Chapter 4

Human placenta derived mesenchymal stem cells (hPDMSCs) — isolation, characterization and islet neogenesis

The term — placenta, comes from the Latin for cake, from Greek flat-slab-like [Online Etymology Dictionary, 2009], referring to its round, flat appearance in humans. The placenta is a highly vascularized, ephemeral organ that connects the developing fetal tissues to the maternal uterine wall by an umbilical cord, which further inserts into the chorionic plate [Yetter, 1998]. In humans, the normal term placenta measures 15 to 20 cm in diameter, 1.5 to 3 cm in thickness and weighs 450 to 600 gm [Rosai, 2004].

The formation of a placenta is prerequisite for successful pregnancy [Zhou et al. 2003]. The placenta develops from the same sperm and egg cells that form the fetus, and functions as a feto-maternal organ with two components, the fetal part - Chorion frondosum, and the maternal part - Decidua basalis. In humans, by days 6–7 after fertilization (during the implantation window), the blastocyst implants and placenta development begins. At this stage, the blastocyst is flattened and composed of an outer wall (trophoblast) that surrounds the blastocystic cavity. The trophoectoderm further give rise to the trophoblastic villi. During the first trimester, trophoectoderm divides into an outer and inner cytotrophoblast layer, which surrounds a central mesenchymal core containing primitive fibroblast and scattered macrophages [Moore and Persaud 1998; Benirschke and Kaufmann, 2000; Rosai, 2004]. The syncytiotrophoblast (otherwise known as syncytium), is a multinucleate continuous cell layer which covers the surface of the placenta thereby contributing to the barrier function of the placenta. The syncytiotrophoblast forms as a result of differentiation and fusion of the underlying cytotrophoblast cells, a process which continues throughout placental development. The umbilical cord vessels, which branch out over the surface of the placenta further divide to form a network covered by a thin layer of cells. This results in the formation of villous tree structures. On the maternal side, these villous tree structures are grouped into lobules called cotyledons (Fig 4.1). The placenta grows throughout pregnancy. Development of the maternal blood supply to the placenta is suggested to be complete by the end of the first trimester of pregnancy (approximately 12-13 weeks).
The cytotrophoblasts attach the placenta into the uterine circulation; as a result, the organ is ideally positioned to perform a wide variety of functions during pregnancy, including gas, nutrients and waste exchange. The perfusion of the intervillous spaces of the placenta with maternal blood allows this transfer of nutrients and oxygen from the mother to the fetus and the transfer of waste products and carbon dioxide back from the fetus to the mother. In addition to the transfer of gases and nutrients, the placenta also has metabolic and endocrine activity. It produces, among other hormones, progesterone, which is important in maintaining the pregnancy; somatomammotropin (also known as placental lactogen), which acts to increase the amount of glucose and lipids in the maternal blood; estrogen; relaxin, and beta human chorionic gonadotrophin (beta-hCG). This results in increased transfer of these nutrients to the fetus and is also the main cause of the increased blood sugar levels seen in pregnancy. This hormone (beta-hCG) ensures that progesterone and oestrogen are secreted; progesterone and oestrogen thicken and maintain the uterine lining as well as inhibit the production and release of more eggs. However after about 2 months the placenta takes on the role of producing progesterone and therefore beta-hCG is no longer needed. Beta-hCG is excreted in urine and this is what pregnancy tests detect. It also produces insulin-like growth factors (IGFs). Placenta also protects the fetus from immune system of mother by means of
placental barrier. To hide itself from attack by the mother’s immune system, the placenta secretes Neurokinin B containing phosphocholine molecules. This is the same mechanism used by parasitic nematodes to avoid detection by the immune system of their host.

Human placenta, besides playing a fundamental and essential role in fetal development, nutrition, and tolerance, may also represent a reserve of progenitor/stem cells. The invasive character of the cytotrophoblasts should be kept in mind when clinical safety of placental derived stem cells and their applications is assessed. Very recently placenta was shown as an important hematopoietic organ, [Bárcena et al. 2009]. Whereby, cells in chorionic villi showed the presence of hematopoietic markers CD34 and CD45. These progenitors are in principle capable of forming all hematopoietic cell lineages of the adult animal. In addition to hematopoietic stem cells, placenta has been reported to contain a population of multipotent stem cells demonstrating some of the characteristics of pluripotent ES cells including expression of stem cell markers c-kit, Thy-1, OCT-4, SOX2, hTERT, SSEA1, SSEA3, SSEA4, TRA1-60 and TRA1-81. These cells resemble mesenchymal stem cells and can be induced to differentiate into hepatocyte-, vascular endothelial-, cartilage- and neural like cells [Strom and Miki, 2003, Fukuchi, et al. 2004, Matikainen and Laine, 2005, Zhang et al. 2006, Strakova, et al. 2008, Alvarez-Silva, et al. 2003, Yen et al. 2005, Miao et al. 2006, Parolini et al. 2008, Chien et al. 2006]. Differentiation of these placenta derived mesenchymal stem cells (PDMSCs) into insulin positive cells [Chang et al. 2007] has raised hopes for use of these cells as an alternative source for therapeutic approach in diabetes.
EXPERIMENTAL DESIGN

In vitro isolation, proliferation and characterization of human placenta derived mesenchymal stem cell (hPDMSCs) culture has been illustrated in scheme 4.1. The mesenchymal nature of the isolated hPDMSCs was confirmed by immunocytochemistry using confocal microscopy and FACS analysis. Human PDMSCs were then tested for multilineage potential. Differentiation of hPDMSCs to pancreatic lineage was studied using serum free medium containing cocktail of Insulin Transferrin Selenium (ITS), GLP1, niacinamide and taurine. Differentiated islet like clusters were then studied for in vitro and in vivo functionalities.

All the materials used and the methods employed to carry out the work have been described in detail in ‘Materials and Methods’ section.

Scheme 4.1: Experimental design for human placenta derived mesenchymal stem cells

Human Placenta collected in transfer medium

Cord blood drained and placenta washed with PBS containing antibiotic

Placenta chopped in pieces

Enzymatic digestion

Digest filtered through muslin cloth

Centrifugation at 1500 rpm

Cell pellet seeded in growth medium- α-MEM containing 10% human umbilical cord blood serum

Cryopreservation

Characterization and Trilineage differentiation

Differentiation to islet-like cell

In-vitro testing (mulin secretion glucose stimulation)

In-vivo studies (Transplantation into STZ diabetic mice)
RESULTS

Isolation and expansion of human placental derived mesenchymal stem cells (hPDMSCs)

The results reported represent the summary of the data obtained using 53 human placentae of which cells were successfully isolated from 39 samples. The isolated cells from placenta at passage 0 demonstrated the mixed population of epithelial and fibroblast like cell (Fig 4.2A). Cell colonies from placental tissue began to appear after 7-10 days of cells isolation. After passing with trypsin, the epithelioid population rapidly disappeared from culture and could no longer be found by the second passage (Fig 4.2B). The 100% confluency was reached after 21 to 25 days of culture. After subjecting cells to different growth media, optimum growth was obtained in the medium α-MEM supplemented with 10% human umbilical cord serum (Fig 4.2C). The fibroblastoid population of cells continued to proliferate, even after 25 passages.

![Image of hPDMSCs](image_url)

**Fig. 4.2:** hPDMSCs exhibiting mixed population of epithelial and fibroblast-like cell at passage 0 (A) and fibroblast like cell appearance at later passages (B). Optimization of growth medium for hPDMSC indicating maximum cell proliferation in alpha-MEM compared to others (C). The growth analysis of these cells showed mean doubling time of 21.84 h (D). Negligible difference in ROS generation in pre-cryopreservation (Pre Cryo) and post-cryopreservation (Post Cryo) was observed (E). Cell cycle analysis at passage3 showed maximum population of hPDMSCs at G1 Phase (F).

The combined data of the cell count showed that the lag phase of these hPDMSCs lasts for around 72h, leading to 120h of log phase (Fig. 4.2D). It was observed that hPDMSCs have a doubling time of 21.84h.
The reactive oxygen species (ROS) was determined in both, cells at passage 3 and cells from the same passage after cryopreservation and revival. The mean fluorescence values of cells without cryopreservation and cells after cryopreservation were found to be 0.754 and 0.755 respectively (Fig 4.2E) revealing negligible difference in ROS generation.

**Immunocytochemistry and Flow cytometry:**
We carefully checked the antigenic phenotype of all the cell preparations by flow cytometric analysis. Immunophenotyping of hPDMSCs revealed these cells to be positive for many markers common to mesenchymal stem cells such as Stro-1 (Fig. 4.3A), vimentin (Fig.4.3B), nestin (Fig. 4.3C), and embryonic Oct 4 (Fig. 4.3D) Flow cytometric analysis of the hPDMSCs showed that they were strongly positive for CD44, CD105, CD117 (Fig 4.3E) and negative for CD10, CD34, CD45, CD166.

![Immunocytochemistry](image)

**Differentiation Studies:**
To promote adipogenic differentiation, hPDMSCs were induced by adipogenic differentiation cocktail containing dexamethasone, 3-isobutyl-1-methylxanthine, insulin and indomethacin. Human PDMSCs cultured with this adipogenic differentiation medium change their morphology from elongate to round shaped within 8 days. This coincides with the accumulation of intracellular droplets. After 16 days in culture, more than 95% of the cells had their cytoplasm filled with lipid rich vacuoles. These large, round intracytoplasmic lipid droplets were stained positive by Oil Red O (Fig 4.4A).
Human PDMSCs differentiated into chondrogenic cell lineage after 3 weeks of incubation in chondrogenic medium. Chondrogenic phenotype in induced hPDMSCs was signaled by the changes in cell morphology, from spindle-shaped to larger round cell aggregates, and by the accumulation of sulfated proteoglycans which are present in cartilage. These proteoglycans in the matrix stained positive with Safranin-O (Fig. 4.4B)

Osteogenic differentiation was induced in hPDMSCs with use of osteogenic differentiation cocktail containing dexamethasone, ascorbic acid 2-phosphate and β-glycerophosphate. On exposure to osteogenic differentiation medium for 3 weeks hPDMSCs showed changes in cell morphology, from fibroblast like to cuboidal shaped as they differentiate and mineralized. Calcium phosphate mineralization, which stained positive by Alizarin red S stain indicate direct evidence of calcium deposits as amorphous accumulation between cells (Fig 4.4C) after 3rd week of osteogenic induction.

When exposed to neuronal differentiation cocktail medium for 21 days hPDMSCs changed their morphology to neuronal cell. These cells stain positive for neuronal markers Map2 and NeuN. (Fig 4.4D).

![Fig 4.4: Multilineage differentiation potential of hPDMSCs : The panels display representative photomicrographs of - (A) adipocytes detected by Oil Red O staining (magnification 20x); (B) chondrocytes detected by Safranin-O staining (magnification 20x); (C) osteoblasts detected by Alizarin Red S staining (magnification 20x); (D) Neuronal lineage by expression of Map2 and NeuN.](image)

**Differentiation to Islets like Cluster and their functional study:**
The induction of hUCMSCs with serum free medium containing cocktail of ITS, nicotinamide, taurine have microscopically showed the progressive cell clustering from day 2 onwards which led to typical islets like clusters (ILCs) formation at the end of day 10 (Fig 4.5A). After 10 days of induction these mature ILC clusters stained positive for the islet- specific DTZ stain (Fig. 4.5B), which is known to selectively stain...
pancreatic beta cells because of their high zinc content, non-islet tissue remained unstained. These clusters were also found positive for insulin and glucagon (Fig. 4.5C) by immunocytochemistry.

These newly generated ILCs showed insulin secretion upon glucose stimulation. Basal insulin secretion was observed around 15 pmol/mL while around 100 pmol/mL for stimulation (Fig. 4.5D). Undifferentiated hPDMSCs showed 18 pmol/mL basal glucose index for the basal while 48 pmol/mL for stimulation index (Fig 4.5A). Also, C-peptide secretions from these ILCs were observed to be around 108.54 pmol/L (Fig 4.5B). While amylase secretion in day 10 supernatant was negligible.

Undifferentiated placental MSCs along with day 4 and day 10 differentiated ILCs when analyzed for the pancreas specific and β cell development transcription factors by Taqman based real-time PCR. The results demonstrate abundance of proinsulin glucagon, somatostatin Ngn3 and Isl1 transcripts in hPDMSCs and day 10 ILCs, while day 4 cell clusters showed abundances of insulin, Ngn3 and Isl1 transcripts (Fig 4.5F).

Fig 4.5: Islet neogenesis from hPDMSCs : hPDMSCs upon exposure to Serum Free Medium (SFM) supplemented with growth factors involved in pancreatic development formed Islet like Clusters (ILCs) (A). These ILCs are positive for DTZ staining (B) and exhibit presence of insulin and glucagon by immunocytochemistry (C). Newly generated ILCs on glucose stimulation showed five fold increase; while undifferentiated hPDMSCs showed around 2.5 fold increase in insulin secretion than the basal level (D). C - peptide secretion was observed only in day 10 ILC culture supernatant (E). Real time Taqman based PCR results showed the abundance of pro-insulin, glucagon, somatostatin, Ngn3 and Isl1 transcripts in undifferentiated hPDMSCs as well as in in day 4 and day 10 ILCs (F).
Islet like clusters transplantation to STZ induced Diabetic animals.

Islet-like cell clusters derived from hPDMSCs and undifferentiated hPDMSCs were transplanted into STZ induced diabetic Balb/c mice with the help of biocompatible capsules. These capsules were transplanted into the intraperitoneal cavity of the experimental mice and they were observed for the period of 30 days.

All the mice transplanted with biocompatible capsule did not showed any sign of graft rejection. Diabetic mice without transplantation (Diabetic control) showed hyperglycemia through out the study period and died after 20 days. Non transplanted-non diabetic mice (sham Tx) showed normal Blood Glucose values. ILCs (ILC Tx) and undifferentiated hPDMSCs (hPDMSCs Tx) transplanted mice showed reduction in BG levels and reversal of experimental diabetes after 20 days, which were maintained at less than 140 mg/dL. Diabetic control mice did not restore normoglycemia and died after day 20 (Fig 4.6C) with constant loss in body weight, while increase in body weight of ILC Tx, hPDMSCs Tx, Sham Tx group (Fig 4.6D) was observed.

To further evaluate the function of the implanted ILCs, we performed an Intra Peritoneal Glucose Tolerance Test (IPGTT) on diabetic control mice, non diabetic control mice and on ILCs transplanted mice. At day 30 (Fig 4.6E), blood glucose in
normal non diabetic control mice showed typical response with elevated glucose concentrations observed at 15min, followed by a return to nadir by 120 min after glucose infusion. Blood glucose concentrations in the ILC-transplanted mice were little elevated as compared to non diabetic control mice, but displayed a similar GTT profile with lower glucose concentration by 120mins of glucose injection. However, the diabetic animals continued to display elevated glucose concentrations beyond 120min. Results indicated that the implanted ILCs produced insulin, matured during the two month period and were indeed responsive to a glucose challenge in vivo.

To confirm whether normal glucose concentrations observed in transplanted mice were due to the hPDMSC derived ILC graft, we surgically removed the grafted capsules after 6 weeks of STZ injection. Hyperglycemia was observed within three days after removal of ILC-grafted capsules and all mice died within 2 weeks of graft removal.
DISCUSSION

Obtaining sufficient human stem cells has been problematic for several reasons. First, isolation of normally occurring populations of stem cells in adult tissues has been technically difficult, costly and yields a very limited in quantity of the desired cell population. Secondly, procurement of these cells from embryos or fetal tissue including aborts has raised many ethical and moral concerns. The widely held belief that the human embryo and fetus constitute independent life has justified a moratorium on the use of such sources for any purpose. Alternative sources which do not violate the sanctity of independent life would be essential for further progress in the use of stem cells clinically. In this context placenta seems to be an ideal source, which is discarded as a waste organ after the birth of the child. It is a natural bioreactor environment in which embryonic like stem cells resides, hence can be exploited as a non-invasive and rich source for mesenchymal stem cells (MSCs). The placenta is best examined in the fresh state immediately after delivery [Langston, et al. 1997] which has been, processed for MSCs within 1-2 h of delivery.

Our results show that mesenchymal stem cells are present in the human term placenta (hPDMSCs). Using routine cell culture techniques, hPDMSCs can be successfully isolated and expanded in vitro. Although the initial cell culture consisted of both fibroblastoid and non-fibroblastoid cell types, only the fibroblastoid population remained after enzymatic digestion and passaging, which is in accordance with the earlier observations by Miao et al [2006] and Yen et al.[2005]. In their undifferentiated state MSCs are spindle-shaped and resemble fibroblasts. Our lab has reported [Phadnis, et al. 2006] earlier that human umbilical cord blood serum (hUCBS) supports attachment, propagation and differentiation of human bone marrow derived mesenchymal cells, which was further confirmed by Shetty et al. [2007]. Hence the growth medium for hPDMSCs (α-MEM) was supplemented by hUCBS instead of the fetal calf serum for better proliferation. We deliberately avoided the use of FCS as a culture supplement in order to obtain a medium free of xenoproteins to make the cells suitable for human transplantation studies. Human PDMSCs have been cultured for more that 27 population doublings without any change in the morphology, MSC characteristics, and multipotent differentiation potential.

The human Placenta derived MSCs expressed neither heamatopoietic markers (e.g. CD45, CD34, CD14) nor endothelial markers (e.g. CD34, CD31). Our observations are supported by earlier studies [Pittenger, 1999, Majumdar et al. 1998]. They do
express a large number of adhesion molecules CD44 and some stromal cell markers along with mesenchymal proteins: Stro-1, vimentin, nestin; surface markers CD 44, CD105, CD 117 confirming their identity as MSCs. Of considerable interest is the fact that in addition to MSC markers, Oct-4 which is found only in ES cells and embryonic germ cells has been found to be expressed in hPDMSCs. Similar observations had been reported previously by some researchers [Shambrott et al. 1998, Thomson et al. 1998, Reubinoff et al. 2000]. The detection of ES cell surface markers on hPDMSCs suggests that these may be very primitive cells. If this is correct, it may well be that the renewal and differentiation capacity of hPDMSCs are more extensive than other adult stem cells. Presence of embryonic characteristic may also be responsible for the beta cell function in hPDMSCs [Chang et al. 2007].

There are no markers which specifically and uniquely identify MSCs and therefore stem cells are defined by their immunophenotypic profile, by their characteristic morphology; and by their extensive capacity for self-renewal while retaining the ability to differentiate into a number of lineages. In vitro, we have observed that these isolated hPDMSCs differentiated into adipocyte, chondrocyte, osteocyte and neuronal lineages as reported previously by many groups [Matikainen and Laine, 2005, Strakova, et al. 2008, Alvarez-Silva, et al. 2003, Yen et al. 2005, Miao et al. 2006, Parolini et al. 2008]. The multilineage differentiation and proliferative capability and the presence of various MSC– and ES cells–markers lend strong support to the presence of a putative stem cell population within hPDMSCs.

Until now many research groups have showed the tri-lineage differentiation potential of hPDMSCs. However, the potential of hPDMSCs to differentiate into insulin-positive cells which functionally secrete insulin in vitro and in vivo had been recently demonstrated by Chang et al. [2007]. The results obtained by this group revealed that the total time taken for the differentiation in to insulin producing spheroid bodies was 4 weeks in presence of serum free medium containing insulin, transferrin and selenium chloride. We have modified the same protocol for time of addition of these pancreatic growth factors (ITS) with addition of GLP1 and nicotinamide. This resulted in the differentiation of PDMSCs into islet like clusters in merely 10 days which is highly desirable for faster enrichment of islets.

The islets obtained were found to be responsive to glucose challenge as evidenced by five fold increase in insulin secretion over basal stimulation (Fig 4.5D) indicating ability of the islet generated to synthesize, store and release insulin in response to
glucose challenge. The undifferentiated hPDMSCs were also showed 2.5 fold increase in insulin secretion upon glucose stimulation confirming the presence of insulin in undifferentiated state.

Presence of insulin in undifferentiated hPDMSCs as well as in ILCs was further confirmed by qPCR and by in vivo studies. Undifferentiated hPDMSCs along with ILCs showed presence of proinsulin transcripts and when transplanted into STZ induced diabetic mice achieved normoglycemia in 15 days. These observations were in agreement with that of Chang et al. [2007] who demonstrated the presence of insulin in undifferentiated hPDMSCs. The presence of insulin gene in undifferentiated hPDMSCs could be explained by the presence of insulin-like growth factor in placenta. Presence of transcripts like isl1, Pdx1 in day 4 cell clusters indicated the correct path towards β cell development. While abundance of insulin, glucagone and somatostatin transcripts in day 10 ILCs indicate the similarity with the islets.

In our present study we have used undifferentiated placental MSCs and ILCs generated from them for transplantation in Balb/C mice (xenotransplantation) with help of biocompatible capsules. Prior to transplantation, the biocompatible capsule were dipped in Chitosan, acetic acid and CaCl₂ as chitosan is known fibrotic growth inhibitor in vitro [Malette et al.1986; Risbud, et al. 2000].

There have been several reports till now regarding the use of alginate based matrices in islet encapsulation [Soon-Shiong et al. 1993]. However, Rha [1984] and Hardikar et al. [1999] have reported the use of chitosan-alginate matrices for islet encapsulation. Chitosan, a cationic polysaccharide obtained by alkaline deacetylation of chitin, a principle component of exoskeleton in organisms like crustaceans [Chandy and Sharma, 1990], is well documented for its anti-bacterial and anti-fungal properties [Allan and Hadwiger 1979; Stossel and Leuba 1984].

A lot of inconsistent results have been published on rodent model [Soon-Shiong, 1994], some demonstrating long-term success, while others reporting severe fibrous outgrowth and graft malfunction within a short period following implantation. Our transplantation studies did not show any signs of graft failure during the tenure of study with the use of Islets/hPDMSCs packed in biocompatible capsules. These capsules showed proper graft functionality upon transplantation suggesting proper glucose sensing, insulin release and normal IPGTT by the encapsulated islets. The microcapsule membrane thus allowed the passage of low molecular weight
substances (mainly glucose and insulin) through the capsular membrane but not of immunocytes and other macromolecules.

Chang et al. [2007] in their transplantation study have observed that upon transplantation of spheroid bodies generated from hPDMSCs (SB-PDMSCs) into STZ induced diabetic mice revert back to normoglycemia while only hPDMSCs transplanted animals remained diabetic. This was in contradiction with our observations. In our in vitro study both undifferentiated hPDMSCs as well as differentiated hPDMSCs (i.e. ILCs) showed reduction in blood glucose concentration while progressive increase in body weight. The hPDMSCs have been shown to be safe with no immune rejection and teratomas formation on xenotransplantation [Keyser, et al. 2007]. IPGTT performed on ILCs and hPDMSCs transplanted STZ treated experimental Balb/C mice further documented reversal of experimental diabetes. This normoglycemic condition was maintained for over 3 months follow up. Hyperglycemia reaccoured after the removal ILCs grafted biocompatible capsules indicating the normoglycemic stage was due to the transplanted ILCs.

The present study showed that the placenta-derived MSC-like cells could be easily isolated and expanded in medium supplemented only with hUCBS without morphological and characteristic changes. Along with the ease of accessibility, lack of ethical concerns, and abundant cell number, hPDMSCs may be an attractive, alternative source of progenitor / stem cells for basic or translational research. Our data on the differentiation capabilities of hPDMSCs are highly promising, and exhibiting the potential to differentiate into islets with capacity of insulin secretion in an undifferentiated state, offering yet another non pancreatic, readily available, noninvasive and inexhaustible source of stem cells for islet generation for cell replacement therapy in diabetes.