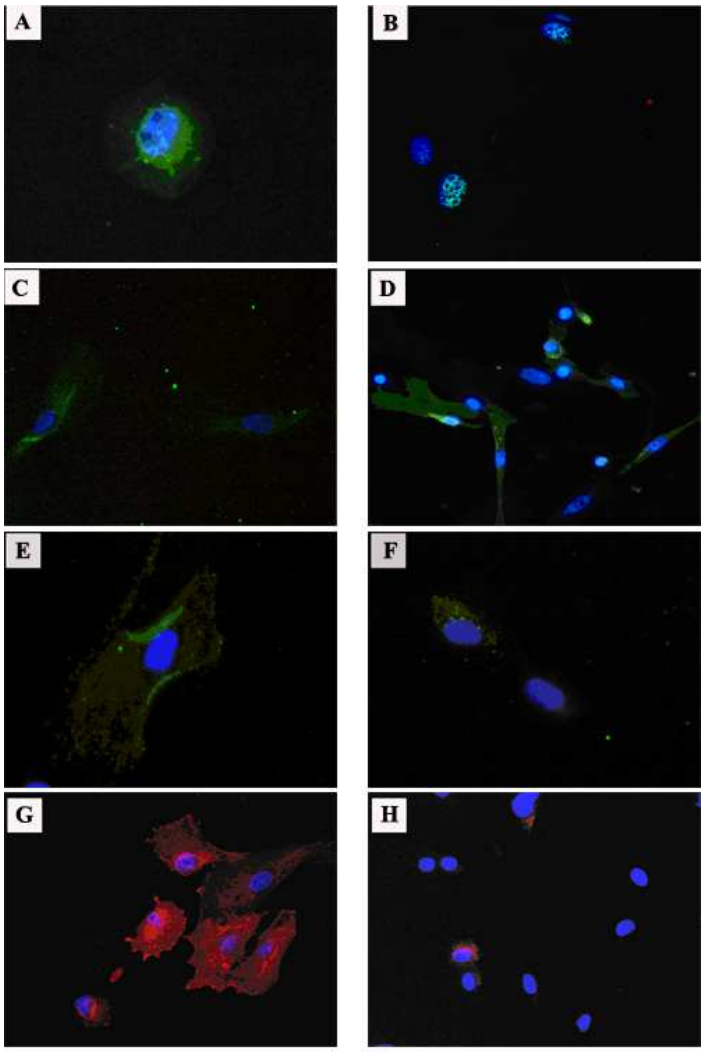


Figure 8.
Immunocytochemical analysis of isolated human placenta-derived cells
Fluorescence images of representative microscopic fields, staining respectively: ***A,*** c-KIT (green); ***B,*** Oct3-4 (green); ***C,*** ABCG2 (green); ***D,*** SSEA4 (green); ***E,*** TRA-1-60 (green); ***F,*** TRA-1-81 (green); ***G,*** CD44 (red); ***H,*** CD34 (red); 400X (all), nuclear stain with DAPI (blue).

ABCG2 belongs to a family of transporter proteins involved in drug resistance. Although its contribute to stem cells hasn't been clarified yet, several hypothesis suggest that it may confer survival properties. Its expression is generally related to early progenitors.

Stage-specific embryonic antigens (SSEAs) are cell-surface molecules that exhibit lineage-restricted patterns of expression during development. SSEA4 acts as a marker of human embryonic stem cells (ES) but it is also expressed on the surface of human embryonal carcinoma (EC) cells, human embryonic germ cells (EG) and mesenchymal stem cells (MSC) at very early differentiation stage[57, 58].

Besides being expressed in embryonic cells, this epitope is also present in human erythrocytes.

Like SSEA4, TRA-1-60 and TRA-1-81 antigens are commonly used as markers of undifferentiated pluripotent human stem cells because they identify embryonic stem cells, embryonal carcinoma cells and embryonic germ cells. The expression of TRA-1-60 on human ES cells is down-regulated upon differentiation.

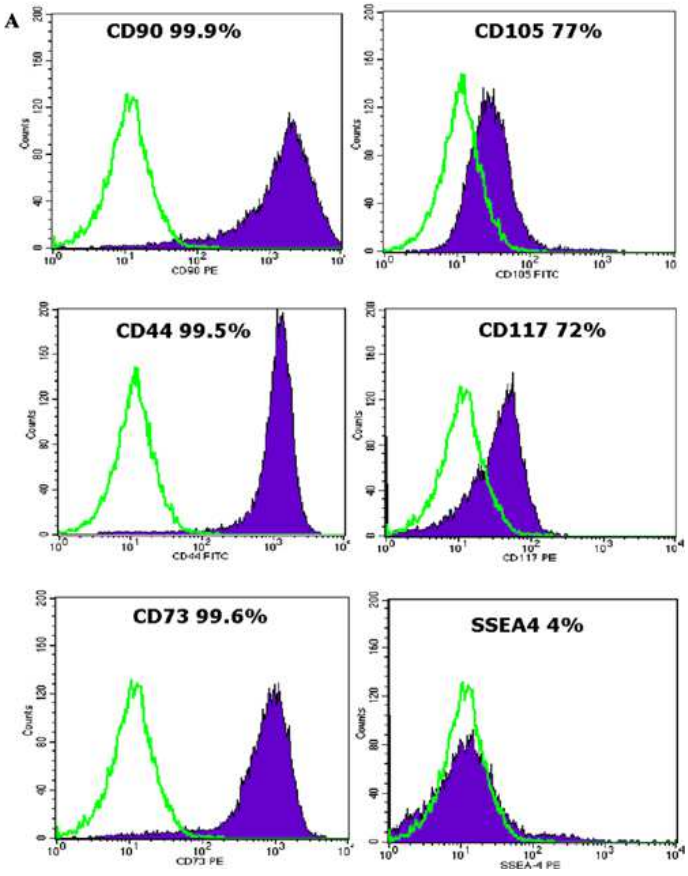
CD44 protein belongs to a family of adhesion molecules involved in cell to cell and cell-matrix interaction. Hyaluronic acid, collagen, laminin and fibronectin are all important ligands of this family of proteins. CD44 gene expression is associated with cells of mesenchymal lineage, stem progenitors and mature cells both.

CD34 antigen is frequently co-expressed with CD133 protein by hematopoietic progenitors, so that these markers are actually employed for primitive stem cells identification and isolation. Taken singularly, these antigens refer respectively to hematopoietic progenitors and mononucleated cells isolated from bone marrow, peripheral and umbilical cord blood. CD34 protein plays an active role during hematopoiesis by facilitating stem cells adhesion to bone marrow microenvironment[59, 60]. Furthermore, CD34 is a typical marker of terminally differentiated endothelial cells, since it is expressed by cells of vessels and microvessels. The importance of this aspect for our discussion will be explained later (see "*Selection of CD34^{neg} fraction of multipotent mesenchymal placenta-derived cells*").

Immunocytochemistry suggested that isolated placenta-derived cells belonged to a population comprising hematopoietic and mesenchymal progenitors. Further characterization with cytofluorimetric analysis was necessary to define prevailing subpopulation. For this reason, cells were tested especially for mesenchymal antigens expression, as well as markers of multipotency, immunogenicity and hematopoietic lineage in order to verify nature, differentiation state and contamination grade. Results are resumed in **Figure 9**.

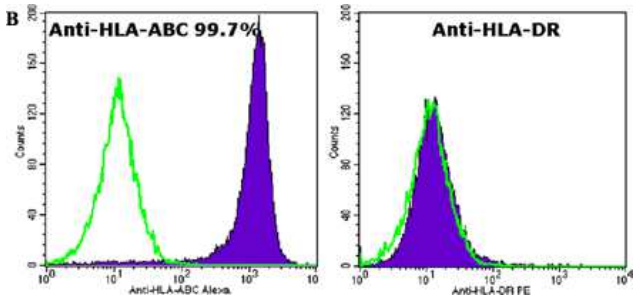
Panel **A** comprises antigens of undifferentiated cells and mesenchymal lineage. In particular, CD117 (c-KIT) and SSEA4 identify cells at a very low state of commitment, while CD90, CD105, CD44 and CD73 are typical markers of undifferentiated mesenchymal stem cells (MSC). CD90 is a glycoprotein of surface belonging to immunoglobulins superfamily, whose expression is exclusive of mesenchymal progenitors, given that hematopoietic ones fail to express it. CD105 (endoglin) is a type I membrane glycoprotein located on cell surface and is part of the TGF-β receptor complex. It has been postulated that endoglin is involved in the cytoskeletal organization by affecting cell morphology and migration[61] and that it regulates trophoblast differentiation[62]. CD73, also known as ecto-5'-nucleotidase, is a plasma membrane protein that catalyzes the conversion of extracellular nucleotides to membrane-permeable nucleosides. The encoded protein is used as a determinant of lymphocyte differentiation.

Figure 9.
Cytofluorimetric analysis of isolated human placenta-derived cells
A, Markers of multipotency and mesenchymal lineage;
B, Markers of histocompatibility antigens;
C, Markers of hematopoietic multipotent cells;
D, Markers of mature blood cells.

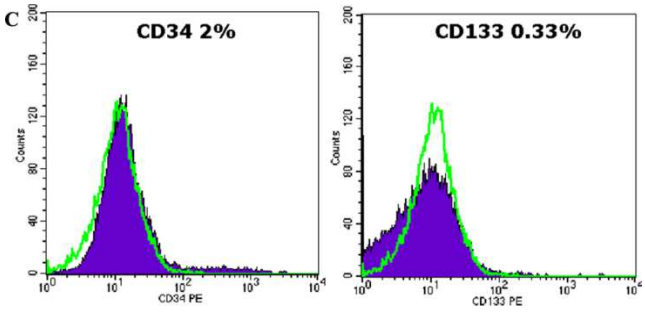


Analyzed population turned out to be bright positive (3-4 log units) for CD90 (99.9%), CD44 (99.5%) and CD73 (99.6%), while it was faint positive (2 log units) for CD105 (77%), CD117 (72%) and SSEA4 (4%). These results were coherent with a scenario describing a population of cells characterized by a low grade of commitment and originated in prevalence from mesenchymal lineage. Since CD117 isn't generally accepted as mesenchymal antigen, its widespread expression revealed contamination with cells of other origin, probably very low committed progenitors of hematopoietic lineage.

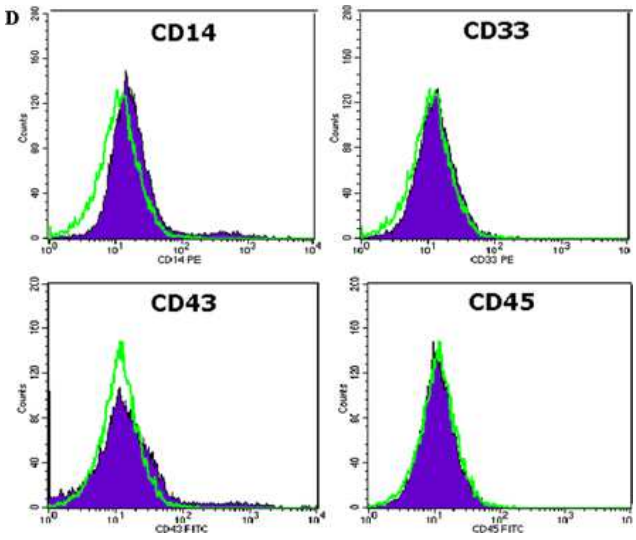
In order to confirm mesenchymal origin of the majority of isolated cells, they were tested for histocompatibility antigens (Panel **B**). Cells didn't express HLA-DR, an antigen of type 2 major complex of histocompatibility, while they were bright positive (99.7%) for HLA-ABC, a series of antigens belonging to type 1 major complex of histocompatibility. These results were in line with the supposed mesenchymal nature of tested cells and underlined that adopted culture conditions contributed to keep a low grade of immunogenicity. This aspect was of particular importance from the point of view of a possible use of these cells in cell therapy procedures, like for example xenogenic grafts and allotransplantations.



Panel **C** shows level of expression of hematopoietic markers CD34 and CD133. Isolated cells that turned out to be positive for these two antigens counted respectively for 2% and 0.33% of total population.



In panel **D** we combined proteins whose expression is confined to undifferentiated or mature blood cells, in this case monocytes (CD14), myeloid progenitors (CD33), T-cells, granulocytes and monocytes (CD43) and leukocytes (CD45). For all considered markers, cells showed no positivity. The latter panels helped us to confirm that isolated population was almost pure, since we could exclude contamination with mature blood cells. On the contrary, expression of CD34 and CD133, even if limited, underlined the presence of a small subpopulation of progenitors of hematopoietic origin, as previously pointed out by CD117 expression.



CD34 antigen presence could also be index of contamination by mature endothelial cells coming from villi microvessels and bigger vessels of placental circulation.

Taken together, results of immunocytochemistry and FACS suggested that at this stage population of fibroblast-like cells was composed by cells expressing antigens of undifferentiated or low committed state, characterized by reduced immunogenicity and in prevalence of mesenchymal origin. In order to better understand provenience of $CD34^{pos}$ contaminating cells, samples of whole placental tissue and of its annexes were collected during isolation procedure, fixed and stained for this antigen (**Fig. 10**).

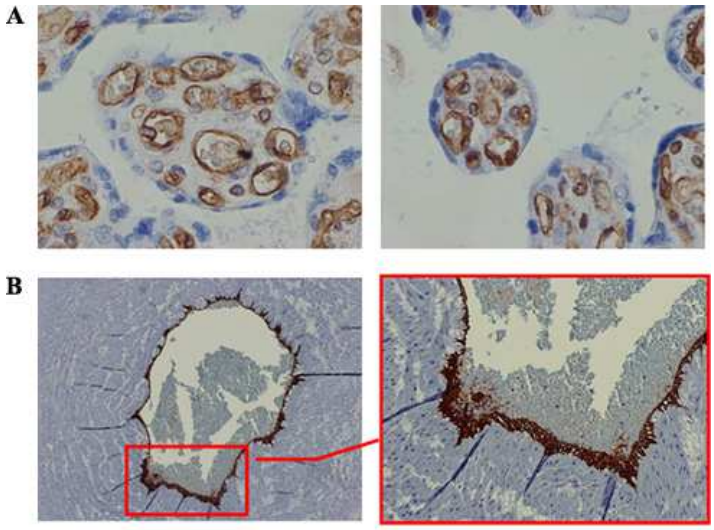


Figure 10.
Evaluation of CD34 expression by placental tissue and its annexes
Histological staining for CD34 (brown) and Alkaline phosphatase (blue)
A, Villi in transverse section, phase contrast images of representative microscopic fields, 40x;
B, Umbilical vein in transverse section, phase contrast images of a representative microscopic field, 40x and 100x (detail).

Figure shows CD34 localization coinciding with mature endothelial cells forming thin internal layer of villi microvessels or with mature blood cells filling the same vessels (**A**). As expected for poorly differentiated cells with stemness properties, cells of syncytio- and cytotrophoblast were positive for alkaline phosphatase staining (blue), while they completely lacked CD34 antigen expression. This marker was however findable in the endothelium covering internal side of big placental vessels, like for example umbilical vein. We could conclude that $CD34^{pos}$ subpopulation of contaminating cells originated during isolation procedure mostly from terminally differentiated endothelial cells of placental circulation vessels and from red blood cells or their progenitors.

Placenta-derived cells in vitro differentiation

In vitro differentiation experiments were performed in order to verify plasticity of isolated population. As several times mentioned before, human placenta-derived cells are thought to be multipotent, to replicate as undifferentiated cells and to have the potential to differentiate to lineages of mesenchymal tissues, including bone, cartilage, fat, tendon, muscle, and marrow stroma[12].

Adipogenic differentiation

Adipogenic differentiation was induced in the expanded cell culture by treatment with 5% FBS, isobutylmethylxanthine (IBMX), dexamethasone, insulin and indomethacin[63]. An important early event of adipogenesis is the exit from the cell cycle[30]. Human MSC generally require hormonal stimulation for several cycles to achieve the commitment to adipogenic lineage. This suggested that only a certain number of cells were sensitive during each hormonal induction. We attempted to enhance differentiation by reduction of serum quantity in order to "synchronize" the cell cycle[64]. With this idea in mind, cells were plated at high density in 5% FBS medium and differentiating agents were only added when cells reached confluence. IBMX plays a positive role in terminal differentiation of adipocytes because it downregulates osteogenic markers by enhancing PPARγ2 and LPL expression. Indomethacin is a non-steroidal anti-inflammatory drug and cyclooxygenase inhibitor that is frequently used as a research tool to study the process of adipocyte differentiation[65]. Treatment of various preadipocyte cell lines with micromolar concentrations of indomethacin in the presence of insulin promotes their terminal differentiation. Insulin is classically viewed as a promoter of adipogenesis because it increases and accelerates triglycerides accumulation. Anyway, high concentrations of insulin have a mitogenic effect, so that we decided to supplement our adipogenic medium with the final insulin concentration of 10µg/ml.

After 28 days of induction, mRNA was analyzed by semi-quantitative Real-Time PCR and lipid droplet deposition was evaluated. Results are shown in **Figure 11**.

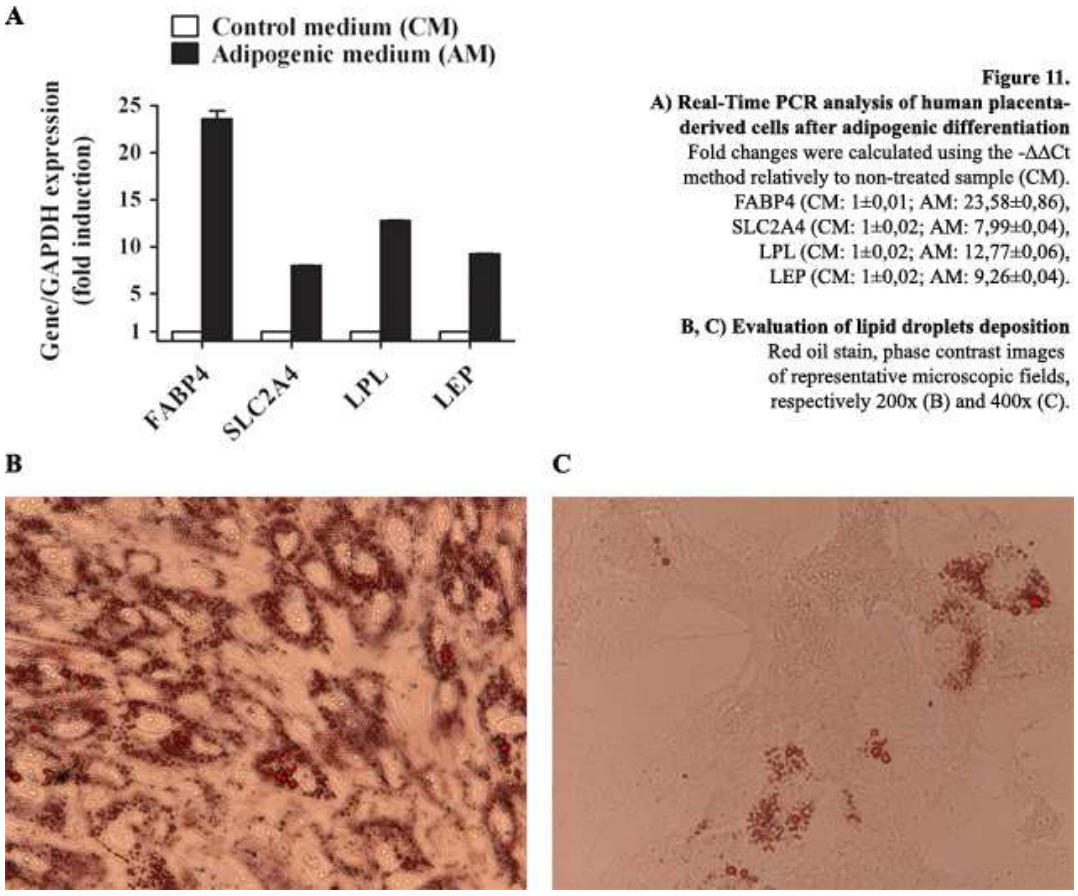


Figure 11.
A) Real-Time PCR analysis of human placenta-derived cells after adipogenic differentiation
Fold changes were calculated using the -ΔΔCt method relatively to non-treated sample (CM).
FABP4 (CM: 1±0,01; AM: 23,58±0,86),
SLC2A4 (CM: 1±0,02; AM: 7,99±0,04),
LPL (CM: 1±0,02; AM: 12,77±0,06),
LEP (CM: 1±0,02; AM: 9,26±0,04).

B, C) Evaluation of lipid droplets deposition
Red oil stain, phase contrast images of representative microscopic fields, respectively 200x (B) and 400x (C).

Adipogenic medium induced a consistent increase in the expression of typical adipocyte markers (**A**) according to model proposed by Rosen et al[66]: FABP4, coding for adipocyte fatty acid binding protein aP2, SLC2A4, coding for GLUT4 protein able to enhance insulin sensitivity, LPL (lipoprotein lipase), a protein involved in lipid accumulation and LEP (leptin), a secreted product of mature adipocytes.

Induction also was underlined by the appearance of lipid-rich vacuoles within cells (**B** and **C**), as pointed out by Oil-Red-O staining. During differentiation period, these lipid droplets continued to develop over time, coalesced, and eventually filled cells, consistently with the acquisition from placenta-derived cells of a mature phenotype.

Osteogenic differentiation

Choice of each differentiating agent composing osteogenic induction medium was well-considered starting from literature data.

Dexamethasone is a synthetic glucocorticoid that acts through nuclear receptors. In primary cultures of multipotent mesenchymal cells, dexamethasone was demonstrated to increase the number of fibroblast colony-forming units. In that cases, number of colonies showing alkaline phosphatase activity was increased, consistently with the improvement of osteogenic differentiation by this glucocorticoid[67]. Bone marrow stromal cells subjected to continuous dexamethasone treatment exhibited higher mRNA expression levels of osteogenic markers such as osteopontin and osteocalcin, suggesting that dexamethasone itself could increase cell responsiveness to other differentiation agents present in osteogenic medium[68]. Glucocorticoids exert both transcriptional and post-transcriptional effects[69]. Further, glucocorticoids enhance vitamin D3 effect on gene expression[70]. Those genes which are upregulated by 1-25 vitamin D3 are transcribed at an increased rate by dexamethasone, while those genes which are inhibited by vitamin D3 remain inhibited in the presence of dexamethasone and D3[71]. It is actually generally accepted that glucocorticoids promote changes in gene expression involved in cell-cell and cell-extracellular matrix signalling mechanisms that support the growth and differentiation of cells capable of osteoblast phenotype development and bone tissue-like organization, while inhibiting the growth of cells that cannot progress to the mature osteoblast phenotype. L-proline and Ascorbic acid are both involved in collagen synthesis, given that the first is the major aminoacid component of collagen α-chains, while the second is a necessary cofactor for activation of enzymes controlling tropocollagen production. L-Ascorbic acid 2-phosphate significantly stimulates cell growth in the presence of foetal bovine serum (FBS) and increases expression of osteoblast differentiation markers[72, 73]. β-glycerophosphate was also added to induction medium because of its role in releasing inorganic phosphates essential for bone mineralization[74].

At the end of 28 days of induction protocol, osteogenic differentiation was evaluated through Real-Time gene expression analysis and Alizarin Red S stain to assess calcium deposition. Results are reported in **Figure 12**.

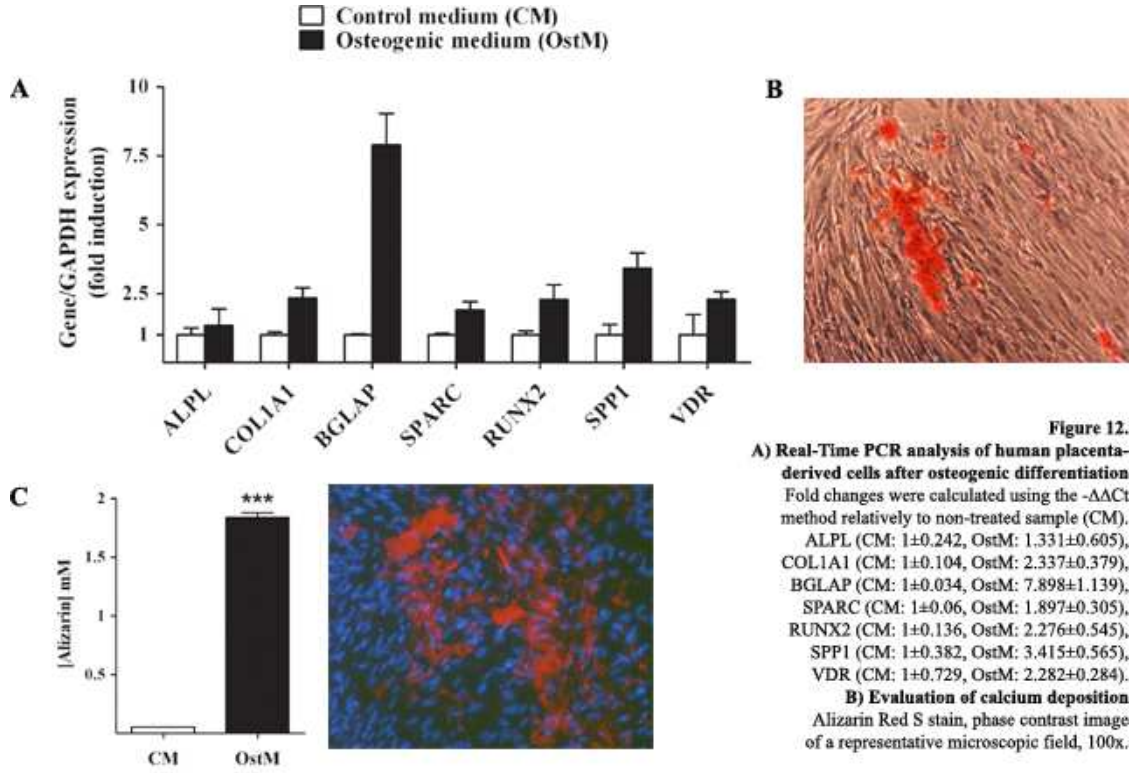


Figure 12.
A) Real-Time PCR analysis of human placenta-derived cells after osteogenic differentiation
Fold changes were calculated using the -ΔΔCt method relatively to non-treated sample (CM).
ALPL (CM: 1±0.242, OstM: 1.331±0.605),
COL1A1 (CM: 1±0.104, OstM: 2.337±0.379),
BGLAP (CM: 1±0.034, OstM: 7.898±1.139),
SPARC (CM: 1±0.06, OstM: 1.897±0.305),
RUNX2 (CM: 1±0.136, OstM: 2.276±0.545),
SPP1 (CM: 1±0.382, OstM: 3.415±0.565),
VDR (CM: 1±0.729, OstM: 2.282±0.284).
B) Evaluation of calcium deposition
Alizarin Red S stain, phase contrast image of a representative microscopic field, 100x.
C) Quantification of calcium deposition
Mineralization generated by cell differentiation was evaluated as described in Materials and Methods.
Data are expressed as mean ± SEM (n=3) and analysed with Student's T test, *** $P < 0.001$
Alizarin Red S stain, fluorescence image of a representative microscopic field, 100x, nuclear stain with DAPI (blue).

Expression of typical genes of mature bone such as collagen α1 chain (COL1A1), osteocalcin (BGLAP), osteonectin (SPARC), transcription factor RUNX2, osteopontin (SPP1) and vitamin D receptor (VDR) resulted slightly increased after exposure to osteogenic medium (**A**). Alkaline phosphatase (ALPL) was the only marker which seemed not to be influenced by differentiating culture conditions. These results are consistent with differentiation model suggested by Bellows et al[75]: such high level of osteocalcin expression is index of late stage of differentiation towards osteoblastic lineage, since early osteoblasts exhibit a lower expression of this marker. Great osteocalcin induction, so that of other constitutive osteogenic antigens, can be mainly due to RUNX2 action.

RUNX2, a member of the Runt-domain family of transcription factors, is expressed in all osteoblasts[76] but also in hypertrophic chondrocytes[77] and early mesenchymal condensation. RUNX2 has two distinct functions in bone formation: an essential role in the differentiation of mesenchymal progenitors into osteoblasts, both in endochondral and intramembranous skeletons and the ability to stimulate hypertrophic chondrocyte differentiation. By stimulating hypertrophic chondrocyte differentiation, RUNX2 primes the cartilage skeleton for its subsequent invasion by osteoblasts and its replacement by a bone specific matrix. Indeed, once chondrocytes become hypertrophic they are destined for rapid cell death and replacement by bone[78].
Starting from these observations, we could hypothesize that RUNX2 induced a network of signals which was finally responsible for induction of all considered osteogenic markers.

Staining with Alizarin Red S pointed out the deposition of calcium crystals on the surface of cultured cells (**B**). Since the amount of calcium deposition was proportional to the quantity of bound Alizarin Red, quantification of dye binding made qualitative evaluation of cell differentiation possible (**C**). Even if calcium deposition wasn't uniform, we could conclude that adopted induction conditions were sufficient to stimulate placenta-derived cells to differentiate towards osteoblastic lineage.
Taken together with Real-Time PCR analysis evidence, our results seemed to suggest the ability of isolated placental cells to achieve complete osteoblast phenotype when stimulated in defined culture conditions.

Chondrogenic differentiation

First attempts to achieve chondrogenic differentiation of placenta-derived cells were performed using classical monolayer cultures. Unfortunately, combination of this kind of culture and a defined induction medium wasn't effective in enhancing expression of chondrogenic lineage specific genes (data not shown). Literature data report that the condensation of prechondrogenic mesenchyme is a critical step of chondrogenesis process during embryonic development. For this reason, we adopted a culture system that facilitates cell-cell interactions in a way analogous to those that occur in precartilage condensation. This system is an adaptation of the ''pellet'' culture system that was originally described as a method for preventing the phenotypic modulation of chondrocytes in vitro[33] and it is based on protocol described by Mackay et al[37]. However, this three-dimensional culture system is not sufficient for the induction of chondrogenesis, so that a defined medium to which were added certain bioactive factors including dexamethasone and TGF-β3 was formulated.

To induce chondrogenic differentiation of MSC, all three mammalian TGF-β subtypes are commonly used[79-81] and several studies demonstrate that all of them are able to enhance chondrogenic induction with comparable results[82]. Despite this, it is well known that transforming growth factor-β3 plays a central role during chondrogenesis by upregulating a number of molecules associated with prechondrogenic mesenchymal condensation. Furthermore, TGF-β3 determines the commitment of bone marrow-derived MSC to mesenchymal chondroprogenitor cells in vitro[12, 37].

This process also requires the synthetic glucocorticoid analog dexamethasone[36]. It is demonstrated that glucocorticoids promote differentiation of chondrocytes and maintain the integrity of the cartilaginous matrix in isolated primary cell populations[83, 84]. We chose to supplement differentiation medium with dexamethasone because of its recognized function in inducing transcription factor SOX9 and COL2A1 expression[85].

As in the case of osteogenic differentiation medium, L-proline and L-Ascorbic acid 2-phosphate were added to chondrogenic medium in order to supply substrates for collagen synthesis.

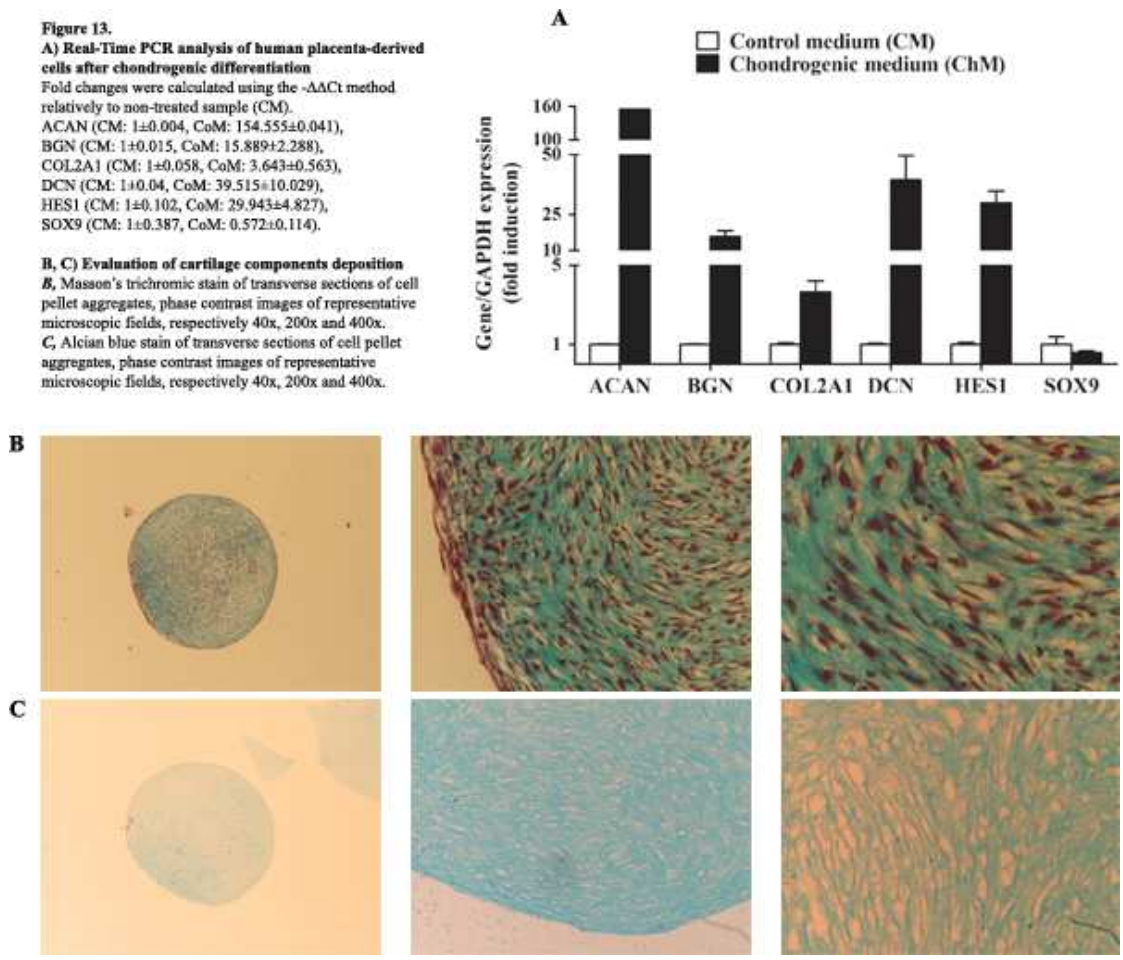
IGF-1 promotes in vitro survival, development and maturation of chondrocytes. It also has positive effects on MSC differentiation towards chondrogenic lineage, so that literature data report a synergic action in combination with TGF-β3 and dexamethasone[86, 87].

Glucose and glucose-derived sugars have been reported to play important roles in the development, maintenance, repair, and remodelling of cartilage[88]. Glucose is also the major energy substrate and the main precursor for chondrocytes synthesis of glycosaminoglycans and extracellular matrix. Moreover, the transport of glucose through the articular cartilage and into chondrocytes is essential for growth, development, maintenance, and structural integrity of the articulating joint[89]. Previous experiments in which pellet cultures were induced to differentiate in medium with low glucose concentration pointed out that in this conditions cells weren't able to form stable aggregates and that they underwent early apoptosis[90]. In the same way, we observed that serum-free medium wasn't suitable for three-dimensional culture system adopted, as it reduced cell viability and prevented formation of compact, round cell aggregates. Starting from these observations, we decided to use high glucose DMEM as basal medium to support chondrogenic differentiation and to supplement it with 1% FBS.

Gene expression with Real-Time PCR and histology of culture aggregates were measured on day 28 after cell induction beginning. **Figure 13** reports results.

Figure 13.
A) Real-Time PCR analysis of human placenta-derived cells after chondrogenic differentiation
Fold changes were calculated using the -ΔΔCt method relatively to non-treated sample (CM).
ACAN (CM: 1±0.004, CoM: 154.555±0.041),
BGN (CM: 1±0.015, CoM: 15.889±2.288),
COL2A1 (CM: 1±0.058, CoM: 3.643±0.563),
DCN (CM: 1±0.04, CoM: 39.515±10.029),
HES1 (CM: 1±0.102, CoM: 29.943±4.827),
SOX9 (CM: 1±0.387, CoM: 0.572±0.114).

B, C) Evaluation of cartilage components deposition
B, Masson's trichromic stain of transverse sections of cell pellet aggregates, phase contrast images of representative microscopic fields, respectively 40x, 200x and 400x.
C, Alcian blue stain of transverse sections of cell pellet aggregates, phase contrast images of representative microscopic fields, respectively 40x, 200x and 400x.



Real-Time PCR showed great increase in expression of gene markers of chondrogenesis, such as aggrecan (ACAN), byglican (BGN), collagen type II (COL2A1), decorin (DCN) and transcription factors HES1 and SOX9.

Decorin and byglican are considered early genes in chondrogenic differentiation, so that their marked expression can be the index of differentiation pathway beginning. Both markers share a structural and functional relationship, because they are actually involved in the formation of cartilage.

Aggrecan is a large, very abundant proteoglycan that is almost unique to cartilage. It forms enormous aggregates by binding to linear chains of the glycosaminoglycan hyaluronan. Its expression is generally related to chondroblast differentiation stage[91].

At this stage, cells also started expressing gene for collagen type II at high levels. The latter form a fiber network that constitute the framework of cartilage.

No transcription factor capable of specifically inducing differentiation to articular cartilage has been identified to date. However, transcription factor SOX9 has essential, non-redundant roles in specifying the commitment and differentiation of mesenchymal cells towards the chondrogenic lineage in all developing skeletal elements[92]. It is expressed predominantly in mesenchymal condensations throughout the embryo in the regions where deposition of cartilage matrix is taking place, suggesting a role in skeletal formation[93]. It activates type II collagen gene expression during chondrocyte differentiation and enhances aggrecan gene promoter activity in a cartilage-derived cell line[94-96]. SOX9 is turned on in chondrogenic and osteogenic mesenchymal cells prior to condensation, remains highly expressed in chondroblasts and prechondrocytes and is turned off when cells undergo prehypertrophy[97].

Transcription factor HES1 is a Notch1 effector, the latter having well known strong inhibitory effect on cartilage differentiation in mice[98]. Downstream HES1 protein is capable of exerting a similar, but weaker, effect. From this point of view, the significant increase in HES1 expression during differentiation of pellet mass system might be important to balance the action of positive transcription factors such as SOX9, thus allowing differentiation to be properly controlled[99].

This could explain the decrease in SOX9 expression pointed out by PCR analysis. We could actually hypothesize that SOX9 was turned on during first days of induction in order to begin differentiation pathway. As cells passed through early chondroblasts and prechondrocytes stages achieving more differentiated phenotype, SOX9 expression was gradually reduced by HES1 action.

On the whole, Real-Time PCR results were coherent with a pattern of expression typical of mature chondroblast/early prehypertrophic phenotype, as during prehypertrophic stage cells express higher levels of RNA for ACAN, COL2A1 and most other early cartilage matrix genes than chondroblasts do[91].

As demonstrated by histological analysis, induced cells showed the features of chondrocytes in their morphology and extracellular matrix. Placenta-derived cells proliferated and underwent condensation to form aggregates. In this form, cells differentiated into chondrocytes, generating a highly organized cartilage-specific extracellular matrix. At little magnification, Masson's trichromic (**B**) staining gave an overall idea of pellets dimensions and cell organization. An evident thin layer of cells with reduced cytoplasm formed a capsule all around pellet structure. Inside, cells were immersed in a matrix showing different composition and density respect to observed zone. Green stain appeared to be more evident in the peripheral one, while at the centre it was less concentrated. This particular stain distribution suggested that prevailing components of the central extracellular matrix were non-collagenous fibres, while collagen ones became more and more abundant as the peripheral zone was reached. On the other hand, Alcian blue staining (**C**) pointed out the accumulation of sulfated glycosaminoglycans and mucopolysaccharides, as previously suggested by marked increase in byglican and decorin expression. Results of histological analysis gave a scenario in which adopted culture conditions seemed to facilitate deposition of fibrocartilage, rather than hyaline one. Cells weren't actually organized in columnar orientation and didn't form characteristic isogenous groups. Moreover, observed cell-matrix ratio was typical of fibrocartilaginous phenotype.

On the whole, we could observe that adopted differentiation technique and culture conditions were useful to get induction of chondrogenic gene expression by isolated placental cells. Cells not only acquired chondroblastic phenotype, they also caused production and deposition of typical cartilage extracellular matrix components, such as collagen fibres and glycosaminoglycans. From this point of view, we obtained a matrix with fibrocartilaginous characteristics, probably due to serum presence in differentiating medium. These results forced us to change culture conditions, in order to find the best which could guarantee hyaline cartilage deposition for applications in articular cartilage regeneration. However, the type of tissue we obtained can be easily employed in musculoskeletar fibrocartilage regeneration procedures.

Preliminary conclusions

Literature data suggest that four regions of foetal human placenta can be distinguished: amniotic epithelial, amniotic mesenchymal, chorionic mesenchymal and chorionic trophoblastic[100]. From these regions, the following cell populations can be isolated: human amniotic epithelial cells (hAEC), human amniotic mesenchymal stromal cells (hAMSC), human chorionic mesenchymal stromal cells (hCMSC) and human chorionic trophoblastic cells (hCTC)[101].

According to what proposed by Dominici et al[14] for bone marrow-derived mesenchymal stromal cells, minimal criteria for defining cells isolated from foetal membranes are:

- ✓ Foetal origin;
- ✓ Adherence to plastic;
- ✓ Formation of fibroblast colony-forming units;
- ✓ A specific pattern of surface antigen expression (**Table 2**)

Positive Markers (≥ 95%)	**Negative Markers (≤2%)**
CD73, CD90, CD105	CD14, CD34, CD45, HLA-DR

Table 2. Definition of commonly recognized mesenchymal cells antigens

- ✓ Differentiation potential toward one or more lineages, including osteogenic, adipogenic, chondrogenic.

On the basis of these important guide lines and relatively to requisites that cells demonstrated to own during described experiments, we were able to reasonably define origin, embryological derivation and commitment grade of our isolated placenta-derived cells.

Chorionic nature of cells was guaranteed through exclusion of amnios from isolation procedure, so that foetal origin requisite was respected. Once cultured, primary cells grew on uncoated plastic surface and proliferated rapidly, showing ability to form fibroblast colony-forming units. On the other hand, immunocytochemistry and flow cytometric analysis revealed mesodermal derivation of cells by pointing out expression of markers that are commonly believed to belong to mesenchymal lineage. Moreover, cells were found to be negative for hematopoietic and mature blood cells antigens.

On the whole, this evidence allowed us to catalogue isolated cells as human chorionic mesenchymal stromal cells (hCMSC).

Finally, in vitro induction experiments gave the proof of multilineage differentiation potential. When subjected to defined induction protocols, isolated mesenchymal cells were able to acquire adipogenic, osteogenic and chondrogenic phenotype, expressing in all cases specific antigens of completely mature or late committed state. With these preambles and given reduced expression of pluripotency antigens such as SSEA4, we could conclude that mesenchymal placenta-derived cells are multipotent cells, at a commitment grade comparable to well characterized hematopoietic progenitors.

As mentioned in Material and Methods section, cell isolation was performed to obtain cells both from placentas of healthy patients and of pregnant women affected by Type I Diabetes. In both cases, cells were tested for origin, embryological derivation and commitment grade with experiments so far described, obtaining comparable results. For the record, all images presented in this section refer to cells isolated from healthy patients.

Following experiments, as described in Materials and Methods and whose results will be reported in the following parts of Discussion section (see "*Results and Discussion, Part 2 and 3*") will be performed on cells from healthy and diabetic patients. In order to facilitate comprehension, we will refer to these cells as **PLN17**, indicating **PL**acenta-derived cells isolated from healthy (**N**ormal) patient, and as **PLD09**, referring to **PL**acenta-derived cells isolated from **D**iabetic patient.

Selection of CD34^{neg} fraction of multipotent mesenchymal placenta-derived cells

In the last part of this Discussion section, PLN17 and PLD09 will be tested for their capacity to differentiate towards endothelial lineage (see "*Results and Discussion, Part 3*"). From this point of view, contamination of isolated cell population with mature endothelial cells as revealed by previous experiments might constitute a problem, thus questioning the validity of eventually positive results.

In order to obtain completely pure cell populations lacking terminally differentiated endothelial elements, PLN17 and PLD09 were subjected to immunodepletion for CD34 antigen. Thanks to this procedure, CD34^{pos} endothelial cells were eliminated and CD34^{neg} fraction of both PLN17 and PLD09 was designed for following experiments.

Before endothelial differentiation protocol, both cell types were characterized again with flow cytometric analysis. Results are shown in **Figure 14**.

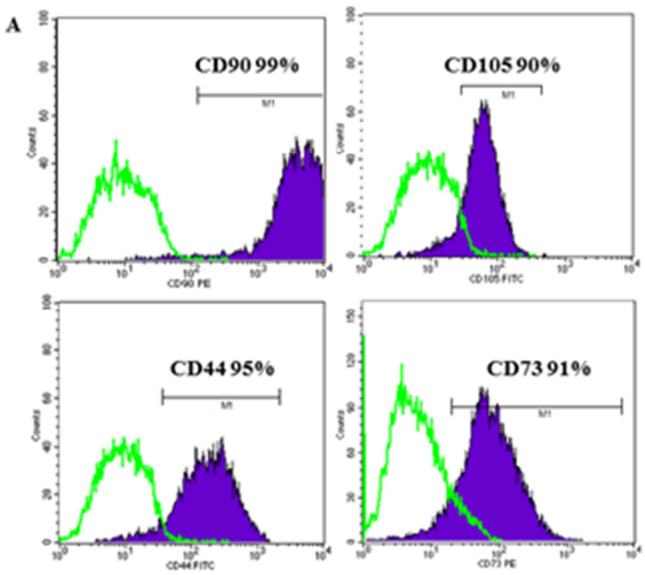
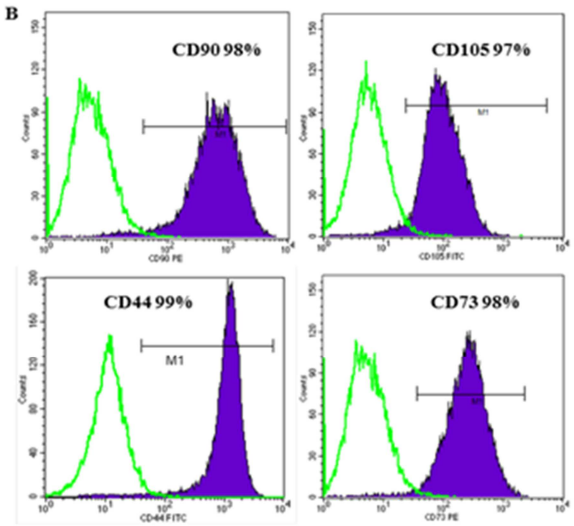


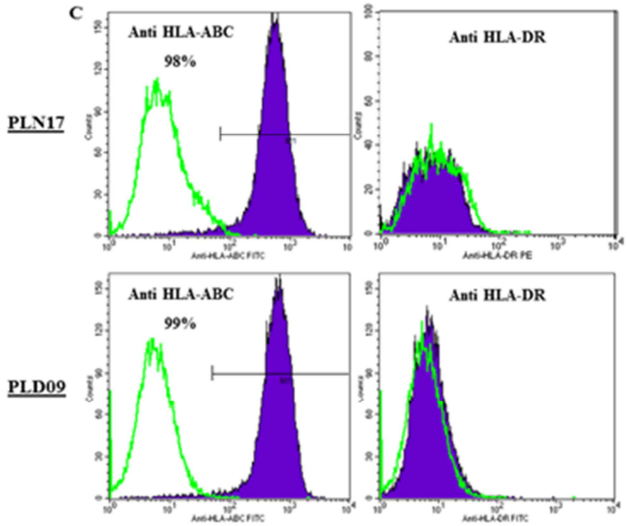
Figure 14.
Cytofluorimetric analysis of isolated multipotent mesenchymal placenta-derived cells PLN17 and PLD09 after CD34 antigen immunodepletion

A, PLN17, Markers of multipotency and mesenchymal lineage;
B, PLD09, Markers of multipotency and mesenchymal lineage;
C, Markers of histocompatibility antigens;
D, Markers of hematopoietic multipotent cells.

Panel **A** refers to PLN17 and shows antigens of multipotency and mesenchymal lineage. Results were comparable with the first cytofluorimetric characterization. CD34^{pos} fraction depletion didn't alter phenotypic expression of isolated cells.

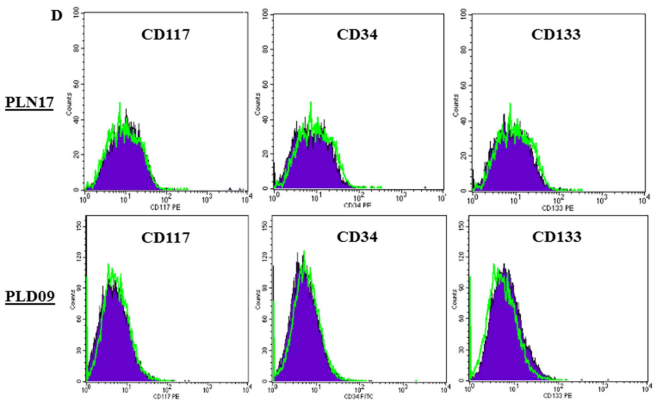


In panel **B**, expression of multipotency and mesenchymal lineage markers by PLD09 is shown. No appreciable differences between PLN17 and PLD09 were evident.



Further demonstrations that selection of $CD34^{neg}$ population had no influence on cells nature and properties came from Panel **C**, in which expression of histocompatibility antigens is reported. As previously described, cells were characterized by a low grade of immunogenicity. More interestingly, PLN17 and PLD09 displayed same pattern of expression.

Finally, antigens of hematopoietic multipotent cells were tested. As shown in Panel **D**,



immunodepletion for CD34 marker was useful. After procedure, each contamination from immature cells of hematopoietic lineage or fully differentiated endothelial cells was eliminated.

A summary of flow cytometric analysis, comparing PLN17 and PLD09 antigenic pattern of expression, is reported in **Table 3**.

Antigen	PLN17	PLD09
CD44	95%	99%
CD73	91%	98%
CD90	99%	98%
CD105	90%	97%
HLA-ABC	98%	99%
HLA-DR	n.d.	n.d
CD117	n.d	n.d
CD34	n.d	n.d
CD133	n.d	n.d

Table 3. Cytofluorimetric characterization of PLN17 and PLD09 antigenic expression

Human umbilical vein endothelial cells

Human umbilical vein endothelial cells (HUVEC) were isolated following protocol described in Materials and Methods.

Cell were cultured on collagen coated plastic surface in a defined maintenance medium containing specific growth factors, such as endothelial cells growth supplement (ECGS) and heparin. After about 10 days in culture, endothelial cells grew in confluent monolayers (**Fig. 15**). Cells were homogenous in size and shape, closely apposed, characterized by the typical cobblestone morphology, with an oval, centrally located nucleus and indistinct cell borders. To test the homogeneity of isolated cellular population, an immunocytochemical staining was carried out to detect positivity for von Willebrand factor and PECAM-1. More than 95% of cells were positive for both antigens (data not shown). Observation of cultured endothelial cells for periods up to 5 passages revealed no evident transformation to spindle-shaped cells, like for example fibroblasts or smooth muscle cells. Once endothelial morphology was verified, cells were used in experiments described in following section.

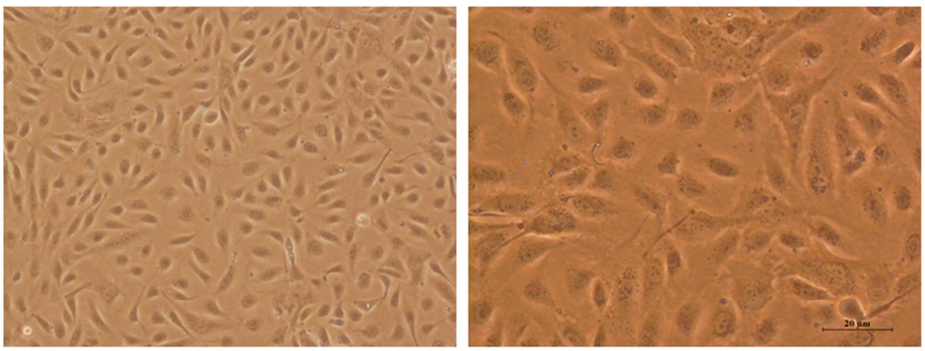


Figure 15.
HUVEC in maintenance culture
Human umbilical vein endothelial cells at confluence, phase contrast images of representative microscopic fields, respectively 100x and 200x

Summary

The presence within bone marrow of a population of mesenchymal stem cells (MSC) able to differentiate into a number of different mesenchymal tissues, including bone and cartilage was first suggested by Friedenstein nearly 40 years ago.

Here we tried to identify cells with MSC-like properties in human term placenta. Placental tissue samples from foetal portion of organ were enzymatically digested and the population of isolated cells was examined for morphology, behaviour in culture, antigenic expression pattern and multilineage differentiation potential.

We found that placenta-derived cells proliferated as plastic-adherent cells with a fibroblast-like morphology and can differentiate into cells of the mesodermal lineage, including adipocytes, osteoblasts and chondrocytes.

Moreover, they were characterized by the expression of several mesenchyme-specific markers, as well as antigens of undifferentiated state and low immunogenicity.

On the whole, these observations allowed us to define the isolated population as human chorionic multipotent stromal cells (hCMSC), accordingly to literature data.

Cells from both placentas of healthy and diabetic donors were isolated, the latter being characterized as previously described. This cell type showed no appreciable differences in culture behaviour, pattern of expression and differentiation potential respect to healthy counterparts. Both cell populations, respectively referred to as PLN17 and PLD09, were depleted of contaminating $CD34^{pos}$ mature endothelial cells, so that they could represent an ideal starting material for subsequent endothelial differentiation protocol.

Human umbilical vein endothelial cells (HUVEC) were also isolated. Morphology and expression of antigens of endothelial lineage were tested, in order to guarantee homogeneity of isolated population. After these evaluations, cells were thought to be suitable for procedures described in the following section of this thesis.

Part 2. Mimicking hyperglycaemia in vitro affects cell proliferation

Introduction - Regenerative Medicine

The artificial generation of tissues, organs or even more complex living organisms was throughout the history of mankind a matter of myth and dream. Philosophers, naturalists and scientists were fascinated by the marvels of regeneration seen in nature, but imitation of nature required that we first understood the basic biology of tissues and organs, including developmental biology. Tissue Engineering and Regenerative Medicine are terms from the biomedical field that deal with the transformation of these fundamental ideas to practical approaches.

Applied by the scientific literature in cases of surgical manipulation of tissues and organs or in a broader sense when prosthetic devices or biomaterials were used, the term "Tissue Engineering" as it is nowadays used was introduced in medicine only in 1987. The commonly recognized definition was as follows: "*Tissue Engineering is the application of the principles and methods of engineering and life sciences toward the fundamental understanding of structure-function relationships in normal and pathologic mammalian tissue and the development of biological substitutes to restore, maintain or improve function.*"[102] The early years of Tissue Engineering were based on cell and tissue culture approaches, so that three principal research lines were developed: isolated cells or cell substitutes, tissue-inducing substances and cells placed on or within matrices (scaffolds).

In the last decades, improvements in cell culture techniques such as study of cellular processes or the ability to genetically manipulate cells and the advent of Biotechnology allowed the field of Tissue Engineering to evolve into a multitasking discipline. From this point of view, genetic engineering, stem cell biology, cloning, biomaterials and biomedical devices can play a major role on their own or can be all comprised in a developing field called Regenerative Medicine.

The terms Tissue Engineering and Regenerative Medicine were used in parallel and as synonymous to each other, even if the second is nowadays regarded to as the natural and logic evolution of the first. The NIH has currently adopted the following definition: "*Regenerative medicine/tissue engineering is a rapidly growing multidisciplinary field involving the life, physical and engineering sciences that seeks to develop functional cell, tissue, and organ substitutes to repair, replace or enhance biological function that has been lost due to congenital abnormalities, injury, disease or aging.*"[103]

Regenerative medicine is an emerging branch of medicine whose goal is to restore organ and/or tissue function using a biological approach. Conceptually, the application of this new discipline to human health care can be thought of as a refinement of previously defined principles of Medicine. Physicians have historically treated the majority of diseases by supporting nutrition, trying to eliminate negative factors and optimizing the environment so that the body can heal itself. In the field of Regenerative Medicine the same thing is accomplished on a cellular level. The harmful tissue is eliminated and cells necessary for repair are then introduced in an environment that has been previously manipulated to guarantee cell survival and to induce body to heal itself.

Recent advances in stem cell technology have shown great promise. The enthusiasm surrounding stem cells is related in part to their potential to treat a broad range of clinical pathologies. Some identified stem cell targets, such as neurological diseases, spinal cord injuries, diabetes and cardiovascular diseases currently have few accepted treatments or no cures. In other conditions, such as bone fracture healing or cartilage repair, stem cells may improve therapies currently in use.

In the future years, Regenerative Medicine will have to face an important challenge of modern Medicine: the use of autologous cells versus allogeneic or even xenogeneic cells. To date, there are many tissue-engineering applications for which appropriate autologous donor cells may not be available, so that cellular therapies need to find new sources of cells, mainly from among progenitor and stem cell populations, in order to customize treatments for individual patients.

Introduction - Glucose, Hyperglycaemia and Diabetes

Glucose is perhaps the most important fuel for human metabolism. Glucose has a central role in metabolism due to the fact that many tissues (e.g., brain, red blood cells, exercising skeletal muscle) require an uninterrupted flow of this sugar to supply their energy needs. Glucose is a flexible metabolite that can be converted to other forms of fuel, including lipids (e.g., fatty acids, cholesterol, steroid hormones), amino acids and nucleic acids. Except for vitamins, essential amino acids and essential fatty acids, all metabolites needed by human body can be synthesized from glucose.

That is the reason why it is also a fundamental component of classic culture media, which contain different concentrations ranging from 1 to 4.5g/L (5.5 - 25mM). It is well known that glucose in the microenvironment of cell culture can influence gene regulation, proliferation and differentiation, as well as apoptosis and cell senescence. Despite this, our knowledge about effects of varying glucose concentration on cells with stemness properties is still lacking, both *in vivo* and *in vitro*. Application of these cells to the field of Regenerative Medicine requires a better understanding of mechanisms regulating glucose uptake and catabolic pathways that contribute to make stem cells metabolically different from more differentiated cell types.

In our discussion we will deal in particular with hyperglycaemia effects on culture behaviour and fate choice of multipotent placenta-derived cells.

It is noteworthy that hyperglycaemia resulting from uncontrolled glucose regulation is the causal link between Diabetes and diabetic complications[104, 105].

Diabetes is a disease caused by impaired glucose metabolism. Approximately 90% of Diabetes cases is due to a defect in insulin production and/or utilization (Type 2 Diabetes Mellitus), while the more severe form (Type 1 Diabetes Mellitus) is caused by a complete loss of the insulin-producing β-cells within the islets of Langerhans of the pancreas. Since the discovery of insulin in 1921, Diabetes has become a treatable condition and the life expectancy of patients has been greatly improved[106]. However, even with diligent blood glucose monitoring and insulin administration, the metabolic abnormalities associated with the disease can lead to many chronic secondary complications, affecting kidneys, peripheral nerves, eyes, heart and vascular system.

Four major molecular mechanisms have been implicated in hyperglycaemia-induced tissue damage: (1) activation of protein kinase C (PCK) isoforms, (2) increased hexosamine pathway flux, (3) increased advanced glycation end products formation (AGE), (4) increased polyol pathway flux[107-109]. Brief episodes of hyperglycaemia cause tissue damage through processes involving repeated acute changes in cellular metabolism. However, exposure to high glucose also causes cumulative changes in long-lived macromolecules, which persist despite restoration of normoglycaemic conditions. Hyperglycaemia-induced overproduction of superoxide is commonly regarded as the link between high glucose and pathways responsible for hyperglycaemic damage[110, 111]. Indeed, Diabetes is typically accompanied by increased production of free radicals and/or impaired antioxidants defence capabilities, indicating a central contribution for reactive oxygen species (ROS) in the onset, progression and pathological consequences of Diabetes[112-114].

In recent years increasing interest concerning relationship between maternal Diabetes and its possible effects on foetus aroused.

Glucose is the principal energy substrate for placenta and foetus and is essential for normal foetal metabolism and growth. Not surprisingly, its supply is regulated by a relatively complex set of mechanisms that tend to keep its metabolism almost constant. Foetal glucose exposure and, consequently, foetal insulin secretion are strictly dependent from mother glucose supply during pregnancy[115].

On the other hand, placental glucose metabolism is not overtly controlled by maternal or foetal hormones, it responds mainly to substrate availability and is affected by substrate excess, as in the case of Diabetes[116]. Placenta is not permeable to insulin, but is endowed with high density of insulin receptors[117, 118]. Insulin effects on placenta began only lately to be elucidated. Several lines of evidence indicated that placenta is sensitive to insulin, whose action is mostly directed towards control of placental growth by stimulating mitogenesis and DNA synthesis, rather than regulating metabolic pathways and glucose fluxes[119, 120]. This particular feature distinguishes placenta from classical insulin target tissues in which the major insulin effect is the stimulation of glucose transport and metabolism. Alterations of placental functions have been described in Type I Diabetes but are less well-understood than in pregnancies complicated by Type II and Gestational Diabetes.

They comprises expression and localization of glucose transporters, glycogen deposition, functionality of glycolytic enzymes and mitochondria.
These observations are particularly important for our discussion with the purpose to elucidate possible effects of maternal Diabetes on multipotent placenta-derived cells. Little is known about cells needs when expanded in culture or about best growth conditions to guarantee stemness properties maintenance, with regard to glucose requirement and metabolism. An eventual employment of these cells in allotransplantations could be an interesting new approach for the cure of diabetes-damaged tissues, but unfortunately it is delayed due to gaps in our knowledge.

As previously mentioned, endothelium of vascular system is one of Diabetes-damaged tissues *in vivo*[121-124]. Hyperglycaemia effects on mature endothelial cells *in vitro* are also well characterized. Along with biochemical changes, high glucose levels also affect cell function. Indeed, endothelial cells in hyperglycaemic conditions display increased permeability[125] and altered basement membrane protein production[122]. As a matter of fact, a direct relationship between high glucose concentration and augmentation of albumin flux through cell monolayer has been pointed out[125]. Moreover, several observations suggest that upregulation of basement membrane and extracellular matrix proteins, such as fibronectin and collagen type IV occurs in hyperglycaemic conditions[126]. This perturbation of basal membrane homeostasis can cause several adverse side effects, given that altered cell metabolism and impaired cell functions, including migration, adhesion and growth, were observed[127].
High glucose has also been shown to alter endothelial cells size and organelles disposition *in vitro*. Total cytoplasmic area was increased in size, to nuclear area's detriment. A higher number of giant and polynucleated cells was observed[128]. Along with actin, several adhesion proteins were affected by high glucose levels. Focal adhesion kinase and paxillin were found to increase over time with exposure to hyperglycaemia, while calcium dependent adhesion molecules (cadherins) were decreased[129].
Hyperglycaemia is widely known to induce apoptosis in endothelial cells. The elevated level of oxidative stress is the main cause of both constant and intermittent exposure to high glucose concentration[130]. Along with oxidative stress, mitochondrial dysfunction and impaired ability of insulin to stimulate the phosphorylation of Akt are suspected causes of apoptosis of hyperglycaemic endothelial cells[131, 132].

In endothelial cells exposed to long term high glucose levels, the singular phenomenon of cellular "memory" was pointed out. Almost 25 years ago Cagliero et al[133]. showed that there was a persistence or "memory" of the induced gene expression of basement membrane components (collagen type IV and fibronectin) long after high glucose levels were normalized in endothelial cell cultures. This fact suggested the possibility of a long lasting deleterious effect of hyperglycaemia on these cells, independent of the actual glucose concentration. More recently, other groups showed that this phenomenon could influence not only expression of high glucose-stress markers, but also endothelial cell proliferation potential. They hypothesized that persisting overproduction of ROS could provide molecular basis underlying hyperglycaemic "memory"[38, 134]. They demonstrated that a common antioxidant like α-lipoic acid, which functions at mitochondrial level, was able to eliminate adverse effects on cell proliferation as well as overproduction of ROS, confirming their hypothesis.

Present knowledge about hyperglycaemia effects on cells with stemness nature is still incomplete and partly controversial. Only recently, a general description of stem cell metabolism has emerged, involving increased glycolytic flux, limited oxidative metabolism and resistance to oxidative damage.

The most commonly cited feature describing metabolism of stem cells and of most proliferative cells is their glycolytic phenotype. This designation stems from Otto Warburg's work in the early 20th century, when he demonstrated that tumours and proliferating tissues undergo aerobic glycolysis, consuming high levels of glucose and converting much of this sugar to lactate[135].

This phenomenon seems to apply to multipotent cells in culture, which are typically maintained in a state of constant proliferation. Direct evidence of this glycolytic phenotype in stem cells has come in the form of enzyme levels and activities analysis[136, 137]. Once in culture, progenitor cells may take one of several fates: quiescence, proliferation and self-renewal, transit amplification and terminal differentiation or programmed cell death. Metabolic needs of cells change significantly with regard to their fate and function. This is particularly true for cells rapidly proliferating in culture, which require ATP, reducing equivalents and biosynthetic intermediates to grow and divide[138].

A schematic representation of stem cell fate choices and metabolic phenotypes is shown in **Figure 16**. In response to stimuli and cues, stem cells can enter a state of proliferation or self-renewal, where they maintain a high glycolytic flux to support biosynthesis. Cells may also be directed to terminally differentiate, where glucose oxidation supports energy generation in mitochondria. Alternatively, cells may undergo programmed cell death or exit the cell cycle and remain quiescent, a state where the relative metabolic fluxes are largely uncharacterized.

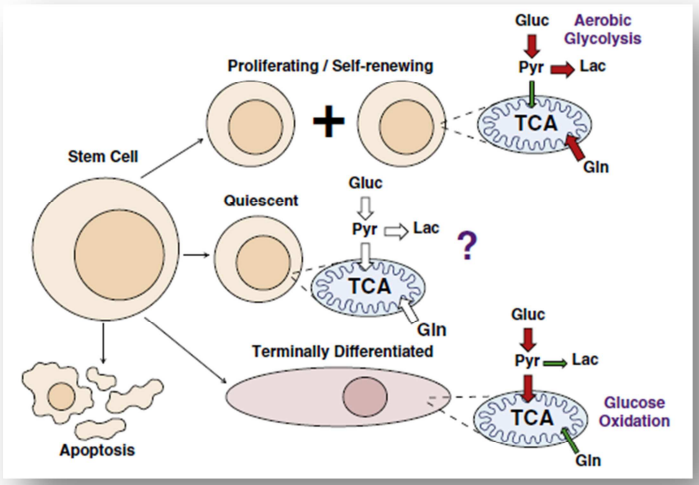


Figure 16.
Schematic representation of stem cell fate choices and metabolic phenotypes.
(adapted from Vacanti et al.[139])

All these indications are important to improve our understanding of metabolic events underlying stemness, but fail to clarify if prolonged exposure to hyperglycaemic conditions affects or impairs all cellular processes related to normal cell metabolism. This fact is of particular relevance in order to establish optimal culture conditions for multipotent cells designed for Regenerative Medicine applications.

As an energetic fuel *in vitro*, glucose is actually a fundamental component of all commercial culture media, so that they are chosen on the basis of glucose content, with regard to single stem cell type requirement. For example, high glucose containing media are generally used for maintenance and proliferation of embryonic stem cells, even if some group pointed out that high glucose concentration isn't necessary for embryoid bodies development[55].

Unfortunately, literature reports controversial evidence about high glucose requirement of adult stem cells. In some cases, a positive role for hyperglycaemia in sustaining cell proliferation and differentiation potential is emphasized[140], while other groups underline a detrimental effect of high glucose concentration on stem cell division, viability, multilineage commitment potential and aging[141-144].

High glucose is not only a component of growth media, but it is also present in media for several different differentiation protocols. From this point of view, a real proof of its active action as an inducing or differentiating agent hasn't been shown yet. Anyway, many consider it as an "assisting" agent to all other classic differentiating agents, such as cytokines and growth factors.

Aim of the study

In the second part of this thesis we aimed to stress the effects of prolonged exposure to hyperglycaemic conditions on cultured mesenchymal multipotent placenta-derived cells. Our interest was dedicated to characterize proliferation rate and survival properties when compared to those of mesenchymal placental cells maintained in normoglycaemic medium. Through definition of cell behaviour in these two different conditions we wanted to deepen previous notions about optimal culture techniques, which can guarantee a successful application of these cells in Regenerative Medicine.

Moreover, we aimed to compare mesenchymal multipotent cells from healthy and diabetic donors, in order to point out eventual discrepancies in cellular responses. This aspect may lead to the discover of new sources of multipotent cells, given that cells from a diseases subject have never been employed in tissue engineering protocols before, for allotransplantations either.

In the third and last part of this thesis, mesenchymal multipotent cells from healthy donor (PLN17) will be finally tested for their differentiation potential towards endothelial lineage. Influence of hyperglycaemia on differentiation will be also verified.

Results and discussion

Effects of prolonged exposure to hyperglycaemic conditions on cultured mesenchymal multipotent placenta-derived cells, both from healthy (PLN17) and diabetic donor (PLD09), were tested through creation of several growth curves. As described in Materials and Methods, cell number at different time points was estimated with Resazurin assay.

It is important to underline that glucose concentrations chosen to mimic normo- and hyperglycaemic states are comparable to those observed *in vivo*. As a matter of fact, sustained hyperglycaemia in cell culture has frequently been used as an *in vitro* model system to mimic diabetes or diabetes-like conditions. Glucose concentration of 25mM used in these studies can realistically be observed in uncontrolled diabetic pregnancies. In healthy individuals venous plasma glucose concentration remains between 60 and 90mg/dl (3.3 and 5.0mM) after overnight fasting and only transiently increases to 120 and 130mg/dl (6.7 and 7.2mM) after a mixed meal[120].

Glucose, given its hydrophilic nature, does not freely cross cell membranes but enters a cell via specific membrane bound transport proteins that permit its facilitated uptake. Several different isoforms of facilitated glucose transporter family members (GLUT) have been found, each with a tissue-specific distribution[145, 146]. From this point of view, our placenta-derived cells, both PLN17 and PLD09, have shown to express GLUT1 and, at a lower level, GLUT3 mRNA when subjected to Real-Time PCR amplification (data not shown). GLUT4 isoform mRNA was also amplified, but its expression was only detectable after several amplification cycles (data not shown).
Expression of GLUT transporters in human placenta were first reported by Bell et al.[147], who noted the presence of mRNA coding for GLUT1 in samples extracted from whole placenta. GLUT1 is a ubiquitous isoform expressed in almost all tissues and regarded as the constitutive form of the transporter. In placenta, distribution of GLUT1 mRNA examined by in situ hybridization demonstrated localization primarily to the syncytiotrophoblast, with lesser quantities found in placental vascular endothelium[148].
In situ hybridization studies also demonstrated that while GLUT3 mRNA was much less abundant than that for GLUT1, it was distributed throughout placental villous tissue[148].

Although GLUT3 mRNA was widely distributed, GLUT3 protein appeared to be expressed only in the vascular endothelium, while lack of its expression in the syncytiotrophoblast layer was pointed out[149].

Many studies have failed to detect significant expression levels of the insulin-responsive GLUT4 isoform in placenta[150-152]. However, Xing et al.[153] demonstrated a GLUT4 signal in intravillous stromal cells of human term placenta that co-localized with insulin receptors, which may suggest that placental glucose metabolism could be stimulated by foetal insulin. It is clear that many of the earlier studies examining insulin effects on placental glucose transport were designed for eliciting responses similar to those observed in well-characterized insulin-responsive target tissues, such as muscle or adipose tissue. As we know now, rapid translocation of GLUT4 from cytoplasmic vesicles to membrane in response to insulin is primarily responsible for the rapid upregulation of glucose transport activity observed in these tissues. In the placenta there is no syncytial GLUT4 to cause such fast response to insulin signal, therefore we can hypothesize that any insulin response from placental cells would take longer time to assess.

On the basis of this evidence and starting from the assumption that our cells would be subjected to long time treatments, we decided to set insulin amount in maintenance medium to that suggested for stem cells, which corresponds to a pharmacological concentration (insulin 10mg/L = 1.72µM).

Growth curves in chronic normo- and hyperglycaemic conditions are shown in **Figure 17**.

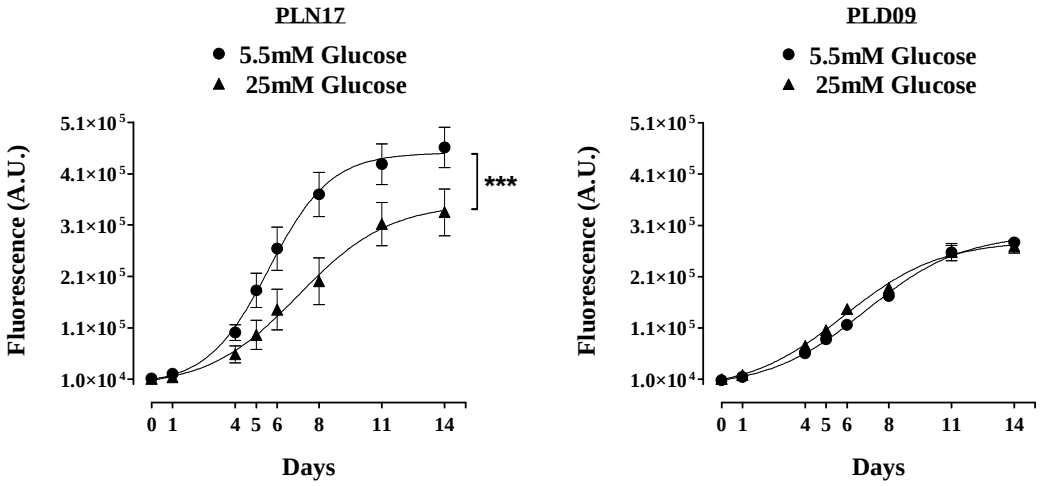


Figure 17.
PLN17 and PLD09 growth curves in chronic normo- and hyperglycaemic culture conditions
Cells growth curves were obtained with Resazzurin assay as described in Materials and Methods.
Briefly, PLN17 and PLD09 were seeded on 96-well plate at the starting density of 1000 cells/well and both cell types were chronically incubated respectively in 5.5mM (LOW) and 25mM (HIGH) glucose medium. Cell number was estimated at defined time points through relevation of Resazurin fluorescence. At the end of experiment, growth curves were analysed with nonlinear regression and fitted to Boltzman sigmoidal curve. Points represent mean ± SEM of 7 independent experiments for PLN17 and 12 independent experiments for PLD09. Statistical analysis was calculated comparing curves with one-way analysis of variance (ANOVA) followed by Bonferroni post test, assuming that distribution of data approximates normal/Gaussian one.
*** $p < 0.001$ *vs.* control (5.5mM glucose, LOW)

The first and most important evidence underlined by these series of experiments was that prolonged incubation in presence of high glucose deeply reduced proliferation potential of placental cells from healthy donor. Comprehensive PLN17 growth curve showed a decrease in cell number starting from fourth day in hyperglycaemic medium after seeding on 96-well plate. This reduction was particularly consistent during exponential growth phase, so that at plateau phase cell were extremely less in number if compared to cells always kept in normoglycaemic medium. It was also clear that the observed discrepancy in cell growth was not caused by a delay due to a prolonged initial lag phase, given that each point on one curve perfectly paralleled the corresponding one on the other curve.

On the contrary, in the case of placental cells from a diabetic patient PLD09, no evident effect of high glucose chronic treatment was noticeable. Curiously, for both curves at each considered time point Resazurin fluorescence level was lower than the that of PLN17 cells.

As described in Materials and Methods, PLN17 and PLD09 growth was also tested after "switch" of glucose concentration. Results are reported in **Figure 18**.

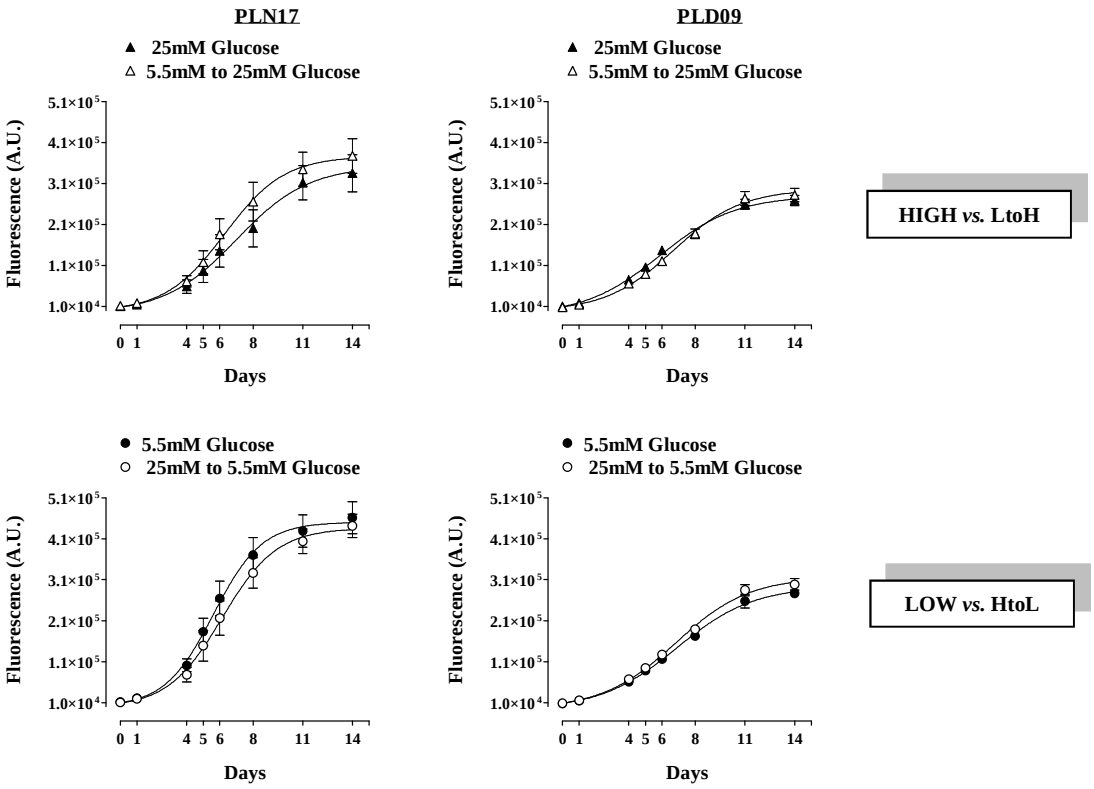


Figure 18.
PLN17 and PLD09 growth curves in conditions of glucose concentration "switch"
Cells growth curves were obtained with Resazzurin assay as described in Materials and Methods.
Briefly, after a first week of incubation in 5.5mM (LOW) and 25mM (HIGH) glucose medium, PLN17 and PLD09 were seeded on 96-well plate at the starting density of 1000 cells/well. For both cell types, glucose concentration was switched respectively from 5.5mM to 25mM glucose (LtoH) and from 25mM to 5.5mM glucose (HtoL). Cell number was estimated at defined time points through relevation of Resazurin fluorescence. At the end of experiment, growth curves were analysed with nonlinear regression and fitted to Boltzman sigmoidal curve. Points represent mean ± SEM of 7 independent experiments for PLN17 and 12 independent experiments for PLD09. Statistical analysis was calculated comparing curves with one-way analysis of variance (ANOVA) followed by Bonferroni post test, assuming that distribution of data approximates normal/Gaussian one.

These experiments pointed out that there was no appreciable difference between treatments for both cell types. When cells were "switched" to high glucose medium (HIGH vs. LtoH), positive effects of previous conditioning week in normoglycaemic conditions were immediately counterbalanced by detrimental effects of hyperglycaemia. In the same way, first week of conditioning in high glucose medium seemed to have no prolonged negative effects on cell growth after "switch" to low glucose concentration (LOW vs. HtoL).

The latter aspect was particularly important with regard to metabolic "memory" phenomenon that was described for other cell types when cultured in hyperglycaemic medium, for example endothelial cells[154]. We hypothesized that placenta-derived cells PLN17 and PLD09 were somehow resistant to high glucose effects in a way that could be proportional to duration of conditioning incubation in hyperglycaemic conditions or to degree of glucose concentration. These hypothesis will be deepened further down by introducing cells sensitive to glucose concentration such as HUVEC as terms of comparison.

First experiments stressed that PLN17 proliferation was severely impaired by prolonged exposure to high glucose, while PLD09 growth didn't seem to be influenced by hyperglycaemic conditions. At that point, we aimed to clarify if negative effects observed were due to glucose itself or to other implications of its presence at high concentration in maintenance medium, such as increased osmolarity. To evaluate these aspects, we repeated the same experiments of growth curves analysis introducing galactose and mannitol as metabolites alternative to glucose.

D-Galactose (**Fig. 19**) is a hexose monosaccharide and glucose epimer. It is not sent to glycolysis prior to conversion in glucose thanks to the action of three enzymes, galactokinase, galactose-1-phosphate uridyltransferase and phosphoglucomutase.

In experiments involving galactose, treatment generally referred to as HIGH indicated a medium containing a final sugars concentration of 25mM, composed by 5.5mM glucose and 19.5mM galactose.

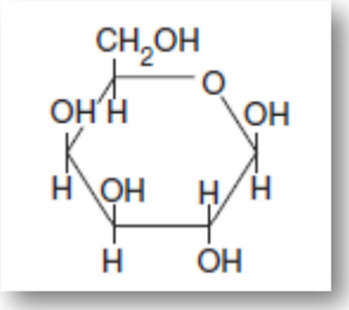


Figure 19.
D – Galactose structure
(Adapted from Garg, Coman, Hales, *Carbohydrate chemistry, biology and medical applications*, Elsevier)

Figure 20 shows PLN17 and PLD09 growth curves in chronic conditions of low glucose or high concentration of sugars. It was immediately evident that glucose replacement with galactose didn't have any influence on cell proliferation for both cell types. As previously pointed out for glucose experiments, the degree of Resazurin fluorescence detected for PLD09 was constantly lower than the one of PLN17.

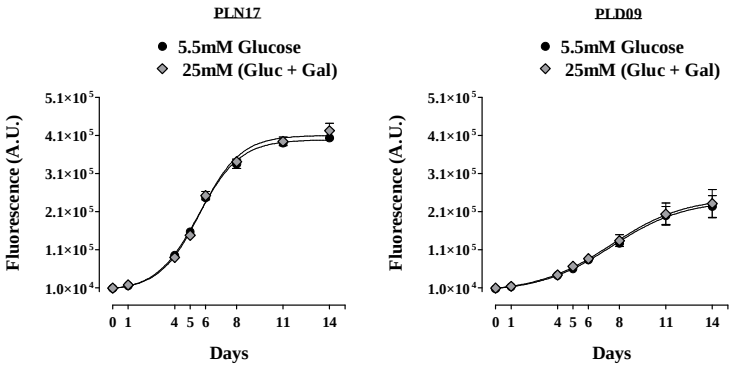


Figure 20.
PLN17 and PLD09 growth curves in presence of Galactose
Cells growth curves were obtained with Resazzurin assay as described in Materials and Methods.
Briefly, PLN17 and PLD09 were seeded on 96-well plate at the starting density of 1000 cells/well and both cell types were chronically incubated respectively in 5.5mM glucose (LOW) and 25mM glucose + galactose medium (5.5mM glucose + 19.5mM galactose, HIGH). Cell number was estimated at defined time points through relevation of Resazurin fluorescence. At the end of experiment, growth curves were analysed with nonlinear regression and fitted to Boltzman sigmoidal curve. Points represent mean ± SEM of 5 independent experiments for PLN17 and 12 independent experiments for PLD09. Statistical analysis was calculated comparing curves with one-way analysis of variance (ANOVA) followed by Bonferroni post test, assuming that distribution of data approximates normal/Gaussian one.

Experiments of sugar concentration "switch" were repeated in presence of galactose both for PLN17 and PLD09 (**Fig. 21**). Results confirmed what just observed. Galactose as energetic fuel instead of glucose had no positive or negative effect on proliferation potential of multipotent placenta-derived cells. Evidence of growth curves analysis in presence of galactose was compatible with a scenario in which glucose was the only responsible of PLN17 proliferation impairment.

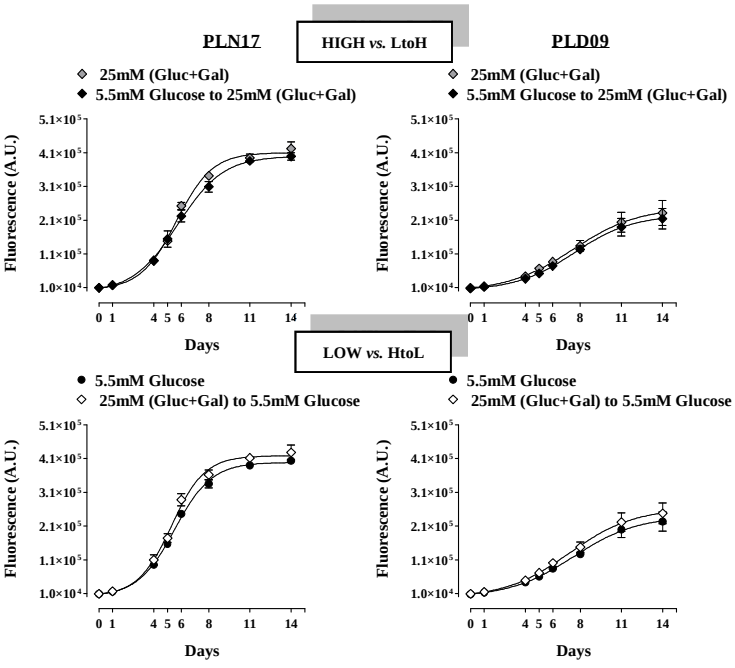


Figure 21.
PLN17 and PLD09 growth curves in conditions of galactose concentration "switch"
Cells growth curves were obtained with Resazzurin assay as described in Materials and Methods.
Briefly, after a first week of incubation in 5.5mM glucose (LOW) and 25mM glucose + galactose medium (5.5mM glucose + 19.5mM galactose, HIGH), PLN17 and PLD09 were seeded on 96-well plate at the starting density of 1000 cells/well. For both cell types, galactose concentration was switched respectively from 5.5mM glucose to 25mM glucose + galactose medium (5.5mM glucose to 25mM gluc+gal, LtoH) and from 25mM glucose + galactose to 5.5mM glucose (25mM gluc+gal to 5.5mM glucose, HtoL). Cell number was estimated at defined time points through relevation of Resazurin fluorescence. At the end of experiment, growth curves were analysed with nonlinear regression and fitted to Boltzman sigmoidal curve. Points represent mean ± SEM of 5 independent experiments for PLN17 and 12 independent experiments for PLD09. Statistical analysis was calculated comparing curves with one-way analysis of variance (ANOVA) followed by Bonferroni post test, assuming that distribution of data approximates normal/Gaussian one.

Thanks to next set of experiments we were able to clarify if observed detrimental effects were caused by increase in medium osmolarity or were consequences of glucose metabolism.

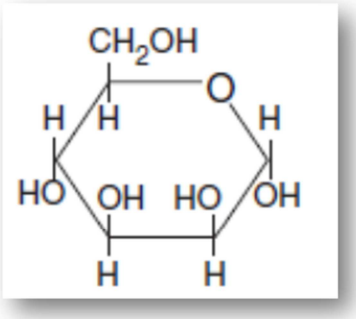


Figure 22.
D – Mannitol structure
(Adapted from Garg, Coman, Hales, *Carbohydrate chemistry, biology and medical applications*, Elsevier)

D-Mannitol is a hexose polyalcohol and a glucose epimer (**Fig. 22**). Mannitol and glucose share a common structure which make them chemically kindred, but cells can only metabolize a small fraction of mannitol present in biological fluids, so that it stays in the extracellular environment contributing to increase osmolarity. Mannitol metabolism is insulin-independent, a feature that makes it different from previously tested sugars.

Before this set of experiments, osmolarity of complete glucose-, galactose- and mannitol-based media was measured in order to emphasize eventual discrepancies between different treatments. Results shown in **Table 4** are expressed as means ± S.D. of 9 independent measures. They stressed that osmolarity of high glucose, galactose and mannitol media were comparable and each of them was significantly different from that of low glucose medium.

	Osmolarity mmol/Kg*
5.5mM Glucose	305.89 ± 2.89
25mM Glucose	338.89 ± 2.16
25mM Galactose (5.5mM glucose + 19.5mM galactose)	338.89 ± 3.06
25mM Mannitol (5.5mM glucose + 19.5mM mannitol)	339.56 ± 2.65

Table 4. Osmolarity determination of complete PLN17 and PLD09 media (* as detected with 5500 Vapor Pressure Osmometer Escor, Delcon)

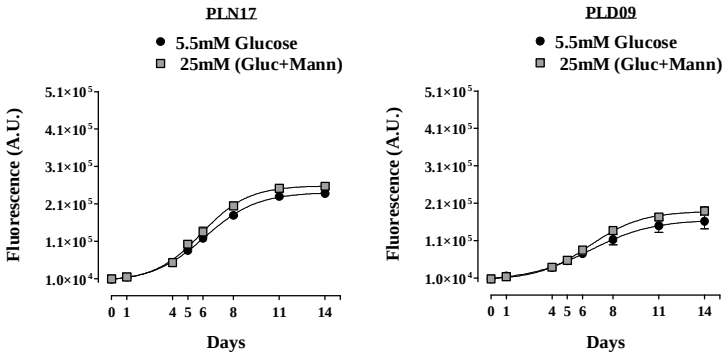


Figure 23.
PLN17 and PLD09 growth curves in presence of Mannitol
Cells growth curves were obtained with Resazzurin assay as described in Materials and Methods.
Briefly, PLN17 and PLD09 were seeded on 96-well plate at the starting density of 1000 cells/well and both cell types were chronically incubated respectively in 5.5mM glucose (LOW) and 25mM glucose + mannitol medium (5.5mM glucose + 19.5mM mannitol, HIGH). Cell number was estimated at defined time points through relevation of Resazurin fluorescence. At the end of experiment, growth curves were analysed with nonlinear regression and fitted to Boltzman sigmoidal curve. Points represent mean ± SEM of 5 independent experiments for PLN17 and 9 independent experiments for PLD09. Statistical analysis was calculated comparing curves with one-way analysis of variance (ANOVA) followed by Bonferroni post test, assuming that distribution of data approximates normal/Gaussian one.

As in the case of galactose experiments, PLN17 and PLD09 proliferation wasn't impaired by mannitol presence in growth medium (**Fig. 23**).

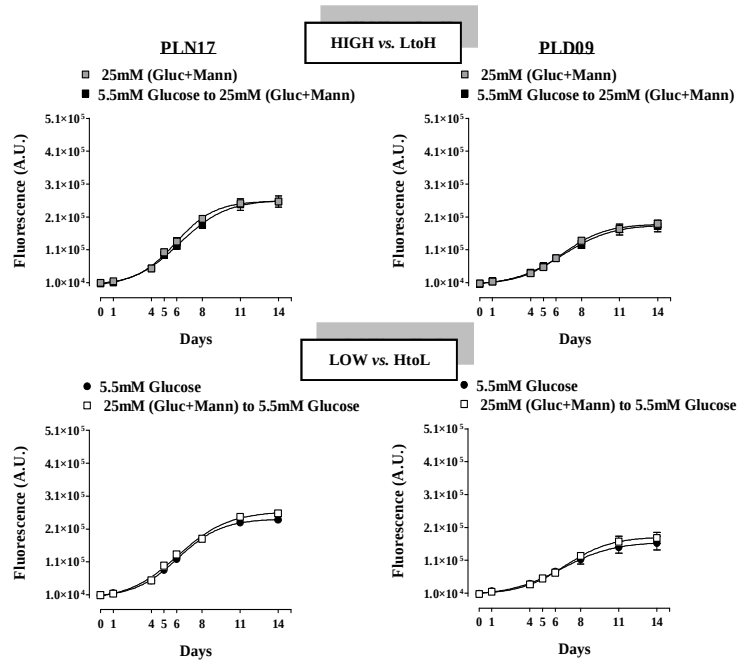


Figure 24.
PLN17 and PLD09 growth curves in conditions of mannitol concentration "switch"
Cells growth curves were obtained with Resazzurin assay as described in Materials and Methods.
Briefly, after a first week of incubation in 5.5mM glucose (LOW) and 25mM glucose + mannitol medium (5.5mM glucose + 19.5mM mannitol, HIGH), PLN17 and PLD09 were seeded on 96-well plate at the starting density of 1000 cells/well. For both cell types, mannitol concentration was switched respectively from 5.5mM glucose to 25mM glucose + mannitol medium (5.5mM glucose to 25mM gluc+mann, LtoH) and from 25mM glucose + mannitol to 5.5mM glucose (25mM gluc+mann to 5.5mM glucose, HtoL). Cell number was estimated at defined time points through relevation of Resazurin fluorescence. At the end of experiment, growth curves were analysed with nonlinear regression and fitted to Boltzman sigmoidal curve. Points represent mean ± SEM of 5 independent experiments for PLN17 and 9 independent experiments for PLD09. Statistical analysis was calculated comparing curves with one-way analysis of variance (ANOVA) followed by Bonferroni post test, assuming that distribution of data approximates normal/Gaussian one.

The same results were obtained when PLN17 and PLD09 were subjected to "switch" of concentration (**Fig. 24**).

The rate of cell proliferation wasn't influenced by any of considered treatments.

Thanks to this evidence we could exclude an involvement of osmolarity in causing reduction of multipotent placenta-derived cells proliferation potential, thus strengthening the hypothesis that only glucose has a negative effect on growth of these cells.

At this point of our discussion it is important to make some specifications about the method we chose to determine cell growth in hyperglycaemic or hyperosmolar state. Resazurin, also known as Alamar Blue, is a redox indicator that measures proliferation quantitatively[155, 156]. The correlation between Resazurin metabolism in the growth medium and the quantity/proliferation of living cells is direct, so that this assay provides an excellent estimation of cell growth. When added to cell cultures, the oxidized form of Resazurin enters the cytosol and is converted to the reduced form Resofurin by mitochondrial enzymes activity. From another point of view, mitochondrial enzymes activity is partly responsible for metabolism of different energy sources contained in growth media, in our case glucose, galactose and mannitol. Given the tight relationship between mitochondrial apparatus and degree of Resazurin reduction, we wondered if employment of such different energy substrates could somehow affect mitochondrial functioning, together with assay responses. To evaluate this hypothesis, for each treatment and for both PLN17 and PLD09, we created standard curves, in which a direct influence of considered substrate on correlation between Resazurin fluorescence and increasing number of cells was searched. Results are summarized in **Figure 25**.

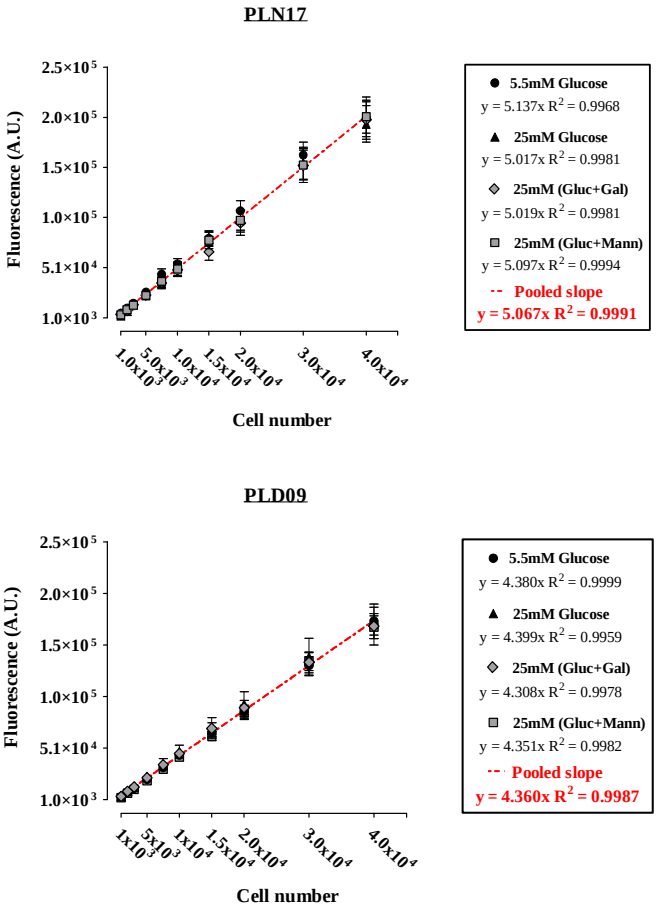


Figure 25.
Validation of Resazurin assay
Resazurin assay as method for the determination of cell growth was validated through creation of standard curves. PLN17 and PLD09 were seeded in 96-well plate at different known numbers of cells and incubated respectively with 5.5mM glucose, 25mM glucose, 25mM glucose + galactose (5.5mM glucose + 19.5mM galactose) and 25mM glucose + mannitol (5.5 glucose + 19.5mM mannitol). Twenty-four hours after seeding cells were incubated with a Resazurin solution as described in Material and Methods and fluorescence was detected. At the end of experiment, curves were fitter for linear regression. Points represent mean ± SEM of 9 independent experiments for both PLN17 and PLD09. Statistical analysis was performed through comparison of slopes so that a pooled slope for both PLN17 and PLD09 was calculated.

We could observe that for both cell types changes in energetic substrate nature didn't affect Resazurin metabolism. This fact was particularly true given that it was possible to calculate for PLN17 and PLD09 equation parameters of a comprehensive standard curve (pooled slope represented in red). Resazurin reduction showed high reproducibility with very low intra- and inter-assay variations. There was also an optimal linear correlation between percentage of Resazurin reduction and increase in cell number, because over a range of $1x10^3$ to $4x10^4$ cells none of R^2 values calculated was lower than 0.99. We could conclude that Resazurin assay was effective in giving true evidence of cell proliferation rate in time, even in the case in which cells were subjected to different treatments. Results were also reproducible, so that a direct comparison between distinct, subsequent experiments was possible.

Validation of Resazurin assay as method to determine cell growth also pointed out that PLN17 and PLD09 differed in metabolism rate of this compound (**Fig. 26**). It was clear that, at the same cell number, PLD09 displayed a reduced ability to convert Resazurin to its fluorescent form if compared to PLN17. This could be the logic explanation for constantly low Resazurin fluorescence observed before for PLD09 in the cases of growth in glucose and galactose containing media. It's meaningful to underline that this fact did not invalidate growth curves analysis in any way, but maked comparison between PLN17 and PLD09 results impossible. For this reason, in our discussion we limited ourselves to analyse effects of hyperglycaemia on proliferation of single cell types, without making any sort of comparison.

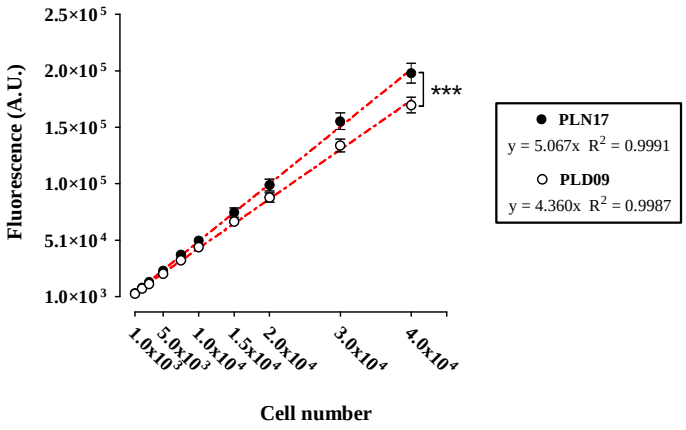


Figure 26.
Validation of Resazurin assay, comparison between PLN17 and PLD09 pooled standard curves
Resazurin assay as method for the determination of cell growth was validated through creation of standard curves. PLN17 and PLD09 were seeded in 96-well plate at different known numbers of cells and incubated respectively with 5.5mM glucose, 25mM glucose, 25mM glucose + galactose (5.5mM glucose + 19.5mM galactose) and 25mM glucose + mannitol (5.5 glucose + 19.5mM mannitol). Twenty-four hours after seeding cells were incubated with a Resazurin solution as described in Material and Methods and fluorescence was detected. At the end of experiment, curves were fitter for linear regression and singular treatments results were pooled in a unique slope both for PLN17 and PLD09. Points represent mean ± SEM of 9 independent experiments for both PLN17 cell types. Statistical analysis was performed through comparison of slopes. *** p <0.001 PLN17 *vs.* PLD09

Previous experiments involving switch of glucose concentration have shed light upon the completely lack of metabolic memory phenomenon that has been several times associated to persistence of high glucose effects, even when normoglycaemic conditions are restored. After having demonstrated that prolonged incubation with high glucose medium resulted in a relevant reduction of proliferation capacities of placental cells from healthy donor (PLN17), we expected to observe that impairment of cell growth would be evident even after cells were incubated again in low glucose medium. On the contrary, cells completely restored their proliferation functionality (HtoL) while, on the other hand, they immediately demonstrated to suffer from detrimental glucose effects when its concentration was increased to mimic hyperglycaemic state (LtoH).

To give an explanation to observed events, we repeated the same analysis of growth curves in low and high glucose medium and after switch of glucose concentration on HUVEC, given that they are generally considered sensitive to variations in glucose amount and that metabolic memory was first described thanks to experiments performed on this kind of cells. Before assessing experiments, osmolarity of complete medium with low or high glucose concentration addition was determined (**Table 5**).

	Osmolarity mmol/Kg*
5.5mM Glucose	271.11 ± 2.71
25mM Glucose	293.20 ± 2.79

Table 5. Osmolarity determination of complete HUVEC media
(* as detected with 5500 Vapor Pressure Osmometer Escor, Delcon)

We could observe that basal osmolarity of 199 medium, commonly used as maintenance medium for HUVEC, was lower than that previously determined for DMEM LOW, the medium we chose to support placenta-derived cells growth in culture. In the same way, the addition of glucose to 199 medium to reach the final concentration of 25mM didn't result in an osmolarity value comparable to that of DMEM HIGH. Despite these observations, we chose basal 199 medium and its hyperosmolar version with increased glucose amount as media to perform our experiments, because they were the most suitable to guarantee optimal viability of cultured endothelial cells. Moreover, as mentioned before, glucose concentration of 25mM could realistically mimic hyperglycaemic conditions as they occur *in vivo*.

Obtained growth curves for HUVEC are shown in **Figure 27**.

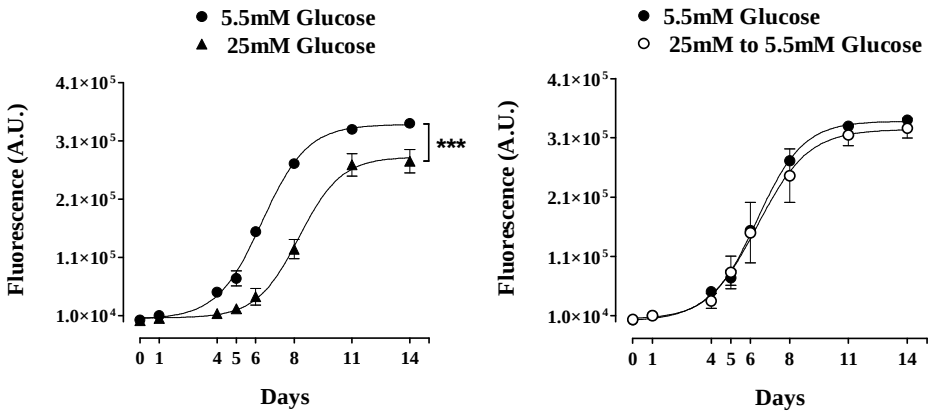


Figure 27.
HUVEC growth curves in chronic normo- and hyperglycaemic culture conditions and after glucose concentration "switch"
Cells growth curves were obtained with Resazzurin assay as described in Materials and Methods.
Briefly, HUVEC were seeded on 96-well plate at the starting density of 1000 cells/well and chronically incubated respectively in 5.5mM (LOW) and 25mM (HIGH) glucose medium (left). In the case of glucose concentration switch, after a first week of incubation in 25mM glucose medium, glucose concentration was switched from 25mM to 5.5mM glucose (HtoL, right). Cell number was estimated at defined time points through relevation of Resazurin fluorescence. At the end of experiment, growth curves were analysed with nonlinear regression and fitted to Boltzman sigmoidal curve. Points represent mean ± SEM of 3 independent experiments. Statistical analysis was calculated comparing curves with one-way analysis of variance (ANOVA) followed by Bonferroni post test, assuming that distribution of data approximates normal/Gaussian one.
*** $p < 0.001$ *vs.* control (5.5mM glucose, LOW)

As expected, we observed a statistically significant reduction of cell proliferation during prolonged exposure to hyperglycaemia. However, also in this case it was impossible to observe the metabolic memory phenomenon, given that switch of glucose concentration from high to low (25mM to 5.5mM, HtoL) restored normal cell proliferation, without any evident persistent effect of previous hyperglycaemic state. Results of glucose switch from low to high concentration showed an impairment of cell growth after medium was changed with high glucose one, thus agreeing in underlining lack of metabolic memory (5.5mM to 25mM, LtoH, data not shown).

These experiments pointed out that chosen high glucose concentration of 25mM was effective in causing marked reduction of proliferative potential for both HUVEC and cells isolated from placenta (PLN17). These effects were only evident for both cell types in the case of chronic incubation (three weeks or more) in hyperglycaemic conditions, while only one week of pre-conditioning with high glucose didn't seem to cause any sort of decay of normal cell growth. We could take additional information about this aspect by repeating experiments performing a longer pre-conditioning period.

In the light of the facts, we could hypothesize that behaviour of placental cells from healthy donor PLN17 when cultured in chronic hyperglycaemia resembles that of high glucose-sensitive cells, such as HUVEC. This equivalence of reactions and behaviour in culture also occurred when glucose concentration was only intermittently changed, as in the case of "switch" experiments. During following tests we dealt with the definition of the precise molecular process responsible for high glucose-caused proliferation impairment.

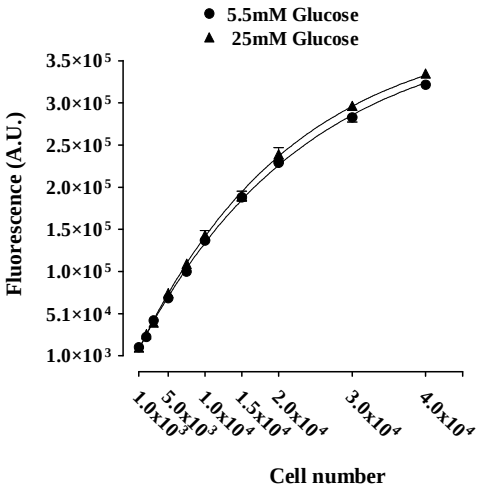


Figure 28.
Validation of Resazurin assay
Resazurin assay as method for the determination of cell growth was validated through creation of standard curves. HUVEC were seeded in 96-well plate at different known numbers of cells and incubated respectively with 5.5mM glucose or 25mM glucose. Twenty-four hours after seeding cells were incubated with a Resazurin solution as described in Material and Methods and fluorescence was detected. At the end of experiment, curves were fitter for exponential growth equation. Points represent mean ± SEM of 3 independent experiments. Statistical analysis was performed through comparison of equation parameters.

In order to validate Resazurin assay as method for determination of HUVEC growth, we repeated standard curves experiments (**Fig. 28**). Curiously, mathematic correlation between Resazurin reduction and increased cell number wasn't linear as previously observed but perfectly fitted an exponential growth curve. Indeed, calculated R^2 values were about 0.99 in the case of growth in both low and high glucose medium. This evidence didn't invalidate use of Resazurin assay, given that a perfect correspondence between cells metabolism of this compound in normo- and hyperglycaemic conditions was clear. Moreover, the exponential relationship between HUVEC cell number and Resazurin reduction was already reported by other groups[157].

After having demonstrated that glucose at high concentration is the only responsible for reduction of both PLN17 and HUVEC proliferative potential, we aimed to clarify the molecular mechanism underlying these events. As explained in the introductive section, many studies identified the increase in cellular oxidative stress state as the molecular link between hyperglycaemia and its detrimental effects. They demonstrated that the augmentation of glucose amount into glycolytic flux and of its metabolites into Krebs cycle were frequently associated to the increase in reactive oxygen species production (ROS), both of mitochondrial and non-mitochondrial origin. Based on these observations, we hypothesized that an alteration in oxidative stress state of both PLN17 and HUVEC could occur as direct consequence of prolonged incubation in hyperglycaemic conditions, thus giving the explanation for observed proliferation impairment. To validate this hypothesis, we evaluated cellular response in the case of addition to medium of the potent antioxidant N-Acetyl Cysteine (NAC). NAC was added to high glucose medium in order to counterbalance hypothesized glucose induction of oxidative stress. Placental cells from diabetic donor PLD09 were also included into experiments. In a following series of tests, reactive oxygen species production was directly evaluated through fluorimetric assay.

Cell growth in presence of NAC antioxidant was compared to that of cells incubated in hyperglycaemic medium (**Fig. 29**). PLN17 and PLD09 growth didn't show to be influenced by NAC presence inside medium. We could affirm that NAC had no positive role in restoring proliferation rate, given that growth curves obtained by incubation in high glucose medium and in high glucose medium with NAC addition were comparable.

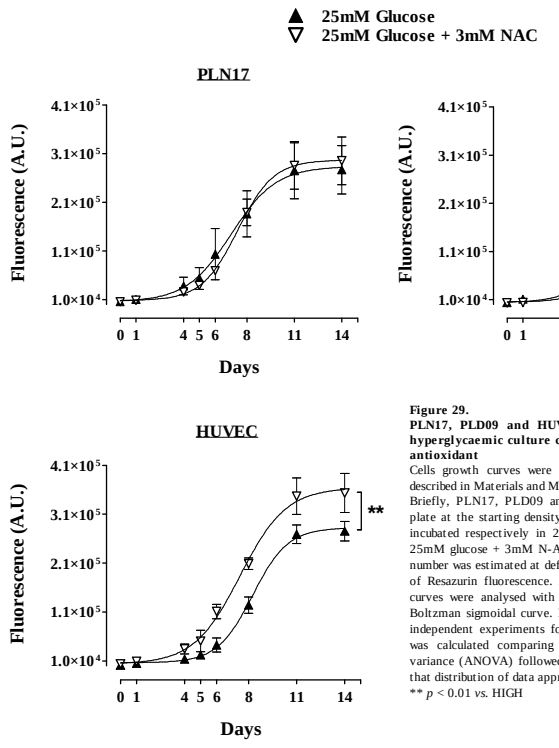
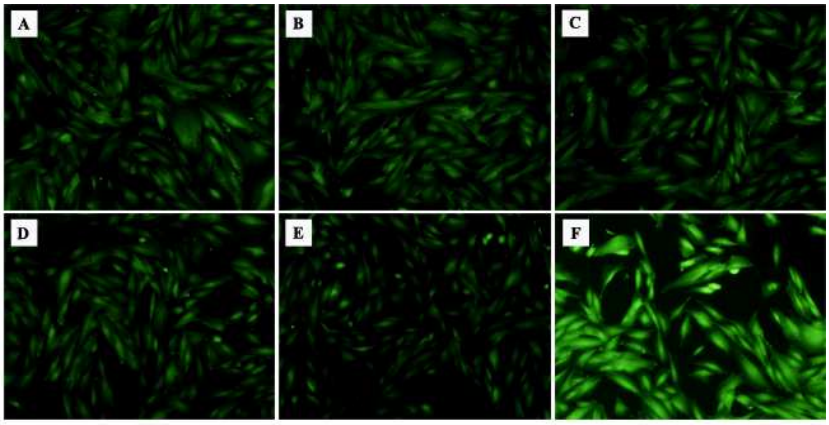


Figure 29.
PLN17, PLD09 and HUVEC growth curves in chronic hyperglycaemic culture conditions and in presence of an antioxidant
Cells growth curves were obtained with Resazzurin assay as described in Materials and Methods.
Briefly, PLN17, PLD09 and HUVEC were seeded on 96-well plate at the starting density of 1000 cells/well and chronically incubated respectively in 25mM glucose medium (HIGH) and 25mM glucose + 3mM N-Acetyl cysteine medium (NAC). Cell number was estimated at defined time points through relevation of Resazurin fluorescence. At the end of experiment, growth curves were analysed with nonlinear regression and fitted to Boltzman sigmoidal curve. Points represent mean ± SEM of 3 independent experiments for all cell types. Statistical analysis was calculated comparing curves with one-way analysis of variance (ANOVA) followed by Bonferroni post test, assuming that distribution of data approximates normal/Gaussian one.
** $p < 0.01$ *vs.* HIGH

These results excluded a direct involvement of oxidative stress in proliferation impairment, but didn't helped us to clarify the role of hyperglycaemia.
HUVEC showed a completely different situation. In this case, NAC presence in growth medium restored proliferative potential, thus suggesting that hyperglycaemia-induced oxidative stress could be the molecular mechanism through which high glucose exerted its action relatively to HUVEC. All in all, we observed the first discrepancy between PLN17 and HUVEC behaviour in culture. As expected, reduction of HUVEC proliferation rate in presence of high glucose could be referred to an increase in cellular oxidative stress, as a direct consequence of long term expose to hyperglycaemia. On the contrary, NAC failed to restore normal PLN17 growth, suggesting that molecular mechanisms of hyperglycaemic damage weren't related to ROS overproduction and impairment of cells redox state.
Results for PLD09 were coherent with what observed so far: given that this cell type didn't show an impairment of normal growth in hyperglycaemic conditions, the lacking occurrence of increase in oxidative stress state could be considered plausible.

Determination of reactive oxygen species production was performed as an ulterior confirmation of what so far hypothesized. As described in Materials and Methods, this test was performed thanks to a fluorimetric assay based on mitochondrial and non-mitochondrial ROS detection from CM-H_2DCFDA dye. PLN17, PLD09 and HUVEC were tested not only in chronic normo- and hyperglycaemic conditions, but also when subjected to glucose concentration switch or cultured in presence of NAC antioxidant. For all experiments, positive controls for ROS production were created by incubating cells with H_2O_2.

Figure 30 and **Figure 31** report results obtained for PLN17 and PLD09 cells.



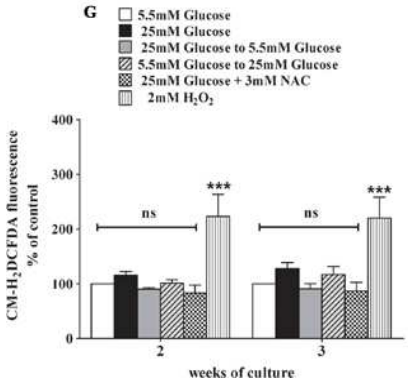
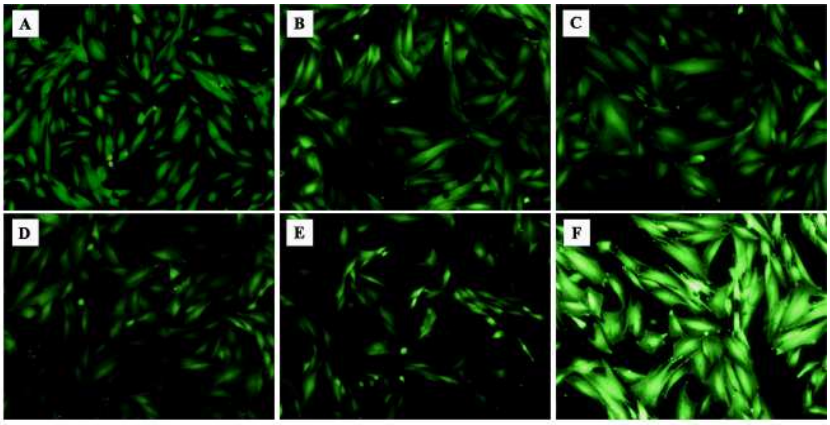


Figure 30.
Evaluation of Ros production by PLN17 with CM-H_2DCFDA fluorescent dye
PLN17 were cultured in LOW (5.5mM glucose, ***A***) or HIGH (25mM glucose, ***B***) glucose for 3 weeks, while for HtoL (25mM glucose to 5.5mM glucose, ***C***) and LtoH (5.5mM glucose to 25mM glucose, ***D***) cells were grown respectively with 25mM or 5.5mM glucose for the first week, then in 5.5mM or 25mM glucose medium for the last 2 weeks. To evaluate the effect of an antioxidant, PLN17 were also treated with HIGH glucose medium with addition of N-acetyl cysteine (25mM glucose + 3mM NAC, ***E***) for 3 weeks. Cells incubated with 2mM H_2O_2 for 20 min were employed as positive control for Ros production (***F***).
CM-H_2DCFDA assay was performed as described in Materials and Methods, for each experimental condition Ros detection was accomplished twice, after 2 or 3 weeks of culture respectively. Images of representative microscopic fields (100x, 3 weeks incubation) are shown. (***G***), Fluorescence of each experimental condition was measured in quadruplicate, data are expressed as percentage of fluorescence of control condition (5.5mM glucose). Bars represent mean ± SEM of 5 independent experiments.
*** p <0.001 *vs.* control, as obtained with two way Anova analysis followed by Bonferroni post test.



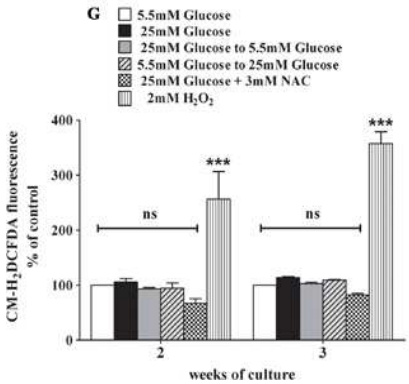


Figure 31.
Evaluation of Ros production by PLD09 with CM-H_2DCFDA fluorescent dye
PLD09 were cultured in LOW (5.5mM glucose, ***A***) or HIGH (25mM glucose, ***B***) glucose for 3 weeks, while for HtoL (25mM glucose to 5.5mM glucose, ***C***) and LtoH (5.5mM glucose to 25mM glucose, ***D***) cells were grown respectively with 25mM or 5.5mM glucose for the first week, then in 5.5mM or 25mM glucose medium for the last 2 weeks. To evaluate the effect of an antioxidant, PLD09 were also treated with HIGH glucose medium with addition of N-acetyl cysteine (25mM glucose + 3mM NAC, ***E***) for 3 weeks. Cells incubated with 2mM H_2O_2 for 20 min were employed as positive control for Ros production (***F***).
CM-H_2DCFDA assay was performed as described in Materials and Methods, for each experimental condition Ros detection was accomplished twice, after 2 or 3 weeks of culture respectively. Images of representative microscopic fields (100x, 3 weeks incubation) are shown. (***G***), Fluorescence of each experimental condition was measured in quadruplicate, data are expressed as percentage of fluorescence of control condition (5.5mM glucose). Bars represent mean ± SEM of 4 independent experiments.
*** p <0.001 *vs.* control, as obtained with two way Anova analysis followed by Bonferroni post test.

PLN17 and PLD09 cells shared similar results. Cell pictures gave a direct, macroscopic confirmation of this fact. Looking at them, it was immediately clear that no relevant differences between treatments and cell types were pointed out by fluorimetric assay. Effectiveness of considered assay in detecting intracellular ROS was confirmed by positivity shown by controls treated with the oxidative agent H_2O_2.

As suggested by previous observations and shown by graphs, it wasn't possible to detect any increase in ROS production by cells kept in hyperglycaemic medium, both after two and three weeks of culture. This evidence was expected for PLD09, as they didn't show any negative influence by high glucose concentration. On the other hand, it gave the final proof that the impairment of PLN17 proliferative potential wasn't due to ROS hyperglycaemic-induced overproduction, as in contrast suggested for many other cells types. This hypothesis was particularly reliable in the light of results achieved for HUVEC (**Fig. 32**).

For these cells, a marked increase in oxidative stress level in the case of prolonged incubation in high glucose medium was clearly detectable, thus giving an explanation to observed cell growth impairment. A further confirmation of oxidative stress involvement was given by statistically significant difference detected between ROS production in high glucose medium and in high glucose with the addition of NAC. NAC reduced oxidative stress at a level which made restoration of cell growth possible. These observations completely confuted our previous hypothesis about the equivalence between PLN17 and HUVEC behaviour in culture.

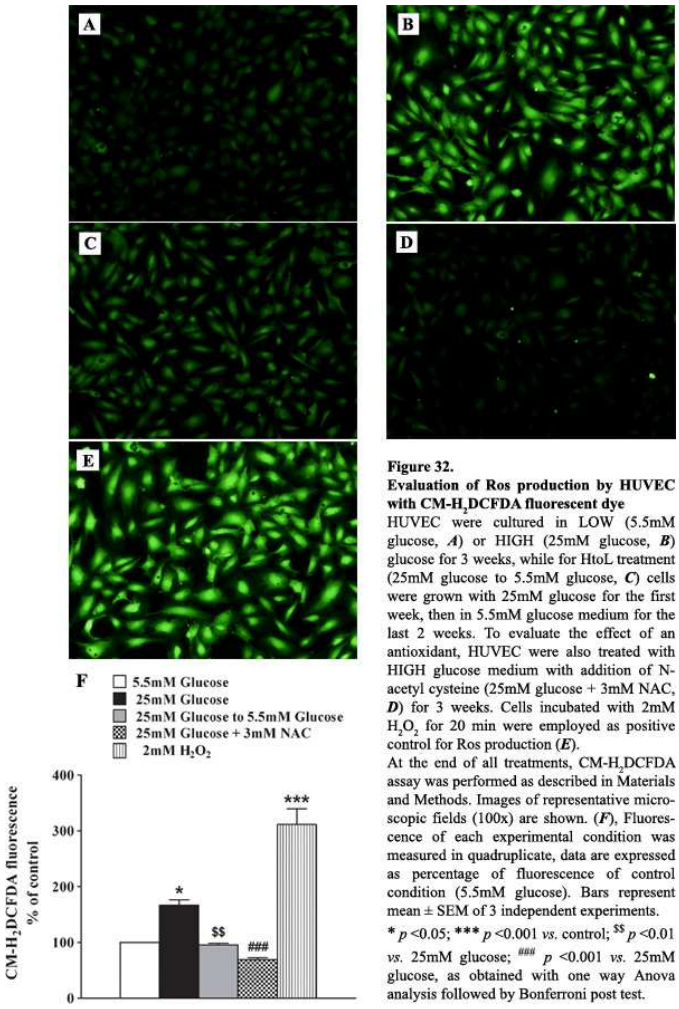


Figure 32.
Evaluation of Ros production by HUVEC with CM-H_2DCFDA fluorescent dye
HUVEC were cultured in LOW (5.5mM glucose, ***A***) or HIGH (25mM glucose, ***B***) glucose for 3 weeks, while for HtoL treatment (25mM glucose to 5.5mM glucose, ***C***) cells were grown with 25mM glucose for the first week, then in 5.5mM glucose medium for the last 2 weeks. To evaluate the effect of an antioxidant, HUVEC were also treated with HIGH glucose medium with addition of N-acetyl cysteine (25mM glucose + 3mM NAC, ***D***) for 3 weeks. Cells incubated with 2mM H_2O_2 for 20 min were employed as positive control for Ros production (***E***).
At the end of all treatments, CM-H_2DCFDA assay was performed as described in Materials and Methods. Images of representative microscopic fields (100x) are shown. (***F***), Fluorescence of each experimental condition was measured in quadruplicate, data are expressed as percentage of fluorescence of control condition (5.5mM glucose). Bars represent mean ± SEM of 3 independent experiments.
* p <0.05; *** p <0.001 *vs.* control; $^{\$\$}$ p <0.01 *vs.* 25mM glucose; $^{\#\#\#}$ p <0.001 *vs.* 25mM glucose, as obtained with one way Anova analysis followed by Bonferroni post test.

In hyperglycaemic conditions, PLN17 didn't behave as high-glucose sensitive cells, given that it wasn't possible to establish a direct correlation between impairment of proliferation rate and hyperglycaemia-induced increase of oxidative stress. On the contrary, it was evident that PLN17 were somehow resistant to ROS overproduction in response to increased glycolytic flux and glucose metabolic degradation. Our attempts to clarify the nature of molecular mechanisms underlying high glucose-induced reduction of placental cells growth will be discussed in following experiments.

Present experiments about determination of ROS overproduction were also effective in giving explanation to lack of metabolic memory phenomenon. As pointed out by graphs for PLN17 and PLD09, "switch" culture conditions didn't show a level of oxidative stress appreciably higher than chronic treatments. This fact was easily comprehensible, given that we just proved that, for placental cells, hyperglycaemic conditions weren't responsible for ROS overproduction related to persistence of high glucose-induced damage. On the other hand, treatment of HUVEC with high glucose medium followed by decrease of substrate concentration (HtoL) showed a statistically significant reduction of oxidative stress level if compared to that of chronic high glucose treatment. This lack of persistence of hyperglycaemic effects was assumed to be the right explanation for metabolic memory failure observed in HUVEC.

The final confirmation of absence of oxidative stress involvement in impairing placenta cells growth came from expression analysis of HMOX1, a gene related to cellular response to oxidative stress (**Fig. 33**). HMOX1 protein functions as an antioxidant enzyme playing a pivotal role in maintenance of cellular homeostasis in response to oxidative stress[158]. HMOX1 is inducible by several different agents whose common feature is to alter redox state of cells by increasing ROS production and/or modifying glutathione levels. It is also well-known that the principal mechanism through which oxidant inducers regulate HMOX1 activity is modulation of gene transcription[159]. For this reason, eventual variations of HMOX1 expression between normo- and hyperglycaemic treatment could be considered as an index of cell response to increased oxidative stress.

Gene expression analysis didn't show induction of HMOX1 transcription when placenta-derived cells were chronically cultured in high glucose medium, whether after two or three weeks of culture. This evidence was compatible with a scenario in which cell redox state wasn't altered by prolonged hyperglycaemic condition. A marked increase of gene expression was only detectable when cells were treated with H_2O_2 to create positive controls. These observations contributed to confirm our hypothesis about the absence of oxidative stress involvement in hyperglycaemia-induced proliferation impairment.

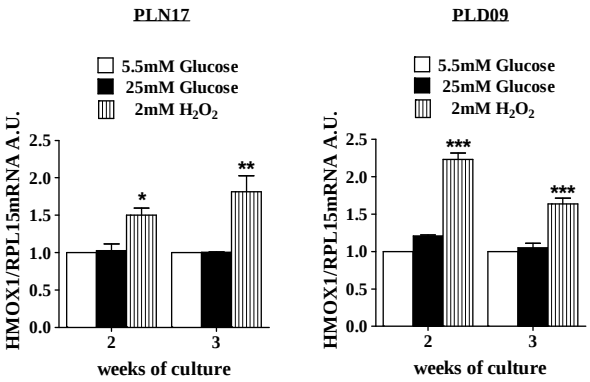


Figure 33.
Real-time PCR analysis of Heme oxygenase-1 expression
PLN17 and PLD09 were cultured in LOW (5.5mM) or HIGH (25mM) glucose medium for 2 or 3 weeks. Positive controls for oxidative stress induction were realized incubating both cell types for 20 min with 2mM H_2O_2. After RNA extraction and reverse transcription, cDNA was employed as template for Real-time PCR with HMOX1 primers. Gene expression was normalized for RPL15 housekeeping gene and calculated using ΔΔCt method as fold change relatively to control condition (5.5mM glucose; =1). Data are expressed as mean ± SEM of 3 independent experiments, each performed in duplicate. *p <0.05; ** p <0.01; *** p <0.001 *vs.* control, as obtained with two way Anova followed by Bonferroni post test.

After having demonstrated that placental cells PLN17 and HUVEC didn't share a common response to hyperglycaemia in culture, we decided to focus on the definition of mechanism through which chronic exposure to high glucose affected proliferation of placenta-derived cells. On the other hand, we aimed to elucidate molecular determinants responsible for observed discrepancies between PLN17 and PLD09 behaviour, when cultured in high glucose medium for long time.

It's important to underline that a total three weeks duration for last and following experiments was chosen in order to reproduce exact duration of growth curves experiments (one week pre-conditioning followed by two weeks of culture maintenance), thus allowing an eventual parallelism of results between different experiments.

On the basis of last and previous evidence, we first hypothesized that observed impairment of placenta-derived cells proliferation wasn't due to side aspects of augmented glucose metabolism, rather to reduced viability of cells during long time incubation. In fact, it is widely known that hyperglycaemia induces apoptosis of some cell types. In this case, hyperglycaemia shouldn't be considered a classical damaging agent because of its role in perturbing cellular metabolic state, but as a culture condition first of all capable of impairing cell survival properties and, gradually, of causing cell death if prolonged over a certain period of time. To test this idea, we performed a LDH measurement assay as described in Materials and Methods, in order to verify the occurrence of hyperglycaemia-induced impairment of cell viability and to evaluate its evolution at different time points during long term culture. If the hypothesis about the increase of cell death in high glucose medium was confirmed, we would expect to detect a significantly higher amount of released LDH for hyperglycaemia-treated cells respect to normoglycaemia-incubated ones. PLN17 and PLD09 were both subjected to this assay, results are shown in **Figure 34**.

Graphs demonstrated that the level of released LDH wasn't significantly different between normo- and hyperglycaemic treatments, whether after two or three weeks of culture. Results were also coherent between PLN17 and PLD09. This observation was expected for PLD09, given that in previous experiments a relevant reduction of cell number hasn't ever been determined for this cell type. For PLN17 the same observation contributed to exclude any possible action of hyperglycaemia on viability and survival properties, suggesting that molecular mechanism through which it exerts its action lies outside of both cell metabolism and cell choice between life and death. Further analysis were required to elucidate mechanism of action of hyperglycaemia on placenta-derived cells.

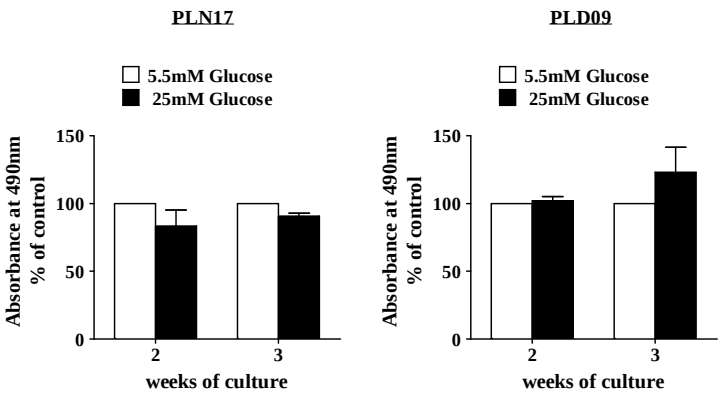


Figure 34.
Evaluation of cell death with LDH assay
PLN17 and PLD09 were cultured in LOW (5.5mM) or HIGH (25mM) glucose for 2 or 3 weeks. For both experimental conditions and cell types, cell death was assessed performing LHD assay as described in Material and Methods. The measurement was repeated twice, after 2 or 3 weeks of culture respectively. Absorbance of both control (5.5mM glucose) and treatment (25mM glucose) was detected in quadruplicate, data are expressed as percentage of absorbance of control condition. Bars represent mean ± SEM of 3 independent experiments. Statistical analysis with two way Anova followed by Bonferroni post test pointed out no relevant difference between control and treatment for both cell types.

Thanks to previous test we clarified that observed impairment of placenta-derived cells proliferation wasn't due to the reduction of living cells, thus suggesting that it could be a matter of decrease of number of actively proliferating cells. In our attempts to discover hyperglycaemia action at a molecular level, we focused to analyse pattern of expression of some proteins involved in regulation of cell cycle. We chose in particular p15 and p21 proteins because of their well-known role as controllers of cell cycle checkpoints in response to several different stimuli.

Both $p15^{INK4b}$ and $p21^{waf1}$ belong to the family of cyclin-dependent kinase inhibitors (CKIs) and play a crucial role in the regulation of the cell cycle in non-transformed cells. They are implicated in suppression of cell proliferation under stress conditions caused for example by growth factor deficiency, DNA damage, heat shock and exposure to heavy metals or antiproliferative cytokines.

p15 regulates progression through the G_1 phase of the cell cycle: by interacting with CDK4 and CDK6 it prevents complex formation between these kinases and D-type cyclins.

p21 was identified as a protein suppressing cyclinE/A-CDK2 activity and was originally considered as a negative regulator of cell cycle and a tumour suppressor. It is now considered that p21 has alternative functions, all of them involved in regulation of fundamental cellular programs: cell proliferation, differentiation, migration, senescence, and apoptosis[160].

Their synergistic role in regulating progression or arrest of cell cycle has been clearly demonstrated in some kind of tumours in which suppression of their expression was associated to lack of control of cell cycle passage through checkpoints, thus causing uncontrolled cell growth[161-163].

Here we investigated p15 and p21 proteins expression levels in response to prolonged hyperglycaemic treatment through Western blot analysis, in order to determine presence or absence of hyperglycaemic-induced cell cycle detriment.

Figure 35 reports images of a representative blot for both proteins (**A**), together with results of densitometric analysis (**B**). PLN17 showed a statistically significant increase of p15 protein expression, both after two and three weeks of culture. The statistically significant enhancement of protein level was achieved also for p21. On the whole, starting from these observations we could deduce that treating placenta-derived cells from healthy donor (PLN17) with chronic exposure to high glucose concentration resulted in an impairment of cell proliferation almost surely due to arrest of cell cycle progression. We hypothesized that cells reduced their proliferation rate instead of completely exit the cycle. The contemporary marked increment of p15 and p21 protein levels in fact was compatible with a scenario in which reduction of proliferation potential occurred as a consequence of arrest in G_1 phase, rather than in G_0 one.

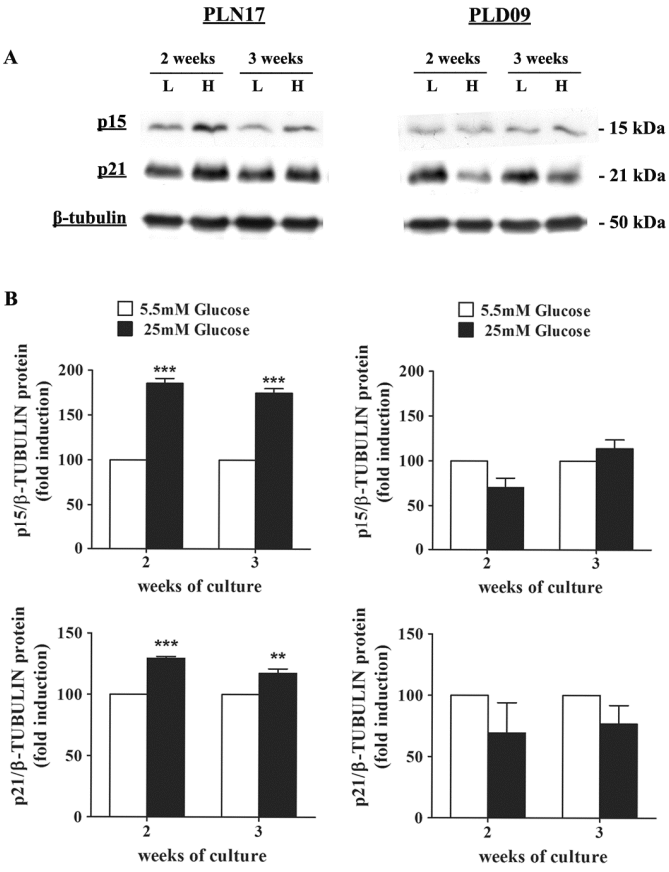


Figure 35.
Western Blot analysis of proteins involved in cell cycle arrest
PLN17 and PLD09 were cultured in LOW (5.5mM) or HIGH (25mM) glucose medium for 2 or 3 weeks. At the end of treatment, cells were lysed as described in Materials and Methods and cell lysates were analyzed for the expression of p15 and p21 proteins. β-tubulin was chosen as loading control. (***A***), images from a representative blot; (***B***), densitometric analysis, data are expressed as means ± SEM of 3 independent experiments. ** p <0.01; *** p <0.001 *vs.* control (5.5mM glucose, =100), as obtained with two way Anova followed by Bonferroni post test.

PLD09 results needed a different discussion. We observed that neither p15 nor p21 showed an increase of protein level for whole experiments duration. On the contrary, a little reduction was detectable for p21, while p15 protein expression after three weeks of culture in hyperglycaemic conditions was comparable to that of control cells. Curiously, blot images showed that p21 protein was much more expressed by normoglycaemic-treated cells both after two and three weeks of culture, thus suggesting that a certain impairment of cell cycle progression was taking place. These observations excluded the occurrence of cell cycle arrest, thus underlining an evident discrepancy between PLN17 and PLD09 response to hyperglycaemic state.

At this point of our discussion we could only hazard some guesses. The first and easier hypothesis was that long term exposure to high glucose medium had no effects, both positive and negative, on placental cells isolated from diabetic donor (PLD09). This could be the direct consequence of cells provenience from diseased individuals characterized by a general impairment of glucose homeostasis. The incoming idea is that exposure to maternal diabetes could have an epigenetic impact not only on foetus as many studies confirmed, but also on placental cells[164]. Furthermore, perturbations in the maternal compartment may affect the methylation status of placental genes resulting in changes of placental function[165]. In the case of PLD09 cells we hypothesized that observed alteration of culture behaviour, if compared to placental cells from healthy donor, could be due to persistence of Diabetes-caused metabolic abnormalities that these cells underwent during gestation. Unfortunately further analysis were needed to confirm this hypothesis.

Summary

At the end of analysis of high glucose, galactose and mannitol effects on proliferation of placenta-derived cells, we observed a relevant reduction of PLN17 growth in hyperglycaemic conditions. We proved that decrease in proliferative potential wasn't due to hyperosmolarity superimposed by augmented glucose concentration, but most reasonably to increased glucose uptake and metabolism. This hypothesis was confirmed by the fact that when medium osmolarity was augmented by glucose substitution with a virtually metabolically inert substrate such as mannitol, no appreciable effect on cell growth was noticed. As an ulterior confirm of what just affirmed, presence of high galactose concentration inside medium didn't altered cell proliferation. Experiments of "switch" of glucose concentration helped us to deepen our knowledge about cells reaction to glucose amount variations in culture media. Differently from expected, placental cells PLN17 didn't shown the metabolic "memory" phenomenon described as the persistence of high glucose effects on culture cell behaviour even after return to normoglycaemic conditions. In fact, a complete restoration of cell proliferative potential occurred when glucose concentration was lowered, while increase in this substrate concentration resulted in an immediate impairment of cell growth. Given that metabolic memory has frequently been observed for high glucose-sensitive cell types such as endothelial cells, we introduced HUVEC as terms of comparison for placenta cells in order to clarify this aspect. HUVEC showed a relevant reduction of proliferation in hyperglycaemic medium, but failed to give a demonstration of persistence of detrimental high glucose effects in the case of normoglycaemia restoration. Apparently, cultured placenta cells PLN17 behave like high glucose-sensitive endothelial cells relatively to chronic hyperglycaemia and short intermittent periods of incubation with high-glucose.

On the opposite side, placenta-derived cells from diabetic donor PLD09 didn't demonstrate to be affected by persistence of hyperglycaemic or hyperosmolar state: their proliferation rate wasn't altered by high glucose, galactose or mannitol concentrations, neither positively nor negatively.

Following analysis dealt with evaluation of oxidative stress status of PLN17, PLD09 and HUVEC in response to hyperglycaemia. We demonstrated that sustained hyperglycaemia didn't cause a relevant enhancement of reactive oxygen species production by both PLN17 and PLD09. Analysis of HMOX1 gene expression confirmed that these cell types didn't activate mechanisms of protection against increased oxidative stress. On the contrary, ROS overproduction by hyperglycaemia-treated HUVEC was evident. These observations completely confuted our previous hypothesis: PLN17 didn't behave as high-glucose sensitive cells when persistently cultured in high glucose medium.

PLN17 and PLD09 were subsequently tested for evaluation of cell death with LDH assay. The analysis showed that there wasn't a relevant raise of cell death rate even after long exposure to high glucose concentration. In our attempt to discover the molecular link between hyperglycaemia and observed impairment of proliferative potential, we finally analysed placental cells for the expression of proteins involved in progression of cell cycle. These experiments demonstrated that amount of p15 and p21 proteins was significantly higher in PLN17 cells when constantly kept in hyperglycaemic conditions. On the other hand, PLD09 confirmed the lack of detrimental effects on their growth by prolonged high glucose exposure, given that no relevant enhancement of p15 and p21 proteins was detectable.

On the whole, in the second part of this Ph.D thesis we demonstrated that hyperglycaemia acts on placenta-derived cells from healthy donor by considerably impairing their proliferative capacities. The observed reduction of cell growth was caused by hyperglycaemia-induced expression of proteins related to regulation of cell cycle progression. We suggested that these events could concur in beginning of cell differentiation program, as will be further discussed in the last section of this thesis.

Part 3. Mimicking hyperglycaemia in vitro affects cell differentiation

Introduction - Regeneration of endothelial tissue

Application of stem cells to achieve vascular repair and regeneration is a relatively new approach of Regenerative Medicine. As mentioned before, endothelial cells are one of the first targets of hyperglycaemic damage, but these cells are also injured by pathologies apart from Diabetes. This is the case of inflammatory diseases such as atherosclerosis and vasculitis, of neoplastic/metastatic diseases or of pathogen infections. In the majority of cases, if damage extent exceeds reparation capacities of endothelial tissue, it doesn't succeed in healing itself, so that substitution with healthy tissue should be taken into consideration. This is especially true for endothelial tissue, given that it is not endowed with an intrinsic regeneration potential comparable to that of cartilage or bone. From this point of view, when common treatments fail to repair a damage, a complete recovery of tissue functionality can be obtained only through tissue regeneration starting from stem cells therapy approaches. That is the reason why the definition of a commonly recognized experimental strategy to differentiate stem cells towards endothelial lineage is becoming more and more important.

During last decades protocols for regeneration of tissues such as epithelium and bone have enormously progressed, while the field of endothelial tissue regeneration has faced many problems. This delay in achieving concrete results is partly due to complications related to nature and organization of endothelium. First of all, it is a really heterogeneous tissue[166, 167]. Macroscopically, vessels of vascular system can be divided in veins, arteries and capillaries. Moreover, inside all of them, endothelial cells display different specializations related to cell localisation, so that morphological and functional discrepancies are even microscopically evident. Endothelial cells directly regulate vascular function, transport of solutes and antithrombotic properties of the blood-tissue interface[168-170]. This evidence suggests that a univocal phenotype for mature endothelial cells can't be defined[171], representing a challenge for the establishment of a universally recognized differentiation protocol.

Molecular mechanisms underlying single cell specialization are thought to be due to interaction with surrounding cellular microenvironment, both direct and mediated by soluble factors. As a matter of fact, during embryogenesis endothelial cells originate from a common precursor called angioblast, but local environmental biomechanics and biochemical forces and contact with adjacent cells are factors responsible for subsequent development and differentiation. The latter aspect suggests that for the definition of an endothelial differentiation protocol three-dimensional culture systems, such as those based on employment of scaffolds or engineered biomaterials, should be taken into consideration.

Until recent years the biggest part of studies on endothelial differentiation has been performed on stem cells from hematopoietic lineage. These cells are considered the best starting point for endothelial induction because they share with mature endothelial cells the same embryological origin: de novo vascular morphogenesis in the embryo is in fact initiated by a progenitor of mesodermal origin, the hemangioblast, capable of giving rise to endothelial and hematopoietic lineages[172].

First evidence of possibility to induce hematopoietic stem cells towards mature endothelium was stressed in 1997 by Asahara and colleagues[173], who described that a population of mononuclear cells isolated from peripheral blood showing positivity for CD34 antigen was able to differentiate into endothelial cells in vitro and incorporate into ischemic tissue at sites of angiogenesis in vivo. Shortly after this first description of the presence of such cells in the circulating blood, Shi et al[174]. cultured a bone marrow-derived $CD34^{pos}$ subset of cells and found that these cells were able to differentiate into mature endothelial cells either.

From this point of view, the discover of cells of mesenchymal origin with multilineage differentiation potential (MSC) could represent a new source of cells to be exploited for endothelial regeneration protocols. In addition, they can be obtained from various different tissues, for example bone marrow, placenta and its annexes, peripheral blood, tendon, cartilage, bone. MSC have been suggested to be immunoprivileged and capable of allogeneic administration in vivo with very few negative consequences if compared to cells of hematopoietic origin[175]. This quality could become the most important aspect in terms of applicability towards endothelium repair and regeneration.

Anyway, before stem cells of mesenchymal lineage could be employed in Regenerative Medicine, scientific proofs of their ability to differentiate in mature endothelial cells are required. In the last ten years several efforts have been made to deepen our knowledge about vasculogenic potential of mesenchymal stem cells and to improve differentiation protocols[176-179]. Unfortunately, none of cited studies dealt with hyperglycaemia role in mesenchymal cells differentiation towards endothelial lineage, so that information and scientific evidence are still lacking.

Aim of the study

In the third and last part of this thesis we aimed to evaluate effects of long term exposure to high glucose on endothelial differentiation potential of mesenchymal multipotent cells isolated from term placenta. Our concentration was dedicated to cells obtained from healthy donors (PLN17). In particular, in the case of a possible future application of these cells to cellular therapies and vascular tissue repair, we wanted to point out an eventual role of hyperglycaemia as differentiating agent relatively to cells with plasticity features. With this in mind, we established optimal culture conditions to induce differentiation, in order to verify mesenchymal placental cells ability and easiness in committing towards endothelial lineage.

Results and Discussion

At the end of last section of this doctoral thesis we succeeded in identify the direct cause of proliferation impairment observed during sustained hyperglycaemic conditions for PLN17 cells. Through western blot analysis we determined the raise of expression of some proteins involved in regulation of cell cycle progression, thus pointing out that hyperglycaemia forced placenta-derived cells to exit cell cycle and reduce proliferation rate. Before proceeding with our discussion, an important specification is necessary. In this section of thesis concerning differentiation potential towards endothelial lineage of placental cells, we didn't analysed cells isolated from diabetic donor. As we mentioned before, the employment of PLD09 cells in differentiation protocols such as endothelial one requires a deeper understanding of their metabolic functionality in order to clarify nature of behavioural discrepancies observed in culture between these cells and their healthy counterparts.

The overall situation outlined by protein analysis suggested that PLN17 didn't exit the cycle to reach a completely quiescent state, rather they slowed down their proliferation as consequence of a change in their metabolic and aging status. From this point of view, we supposed that exit from the active cell cycle wasn't due to a progressive increase of cellular senescence. It is nowadays well-known that after a finite number of divisions, primary cultures enter a state of replicative senescence in which they are growth-arrested and refractory to further mitogenic stimulation. In the past years many studies demonstrated that oxidants are important in the development of replicative senescence, given that a sustained raise in cellular oxidative stress was found to function as a common trigger for activation of the senescence program[180, 181]. Thanks to previous experiments about detection of ROS overproduction, we demonstrated that isolated placental cells didn't undergo enhancement of oxidative stress level, thus excluding possibility that reduction of proliferation was caused by progression towards senescent phenotype.

Here we hypothesized that PLN17 cells abandoned the active proliferating state to start differentiation program in response to prolonged hyperglycaemic treatment. We wondered if hyperglycaemia itself could be considered as a differentiating agent able to act on cells with stemness features by creating the right microenvironmental conditions to begin cell commitment and support its progression.

It is noteworthy that high glucose media are commonly used in differentiation protocols given that many studies reported that high glucose concentrations can positively affect cell commitment program by enhancing the action of classical differentiating agents or supporting cell metabolic needs. No study about a possible function of hyperglycaemia as a direct differentiating agent has been reported to date.

To deepen our knowledge about latter aspect, we performed a series of differentiation experiments which aimed on the one hand to investigate a positive role for hyperglycaemia in beginning endothelial commitment of placental cells, while, on the other, to verify the ability of same cells to differentiate towards this lineage. Experimental protocols were based on incubation of PLN17 $CD34^{neg}$ population in hyperglycaemic medium in the absence or presence of growth factors generally involved in endothelial commitment.

First evidence of a possible role for sustained hyperglycaemia in starting placental cells commitment towards endothelial lineage came from expression analysis of a series of endothelial specific genes with Real-Time PCR (**Fig. 36**). In this case, cells were only treated respectively with low or high glucose maintenance media in the absence of endothelial lineage-specific growth factors and gene expression was assessed after a chronic incubation period of three weeks.

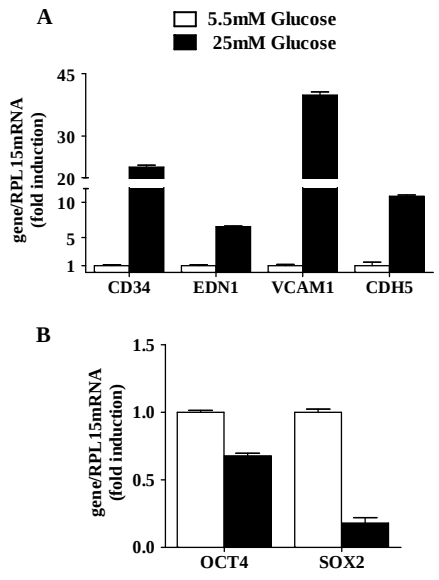


Figure 36.
Real-time PCR analysis of spontaneous endothelial differentiation caused by maintenance in high glucose medium
PLN17 were cultured in LOW (5.5mM) or HIGH (25mM) glucose medium for 3 weeks. After RNA extraction and reverse transcription, Real-time PCR was performed to evaluate the expression of endothelial lineage markers (***A***) and stemness transcriptional factors (***B***). Gene expression was normalized for RPL15 housekeeping gene and calculated using ΔΔCt method as fold change relatively to control condition (5.5mM glucose, =1). The experiment was repeated twice with comparable results, bars represent mean ± SEM.

Panel A clearly showed the spontaneous relevant increase of expression of endothelium specific antigens CD34, EDN1, VCAM1 and CDH5 in response to long term exposure to hyperglycaemia in vitro.

The protein encoded by EDN1 gene is proteolytically processed to release a secreted peptide termed endothelin-1. This peptide is a potent vasoconstrictor and is produced by mature vascular endothelial cells.

Vascular cell adhesion protein 1 (VCAM1), also known as cluster of differentiation 106 (CD106) is an adhesion molecule and a major mediator of the inflammatory response. It is expressed on activated microvascular endothelial cells in response to signals arising from immune activation in infections, graft rejection, tumour recognition and killing.

Protein encoded by CDH5 gene belongs to a family of calcium dependent cell adhesion proteins, which preferentially interact with themselves in a homophilic manner to connect cells. CDH5 cadherin, also known as VE-cadherin (Vascular-Endothelial), may play an important role in endothelial cells biology through control of cohesion and organization of intercellular junctions.

Considered markers are all expressed by mature endothelial cells with varying distribution between different regions of vascular system. The enhancement of expression of these genes was extremely relevant if compared to that of cells incubated in control conditions, so that it constituted a preliminary index of a positive role for hyperglycaemia in beginning endothelial commitment.

In **Panel B** gene expression of two important transcription factors was analysed. Both OCT4 and SOX2 are transcription factors of stemness lineage and their expression is commonly elevated in cells at a low degree of commitment.

OCT4 was the first gene to be identified as a master regulator of pluripotency in embryonic stem cells and its expression profile suggests that it may regulate cell fate during early developmental control[182]. In cells with stemness features OCT4 appears to regulate cell fate in a dosage-dependent manner, so that only an optimal amount can sustain self-renewal[56].

SOX2 often cooperates with OCT4 to regulate gene expression. Recent studies demonstrated that SOX2 is necessary for the regulation multiple transcription factors that affect OCT4 expression, thus stabilizing stem cells in a multipotent state by maintaining the required level of OCT4 expression[183, 184].

In this case, the observed reduction of expression of OCT4 and SOX2 genes could validate the idea that placental cells in prolonged hyperglycaemic conditions leave the undifferentiated state to commit to a more mature phenotype.

The analysis the spontaneous endothelial differentiation of PLN17 cells during persistent incubation in high glucose medium also pointed out that gene expression of two endothelial tissue-specific transcription factors was affected. Results of Real-Time PCR analysis for ETS1 and GATA2 genes are reported in **Figure 37**.

Although many transcription factors play important roles in vascular development, none appear to be as centrally involved in transcriptional programs controlling endothelial cell development as ETS proteins. To date, all characterized endothelial enhancers and promoters contain multiple essential ETS binding sites and ETS motifs are strongly associated with endothelial genes throughout the human genome[185]. Within endothelial tissue-specific genes whose expression is controlled by ETS1 we can include gene encoding for VEGF and its receptors VEGFR1 and 2, CDH5, ICAM1, VWF and angiopoietins receptors TIE1 and 2[186]. ETS1 expression during endothelial cells differentiation and development is thought to be induced by several different growth factors such as FGF2, EGF, HGF, VEGF[187].

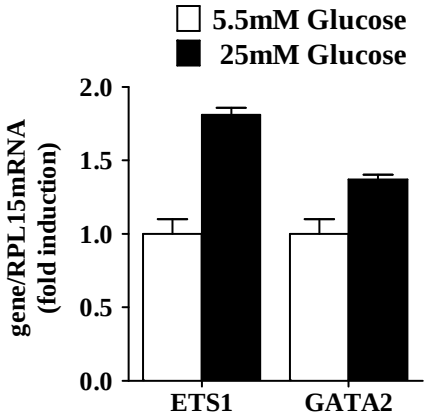


Figure 37.
Real-time PCR analysis of spontaneous endothelial differentiation caused by maintenance in high glucose medium
PLN17 were cultured in LOW (5.5mM) or HIGH (25mM) glucose medium for 3 weeks. After RNA extraction and reverse transcription, Real-time PCR was performed to evaluate the expression of endothelial specific transcriptional factors. Gene expression was normalized for RPL15 housekeeping gene and calculated using ΔΔCt method as fold change relatively to control condition (5.5mM glucose, =1). The experiment was repeated twice with comparable results, bars represent mean ± SEM.

The zinc finger transcription factor GATA2 is also known to play an important role in the activation and maintenance of endothelial gene expression. GATA2 is the most abundantly expressed GATA factor in endothelial cells and numerous endothelial enhancers contain GATA binding sites, which are bound directly by GATA2. Several studies support the notion that GATA2 is an early regulator of endothelial development and that this transcription factor may be involved in the specification of stem cells towards endothelial lineage[188].

It is also well-known that the expression of endothelial-specific genes is controlled synergistically by multiple transcription factors that have overlapping expression in the endothelium. For example, several characterized endothelial enhancers contain conserved binding sites for ETS and GATA family members, suggesting that these considered transcription factors may function together in the activation of these genes[189].

Given the widely recognized importance of ETS1 and GATA2 in determining stem cells fate towards endothelium, we noticed that their expression was only poorly increased by hyperglycaemic treatment. This observation led us to the supposition that high glucose persistence might have a role in starting endothelial commitment by making placental cells more permissive and sensitive to environmental stimuli, rather than as differentiating agent itself. On the basis of this hypothesis, it was clear that a complete maturation of cells could be achieved only through treatment with specific growth factors, such as VEGF.

In this case, before proceeding with differentiation experiments, we wanted to determine the general degree of cells responsiveness to a possible VEGF treatment by evaluation of the expression of VEGF receptor VEGFR2 in normo- and hyperglycaemic conditions. In fact, while VEGF binds to two receptor protein tyrosine kinases, VEGFR1, encoded by FLT-1 gene and VEGFR2, encoded by KDR gene, most biological functions of VEGF are mediated via VEGFR2 and the role of VEGFR1 is currently less known.

VEGFR2 expression was determined on the whole population of PLN17 cells with cytofluorimetric analysis after isolation, recovery in low glucose maintenance medium and depletion of $CD34^{pos}$ fraction. Expression of KDR gene was also tested after three weeks of chronic incubation in high glucose medium, in order to discover the role played by hyperglycaemia in altering it. **Figure 38** reports both series of results.

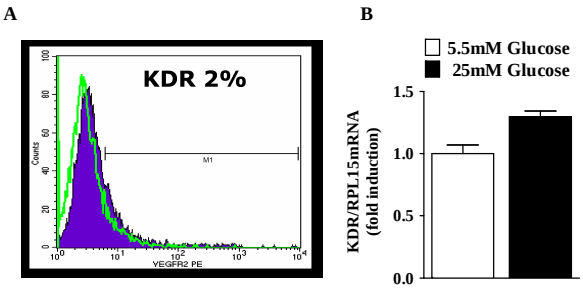


Figure 38.
Evaluation of basal KDR expression and after long time treatment with high glucose medium
Basal expression of VEGF receptor KDR by PLN17 was detected with flow cytofluorimetry (***A***). Before analysis PLN17 were expanded in maintenance medium (see Materials and Methods) for 7 days. Real-time PCR analysis of KDR expression after 3 weeks incubation in LOW (5.5mM) or HIGH (25mM) glucose medium (***B***). After RNA extraction and reverse transcription, Real-time PCR was performed as described in Materials and Methods. Gene expression was normalized for RPL15 housekeeping gene and calculated using ΔΔCt method as fold change relatively to control condition (5.5mM glucose, =1). The experiment was repeated twice with comparable results, bars represent mean ± SEM.

Panel A showed that the fraction of PLN17 population positive for VEGFR2 expression counted only for 2%. This was an expected result, given that VEGFR2 and CD34 were found to be co-expressed by subpopulations of multipotent cells endowed with endothelial differentiation potential. In this case, it was possible that depletion of $CD34^{pos}$ cellular fraction also contributed to reduce the amount of VEGFR2 positive cells.

The analysis of KDR gene expression showed in **Panel B** stressed that only a little increment relatively to control cells was detectable after three weeks of chronic hyperglycaemia incubation. Taken together this piece of evidence outlined that the overall PLN17 cells responsiveness to treatment with VEGF would be too scanty and could extremely impair the efficiency of endothelial differentiation induced by this growth factor.

To face this problem we tried to stimulate expression of VEGF receptor 2 indirectly by treating cells with a mixture of differentiating agents which were demonstrated to act as positive inducers of ETS1 expression. As previously mentioned in fact, ETS1 is considered the principal transcription factor responsible for KDR gene induction in developing endothelial cells, so that a direct correlation between ETS1 increase of expression and enhancement of VEGFR2 transcription rate was detected[190, 191].

To achieve this purpose we planned a preliminary set of experiments in which PLN17, previously kept in normoglycaemic conditions, were subjected to a short treatment (3 and 24 hours) with a combination of 10ng/ml FGF2 and 10ng/ml EGF in high glucose medium. It is important to underline that for this incubation FGF2 concentration was enhanced with respect to that of maintenance medium (5ng/ml), in order to reach the amount considered proper to support a differentiating milieu.

Soluble EGF was also added to pre-conditioning medium because it was shown to increase paracrine secretion from mesenchymal stem cells of potent vasculogenic growth factors such as VEGF and HGF[192].

At the end of incubation, gene expression analysis for ETS1 and KDR was performed with Real-Time PCR (**Fig. 39**). For this series of tests, results were compared to that of control cells always maintained in high glucose medium, in order to give a direct evidence of growth factors action and to distinguish it from hyperglycaemia-induced alterations of expression. Graphs showed a relevant increment of ETS1 expression (8 folds) after only three hours of incubation with considered growth factors, while, as generally expected for a transcription factor, the expression was reduced to the half at 24h of treatment.

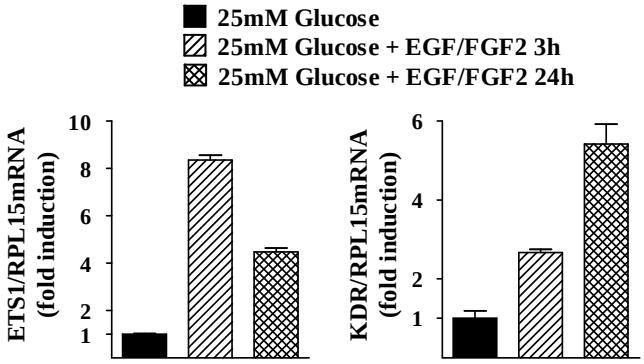


Figure 39.
Real-time PCR analysis of trascription factor ETS1 and VEGF receptor KDR expression after exposure to EGF and basic FGF
PLN17 were treated in HIGH (25mM) glucose medium with addition of 10ng/ml EGF and 10ng/ml FGF2 for 3 or 24 hours. After RNA extraction and reverse transcription, cDNA was employed as template for Real-time PCR with ETS1 and KDR primers. Gene expression was normalized for RPL15 housekeeping gene and calculated using ΔΔCt method as fold change relatively to control condition (25mM glucose, =1). The experiment was performed twice with comparable results.

Results obtained for KDR gene were coherent with those observed for ETS1 and encouraging for our purpose. We observed a constant increase of VEGFR2 expression that could be reasonably due to corresponding ETS1 trend and that reached the maximum level (about 6 folds of induction) after 24 hours of incubation. We could conclude that the pre-conditioning incubation in high glucose medium with the addition of FGF2 and EGF was effective in prompting PLN17 cells to a state in which reduction of proliferation rate together with increment of VEGF receptor 2 expression could contribute to the success of following endothelial differentiation protocol.

In the light of last results, endothelial experimental protocol was established as a two-steps experiment. As mentioned in Materials and Methods, in the first step PLN17 were treated with a 24h incubation with FGF2 and EGF in high glucose medium to increase number of cells expressing VEGF receptor 2. Finally, in the second part of endothelial differentiation protocol cells underwent a long incubation in presence of a specific induction medium containing respectively low or high glucose concentrations, 2% FBS, 10ng/ml FGF2 and 50ng/ml VEGF as differentiating agents.

VEGF is a major regulator of neovascularization under physiological and pathological conditions, where neovascularization is indicated as the *de novo* formation of immature vessels thanks to differentiation of progenitor cells. There are five members of the VEGF family, VEGF-A, VEGF-B, VEGF-C, VEGF-D and placenta growth factor PlGF. Notably, several VEGF-A alternatively spliced isoforms exist, including $VEGF_{121}$, $VEGF_{145}$, $VEGF_{148}$, $VEGF_{165}$, $VEGF_{183}$, $VEGF_{189}$ and $VEGF_{206}$, which differ in length of amino acid residues chain and have been identified in humans[193, 194].

VEGF-A is a pivotal mediator of early vascular formation and it additionally has a vital regulatory role in post-natal angiogenesis through its abilities to induce endothelial cells differentiation, proliferation, migration and to promote their adhesion to primitive vascular structures[195]. This growth factor, in particular isoform 165, was added to endothelial differentiation medium together with FGF2 because of their well-documented synergistic action in promoting maturation of vascular system *in vivo*[196].

Recent findings have endorsed the view of an indirect contribution of fibroblast growth factor 2 signalling to vascular development. A study using embryoid bodies demonstrated a non-immediate role played by FGF2 in vasculogenesis, as vascular endothelial growth factor supplementation was sufficient to promote vascular development of embryoid bodies[197].

During a total period of induction of 21 days, PLN17 mRNA was extracted both after two and three weeks and subjected to evaluation with Real-Time PCR. Results of treated samples were compared to that of control cells cultured in maintenance media with addition respectively of low or high glucose concentrations. Several different markers of endothelial lineage were tested in order to verify the acquisition of a specialized, more mature phenotype (**Fig. 40**).

In **Panel A** we reported results for endothelial markers previously analysed during evaluation of spontaneous differentiation in response to hyperglycaemia.

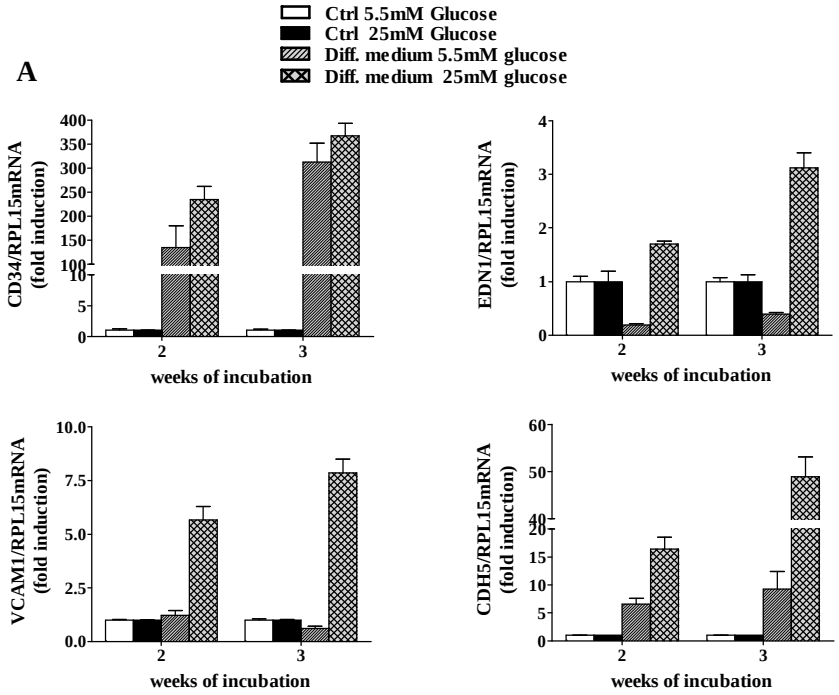
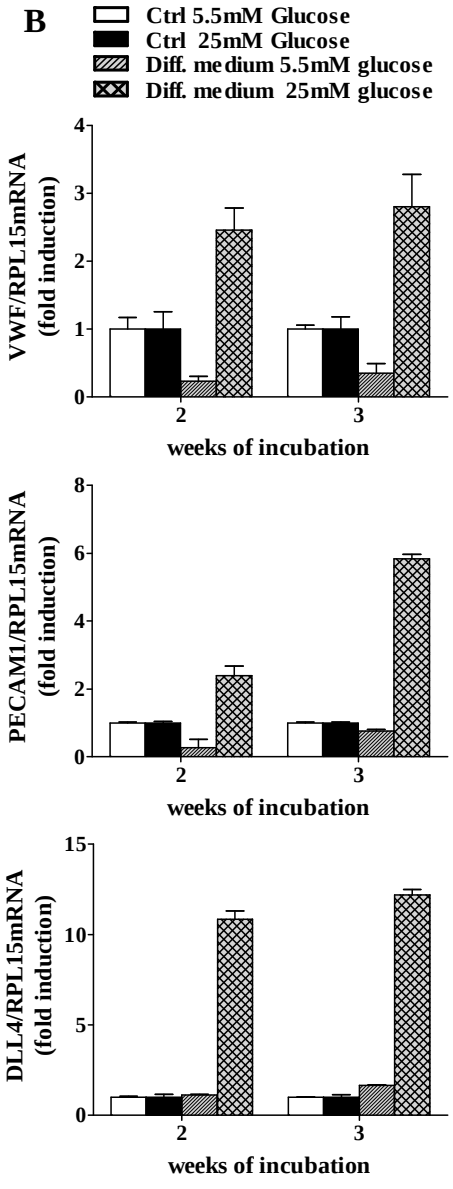


Figure 40.
Evaluation of endothelial differentiation with Real-Time PCR
PLN17 were induced to differentiate into mature endothelial cells as described in Materials and Methods. Briefly, cells were cultured in two defined media, each of them containing 10ng/ml FGF2, 50ng/ml VEGF and LOW (5.5mM) or HIGH (25mM) glucose. Cells grown in LOW or HIGH glucose medium in the absence of growth factors were used as negative controls. RNA extraction was accomplished at two different time points, respectively after 2 or 3 weeks of induction. After reverse transcription, Real-time PCR was performed to evaluate the expression of endothelial lineage markers. (***A***), (***B***), constitutive and inducible antigens of mature endothelial cells; (***C***), vegf and his receptor KDR; (***D***), angiopoietins and their specific receptors TIE1 and TIE2. Gene expression was normalized for RPL15 housekeeping gene and calculated using ΔΔCt method as fold change relatively to control conditions (5.5mM and 25mM glucose, both =1). The experiment was repeated twice with comparable results, bars represent mean ± SEM.

First of all, we could observe that the addition of growth factors specific to cause induction towards endothelial lineage resulted in an extremely significant increase of almost all considered markers, except for EDN1 and VCAM1. In the case of EDN1, the enhancement of expression was comparable to that observed in figure 36. On the contrary, VCAM1 showed a marked reduction of expression, even if it was on the whole much more abundant than that of cells differentiated with low glucose medium. We observed an overall enhancement of expression of considered markers when cells were treated with specific growth factors with respect to both control conditions. For differentiation protocol performed in normoglycaemia, results pointed out that EDN1 and VCAM1 expression was even impaired by incubation with growth factors. The most convincing results were those of CD34 and CDH5 genes, whose expression was

respectively about 350 and 50 folds higher than controls after three weeks of differentiation. We concluded that placenta-derived cells left the undifferentiated state to commit towards endothelial lineage in response to incubation in presence of FGF2 and VEGF. The degree of commitment achieved was more advanced when differentiation protocol was performed in sustained hyperglycaemic state.

In **Panel B** we analysed expression of important constitutive markers of endothelium, such as von Willebrand factor (VWF) and platelet-endothelial cell adhesion molecule (PECAM1). We also tested expression of endothelial-specific Notch ligand DLL4.



Von Willebrand factor is a glycoprotein produced uniquely by endothelial cells and megakaryocytes. It is heterogeneously distributed throughout the vasculature, while its transcriptional control occurs in response to tissue microenvironment and is responsible for local variations in endothelial cell levels. Some studies reported that FGF2 and VEGF, potent angiogenesis inducers, up-regulate expression of VWF mRNA and protein in cultured cells with a synergistic effect[198].

Human PECAM1, also known as CD31, is a transmembrane glycoprotein that belongs to the cell adhesion molecule family. Most, if not all, activities associated with PECAM1 can be attributed to homophilic PECAM1-PECAM1 interactions. Among these are leukocyte extravasation, bone marrow hematopoiesis, and vascular development, both during embryogenesis and adult neo-vascularization.

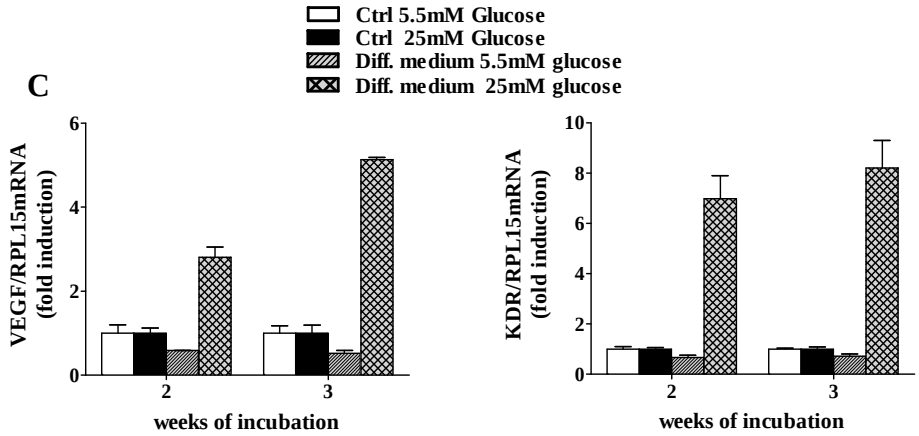
In vivo, vessel type-specific expression patterns were found for VWF and CD34, while PECAM1 was homogeneously and strongly expressed throughout the vascular system[199].

Some studies demonstrated that VEGF can induce gene expression of Delta-like 4 (DLL4), a well-known Notch1 ligand[200]. Notch and its ligands play critical roles in cell fate determination. Expression of Notch and DLL4 in vascular endothelium have suggested a critical role for Notch signalling in vasculogenesis and angiogenesis.

Differently from previous results, here we noticed that differentiating treatment with low glucose medium was completely ineffective in stimulating placental cells differentiation, given that the expression of all considered markers resulted impaired or unchanged respect to control cells.
On the contrary, differentiating treatment in presence of high glucose concentration confirmed its ability to induce cell differentiation. The increment of expression of VWF and PECAM1 constitutive antigens was respectively of about 3 and 6 folds at the end of induction period. For both genes, it didn't reach the same level of previously considered markers, however it was particularly important in order to define the degree of commitment achieved by cells. This is the case of markers whose expression isn't commonly activated during early phases of cellular commitment, so that their presence could be an index of an intermediate maturation status. Once again we could stress the importance of the hyperglycaemic milieu in sustaining endothelial differentiation of placenta-derived cells.

In **Panels C** and **D** we completed the analysis of PLN17 cells endothelial differentiation thanks to the evaluation of other important antigens of endothelium function.

We were able to demonstrate that placental cells began to produce, and presumably secrete, VEGF when kept in hyperglycaemic conditions. Moreover, the expression of receptor VEGFR2 was sensitively increased by pre-treatment with FGF2 and EGF in high glucose medium and remained sustained even after two or three weeks of induction (**C**). This aspect was of pivotal importance from the point of view of differentiation progression. It is noteworthy in fact that autocrine VEGF signalling is required for maintenance of vascular homeostasis through supporting of endothelial function[201], while paracrine VEGF signalling occurs in a differentiation-dependent manner and plays a positive role in enhancing differentiation progression[202]. These results suggested that cells, once achieved a good degree of commitment, were able to sustain and reinforce their own differentiation.

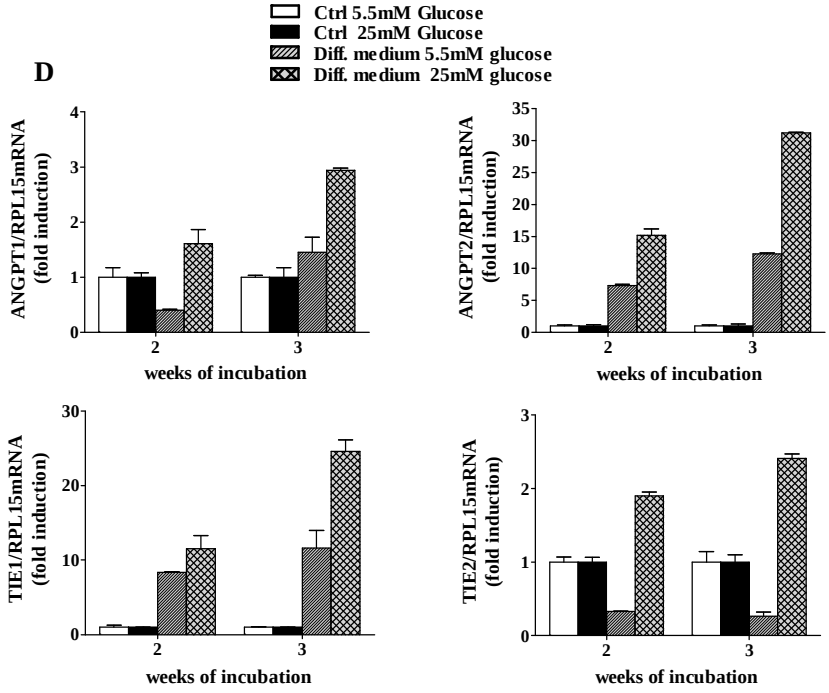


Finally, we evaluated changes of expression of genes encoding for angiopoietins ANGPT1 and ANGPT2 and their receptors TIE1 and TIE2 (**D**).

Angiopoietins are proteins with important roles in vascular development and angiogenesis. All angiopoietins bind with similar affinity to an endothelial cells-specific tyrosine-protein kinase receptor called TIE2[203]. It plays a critical role in mediating reciprocal interactions between endothelium and surrounding matrix and mesenchyme. This protein also contributes to blood vessels maturation and stability. Modulation of TIE2 receptor activity by its angiopoietin ligands is crucial for angiogenesis, blood vessels maturation and vascular endothelium integrity.

It has been proposed that angiopoietins 1 and 2 function as pro- and anti-angiogenic factors, owing to their respective agonist and antagonist signalling action through TIE2 receptor[204].

TIE1 is an orphan receptor tyrosine kinase that is expressed almost exclusively in endothelial cells and that is required for normal embryonic vascular development. Recently it has been suggested that molecular balance between receptors tyrosine kinases TIE1 and TIE2 is dynamically controlled by VEGF and regulates angiopoietins signalling[205].



In graphs we could observe that hyperglycaemic treatment with the addition of growth factors confirmed to be the best endothelial differentiation protocol for placenta-derived cells once again. In this case also, the increment of expression of considered antigens was consistent and higher than that reached through differentiation in normoglyceamic conditions.

At the end of last set of experiments we pointed out that when placenta-derived cells PLN17 were incubated for up to 2-3 weeks in the presence of VEGF, FGF2 and high glucose concentration (25mM), they began to express a variety of endothelial lineage surface markers, such as VEGFR2, PECAM1, VE-Cadherin, VWF, VCAM1, TIE1 and TIE2 and to secrete a set of endothelial specific signalling molecules such as VEGF and angiopoietins.

The molecular mechanism through which an immature cell acquires a mature endothelial phenotype has been clarified during last decades. Now we know that the appearance of endothelial-specific molecules occurs at different times after the onset of cell differentiation, "mimicking" *in vivo* vasculogenesis steps and suggesting that endothelial cell commitment follows sequential maturation stages[206]. Interestingly, the endothelial developmental pattern observed in culture for stem cells and endothelial progenitors follows a sequence of events similar to that occurring in the embryo during the establishment of vascular system. *In vitro*, this process can be optimized by the presence of well-known pro-vasculogenic growth factors such as VEGF, FGF2, HGF, PDGF and TGF-β[207]. An early stage defined by VEGFR2 and TIE2 expression may reflect commitment towards the endothelial lineage. In the intermediate state PECAM1 is induced. Later maturation steps were characterized by the appearance of VE-cadherin and TIE1 antigens[208].

Our data indicated that differentiating PLN17 cells began the commitment program and could acquire expression of markers of all considered stages of differentiation. With the kind of analysis we adopted we weren't able to define the temporal sequence through which endothelial markers appeared, anyway we could notice that placenta-derived cells subjected to differentiating treatments expressed almost all antigens we tested at a high levels relatively to control conditions. On the basis of these observations, it was quite difficult to establish the exact maturation grade reached by PLN17 cells.

To deepen this aspect, we performed an ulterior test in which cells differentiated in both low and high glucose media were detached from plastic surface at the end of the third week of induction and seeded in a three-dimensional model on Matrigel in order to verify their ability to form vessel-like structures (**Figure 41**).

This test underlined that the appearance of such structures was limited in the case of normoglycaemia-treated cells, even after seven days of incubation in presence of Matrigel matrix (**F**). At this stage in fact, cells created only few contact points and the lack of well-defined tube-like structures was evident. For hyperglycaemia-treated cells we could observe the first formation of small cell aggregates three days after seeding on Matrigel (**B**). These structures suggested that cells began a spatial re-organization in which direct contact points between different cells were visible. At the end of seven days of incubation in three-dimensional system, cells completed their migration and distribution in the space reached a good level of organization, so that a vessels network of little dimensions and limited organized structure appeared (**C**).

This evidence excluded the complete maturation of cells and led us to hypothesize that the combination of specific endothelial growth factors and hyperglycaemia prompted PLN17 cells to acquire an intermediate differentiation status.

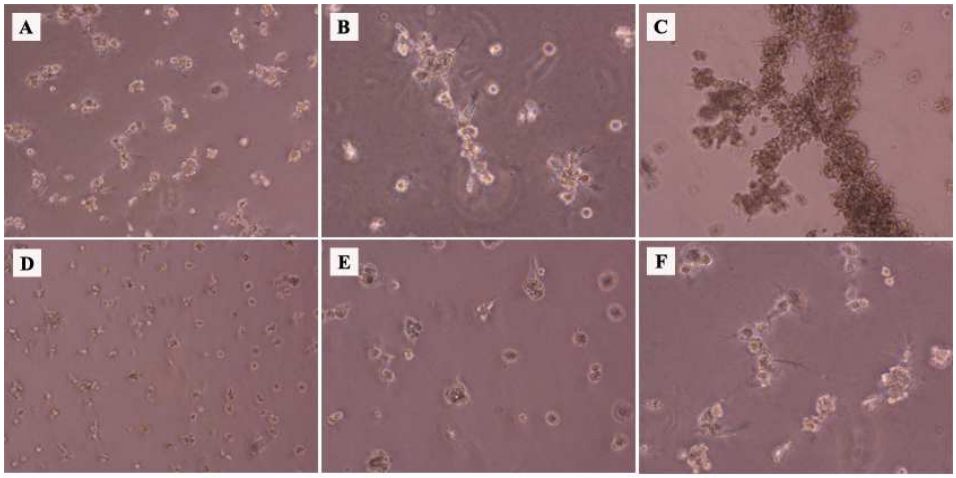


Figure 41.
Capillary formation assay on PLN17 after endothelial differentiation
PLN17 were differentiated in endothelial cells both in normo- and hyperglycaemic conditions as described in Material and Methods. At the end of 21 days of induction, cells were detached and seeded in a three-dimensional model on Matrigel matrix and incubated again with the previous induction medium. The ability to form vessel-like structures was monitored daily during seven days of incubation. Phase constrast images of representative microscopic fields; ***A***, differentiation in high glucose medium 24 hours (100x), 3 days (***B,*** 200x) and 7 days (***C,*** 100x) after seeding. ***D***, differentiation in low glucose medium 24 hours (100x), 3 days (***E,*** 200x) and 7 days (***F,*** 100x) after seeding.

By comparing differentiation protocols in normo- and hyperglycaemic media it was clear that the increment of expression was constantly higher in the second case, so that we could conclude that endothelial commitment was sustained and facilitated by the presence of high glucose concentration. We admitted that proofs about a direct role of hyperglycaemia as differentiating agent weren't sufficient to confirm such previous supposition. On the other hand, results obtained suggested that hyperglycaemia contributed to create an ideal microenvironment to induce mesenchymal multipotent cells isolated from placenta to commit towards endothelial lineage. Reasonably, high glucose treatment acted by inducing the exit from the active proliferating state and by increasing cell responsiveness to incubation with growth factors.
On the whole, we demonstrated that placenta-derived MSC were endowed with endothelial differentiation potential and suitable for basic and clinical studies aimed at the development of vasculature-directed Regenerative Medicine.

Summary

At the end of the third and last part of this doctoral thesis we demonstrated that mesenchymal multipotent cells isolated from human term placenta (PLN17) were able to abandon the undifferentiated state to commit towards endothelial lineage. Cell differentiation was achieved through the action of specific growth factors such as FGF2 and VEGF. Hyperglycaemic condition of culture was also found to be important to begin and sustain cell differentiation. Thanks to a series of experiments in which cells were treated by incubation with growth factors and respectively low or high glucose concentrations, we demonstrated that normoglycaemia slowed down the progression of differentiation, while hyperglycaemia increased the expression of all considered markers of mature endothelium. We affirmed that hyperglycaemia couldn't be considered a differentiating agent itself. However, it prompted cell towards endothelial commitment by creating environmental culture conditions that augmented cell responsiveness to growth factor treatment. At the end of experimental protocols, cells showed an intermediate degree of differentiation, which was also confirmed by the newly acquired ability to form little tube-like structures when seeded on three-dimensional susbtrate (Matrigel).

Conclusions

A growing crisis in organ transplantation and an aging population have driven the search for new and alternative therapies. Despite growing numbers of donors, the availability of suitable organs and tissues is still insufficient. Recent advances in Tissue Engineering and Regenerative Medicine hold the promise of custom-made medical solutions for all most widespread pathologies such as diabetes, cardiovascular failure, Parkinson's and Alzheimer's diseases, osteoporosis. Regenerative Medicine focuses on strategies to repair, regenerate and/or replace tissues and organs. The goal in each of these cases is to restore tissue and organ function through the delivery of cells, signalling molecules and/or support structures.

Cells are the machinery that promote tissue regeneration and, specifically, stem cells are a useful source for transplantation and Tissue Engineering. These cells, however, may originate from a variety of locations and be at varying levels of commitment.

This doctoral thesis demonstrated that term human placenta is an organ endowed with stem elements of different origin and nature. We established an experimental protocol to allow an easy and effective isolation of such elements from foetal region of placenta, with particular regard to cell originated from mesenchymal lineage. Both placentas from healthy donors and diabetic patients were collected and their cells isolated. These cells were expanded in culture as a plastic adherent homogeneous population of $CD34^{neg}$ fibroblast-like cells displaying high proliferative potential and clonal properties. We referred to cells from healthy donors ad PLN17 and from diabetic patient as PLD09. They both expressed a unique pattern of mesenchymal and multipotency antigens which excluded contamination from other undifferentiated or mature cell types (hematopoietic stem and endothelial cells) and contributed to define them as human Chorionic Mesenchymal Stem Cells (hCMSC). Multipotency properties and differentiation potential were demonstrated through in vitro induction of adipogenic, osteogenic and chondrogenic commitment. Given the intrinsic plasticity showed, mesenchymal multipotent placenta-derived cells could be employed for regeneration of hard tissues. In this case, more information about interaction between these cells and biomaterials for implantology were needed in order to optimize culture conditions and differentiation protocols.

Mesenchymal multipotent cells from human placenta also displayed reduced immunogenicity and immunomodulatory properties if compared to well-characterized mesenchymal stem cells from bone marrow.

Regenerative strategies relying on differentiation of autologous cells are commonly based on isolation of elements with stemness features from patient's own bone marrow. Unfortunately, the fraction of mesenchymal stem cells in human bone marrow is small and accounts only for 0.01-0.001% of whole population of nucleated cells, so that when isolation of such progenitor cells is impossible or ineffective, the employment of cells from unrelated allogeneic donor become a choice. In all these cases, multipotent cells from human placenta could represent a valid alternative, given their permissive immunogenicity status and that size of organ allows isolation of higher amount of cells.

In the second and third parts of this doctoral thesis, effects of mimicking hyperglycaemia *in vitro* on placenta-derived cells were analysed. The prevailing literature accounts for both detrimental and beneficial effect of high glucose on mesenchymal stem cells, leading to perplexity. Anyway, this aspect of mesenchymal cell biology needs to be deepened in order to define optimal maintenance and differentiation protocols. Thus, this study evaluated the effects of sustained hyperglycaemia on proliferation and differentiation properties of mesenchymal multipotent cells from human placenta. In particular, we focused on commitment towards endothelial lineage. During last decades Tissue Engineering reached some biological limitations, partly due to the observation that mechanistic approach of biomaterials failed to take into consideration the issue of regenerated-tissue vascularization. When the centrality of angiogenesis for homeostasis, bioassimilation and biointegration of tissue engineered-constructs became clear, the need of cells capable to differentiate in endothelium supporting neo-vascularization process became impellent. Here we demonstrated that placental multipotent cells from healthy patient (PLN17) could be effectively differentiated in endothelial cells.

We provided proofs for the impairment of cell proliferation potential caused by prolonged exposure to hyperglycaemia and demonstrated that these events created a microenvironment able to increase cell responsiveness to treatment with endothelial-specific growth factors. In fact, placental cells incubated with high glucose (25mM), VEGF and FGF2 committed towards endothelial lineage until they reached an intermediate differentiation status. Thus, we concluded that hyperglycaemia *in vitro* might not be a barrier for the effective functional improvement of placental cells transplantation, but, on the contrary, it could function by supporting cells metabolic needs during differentiation and enhancing growth factor action.

Placenta-derived cells isolated from diabetic patient (PLD09) were also tested to evaluate effects of hyperglycaemic treatment, in order to find out eventual discrepancies relatively to cells from healthy donor. Their proliferation potential didn't show to be influenced by prolonged incubation in high glucose medium and cells didn't respond to all subsequent treatments performed to elucidate the molecular mechanism underlying the lack of hyperglycaemia-induced impairment of proliferation. We concluded that further tests were necessary to point out the biological behaviour of these cells when kept in culture.

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