Chapter 1

REVIEW OF LITERATURE AND INTRODUCTION

“Man may be the captain of his fate, but he is also the victim of his blood sugar”

[Oakley, 1962]

Diabetes mellitus (DM) is a clinically and genetically heterogeneous group of disorders, usually due to a combination of hereditary and environmental factors [Tierney, et al. 2002] characterized by abnormally high blood glucose levels due to defects in either insulin secretion (Type 1 DM) or insulin resistance (Type 2 DM) of the body’s cells to the action of insulin, or due to combination of these [Rother, 2007]. Diabetes mellitus, long considered a disease of minor significance to world health, is now taking its place as one of the main threats to human health in the 21st century [Zimmet, 2000 and Wild et al. 2004]. Changes in human behavior and lifestyle over the last century have resulted in a dramatic increase in the incidence of diabetes worldwide. The past two decades have seen an explosive (almost 46%) increase in the number of people diagnosed with diabetes worldwide, [Amos, et al. 1997, King, et al. 1998, Zimmet, et al. 2001 and Wild, et al. 2004] with more than 40% in India [Ramachandran, et al. 2001]. The global prevalence of diabetes is shifting significantly from the developed countries to the developing countries [Wild, et al., 2004].

DM is currently a chronic disease without a cure; however, type 2 diabetes can be managed with a combination of dietary treatment, medication, exercise, and insulin supplementation. A sustained C-peptide production and successful insulin independence continuously for five years after pancreatic islet transplantation in type 1 diabetic patients has showed a ray of hope [Ryan, et al. 2005]. Since then, the islet transplantation is increasingly being used as a cell replacement therapy for type 1 diabetes [Limbert, et al. 2008]. However, the need for ongoing immunosuppressive therapy and the scarcity of donor islets have precluded the widespread adoption of islet transplantation. Although xenotransplantation (for example, porcine islets) could provide a virtually inexhaustible source of islets for transplantation [Cozzi and Bosio, 2008, Dufrane and Gianello, 2008] the concern about infection by animal retroviruses and certain ethical issues limit the use of this potential source. Hence, there is a need to look for new sources of islet tissues to meet the potential demand for islet cell transplantation.
The Pancreas
The pancreas was first identified by Herophilus (335-280 BC) a Greek anatomist and surgeon. Only a few hundred years later, Ruphos, another Greek anatomist, gave the pancreas its name. The term "pancreas" is derived from the Greek pan, "all", and kreas, "flesh" [Harper, 2001].

Pancreatic development and organogenesis
The pancreas develops from the embryonic foregut and is therefore of endodermal origin. Pancreatic development begins with the formation of ventral and dorsal buds. Differential rotation and fusion of the ventral and dorsal pancreatic buds results in the formation of the definitive pancreas [Carlson, 2004]. Differentiation of cells of the pancreas proceeds through two different pathways, corresponding to the dual endocrine and exocrine functions of the pancreas. Under a microscope (Fig 1.1),

![Histology of human pancreas- H&E staining. InD - intralobular duct, IrD - interlobular duct, CT - connective tissue, BV - blood vessel, IL - islet of Langerhans.](http://www.medicalhistology.us/twiki/pub/ChapterThirteen/Slides/b36_interlobular_duct_pancreas_10x_labelled.jpg)

H&E stained sections of the pancreas reveal two different types of parenchymal tissues. Darker stained acinar cells belong to the exocrine pancreas and secrete digestive enzymes into the gut via a system of ducts, where as lightly staining clusters of endocrine cells are called 'islets of Langerhans', which are compact spheroidal clusters, embedded in the exocrine tissue. A healthy adult human pancreas, constitute one to two million Islets (~1 to 1.5% of the mass of the pancreas) [Elayat, et al. 1995 and Bonner-Weir, et al. 2000].

Development of the exocrine acini progresses through three successive stages. These include the predifferentiated, protodifferentiated, and differentiated stages, which correspond to undetectable, low, and high levels of digestive enzyme activity,
respectively. Progenitor cells of the endocrine pancreas arise from cells of the protodifferentiated stage of the exocrine pancreas [Carlson, 2004]. Under the influence of neurogenin-3 (Ngn3) and Isl-1, but in the absence of Notch receptor signaling, these cells differentiate to form two lines of committed endocrine precursor cells. In the early embryonic development; beta (β)-cells originate from a distinct population of Ngn3-positive progenitor cells [Edlund, 2002, Gradwohl, et al. 2000]. Subsequently, during the late fetal gestation period, there is a massive increase in the β-cell mass, major source due to neogenesis from non-endocrine Ngn3-positive progenitor cells [McEvoy, et al. 1980, Eriksson and Swenne, 1982, Swenne and Eriksson, 1982].

During fetal development, the process that guides pancreatic precursor cells to form each of the distinct endocrine cell types involves the sequential expression of a number of pancreatic transcription factors, including Nkx2.2, Nkx6.1, Pdx1, NeuroD1, Ngn3, Pax4, and Pax6. [Smith et al. 1991]

Alpha (α) and gamma (γ) – cells, which produce the peptides glucagon [White, 1999] and pancreatic polypeptide (PP) respectively were formed under the influence of Pax-6; while Pax-4 produces beta (β) - and delta (δ)-cells, which secrete insulin, and also an insulin antagonist called amylin [Leahy and Cefalu, 2002] and somatostatin [Costanzo, 2003] respectively. More recently, a fifth peptide hormone, ghrelin has been identified in the human islet. Ghrelin is produced mainly in the stomach and functions to increase the secretion of growth hormone and regulate food intake and energy balance [Kojima, et al. 2001]. Ghrelin is expressed in epsilon (ε)-cells in the human islet. Expression of ghrelin has variably also been reported to be in α cells [Date, et al. 2002], β cells [Volante, et al. 2002], or in a unique islet cell type [Wierup, et al., 2002] leading to controversy. The function of ghrelin within the
islet is also unknown, but it may have a paracrine role in regulating insulin secretion. A proportion of the adult islet cells make peptide YY in addition to their principal product [Ali-Rachedi, et al. 1984].

The respective hormones from α cells (15-20% of total islet cells), β cells (65-80%), δ cells (3-10%), PP cells (3-5%) and ε cells (<1%) are secreted directly into the bloodstream. Of all these, β-cells, make up the majority of cells in the islets. The polypeptide hormone insulin is among the best studied hormones, having been the first protein for which the complete amino acid sequence was determined and the first hormone to be molecularly cloned [German, 2004]. Insulin can be detected in the fetal circulation by the fourth or fifth month of fetal development [Carlson, 2004]. Insulin is of profound importance in the regulation of carbohydrate, fat and protein metabolism,

Diabetes mellitus

Type 1 DM patients are insulin dependant as a result of autoimmune destruction of pancreatic β-cells. In contrast, type 2 DM is mainly caused by a combination of insulin resistance and inadequate insulin secretion. It is now well established that in type 2 DM, β-cell mass is reduced by 50% [Limbert, et al. 2008]. In both the forms of DM there is certainly an imbalance between β-cell birth and death.

Beta-cells death and/or dysfunction

The β-cells death and/or dysfunction would result in an insufficient amount of insulin that leads to high glucose levels in the blood, known as Diabetes mellitus [WHO, 1999]. Diabetes occurs when pancreatic β-cell performance is compromised, either due to loss of β-cell mass caused by an autoimmune attack (type 1) or reduced β-cell mass and or function (type 2), leading to absolute or relative insulin deficiency [Leahy, 2005]. Regulation of the β-cells mass appears to involve a balance of β-cell replication and apoptosis but, at the molecular level, pancreatic β-cell loss by apoptosis appears to play an important role in the development of insulin deficiency and the onset and/or progression of the disease [Lupi and Del Prato, 2008]. When islets are maintained in the presence of high glucose, they may release interleukin-1 β (IL-1 β) and undergo apoptosis [Maedler, et al. 2002]. Genetically engineered mice, which lacked insulin receptors specifically in β-cells, exhibited considerably decreased β-cell mass and developed diabetes [Zulewski, et al. 2001, Otani, et al. 2004]. The rapid degradation of carboxypeptidase E plays a significant role in β-cell death in response to the free fatty acid palmitate. These newly identified targets of β-
cell lipotoxicity present novel avenues for research and therapeutic intervention [Johnson, 2009]. The development of strategies to avoid β-cell mass reduction, both in vivo and in vitro, could prove a promising portion for cell based therapy of type 1 and type 2 diabetes.

**Treatments for Diabetes mellitus**

The year 2008 has been marked by tremendous activity and possibilities for improvement in the treatment of patients with type 1 as well as type 2 diabetes.

**Insulin therapy**

Insulin therapy for diabetes has been utilized over the last 85 years, since the first patient was treated in 1922. Although, the most advanced insulin preparations and intensified insulin regimens can improve blood glucose level, exogenous insulin administration cannot ensure continuous blood glucose control and prevention of the onset of chronic deleterious complications [Kaestner, 2007].

The existing therapies with exogenous insulin or hypoglycemic agents for diabetes are frequently inadequate, resulting in significant morbidity and mortality to patients, as they do not offer a cure, and fail to prevent the secondary complications associated with diabetes [Nathan, 1993]. Hence, investigators are exploring alternative treatments to substitute exogenous insulin therapy. It is now well accepted that the cure for type 1 diabetes and for many cases of type 2 diabetes requires either regeneration or replacement of insulin producing cells. Finding a functional substitute for the ‘missing -cell’ or restoring regeneration capacities is a major goal in the field of diabetes research.

**Human pancreatic islet transplantation**

In 1967, Lacy introduced the idea of islet transplantation [Trucco, 2005]. However, it did not become a successful reality for human treatment until 2000, when Shapiro and associates introduced the Edmonton protocol: collecting islets from 2-4 donor pancreata in combination with glucocorticoid-free immunosuppressive regimen [Shapiro, *et al.* 2000]. The results for Edmonton protocol established a landmark towards a cure for diabetes. Compared to whole pancreas transplantation, the transplantation of human pancreatic islets is technically easier, has lower morbidity, and permits storage of islet graft in tissue culture or cryopreservation for banking [Nanji, *et al.* 2006]. The low morbidity of the procedure and the potential for inducing
tolerance to the grafted tissue define islet transplantation as a promising strategy for correcting diabetes in young patients, including children [Hathout, et al. 2003]. Transplantation of a sufficient number of pancreatic islets can normalize blood glucose levels and may prevent the devastating complications of diabetes [Shapiro et al. 2000, Street et al. 2004]. Upon larger number of islets transplantation, the C-peptide levels in serum increases and the exogenous insulin requirement decreases, resulting in a greater probability of insulin independence [Bertuzzi and Ricordi, 2007]. However, in spite of the progress achieved, islet transplantation does not offer an adequate solution for a permanent cure of hyperglycemia with significant long-term clinical benefit for all diabetic patients in need. The islets isolated from pancreas of donors are in short supply and caters only small percentage of patients [Chatenoud, 2008] and the number of islets required to achieve independence from insulin injections is very high and resources of human donor organs to provide islet grafts are limited. About 850,000 (11,000 islet equivalent/kg body weight) islets are required to achieve successful transplantation outcomes with the Edmonton protocol. With islet auto-transplantation after total pancreatectomy, it has been estimated that a minimum of 300,000 islets are necessary to achieve insulin independence in 70% of recipients [Pyzdrowski, et al. 1992]. In a 5-year follow-up study after clinical islet transplantation, only a minority of patients (10%) maintained insulin independence and the average duration of insulin independence lasted only for 15 months [Ryan, et al. 2005]. In addition, most allogenic grafts usually last for no more than 2 years and islet transplantation has significant side effects due to the accompanying immunosuppressive therapy.

Nevertheless, advances in procurement techniques from cadaveric donors and improvements concerning less toxic and more potent immunosuppression techniques should progressively lead to lower islet requirements to control glycemia [Rood, et al. 2006]. Thus, achieving successful single donor islet transplantation is currently a major challenge. The results obtained through human pancreatic islet transplantation were an encouraging advancement in the efforts to generate new sources of insulin producing cells and to develop new therapeutic strategies [Weir and Bonner-Weir, 2004, Ahren, 2005].

**Islet (β-cell) replication and / or regeneration**

Therapies that increase functional β-cell mass may offer a cure for diabetes. Efforts to achieve this goal encompass several directions. In principle, it could be achieved by regeneration and/or replication of the patient’s own β cells. The regeneration of
pancreatic β-cells *in vivo* could be a potential therapeutic approach for diabetes treatment emerging from research bench; it might also be a big step to cure diabetes finally [Trucco, 2005, Yamaoka, 2002, Suarez-Pinzon, *et al.* 2008].

The sources of new adult β-cells are debatable with the extent to which they contribute in β-cell mass / turnover and expansion. Beta cells, in our body have the ability to undergo continuous turnover, with lower growth rate [Teta, *et al.* 2005 and Chen, 2007], measured to be between 0.5 to 2% [Swenne, *et al.* 1984]. Today, most investigators agree that new adult β-cells, for the most part, are replicated from proliferation of pre-existing β-cells and only a small number of new adult β-cells neogenesised from stem/progenitor cells under some circumstances. The β-cell mass continues to increase throughout the neonatal period, both by replication of differentiated β-cells and neogenesis from stem/progenitor cells [Bouwens, *et al.* 1994 Swenne, 1992], but has also been observed in the regenerating adult pancreas [Smith, *et al.* 1991, Hardikar and Bhonde, 1999]. Very recently, Fiaschi-Taesch *et al.* have shown that cdk-6 and a D-cyclin partner can be used to markedly accelerate replication of human beta cells in vitro. Most importantly, combined over expression of cdk-6 with cyclin D1 also leads to human β-cells replication *in vivo*, and results in enhanced human islet engraftment and function in an *in vivo* transplant diabetes model [Fiaschi-Taesch, *et al.* 2009].

The two basic mechanisms for expansion of β-cell mass during late stages of fetal development, replication of pre-existing β-cells, and differentiation from pancreatic duct epithelium, also exist during adult life [Soria, *et al.* 2000] and may contribute to the regulation of islet mass in the adult [Montanya, *et al.* 2000]. Some evidences suggest that new adult β-cells could be neogenesised from progenitor cells residing in the epithelium of the pancreatic ducts [Pavlovic, *et al.*1999, Bonner-Weir, *et al.* 2004, Katdare, *et al.* 2004]. Therefore, β-cells continuously undergo apoptosis at the end of their life span, albeit quite slowly, when they are possibly replaced by newly generated “mesenchymal-epithelial transition” cells from the ducts [Tosh and Slack,
2002]. After stimulation, intra-islet progenitors can generate newly formed β-cells within 2-3 days [Zulewski, et al. 2001]. These mesenchymal-type cells, which exhibit no insulin expression, can then be induced to differentiate into insulin-expressing islet-like cell aggregates, which reestablish the epithelial character typical of islet cells [Gershengorn, et al. 2004].

There are reports indicating that either ductal cells or intra-islet cell progenitors can produce insulin producing cells as well as other pancreatic cell types in vitro [Bonner-Wier, et al. 2000, Banerjee and Bhonde, 2003, Lechner and Habener, 2003 and Noguchi, et al. 2006]. In experiments, both cell types could be expanded to some extent during in vitro culture, and then differentiate into insulin producing cells. However, the amount of insulin released, and their glucose responsiveness, seems to be reduced when compared with normal isolated islets [Zulewski, et al. 2001, Bonner-Wier, et al. 2000].

Other studies, providing supportive evidence to the neogenesis theory suggest that new β-cells originate from intra-islet progenitor cells, which have a high replicative potential [Banerjee and Bhonde, 2003, Xu, et al. 2006, Teng, et al. 2007, and Jetton, et al. 2008]. Also, it has been proved that endogenous β-cell regeneration may be achieved if the autoimmune diseases can be halted early, or soon after diabetes onset, by regeneration compatible drugs [Nir, et al. 2007]. Considering the potential of these pancreatic duct cells to serve as progenitors for new β-cells in the adult, their manipulation constitutes a very promising therapeutic approach for diabetes.

Different models have been used to explore which factors contribute to islet cell proliferation and neogenesis, for example, partial pancreatectomy, streptozotocin treatment, and cellophane wrapping. These studies resulted in the identification of factors that may be useful to drive in vitro differentiation, although it has not yet been clearly established as to which of these are critical for the survival, proliferation, and differentiation of pancreatic β-cells.

The β-cell regeneration in vivo is providing novel potential therapeutic approach to replace the β-cells lost due to autoimmune destruction in type 1 diabetes, or restore the β-cell mass and functions damaged due to the failure of compensation and β-cell apoptosis in type 2 diabetes.
The reports also suggested that β-cells were transdifferentiated from hepatocytes [Sapir, et al. 2005] and pancreatic acinar cells [Lipsett and Finegood, 2002, Bonner-Weir, et al. 2008] represent additional pathways that may lead to adult β-cell formation. However, Desai and colleagues traced the lineage of β-cells and showed that β-cells did not arise from acinar cells. Instead, they found that acinar cells gave rise to more acinar cells [Desai, 2007]. Recently, using a strategy of re-expressing key developmental regulators in vivo, Zhou and his colleague identified a specific combination of three transcription factors (Ngn3, Pdx1 and Mafa) that reprograms differentiated pancreatic exocrine cells in adult mice into cells that closely resemble β-cells [Zhou et al. 2008].

In vitro, there is strong evidence that new pancreatic islets can be derived from progenitor cells present within the ducts and islets [Cornelius, et al. 1997, Ramiya, et al. 2000, Gao, et al. 2003, Banerjee, et al. 2005], in a process called “neogenesis”. Furthermore, newly generated islets, from ductal precursor cells, have been shown to restore normoglycemia, when transplanted into experimental diabetic mice [Katdare, et al. 2003]. In a recent important study, experimentally induced damage to the mouse pancreas resulted in the up-regulation of progenitor cells with the capacity to differentiate into different islet endocrine cells, indicative of the existence of multipotent pancreatic progenitor cells [Xu, et al. 2008]. Putative progenitor cells have been localized previously to the exocrine pancreas [Efrat, 2008, Zhao, et al. 2007] to pancreatic ducts [Bonner-Weir, et al. 2000, Yatoh, et al. 2007] and to endocrine islets [Zulewski, et al. 2001, Davani, et al. 2007], suggesting a widespread distribution throughout the pancreas. The prospect of isolating, expanding and differentiating adult pancreatic progenitor cells to a β-cells phenotype has obvious therapeutic potential but at present the precise identity of these cells remains elusive.

Stem cell Therapy as a source for islet neogenesis

Stem cells have the ability to self-renew indefinitely by asymmetric cell division and a potential to differentiate into one or more specialized cell-types [Gordon and Blackett, 1998, Scheffler, et al. 1999, Weissman, 2000]. Stem cells of embryonic or adult origin have become the favorable and attractive target of research in the biomedical sciences as they offer solution to overcome the technical difficulties associated with conventional cell therapy by their ability to proliferate, replicate in a controlled laboratory conditions and to differentiate into a wide range of tissue types.
In developing a potential therapy for patients with diabetes, the stem cell system needs to meet several criteria. For diabetes therapy, it is not clear whether it will be desirable to produce only β-cells or whether other types of pancreatic islet cells (PP cell, δ cells) and acinar cells are also essential. Recent trends indicate that isolated β cells cultured in the absence of the other islet cells (α, δ, PP) are less responsive to changes in glucose concentration than intact islet clusters made up of all islet cell types [Burns, et al. 2004]. Perhaps the most important issue is the choice of the appropriate sources of stem cells.

Embryonic Stem Cells:
October 2008, marks the 10th anniversary of the first reported derivation of human Embryonic Stem (ES) cells [Thomson, et al. 1998]. The report was met with much acclaim as it was quickly recognized that the ability of these pluripotent stem cells derived from the inner mass of the mammalian blastocyst to differentiate indefinitely into all cell types/germ layers of the human body; could provide a source of cells both in vivo and in vitro for replacing tissues lost to injury and disease - the ultimate goal of regenerative medicine.

Regardless of the species, undifferentiated ES cells express several cell surface markers; these include stage specific embryonic antigen, SSEA1 in mouse and SSEA-3 and SSEA-4 in human, as well as tumor recognition antigens TRA-1-60 and TRA-1-81 in human [Enver, et al. 2005, Adewumi, et al. 2007, Fong, et al. 2009]. In
the uncommitted state both mouse and human ES cells express alkaline phosphatase, the POU-domain transcription factor Oct-4, and telomerase [Adewumi, et al. 2007]. When removed from the specific culture conditions required to maintain an undifferentiated state, ES cell lines from both species can spontaneously differentiated in vitro to form a variety of cell types derived from each of the three germ layers. Even without the addition of any exogenous growth factors, ES cells allowed to differentiate will spontaneously form neurons [Bain, et al. 1995, Wichterle, et al. 2002], cardiomyocytes [Maltsev, et al. 1994, Wobus, et al. 1997], muscle cells [Rohwedel, et al. 1994], hematopoietic cells [Kaufman, et al. 2001] pancreatic precursors cells [Kahan, et al. 2003, Zulewski, 2008] and many other cell types [Doetschman, et al. 1985, Keller, 1995, Baker and Lyons, 1996, Yamane, et al. 1999]. Although these procedures provide a starting point for producing specific cells for possible applications, whether in regenerative medicine, or as tools for drug discovery or as disease models, little is understood about the underlying mechanisms. Even if, limited success has been achieved by manipulating culture conditions to drive differentiation of human ES cells along particular lineages, there is little evidence to support the view that such conditions specifically direct differentiation rather than select for propagation or survival of cells generated by spontaneous processes. A number of chemical agents and growth factors such as retinoic acid or bone morphogenic protein (BMP) are well known to have physiological roles in embryonic development and have also been used to promote differentiation of ES cells in culture [Schuldiner, et al. 2000]. However, even in these cases, there is a tendency for differentiation to follow broad lineages such as ectodermal or mesodermal; the cell types generated also tend to be heterogeneous [Gokhale and Andrews, 2009].

Actually, insulin secretion in differentiated ES cells never exceeded 1.6% of the amount that a β-cell typically secretes. This is about the same production level found in non-β insulin producing cells, such as fetal liver cells or certain neuronal cells [Alarcon, et al. 1998, Devaskar, et al. 1994].

Identification of a pancreatic progenitor cells would be an important step to isolate large numbers of cells that could be readily differentiated into islets or β-cell. Based on the expression of many neuronal cell markers, a neuroectodermal origin of pancreatic endocrine cells has been hypothesized for many years. It was suggested that the intermediate filament protein nestin, which is expressed in neuronal precursors might be marker of islet progenitor cells [Hunziker and Stein, 2000]. Several groups have used protocols designed to enrich for neural precursors, characterized by expression of nestin, in attempts to coax ES cells to adopt a pancreatic islet fate [Lumelsky, et al. 2001, Zulewski, et al. 2001]. However, Lumelsky and coworkers [Lumelsky, et al. 2001] observed that using a differentiation protocol for neuronal progenitor cells, the differentiation of nestin-positive ES cells into β-like cells, had a limited effect. Only very few cells were insulin-positive and the resulting cell aggregates did not express the Pdx-1 (pancreatic development homeobox 1, a homeodomain protein absolutely required for pancreatic development in both human and mice) and the majority of cells exhibited a neuronal phenotype. There is some controversy about the use of nestin as a progenitor marker and its significance in islet neogenesis. Some authors considered nestin as a neuroepithelial precursor marker (neural cell adhesion molecule), which is also essential in multipotential progenitor cells of pancreas, present both in rat and in human pancreatic islets (called nestin-positive islet-derived progenitors) [Huang and Tang, 2003, Zulewski, et al. 2001]. Others have reported that nestin only marks a population of mesenchymal or endothelial cell types, thereby excluding role of nestin in islet cell development [Selander, et al. 2002, Klein et al. 2003]. Recently, it has been demonstrated that the nestin positive cells in the pancreas of mice of different ages is immunolocalized of with reference to insulin and glucagon positive cells. [Dorisetty, et al. 2008]

The proliferative capacity of ES cells is attractive but their applications are limited due to risk of teratocarcinoma formation [Blyszczuk, et al. 2004, Hori, et al. 2002, Sipione, et al. 2004]. The major concern with ES cells is considerable ethical issues regarding the use of human ES cells followed by their pluripotency and plasticity, to create a mixture of many different cells failing to produce the homologues population of fully
differentiated β-cells required for transplantation therapy [Zulewski, 2006]. Moreover, considering that ES cells are by definition immortal, their poor survival when differentiated was unexpected. Even though ES cells would seem to constitute an ideal source for cell replacement therapies for many human diseases, recent events have shown that some spectacular results have to be reanalyzed very carefully [Mandavilli, 2005].

The great advantage of ES cells over other stem cells is that they can generate many potentially useful cell types - but that is also their disadvantage. To use ES cells effectively in regenerative medicine, or in other applications, such as disease modeling or drug discovery, applications that are often overlooked in popular discussion, it is essential that we understand how to control their differentiation. This is necessary if one is to expand cultures of the undifferentiated cells to a usable scale, free of potential pathogens. It is also necessary if one is to produce specified cell types, free of other unwanted cells, and if the genetic fidelity of the cells is to be preserved, a factor that was not immediately apparent when these cells were first derived. These issues present substantial challenges [Gokhale and Andrews, 2009]. Nevertheless, in diabetes research, human ES cells may help to decode some crucial steps in vitro, since almost all the data available on pancreas development were obtained from animal models. To activate the differentiation programs, ES cells are forced to aggregate into embryonic bodies (EBs) by culturing in suspension and in the absence of leukemia inhibitory factor (LIF). These unique properties make ES cells of great interest as a source to obtained insulin producing cells for diabetes treatment [Santana, et al. 2004].

Recent success in generating insulin-secreting islet-like cells from human embryonic stem (ES) cells, in combination with the success in deriving human ES cell - like induced pluripotent stem (iPS) cells from human fibroblasts by defined factors, have raised the possibility that patient - specific insulin - secreting islet-like cells might be derived from somatic cells through cell fate reprogramming using defined factors [Takahashi, et al. 2007]. Although the precise relationship of iPS and ES cells remains to be explored in detail, the advent of iPS cell technology circumvents the ethical, legal and logistical problems associated with the need for human embryos to derive ES cell lines, and has started a democratization process that should see a substantial expansion in the study of pluripotent cell biology. With all these results in mind, insulin producing, C-peptide and glucagon positive islet-like clusters (ILCs) from the iPS cells were derived from human skin cells by retroviral expression of
Oct4, SOX2, c-MYC, and KLF4 under feeder-free conditions [Tateishi, et al. 2008]. The major hurdles that must be overcome to enable the broad clinical translation of these advances include teratoma formation by ES and iPS cells, and the need for immunosuppressive drugs. Thus, the creation of human - animal hybrid embryos by iPS - proposed as a way to generate embryonic stem cells without relying on scarce human eggs - has met with legislative hurdles and public outcry. But a very recent report [Young, et al. 2009] suggests that the approach has another, more fundamental problem: it may simply not work - as these hybrid embryos fail to grow beyond 16 cells. Hence, it is not yet sure whether iPS cells lives up to all the great hopes given to them.

Adult Stem Cells:

Several adult tissues, including blood, epidermis, liver, enterocytes and spermatogonia are replenished throughout the life. This observation of the regenerative potential of adult tissue led to the concept of adult stem cells. Adult stem cells are rare cells with low proliferation capacity, which according to present hypotheses reside in stem cell niches and give rise to a transient amplifying cell pool that differentiates, thereby regenerating the respective tissues [Ebert, et al. 2006] except germ layer and further classified as hematopoietic stem cells [HSC] and mesenchymal stem cells (MSC).

Although MSCs were originally isolated from bone marrow [Pittenger, et al. 1999], recent years have witnessed an explosion in the number of adult stem cell populations isolated and characterized. Every tissue or organ, from adipose tissue [Zuk, et al. 2001], placenta [In’t Anker, et al. 2004], amniotic fluid [In’t Anker, et al. 2003], umbilical cord blood [Bieback, et al. 2004, Kogler, et al. 2004] etc. exhibits stem cell population. Some studies have suggested that there may be a greater degree of plasticity, perhaps even pluripotency, associated with adult stem cells than was previously believed. The multilineage differentiation potential of MSCs derived from variety of different species has been extensively studied in vitro since their first discovery in 1960 [Friedenstein, et al. 1966]. An excitement ensued when, in 1998, Ferrari et al. showed transplantation of genetically marked bone marrow into immunodeficient mice migrated into areas of induced muscle degeneration, undergo myogenic differentiation, and participated in the regeneration of the damaged fibers. Subsequently, numerous publications [Joshi and Enver, 2002, Goodell, 2003, Raff, 2003] have described various events, in which adult stem cells from one organ give rise to cell type characteristics of different organ. The capacity to differentiate into
multiple mesenchymal lineages including cartilage [Kadiyala, et al. 1997, Johnstone and Yoo, 1999], bone [Bruder, et al. 1997 and 1998, Kadiyala, et al. 1997, Richards, et al. 1999], and adipose tissue [Prockop, 1997, Dennis et al. 1999] is being used as a functional criterion to define MSCs. This ability has rendered MSC an ideal candidate cell source for clinical tissue regeneration strategies, including the augmentation and local repair and regeneration of specific lineage. Recent studies indicated the identification of pluripotent cells that not only differentiate into cells of mesoderm lineage, but also into endoderm and neuroectoderm lineages, including neurons [Sanchez-Ramos, et al. 2000], hepatocytes [Schwartz, et al. 2002], and endothelia [Caplan and Bruder, 2001].

Individual colonies derived from single MSC precursors have also been reported to be heterogeneous in terms of their multilineage differentiation potential. Only one third of adherent bone marrow derived MSC clones are pluripotent [Pittenger et al. 1999]. Furthermore, nonimortalized cell clones examined by Muraglia et al. in 2000 demonstrated that 30% of the in vitro derived MSC clones exhibited a tri-lineage (osteogenic/chondrogenic/adipogenic) differentiation potential, while the reminder displayed a bi-lineage (osteogenic/chondrogenic) or uni-lineage (osteogenic). These observations are consistent with other in vitro studies using conditional immortalized clones. [Majumdar et al. 1998, Dormady, et al. 2001, Osyczka et al. 2002]. It has also been observed that only 58.8% of the single colony derived clones had the ability to form bone within Hydroxyapatite-tricalcium phosphate ceramic scaffolds post implantation in immunodeficient mice [Kuzenetsov et al. 1997]. All these results indicate heterogeneous nature of clonally derived MSCs with respect to their developmental potential.

At present, there is no specific marker or combination of markers have been identified that specifically defined MSCs [Baksh et al. 2004, Nauta and Fibbe, 2007]. Phenotypically, ex vivo expanded MSCs express a number of non specific markers, including CD29, CD44, CD73, CD90, CD105, CD166 [Pittenger, et al. 1999, Deans and Moseley, 2000]. Despite this controversy of what defines a mesenchymal stem cell, there is general agreement that MSCs lack typical hematopoietic antigens, namely CD14, CD34 and CD45 [Pittenger, et al. 1999].

With specific regards to pancreatic lineages there has been a report of differentiation/transdifferentiation by Ianus and colleagues [Ianus, et al. 2003] who studied in vivo differentiation of adult bone marrow derived cells into pancreatic
endocrine cells. Their results suggested that a population of cells within the bone marrow has the capacity to transdifferentiate into cells that can populate and perhaps function within, the endocrine pancreas. One study using streptozotocin (STZ) – induced pancreas damage demonstrated that pancreatic cell proliferation was induced after bone marrow transplantation [Hess, et al. 2003]. The stem cells from bone marrow are capable of producing a whole spectrum of cell types; highlighting the opportunity to manipulate these cells for therapeutic use as they have been shown to cross the lineage boundaries [Serakinci and Keith, 2006]. It has been shown that the human bone marrow derived mesenchymal stem cells could be induced to differentiate into functional insulin producing cells using Pdx-1, delivered by recombinant adenovirus. More recent reports suggest that bone marrow stem cells reversed experimental diabetes *in vivo* by enhancing the regeneration and survival of endogenous β-cells rather than repopulating the islets with trans-differentiated β-cells [Hasegawa, et al. 2007, Urban, et al. 2008]. Several *in vitro* studies have demonstrated that rodent bone marrow stem cells can adopt an insulin-expressing phenotype [Tanget, et al. 2004], and driving the phenotype of human bone marrow stem cells by the forced expression of β-cells transcription factors generated cells capable of glucose responsive insulin secretion [Karnieli, et al. 2007]. Banerjee and colleagues have demonstrated reversal of experimental diabetes in STZ diabetic mouse model by multiple injections of unfractionated bone marrow leading to induction of pancreatic regeneration, which is highly promising [Banerjee, et al. 2005], although the mechanism is not suggested. These studies point towards futuristic therapeutic approach of auto transplantation of bone marrow to cure diabetes.

Apart from bone marrow the mature liver has been shown to serve as a potential source of tissue for generating functional endocrine pancreas. This may allow the diabetic patient to be the donor of his or her own therapeutic tissue; thus alleviating both the needs for allo-transplantation and the subsequent immune suppression [Meivar-Levy and Ferber, 2006]. There have been sporadic reports that progenitor/stem cells from other tissues can be induced to differentiate into insulin expressing cells, including cells localized to intestinal epithelium, dermis, spleen, salivary gland and blood monocytes, endometrium / menstrual blood [Meng, et al. 2007]. These studies have not always proved to be reproducible, and have been reviewed recently [Burke, et al. 2007, Limbert, et al. 2008, Efrat, 2008]. Thus, with the ability to differentiate into multiple lineages (multipotent) and immunosuppressive effects, adult stem cells became an attractive alternative to human ES cells in
regenerative medicine and cell-based therapy against various human diseases including type 1 diabetes [Liu and Han, 2008]. In particular, adult stem cells have many attractive features as a source of functional transplantable cellular material when compared to ES cells.

Fetal stem cells, comprising the final broad stem cell class, have a comparatively recent history. They can be isolated from two distinct sources, the fetus proper and the supportive extra-embryonic structures. Stem cells derived from the extra-embryonic sources are particularly interesting due to their potential clinical utility. Over the past decades fetal stem cells have been isolated from multiple extra-embryonic tissues, reminiscent of gradual broadening of stem cell sources seen in the adult. Amniotic fluid, amnion, umbilical cord blood, Wharton’s jelly and placenta have all generated putative stem cells. These tissues, collectively also known as the afterbirth (after the fetus delivery, placenta begins a physiological separation for spontaneous expulsion afterwards along with all umbilical cord, amnion etc. therefore, called the afterbirth) are routinely discarded at parturition as a biological waste. However, this situation may change in the near future, as a growing number of reports demonstrated greater potential of these tissues as ‘store house’ of stem cells which makes it a valuable alternative stem cell source with fewer concerns in terms of ethical controversy and moral issues [Marcus and Woodbury, 2008] of the resident stem cell populations.

The first isolated fetal stem cells were hematopoietic, derived from human umbilical cord blood. The isolated cells were capable of long-term self-renewal and differentiation to multiple hematopoietic lineages [Knudtzon, 1974, Ueno, et al. 1981]. Clinically, cord blood stem cells were successfully employed in a bone marrow transplant in 1988 [Broxmeyer, et al. 1989]. Stem cells from extra-embryonic tissue expressed a number of mesenchymal cell surface markers, including CD90 and CD105. Subsequent work demonstrated the expression of Oct4 within a subset of most of the extra-embryonic stem cells. This is important, as Oct4 expression is associated with pluripotent cells such as embryonic germ cells and ES cells [Prusa, et al. 2003, Mitchell, et al. 2003, Weiss, et al. 2006]. This observation keeps extra-embryonic stem cells poised to join embryonic and adult stem cells. Following in vitro expansion, the isolated cells were capable of differentiating in vitro into chondrocytes, adipocytes and osteocytes. In addition to tri-lineage differentiation, stem cells derived from umbilical cord [Yang, et al. 2008], cord blood [Laitinen and Laine, 2007, Koblas, et al. 2005, Denner, et al. 2007], placenta [Poloni, et al. 2008], amniotic membrane
[Miki, et al. 2007] have shown potential for differentiation into insulin producing β-cells and have been considered as surrogate β-cells source for islet transplantation. Most importantly, an emerging body of data indicates that MSCs possess immunomodulatory properties [Rasmusson, 2006, Uccelli, et al. 2006, Alma, et al. 2007, Le Blanc and Ringdén, 2007] and may play specific roles as immunomodulators in maintenance of peripheral tolerance, transplantation tolerance, autoimmunity, tumor evasion, as well as fetal-maternal tolerance. These observations have further raised clinical interest in adult and its counterpart fetal MSCs.

Recent advance in adult/fetal stem cell technologies and basic biology has accelerated therapeutic opportunities aimed at eventual clinical applications. First, the extracorporeal nature of these stem cell sources facilitates isolation, eliminating patient risk that attends other adult stem cell isolation. Most significantly, the comparatively large volume of extra-embryonic tissues and ease of physical manipulation theoretically increases the number of stem cells that can be isolated. Second, ethical questions surrounding the use of human embryonic stem cells are essentially absent from the discussion of adult stem cells. Third, because they can be isolated from the prospective patient, they would be genetically matched, thus eliminating the need for immunosuppressive therapies. Fourth, the putative ability of extra embryonic stem cells to differentiate into cells from multitude of lineage suggests their use in treating a variety of diseases. Stem cells from extra-embryonic tissues represent an emerging area of research that bears tremendous potential with broad application to many different areas including normal and pathological development, assisted reproductive technology procedures and regenerative medicine [Hemberger, et al. 2008].

The extra-embryonic/adult stem cells have opened up the possibility of ‘fixing’ a particular genotype (either normal or diseased) in pluripotent stem cells. Isolation from tissues normally discarded at birth facilitates easy harvest and overcomes ethical concerns. The cells isolated from these tissues grow well in culture, appear capable of differentiation to multiple cell types and may be less likely to be rejected following transplantation. All these studies prompted us to examine the stem cell potential of the biological waste tissues of human origin such as umbilical cord, placenta, amnion and endometrium and to test their ability to differentiate into glucose responsive insulin producing islet like clusters for possible cell replacement therapy in diabetes as well as to create an islet model for in vitro studies.
With this rationale in mind the present study was designed to serve the following objectives:

1. To isolate, propagate and characterize mesenchymal stem cells from human umbilical cord, placenta, endometrium and amnion, which are generally discarded as a biological waste.

2. To assess multilineage differentiation potential of isolated mesenchymal stem cells from all the above sources under the influence of differentiation cocktails with special emphasis on pancreatic lineage.

3. To test the functionality of islet like clusters derived from mesenchymal stem cells of above sources *in vitro* and *in vivo* in terms of insulin secretion in response to glucose challenge.