AN *IN VITRO* STUDY OF MESENCHYME–ISLET CELL INTERACTIONS IN ISLET NEOGENESIS: A MODEL FOR TISSUE REPLACEMENT THERAPY IN DIABETES MELLITUS

by

Juziel Kampando Manda

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Supervisor: Dr. Venant Tchokonte-Nana, PhD

Co-Supervisor: Prof. Benedict John Page, PhD

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DECLARATION

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Dedication

To my parents.

Without your tireless support and endless encouragement, I would not have gone further with my education.

Abstract

Shortages of donor islets, immune rejection, and the need for life-long immuno-suppressors remain the clinical challenges of islet transplantation in the treatment of diabetes mellitus. An alternative to these challenges is the *in vivo* generation of beta cells within the patient's pancreas. The animal model of pancreatic injury has been reported to be a potential source of islet cells for tissue replacement therapy in type 1 diabetes mellitus. However, the *in vitro* regenerative capacity of endogenous beta cells in this model needs more investigation. This study investigated, *in vitro*, the effect of pancreatic duct ligation (PDL)-induced islet/duct-mesenchymal stromal cells (MSCs) interactions on islet and duct cells development and assessed the long-term transplantation outcome of islet-mesenchymal cells isografts. Islets, duct fragments, and MSCs were isolated from post PDL tissues harvested from eighty adult male Wistar rats (250 - 300g) 24- and 120 h following duct ligation. Islets or duct fragments were cultured with or without MSCs ([Islet/MSC+ or Islet/MSC-] or [PEDC/MSC+ or PEDC/MSC-]). Development of islets and duct fragments in culture were evaluated morphologically and by immunocytochemistry using antibodies against Pdx1, Ngn3, CK7 and insulin. Islets were also transplanted with or without MSCs (Islet/MSC+ or islet/MSC-) in diabetic animals (n = 40). Isografts survival and function were evaluated by monitoring blood glucose levels, and immunohistochemistry of graft tissues were studied. Results showed activation of $Pdx1^+$ islet cells in both cultures with or without MSCs, however, expansion of $Pdx1^+$ cells were promoted in the presence of MSCs and this was followed by activation of Ngn3 expression and expansion of $Ngn3^+$ cells, which was maintained in islet cells up to 4 weeks. This resulted into low levels of insulin expression in islet-like aggregates formed between the third and the fourth week. Co-

culturing of duct fragments with MSC similarly resulted into maintenance of endocrine precursors that expressed Ngn3, which later formed islet-like aggregates. In cultures with MSCs, duct epithelial cells developed growth areas with cells that co-expressed CK7 and Ngn3 in periductal cells. When periductal cells formed islet-like aggregates, Ngn3 co-expressed with insulin in islet-like cell clusters closer to ducts. Transplantation of early harvested (24 h PPDL) islets showed better curative capacity than late (84 h PPDL) islets. The average glucose levels were lower throughout the 5 weeks monitoring period in 24 h PPDL transplanted rats. The average time to reverse hyperglycemia in 80% of the 24 h PPDL transplant group was 32 ± 2 days (~4.5 weeks), while only 20% in the 84 h PPDL transplant group attained normoglycemia at 61 ± 2 days (~9 weeks) (p = 0.0011) post transplantation. Graft survival rate was higher in islets co-transplanted with MSC (Islet/MSC+) compared to grafts transplanted with islets alone (Islet/MSC-). Islet morphology and distribution of beta cells was normal in Islet/MSC+ similar to the endogenous islets in the pancreas. In conclusion, MSCs promote the expansion of Pdx1+ cells and maintain the expression of Ngn3 in islet cells and duct–derived neogenetic cells. MSCs prolong graft survival and improve the capacity of early harvested post PDL islets to reverse hyperglycemia; this novel observation may be applicable to clinical transplantation.

Opsomming

'n Tekort aan skenker eilandjies, immuun verwerping, sowel as die behoefte aan lewenslange immuno-onderdrukkers is van die grootste kliniese uitdagings in eiland oorplanting in die behandeling van diabetes mellitus. 'n Alternatief tot hierdie uitdagings is die *in vivo* generering van betaselle in die pankreas van die pasiënt. 'n Dier model vir pankreas beserings is voorheen berig as 'n potensiële bron van eiland selle vir weefsel vervangingsterapie in tipe 1-diabetes mellitus, maar verdere ondersoek met betrekking tot die *in vitro* regeneratiewe kapasiteit van endogene betaselle in hierdie model is nodig. Hierdie studie ondersoek, *in vitro*, die effek van post-pankreatiese buis afbinding (PPBA) - gëinduseerde eiland / buis-mesenkiemale stroma sel (MSS) interaksies op eiland en buis sel ontwikkeling en beoordeel die langtermyn oorplanting uitkoms van eiland-mesenkiemale selle. Eilandjies, buis fragmente, en MSS is geïsoleer vanaf tagtig volwasse mannetjie Wistar rotte (250 - 300g) en geoes 24- en 120- ure nadat buis afbinding voltooi is. Eilandjies of buis fragmente was gekweek in die aan- of af-wesigheid van MSS (eiland / MSS + of eiland / MSS -). Die ontwikkeling van eilandjie en buis fragmente in kultuur, is met behulp van morfologie asook die aanwesigheid van teenliggaampies vir Pdx1, Ngn3, CK7 en insulien, wat deur immunositochemie evalueer is. Eilandjies is ook in diabeet rotte (n = 40) oorgeplant in in die aan- of af - wesigheid van MSS (eiland / MSS + of eiland / MSS -). Oorplantingsoorlewing en funksie is evalueer deur bloed glukose vlakke te asseseer en ent weefsel immunohistochemie te bestudeer. Resultate het die aktivering van $Pdx1^{+}$ eiland selle in beide die aan- of afwesigheid van MSS aangetoon. Uitbreiding van $Pdx1^{+}$ selle is bevorder in die aanwesigheid van mss wat gevolg is deur 'aktivering van Ngn3 uitdrukking asook uitbreiding van $Ngn3^{+}$ selle, wat in eiland selle is in stand gehou is tot en met vier weke. Dit het gelei tot lae

vlakke van insulien uitdrukking in eiland-agtige aggregate wat gevorm het tussen die 3de en die 4de week. Medekweking van buis fragmente met MSS het tot die instandhouding van endokriene voorlopers wat Ngn3 uit druk, en later eilandagtige aggregate gevorm het, tot gevolg gehad. In kulture met MSS, ontwikkel buis epiteelsellle groeigebiede met selle wat beide CK7 en Ngn3 uitdruk in peribuis selle. Nadat peribuis selle eiland-agtige aggregate vorm, is Ngn3 saam met insulien uitgedruk binne eiland-agtige sel trosse naastens aan buisies. Oorplanting van vroeë oes (24 uur PPBA) eilandjies het beter genesende vermoë as laat (84 uur PPBA) eilandjies getoon. Die gemiddelde glucose vlakke was laer deur die 5 weke-lange moniteringstydperk in rotte waarin oorplanting 24 uur PPBA uitgerig is. Die gemiddelde tyd om hiperglisemie om te keer in 80% van die 24 uur PPBA oorplanting groep was 32 ± 2 dae (~ 4.5 weke), terwyl slegs 20% in die 84 uur PPBA oorplanting groep normoglisemie bereik het op 61 ± 2 dae (~ 9 weke) ($p = 0,0011$) na oorplanting. Ent oorlewing was hoër in eilandjies wat in die teenwoordigheid van MSS (eiland / MSS +) in vergelyking met ente wat oorplant is met eilandjies alleen (eiland / MSS -). Eilandjie morfologie en verspreiding van betaselle was normaal in eiland / MSS^+, soortgelyk aan die endogene eilandjies in die pankreas. Ten slotte, MSS bevorder die uitbreiding van $Pdx1^+$ selle en handhaaf die uitdrukking van Ngn3 in eiland selle en-buis afgeleide neogenetiese sell. MSS verleng ent oorlewing en verbeter die kapasiteit van die vroeë oes PPBA eilandjies om hyperglisemie om te keer; hierdie waarneming mag van toepassing wees op kliniese oorplanting.

Peer reviewed manuscripts and abstracts arising from this Thesis

Peer-reviewed published paper:

- **Manda J.K.**, Page B.J. Tchokonte-Nana V., 2017 Mesenchymal cells are required for epithelial duct cell-to-beta cell maturation and function in an injured adult pancreas in the rat. *Acta Histochemica*.

Paper submitted in peer-reviewed Journal:

- **Manda J.K.,** Page B.J., Tchokonte-Nana V. Immunohistochemical characterization of Pdx1, Ngn3 and Insulin protein expressions in islet-MSC *in vitro* interactions: A morphochronological evaluation. Article under review for publication with *Folia Histochemica et cytobiologica*, Manuscript # 54910.

Manuscript for publication

- Tchokonte-Nana V., **Manda J.K,** Page B.J., Early islet and mesenchymal cells from the ligated splenic part of the pancreas improve syngeneic engraftment and islets function in diabetic rats.

Peer reviewed conference abstracts:

- **Manda J.K.,** Tchokonte-Nana V. Page B.J Mesenchymal stromal cells (MSCs) isolation: Modified method for PDL tissue. International Islet Society Annual conference. Spiers hotel, Stellenbosch, Cape Town, South Africa, June, 2014. (Oral presentation)

- **Manda J.K.,** Tchokonte-Nana V. Page B.J. *In vitro*, PDL stimulated duct fragments generate islets and exocrine cells. International Islet Society Annual conference. Sydney, Australia, June, 2015 (Poster Presentation)

- **Manda J.K.,** Tchokonte-Nana V. Page B.J. *In vitro*, expansion of islets from injured adult pancreas involves epithelial-mesenchymal transition. Annual Academic day, Stellenbosch University, Faculty of Medicine and Health Sciences, Tygerberg, Cape Town, August, 2015 (poster presentation).

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ABBREVIATIONS AND ACRONYMS

ActRIIA, B	Activin receptor type II A, B
ALK3	Activin receptor-like kinase 3
Arx	Aristaless homeobox
BGL	Blood Glucose Level
BMP 4/7	Bone morphogenetic protein 4/7
BrdU	Bromodeoxyuridine
CA/TD	Centroacinar cells/Terminal duct cells
CAQIA	Computer-assisted quantitative image analysis
CD-45	Cluster of differentiation - 45
cDNA	Complementary Deoxyribonucleic Acid
Cdx 1 / 2	Caudal homeobox 1 / 2
Chg	Chromogranins
CK-7	Cytokeratin 7
c-Myc	Cellular- myelocytomatosis oncogene
Cpal	Carboxypeptidase A1
DAPI	4, 6-diamindino-2-phenylindole
DE	Definitive endoderm
DL1	Delta-like gene 1
DMEM	Dulbeco's modified minimum essential medium
DNA	deoxyribonucleic acid
E8.5	Embryonic day 8.5
EDTA	Ethylenediaminetetraacetic acid

EGF	Epidermal growth factor
EphrinB2	Eph family receptor interacting proteins B2
ESCs	Embryonic stem cells
FACS	Fluorescence activated cell sorter
FBS	Fetal bovine serum
FGF4, 10	Fibroblast growth factor 4, 10
FGFR2B	Fibroblast growth factor receptor type 2B
FITC	Fluorescein Isothiocynate
FoxA1	basic helix-loop-helix (bHLH) Forkhead box 1A
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GATA 4	Transcription factor characterized by its ability to bind to the DNA sequence "GATA" 4
GIT	Gastrointestinal tract
GK	Glucokinase
GLP-1	Glucagon-like Protein 1
GLUT-2	Glucose transporter 2
Hb9/Hlxb9	Homeobox gene-9 motor neuron-specific
HBSS	Hanks balances salt solution
HES1	Hairy/ Enhancer-of-split 1
HGF	Hepatocye growth factor
Hhex	Hematopoietically expressed homeobox
HNF1β	Hepatocyte nuclear factor 1β Homeobox 1

hPSCs	Human pluripotent stem cells
IAPP	Islet amyloid polypeptide
ICC	Immunocytochemistry
IgG	Immunoglobulin
IHC	Immunohistochemistry
Insm1	Insulinoma-associated antigene 1
IPGTT	Intraperitoneal glucose tolerance test
iPSCs	Induced pluripotent stem cells
IsL1	LIM homeodomain protein islet 1
MAF-A/B	V-maf Musculoaponeurotic fibrosarcoma oncogene family protein homologue A/B
Mnx1	Motor neuron and pancreas homeobox 1
MPCs	Multipotent progenitor cells
MSCs	Mesenchymal stromal cells
Myt1	Myeline transcription factor 1
NeuroD1/Beta2	Neurogenic of differentiation 1 gene/ beta-cell E-box transactivator 2
NGN3	Neurogenin 3
NGS	Normal goat serum
NKx6.1	NK6 homeobox gene 1
OC-1	Onecut 1
PAX 4, 6	Paired box genes 4, 6
PBS	Phosphate buffered saline
PBX1	Pre-B-cell leukemia homeobox 1

PCR	Polymerase chain reaction
PDL	Pancreatic duct ligation
PMSCs	Pancreatic mesenchymal stromal cells
PPDL	Post pancreatic duct ligation (portion of the pancreas distal to the ligation point)
PDX1/IPF1/IDX1	Pancreatic and duodenal homeobox-1/Insulin Promoter Factor-1/Islet duodenal
PE	Pancreatic endoderm
PITCH 1	Mesenchymal Patched homolog 1
PKC	Protein kinase C
PrE	Primitive endoderm
Prox1	Prospero-related Homeobox 1 gene
PTF1α	Pancreas specific Transcription Factor 1α
RA	Retinoic acid
RBP-J_K	Recombination signal binding protein for immunoglobulin J –kappa region
Rfx6	Regulatory factor X-box binding protein
RIP	Rat insulin promoter
RNA	Ribonucleic Acid
RPM	Revolution per Minute
RPMI-1640	Roswell park memorial institute -1640
SD	Standard deviation
SEM	Standard error of mean
Shh	Sonic hedgehog

SOC	Sham operated control
Sox 17	Sex determining region on Y (SRY)-box containing gene 17
SPSS	Statistical Package for Social Science
STF-1	Somatosatin transcription factor 1
STZ	Streptozotocin
SURI	Sulfonylurea
TBR-1	Transforming growth factor receptor type 1
Tgfβ	Transforming growth factor β
VEGF-A	Vascular endothelial growth factor- A

Chapter 1 Introduction

1.1 Literature Review

Diabetes mellitus is characterized by total or relative loss in insulin producing beta cells, classified as Type 1(Insulin dependent) and type 2 (Maturity onset), respectively. In diabetic patients, the body produces insufficient amounts of insulin to regulate blood glucose levels. Current treatment regimens include daily oral intake of hypoglycemic drugs and injections of exogenous insulin. Despite remarkable progress in the treatment of diabetes, *in vivo* generation of new insulin-producing beta cells, to replace the lost or replenish insufficient numbers of beta cells appears to be of great potential. Therefore, the search for new sources of beta cells offers hope as new therapeutic measures for treatment of diabetes mellitus.

Many studies currently focus on development of strategies to, either expand existing beta cells or, generate new beta cells from exocrine tissue of the pancreas (Bonner-Weir et al. 1993; Gu, Sarvetnick 1993; Rosenberg 1995; Bonner-Weir, Weir 2005; Suarez-Pinzon et al. 2005; Xu et al. 2008a; Rooman, Bouwens 2004, Inada et al. 2008). Understanding how insulin-producing beta cells are generated within the pancreas may help in the development of regenerative and cellular therapies for type 1 and type 2 diabetes mellitus. However, in order to develop protocols allowing *in vitro* generation or *in vivo* re-generation of beta cells, it is important to understand the molecular regulatory factors that control the development of the embryonic pancreas as well as the adult pancreatic beta cells in an *in vivo* model.

1.2 Embryonic development of the pancreas

1.2.1 Early development of the pancreas

Pancreas organogenesis involves many complex tissue interactions that control and regulate the specification and differentiation of the progenitor cells. This process is controlled by a network of signaling pathways and their transcription factors (Wilson, Scheel & German 2003).

1.2.1.1 Development of the definitive endoderm

The first step in pancreas development is the specification of the primitive endoderm (PrE) from pluripotent stem cells arising from the inner cell mass of the blastocysts (3-5 days post fertilization in mice) (Kanai-Azuma et al. 2002; Guney, Gannon 2009). Primitive endodermal cells are precursors recognized by the expression of sex determining region on Y (SRY)-box containing gene 17 (Sox17) that subsequently differentiate into visceral and parietal endodermal layers (Kanai-Azuma et al. 2002; Guney, Gannon 2009).

Following PrE specification, the blastocyst gastrulates to give rise to three germ layers: endoderm, mesoderm and ectoderm. The definitive endoderm (DE) is formed during this process when cells of the epiblast leave the inner cell mass through the primitive streak. In mice, DE cells are formed between embryonic (E) day 6.5 and E7.5 and is regulated by key factors such as Wnt/beta-catenin, Nodal, Transcription factor 4 and 6 that bind to the DNA sequence "GATA" (GATA-4/6), the basic winged helix-loop-helix (bHLH) forkhead box A1 (FoxA1) and FoxA2 (previously known as hepatocyte nuclear factor 3α and β (Hnf3α and β) respectively) (Fig. 1.1) (Shimosato, Shiki & Niwa 2007).

Before organogenesis, all endodermal cells are uncommitted to specific cell lineages. Later, DE becomes patterned into anterior and posterior DE. The anterior DE will form the foregut

endoderm from which the pancreas, liver and lungs will bud out, while the posterior DE will form midgut and hindgut which will eventually give rise to small and large intestines (Dominguez-Bendala, Inverardi & Ricordi 2011). A report by Wells et al. (2000) showed that mesectoderm cells produce fibroblast growth factor (Fgf) 4 that induces posteriorization of the definitive endoderm. Fibroblast factor 4 induces posteriorization by suppressing the anterior endodermal transcription factors NK domain homeobox (Nkx) 2.1 and *Antennapedia*/Ftz, class hematopoietically expressed homeobox (Hhex). These factors control the formation of foregut endoderm, and activates the expression of posterior endodermal transcription factors: the pancreatic and duodenal homeobox 1 (Pdx1) and caudal homeobox 1 / 2 (Cdx1/Cdx2). Pdx1 and Cdx1/Cdx2 define the pancreatic field (Wells, Melton 2000, Dessimoz et al. 2006). Additionally, there is evidence that retinoic acid (RA) secretion from paraxial mesoderm controls anterior-posterior patterning in gut organs and promote the allocation of the pancreas (Stafford, Prince 2002; Chen et al. 2004; Bayha et al. 2009).

1.2.1.2 Specification of the pancreatic endoderm (PE)

As a second specification step towards pancreas outcome, the gut endoderm becomes specified into organ fields through a series of patterning events that will eventually give rise to tissue and glands that line gastrointestinal tract (GIT) (Slack 1995). These processes are regulated by extrinsic factors secreted by neighbouring mesodermal derivates as well as factors intrinsically produced by the endodermal cells themselves. Through these events, two pancreatic fields are specified within the gut definitive endoderm: the anterior and posterior pancreatic fields (Slack 1995). The Pancreas develops from the posterior and anterior foregut endoderm fields as dorsal and ventral pancreatic buds respectively (Kim, Hebrok 2001) (Fig.1.1).

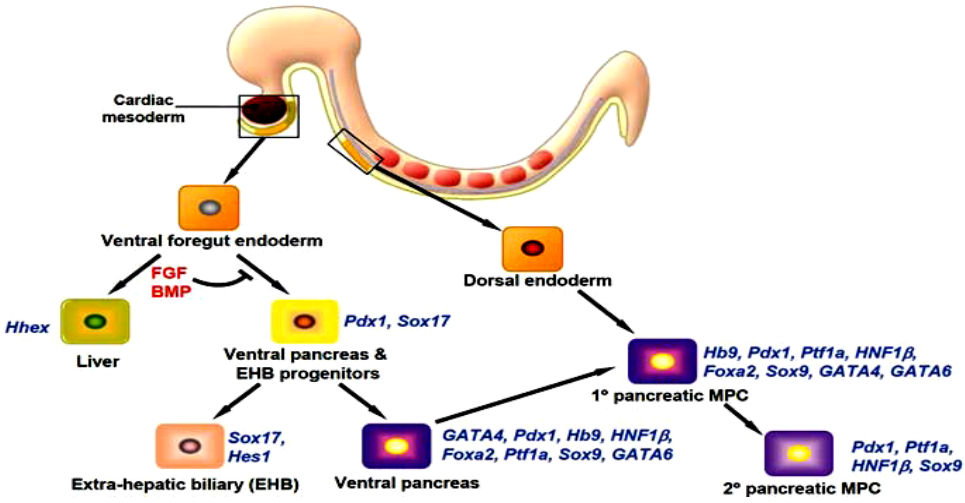


Figure 1.1. Early stage of embryo development at E8.5 (saggital view) showing key transcription factors controlling specification of the dorsal and ventral pre-pancreatic endoderm. *Adapted and modified from Pan, Wright 2011.*

These buds are formed from multi-potent progenitor cells that give rise to all cell types in the pancreas. Initially, three primordia buds arise from the definitive gut endoderm. The dorsal bud arises first from the dorsal side, followed by two ventral buds, which arise from the ventral side (Lammert, Cleaver & Melton 2001). At E9.5 (22 – 25 somites stage in mice or 26th day of gestation in humans), a recognizable dorsal pancreatic bud appears as an epithelial thickening of the dorsal endoderm, indicating the beginning of pancreatic morphogenesis (Slack 1995) (Fig. 1.2C).

1.2.1.3 Early tissue interactions in pancreatic endoderm specification

The period just before specification of the dorsal pancreatic bud at E9.5, the uncommitted endodermal cells in posterior pancreatic field receives interactive inductive signals from the mesectoderm. Following this, the dorsal pancreatic bud recieves permissive signals from the endothelium and notochord that specifies the location of the pancreatic epithelium, and finally

the proliferative signals from mesenchyme that regulate the size of the pancreatic domain (Kim, Hebrok & Melton 1997; Lammert, Cleaver & Melton 2001; Jorgensen et al. 2007). The mesectoderm secrete inductive signals, such as Fgf4, which induce competency in the dorsal foregut endodermal cells to interact with permissive pro-pancreatic signals from the notochord (Wells, Melton 2000).

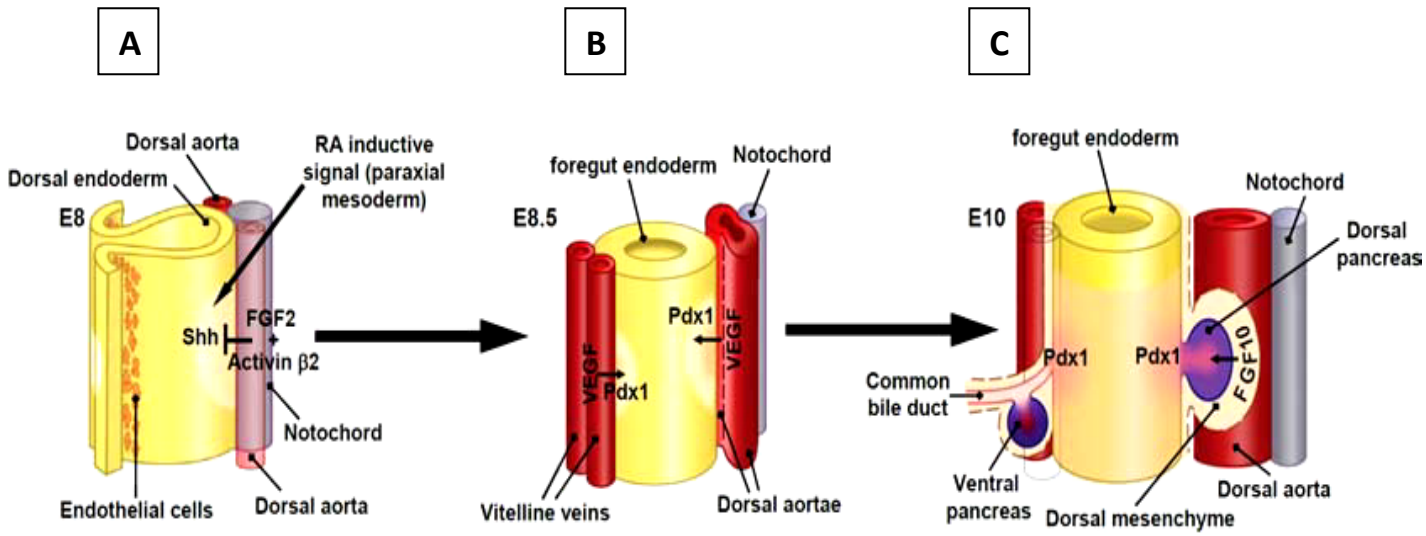


Figure 1.2. Schematic diagram showing early patterning of the foregut endoderm: The pancreatic domain is established by signaling from adjacent mesodermal derivatives. A: Fgf2 and Activinβ2 from notochord represses Sonic hedgehog (Shh). *Adapted from Pan, Wright 2011.*

(i) Notochord–endoderm interactions

The notochord secretes permissive signals, such as activin-βB and Fgf2, which block the endodermal expression of sonic hedgehog (Shh), a member of the Hh signaling family, that regulates the formation of the stomach and the duodenum (Fig 1.2A) (Hebrok, Kim & Melton 1998, Hebrok et al. 2000). Repression of Shh in the pre-pancreatic endoderm leads to activation of Pdx1 and pancreas specific transcription factor 1α (Ptf1α), and permits the mesenchymal patched homolog 1 (PTCH1) to activate lineage specification of the pancreas (Hebrok, Kim &

Melton 1998). The expression of Shh in pancreas epithelium inhibits pancreas formation and controls organ specification by generating a molecular boundary at the foregut and midgut border. In mice, the notochord maintains contact with the dorsal pre-pancreatic endoderm until about E8.5 (13-somite stage in mice and 3 weeks in humans) (Kim, Hebrok & Melton 1997) (Fig.1.2B).

Prior to the 13-somite stage, when dorsal pre-pancreatic endoderm is cultured alone, Pdx1, islet-1 (Isl-1) and paired box 6 (Pax6) are not activated. However, when co-cultured with notochord, Pdx1, Isl-1 and Pax6 are activated. This suggests that prior to the 13-somite stage, the dorsal pre-pancreatic endoderm cells are still uncommitted to differentiate to a pancreatic fate autonomously, but require inductive signals from the notochord for commitment to pancreatic fate (Kim, Hebrok & Melton 1997). After suppression of Shh, pancreas differentiates into cells that express Pdx1 and Ptf1α/p48. However, before expression of Pdx1 and Ptf1α, several transcription factors that pattern the gut DE toward pancreatic lineage are activated including, Hnf6 (onecut1), Hlxb9 (hb9), Hnf3b (Foxa2), Hhex (Hex) and vHnf1 (also known as Tcf2 and Hnf1β) (Fig.1.1) (Shimosato, Shiki & Niwa 2007, Gittes 2009).

One of the first transcription factors to be expressed before Pdx1 is a motor neuron-specific homeobox gene-9 (Hlxb9) (Li et al. 1999). The Hlxb9 gene encodes the homeobox transcription factor Hb9 that is also expressed in the notochord and the dorsal pancreatic bud and is expressed before Pdx1. At this early stage, Hb9 is activated in the early pancreatic buds (Li et al. 1999). Later, Hlxb9 is activated in pancreatic buds during delamination and migration of the epithelial cells. In Hlxb9 mutant mice, Pdx1 is downregulated and the dorsal pancreatic bud is repressed,

while the ventral pancreas develops normally, suggesting that Pdx1 functions downstream of Hlxb9 in the dorsal but not the ventral pancreatic bud (Li et al. 1999).

Hnf family: Also working upstream of Pdx1, are members of Hnf family of transcription factors: Hnf6 (also known as Onecut1), FoxA2 and vHnf1 (Shimosato, Shiki & Niwa 2007, Gittes 2009). Hepatocyte nuclear factor 1β induces the expression of Hnf6 (Barbacci et al. 1999, Pierreux et al. 2006, Poll et al. 2006) which, in turn, induces the expression of Hnf3β/Foxa2 which along with Foxa1 are transcriptional regulators of Pdx1 during pancreas specification (Wu et al. 1997, Gao et al. 2008). The expression of Hnf6 is detected early in the epithelial precursor cells of the exocrine and endocrine pancreatic beta cells. Hepatocyte nuclear factor 6 is also needed for proper Ngn3 expression (Jacquemin et al. 2000).

Pancreatic duodenal homeobox-1 (Pdx1): Cells of the endodermal region that get committed to a pancreatic fate, first activate the homeodomain transcription factor Pdx1, also known as insulin promoter factor 1 (IPF1) (Leonard et al. 1993; Ohlsson, Karlsson & Edlund 1993; Ahlgren et al. 1998), Islet/duodenum homeobox 1 (IDX-1) or Somatostatin transcription factor-1 (STF-1) (Leonard et al. 1993; Miller, McGehee & Habener 1994). Pancreatic and duodenal homeobox 1 is a member of the parahox group of homeodomain transcription factors that is expressed in the endodermal region committed to a pancreatic fate (Brooke, Garcia-Fernandez & Holland 1998). Pancreatic and duodenal homeobox 1 expression is observed in early pancreatic progenitors on E8.5 in mouse embryos (E9.5 in rats, Fig.1.2B). In mice or humans lacking Pdx1 gene, the pancreatic bud is initiated, but it does not develop further (Offield et al. 1996; Ahlgren et al. 1998), suggesting that Pdx1 is necessary for the full development of the pancreatic buds. This data therefore suggests that Pdx1 is not the only factor that is needed in specification of the early pancreatic buds. As mentioned above, it is possible that other factors such as Hlxb9, which

is expressed in the dorsal bud before Pdx1 might be involved in the initiation of the early pancreatic buds (Li et al. 1999, Hadjantonakis, Papaioannou 2001).

However, although Pdx1 is required for pancreas organogenesis, this gene is also expressed in the duodenum, distal stomach and the common bile duct as well. In addition, a report published by Spence et al. (2009), demonstrated that Pdx1 is also co-activated with Sox17, a transcription factor that is expressed in the bipotential ventral foregut domain that gives rise to both the pancreatic and the biliary primordia (Fig.1.1 &1.2C). Sex determining factor (SRY)-homeobox 17 seems to inhibit development of ventral pancreatic bud and therefore, ventral pancreatic bud is only formed when Sox17 is repressed (Spence et al. 2009). Sex determining factor homeobox 17 is persistently upregulated in the biliary tract and creates a molecular boundary between pancreas and the liver. The question of why Pdx1 is co-expressed with Sox17, an inhibitor of ventral pancreatic bud development, is not clearly understood. In addition, Pdx1 expression also overlaps with p48/PTF1α, a transcription factor that is activated shortly afterwards in the pancreatic anlagen. As development proceeds, p48 expression becomes restricted to the acinar and ductal compartments. In homozygous p48 mutant mice, all exocrine and most endocrine tissue do not develop and neonates die afterbirth (Krapp et al. 1998, Kawaguchi et al. 2002). Therefore, Pdx1 is regarded as a marker of early pancreatic progenitor cells. Also co-expressing with Pdx1 is Sox9 that is expressed between E9.5 and E12.5, suggesting that Sox9 marks the progenitor population in the pancreatic bud and promotes proliferation and their survival (Fig.1.1) (Seymour et al. 2007)

(ii) Dorsal aorta endothelium-endoderm interactions

At the 13-somite stage in mice, the paired dorsal aortas fuse at midline displacing and separating the notochord from the endoderm (Fig 1.2B, C). Signals from the endothelial cells of the aorta have been shown to promote outgrowth of the dorsal bud. When mouse dorsal pre-pancreatic endoderm was cultured in the presence of dorsal aorta, Pdx1 and insulin expressions were induced (Lammert, Cleaver & Melton 2001, Yoshitomi, Zaret 2004). In a follow-up study, transgenic mice that expressed vascular endothelial growth factor A (VEGF-A) under Pdx1 promoter displayed hyperplastic islets and ectopic insulin+ cells in the stomach (Lammert, Cleaver & Melton 2001), a finding that clearly indicates the importance of endothelial signals in the initiation of the pancreas. During this stage, the first endocrine cells detected are glucagon–producing cells (alpha cells) as early as E9.5 (Pictet et al. 1972; Herrera et al. 1991; Teitelman et al. 1993; Upchurch, Aponte & Leiter 1994).

(iii) Mesenchyme-epithelial interactions

After depending on signals from the aorta, mesenchymal cells condense around the evaginating epithelium, separating the aorta from the epithelium (Fig. 1.2C). There is increasing evidence that continued interactions with adjacent mesenchymal tissues promote organogenesis of the pancreas and their subsequent specification steps (Pictet et al. 1972). Mesenchymal–epithelial interactions are regulated by several families of signaling molecules. Studies in knockout animal models have identified the roles of individual signaling pathways, however, the complex interactions between these signaling pathways demands more investigation.

Mesechyme-derived fibroblasts growth factors (Fgf) family (Fgf 1, 2, 7 and 10): During this early stage of development, Fgfs are some of the factors that are expressed in the epithelial-

mesenchymal interface region where they mediate many developmental processes, particularly in regulation of branching morphogenesis of the early pancreatic bud (reviewed in Hogan 1999). Fibroblast growth factors are ligands that signal by binding to tyrosine kinase F**g**f receptors. In the preceding stages of bud development, members of the Fgf family, Fgf1, 7 and 10 were found to be highly activated in the mesenchyme while their receptor, Fgf receptor type 2B (FGFR2B) were expressed in the epithelium. Secretion of ligands of the Fgf family members, Fgf2 and Fgf10, by the notochord and dorsal mesenchyme initiates and maintains critical transcription factors that specify the pancreatic primordium (Fig.1.2C) (Jacquemin et al. 2006, Bhushan et al. 2001). The activated Fgf10 in the pancreatic mesenchyme and its receptor FGFR2 in the epithelium (Bhushan et al. 2001, Dichmann et al. 2003, Seymour et al. 2012) regulate proliferation of the pancreatic progenitors. In Fgf10 knock-out mice, growth and branching of the pancreatic primordium is heavily affected (Bhushan et al. 2001).

During later stages, Fgf10 activates Notch signaling, which promotes proliferation and inhibits differentiation of endocrine precursors (Hart, Papadopoulou & Edlund 2003; Norgaard, Jensen & Jensen 2003; Miralles et al. 2006). In addition, Fgf10 was shown to regulate the expression of Sox9 and Ptf1α in the epithelium (Jacquemin et al. 2006, Seymour et al. 2012).

The Dorsal pancreatic bud: As the mesenchyme thickens round the dorsal pancreatic bud at E9.5, LIM homeodomain protein islet-1 (Isl-1) is specifically activated in the mesenchyme surrounding the dorsal bud and is required for mesenchyme and dorsal pancreatic bud formation (Ahlgren et al. 1997). However, Isl-1 is not expressed in the ventral bud. Accordingly, in Isl-1 deficient mice, dorsal mesenchyme fails to form and islet cells differentiation, proliferation and survival in the endoderm is dramatically affected (Ahlgren et al. 1997). Additional studies went

further to show that deletion of aldehyde dehydrogenase family 1 subfamily 2 (Aldh1a2 also known as RALDH2), an enzyme that synthesizes RA in mice, abolishes formation of dorsal pancreas and early glucagon-expressing cells (Martin et al. 2005, Molotkov, Molotkova & Duester 2005), similar to the phenotypes that are observed in Islet-1 (Isl-1) (Ahlgren et al. 1997) and N-cadherin (Esni et al. 1999) null mice. These results clearly suggest that mesenchymal-derived factor, Isl-1, is required for the development of the dorsal pancreas (Ahlgren et al. 1997). Moreover, mesenchymal cells produce Ephrin B2, and through its receptor, EphB, induces development of the microlumens in the pancreatic epithelial bud (Villasenor et al. 2010).

The ventral pancreatic bud: Later, at around E10.5 (6 days after evagination of dorsal bud in humans), two ventral pancreatic buds appear in close contact with cardiac mesenchyme and two vitelline veins (approx. 30 somites stage) [for review see (Zorn, Wells 2009)]. Soon after evagination, one of the ventral buds degenerates (as one of the vitelline veins also regresses), whereas the remaining ventral bud later fuses with the dorsal bud following rotation of the gut tube at around E12.5 (E37.5 in humans) (Lammert, Cleaver & Melton 2001, Jorgensen et al. 2007). Development of the ventral pancreas is regulated by signals from the lateral plate, the cardiac and the septum transversum mesenchyme (Fig. 1.1). As the ventral bud evaginates into cardiac mesenchyme, it comes into direct contact with the lateral plate mesoderm. Lateral plate mesoderm produces instructive signals, such as activins, RA and bone morphogenetic proteins 7 (BMP7) that induce the expression of pancreatic markers similar to the roles of RA and activins in the dorsal bud (Fig.1.2A) (Kumar et al. 2003).

In addition, signals from cardiac mesenchyme and septum transversum mesenchyme determine the fate of this bipotential ventral endoderm sheet into pancreas or liver progenitors. Increased

Fgf signals secreted from cardiac mesoderm inhibits the development of the pancreas and promotes liver development (Wandzioch, Zaret 2009). This process is regulated by a delicate balance between BMP4/7, Fgf and transforming growth factor (Tgf) β secreted by the septum transversum (Wandzioch, Zaret 2009). Work published by Wandzioch and colleagues further showed that activation of BMP through its receptor activin receptor-like kinase 3 (ALK3) by the septum transversum mesenchyme induces the endodermal expression of transcription factor 4 binding to DNA sequence GATA (GATA4) and inhibits ventral pancreatic bud development but promotes liver bud development by activating liver-specific genes in the 3-4 somite stage embryos (Wandzioch, Zaret 2009). However, hours later (in the 5-6 somite stage), the study showed that expression of Noggin (NOG) inhibits mesenchyme-derived BMP signaling and induces Pdx1 expression, reversing the inhibitory effects of BMP (Wandzioch, Zaret 2009). Therefore, formation of the ventral pancreas is regulated by different signaling pathways, with opposing effects, which arise from the surrounding mesenchymal tissues. In an effort to generate therapeutic beta cells for diabetes mellitus, RA, Fgf10, Protein kinase C (PKC) activators, Shh (cyclopamine) and BMP inhibitors have been used in directed differentiation protocols to induce pancreatic epithelium from DE derived from human pluripotent stem cells (hPSCs) (D'Amour et al. 2006; Kroon et al. 2008; Nostro et al. 2011; Cheng et al. 2012; Rezania et al. 2012)

Phases of pancreas development: Once the organ is specified, the pancreas epithelium thickens and expands into the surrounding mesenchyme. Formation of the rodent pancreas occurs in two phases of development overlapping each other. The first phase of development occurs between E9.5-E12.5 in a mouse. Starting from E10.5, the epithelia of the two buds, which are still partially differentiated, undergo the first phase of developmental transition. At this phase of

development, a few insulin-producing cells (beta cells), often co-expressing the glucagon hormone, appear in a cluster–budding process as the first differentiated cells (Pictet et al. 1972; Teitelman et al. 1993; Villasenor et al. 2010; Kesavan et al. 2009). Although alpha cells (glucagon–producing cells) and a few cells co-expressing insulin and glucagon can be detected as early as E9.5, beta cells and other hormone-secreting cells that are fully differentiated become first evident in the secondary transition at E13.5 (Hashimoto et al. 1988, Alpert, Hanahan & Teitelman 1988, Sander et al. 2000). This first transition stage is characterized by active progenitor proliferation to generate a stratified epithelium followed by formation of microlumens that later coalesce. By E12.5, the stratified epithelium undergoes expansion, plexus remodeling and branching morphogenesis into a ductal tree, that finally forms two primordial pancreas organs with ductal epithelium that consists of predominantly undifferentiated cells.

Role of transcription factors: At E12.5, the dorsal and ventral buds fuse together. The majority of these early progenitors express the transcription factors such as Pdx1, Ptf1α, carboxypeptidase A1 (Cpa1), c-Myc, Sox9, Hnf1b, hairy/enhancer-of-split 1 (Hes1) and Nkx6.1 (Jonsson et al. 1994; Apelqvist et al. 1999; Sander et al. 2000; Haumaitre et al. 2005; Seymour et al. 2007; Hald et al. 2008). Based on their cellular markers, these early $Pdx1^{+}$, $Ptf1a^{+}$, $Cpal^{+}$, $Hes1^{+}$, $Sox9^{+}$, $Nkx6.1^{+}$ and $Hnf1b^{+}$ pancreatic epithelial cells are regarded as pancreatic multipotent progenitor cells (MPCs) [comprehensively reviewed in Gittes 2009, Kopp et al. 2011, Pan, Wright 2011, Seymour, Sander 2011]. Mutant mice deficient in any one of these factors show varying degrees of hypoplasia or agenesis of the pancreas, despite initiating the bud, suggesting that initiation of pancreatic program is not completely inhibited by the absence of one single factor [reviewed in (Gittes et al. 1996, Pan, Wright 2011, Seymour, Sander 2011, Mastracci, Sussel 2012,)].

Although pancreatic budding is initiated, evidence show that these factors are required for driving these progenitors cells into pancreatic fate. Deletion of Ptf1α, for example, converts pancreatic-fated progenitors to duodenal and common bile duct endoderms (Kawaguchi et al. 2002, Burlison et al. 2008), while deletion of both Ptf1α and Pdx1 converts liver progenitors to pancreatic lineage, suggesting that Pdx1 and Ptf1α can change the destiny of the non- pancreatic genes from non-pancreatic cells to pancreatic lineage genes (Horb et al. 2003, Jarikji et al. 2007,). However, the mechanism by which Ptf1α and Pdx1 activate non-pancreatic genes in non-pancreatic cells to pancreatic genes needs more investigation. A better understanding of how these factors contextually activate pancreatic gene expression in non-pancreatic cells can help us develop novel strategies to re-program, for example, fibroblasts directly into pancreatic cells as revealed recently for cardiomyoctes and neurons (Thier et al. 2012, Zhu, Wang & Ding 2015). Around E12.5, a subset of these early pancreatic progenitors upregulate Cpa1 and Neurogenin 3 (Ngn3), markers associated with pre-acinar and pre-endocrine domains respectively (Jorgensen et al. 2007, Zhou et al. 2007).

Pancreatic epithelium and organ patterning/specification: Between E13.5 and E16.5, the pancreatic epithelium goes through a secondary transition stage of organ domain patterning. This stage is characterized by growth, branching and specifications of multi-potent progenitors toward differentiated lineages (Rutter et al. 1968, Pictet et al. 1972,). Organ domain pattering gives rise to a network of branched epithelia that is spatially segregated into ‘tip’ (from which acinar cells originate) and ‘trunk’ (from which endocrine and duct cells originate) domains that express specific cell-intrinsic markers (Fig.1.3) (Zhou et al. 2007, Villasenor et al. 2010,).

Specification of multipotent progenitors to different lineages occurs by initiation and expression of specific gene profiles regulated by distinct transcription factors that are expressed in spatial and temporal manner. Lineage tracing studies demonstrated that multipotent progenitor cells reside at the tip of the branching epithelium and are marked by the expression of Pdx1, Ptf1a, Cpal, and c-Myc (Hebrok, Kim & Melton 1998, Zhou et al. 2007, Schaffer et al. 2010). These multipotent progenitors differentiate into acinar, duct, and endocrine cells. After E14.5 of development, Ptf1a and its target Cpa1 become restricted to the tip and drive these cells to exocrine fate (Zhou et al. 2007, Schaffer et al. 2010). Cells in the trunk sub-compartment are marked by Hnf1b, Sox9, Foxa2, Prox1, Tcf2, Hes1 and Nkx6.1/6.2, and differentiate into endocrine and duct cells (Solar et al. 2009, Kopp et al. 2011, Pan, Wright 2011, Kopinke et al. 2012, Schaffer et al. 2013,).

From this stage and thereafter, multipotent progenitors in the trunk sub-compartment specify towards differentiated lineages (Villasenor et al. 2010). Nkx6.1/6.2 and Ptf1α act as major regulators of tip/trunk compartmentalization process. Nkx6.1/6.2 segregate to trunk cells where it promotes trunk identity together with Notch, while Ptf1α similarly regulate acinar cell development in the tip compartment (Schaffer et al. 2010, Schaffer et al. 2013, Afelik, Jensen 2013).

1.2.2 Specification and differentiation of endocrine pancreas from ductal trunk epithelium

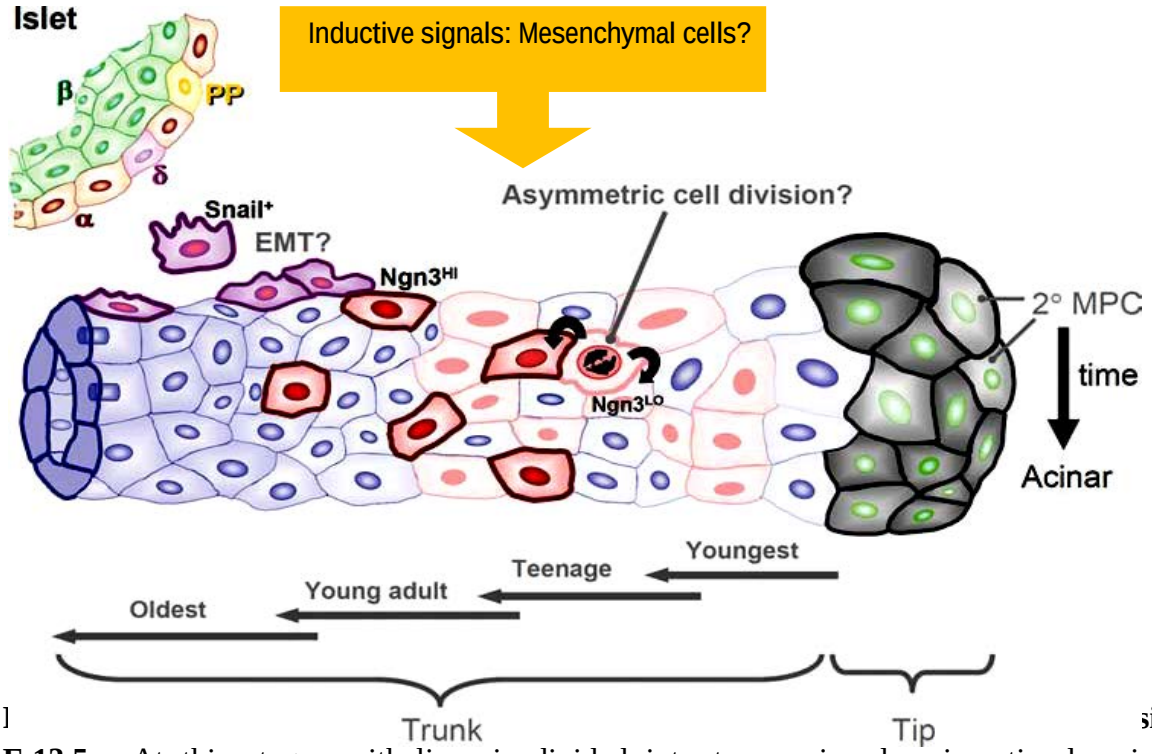
Following the expression of Pdx1, the next step is the differentiation of the pancreatic buds into endocrine and exocrine cells. Experiments that use lineage tracing systems have shown that Pdx1-expressing progenitor cells give rise to both endocrine and exocrine cells (Herrera 2000, Gu, Dubauskaite & Melton 2002). While tip cells are fated to generate acinar cells, the trunk compartment is bipotential and gives rise to duct and endocrine cells (Fig. 1.3) (Solar et al. 2009,

Kopp et al. 2011, Kopinke et al. 2012). In mice embryos, differentiation of endocrine cells occurs in two waves. The first wave occurs during the first epithelial transition stage between E9.5 and E13.5 and forms glucagon- and insulin-positive cells plus the other cells co-expressing glucagon and insulin but these cells never contribute to adult islet cells (Herrera 2000). The second wave of endocrine differentiation occurs during secondary epithelial transition (E13.5-E15.5) and these cells forms adult islets (Offield et al. 1996).

Differentiation of endocrine progenitors within the trunk epithelium relies on expression of transcription factors Pdx1 and Hnf6 (Offield et al. 1996), and this occurs by progressively de-activating the expression of Hnf1b and Sox9. Concomitantly, at E13.5, the pro-endocrine basic helix-loop-helix (bHLH) transcription factor Neurogenin 3 (Ngn3) is transiently activated in a subset of cells in progenitor cords. Neurogenin 3 activation in the pro-endocrine progenitor cells leads to the differentiation of the endocrine cells and results into separation of endocrine cells from duct lineages.

There is now evidence that timing and Ngn3 expression levels are crucial in driving primitive ductal epithelial cells entry into either, exocrine or endocrine lineage and fate allocation. Work done by Wang *et al*. (2010) demonstrated that epithelial trunk cells with high Ngn3 expression levels become endocrine-committed, while those with low Ngn3 levels remain in the primitive ducts (or progenitor cords) as precursors (Fig.1.3). Trunk epithelial cells that do not activate Ngn3 eventually differentiate into duct cells (Fig 1.3) (Magenheim et al. 2011a; Beucher et al. 2012). Although levels of Ngn3 are important in triggering endocrine fate, how this occurs is not clearly understood.

Some reports show that Ngn3 expression appears to be induced by upstream transcription factors such as Hnf3β, Hnf1β, Sox9, Pdx1 and Hnf6 that have been detected to bind to Ngn3 distal regulatory region (Jacquemin et al. 2000; Lee et al. 2001; Oliver-Krasinski et al. 2009; Kim et al. 2012; Shih, Wang & Sander 2013). However, little is known on how these factors regulate Ngn3 activation. It is also not clearly understood why Ngn3 is expressed in only a subset of cells



] **sition at E.13.5.** At this stage, epithelium is divided into two major domains: tip domain (grey) containing multipotent progenitor cells (MPC), and a trunk region containing the endocrine/ duct bi-potential progenitor pool. Cells scattered within the trunk express different levels of Ngn3, with $Ngn3^{LO}$ cells (light pink) (precursors but not yet committed). $Ngn3^{LO}$ undergo asymmetric cell division that give rise to one $Ngn3^{HI}$ (darker nucleus, red border) daughter cell that becomes endocrine-committed and rapidly express Snail2 (purple cells) and migrate away via epithelial–mesenchymal transition (EMT), before aggregating to form the endocrine islets. *Adapted and modified from Pan , Wright 2011*

within the primitive duct epithelium, while in majority of these cells, the gene is repressed. Factors arising from the mesenchyme have been postulated to participate in lineage selection (Fig.1.3).

1.2.2.1 Mesenchymal-epithelial interactions in specification of endocrine versus exocrine

Transforming growth factor-β isoforms (Tgf-β isoforms): In mice, three members of the superfamily transforming growth factor-β (Tgf–β) *isoforms Tgf-β1, -β2 and –β3*, are specifically expressed in the epithelium of the developing pancreas at E12.5 and later localized to acinar cells, while their receptors, transforming growth factor receptor type I (TBR-I or Alk5) and TBR-II are expressed in the mesenchyme and the epithelium but later localize to duct epithelium (Crisera et al. 1999; Crisera, Longaker & Gittes 1999; Crisera et al. 2000; Tulachan et al. 2007). There is evidence that Tgf-β isoforms participate in pancreatic lineage selection. In early pancreatic epithelium, it enhances proliferation and accumulation of periductal endocrine cells (Tulachan et al. 2007), while in late gestational periods, it restricts the recruitment of ductal or periductal cells into endocrine lineages (Fig. 1.3) (Crisera et al. 1999; Crisera, Longaker & Gittes 1999; Crisera et al. 2000; Tulachan et al. 2007)

1.2.2.1.1 Role of Activins in lineage specification

In addition to determining lineage specification and early branching of epithelial cells in the pancreatic bud (Ritvos et al. 1995, Kim et al. 2000), notochord-secreted Tgf-β sub-family signals such as activin and other growth differentiation factors, determine endocrine and exocrine lineage specification during the secondary transition stage (Ritvos et al. 1995, Kim et al. 2000, Maldonado et al. 2000). In a study by Mirales et al. (1998), Tgf-β was shown to activate matrix metalloproteinase MMP-2 through type II activin receptors; ActRIIA and ActRIIB (Fig. 1.3),

which promotes migration and formation of islets and suppresses exocrine/acinar differentiation (Demeterco et al. 2000). These receptors are widely upregulated in the embryonic pancreatic epithelium and later in islets of adult pancreas (Kim et al. 2000). In type II activin receptor-deficient mice, small islets develop (Yamaoka et al. 1998), suggesting that Tgf-β signaling, mediated by activin receptor, regulates islet differentiation (Kim et al. 2000). There is also evidence that activin enhances insulin-producing cells differentiation by inhibiting aristaless homeobox (Arx) (a transcription factor required for differentiation of alpha cells) and pro-glucagon in human islets and pancreatic duct cells (Mamin, Philippe 2007). Additional report shows that activin also induces Ngn3 through a non-canonical target of activin, p38 mitogen activator protein K (MAPK) (Ogihara et al. 2003).

1.2.2.2 Endocrine versus exocrine differentiation within the duct epithelium

(i) The notch pathway

Within the primitive duct epithelium, it is reported that endocrine versus exocrine differentiation is regulated by lateral inhibition mediated by Notch signaling pathway (Apelqvist et al. 1999; Jensen et al. 2000b; Afelik, Jensen 2013; Shih, Wang & Sander 2013). Notch signaling controls the fates of cells through local cell interactions in many cellular processes and tissues including the developing pancreas [reviewed in (Lammert, Brown & Melton 2000; Nam, Aster & Blacklow 2002)]. When stimulated by Hnf6 (Jacquemin et al. 2000), committed endocrine progenitors expressing Ngn3 autonomously activate the expression of Notch ligands: Jagged 1-2 (Jag1-2), and Delta-like gene 1 and 3 (Dll-1 and -3) that bind to Notch receptors (Notch 1–3) in neighbouring cells and activate the Notch signaling pathway (Heremans et al. 2002). Activated Notch translocates to the nucleus and binds to its downstream target, Hes1. Activated Hes1 represses Ngn3, and therefore prevents widespread Ngn3 activation and subsequent endocrine

differentiation (Ahnfelt-Ronne et al. 2012). In addition, Hes1 maintains a pool of proliferative progenitor cells within the duct epithelium by repressing the cyclin-dependent kinase p57, the cell cycle inhibitor in multipotent progenitor cells (Georgia et al. 2006). Recently, Hes1 was further shown to promote Ngn3 protein degradation (Qu et al. 2013). In addition to activating Hes1, Notch signaling also regulates Sox9 expression that specifies duct cells (Kageyama, Ohtsuka & Kobayashi 2007, Shih et al. 2012). Therefore, duct epithelial progenitor cells in which Notch is upregulated are capable of continued proliferation while cells in which Notch is downregulated, express Ngn3, exit the cell cycle, and proceed to differentiate into endocrine cells. Epithelial cells in which Ngn3 is activated marks the endocrine progenitors (Gu, Dubauskaite & Melton 2002; Herrera 2002). Expression of Notch receptors (Notch1–3), ligands (Jag-1, Jag-2, Dll-1, and Dll-3), and target Hes1 has been observed in undifferentiated cells within the mouse embryonic pancreas but not in differentiated endocrine cells in the embryonic or adult pancreas (Murtaugh et al. 2003; Hald et al. 2003; Esni et al. 2004) .

In the developing pancreas the role of the Notch-Hes1 pathway is to promote precursor cell replication and prevent premature endocrine differentiation (Murtaugh et al. 2003; Hald et al. 2003; Esni et al. 2004). As evidence to support this, murine models, deficient of Notch signaling molecules such as Dll-1, recombination signal binding protein for immunoglobulin J kappa region (Rbp-Jk) and Hes1, show accelerated differentiation of endocrine cells thereby depleting the population of progenitor cells, resulting into hypoplastic pancreas (Apelqvist et al. 1999; Jensen et al. 2000b). At the end of this phase , cells express a group of transcription factors such as Isl-1, Pax6 (St-Onge et al. 1997), Pax4 (Sosa-Pineda et al. 1997), Nkx2.2 (Sussel et al. 1998), Beta2/NeuroD (Naya et al. 1997) and Ngn3 (Gradwohl et al. 2000, Schwitzgebel et al. 2000).

which mark them as the endocrine cell precursors, In principle, the endocrine cell precursors can differentiate into different endocrine islet cells.

Between E13.5 and E15.5, high levels of Ngn3 expression occur in the cells of the trunk epithelium and signifies the differentiation and expansion of the endocrine cells. Ngn3 is required for endocrine cell differentiation as Ngn3-null pancreas is devoid of endocrine cells while exocrine tissue is normal (Gradwohl et al. 2000). In *in-vitro* and during *in-vivo* regeneration or transplantation, inhibitors of Notch, in addition to other growth factors, have been used in directed differentiation protocols to generate Ngn3+ cells after induction of the pancreatic epithelium derived from hPSC *in vitro*, (D'Amour et al. 2006; Kroon et al. 2008; Nostro et al. 2011; Cheng et al. 2012; Rezania et al. 2012).

(ii) Neurogenin 3 (Ngn3)

The basic helix-loop-helix (bHLH) transcription factor Ngn3 was shown to give rise to all four types of endocrine cells of the pancreas (Jensen et al. 2000a). Neurogenin 3 is exclusively and transiently expressed in cells scattered within the pancreatic epithelium, while in differentiated endocrine cells the gene is downregulated (Schwitzgebel et al. 2000). Neurogenin 3 is required for the development of the endocrine pancreas, and mutant mice in which Ngn3 is deleted are born without pancreatic endocrine cells (Gradwohl et al. 2000). There is also evidence from cell-tracing studies that Ngn3-positive cells are endocrine precursors (Gu, Dubauskaite & Melton 2002). Thus, Ngn3 can be used as a marker to trace the onset of endocrine cell differentiation in the pancreas. When Ngn3 is ectopically activated in the gut epithelial cells, differentiation of glucagon- and somatostatin-producing cells are induced, at the expense of insulin-producing ß-cells (Grapin-Botton, Majithia & Melton 2001). Downstrean, Ngn3 proteins bind to the promoter

for Neurogenic differentiation 1 (NeuroD1), [also known as beta-cell E-box transactivator 2 (Beta2)] and activate transcription of the gene (Huang et al. 2000). During pancreatic development, expression of NeuroD1/Beta2 occurs slightly later than Ngn3, and its expression, unlike Ngn3, persists in differentiated islet cells where it regulates differentiation of islet cells and the expression of the insulin gene (Naya et al. 1997).

1.2.2.3 Specification of endocrine cell subtypes

While Ngn3 upregulation in the epithelial trunk progenitor cells signifies endocrine differentiation (Sander et al. 2000; Zhou et al. 2007; Stanger, Tanaka & Melton 2007), it is not completely understood how and when Ngn3+ cells make their choices into appropriate hormone cell type. Equally important is the question of whether each high Ngn3–expressing cell is multipotent or unipotent. Some studies report that each high Ngn3-expressing cell can differentiate into any of the four possible subtypes of the endocrine cells (Deltour et al. 1991; Wilson, Scheel & German 2003; Johansson et al. 2007; Scharfmann et al. 2008), while clonal analysis studies suggest that each high Ngn3+ endocrine progenitor may be unipotent and postmitotic cell that is already pre-committed to specific endocrine lineage such as alpha cells, beta cells, delta cells, PP cells and epsilon cells. i.e. a single high Ngn3+cell is a precursor of either a beta cell or an alpha cell but not a precursor of both at the same time (Gu, Dubauskaite & Melton 2002; Desgraz, Herrera 2009; Miyatsuka et al. 2011).

However, some studies that used a conditional Ngn3 transgene, “Ngn3 addback,” showed that differentiation of each endocrine cell type from high Ngn3+ endocrine progenitors occur at specific time points and through sequential competence states that arise within the duct cells themselves, and lead to generation of alpha cells first (E9.5), then beta cells (E10.5) and δ-cells

(E14.5), followed by PP-cells (E18.5) (Johansson et al. 2007). The time point for differentiation of ε cells is unknown. Although these findings postulate a time frame for islet cell allocation, the basis of these cell-intrinsic competence periods and the mechanism that controls this change in developmental potential have not been established. Furthermore, the origin of the signaling molecules guiding Ngn3-expressing progenitor cells to differentiate towards a specific endocrine cell type is currently unknown. The mechanism may likely involve several regulatory factors and so many cell intermediates that may change according to the condition.

1.2.2.4 Molecular basis in specification of endocrine cell subtypes

Following activation of Ngn3, several transcription factors have been identified to function in endocrine subtype lineage specification, differentiation and maturation. The identified transcription factors expressed downstream of Ngn3 include NeuroD1/Beta2, Nkx2.2, Nkx6.1, Pdx1, Arx, Motor neuron and pancreas homeobox 1 (Mnx1), Foxa1, Foxa2, V-maf musculoaponeurotic fibrosarcoma oncogene family, protein A (MafA), V-maf musculoaponeurotic fibrosarcoma oncogene family, protein B (MafB), Regulatory X-box binding 6 (Rfx6) , insulin associated 2 (IA2), Paired box-containing gene 4 (Pax4), Pax6 and Insulinoma-associated antigen 1 (Insm1), Myeline transcription factor 1 (Myt1) and Snail2/Slug (Rukstalis, Habener 2007, Rukstalis, Habener 2009, Seymour, Smith et al. 2010, Soyer et al. 2010, Sander 2011). However, the mechanism by which these transcription factors specify the changes that contribute to allocation of distinct islet cell subsets is not clearly understood. It is now postulated that specification and differentiation of each endocrine cell type is regulated by a set of “transcription factor codes” that are specific for each cell type (Pan, Wright 2011) (Fig. 1.4). Using a loss-of-function approach, these endocrine cell-type-specification regulatory factors have been divided into three classes based on the phenotypes of the mutant models used: Class 1-

endocrine precursor differentiation factors; Class 2- lineage allocation factors and Class 3 - cell-type maturation factors (Collombat et al. 2006), reviewed in (Pan, Wright 2011).

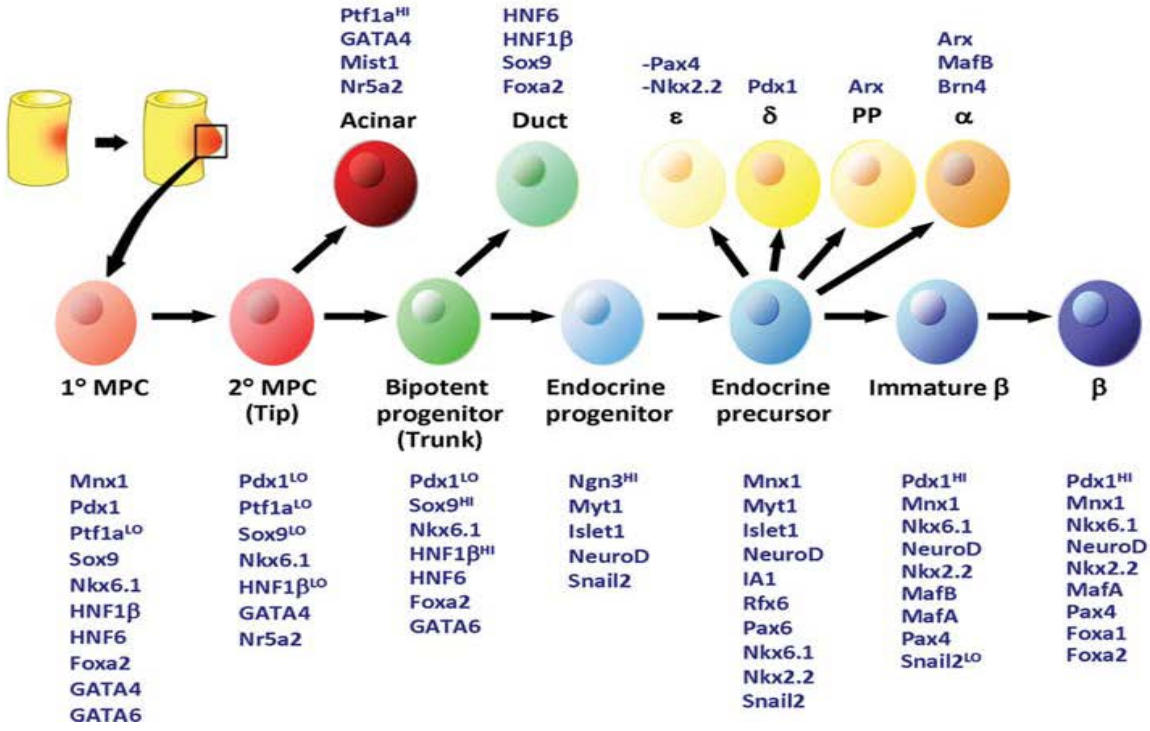


Figure1.4. Shematic flow diagram showing key transcriptional factors activated at each stage of endocrine pancreas development *Adapted and modified from Pan, Wright 2011*

Class 1: Endocrine precursor differentiation factors

Endocrine fate determining factors are mostly downstream targets of Ngn3, including NeuroD1/Beta2 (Huang et al. 2000), Isl-1, Insm-1, Rfx6 and Nkx6.1 (Pan, Wright 2011). These factors are activated early during specification of endocrine subtypes and often co-express with Ngn3. Their main role is to ensure the formation of proper numbers of endocrine cells by stimulating proliferation of endocrine cell progenitors and their intermediates, and allow these progenitors to enter a single-hormone producing state.

(i) NeuroD1/Beta2: NeuroD1/Beta2 is basic helix-loop-helix (bHLH) that is first expressed in pancreatic endodermal cells at E9.5. During secondary transition at E15.5, the gene is

restricted to all endocrine cells except for δ-cells (Chu, Nemoz-Gaillard & Tsai 2001; Anderson et al. 2009a; Anderson et al. 2009b). Mutant mice, in which NeuroD1 is deleted, the numbers of all endocrine cell types are reduced (Naya et al. 1997), particularly beta cells, fail to form islets and die afterbirth due to beta cell apoptosis similar to the phenotypes seen in Isl-1, Insm-1, Rfx6 null mice (Mellitzer et al. 2006; Gierl et al. 2006; Du et al. 2009; Smith et al. 2010; Soyer et al. 2010). Additionally, another report has shown that Isl-1 is required for islet cells differentiation. Mice in which Isl-1is deleted are born without endocrine cells (Ahlgren et al. 1997), and its expression was found to be critical for development of exocrine tissue of the dorsal bud as well, suggesting that morphogenesis of the dorsal and ventral pancreatic buds are regulated by different signaling molecules.

(ii) NKx6.1: Nkx6.1 is first expressed in the pancreatic epithelium from E10.5 and at E15.5. The gene is co-localised with Ngn3+ immature progenitors where it induces proliferation of these cells (Jorgensen et al. 2007). Later, Nkx6.1 is upregulated in Pdx1+ mature beta cells where Ngn3 is downregulated. Nkx6.1 promotes beta cell fate specification from Pdx1+ cells. Deletion of Nkx6.1 causes marked reduction in the number of Ngn3- and insulin-positive cells in mutant mice (Jensen et al. 1996).

Class II: Endocrine progenitor lineage specification factors

Lineage specific transcription factors controls the allocation of the Ngn3+ progenitors and their intermediates towards a specific endocrine cell type. These factors include Nkx2.2, Pdx1, Pax4 and Arx. Deletion of any one of these factors does not change the number of endocrine types, but severely alters the fractional islet allocations by causing lineage-switching.

(i) Nkx2.2: During endocrine differentiation, Nkx2.2 is activated in alpha-, beta- and PP cells, exclusive of the somatostatin-producing delta cells (Sussel et al. 1998). In Nkx2.2 mutants, beta

cells and big fractions of alpha- and PP cells are replaced by epsilon cells (Sussel et al. 1998, Prado et al. 2004). In addition, beta cells are born immature lacking beta cell specific maturation factors, glut2 and glucokinase.

(ii) Pax4 – Arx: Reports show that paired box, Pax4, is a key transcription factor in regulation of insulin producing beta cells and somatostatin producing delta cells differentiation (Sosa-Pineda et al. 1997). Increase in Pax4 expression in Ngn3+ precursor cells marks the beginning of beta cell differentiation. In Pax4 knockouts, beta- and alpha cells are lacking while alpha- and epsilon- dramatically increase to compensate for their absence. Pax4 also inhibits Arx, a factor that specifies alpha cells (Collombat et al. 2003; Collombat et al. 2005). Concomitantly, in Arx mutants, alpha cells are lost, while delta/beta cells increase. Thus, Pax4 and Arx specify beta- and alpha cells respectively. Activation of Pax6 in precursor cell drives these cells to differentiate into glucagon secreting delta cells and maintains a glucagon-producing cell fate (St-Onge et al. 1997).

(iii) Pdx1: In addition to promoting expansion of early progenitor cells in the pancreatic buds, Pdx1 promotes beta cell proliferation and survival during secondary transition stage. Deletion of Pdx1 at this stage, leads to reduction in beta cells while alpha/delta cells increase in number (Gannon et al. 2008). In mature beta cells, Pdx1 activates the expression of insulin, Glut-2 (Ohlsson, Karlsson & Edlund 1993) and Islet amyloid polypeptide (IAPP) (Watada et al. 1996). Thus, Pdx1 is activated at all stages of pancreatic development. In the early stages of embryonic development, Pdx1 is required for pancreas development, while in the in fetal stages of development the gene is required for specific beta cell fate differentiation. In an adult pancreas, Pdx1 activates the expression of the insulin gene and for the differentiation of the mature

pancreas (Ohlsson, Karlsson & Edlund 1993). Most of these factors are expressed in a spatial and temporal manner during development.

Class III: Endocrine cells maturation factors

In addition to endocrine fate determination and lineage specification factors, a third set of regulatory factors control the morphological and functional maturation of differentiating endocrine cells. Transcription factors involved in endocrine cell maturation include MafA/B, Foxa1, Foxa2 and NeuroD1. Loss of these factors does not affect cell lineage specification, but cells display abnormal functions after birth

(i) MafA/B: The basic leucin zipper transcription factors MafA and MafB are activated late in differentiating endocrine cells. MafA is required for acquiring and maintaining the maturity state of beta cells by activating Pdx1, Nk6.1, Insulin, GLUT-2, Slc30a8 (zinc transporter) and G6pc2 (glucose-6-phosphate catalytic subunit 2 protein) genes which are important for beta cell function (Aramata et al. 2005; Zhao et al. 2005; Artner et al. 2006; Raum et al. 2006). Beta cells arise from immature MafB+ cells which give rise to MafB+/MafA+ cells , then finally to mature MafA+ beta cells, a process that results into activation of Pdx1 (Nishimura et al. 2006; Artner et al. 2010). MafA interacts with Pdx1 and NeuroD1 and bind to insulin promoter to activate transcription of the insulin gene (Olbrot et al. 2002; Aramata et al. 2005). MafB later becomes restricted to alpha cells. Therefore, MafB is required for both beta- and alpha cell differentiation and maturation. MafA mutants become glucose intolerant after birth (Zhang et al. 2005)

(ii) Foxa2/Hnf3β: The bHLH Foxa2 promotes maturation of endocrine cells (Lee et al. 2005) by activating Pdx1 that activates the expression of insulin and GLUT-2 in beta cells (Gerrish et al. 2000, Sund et al. 2001). Deletion of Foxa2 also reduces the number of alpha cells indicating

that Foxa2 is also required for development and activation of glucagon in alpha cells (Lee et al. 2005).

1.2.2.4.1 Formation of islets of Langerhans

As development proceeds, committed high-Ngn3-expressing endocrine precursor cells and their immediate downstream intermediates, exit the cell cycle at around E14.5 and delaminate from the ductal epithelium through epithelial–mesenchymal transition (EMT) by rapidly activating Snail2 (Fig. 1.3) (Rukstalis, Habener 2007), before aggregating into islets of Langerhans (Fig. 1.3) (Pan, Wright 2011; Miyatsuka et al. 2011; Gouzi et al. 2011).

The final stages of islet development and maturation is characterized by formation of blood vessels and nerves (Pictet et al. 1972; Burris, Hebrok 2007). The development of blood vessels within the islets is promoted by cellular interactions between vascular endothelial cells and endocrine cells, which are critical for the development of functional islets (Lammert, Cleaver & Melton 2001; Lammert et al. 2003). This is followed by migration of neural crest cells to the aggregates of endocrine cells around E12.5 to innervate islets (Burris, Hebrok 2007; Nekrep et al. 2008). Development of sympathetic innervations is critical for regulating beta cell secretory functions and survival in mature islets. Toward the end of gestation, these endocrine cell aggregates eventually disperse between acini and ducts (Pictet et al. 1972; Slack 1995). Shortly before birth at E18.5, pancreatic polypeptide cells (PP-cells) differentiate. In mice, typical rodent islet architecture, with insulin-secreting cells located at the center and non-beta cells in the periphery, is clearly seen late in gestation (E18.5) (Herrera et al. 1991).

1.2.2.5 Physiological and morphological maturation of beta cells

Physiological and morphological maturation of beta cells is important in order to achieve effective glycemic control. A defective beta cell maturity or a failure of beta cell expansion promotes development of a metabolic disease, such as diabetes mellitus (Georgia, Bhushan 2004; Butler et al. 2007). After weaning, beta cells undergo two main events to achieve maturity: 1) physiological maturity through development of the glucose sensor machinery by increasing dense core sensory granules (Kim et al. 2006) and 2) morphological maturity by remodeling to establish an appropriate beta cell mass proportional to individual's body weight (Georgia, Bhushan 2004; Butler et al. 2007).

To achieve beta cell glucose stimulus-secretion coupling, beta cells activate several genes including proinsulin and insulin (genes that maintain identity of islet cells); Glucose transporter 2 (GLUT-2) and glucokinase (GK) (genes that senses blood glucose concentration); Pdx1, MafA and NeuroD1(Tfs for development and maturation); chromogranins (Chg) A and ChgB, and islet amyloid polypeptide (IAPP) (for formation of secretory granules); SUR1 (Sulfonylurea regulatory receptor in K (ATP) channels e.t.c (Kim et al. 2006; Aguayo-Mazzucato et al. 2011). Meanwhile, to achieve morphological maturation, beta cells activate several genes, such as Cyclin dependent kinase 4, CyclinD2 and FoxM1 (Seymour, Sander 2011).

In summary, the genetic cascade in endocrine development is particularly important in order to understand the pathology of diabetes. Understanding how these factors are regulated or whether such an embryonic pathway can be reactivated in adult pancreas, could be used in designing therapeutic regenerative protocols for the cure of diabetes.

1.3 Role of epithelial-mesenchymal interactions in development of the pancreas as revealed by embryonic explant experiments

Mesenchymal stromal/stem cells (MSCs) are a group of heterogeneous multipotent cells that are capable of differentiating into various mesodermal tissues such as bone, cartilage, adipose tissue and muscle. Various studies have attempted to isolate these cells from various postnatal tissues, including the bone marrow and characterize these cells based on their ability to stick on plastic plates when cultured under normal standard conditions (reviewed in Mafi et al. 2011; Gopurappilly, Bhat & Bhonde 2013a; Scuteri et al. 2014). Most of the data regarding their phenotypic properties is based on analyses of isolated cells that have been expanded *in vitro* and whereas their *in vivo* phenotype and role are largely unknown (reviewed in Mafi et al. 2011; Gopurappilly, Bhat & Bhonde 2013a; Scuteri et al. 2014).

In classic explant culture experiments performed more that 6 decades ago, pancreatic epithelial tissue explants from embryonic pancreas display low levels of differentiation when cultured without MSCs (Golosow, Grobstein 1962). In the same study, when pancreatic epithelial and MSC tissues were cultured side by side separated by Millipore filter, differentiation of the pancreatic epithelium was induced. Results from these studies clearly demonstrate that MSC tissue secretes soluble inductive factors that are required for development of the pancreas (Golosow, Grobstein 1962). In these earlier studies, however, these secreted soluble factor(s) were not identified. Identification of key MSC-derived factors required during pancreas organogenesis is important in development of protocols to direct cell differentiation through particular pathways.

In addition, these earlier *in vitro* studies focused on the development of the exocrine pancreas rather than the endocrine pancreas (Wessells, Cohen 1967; Pictet et al. 1972). However, the few *in vitro* studies done about two decades ago that focused on the role of the MSCs in differentiation of endocrine pancreas obtained contradictory results. Findings from Ruttter and his group showed differentiation of the epithelium into endocrine tissue in the absence of MSCs (Rutter et al. 1978; Gittes et al. 1996), while others did not observe any endocrine cell development in the absence of MSCs (Wessells, Cohen 1967; Andrew, Rawdon & Alison 1994). Similar observations on endocrine development in the absence of MSCs were made by Gittes et al. (Gittes et al. 1996). In their experiment, when pancreatic allografts were transplanted under the kidney capsule with surrounding MSCs, they differentiated into mature pancreatic tissue, forming ducts, acini and endocrine tissues. On the other hand, allografts of epithelial tissue isolated without MSCs formed only dense spheroid bodies of pure islets suggesting that embryonic MSC factors required for the differentiation of the exocrine pancreas differs from those needed for differentiation of the endocrine part. However, soluble signals involved in repression of endocrine and induction of exocrine tissue during these interactions have not been investigated. One candidate molecule could be Follistatin, which was found to regulate the relative propotions of endocrine versus exocrine during pancreas development (Miralles, Czernichow & Scharfmann 1998). In addition, these experiments did not examine the influence of early MSC-derived factors secreted way before isolation of the tissues for culture.

Taken together, findings from these studies show that molecular signals secreted by the notochord control the development of the dorsal pancreas in the early stages of embryonic development (Golosow, Grobstein 1962; Kim, Hebrok & Melton 1997; Wells, Melton 2000). In later stages, proliferation and differentiation of the pancreatic progenitor cells are controlled by

MSC-derived factors that determine the proportion of endocrine versus exocrine tissue (Golosow, Grobstein 1962). Although some studies have reported the important role of signals from MSCs in the control of development of exocrine tissue, their role in the development of endocrine tissue is still not very clear. Whether these extrinsic factors secreted by tissues surrounding islets during pancreas development are also activated in normal or injured adult pancreas such as pancreatic duct ligation (PDL) is also not known. Understanding their roles in pancreatic endocrine cell proliferation and differentiation can help us to devise new regenerative therapeutic strategies in adult pancreas, against the deficiency of beta cells that lead to type 1 and type 2 diabetes mellitus and hence develop appropriate therapeutic solutions to these diseases.

1.3.1 Epithelial-MSCs interactions in mature pancreas

As stated in section 1.3 above, the question of whether the epithelial-MSCs interactions play any role in maintenance of beta cell mass in adult pancreas under normal physiological or injured conditions is not well known. In adult tissue, MSCs are commonly described as a heterogeneous population of adult fibroblast-like, multipotent cells characterized by their ability to adhere to plastic substrate and differentiate into tissues of mesodermal origin, such as adipocytes, chondroblasts, and osteoblasts when cultured *in vitro* (Friedenstein et al. 1974a; Pittenger et al. 1999). It was reported that the relative proportion of MSCs to epithelium changes during pancreas development. More MSC tissue is found in fetal pancreas than in neonates. A recent histological study of fetal mice pancreas showed that relative MSC area is significantly reduced to as low as 11% at E15.5 and 6% of the pancreatic area at E18.5 (Landsman et al. 2011). In adult pancreas, the epithelial compartment is the major component of the pancreatic tissue that comprises endocrine cells, acinar cells and duct cells. The mesenchymal cell compartment is very small and scattered amongst the epithelial tissue, largely composed of endothelial cells and

pancreatic fibroblasts. In addition, present in smaller numbers within the MSC tissue are cell types such as stellate and vascular smooth muscle cells (Hogan 1999). Despite such dramatic reduction in their portion in mature pancreas, embryonic MSCs are, however, still detected throughout pancreas development and maturation (Hogan 1999). Whether this developmental reduction in MSC area reflects a related reduction in beta cell replication and /or neogenesis in adult pancreas is not known.

In a normal adult mouse model, β- cells growth and/ or regeneration occurs very slowly via replication of existing beta cells or neogenesis (generation of new beta cells from precursor cells). A study by Teta et al. (2005) reported that beta cells are long-lived, and in one-year-old mice, only 1 in 1400 beta cells divide in a 24 h period (Teta et al. 2005). The question of whether the epithelial-MSCs interactions play any role in maintenance of beta cell mass in adult pancreas under normal physiological or injured conditions is not clearly understood. Using explant systems to address the role of the MSCs in adult pancreas have been difficult because the mature pancreas epithelium is highly branched into the surrounding MSCs. This prevents clean physical separation of these two layers.

1.3.2. Mesenchymal stromal cells (MSC) isolation and characterization in culture

Findings by Friedenstein and colleagues (Friedenstein et al. 1974b; Pittenger et al. 1999) led to identification of the bone marrow– derived fibroblast-like cells with chlonogenic differentiation potential. After discarding the supernatant containing non-adherent hematopoietic stem cells from low density cultures of bone marrow on plastic culture dishes, Friedenstein and colleagues were able to identify the plastic adherent cells or colony-forming unit fibroblasts, which were later called mesenchymal stromal cells (MSCs) by Owen (1988). Although there are a number of

protocols available to isolate rat MSCs, each isolation protocol can vary in the yield, purity, quality, and the ability of the cells to proliferate. It is interesting to note that since the first description of MSCs by Friedenstein et al.(1966), researchers have used this method of adherence of fibroblast-like cells to the plastic substrate of a cell culture plate as s standard method when isolating cells recovered from umbilical cord, adipose, pancreas or bone marrow tissues etc (Pittenger et al. 1999). Other reports, however, proposed the negative selection method to exclude non-adherent hematopoietic stem cells, or using positive selections for some of the MSC markers in order to purify MSCs (Tyndall, Furst 2007, or a review Mafi et al. 2011).

1.3.3. Characterisation of MSCs in culture

Until now, no specific singular marker has been identified to characterize the mensenchymal stromal cells (MSCs). As a result, researchers use a set of positive and negative markers to phenotypically characterize these cells. MSCs express variable levels of CD105, CD90/Thy1 and CD73 and absence of markers such as CD34, CD45 and CD14 (reviewed in Mafi et al. 2011). A number of other cell surface markers have also been identified, although, some study used their differentiation potential into several mesenchymal lineages as a creteria to identity these cells (Prockop 1997). However, the specificity of some of these markers varies when identifying MSCs isolated from humans versus mice/rats (reviewed in Mafi et al. 2011). To date, no study has succeeded in the identification of MSCs *in vivo*.

The main criterion currently used to characterize and identify MSCs is their capacity for self-renewal and differentiation into tissues of mesodermal origin, combined with a lack in the expression of certain hematopoietic molecules. In 2006, the international society for cellular therapy (ISCT) developed criteria for characterizing MSCs. Firstly, MScs should possess

plastic adherent properties under normal culture conditions and has a fibroblast-like morphology. Secondly, MSCs should be capable of differentiating into osteoblasts, adipocytes and chondrocytes *ex-vivo*. Thirdly, MSCs should demonstrate positive markers such as CD105, CD90/Thy1 and CD73 and absence of markers such as CD34, CD45 and CD14 (reviewed in Mafi et al. 2011).

1.3.4 Pancreas-derived mesenchymal stromal cells (PMSCs)

Protocols for the isolation of PMSCs from porcine fetal pancreas (Cao et al. 2011), human fetal and adult pancreas (Hu et al. 2003), mice pancreas (Mafi et al. 2011, Gopurappilly, Bhat & Bhonde 2013a, Scuteri et al. 2014) have been published. In these studies, it was shown that PMSCs are positive for CD44, CD29, CD 73 (Ecto-5'- nucleotidase) CD 105 (Endoglin), CD 166 (ALCAM), CD 90 (Thy 1), CD 44(H-CAM), CD 49a (VLA-1) but are negative for CD45, CD 14, CD 19, HLA-DR, CD 34, and CD PECA-1) (markers of hematopoietic cells) and CDI3 but negative for CD34 and HLA-DR. These findings demonstrate that PMSCs can be isolated from pancreas based on their adherent ability, their capability of self-renewal and their multipotent potentials.

In summary, evidence from rodent research has remarkably enhanced our understanding of the processes and factors that regulate pancreas development. Development of the pancreas from primitive gut endoderm requires complex interactions of molecular factors, some of which play important roles in proliferation, migration, differentiation and growth of exocrine and endocrine pancreas. Morphogenesis and further differentiation of the pancreas requires the presence of extrinsic factors from notochord and mesenchyme. Key factors in endocrine development include Pdx-1, Ngn3, Pax4 and Pax6 (Fig. 1.4). Although these transcription factors are required

for pancreatic organogenesis, the mechanism by which their expression is regulated is not fully understood. Identifying and characterizing the genetic cascade of regulatory molecules in endocrine development is particularly important in order to understand the pathology of diabetes. Understanding how these factors are regulated or whether such an embryonic pathway can be reactivated in adult pancreas can help us in designing therapeutic regenerative protocols for the cure of diabetes mellitus.

1.4 Diabetes mellitus and cell replacement therapy

Diabetes mellitus is the major chronic metabolic disease that globally affects an estimated 422 million adults, according to the latest 2016 data from the World Health Organization (WHO) (WHO Global Report on Diabetes. Geneva, 2016). Prevalence for diabetes mellitus for all ages worldwide was estimated to be 2.8% in 2000 and 4.4% in 2030 (Wild et al. 2004). Although diabetes is associated with developed countries, the incidence is also increasing in developing countries (Longo-Mbenza et al. 2010). Some contributing factors to this increase include aging, urbanization, population growth, increasing prevalence of obesity and physical inactivity. The prevalence estimates for the age groups 20-79 years in South Africa alone was put at 7% in 2015 (IDF Diabetes Atlas Group 2015). In developing countries, the disease has worst outcomes, largely because the poor cannot access or afford preventable services and treatment. Such increase in prevalence requires urgent global attention in order to reduce increase in deaths because of complications associated with the disease.

There are two forms of diabetes mellitus, namely, type 1 and type 2 diabetes mellitus. Type I diabetes comprises 5–10% of all diabetic mellitus cases while type 2 diabetes , 90–95% of all diabetic mellitus cases (Shapiro et al. 2000; Shapiro et al. 2006). The etiology underlying the

disease is complex. In Type 1 diabetic patients, insulin producing β- cells are selectively lost due to autoimmune reaction while in type 2, beta cell functions are relatively impaired coupled with peripheral resistance to insulin action. In addition, type 2 diabetes results into gradual decrease in beta cell mass with the progression of the disease (Butler et al. 2003), also requiring replacement with new β- cells.

The inability of the body to produce sufficient amounts of insulin, the hormone that regulates the level of blood glucose, leads to chronic hyperglycemia, which affects multiple organs such as the eyes, kidneys, heart, nerves, and blood vessels. If untreated, the hyperglycemic condition may lead to complications such as micro- and macro-vascular damage, blindness, amputation and/or death (Fuller et al. 1983).

1.4.1 Novel treatments for diabetes mellitus

Current treatment regimens of diabetes require daily oral intake of hypoglycemic agents (for type 2) combined with injections of exogenous insulin (for type 1) in order to compensate for insulin deficiency. However, this treatment regimen does not mimic the biological mechanism of controlling the daily fluctuations in systemic blood glucose. Therefore, researchers are investigating novel therapies for treating diabetes by beta cell generation *in vitro* and transplantation or re-generation *in vivo* to restore beta cell mass. Generation or replacement of new insulin producing beta cells would provide a real therapy that would mimic the biological mechanism capable of restoring normoglycemia. Whole cadaveric pancreas transplantation has provided effective treatment to patients with serious complications of diabetes for the past four decades (Bonner-Weir, Weir 2005b). The risks associated with this major surgical procedure, however, prohibit it from being recommended as a treatment for diabetes (Burke, Ciancio &

Sollinger 2004; White, Manas 2008). Islet transplantation, a less invasive procedure, represents a substitute to whole organ transplant and insulin therapy (Shapiro et al. 2000; Shapiro et al. 2006). The use of this procedure is also limited due to shortage of donors, immune rejection, and the need for life-long immuno-suppressors (du Toit et al. 1998; Muller et al. 1998; Inverardi, Kenyon & Ricordi 2003; reviewed in Burke, Ciancio & Sollinger 2004; Ricordi, Strom 2004). An alternative strategy to islet transplantation is the *in vivo* generation of beta cells within the patient's pancreas (Bonner-Weir, Weir 2005a).

1.4.1.1 Beta cell regeneration in adult pancreas

Several attempts to generate endogenous new beta cells for therapeutic purposes using various animal models of pancreatic injuries have been reported (Fig. 1.5). These studies currently focus on strategies that promote either the expansion of existing beta cells within the pancreas or the generation of new beta cells through neogenesis within the pancreas. Neogenesis debate has recently intensified with many cell-tracing studies reporting inconsistent results about the sources of these new beta cells (Fig.1.5) (Bonner-Weir et al. 2010, Kopp et al. 2011, Furuyama et al. 2011, Criscimanna et al. 2011). Until now, the question of whether facultative beta cell progenitors exist in adult pancreas is still under intense investigation. Factors that trigger generation of new beta cells in injured pancreas of experimental models have not been clearly elucidated. This study was developed to address this conundrum.

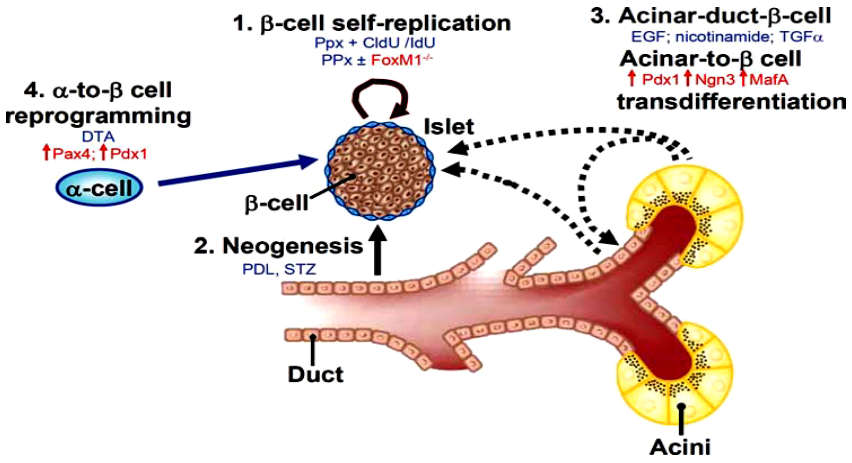


Figure 1.5. Model showing sources of beta cells during regeneration.

Adapted and modified from Pan, Wright ***2011.***

As stated previously, in a normal adult mouse model, replication of existing beta cells occurs very slowly, however, under certain experimental conditions, high levels of growth and neogenesis of beta cells have been reported (Peters, Jurgensen & Kloppel 2000; Bouwens 2006). The most important of these models are: duct ligation in adult rats (Hultquist, Joensson 1965; Wang, Kloppel & Bouwens 1995; Xu et al. 2008a), partial (90%) pancreatectomy in rats (Bonner-Weir et al. 1993a), cellophane wrapping in newborn hamster rats and selective beta cell destruction by streptozotocin/alloxan perfusion in adult mice (Bonner-Weir et al. 1981; Cantenys et al. 1981; Rosenberg, Brown & Duguid 1983; Bonner-Weir et al. 1993a; Waguri et al. 1997). In these animal models, endocrine cell regeneration was reported. Although the existence of stem /progenitor cell is still questionable and controversial (Solar et al. 2009; Kopp et al. 2011; Cavelti-Weder et al. 2013), various sources of islet neogenesis were proposed.

In young adult rats, when 90% of the pancreas is resected, a significant regeneration of both endocrine and exocrine pancreas occurs (Bonner-Weir et al. 1993a; Hayashi et al. 2003). In this

model, replication of pre-existing and contributions from beta cell neogenesis from ductal epithelium were also observed to a lesser extent. However, the signals that trigger the replication capacity of the pre-existing beta cells in this model are not well understood. Blaschuk et al. (1983) showed that a cell aggregating factor, clusterin, is highly expressed in the pancreatic regenerating tissues of this rat model, suggesting an important role for clusterin in regulation of neogenesis in the pancreas (Min et al. 2003).

Waguri et al. (1997) also established a transient diabetic mouse model by selectively perfusing the body and tail of the pancreas with alloxan. Following alloxan perfusion, proliferation and differentiation of islet cells were observed in the pancreas. Similarly, Peters *et* al. (2000) detected beta cell regeneration occurring by two mechanisms: replication of pre-existing beta cells in non-alloxan perfused parts and neogenesis from duct cells in beta cell-depleted parts of the pancreas. Regeneration of beta cell is also observed in streptozotocin (STZ)-treated neonatal rats (Bonner-Weir et al. 1981; Cantenys et al. 1981; Wang, Bouwens & Kloppel 1996). In these neonatal STZ-treated rats, Ngn3 was upregulated, though with contradicting expression patterns i.e. islet cells or duct cells. The general conclusion was that these cells differentiated from duct epithelial precursor cells.

Further supporting evidence came from studies of transgenic mice, in which interferon gamma (IFN- γ) transgene expression was driven by an insulin promoter (Gu, Sarvetnick 1993). IFN- γ expression in islets induced tissue destruction, similar to a pancreatic duct ligation model. This destruction consequently resulted in islet neogenesis from the duct epithelium from which new islets budded off. Similar results were obtained in the pancreas of transgenic mice over-expressing two growth factors, gastrin and Tgf-α (Wang et al. 1993), where beta cell neogenesis

was observed in the ductular epithelium expressing Pdx1 (Gu, Sarvetnick 1993; Wang et al. 1993). In adult hamsters, wrapping the head of the pancreas with cellophane for 8 weeks also induced increase in islet cell mass by 2.5-fold (Rosenberg, Brown & Duguid 1983). Similarly, a noticeable increase in endocrine cell volume as well as ductal cell proliferation was observed in cellophane wrapped head of Velvet monkey pancreas (Wolfe-Coote et al. 1998). In addition, temporarily squeezing the pancreatic main duct for 60 seconds, induced Ngn3 expression in Wistar rats, suggesting that signals that promote generation of duct and endocrine cells seem to be transmitted rapidly (Ferris, Woodroof 2005)

Evidence from rodent models of duct ligation has provided additional support on pancreas regeneration after injury. Many published reports show that injury caused by partial ligation of the pancreatic duct (PDL) induces rapid generation of new islets in the post-ligated pancreas. In earlier studies of adult PDL rat models by Hultquist et al., (1979), and later by Wang et al., ((1995), destruction of exocrine tissue in the post-ligated portion of the pancreas by duct occlusion induced rapid regeneration of exocrine acini by ductular structures and neogenesis of islet cells in the post-ligated portion while the unligated portion remained intact. When these rats were injected with bromodeoxyuridine (BrdU), double increase of BrdU-positive beta cell populations, located near ducts, were observed within the first week after ligation suggesting that these cells differentiated from precursors located within the ductal epithelium and not from pre-existing beta cells (Wang, Kloppel & Bouwens 1995; Xu et al. 2008a). Confirming beta cell neogenesis from ducts in this model, the new cells were found to express Ngn3. The general conclusion was that beta cell neogenesis from exocrine duct cells is the major mechanism of regeneration. In a separate experiment, intermediate cells, which co-expressed insulin and

amylase granules, were observed a day after duct occlusion, indicating neogenesis of beta cells from acinar cells (Bertelli, Bendayan 1997).

In summary, it appears that one major and dominant mechanism to regenerate insulin-producing cells, under injured and physiological conditions, is by replication of the pre-existing beta cells (Fig. 1.5) (Dor 2006; Brennand, Huangfu & Melton 2007). However, when special types of injuries are applied, beta cell neogenesis is also activated amidst controversy about the origin of these new cells. Some studies suggest that these cells arise from duct epithelium (Xu et al. 2008a; Collombat et al. 2009; Collombat et al. 2010; Lee et al. 2010), while others suggest that intra-islet progenitors as well as centroacinar cells are sites of islet neogenesis (Fernandes et al. 1997; Guz, Nasir & Teitelman 2001; De Haro-Hernandez, Cabrera-Munoz & Mendez 2004; Liu et al. 2010; Tchokonte-Nana 2011a). However, although most studies report of beta cell neogenesis from duct epithelium, these ductal precursors have not been fully characterized.

Firstly, some studies indicate that the adult pancreas contain a pool of duct-lining precursor cells that have the capacity to differentiate into endocrine cells and give rise to functional beta-cells (Bonner-Weir, Sharma 2002; Li et al. 2002; Hayashi et al. 2003; Xu et al. 2008a; Inada et al. 2008; Collombat et al. 2009; Liu et al. 2010), while others suggest that all duct cells are precursors which can be reactivated upon stimulus by specific regulatory factors.

Secondly, even though the general conclusion from most of the animal models of pancreas injury discussed above, indicates that beta cells are regenerated by neogenesis from precursor cells, the mechanism to regenerate either by neogenesis, or by replication of pre-existing beta cells, seem to depend on the type of injuries applied. In uninjured mice, more evidence support

the replication of pre-existing beta cells (Dor et al. 2004; Dor 2006). Similarly, in partial pancreatectomy, no evidence of beta cell neogenesis was observed (Dor et al. 2004; Lardon, Bouwens 2005; reviewed by Bonner-Weir, Weir 2005b). Lee et al. (2006) also failed to find Ngn3 (marker of precursor cells) expressing cells in pancreas following partial pancreatectomy. Nevertheless, these studies did not examine the specific regions of the pancreas damaged during surgery, where neogenesis might have occurred (Sharma et al. 1999).

Thirdly, the severity and type of injuries applied to these animal models differed between studies. It is logical to validate conclusions from these studies with evidence from genetic-lineage-tracing techniques and *in vitro* studies. Despite controversial results from these *in-vivo* studies, it is possible that beta cell neogenesis can be induced *in vitro*. For example, when isolated human duct cells are cultured with Ngn3, low levels of islet-specific genes, including insulin, are expressed (Heremans et al. 2002; Gasa et al. 2004). Similar results were obtained when signals from fetal pancreas were applied on cultured human duct cells (Hao et al. 2006) and cultured rat duct cells (Dudek et al. 1991), suggesting that beta cell neogenesis can be reactivated in adult pancreatic ducts, similar to what is observed in an embryo (Piper et al. 2004; Pierreux et al. 2006). Additional evidence on beta cell neogenesis from exocrine tissue came from a study that combined *in vitro* culture with genetic-lineage tracing experiments (Minami et al. 2005). Results from this study demonstrated that adult acinar cells could be induced to transdifferentiate into a beta cell fate, consistent with results from independent work of Means et al. (2005).

1.4.1.2 Factors regulating beta cell neogenesis during regeneration

Factors regulating neogenesis from duct epithelium, however, are largely unknown. It is possible that more than one factor, (extrinsic and intrinsic) acting in a sequence similar to the events that occur during fetal development, are required for beta cell neogenesis in an adult. The mechanism of action for such a complex regulatory pathway in adulthood is unknown.

In a study by Xu et al. (1999), it was demonstrated that exendin-4, an analog of glucagon-like-protein 1 (GLP-1) increased islet cell mass in adult animals subjected to subtotal (90%) pancreatectomy. Similarly, Hui et al. (2001) demonstrated that GLP-1 treatment induced neogenesis of islets in Wistar rats. A more recent study demonstrated that Survivin (Birc5), an inhibitor of apoptosis protein (IAP) gene family, is required for beta cell mass expansion in adult mice subjected to duct ligation (Wu et al. 2012).

Another factor associated with islet neogenesis is a MSC-derived hepatocyte growth factor (HGF that induces cell proliferation, cell motility, and morphogenesis in many cell types. Hepatocyte growth factor and its receptor c-met are expressed in the islet cells of many species. *In vitro,* infusion of HGF in the human fetal and adult pancreatic beta cells stimulates proliferation and promotes insulin secretion (Otonkoski et al. 1994; Otonkoski, Mally & Hayek 1994; Hayek et al. 1995; Otonkoski et al. 1996). In genetically engineered mice, in which HGF is expressed under the rat insulin II promoter (RIP-HGF), over-expression of this transgene resulted in an increase in beta cell mass (Garcia-Ocana et al. 2000; Nakano et al. 2000). Transgenic RIP-HGF mice present with plasma glucose levels that are lower than their normal littermates and have higher fluctuating levels of insulin. In an *in- vivo* study, Nakanobet al. (2000) showed that intra-

peritoneal administration of HGF ameliorated hyperglycemia in diabetic mice transplanted with small numbers of pancreatic islets. Later, the importance of HGF/c-met signaling pathway in regulation of islet growth and function was shown in conditional ablation of c-met in beta cells (Dai et al. 2005). In this study, conditional ablation of c-met resulted into hypoplastic islets, low levels of insulin, and impaired glucose tolerance. These studies, therefore suggest that extrinsic factors from the stimulated surrounding tissue can induce beta cell neogenesis in these animal models.

Regenerating genes (Reg) have also been identified in partial pancreatectomy mouse models through a differentiatial cDNA screen on regenerative pancreas (Terazono et al. 1988; Unno et al. 1992). When Reg genes were cloned both *in vivo* and *in vitro*, beta cell growth was promoted (Watanabe et al. 1994). In cultures of human fetal islet cells, high levels of Reg proteins were detected in proliferating beta cells and low levels were found in mature beta cells (Otonkoski, Mally & Hayek 1994) suggesting an important role of Reg proteins in proliferation of beta cells. In cellophane wrapping and STZ-treated animal models, Islet neogenesis-associated protein-pentadecapeptide (INGAP-PP) were shown to be expressed in proliferating and subsequent differentiating islet cells from ducts (Rafaeloff et al. 1997; Rosenberg et al. 2004), suggesting that these proteins might be involved in neogenesis of islet cells from ducts during regeneration of the pancreas in this model.

Other hormones and beta cell neogenesis: Gastrin and its receptors are largely produced by G-cells of the gastric mucosa. However smaller amounts are also detected in the fetal pancreas during islet neogenesis. In a duct ligated rat model, gastrin is expressed in the ductal complexes of pancreas of the post-ligated tail pancreas (Wang et al. 1993). In a separate study, gastrin infusion stimulated proliferation of acinar cells, ductal cells and enhanced beta cell neogenesis

from exocrine pancreas, suggesting that gastrin is an additional factor that can promote duct ligation-induced islet neogenesis (Rooman et al. 2001, Rooman; Lardon & Bouwens 2002; Rooman, Bouwens 2004). In alloxan-treated mice, gastrin induced islet regeneration in pancreas when combined with EGF (Rooman, Bouwens 2004; Song et al. 2015). In double-transgenic mice established by Wang et al. (1993), it was shown that over-expression of gastrin and TGF-α stimulated beta cell differentiation from duct epithelium.

Growth factor, betacellulin (BTC), was found to induce beta cell replication and neogenesis that ameliorated glucose intolerance in alloxan- induced diabetic mice (Yamamoto et al. 2000). *In vitro*, BTC, acting in coordination with activin, stimulated differentiation of AR42J cells into insulin producing cells (Yamada, Kojima 2005; Halban et al. 2010). Similar roles of BTC in promoting beta cell regeneration acting through multiple steps to in combination with activin were reported *in vivo*, suggesting BTC as an effective treatment for diabetes (Yamada, Kojima 2005)

Nicotinamide (NIC), also known as niacinamide or nicotinic acid amide, is an amide of nicotinic acid or vitamin B_3 and an inhibitor of poly (adenosinediphosphateribose) synthetase/polymerase that ameliorated glucose intolerance in STZ- and alloxan-induced diabetic animals by stimulating proliferation and differentiation of endocrine cells (Fig. 1.5) (Yamamoto, Uchigata & Okamoto 1981). In fetal pancreatic cells, NIC stimulated endocrine differentiation (Otonkoski et al. 1997).

1.4.1.3 Beta celll replacement therapy

In addition to islet transplantation, which is limited by shortage of donors, immune rejection, and the need for life-long immuno-suppression, research has intensified to find other alternative beta cell sources in order to efficiently treat the consequences of type 1 and type 2 diabetes mellitus such as: expansion of pre-existing beta cells, differentiation of embryonic stem cells into beta cells and transdifferentiation from non beta cells (Desgraz, Bonal & Herrera 2011; Guo et al. 2013). The use of embryonic stem cells (ESC), induced pluripotent stem cells (iPSC), adult pancreatic stem cells and non-pancreatic adult cells has been identified as an attractive strategy for the differentiation and expansion of insulin producing cells *in vitro* to obtain a large number of beta cells for transplantation in diabetic patients, reviewed in Desgraz, Bonal & Herrera 2011.

(i) Differentiation of insulin producing cells from adult pancreatic stem cells

The presence and location of adult pancreatic stem cells is still under intense investigation. Two populations of stem/progenitor have been identified: (1) intra-islet stem/progenitor cells (Ramiya et al. 2000; Liu et al. 2010) and (2) the pancreatic ductal epithelial cells expressing cytokeratin 19 (CK19) and Pdx1 (Ramiya et al. 2000; Liu et al. 2010) that can potentially differentiate into endocrine cells.

Intra-islet stem cells: Precursors of beta cells have been identified within the pancreatic islets that express a neural stem cell marker, nestin. Nestin is an intermediate filament that is also detected in vascular endothelium and pancreatic stellate cells (Lardon, Rooman & Bouwens 2002, Klein et al. 2003). When these multipotential cells were isolated and cultured in a media favouring neural stem cell proliferation, they differentiated into cells expressing hepatic markers, pancreatic exocrine and endocrine genes (Zulewski et al. 2001). Data from this study suggested

that nestin-positive cells might be involved in islet neogenesis. A similar result was reported where cultured nestin expressing cells from adult mouse pancreas gave rise to neural and pancreatic lineages (Seaberg et al. 2004). However, lineage-tracing studies refuted these conclusions. On the contrary, a study on early human pancreatic development showed that nestin is not expressed in endocrine progenitor cells (Piper et al. 2002). This was supported by additional evidence from "pulse" and "chase" experiments in mice, which showed that nestin positive cells are not pancreatic islet cells progenitors (Treutelaar et al. 2003; Delacour et al. 2004).

Pancreatic ductal epithelial stem/progenitor cells: In many studies, new beta cells have been generated *in vitro* from cultured pancreatic duct cells amid debate about the origin and identity of these stem cells in adult pancreas (Ramiya et al. 2000; Bonner-Weir et al. 2000; Yao et al. 2004; Suarez-Pinzon et al. 2005; Hao et al. 2006). Epithelial cells, isolated from the pancreatic duct of prediabetic non-obese diabetic (NOD) mice, generated the first new islet cells *in vitro* (Ramiya et al. 2000). Cultured cells from these studies formed islet-like structures that expressed insulin and glucagon in levels that could reverse hyperglycemia in STZ-induced diabetic mice.

Bonner-Weir et al. (2000) also generated *in vitro* islet cells from human pancreatic duct cells when mixed with cells derived from non-endocrine fractions after human islet isolation. When cultured in Matrigel and supplemented with nicotinamide, the expanded monolayer culture cells were able to differentiate into three-dimensional cysts from which the islet cells budded off. However, the islet-like cells from these cultures expressed lower levels of insulin gene (5%) than the one detected in isolated pure islets, but they were able to release insulin in response to glucose.

In some studies, human pancreatic ductal cells differentiated into beta cells when co-transplanted with human fetal pancreatic tissues (Yao et al. 2004; Hao et al. 2006). In addition to nicotinamide, a combination of growth factors such as epidermal growth factor (EGF) and gastrin stimulated neogenesis of islets in rodents (Wang et al. 1993; Rooman, Lardon & Bouwens 2002). Similar results were obtained by Suarez-Pinzon et al. (2005) when both factors were supplemented on cultured human adult islet cells. The general conclusion from these studies suggested that increase in beta cell mass resulted from beta cell neogenesis from ducts rather than from replication of mature differentiated beta cells from islets. Ectopic expression of Ngn3 in human ductal cells triggered endocrine neogenesis from ducts cells (Heremans et al. 2002).

Nevertheless, findings from these studies suggest that generating functional beta cells from pancreatic cells within the adult organ other than from other possible sources is a possible therapeutic option to prevent the side effects of immunosuppressive agents. Identification and characterization of cell types that express Pdx1 and Ngn3 might provide as a possible source of precursor cells of all islet cell types (Jensen et al. 2000a; Wilson, Scheel & German 2003).

(ii) Transdifferentiation of adult pancreatic cell types

Transdifferentiation of various pancreatic cell types has been reported in several studies using animal models (Beattie et al. 1999; Rooman, Lardon & Bouwens 2002; Lardon et al. 2004; Baeyens et al. 2005). In animal models of pancreatic duct occlusion, adult acinar cells were capable of transdifferentiating into ductal epithelial cells when stimulated by gastrin (Rooman,

Lardon & Bouwens 2002). Similar results were obtained *in vitro* (Lardon et al. 2004). After stimulation with specific growth factors, both *in vivo* and *in vitro*, these pancreatic ductal cells transdifferentiated and gave rise to new islets (Rooman, Lardon & Bouwens 2002; Baeyens et al. 2005). On the other hand, other studies have reported de-differentiation of islet cells into a more primitive proliferative epithelial phenotype.

In vitro, human islet cells transdifferentiated into precursor-like cells that expressed Pdx-1/Ipf-1 suggesting that these cells were still not fully differentiated (Beattie et al. 1999). Similarly, a study by Gershengorn et al. (2004) reported a transdifferentiation of human islet cells, through epithelial-mesenchyme transition, into fibroblastoid cells. Upon expansion, these cells formed back aggregates of islet cells that expressed low levels of endocrine genes. When supplemented with betacellulin, adult human islet cells expanded and re-differentiated into beta cells (Ouziel-Yahalom et al. 2006). However, a lineage-tracing study by Chase et al. (2007) challenged the results of the above two studies, and conclusively showed that the fibroblast-like cells were derived from mesenchyme stem cells and not from endocrine cells (Chase et al. 2007; Tchokonte-Nana 2011).

Centroacinar/terminal duct (CA/TD) cells: The question of whether centroacinar and terminal duct cells can be sources of new beta cells in experimental injured pancreas has been suggested by some studies (Tchokonte-Nana 2011). However, these pancreatic cells are not fully investigated and their molecular characteristics not well defined (Leeson, Leeson 1986; Pour 1994). Few studies of pancreatic injury models reported of proliferation of centroacinar cells after injury such as in pancreatectomy, or treatment with caerulein or Streptozotocin (STZ) (Gasslander et al. 1991; Hayashi et al. 2003; Nagasao et al. 2003). However, these cells were not clearly characterized in these studies. Only seven years ago were these cells characterized by

immunhistochemistry and were found to express high levels of class 1 aldehyde dehydrogenase (ALDH1) enzymatic activity (Rovira et al. 2010) which allowed their isolation and characterization by fluorescence-activated cell sorter (FACS). *In vitro*, the same study demonstrated that CA/TD cells were able to differentiate into endocrine and exocrine cell types.

In addition, in caerulein-induced pancreatitis, $ALDH1^+$ CA/TD cells increased their cell mass, when compared to normal duct cells as controls suggesting clearly that CA/TD cells possess characteristics of progenitor cells (Rovira et al. 2010). Similarly, a lineage tracing study of Bmi1+ cells identified a subpopulation of acinar-derived cells that were self-renewing and were able to maintain homeostasis of the pancreas (Sangiorgi, Capecchi 2009). As previously suggested (Hayashi et al. 2003; Nagasao et al. 2003; Tchokonte-Nana 2011) these cells need further exploration to ascertain whether CA/TD cells are able to contribute to the regeneration of the endocrine pancreas.

(iii) Differentiation of beta cells from embryonic stem cells (ESCs)

In the last two decades, research has focused towards developing efficient protocols to differentiate human embryonic stem cells (ESCs) into insulin secreting cells because these cells have high proliferation, self-renewal and regulated differentiation capacity. Diffrentiation of insulin beta cells from mouse ESCs were first reported (Lumelsky et al. 2001). However, these cells secreted low levels of insulin in response to glucose and were not regarded as true beta cells.

Five years later, D'Amour and his group developed a protocol that used Wint and Tgf-βs signaling pathways and growth factors such as activin A, Fgf10 and RA, to differentiate, with high efficiency, hESCs into definitive endodermal cells that expressed Sox 17, Brachyury

protein, CXC-kemokine receptor type 4 and Cerberus (Chen et al. 2004; D'Amour et al. 2006, Zorn, Wells 2009) which later differentiated into pancreatic lineage expressing Pdx1. Similar protocols were published that used RA and inhibitors of Shh and BMP to induce pancreatic lineage, recapitulating the *in vivo* signaling pathway that specifies the pancreas during development (Kroon et al. 2008a; Mfopou et al. 2010a; Nostro et al. 2011; Xu, Browning & Odorico 2011; Rezania et al. 2012). These *in vitro* generated progenitors expressed Pdx1, Sox9, Nkx6.1 and Ptf1α , and were able to differentiate into endocrine, acinar and ducts when engrafted into mice (Kroon et al. 2008b; Kelly et al. 2011; Rezania et al. 2012; Schulz et al. 2012; Xie et al. 2013). However, the therapeutic option of these progenitors has not been tested in humans because of risk of teratoma development (Wakitani et al. 2003) and stringent ethical issues to be followed when procuring these cells (Fischbach, Fischbach 2004).

Interestingly, some reports showed that many *in vitro* studies have generated immature beta cells from embryonic stem cells (Guo, Hebrok 2009; Mfopou et al. 2010b). Embryonic stem cell-derived beta cells produced under culture conditions secret low levels of insulin, respond very little to glucose stimulation and express more than one hormone in the same cell (D'Amour et al. 2006; Jiang et al. 2007), suggesting that these cells are still not fully differentiated. One possible explanation for such results is that many laboratories use protocols (culture conditions) that do not mimic the cellular environment *in vivo*, that require intercellular interactions between MSCs, endothelial and epithelial cells of the pancreatic bud similar to what occurs during organogenesis of the pancreas. During pancreas organogenesis, different cells from all three embryonic germ layers interact and respond to reciprocal inductive signals that direct cell specificity and fate (Kim, Hebrok & Melton 1997; Wells, Melton 2000; Lammert, Cleaver & Melton 2001; Lammert

et al. 2003; reviewed in Cleaver, Melton 2003). Such components are not supplied in the majority of hESC differentiation protocols.

Furthermore, most *in vitro* differentiation protocols grow cells in two-dimensional cultures as monolayers (Segev et al. 2004). Such protocols do not mimic the three-dimensional interactions between islet, epithelial and mesenchyme cells as they occur *in vivo*. Thus, the efficiency of beta cell specification and maturation can be enhanced by developing co-culture systems that include pancreatic mesenchyme or endothelial cells in the cultures (Guo, Hebrok 2009; Sneddon, Borowiak & Melton 2012). In such cultures, inclusion of pancreatic MSCs and/or endothelial cells might provide additional soluble factors necessary for the proliferation and functional maturation of islet cells (Guo et al. 2013). Pancreatic islets consist of many cell types such as endocrine cells, neuronal cells, and vascular endothelial cells that form a complex interactive structure. The inductive cellular interactions that occur between endothelial cells, endocrine and other non-endocrine cells, play important roles for insulin sensing and secretion (Hauge-Evans et al. 1999; Ballian, Brunicardi 2007; Pierreux et al. 2010; Magenheim et al. 2011b; Shih, Wang & Sander 2013). But, addition of extracellular matrix, *in vitro*, promotes beta cell aggregation and angiogenesis (Hoeben et al. 2004) and may promote β -cell physiology when transplanted into diabetic patients.

1.5 Research question

Over the past decades, research has attempted to identify dedicated adult pancreatic progenitor cells and direct differentiation of these embryonic-like stem cells, with the aim of producing insulin secreting beta cells for use in the treatment of diabetes mellitus. The intrinsic molecular mechanisms that specify pancreatic beta cells have been the primary focus of such research.

Extrinsic factors, however, are also necessary for beta cell neogenesis *in vivo*. The lack of a better understanding of how extrinsic factors from tissues surrounding islets induce *in vitro* beta cell differentiation has been a hindrance in advancing *in vivo* differentiation studies.

In pancreatic injury models, injury to the pancreas by occlusion/ligation (PDL) of the main duct induces neoformation of small beta cell clusters - expressing Ngn3, an earliest islet-specific transcription factor in embryonic development - with increase in associated numbers of beta cells in the post-ligated tail pancreas (Wang, Kloppel & Bouwens1995; Page et al. 2004; Xu et al. 2008a; Inada et al. 2008; Murtaugh, Kopinke 2008; Solar et al. 2009). In addition, MSC tissue and ductal structures proliferate and come into close contact with islet cells in the post PDL (PPDL) tail pancreas. Such small clusters of islet cells, located close to ductal structures, later migrate and become interspaced within the enlarged fibrous septa and adipose tissue (Xu et al. 2008a; Tchokonte-Nana 2011; Chintinne et al. 2012).

Furthermore, several other pancreatic injury models have reported of proliferation of MSCs in adult pancreas, besides islet regeneration (Xu et al. 2008a; Tchokonte-Nana 2011). Whether the MSCs-ductal epithelial and/or pre-exisiting islet cells interactions play any important role in signaling for increased beta cell mass during these conditions of injury has not yet been reported. It is also not clear whether they play a role in the normal healthy adult pancreas where many studies report beta cell mass increase by replication of pre-existing cells rather than neogenesis (Dor et al. 2004;Teta et al. 2005; Teta et al. 2007). In these pancreatic injury models, cellular interactions between the newly formed beta cells and the surrounding tissue may play a crucial role in proliferation and differentiation of beta cells similar to what is observed during embryonic pancreatic development (Pictet et al. 1972). In this regard, a study on the lineage-specific

morphogenesis in normal pancreas postulated the importance of epithelial-MSCs interactions (Gittes 2009).

The importance of signals from the pancreatic MSC tissue have been shown to direct a pancreas-specific fate in the chick (Kramer et al. 1987). Signals from the MSCs may direct epithelial duct cells or pre-existing slet cells toward a beta-cell neogenesis or may be required for the maintaince of specification or to direct proper timing of differentiation, proliferation, or migration of beta cells. In fetal rats, intestinal MSCs when combined with endodermal cells induced the differentiation of the main epithelial cell types including endocrine cells (Kedinger et al. 1986), suggesting an important role MSCs plays in differentiation of endocrine cells. The possibility that MSCs underlying the pancreatic islet may be responsible for beta cell neogenesis in PDL model is based on evidence from studies on fetal rats and chick embryos (Haffen et al. 1983; Kedinger et al. 1986; Yasugi 1993). In these studies, endocrine cells were generated from epithelial cells found in places where endocrine cells are not normally found (Haffen et al. 1983; Kedinger et al. 1986; Yasugi 1993). Earlier studies by Yasugi (1993) found glucagon cells in avian allantoic epithelium associated with intestinal or pancreatic MSCs while Haffen et al. (Haffen et al. 1983), noticed endocrine cells in gizzard epithelium associated with intestinal MSCs. Recently, Tchokonte-Nana (2011), observed fibroblast mitotic cells in the early hour of PPDL, suggesting that MSCs replication precedes endocrine formation (Puri, Hebrok 2010) an implication of the requirement of an early extracellular signaling pathway in endocrine cell fate specification.

Futhermore, islet transplantation has therapeutic potential to restore normoglycemia in type I diabetes (Shapiro et al. 2000; Ryan et al. 2001). Although, it represents a less invasive approach for beta cell replacement compared to whole pancreas transplantation, patients still experience a

decline in islet cell survival during the post transplantation period, despite the use of glucocorticoid-free immunosuppressive agents to minimize tissue rejection (Shapiro et al. 2000). In rodent models of pancreatic injury, regeneration of the pancreas has been reported to be a potential source of islet cells for tissue replacement therapy in type 1 diabetes mellitus. Earlier transplantation studies that used adult post pancreatic duct ligation (PDL) tail pancreas as donor tissue isolated 84 hrs post ligation, found that post PDL-tissue has the same efficacy as fetal islet cells after transplantation (Page et al. 2004). However, the graft survival was not sustained in recipient animals with only 40% of the treated animals achieving a short-term normoglycemia. Later studies conducted by the same laboratory examined the morphochronobiology of post PDL pancreas tissues and observed maximum exocrine tissue destruction at 72 and 84 h following ligation (Tchokonte-Nana 2011). These times correspond to the period when donor tissue was harvested in the study by Page et al. (2004). This may suggest that destruction observed in PDL tissues have contributed to the graft failure. Because of this, Du toit and co-workers (du Toit et al. 2013) proposed that an early transplantation time of 12 – 24 h post PDL tissue could sustain isograft function and survival.

1.5.1 Hypothesis

The interaction between the pre-existing islet and/or duct cells and MSCs in the duct-ligated pancreas induces beta cell neogenesis, and can revert diabetes mellitus when transplanted early.

1.6 Aims and objectives of the study

1.6.1 Aims :

- To investigate whether culturing PDL activated-islet cells with pancreas–derived MSCs induces islet cell neogenesis, increase beta cell number and function *in vitro.*

- To investigate whether early transplantation of the *in vitro* co-cultured PDL-induced islet cells with MSCs affects the transplantation outcome in STZ-induced diabetic rats.

1.6.1.1 Objectives

- To isolate and characterise islet cells and MSCs for culture from PDL and sham operated control (SOC) rat pancreas using collagenase enzyme.
- To co-culture isolated islets and MSCs from PDL and sham operated control (SOC) tissues to determine the effect of islet-MSCs interaction on beta cell neogenesis.
- To determine the expression patterns of Pdx1, Ngn3, with insulin in cultured islet cells using immunocytochemistry (ICC) and immunohistochemistry (IHC).
- To isolate and characterize pancreatic duct fragments using collagenase enzymes from PDL and sham operated control (SOC) rat pancreas for co-culture.
- To co-culture isolated pancreatic duct fragments and MSCs from PDL and sham operated control (SOC) tissues to determine the effect of duct-MSCs interaction on beta cell neogenesis.
- To determine the expression of CK7, Ngn3 and insulin in cultured duct cells to investigate the effect of MSC-pancreatic duct fragments on beta cell neogenesis.
- To transplant islets and MSCs in streptozotocin (STZ)- induced diabetic rats and assess the blood glucose profile of diabetic and non-diabetic rats before and after transplantation of islet cells isolated 24-hr and 84 hr following ligation.
- To determine the expression patterns of insulin and proliferation marker, Ki67, in isografts of diabetic and non-diabetic rats by immunohistochemical method.

Chapter 2 Materials and methods

2.1 Ethical issues

The Ethics clearance was obtained from the Stellenbosch University Animal Ethics Committee: ethics number SU-ACUM13-00036. This study complied with the guiding principles laid down by animal welfare organization and the society for the prevention of cruelty to animals (SPCA), and all the recommendations of the declaration therein, as well as, the accepted standards for the use of animals for research and teaching as reflected in the South African National Standards 10386: 2008, and supervised by qualified veterinarian.

2.2 Choice of laboratory animals

Rodent models have been used in research for decades to study human diseases because of the similarities in morphological, physiological, molecular, genetic and pathological events that take place in the pancreas between humans and rodents. In this study, rats (*Rattus norvegicus*) were used because of the following reasons: (i) they breed fast and are readily available as inbred lines at Stellenbosch animal unit and (ii) they have larger pancreas than mice (*Mus musculus*), making it easier to have access to the pancreatic duct for ligation.

2.2.1 Housing and Caging

A total of 220 animals were housed in clean laboratory rodent cages. Ten animals were housed in each cage. After surgery, each cage contained one post–surgery animal. All post-surgery animals were kept under a 60 watt lamp necessary for quick recovery and to encounter hypothermia. All animals were kept in a thermally controlled envirnment and had free access to water, and standard pelleted rat chow (Epol. Midrand , RSA) throughout the experiments.

2.2.2 Experimantal design

A total of 220 randomly selected adult healthy male Wistar rats were obtained from the Animal Unit of the Faculty of Medicine and Health Sciences, University of Stellenbosch. The rats were weighed and divided into two main groups: (i) Cell culture group (n = 80) and (ii) Islet transplantation group (n = 140) (Fig 2.1).

2.2.2.1 The cell culture group

In this group, 80 animals were randomly distributed equally into two experimental groups and two control groups. The experimental groups were composed of 24 h post pancreatic duct ligation (PPDL) and 120 h PPDL. The choice of post-PDL times of 24- and 120 h is based on evidence from previous studies by Tchokonte-Nana (2011) that chronologically documented the cellular mechanisms in a post PDL pancreas and reported that a high peak of exocrine destruction occurs at 72 h. The remodeling of these cells following a pancreatic duct ligation were observed from 84 h and reaching a healthy state at 120 h. In addition, a recent study showed that optimal efficiency of the co-expression of endocrine developmental genes with insulin occurs at 24 h after pancreatic duct ligation (du Toit et al. 2015). To test this, the pancreatic duct of the animals was ligated (PDL) or sham operated, and the post pancreatic ligated (PPDL) tissue portions were removed at 24 h and 120 h prior to cell isolation and culture. Islet from sham operated portion of the pancreas served as control (SOC) to the islet from the PPDL tissue portions at the corresponding time periods.

2.2.1.2 Islet transplantation animal groups

A total of 140 animals were randomly distributed into three subgroups of

(i) **Donor islets (n = 80)**: these animals were further divided into 24 h PPDL (n = 40), 84 h PPDL (n = 20) and Normal group (n = 20). In the former groups (PPDL), animals underwent PDL, and islets were harvested at 24- and 84 h respectively, while in the later group (Normal/Control group) islets were harvested without PDL. All these islets from the three groups served as donor islets to recipient animals.

(ii) **STZ-diabetic recipients (n = 40):** After chemically inducing diabetes in this group, the animals were randomly grouped as 24 h PPDL recipients (n = 20), 84 h PPDL recipients (n= 10) and normal islet recipients (n = 10) corresponding to the islet types of donors. The 24 h PPDL recipients were further subdivided into Islet/MSC- recipients (n = 10) and Islet/MSC+ (n = 10), depending on whether islet cells were combined with mesenchymal cells (MSCs) or not.

(iii) **Control animals (n=20):** These animals were randomly divided into 2 equal groups where diabetes was induced in one group (n = 10), while the other group was untreated. Both groups did not receive islet transplantation and served as controls to recipient animals.

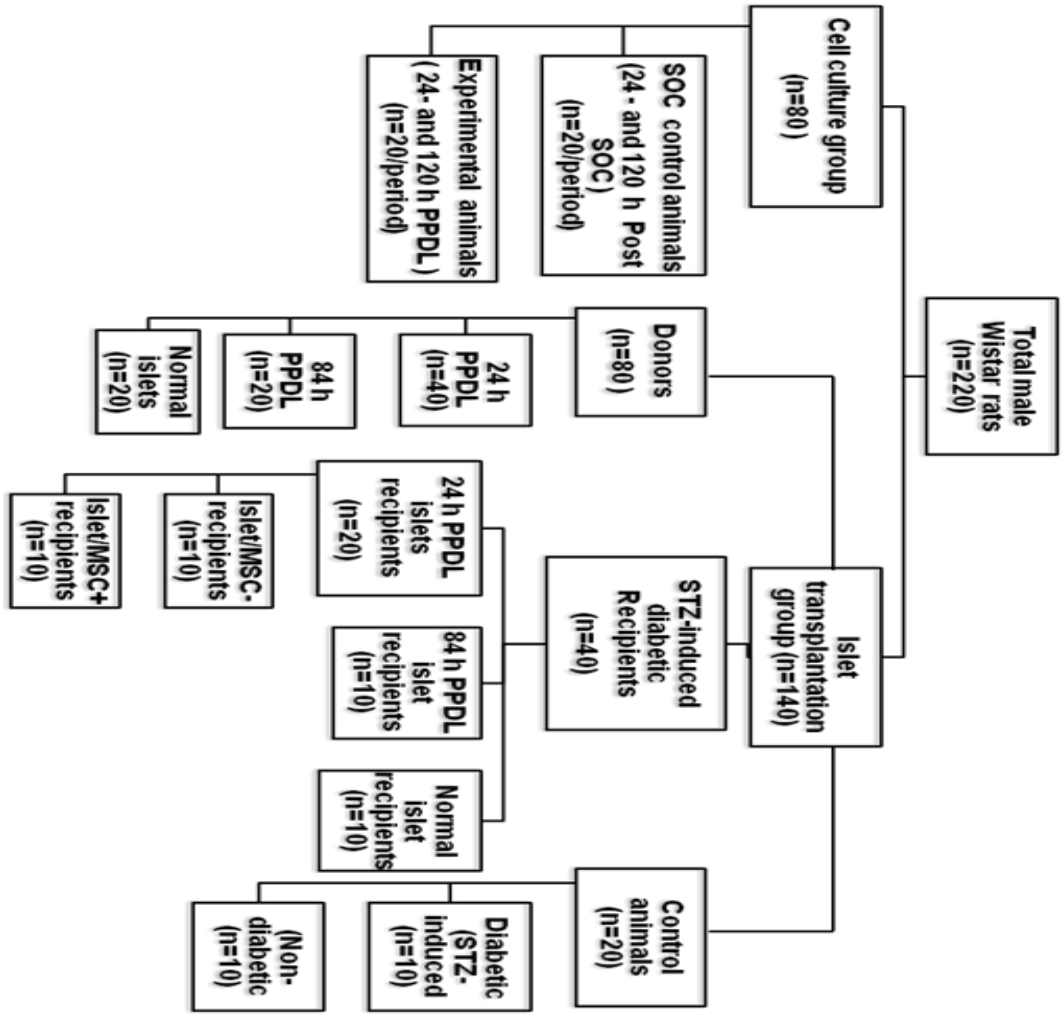


Figure 2.1. Animal samples used in this study. PPDL = post pancreatic duct ligation; SOC = sham operated Control.

2.3 Experimental Procedures

2.3.1 Pancreatic duct ligation in experimental group

All pancreatic duct ligation (PDL) procedures were done in the fully equipped animal theater at Stellenbosch University Central Animal Unit, Tygerberg campus. On the evening prior to PDL surgery, animals were weighed (250-350 g) and housed in a clean cages placed in thermally controlled temperature with free access to water but not food to put them in a fasting condition before surgery.

On the day of PDL surgery, animals were anaesthetized with 5% halothane (Isofor) (Safeline pharmaceuticals (PTY) LTD Roodepoort, South Africa) vaporized in O_2. Complete anaesthesia was verified by pinching the foot and the tail to make sure that there no spontaneous movements or withdrawal responses. Thereafter, the animal was carefully transferred to the surgical table workstation under a dissecting microscope. Before opening the abdomen, animals under anaesthesia were placed in supine position and the abdominal fur (1 cm form each side of the linea alba) was shaved using an electric shaver and the skin was then cleaned with providone-iodine solution containing antiseptic betadine (10 mg/mL) (Adcock Ingram Pharmaceuticals, Industria, Johannesburg, RSA).

After pinching the abdominal skin and foot again, an initial laparotomy incision of about 2 cm was made at the linea alba of the anterior abdominal wall cutting through the epidermis, dermis and the underlying peritoneal membrane and the abdominal cavity was accessed. Care was taken to avoid cutting the internal organs. After opening the abdomen, the stomach and duodenum were pulled out using forceps and reflected superolaterally to expose the tail of the pancreas, which is attached to the spleen. Cotton buds soaked in HBSS were used to prise the pancreas to identify the pancreatic splenic duct (i.e. duct to the splenic lobe of the pancreas).

After identifying the confluence of the splenic and the common bile ducts, a single suture was made at about 1/3 distal to the junction to occlude the duct using a 5/0 Dexon polycaprolate coated braided polyglycolic acid re-absorbable suture material (Fig. 2.2) (Davis and Geck, Cyanamid, Hampshire, United Kingdom). Care was taken not to damage the blood vessels that run close to the splenic duct of the pancreas.

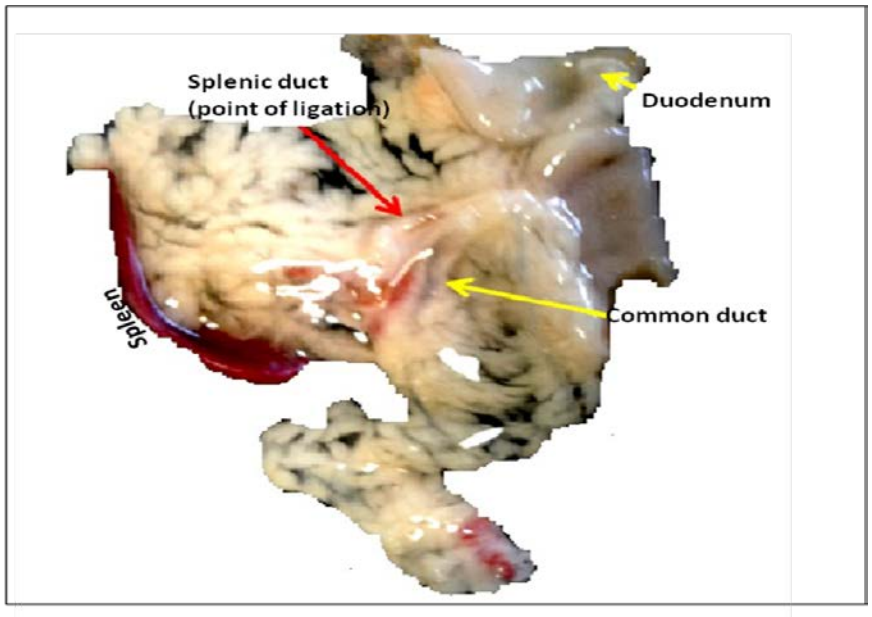


Figure 2.2. Picture of dissected rat pancreas, showing the point of ligation (red arrow) in the splenic lobe.

Following ligation, post-PDL dehydration was prevented by introducing approximately 2 mL of warm saline solution in the abdominal cavity. The abdominal cavity was closed in two separate layers using resorbable 5/0 sterile white braided silicone treated polyester (Davis and Geck, Isando, South Africa) sutures soaked in saline solution. The peritoneum and abdominal muscles were sutured first, followed by the skin. To prevent infection, each animal was injected with 1 mL of Baytril (Smith-Kline Beecham Pharmaceuticals, Midrand, South Africa) and 0.5 mg streptomycin (Novo-Strep 5g/15 mL, Novo Nordish (Pty) Ltd, Johannesburg, South Africa) under the skin of the abdomen in a single dose. After swabbing the wound with an antiseptic rub (Beige Pharmaceuticals Pty. Ltd., Edenvale, South Africa) to minimize scratching, animals were returned to clean laboratory rodent cages. Directly after surgery and the following mornings, animals received an antibiotic Baytril (Bayer (PTY) LTD. Insando, South Africa) to prevent infection and a painkiller, Temgesic (Schering-Plough (PTY) LTD., Woodmead, South Africa).

To counter hypothermia, animals were placed under a 60-watt lamp necessary for rapid recovery. Temperature was controlled in the housing facility and animals had free access to water and standard rat chow (Epol, Midrand, South Africa) under the supervision of a qualified veterinarian until they were euthanized at designated time periods.

2.3.2 Sham operation control group (SOC)

In this group, a surgical procedure, similar to the ligation group in section 2.4.1, was followed except that the duct was not ligated. Animals were anaesthetized with 5% halothane vaporized in O_2 and prepared for surgery. After laparotomy incision was made at the linea Alba of the anterior abdominal wall, the pancreas was just touched twice with a wet cotton swab without ligating the duct. The same post surgery treatment regimen was used, as described in section 2.4.1.1, to prevent infection and hypothermia.

2.4 Tissue collection

2.4.1 Pancreatic tissue harvest

The day of tissue harvest corresponded to time periods 24- and 120 h post PDL (PPDL) in PDL group and an equivalent time period of 24- and 120 h post sham operation (SOC). All surgical procedures were carried out under asceptic conditions in the animal theater as follows. Animals were anesthetized with 5% halothane (Isofor) vaporized in O_2 as previously described in section 2.3.1.1. Complete anaesthesia was verified by pinching the foot and the tail to make sure that there are no spontaneous movements or withdrawal responses. Afterwords, the animal was carefully transferred to the surgical table workstation under a dissecting microscope. With the rat in supine position, the whole abdominal fur was wetted with 70% ethanol before opening the abdomen to avoid fur contaminating the exposed abdominal organs during subsequent steps. A

V-shaped incision was made using a standard pattern scissors, starting from the genital area to the most extreme lateral sides of the ribcage in order to expose all organs in the peritoneal cavity. The abdominal skin, muscles and peritoneum were then reflected over the ribcage to expose contents of the abdominal cavity. Using cotton swabs, the stomach and bowels were pulled to the left side of the rat to expose the pancreas. Following quick exsanguinations through excision of the heart using a sharp pointed pair of scissors, the duodenum, pancreas and spleen were dissected together a shown in Figure 2.2. Then, the pancreas was resected from all points of attachments. In order to attain a maximum yield of islets, the entire pancreas was removed first before excising the tail pancreas distal to the ligation point. For maximum collagenase enzyme activity, the resected tail portin of the pancreas was briefly rinsed in cold Hank's balanced saline solution (HBSS) (Life technologies) to remove any blood. Collagenase A (Sigma) was prepared at final concentration of 1.5 mg/mL in cold HBSS. Then each lobe of splenic portions of the pancreases distal to the point of ligature in the experimental animals or the pancreatic tail portions in the control animals were injected with 2 mLs (1.5 mg/mL) Collagenase A (Sigma) as shown in Figure 2.3.

In experimental animals, the PPDL portion of the pancreas was resected and placed in a sterile 15 mm- petri dish. In sham operated control (SOC) animals, a similar sized tail portion of the pancreas was also surgically resected. After 5 minutes, the pancreata were sliced into 2 mm pieces and quickly transferred into conical tubes containing 10 mLs of cold Collagenase A (1.5 mg/mL in HBSS) ready for islet, ducts and mesenchymal cells isolation. The animals were then euthanized by cardiac puncture to allow excess bleeding and the carcasses disposed of in the cold room for incineration.

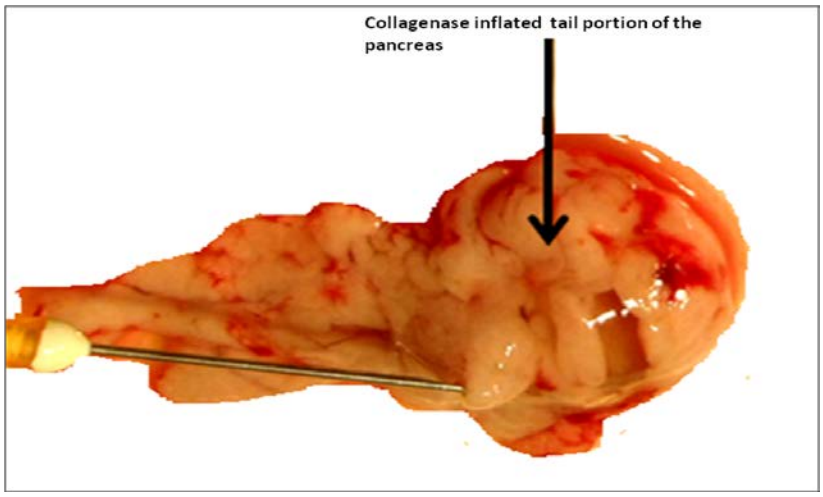


Figure 2.3. Picture shows inflation of the splenic portion of the dissected pancreas with collagenase enzyme.

2.5 Tissue Culture

2.5.1 Experiment 1: Co-culture of PPDL islet cells with pancreas–derived MSCs

Experiment 1 describes the methodology taken to isolate islets and MSCs, and co-culture in order to investigate whether PDL activated-pre-existing islet cells cultured with pancreas–derived mesenchymal stromal cells (MSCs) induces islet cell neogenesis and increase in beta cell number and function.

To assess whether MSCs induce de-differentiation in quiescent mature islet cells or de-differentiated islet cells (Fig.2.4 A), we performed an *in vitro* experiment as shown in Fig 2.4 B. If MSCs stimulate replicating islet cells to serve as pancreatic progenitors after PDL, then we should see a loss of differentiated phenotype in mature endocrine cells and our less-differentiated endocrine precursor-like cells should originate and be continuous with re-differentiong mature endocrine cells in our co-cultures in this study. The immunostaining (ICC) of the proliferating endocrine cells in co-cultures with MSCs should show the expression of Pdx-

1 – the earliest known pancreas specification marker, and the expression of Ngn3 (a cell marker expressed in endocrine precursors). This will present a molecular profile similar to pancreatic progenitors, while the re-differentiating beta cells (insulin^{+} cells) which derived from these progenitor cells should follow a maturation pathway similar to that, which occurs during embryonic development. Therefore, the immuno-expression of Pdx1 and Ngn3 on proliferating endocrine cells in co-cultures with MSCs served as a guide to ascertain the pathway of endocrine development as it occurs during embryonic development.

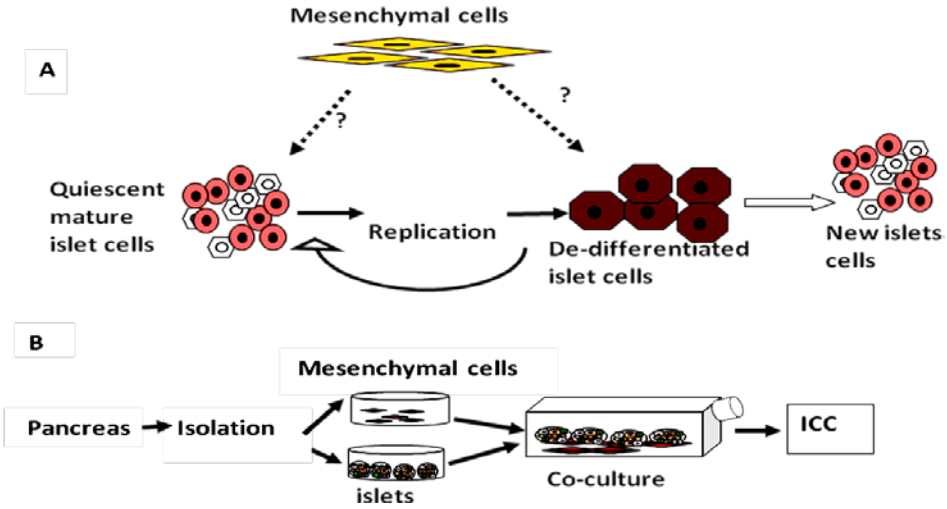


Figure 2.4. Experimental approach to test the hypothesis of MSCs as stimulants of beta cell neogenesis from pre-existing islet cells. (A) Our hypothesis is that with MSCs stimulation, existing islet cells regress to a lesser differentiated phenotype and act as progenitors to form new islets. (B) Islets and mesenchymal cells were enzymatically isolated from PPDL pancreas and co-cultured.

2.5.1.1 Pancreatic tissue digestion and washing

Stage1: Isolation of islets of Langerhan

The pancreatic tissue was digested with Collagenase A enzyme to isolate Islets of Langerhans from the pancreas, as described in previous protocols (Bertera et al. 2012, Gotoh et al. 1985)

with some modifications. All subsequent steps were carried out in a tissue culture hood to ensure that islets remain sterile for cell culture. After harvesting, the enzyme infused organ was then put into a sterile 25 cm^2 flask and kept on ice. In order to achieve a substantial yield of islets, up to three (3) pancreata were pooled in one flask in which 15 mLs collagenase solution was supplemented.

Stage II: Digestion of Pancreatic tissue

Optimization of Colagenase A dilutions

Pancreatic duct ligation (PDL) causes destruction of exocrine tissue in post ligated pancreas. The first goal was to develop and optimize a protocol for isolating islets from a PDL-induced distorted pancreas for *in vitro* and *in vivo* transplantation studies. Many islet isolation protocols use collagenase enzymes to digest pancreatic connective tissues. However, it is reported that these enzymes are variable from different manufacturing companies and even between each lot (Wolters et al. 1992). A report by de Groot (de Groot et al. 2004) also indicates that some rodent strains lend themselves to islet isolation less well than others. To obtain viable purified islets from PPDL pancreas that respond similar to their function *in vivo,* harvested tissues were first digested in varying concentrations (1.4mg/mL, 1.5 mg/mL and 2.0 mg/mL) of the collagenase A. The 1.5 mg/ mL Collagenase A concentration was optimal to the Wistar rat's pancreas exposed to PDL as illustrated in Fig 3.1. Hence, 1.5 mg/mL concentration was used in all digestion experiments.

After optimization, the collagenase infused and excised pancreata were incubated for 30 minutes at 37^{o}C in a shaking water bath. Every 5 minutes, for 30 minutes, the flask was immediately removed from the incubator and vigorously hand shaken for 6–12 seconds to dissociate the tissue into a homogenate. In some cases, shaking was done for every 3 minutes. This step is critical for

optimal yield of the islets. For this reason, shaking was stopped only when the tissue was completely broken up to release islets. Care was also taken not to over-shake, to avoid breaking the islets into single cells. After 30 minutes, digestion was stopped by quickly placing the conical tube in ice and diluting the homogenized pancreatic tissue with 20 mL ice cold HBSS. Care was taken to dilute the digest quickly to avoid unwanted islet digestion.

Stage III: Washing of digested pancreatic tissue

Digested tissue particles were first allowed to settle down by gravity for 5 minutes before the supernatant was removed. The digested tissue was then washed in HBSS wash buffer two times by centrifuging for 2 minutes at 290 x *g* each time to form a pellet and decanting the supernatant to remove small, light-weight tissue particles such as fats. After the second wash, each digest was poured through a large sink strainer size 40 (420 μm) into a 500 mL beaker and forcefully pipetted off the screen with wash buffer. After straining, the digest were dispensed into 50 mL conical tubes (3 pancreata per tube) and washed again by centrifugation as described above. Without loosening the pellet, an estimated 45 mL of the wash supernatant was carefully removed and poured in separate flask (for further purification of mesenchyme cells) without disturbing the loose pellet. At this stage, the pellet contains the islets and other heavier undigested fragments. After re-suspending the pellet in the remining wash buffer in conical tubes, the tubes were filled again and the washing steps were repeated until the supernatant appeared clear of the fine tissue fragments.

StageIV: Islets purification on discontinuous Ficoll gradient

After the last supernatant wash was removed, the pellet was re-suspended and divided into smalller 17 × 100 mm conical tubes with caps (Cell Star), each containing an equivalent of 2 organs per tube and centrifuged gently at 290 x *g* for 1 minute. After decanting the supernatant,

tube were inverted with opening facing down the paper towel to remove as much liquid as possible from the pellet to avoid diluting the 25% Ficoll solution. Ficoll solutions were prepared from Ficoll type 400 (Sigma Aldrich) (Appendix A, Table A1) and construction of a discontinuous density gradient was done by first re-suspending (by vortex) the pelleted pancreatic tissue in 4 mL of the 25% Ficoll stock solution followed by subsequent layering of 2 mL each of the other gradients (23%, 20.5%, and lastly 11%) in order of highest density to lowest, over the 25% Ficoll/tissue mixture. A total of 10 mL with the pancreatic tissue suspended in the 25% fraction at the bottom was contained in each tube (Fig. 2.5)

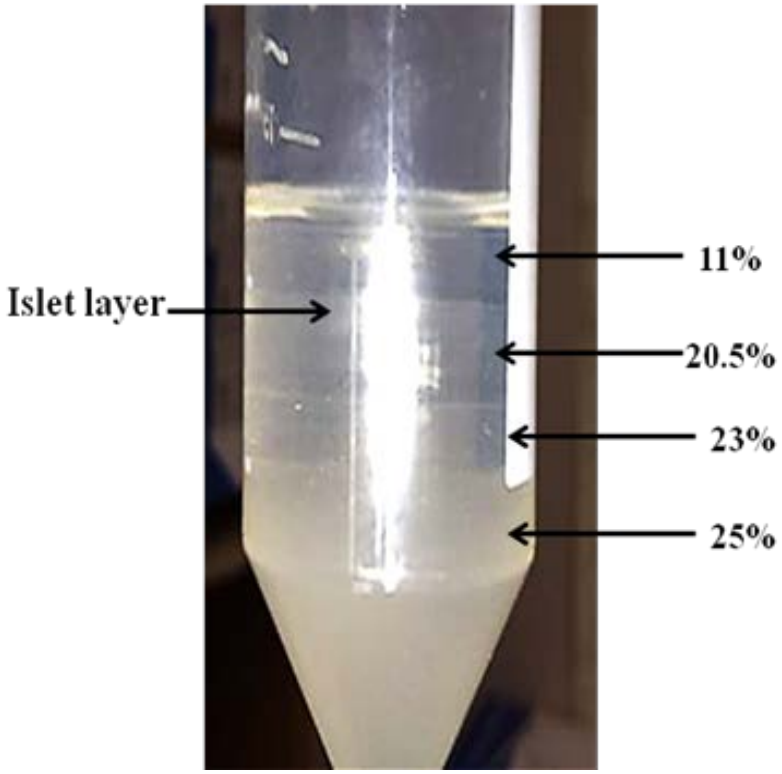


Figure 2.5. Discontinuous ficoll gradient layers.

Without disturbing the layers of the Ficoll density gradient, the preparation was carefully centrifuged for 25 minutes at 800 x *g*. Islets were usually observed at the 11%–20.5% and/or the 20.5%–23% interfaces. Each Ficoll layer was separated and put into 15 mL conical tubes,

washed with HBSS, and centrifuged for 5 minutes at 800 x *g*. After removing the supernatant, islets were washed again and allowed to settle by gravity to remove the last traces of Ficoll. After removing all but I mL, the supernatant containing the islets was transferred to a sterile, non-tissue culture-coated 60mm Petri dish (Fisher Scientific, Pittsburgh, PA, USA) containing 5-6 mL of complete medium (RPMI 1640) (Life Technologies). All Ficoll layers in separate tubes were examined for islets before collecting all the supernatant into another flask for further mesenchyme purification. Islet purification on Ficoll density gradient solution was done quickly because Ficoll is toxic to islets.

Stage V: Islets morphological assessment and picking

To separate from any remaining exocrine tissue, islets were finally isolated physically under the dissecting microscope (Nikon, Japan) by pipetting, and isolation was based on the morphological features of the islets. Pure islets are morphologically round and smooth and golden brown under the dissecting microscope (Nikon, Japan), while rough ones contained impurities of mesenchymal/exocrine cells and were excluded in pure cultures of islets (i.e. cultures without mesenchyme) but included in cultures of islets plus mesenchyme. Islets number and purity were morphologically assessed *in vitro* by dithizone staining (Sigma-Aldrich). Isolated islets were counted and kept in complete media (RPMI 1640) at 37^{o}C in a humidified atmosphere of 95% air and 5% CO_2 ready for culturing or transplantation. Furthermore, islet viability was determined by their capacity to proliferate in culture or revert diabetes *in vivo*.

2.5.1.2 Mesenchyme stromal cells isolation from pancreatic digest

Stage I: Further digest of pancreatic tissue

The supernatants from islet isolation were washed thrice with HBSS. After washing, the supernatant was further subjected to Dulbecco's modified minimum essential medium (H-DMEM [DMEM-with high glucose, HEPES] supplemented with 1 mg/mL collagenase type A (Sigma) and 2% BSA fraction V (Sigma) for futher digestion. The islet depleted pancreatic tissue was further digested by incubating at 37° C in a water bath for 5 minutes before it was transferred to a 50 mL conical tube to quench digestion in 25 mLs of chilled H-DMEM supplemented with 10% fetal bovine serum (Sigma). The digest was later centrifuged at 290 x *g* for 10 min. After removing the supernatant, the pellet was re-suspended in T25 culture flasks containing H-DMEM.

Stage II: Mesenchymal stromal cells separation

Mesenchymal stromal cells (MSCs) were isolated based on their ability to adhere to plastic dishes as previously described (De Schauwer et al. 2011). Therefore, the remaining collagenase digest, obtained after islet and duct isolation, was incubated at 37°C in an atmosphere comprising of 5% CO_2 for 48- 72 hours. After culturing the tissue digest for 48 to 72 h, the supernatant was removed and flasks were washed in H-DMEM (Life Technologies) media to remove the remaining islet and exocrine cells. Fibroblastoid-like cells (MSCs) seen emerging and attaching to the surface were passaged every third to fourth day once they acquired 80-90% confluency before co-culturing with isolated islet cells under a microscope. Mesenchymal stromal cells (MScs) were propagated and expanded using H-DMEM. In this study, MSCs were not analysed for the presence of surface markers by flow cytometry due to financial limitations and lack of antibodies on the market that specifically react with rat tissues.

2.5.1.3 Passaging (sub-culturing) of MSCs

Once reached confluence, MSCs were sub-cultured as follows. After carefully removing culture medium, MSCs were washed twice with phosphate buffered saline (PBS) (Appendix A, Table A2) to remove dead cells and traces of culture medium, which can inhibit the action of trypsin. Adherent MSCs at the bottom of the T25 flask were first trypsinized by adding pre-warmed 0.25% trypsin-EDTA (Appendix A, Table A3) to cover all surfaces of the flask and incubated at 37°C for 5 minutes. Morphological changes of cells were then observed under an inverted Nikon Eclipse TE2000-S microscope (Nippon Kogaku KK, Japan). When cells began to assume a rounded shape, the flask was gently tapped to remove the cells from the surface. In some cases, incubation time was increased when some cells were still attaching to bottom of the culture flask after first gentle tapping. Cells from the surface were later washed by gently pipetting 4 to 5 times with fresh culture medium until all cells are suspended with no clumps. Cells in suspension were thereafter sub-cultured in a 1:5 split ratio by taking 1 mL of cell suspension and adding to a new T25 culture flask containing 4 mLs of complete fresh medium to make a total of 5mLs of cell suspension. T25 culture flasks containing 5mLs of MSCs suspension in RPMI1640 supplemented by 1% Pen-streptomycin were incubated at 37˚C in a humidified atmosphere of 95% air and 5% CO_2. Media was changed every 2 days. All the transfer of medium was done inside the lamina flow tissue culture hood under aseptic conditions.

2.6 Islet–mesenchymal stromal cells co- culture

The first set of experiments was designed to test if direct mesenchymal–islet contact is necessary to induce trophic effects on islets in culture. In addition to direct contact, we also designed the second experimental set to test if MSCs secrete soluble molecules that mediate the trophic effects on neighbouring islet cells. Therefore, two co-culture configuration systems were used in this

study: direct and indirect contact islet-mesenchymal stromal cells monolayer co-culture systems. As mentioned in section 2.6.4.3, isolated MSCs were first expanded before co-culturing with islets. Approximately 200,000 MSCs of passage 3-6 were seeded into Cell Star 35-mm Petri dishes and cultured for 24 h to form a confluent monolayer for the direct and indirect contact islet-MSC monolayer co-culture system. The islets were transferred into culture conditions using sterile “slick” pipette tips. Since islet cells on plastic in the presence of FBS "de-differentiate" by default (become mesenchymal), in our co-culture experiments, mesenchymal and islet cells were thereafter, maintained under serum free 50% RPMI 1640 + 50% H-DMEM culture medium. Islet cell growth was analyzed by direct cell count under the Nikon Eclipse TE2000-S fluorescent microscope (Nippon Kogaku KK, Japan).

2.6.1 Direct contact islet–MSC co-culturing

For the direct contact system, 50 fresh islets were seeded directly onto 200,000 adherent MSCs monolayer. To preserve morphology of islets, some samples for *in vitro* analysis were cultured on 4 well cell culture slides as shown in Fig. 2.6.

2.6.2 Indirect contact Islet-MSC co-culturing

For the indirect transwell co-culture system, cell culture inserts with a semi-permeable membrane pore size of 1.0 µm (Millicell) were inserted into each 35-mm non-treated Petri dish (Cell Star -Greiner Bio-One) containing approx. 200,000 MSCs, and 50 fresh islets were added into the inserts as shown in Fig. 2.7. Serum free RPMI 1640 medium (supplemented with 100 U/mL penicillin/0.1 mg/mL streptomycin) was used for all co-culture configurations. Control islets were cultured alone as groups of 50 also in serum free RPMI-1640 medium supplemented with 100 U/mL penicillin/0.1 mg/mL streptomycin in non-treated 35-mm Petri dishes.

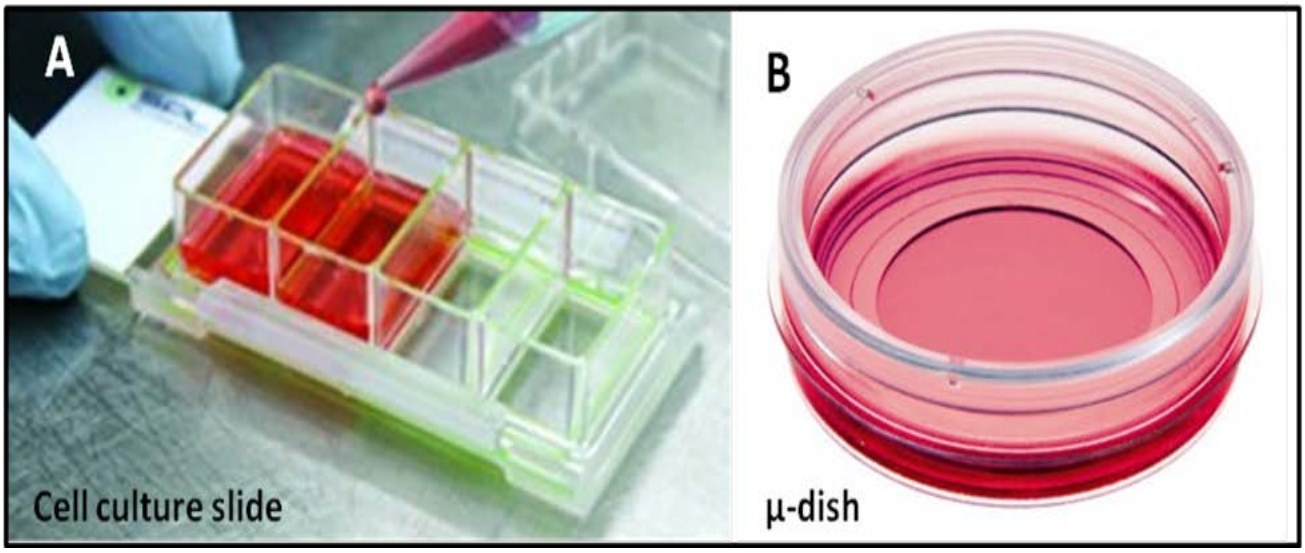


Figure 2.6. Cell culture slide and 35 mm cell culture petri dish.

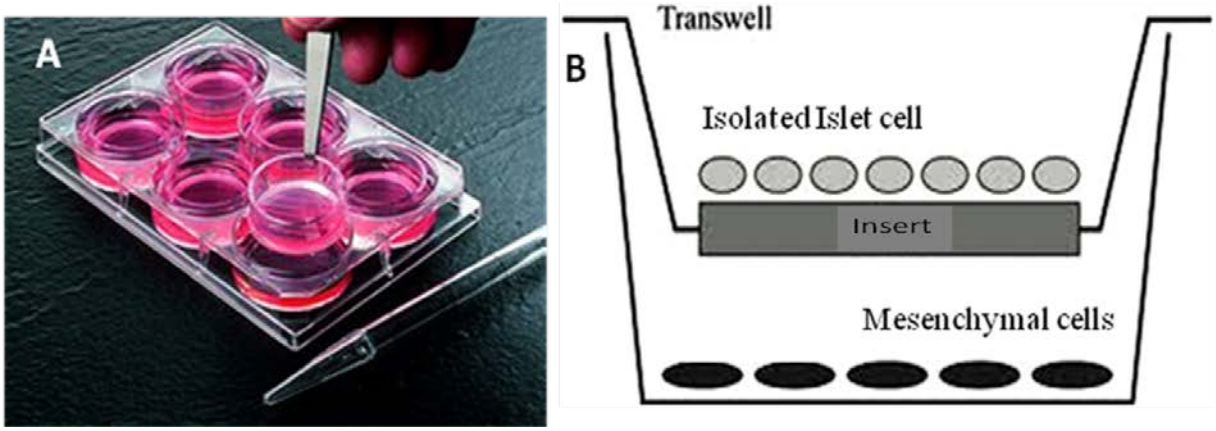


Figure 2.7. Transwell for indirect islet and mesenchymal stromal cells co-culture. (A) Six-well cell culture inserts. (B) Illustrated view of indirect co-culture of islets and mesenchyme**.**

2.6.3 Passaging (sub-culturing) of islet and MSCs in co-cultures

In our co-cultures, islets and mesenchymal cells were sub-cultured every time cells reached confluence for a period of 4 weeks using serum free 1640 RPMI culture medium. A similar method of sub-culturing was used as described in section 2.6.4.3, for cultures of islets only using

serum free culture medium. Once reached confluence in each petri-dish, islets were first counted before trypsinization. After carefully removing culture medium, the composite islets and mesenchymal cells were rinsed twice in phosphate buffered saline (PBS) to eliminate debris and traces of culture medium, which can inhibit the action of trypsin. For direct contact monolayer configuration, some islets formed loose attachments with MSCs but could be removed for sub-culturing by gently pipetting. Adherent composite islet-MSC cells on petri dishes were mildly trypsinized to lift the cells from the dish. To detach adherent islets and mesenchymal cells at the bottom of the T25 flask, pre-warmed 0.05% trypsin-EDTA (Appendix A, Table A2) was added to cover all surfaces of the flask before incubation at 37° C for 5 minutes. Detached cell aggregates (islet/MSC) from the surface were lifted up by gentle tapping the flask, followed by gently pipetting 2 to 3 times with PBS until all islet cell aggregates cells were suspended. Islets co-cultured with MSCs (Islet/MSC+) were sub-cultured as follows: Islets that were loosely attached to petri dishes were pipetted, and transferred into new petri-dishes and then counted. All islet aggregates were pipetted, counted and transferred into new T25 culture flask.The remaining MSC cells in suspension were thereafter, sub-cultured in a 1:5 split ratio by taking 1 mL of MSC cell suspension and adding to a new T25 culture flask containing 4 mLs of complete fresh medium to make a total of 5mLs of cell suspension. Twenty-five islets were then, seeded on T25 culture flasks containing 5mLs of MSCs suspension in RPMI1640, supplemented by 1% Pen-streptomycin and incubated at 37 ˚C in a humidified atmosphere of 95% air and 5% CO_2. The medium was changed every 2 days. All the transfer of medium was done inside the lamina flow tissue culture hood under aseptic conditions.

2.7 Immunocytochemistry (ICC) of cultured islets

2.7.1 Preparation of islet and mesenchymal stromal cells suspension for ICC

Results from our pilot study showed that when islets were cultured together with MSCs in suspension co-culture, MSCs adhered to the surface of the islet within 6 h to form composite MSC/islets. All islets were handpicked for *in vitro* analysis at designated times. For direct contact monolayer configuration, islets formed loose attachments with MSCs but could be removed for *in vitro* analysis or transplantation experiments by gently pipetting.

For adherent composite islet/MSC cells on petri dishes, mild trypsinization was done to lift the cells from the dish. After carefully removing culture medium, islets and mesenchymal cells were rinsed twice in phosphate buffered saline (PBS) to eliminate debris and traces of culture medium, which can inhibit the action of trypsin. To detach adherent islets and mesenchymal cells at the bottom of the T25 flask, pre-warmed 0.05% trypsin-EDTA was added to cover all surfaces of the flask and incubated at 37° C for 5 minutes. Detached cell aggregates (islet/MSC) from the surface were lifted up by gentle tapping the flask, followed by gently pipetting 4 to 5 times with PBS until all islet cell aggregates were suspended. Cells in suspension were later washed twice in PBS and pelleted by centrifugation at 210 x *g* for 1 minute and then re-suspended in PBS at an estimated concentration of 20 islets/µl. Cells cultured on slides were also washed twice in PBS before fixing them in 4% PFA without cytospining.

2.7.2 Cytospin slide preparation

In a direct contact monolayer configuration, some islets formed loose attachments with MSCs and could easily be lifted up on cell culture slides and plastic dishes (Fig. 2.6 A and B).

Therefore, care was taken to avoid loss of islets that could result into unrepresentative samples at the end of the ICC process. For the samples to be representative, all islets and/or composite Islet/MSC were cytospinned in order to stick to the slides. Cytospin pre-coated slides (SPL life Sciences, Korea) were positioned to a slide holder with filter cards and sample chamber, and then attached to cytocentrifuge rotor (Appendix B). After preparation of the sample chamber, 0.1 mL of cell suspension was added to the chamber and centrifuged at 210 x *g* for 14 minutes.

2.7.3 Immunolabeling

After removing the slides from the chambers, cells were fixed by incubating in 4% paraformaldehyde (PFA) diluted in PBS for 20 minutes at room temperature (RT) on two-well chambered slides to preserve tissue morphology and retain the antigenicity of the target molecules. Cells cultured on slides were fixed without cytospining. After rinsing the cells 3 times with PBS, Slides were placed in a polypropylene Coplin staining jar filled with 10 mM Sodium citrate buffer (pH 6) (Life technologies) in a water bath. Slides were briefly boiled in 10 mM Sodium citrate buffer and then maintained at 95 °C for 10 minutes. After incubation, the Coplin jar with retrieval solution and slides were removed from the water bath, and allowed to cool down on bench top for about 30 minutes at room temperature before gently rinsing the sections in distilled water followed by PBS for 5 minutes each. After heat–induced epitope retrieval, slides were subjected to blocking and primary antibody incubation.

After rinsing the cells twice in PBS, cells were blocked by incubating in 2% bovine serum albumin (BSA) (Sigma) for 1 h at room temperature in a humidified chamber to minimize nonspecific binding of antibodies. The slides were then incubated overnight at 4^{o}C in diluted primary antibodies in a humidified chamber (see Appendix A, Table A4 for antibodies and their

corresponding dilutions). For double immunolabeling, cells were incubated with a mixture of two primary antibodies in their corresponding dilutions. To verify the specificity of the staining generated by primary antibodies, appropriate negative controls were included in the experiment where only secondary antibodies were not added.

After rinsing the cells in 2% BSA in PBS, the antigen–antibody complexes were revealed after incubating the slides in fluorophore-conjugated secondary antibodies for 1 hour at room temperature in a humidified chamber using the corresponding dilutions with primary and secondary antibody diluent solutions (Leica) (Appendix A; Table A4 and Table A5). In double immunolabeling, a mixture of two corresponding secondary antibodies was applied. The secondary antibodies (fluorochrome-conjugated goat anti-rabbit and goat anti-mouse IgG) used in this study include: Fluorescein isothiocyanate (FITC), Alexa Fluor 594 and Alexa Fluor 488 (ABCAM) (Appendix A Table A5). Cells were nuclear stained by 300 ng/mL DAPI (4',6-diamidino-2-phenylindole) solution in 1% BSA for 10 minutes before cover-slipping using DPX as a mounting medium. The primary and secondary antibodies and their dilution factors used in this study are presented in Table A4 and Table A5 in the appendix A. The immunofluorescence reactions in cells were visualized by using a Nikon Eclipse TE2000-S fluorescent microscope (Nippon Kogaku KK, Japan) quipped with appropriate filters and camera linked to computer using NIS-Elements basic research (BR) 3.2 software.

2.8 Experiment 2: Duct-mesenchymal stromal cells (MSCs) co-culture

Experiment 2 describes the methodology employed to isolate duct fragments and MSCs to investigate whether pancreatic-derived mesenchymal stimulate neogenesis in pancreatic duct fragments epithelial cells in culture.

To investigate the response of adult pancreatic duct epithelial cells to MSCs (Fig 2.8 A), we also performed an *in vitro* experiment (Fig. 2.8 B). Immuno-expression of CK7, Ngn3 and insulin on proliferating duct-derived cells in co-cultures with MSCs should provide evidence of neogenesis that occurs during embryonic development.

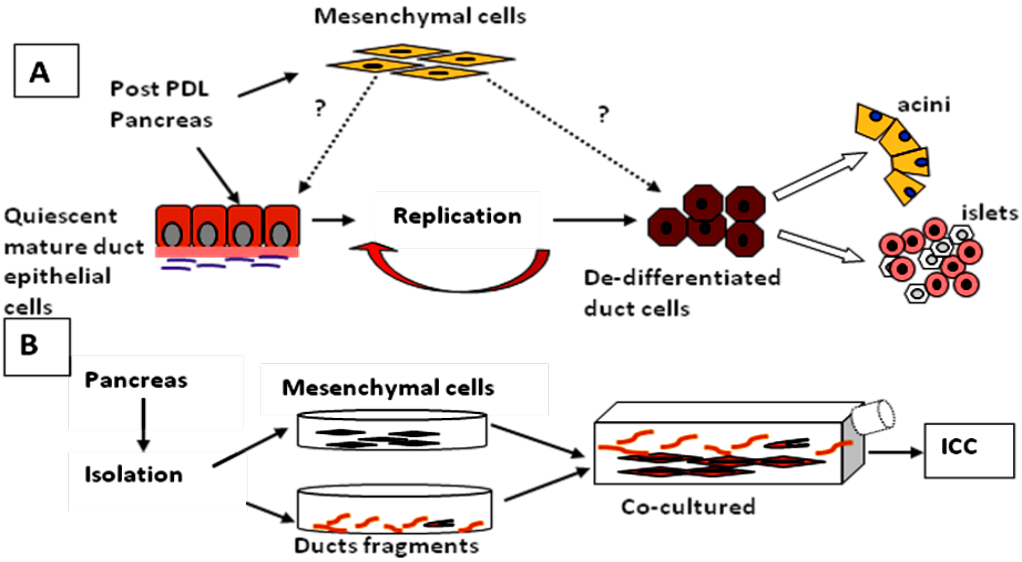


Figure 2.8. Experimental approach to test the hypothesis of MSCs as stimulants of beta cell neogenesis from duct epithelial cells. (A) Our hypothesis is that with MSCs stimulation, duct epithelial cells regress to a lesser differentiated phenotype and act as progenitors to form new insulin producing cells. (B) Duct fragments and MSCs were enzymatically isolated from PPDL pancreas and co-cultured.

2.8.1 Isolation of pancreatic duct fragments using collagenase

The pancreatic duct fragments were isolated using the following methods:

2.8.1.1 Collagenase digestion

Pancreatic duct fragments were isolated using a modified protocol from that used for islet isolation (Bertera et al. 2012, Gotoh et al. 1985, Pipeleers et al. 1981, Githens et al. 1981, Githens et al. 1980). Just like islets, all isolations of ducts were done under sterile condition in a cell culture hood with filtered air lamina flow. Pancreatic tissues harvested from 250- 350 g Wistar rats were first washed with 1X HBSS to remove blood because blood affects the enzyme activity of Collagenase A. After thorough washing, each lobe of the pancreas was inflated by injecting with 2 mLs of Collagenase A (1.5 mg/mL in HBSS) (Sigma) and kept in a petri dish for 5 minutes before mincing. The inflated pancreas was then minced into 2 mm pieces using a sharp scissors in a petri dish and then quickly returned in a vial containing 5 mLs of 1.5 mg/mL Collagenase A (prepared using 1X Hanks balanced salt solution (HBSS)). The vials were then capped and incubated for 30 minutes in a water bath at 37^{o}C with intermittent shaking every 5 minutes to yield a mixture of nerves, blood vessels, ducts, acinar cell clusters and islets. Enzyme digestion was stopped by transferring the digest into a conical tube and diluting it with 20 mLs of cold ice HBSS. Some ducts fragments were obtained from the digested pancreatic tissue samples left over after islet isolation.

2.8.1.2 Ducts fractionation on sieve

As an adaptation, fragments of ducts were isolated from the digest by shaking and vigorously pipetting the digest and then sieved it on mesh stainless–steel sieve (pore size 200 x 300 µm) that was pre-wetted (Merk Millipore). After elution of the fluid and small duct fragments through the sieve, tissues in the supernatant were then left for 2 minutes for lymph nodes and large blood

vessels to sediment, which were later harvested and transferred in separate tube containing 20 mLs of HBSS. The fragments and residues left on the sieve were also harvested by inverting the sieve and rinsing it with another 20 mLs of HBSS.

2.8.1.3 Ducts fractionation on discontinuous Ficoll density gradients

To sediment the remaining exocrine acinar tissue from less dense ductal structures, the supernatant was re-suspended in conical tube by vortexing in HBSS containing 25% Ficoll density cushion. This cushion was overlaid by 2 mLs of each of the 23%, 20.5% and 11% Ficoll and finally 1X HBSS. The tubes were later centrifuged at 800 x *g* for 25 minutes. The floating tissue fraction, with ducts of different sizes ranging from main ducts to intercalated ducts, were collected with a Pasteur pipette and re-suspended in 15 mLs of HBSS and pelleted by centrifuging at 453 x *g* for 5 minutes. The pellet was finally re-suspended in 10 mLs of RPMI 1640 culture medium in a petri dish, ready for isolation.

2.8.1.4 Isolation of specific fragment types

Identification of the duct fragments in the suspension was done under a dissecting microscope (Nikon, Japan), relying on specific characteristic gross morphological features and were hand-picked using a pipette. Specific characteristic morphological features that allowed for the isolation of pancreatic duct fragments for tissue culture were the following: the main intra-pancreatic pancreatic duct showed large lumen with thick columnal epithelial lining frequently disrupted with tubular evaginations (Githens et al. 1981), while intra-lobular pancreatic ducts were lined by simple low columnar to cuboidal epithelium.These pancreatic ducts were frequently lined by loose connective tissue. However, some small pancreatic ducts were simply lined by simple cuboidal epithelium. Intercalated pancreatic ducts, which were often attached to

larger pancreatic ducts, were recognized by a single layer of thick squamous epithelium; presenting straight unbranched duct with smooth outer lining, terminating in centroacinar cells.

Isolated duct fragments were transferred in RPMI 1640 culture medium in T25 tissue flask and left for 24 hours to recover from the effects of digestion and isolation.

2.8.2 Co-culturing of duct fragments with mesenchymal cells

As only a smaller number of 5 to 6 ducts were cultured per dish (unlike 50 islets/200,000MSCs/dish cultured in section 2.7.1), and equivalently smaller number of approximately 150,000 MSCs of passages 3-6 were seeded on each Cell Star 15-mm Petri dishes (Greiner Bio-One), and cultured for 24 h to form a confluent monolayer for the direct contact duct-MSC monolayer co-culture system. For direct contact system, 5-6 fresh duct fragments were seeded directly on the MSC monolayer. The Co-culture system used serum free RPMI-1640 medium supplemented by 1% Penicillin-streptomycin (10,000 units/mL penicillin/ 10,000 µg/mL Streptomycin) (GIBCO). Control ducts were cultured alone without mesenchymal cells in groups of 5-6 ducts per 15-mm petri dish also in serum free RPMI-1640 culture medium. Culture medium was changed every 2 -3 days. Growth morphological changes of duct fragments in culture were investigated under the microscope and pictures were taken every 2 days using an inverted Nikon Eclipse TE2000-S microscope (Nippon Kogaku KK, Japan). All duct fragments including those that were loosely attached to the MSC monolayer and smaller ducts that were completely surrounded by MSC were pipetted for an *in vitro* analysis.

2.9 Experiment 3: Islet transplantation under kidney capsule

Experiment 3 describes the methodology taken to isolate and graft early (24 h) and late (84 h) PPDL islets with MSCs under the renal capsule in order to evaluate the efficacy of PPDL islets in restoring normoglycemia in STZ-induced diabetic rats.

2.9.1 Islet and MSCs transplantation in STZ-induced diabetic rats

2.9.1.1 Induction of diabetes

Diabetes Mellitus was induced by a single dose of 65 mg/kg of streptozotocin (STZ) in inbred syngeneic male Wistar rat recipients (250-350 g) to minimize immune-mediated graft rejection.

Streptozotocin-induced diabetic recipients (n = 40) received 65 mg/kg streptozotocin (STZ) (after establishing a dose curve) (Sigma, St Louis, USA. Product Catalogue no S0130) dissolved in 0.1 M citrate buffer (pH 4.5) via abdominal peritoneal cavity under anesthesia as described in section 2.4.1. Only animals with blood glucose level (BGL) > 11 mMol/L for three consecutive days were included in the study. Diabetic control recipients (n = 10) received same dosage of STZ while normal control animals (n = 10) received saline (placebo) (SABAX, Port Elizabeth, South Africa). Blood glucose levels were measured three times a week using a blood strip sensored glucose meter (Gluco-plus INC, 2323 Halper, Quebec, Canada).

2.9.1.2 Donor islets

Pancreatic endocrine islets were isolated from 24 h PPDL (n = 40), 84 h PPDL (n = 20) and normal islet (n = 20) donor groups. The 24 h PPDL and 84 h PPDL groups received pancreatic duct ligation, while the normal islet group received sham operation using the same protocol as described in sections 2.3.1 up to 2.3.2. Islets were hand-picked under dissecting a microscope.

Purity of islets was verified by dithizone (Sigma-Aldrich) staining and were counted and distributed into groups of 300/recipient for transplantation.

Mesenchymal stromal cells were isolated following ligation based on their ability to adhere to plastic dishes as described previously (Rackham et al. 2013).

2.9.2 Transplantation procedure

In order to avoid normal engraftment alterations caused by immune cell reactions, islet transplantations were carried out on inbred syngeneic Wistar male rats.

2.9.2.1 Animal preparation and transplantation

As stated in sections 2.9.1.1 only animals with fasting blood glucose level (BGL) >11 mMol/L for three consecutive days were used as recipients. Recipient rats were anesthetized, shaved, and cleaned with providone-iodine solution containing antiseptic betadine (10 mg/mL) (Adcock Ingram Pharmaceuticals, Industria, Johannesburg, RSA). After identifying the position of the kidney, a small para-lumbar incision was made on the left side of the animal to expose the kidney. Using a saline soaked cotton swab, the kidney was moistened to prevent drying. Then using the silicone tube as an adapter, the polyethylene tube (PE50) distal end was attached to a Hamilton screw drive syringe containing a pipette tip. With the aid of a Zeiss OPMI-1 operating microscope equipped with a zoom and a focus adjustment (Carl Zeiss, AG, Oberkochen, Germany), a small shallow cut of the capsule was made on the right side of the kidney, through which islet cells were delivered.

Three separate transplant combinations were used.

First combination: Equal halves of STZ-induced diabetic recipient rats (n=10) separately received under the kidney capsule, 300 islets/recipient without MSCs or with MSCs (300 islets/200,000 MSCs/recipient) from 24 h PPDL syngeneic donors.

Second combination: Ten (n=10) STZ- induced diabetic rats were transplanted with islets (300 islets/recipient) obtained from 84 h PPDL syngeneic donors.

Third combination: Ten (n = 10) STZ-induced diabetic rats received islets (300 islets/recipient) from unligated health control syngeneic donors as illustrated in Figure 2.1.

To deliver the islets, the beveled end of PE50 tubing carrying islet cells, was carefully inserted under the capsule while gently swabbing the capsule with normal saline to avoid tearing. Islets were then slowly placed under the renal capsule. Under Isofor anaesthesia, approximately 300 islets per diabetic recipient were delivered under the superior pole of the left renal capsule (Fig. 2.9). After delivering the islets, the kidney incision was cauterized with low heat to maintain homeostasis. After retroperitoneally placing back the kidney, the muscles and skin were sutured using silk sutures. Rats were immediately treated with Batryl and Temgesic and placed in a cage on a heating pad. Animals were given free access to food and water after transplantation and under the care of a veterinarian until euthanized 67 days later. All recipients of islet grafts were included in the study.

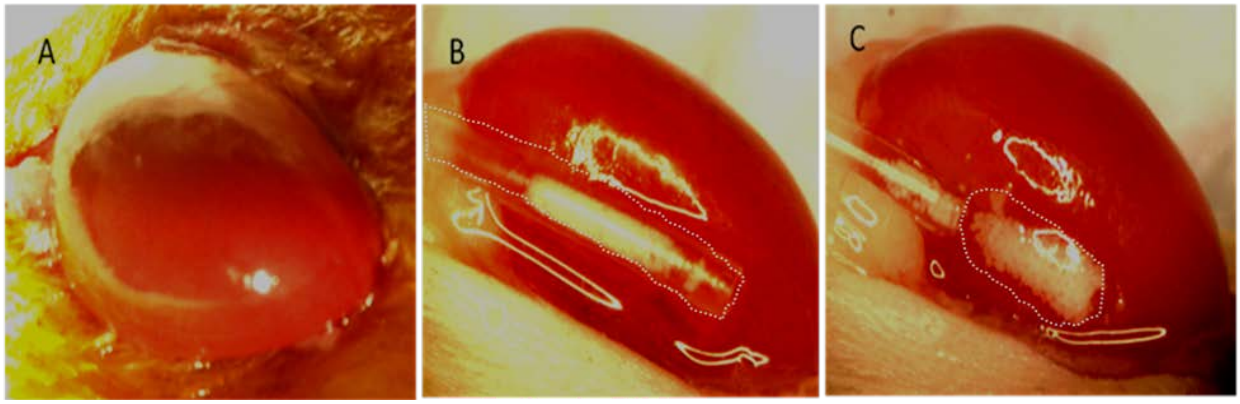


Figure 2.9. Transplantation of islets under the kidney capsule. (A) Kidney was popped out just before transplantation. (B) PE tubing inserted under kidney capsule with 300 islets from syngeneic donor (*dotted line*). (C) Islets deposited under the kidney capsule (*dotted line*).

2.10 Assessment of islet function under the kidney capsule.

Islet functionality was assessed by monitoring body weight and glucose concentrations every 3- to 4 days through the tail vein. Blood glucose levels were measured using a strip sensored blood glucose meter (Gluco-plus INC, 2323 Halper, Quebec, Canada). Transplanted islets were considered to have engrafted when blood glucose levels (BGL) of <11 mMol/L were attained within a period of 5 to 10 days and islet grafts survival was defined as the number of days before recurrence of diabetes. The day of diabetes occurrence was defined as the first two consecutive days of fasting blood glucose readings >11 mMol/L. Reversal of chemically induced diabetes *in vivo* was defined as fasting blood glucose concentrations of <11 mMol/L maintained for a period of 30 days or longer. Rats were euthanized later and kidneys bearing grafts were harvested for histological analysis.

2.10.1 Isografts and pancreas harvesting

Kidneys bearing grafts were harvested and immunohistochemically analysed 69 days post-transplantation. Five animals of each group (diabetic and non diabetic) were nephrectomized

under anaesthesia to perform histology and immunohistochemistry studies. To investigate whether reversal of diabetes is due to functional allografts or regeneration of the pancreas, both the graft bearing kidneys and pancreas were harvested for histochemical analysis.

2.11 Immunohistochemistry of islet grafts

2.11.1 Preparation of tissue sections

To preserve tissue morphology and retain the antigenicity of the target molecules, all harvested kidney allograft and pancreatic tissues for immunohistochemistry were fixed in 4% paraformaldehyde in PBS for 24 h at 4 °C. After fixation, tissues were trimmed and placed in labelled plastic cassettes and dehydrated in a series of graded alcohols using an automated tissue processing machine (Appendix B). After dehydration, tissues were embedded in molten paraffin wax (at 60 °C). After paraffin solidification, tissue blocks were kept at 4 °C until section.

2.11.2 Sectioning

Using a hand-driven rotary microtome, tissues were sectioned at 5 μm and floated on water bath containing de-ionized distilled water at 50 °C. Water was changed between blocks of different groups to avoid antigen contamination. Tissue sections were then mounted on positively charged slides (BIO-SCAN). Once mounted, the slides were dried by incubating them in an oven at 60 °C for 2 h and then stored at room temperature until ready for staining.

2.11.3 Immunochromogenic staining assay

Tissue section from isografts were first de-paraffinized and rehydrated by immersing slides in xylene followed by a series of graded alcohols in descending order of concentration (Appendix B). Care was taken not let tissue dry until antigenicity is revealed.

2.11.4 Heat-induced antigen retrieval

To retrieve the antigenic epitope in the tissue sections, an electric water bath was used. This was achieved by placing a polypropylene coplin staining jar filled with 10 mM sodium citrate buffer (pH 6) (Life technologies) in a water bath. Slides were briefly boiled in 10 mM sodium citrate buffer and then maintained at 95 °C for 10 minutes. After incubation, the coplin jar with retrieval solution and slides were removed from the water bath, and allowed to cool down on bench top for about 30 minutes at room temperature before gently rinsing the sections in distilled water followed by PBS for 5 minutes each. After heat–induced epitope retrieval, slides were subjected to blocking and primary antibody incubation.

2.11.5 Detection system

Leica BOND polymer refined detection kit (Leica, Novolink™ polymer detection system - Cat No: DS 9800) was acquired to immuno-label the slides. Slides were immuno-labeled following the manufacturer's protocol (Appendix B). After rinsing tissues samples in PBS, tissues were incubated with 1-3 drops of "peroxidase block" (0.3% H_2O_2 in methanol) (refine kit) for 10 minutes to quench endogenous peroxidase activity, followed by rinsing with PBS.

After washing in PBS, slides were blocked for 1 h in 2% BSA in PBS at 4°C. The sections were incubated overnight (12-16 hours) at 4^{o}C in primary antibodies anti-Ki67 and anti- insulin

diluted as described in Table A4. After incubation, slides were washed in PBS followed by incubation in “post primary block” (Novolink Kit) for 30 minutes at room temperature. After washing in PBS, slides were incubated in “novolink polymer” (Novolink Kit) for 45 minutes at room temperature. After washing in PBS, slides were incubated in 3,3’ Diaminobenzidine (DAB, brown precipitate) chromogen substrate (Refine Kit) diluted 1: 20 in DAB substrate buffer polymer and finally nuclei counterstained with hematoxylin for 5 minutes.

After washing in PBS, followed by distilled water for 5 minutes, tissue sections were dehydrated by moving slides in a series of graded alcohols as described above (Appendix B) Tissue sections were later cover-slipped using DPX as mounting medium and stored until viewing day.

2.12 Computer-assisted quantitative image analysis (CAQIA)

2.12.1 Microscope slide viewing and image capturing.

Development of islets in culture was monitored using an inverted Nikon Eclipse TE-2000S microscope (Nippon Kogaku KK, Japan) and images were taken directly in culture dishes every 2-3 days to assess any morphological changes in islet growth.

Fluorescent stained tissue slides from islet cells of control [SOC/Normal], post-PDL [24 and 120 h] cultured with or without MSC on different days in culture viewed under an inverted Nikon Eclipse TE-2000S fluorescent microscope (Nippon Kogaku KK, Japan) equipped with colour camera and linked to a computer using NIS-Elements BR 3.2 software. Slides were subjected to fluorescence light for less that 15 seconds to minimize bleaching and exposure time for image capturing was set at 300 milliseconds or 1 second when automated. During image acquisition, tissue slides immunofluorescent images were taken at x40 magnification. General morphology of

islets and ducts samples was assessed to determine any changes introduced by immunofluorescence procedures. Captured images were later quantitatively analysed.

For graft tissue sections, immunochromogenic stained slides were also grouped according to animal treatment (STZ-induced diabetic/non diabetic and transplanted/not transplanted). These slides were viewed under Zeiss Microscope (Carl Zeiss Vision GmbH, Zepplinstrasse 4, 85399 München- Hallbergmoor, Germany) equipped with colour camera linked to a computer using ZEN lite 2012 software. Immunochromogenic images were taken at x40 magnification and quantitatively analysed using ZEN lite 2012 software.

2.12.2 Quantitative analysis of cultured islets and duct fragments

To quantify the absolute numbers of *Pdx1*-, *Ngn3*- and *Insulin*-expressing cells in cultures, all immunostained slides of islets were digitized. Digital images obtained from the slides were segmented and features of positive labelled cells for specific antigens (e.g. insulin, Pdx-1, Ngn-3 etc) were programmed using the automeasurement software of NIS-Elements (Basic Research [BR] 3.2). On each image, the surface areas of *Pdx1, insulin* and *Ngn3* staining were quantified using NIS-Elements BR3.2. Fifty to forty islets were analyzed per culture condition (Pdx1, Insulin or Ngn3) and the stained areas were added up (Appendix C, Tables C1, C2, C3).

To quantify the maintenance of Ngn3 expressing cells, we counted the number of Ngn3 expressing cells among 1,000 insulin expressing cells. The percentage of de-differentiated Ngn3-expressing cells was then calculated (Appendix C, Table 2).

To quantify growth of islets in culture, digital images were obtained and the surface areas of the islets per condition were measured and areas of all islets were added up.

2.12.3 Assessment of graft morphology

For each harvested graft bearing kidney, five to eight tissue sections (5 µm thick) obtained from different regions of the graft were analysed for beta cell density using insulin immunostaining. To evaluate graft morphology, total endocrine area was measured per graft section using digital morphometry (Zen lite 2012 software) linked to Zeiss Microscope (Germany). Endocrine cell density was assessed by measuring the number and area of individual endocrine aggregates as described elsewhere (Figliuzzi et al. 2009, Rackham et al. 2013). An individual endocrine cell aggregate was defined as an area occupied by insulin-positive cells separated from any other adjacent insulin-positive by > 50 µm of insulin-negative non-endocrine tissue. Total endocrine area was defined as the sum of the areas of all endocrine aggregates within an individual graft section. Islet graft was demarcated as an area of endocrine and non-endocrine between the renal parenchyma and renal capsule (Appendix C, Table C4).

To assess engraftment of islets, we counted the frequency of Ki67 nuclei among 1,000 insulin β-cells per graft bearing kidney. Cell counts were done on a minimum of 1000 cells per sample, and percentages were calculated as number of positive cells per 1000 (Appendix C, Table C5)

2.13 Statistical analysis

All the digital images were analysed and data was exported to MS Excel (Microsoft Inc, USA) and the Statistical Package for Social Sciences (SPSS) version 18.0 for Window (SPSS Inc, Chicago, IL, USA) was used for statistical analysis. Each data point is the mean of at least 50 islets. The data was presented as Mean ± SEM. Statistical analysis used student's *t*-test to identify differences in islets development cultured under different conditions as well as in glycemic levels between groups of rats and statistical significant difference was determined at

p≤0.05. Kaplan-Meier survival curve was used to identify differences in time of cure between groups. A log rank *p* value of $p < 0.05$ was considered significant, (n = 10 animals/group).

Chapter 3 Results

3.1 Development of islets cells in cultures

3.1.1 Islets obtained from post-PDL pancreas

3.1.1.1 Modification and adaptation of protocol for islet isolation for culture

Results of digesting the pancreatic tissue in varying concentrations of Collagenase A, isolating islets from non-islet tissue and counting them in RPMI 1640 culture medium to maintain viability are shown in Table 3.1 and in Fig. 3.2.

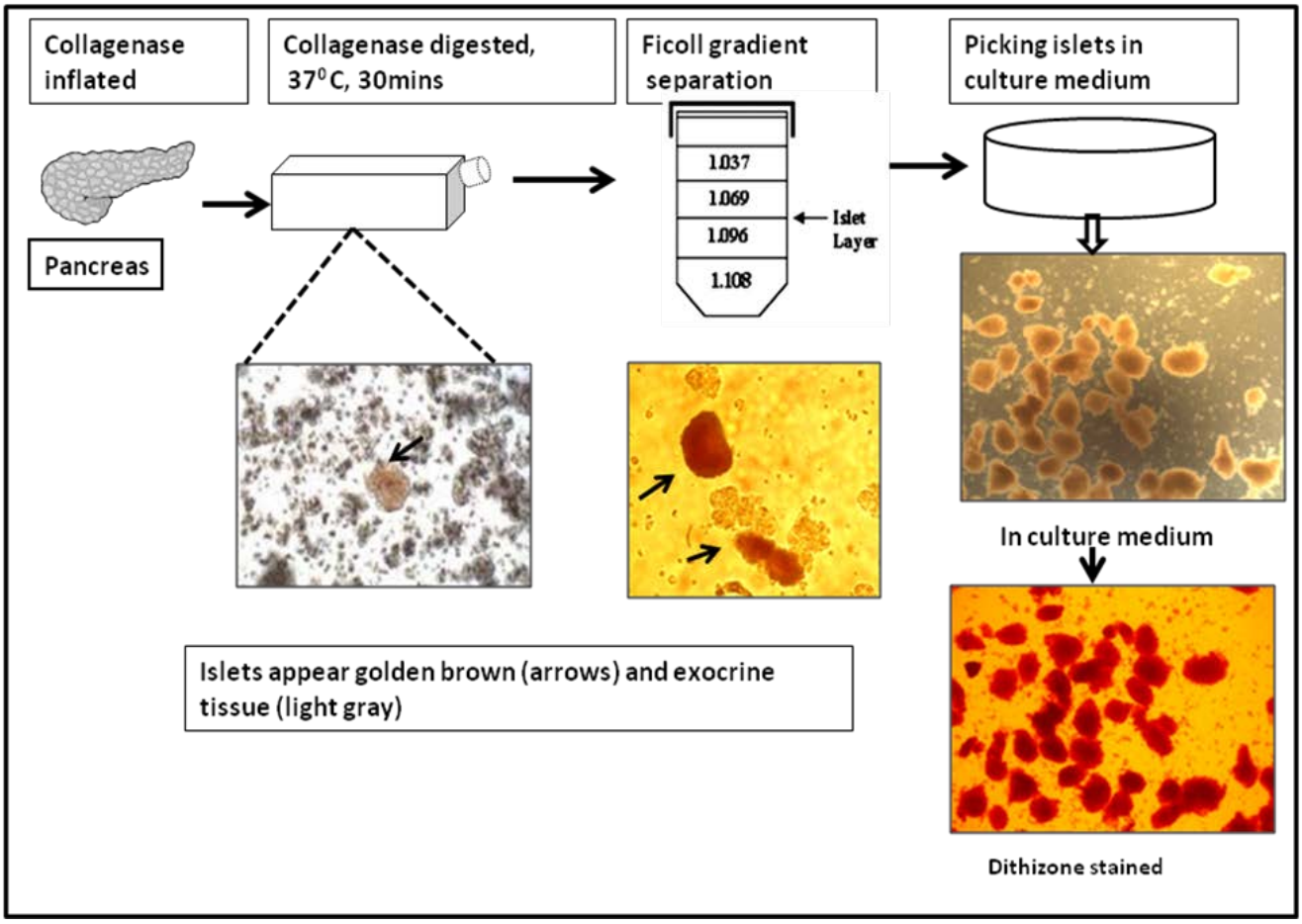


Figure 3.1. SCHEMATIC REPRESENTATION OF ISLET ISOLATION PROCEDURE.

Strain	Sample size (n)	Weight (g)	Collagenase A (mg/mL)	Digestion time (min)	Av. Islet Yield
	3	180-250	1.4	20-30	174
	3	180-250	1.5	20-30	265
	2	180-250	2.0	20-30	316*

** Isolated islets were fragmented and small in size*

Table 3.1. Number of islets isolated from PPDL pancreatic tissue

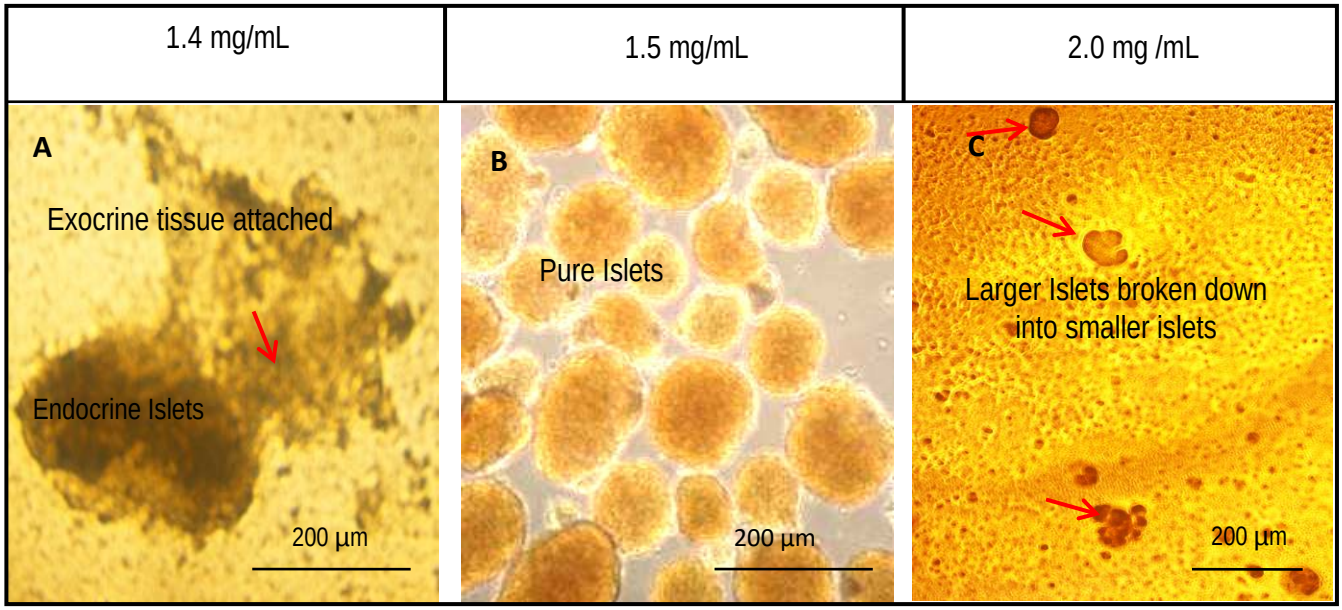


Figure 3.2. Morphological features of endocrine islets recovered from pancreatic digest using varying concentrations of Collagenase A. In A *arrow*s indicate exocrine tissue attached to islet while in B *arrows* indicate small islet cells

3.1.1.2 Morphology and Number of islets recovered

The number and size of the islets varied with Collagenase A concentration. Individual rat islets from a PPDL pancreas may range from 80 to over 400 microns in size (Fig. 3.2 B). Higher

average yield and normal sizes of islets were obtained at collagenase concentration of 1.5 mg/mL and digestion time of 30 minutes (Table 3.1 & Fig. 3.2 B). Numerous and fragmented smaller islets were obtained when Collagenase A concentration was increased to 2.0 mg/mL, digesting at the same period of time (Table 3.1 & Fig 3.2 C.).

Reducing the concentrations of Collagenase A to 1.4 mg/mL resulted into incomplete digestion with islets still attached to exocrine tissue (Fig. 3.2 A). However, a 1.4 mg/mL Collagenase A enzyme concentration was optimal to yield, at least healthy islets from 84 h and 120 h PPDL pancreatic tissue. Results showed that 84- and 120 h PPDL pancreatic tissues were very sensitive to enzyme digestion because the tissue, according to previous studies (Tchokonte-Nana, 2011), is undergoing massive exocrine destruction during this period. The present study has in addition found that it also affects the morphology and integrity of islets. Results also indicate that islets from the 84- and 120 h PPDL groups could easily disintegrate into smaller cellular aggregates at 1.5 mg/mL collagenase A enzyme concentration similar to what was observed with normal control and 24 h PPDL tissues when digested with 2.0 mg/mL of the enzyme as shown in Fig. 3.2 C.

3.1.1.3 Effect of protocol modification

This study used a modification of the protocols from Goto et al. 1985 and De Haan et al., 2004. This modified protocol allowed collagenase to interact more closely with tissues surrounding islets and resulted into higher islet yield. In young male Wistar rats (150-200 g), total number of islets increased with age. More islets were recovered from bigger animals (250-350 g). It was found that in Wistar rats, higher yields of pure and viable islets could be achieved when harvested PPDL pancreas is first injected with 1.5 mg/mL of Collagenase A, minced and

digested for 30 minutes. Results also indicated that Collagenase A of 1.5 mg/mL dissolved in Hank's balanced salt solution instead of H-DMEM containing 10 mMol HEPES, and a digestion time limited to 30 minutes at 37°C proved essential for reproducibility as shown in Table 3.1.

3.1.1.4 Purity and viability of islets recovered

Purity of islets was verified by dithizone staining (red) which specifically binds to zinc found in insulin granules (Fig. 3.3). Results showed that islet cells were viable and showed no areas of hypoxia (darker area at the center) (Fig. 3.2 B&C). More rounded healthy islets were recovered from collagenase digest of 1.5 mg/mL than other concentrations (Fig. 3.2 B).

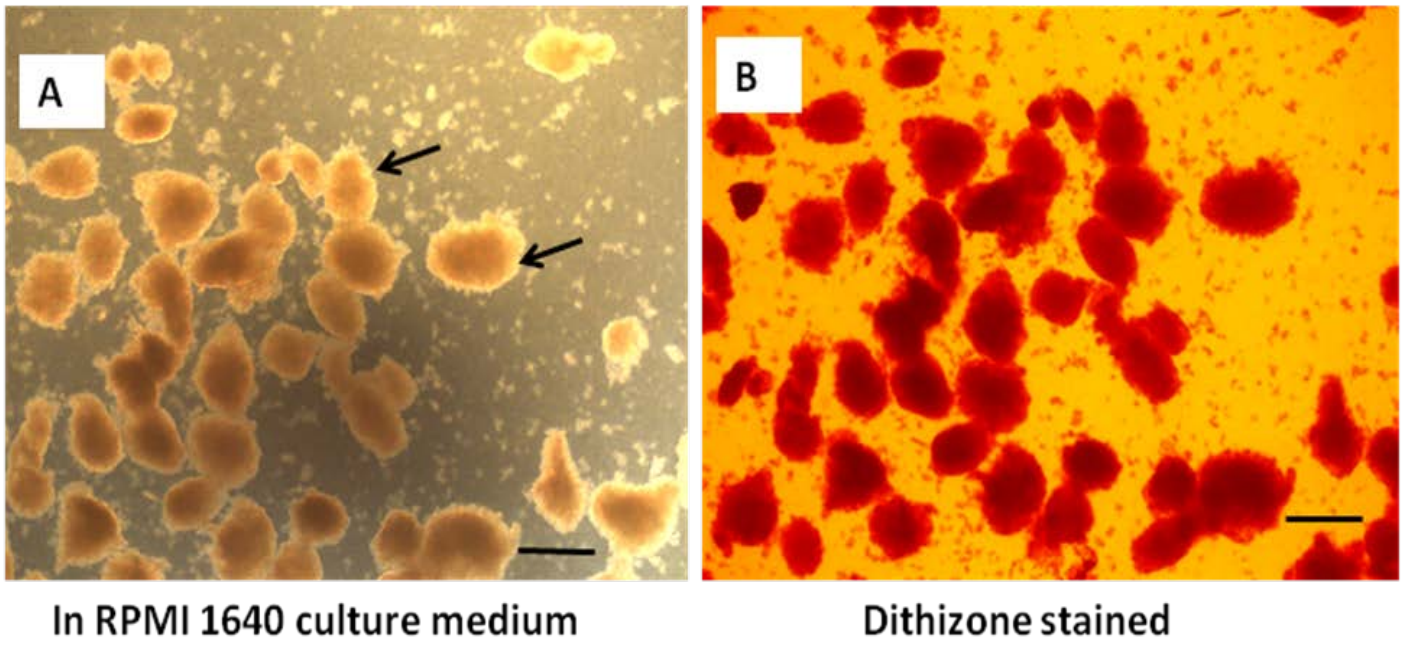


Figure 3.3 Pancreatic islets during purification procedure. (A) Isolated islets (arrows) kept in RPMI 1640 cell culture medium and (B) islets after dithizone staining (red) which specifically binds to beta cell insulin granules. Scale bars = 150 µm

3.1.2 Mesenchymal stromal cells (MSCs) obtained from PPDL pancreas.

Adult PPDL pancreatic tissue prepared from islet-depleted digests was cultured for 48- 72 h as described in Section 2.6.5. After 48 h, the supernatant was removed and the resultant adherent cells usually composed of cellular aggregates primarily of MSCs and some few trapped ductal and exocrine cells were observed (Fig. 3.4B). After 2 days, growing fibroblastic–like MSCs, presenting with narrow cell bodies and long cytoplasmic prolongations, were observed (Fig. 3.4B). These results show that fibroblastoid-like MSCs can be successfully isolated from the PPDL rat pancreas within 48 h of culture by using RPMI 1640 medium (Fig. 3.4B).

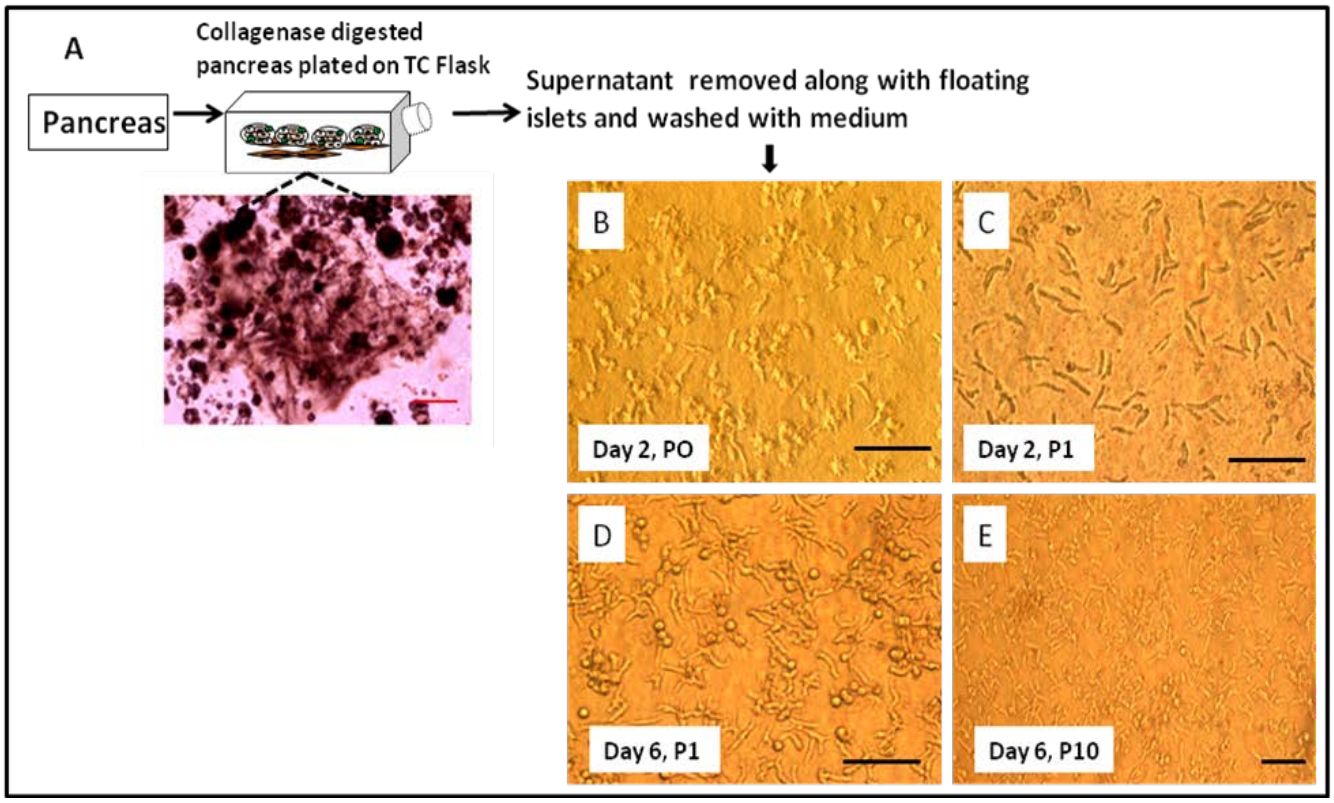


Figure 3.4. Morphology of isolated mesenchyme-like cells from PPDL rat pancreas. Panel A: Schematic representation of isolation procedure: Pancreas digested in Collagenase P was plated in RPMI 1640 culture medium and adherent cells appeared that were propagated after the 48 h. Panels B-E shows morphology of isolated fibroblast-like cells and expanded at different passages: P0 = before passaging; P10 = passage 10; Scale bar = 50µm.

After detaching cell monolayer from the bottom of the petri dish by trypsin EDTA treatment, fibroblast-like cells were allowed to expand in cultures as adherent cells and reached confluence by day 4. These cells were passaged again and reached confluence within 6 days (Fig 3.4D, E) for a total of 24 days in culture medium. The composition of these cells was verified by immunocytochemistry through negative screening by staining for the presence of endocrine, duct and exocrine cell contaminants. Though not a very accurate method of characterizing MSCs than the use of flow cytometry, it was noted that during the initial culture period (passages 1 to 2), some morphological heterogeneity in the adherent cell fraction were observed (Fig. 3.5A, B, C). However, as the cultures were subsequently passaged, homogeneity in morphology of the MSC cells was gradually achieved with fibroblast/flat cells, bearing nuclei rich in nucleoli, predominating (Fig. 3.5 D-I).

To recapitulate the regenerative process that occurs in a PPDL pancreas, mesenchymal–like cells were consistently maintained in a proliferative/undifferentiated state for more than 10 passages (68 days). During this period, we observed a 10-fold increase in cell numbers between successive passages. However, their proliferation rate drastically reduced by 3-fold beyond passage 7 (42 days) and morphologically, these cells began to flatten towards passages 8-10 (Fig 3.5 G, H). Thus, cells from passages 3 to 6, which were morphologically similar to that of MSCs (Fig. 3.4 D-E), were used for islets and MSCs co-culture experiments in order to maintain uniform results.

3.1.3 Islet development from co-culture of PPDL islets and MSCs

3.1.3.1 Development of Islets in direct co-culture with or without MSCs

To determine whether MSCs induce neogenesis in cultured islets, islets and mesenchymal cells were isolated from rat tail pancreas 24 h PPDL and sham operation (SOC), and cultured islets in

direct apposition with or without MSCs in serum free RPMI 1640 culture medium. On this basis, islet development was analysed by assessing the morphological changes and the expression of Pdx1, Ngn3 and insulin in islet cells cultured with or without MSCs as shown in Fig. 3.6.

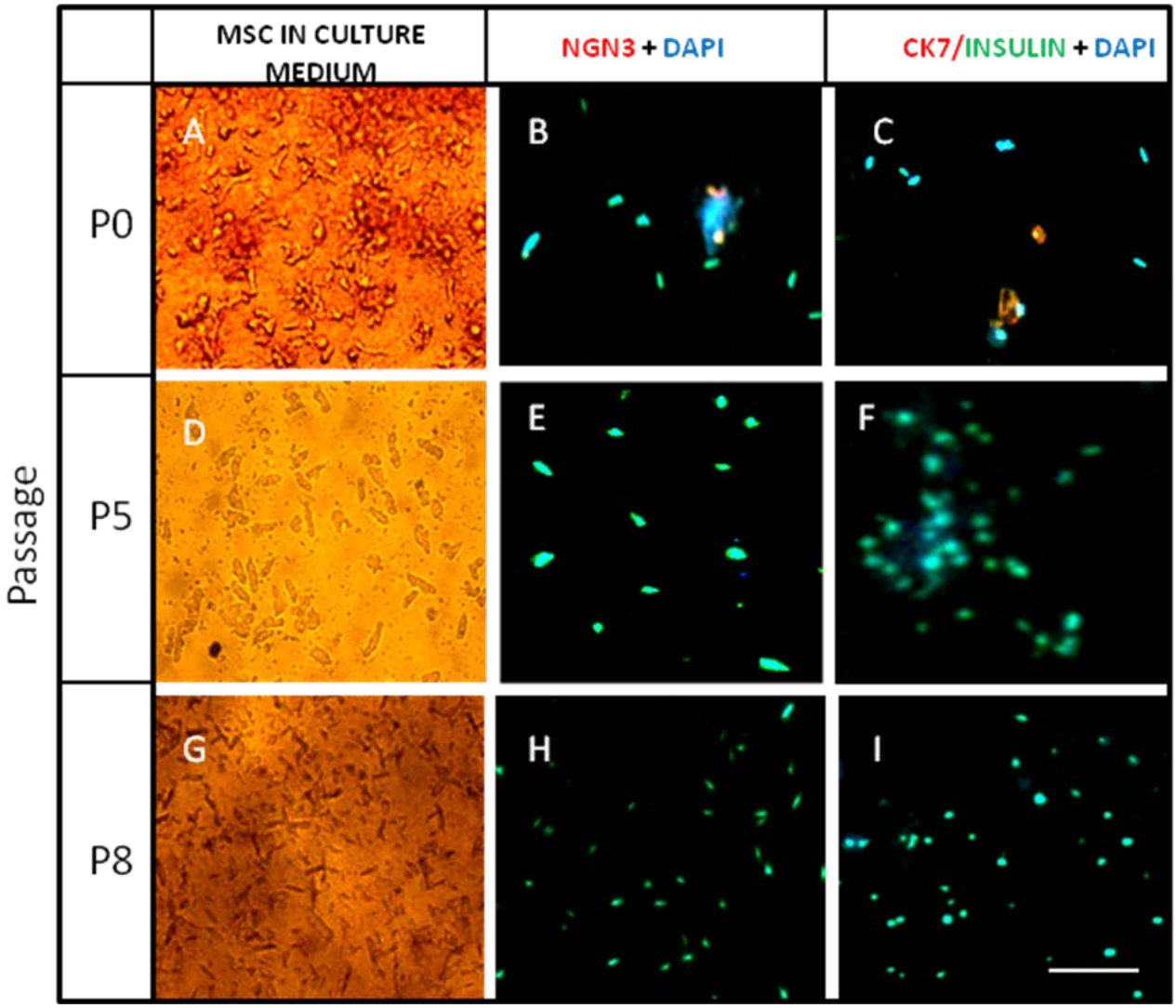


Figure 3.5. Morphology of expanded adherent fibroblastic-like cells at different passages. Purity of adherent fibroblastic-like MSCs was verified by immunostaining for contaminants (negative selection). Left Panels: Mesenchymal stromal cells in culture medium at different passages. In Panel B, < 2% of cell population stained positive for Ngn3 (red/orange), Panel C, < 1% stained positive for CK7(red/orange)/insulin (green) before passaging (P0-day 2). In Panels E,F,H & I, more than 99% of the cells stained negative for Ngn3, CK7 and insulin (P5- day 1, P8- day 6 (confluent culture) Nuclei stained with DAPI. Scale bar = 100µm**.**

3.1.3.2 First week of islets development in culture with and without MSCs

Figure 3.6 shows islets in culture with and without MSCs of sham operated controls (SOC) and 24 h splenic duct ligated tissues. As shown in Figure 3.6, in 24 h PPDL islet cultures without MSCs, islets with smooth edges were clearly observed after 1 day of culture (Fig.3.6 I). After 3 days, small protrusions (lobules) of proliferating cells from the edges of islets were seen, either as attached to the islets, or as individual structures (Fig. 3.6 J**)**. Similar results were observed in cultures of islets only isolated from sham operated normal controls (SOCs) cultured alone (Fig.3.6 A, B). Between days 5 and 7, these proliferating cells in 24 h PPDL islet cultures became elongated, at the same time, some became delaminated and migrated outwards giving a serrated morphology of the islets (Fig. 3.6 K, L) which was similarly observed in control SOC islets cultured alone (Fig.3.6 C, D).

When cultured with MSCs, in contrast, 24 h PPDL islet cells proliferated rapidly and by day 3 of culture, these cells expanded into MSCs with proliferating cells from the edges of the islets migrating outward and expanded over MSCs up to day 7 (Fig 3.6 M-P). Comparable morphological changes in islet development were also observed in control SOC islets cultured with MSCs during the same time period (Fig. 3.6 E-H). The morphological results of the first week, therefore, show that the proliferative capacity of the islets is activated in both cultures with or without MSCs and PDL does not affect their initial proliferative capacity of islets.

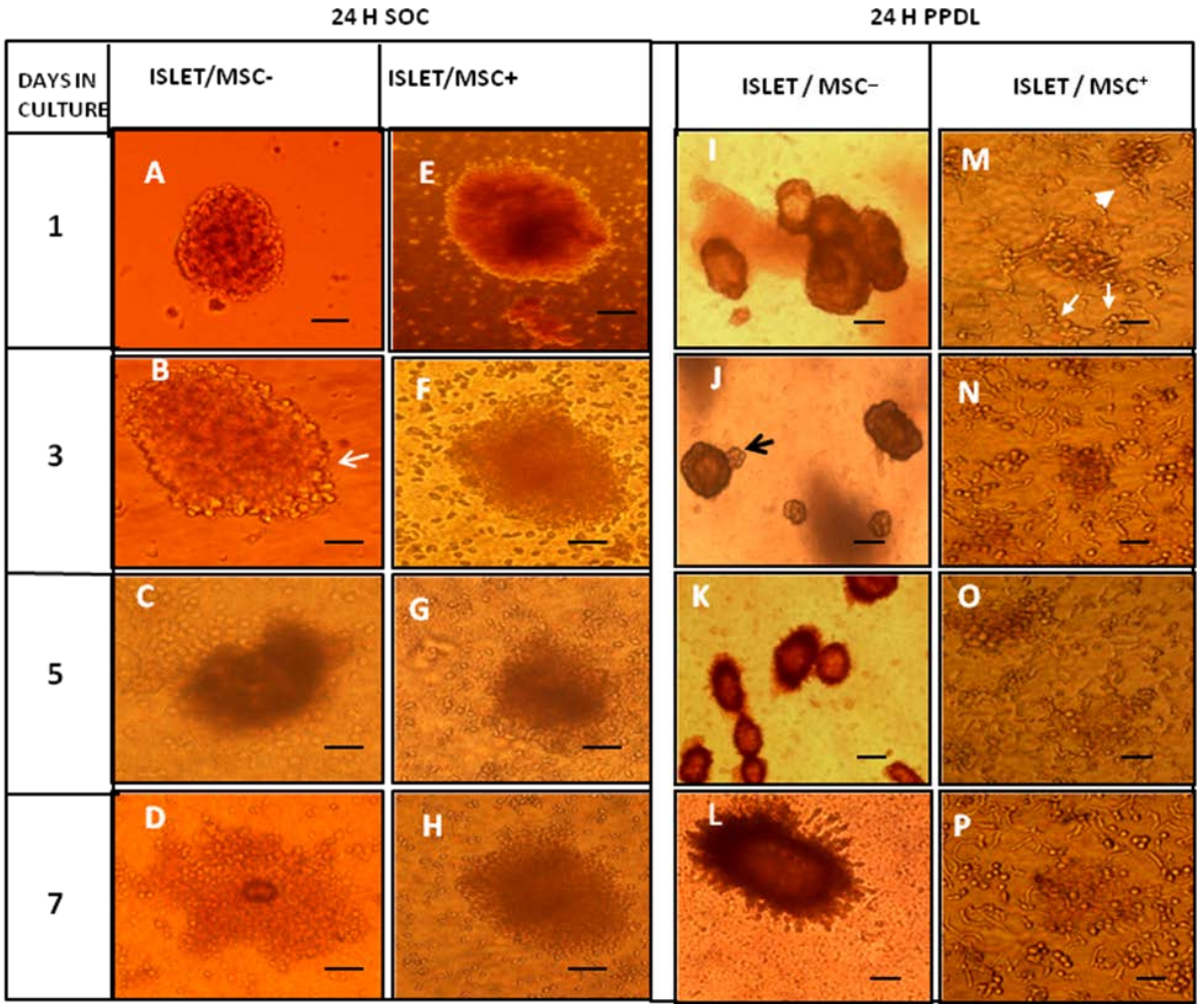


Figure 3.6. Early development of isolated rat islets cultured by direct configuration. Islets and MSCs were isolated from rat pancreas 24 h post pancreatic duct ligation (PDL) and sham operated control (SOC) respectively and cultured with (PDL islets + PDL MSCs versus SOC islets + SOC MSCs) or without mesenchymal stromal cells (SOC islets only or PDL islets only) for different periods. A-H: Representative pictures in culture medium after 1 (A, E, I and M), 3 (B,F, J and N), 5 (C, G, K and O) and 7 (D, H, L and P) days of culture. In M and N, islets are completely surrounded MSCs (white arrows). In M, proliferating islet cells migrate outward (white arrows) (as observed on live cultures under inverted microscope) to form new aggregates (black arrows), (n = 10 animals/group), Bar = 100µm.

To determine whether MSCs induce neogenesis in proliferating islet cells in culture at this stage of islet development, the expression pattern of two transcription factors Pdx1 and Ngn3 that are

activated in pancreatic and endocrine progenitors during early embryonic development were analysed by immunocytochemistry. Pdx1 is first expressed in endodermal cells committed to form pancreatic bud and later is restricted to mature beta cells where it activates the insulin gene (Ohlsson, Karlsson & Edlund 1993). Ngn3 is expressed in endocrine precursor cells (Gu, Dubauskaite & Melton 2002). The study also assessed the insulin gene expression in mature beta cells.

As shown in Fig. 3.7 A&B, in cultures without MSCs, surprisingly, few Pdx+ cells were first detected after 3 days of culture in the slides that were analysed and by the end of first week, a marginal increase in Pdx1 positive cells, mostly located on the peripheral of the islets, were recorded (Fig. 3.7 A-D). In the presence of mesenchymal cells, in contrast, more Pdx1 expressing cells were detected from day 3 of culture than those cultured without MSCs and their numbers were significantly increased from day 3 up to Day 7 (p = 0.01753) when compared to cultures without MSCs (Fig. 3.7 F, G, H & K).

Since Pdx1 is also expressed in mature beta cells where it activates the insulin gene (Ohlsson, Karlsson & Edlund 1993), analysis of insulin expression was carried out in the first week to assess whether insulin gene is also activated in $Pdx1^{+}$ cells in the absence or presence of MSCs. As shown in Fig. 3.8 (A-E), cells co-expressing Pdx1 and insulin proteins were detected throughout the first week of culture up to day 9. Insulin expression was increased in cultures without MSCs throughout the week; however, very low numbers of $Ngn3^{+}$ cells were detected throughout the first week of culture (Fig. 3.7 A-E, L) in the absence of MSCs.

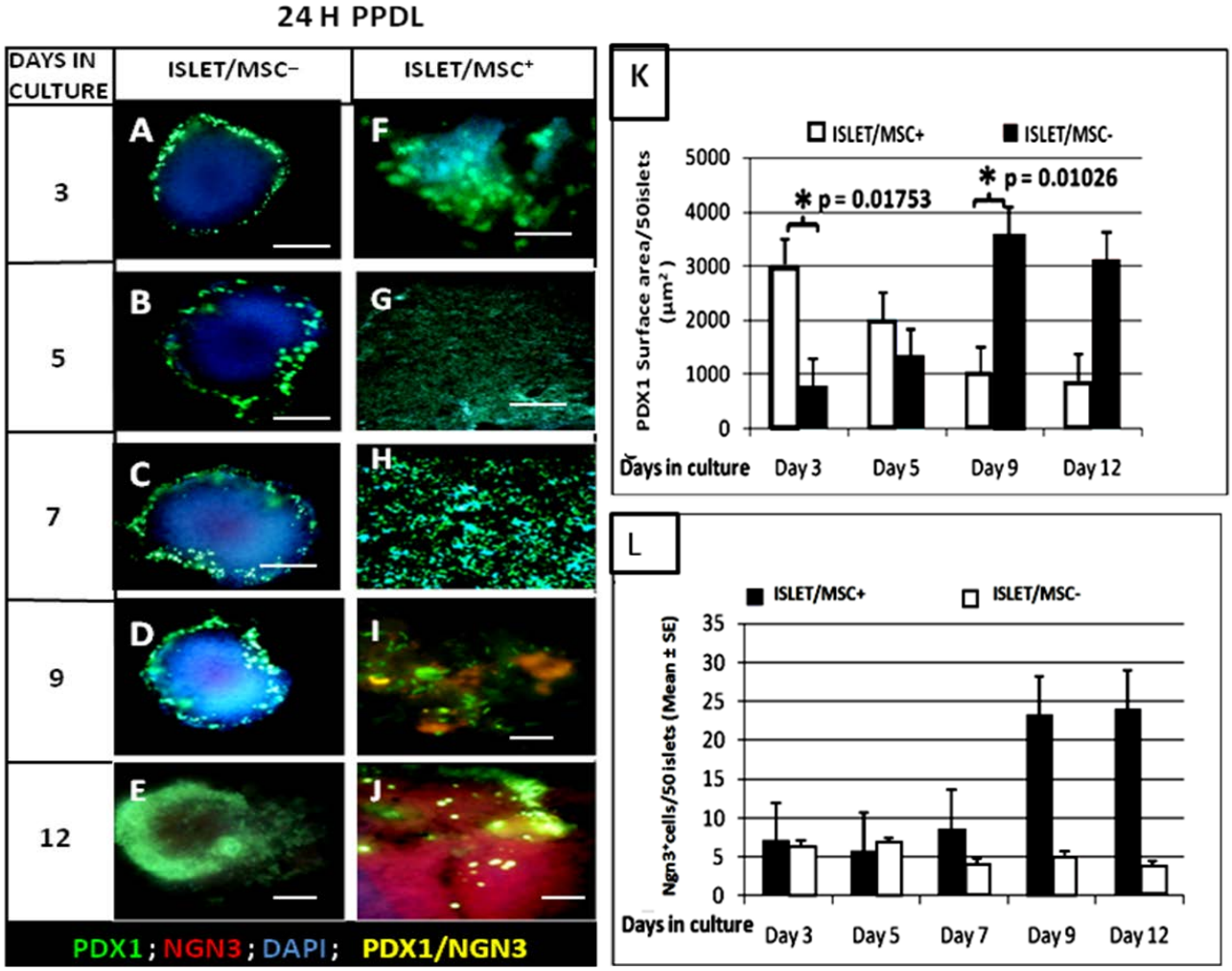


Figure 3.7. Expression of Pdx1 and Ngn3 during early development of islets cultured by direct contact with or without mesenchyme(MSCs). Islets and MSCs were isolated from rat pancreas 24 h post pancreatic duct ligation (PDL) and cultured with or without MSCs for different periods. Immunocytochemistry analysis of islets cells cultured with (F-J) or without (A-E) MSCs for different days of culture. Islet cells development was evaluated by anti-Pdx1 (green) and anti-Ngn3 (orange/red). Nuclei were counter-stained in blue with DAPI. K-L: Graphs show $Ngn3^+$ cell numbers and absolute surface areas occupied by $Pdx1^+$ cells in cultures with or without MSCs. For each culture condition, forty (40) to fifty (50) islets were analyzed. Data are means ± SE. $^{**}p < 0.05$, (n = 10 animals/group). Scale bar = 100µm

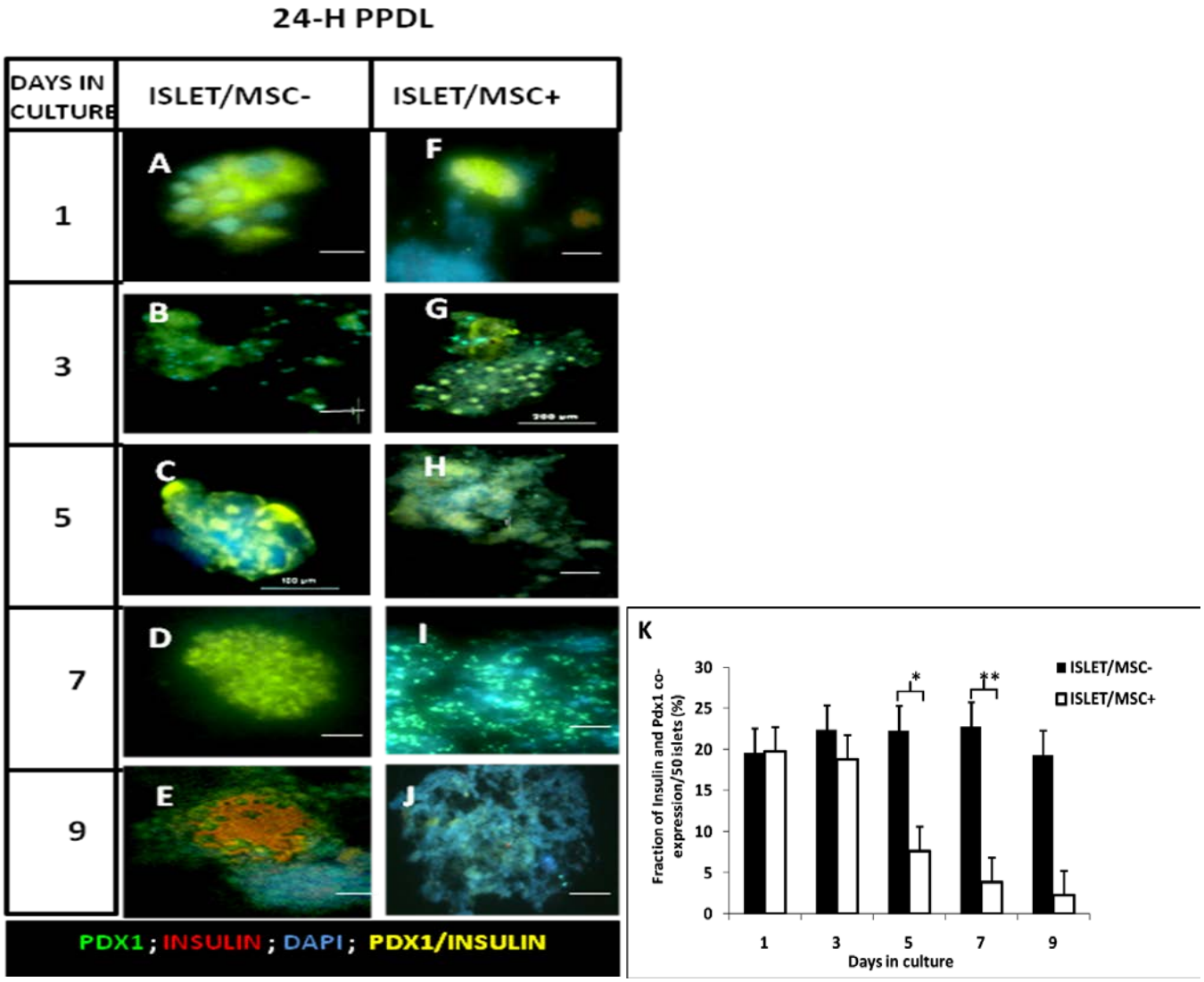


Figure 3.8. Co-expression of Pdx1 and Insulin during early development of islets cultured by direct contact with or without MSCs. Immunocytochemistry analysis of whole mount islets cells isolated from rat pancreas 24-hr post pancreatic duct ligation (PDL) and cultured with (E-G) or without (A-D) MSCs for different days of culture. Islet cells development was evaluated by anti-Pdx1 (green) and anti-insulin (orange/red). Nuclei were counter-stained in blue with DAPI. K: Graph shows absolute surface areas occupied by $Insulin^{+}$ and $Pdx1^{+}$ cells that developed over different days of culture with (Islet/MSC+) or without (Islet/MSC-) MSCs. For each chlture condition, forty (40) to fifty (50) islets were analyzed. Data are means ± SE. $^{**}p < 0.05$ (p = 0.0481), (n = 10 animals /group), Scale bar = 100µm.

In the presence of MSCs, Pdx1 co-expressed with insulin up to day 7, similar to cultures without MSCs (Fig. 3.8 F-I).However, insulin expression gradually decreased by day 7 (Fig. 3.8 J). Very low numbers of $Ngn3^{+}$ cells were also detected in cultures with MSCs up to day 7 (Fig. 3.7 L). At the end of first week of culture, islets were composed of largely $Pdx1^{+}/Ngn3^{-}$ cells in both cultures with or without MSCs (Fig 3.7 A-C, G-H). However, the number of $Pdx1^{+}$ cells was significantly higher in cultures with MSCs compared to cultures of islets only in the first 3 days of culture ($p= 0.01753$, Fig. 3.7 K). Similar results were obtained when compared to cultures of 24 h PPDL islets + 24 h PPDL MSCs versus cultures of 24 h post SOC islets + 24 h post SOC MSCs controls in which significantly higher numbers of Pdx1+ cells were observed in cultures with MSCs than in cultures of islets only, as shown in Fig. 3.9. In 24 h post SOC islets, Ngn3+ cells were not detected throughout the first three days of culture both, with or without SCs, possibly suggesting that in the early hours of PPDL, Ngn3 is induced by factors other than those arising directly from MSCs.

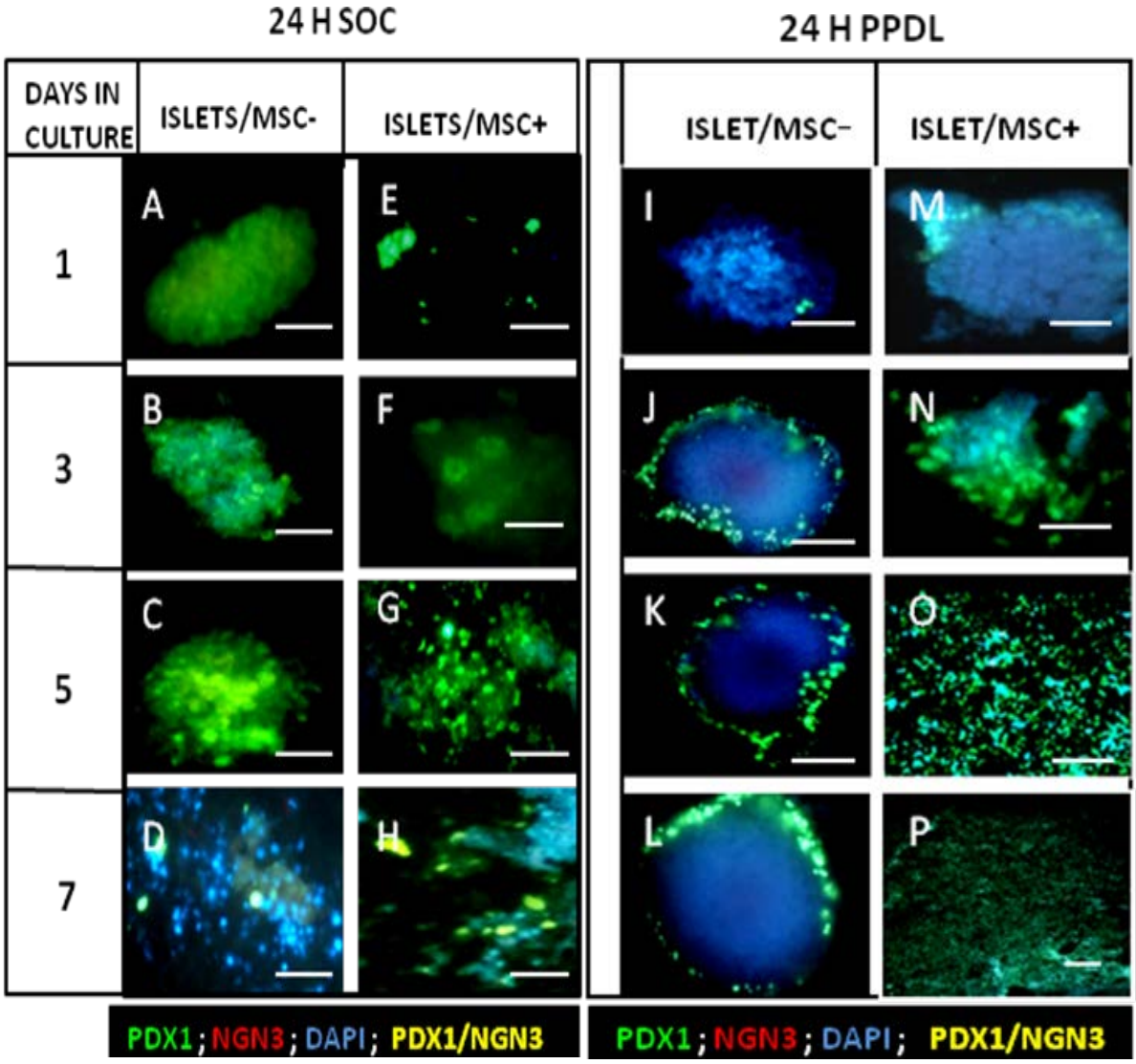


Figure 3.9. Expression of Pdx1 and Ngn3 during early development of islets cultured by direct contact with or without MSCs. Immunocytochemistry analysis of whole mount islets cells isolated from rat tail pancreas 24 h post pancreatic duct ligation (24 h PPDL) and sham operation (24 h SOC) and cultured without (A-D) for SOC and I-L) for PDL or with MSCs (E-H) for SOC and (M-P) for PDL. Islet cells development was evaluated by anti-Pdx1 (green) and anti-Ngn3 (red). PDX1/NGN3 (orange). Nuclei were counter-stained in blue with DAPI.), Scale bar = 100µm

3.1.3.3 Second week of islet development in culture with and without MSCs

As 24 h PPDL islet cell culture with or without MSCs continued for the second week, differences in rates of proliferation were observed. In cultures without MSCs; rapid islet cell proliferation and elongation was observed between day 9 and 11 (Fig. 3.10 I, J) Elongated islet cells formed a mesenchyme-like sheet of proliferating cells (Fig. 3.10 I, J). However, with continued culturing, islet cells did not continue expanding in the absence of MSCs and failed to reach confluency, even after passaging twice on days 11 and 14, instead, few small spheroid clusters of cells began to form within the amorphous mesenchyme-like sheet of cells on day 13 (Fig. 3.10) K), and became clearly visible on day 15 as large diffusible spheroid bodies (Fig. 3.10 L).

In the presence of MSCs, however, rapid expansion of islets cells was observed. Most islets that were in contact with the monolayer of MSCs expanded extensively over the MSCs (Fig 3.10 N, O). By day 11, proliferating islet cells changed their morphology into elongated shape similar to what was observed in cultures without MSCs. After similarly passaging on days 11 and 14, continued culturing of these cells with MSCs, however, promoted more expansion of up to 80% confluence by day 15 (Fig 3.10 P). The expanding islet cells did not form spheroid bodies by the end of second week, but appeared as an amorphous sheet of loose cells spreading over MSCs.

Results of the SOC islets and SOC Islet/MSCs development in the second week of cultures also showed similar morphological features. In SOC islets cultured alone, islet-like aggregations were similarly observed between 13 and 15 days of culture (Fig 3. 10 C, D) while SOC islet/MSCs

cultures showed just few aggregations within the expanding mass of islet cells during the same time period (Fig. 3.10 G,H).

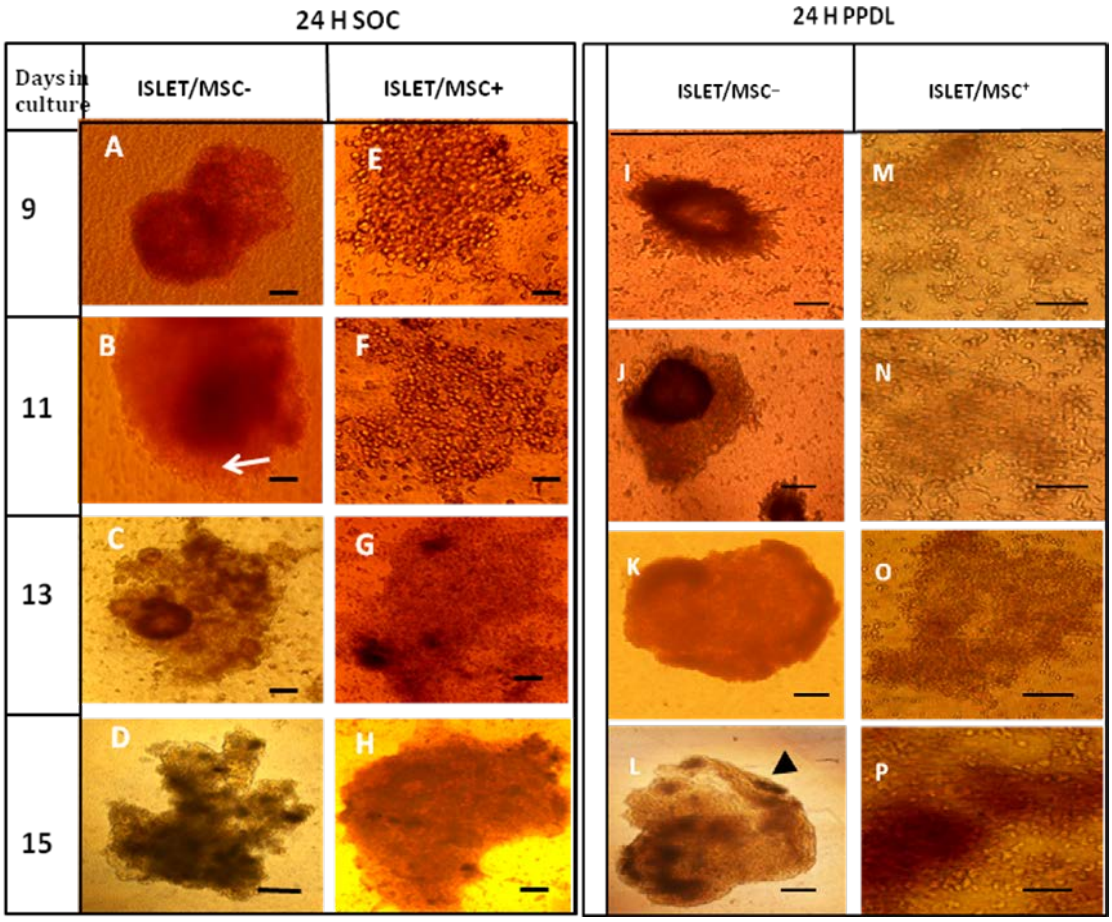


Figure 3.10. Further development of islets cultured by direct configuration. Islets and mesenchymal cells (MSCs) were isolated from rat pancreas 24 h post SOC and pancreatic duct ligation (PDL) respectively and cultured with (SOC islets+ SOC MSCs versus PDL islets+ PDL MSCs) or without mesenchymal cells (SOC islets only or PDL islets only) for different periods. A-H: Representative pictures in culture medium after 9 (A, E, I and M), 11 (B, F, J and N), 13 (C, G, K and O) and 15 (D, H, L and P) days of culture. Proliferating islet cells migrate outward to form new aggregates (arrow head), (n = 10 animals/group were analysed), Bar = 100µm.

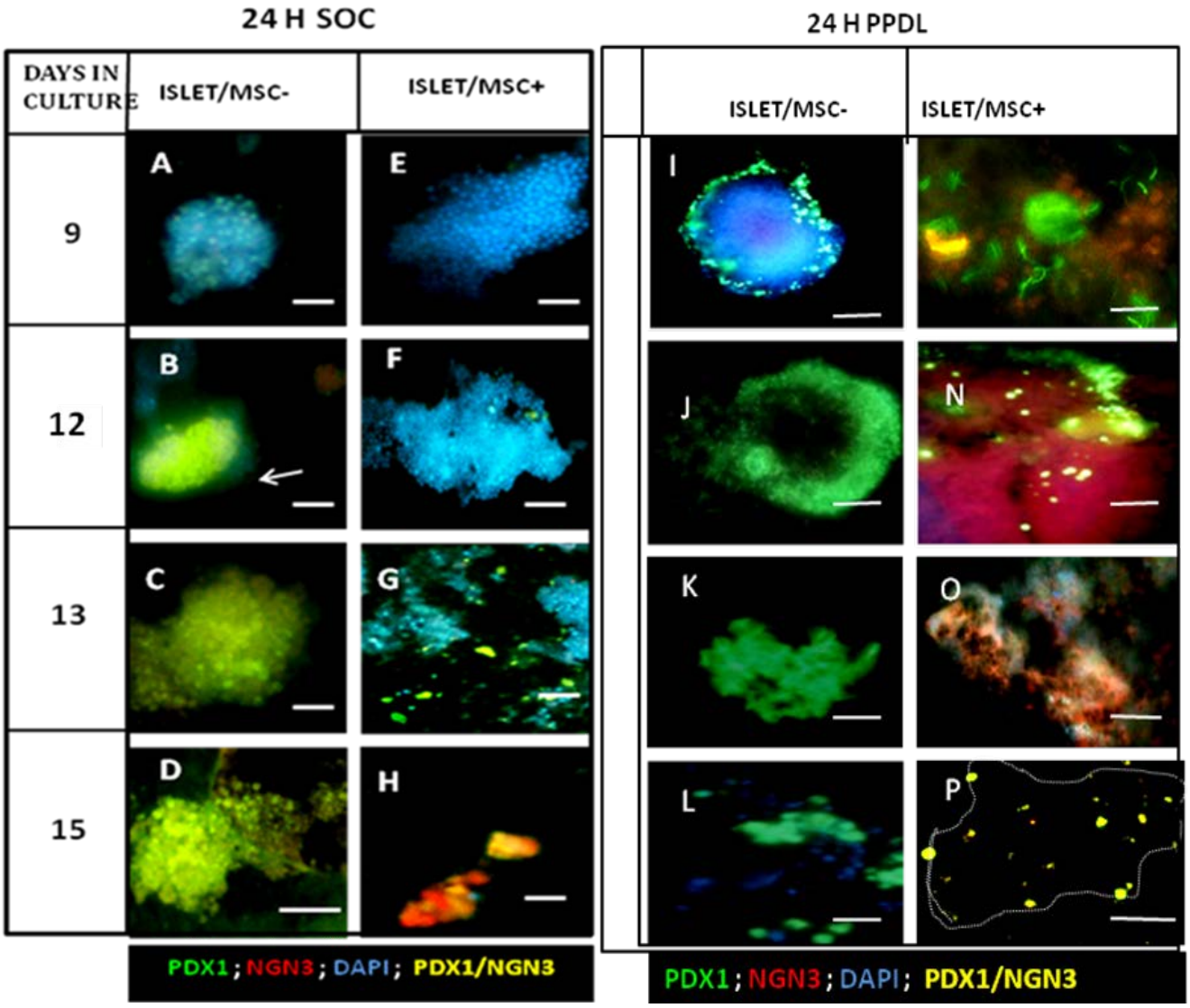


Figure 3.11. Expression of Pdx1 and Ngn3 during the second week of development of islets cultured by direct contact with or without mesenchyme. Islets and mesenchymal cells(MSCs) were isolated from rat pancreas 24 h post SOC and pancreatic duct ligation (PDL) respectively and cultured with (SOC islets+ SOC MSCs versus PDL islets+ PDL MSCs) or without mesenchymal cells (SOC islets only or PDL islets only) for different periods. Immunocytochemistry analysis of 24 h PPDL islets cells cultured without (A-D) or without (A-D) with (E-H) for 9 (A and E), 12 (B and F), 13 (C and G) and 15 (D and H) days of culture. I-P: Immunocytochemistry analysis of 24 h SOC islets cells cultured without (I-L) or with (M-P) MSCs for same period of time. Islet cells development was evaluated by anti-Pdx1 (green) and anti-Ngn3 (orange/red). Nuclei were counter-stained in blue with DAPI. (n = 10 animals/group were analysed), Bar = 100µm.

Analysis of the expression of Ngn3, Pdx1 and insulin in 24 h PPDL islets in the second week of culture showed that in cultures without MSCs, Pdx1 expression continued to be expressed in the peripheral islet cells and increased in the second week up to day 13 (Fig. 3.11 I, J, K).

Quantitatively, the number of $Pdx1^+$ cells was significantly higher in cultures without MSCs compared to cultures with MSCs at days 9 and 12 ($p = 0.01026$) (n = 10) (Fig. 3.7 K). In cultures of islets only, Ngn3 expression reached undetectable levels in most of the slides analysed on days, 9 and 12, however, insulin, often co-expressing with Pdx1, continued to be expressed in significantly high levels compared to cultures with mesenchyme ($p = 0.0481$), ($p < 0.05$) (n = 10) (Fig. 3.8 D, E & K).

In the presence of MSCs, however, Pdx1 expression gradually decreased in the second week, and this was followed by activation of Ngn3 on day 9 such that by day 12 (Fig.3.7 I, J & L and Fig. 3.11 M, N), the number of $Ngn3^+$ cells was significantly higher when compared to cultures of islets only ($p = 0.00102$) (n = 10) (Fig. 3.7 L). Concomitantly, insulin expression decreased to very low levels by day 9 such that by day 12, the insulin proteins were almost undetectable in cultures with MSCs (Fig. 3.8 I, J & K).

Immunostaining analysis in cultures of 24 h SOC islets only showed continual expression of Pdx1 in second week, however, in 24 h SOC islet/MSCs, a complete down-regulation of both Pdx1 and Ngn3 was observed between 9 and 12 days in the second week (Fig 3.11 E, F), but Ngn3 and Pdx1 expression re-appeared between 13 and 15 days of culture (Fig.3.11 G, H).

Taken together, the results show that Pdx1 is activated in islet cells cultured both, with or without MSCs during the first week of culture. However, the $Pdx1^+$ cells expanded more in

cultures with MSCs than without MSCs and this was followed by activation of Ngn3 which continued to expressed up to second week (Fig. 3.7 H, I & J) and Fig.3.11 M, N).

3.1.3.4 Third and fourth week of Islet development in culture with and without MSCs

Since islet cells cultured with MSCs did not form islet-like structures by the end of the second week, the 24 h PPDL islets were cultured further up to four weeks and the results were compared with those that were cultured in the absence of MSCs as shown in Fig. 3.12. Morphologically, in islets cultured without MSCs, proliferation of islet cells drastically reduced after day 17 (Fig. 3.12 A). No more increase in islet size was observed during third and fourth week of culturing islets only, even after passaging on day 24 (Fig. 3.12 H). Instead, most individual elongated islet cells acquired an almost round shape and continued aggregating to form huge spheroid bodies that began to bud off as early as 21 day of culture. (Fig. 3.12 B). Islets failed to aggregate into smaller clusters but appeared as a dark unified mass of cells in the absence of MSCs.

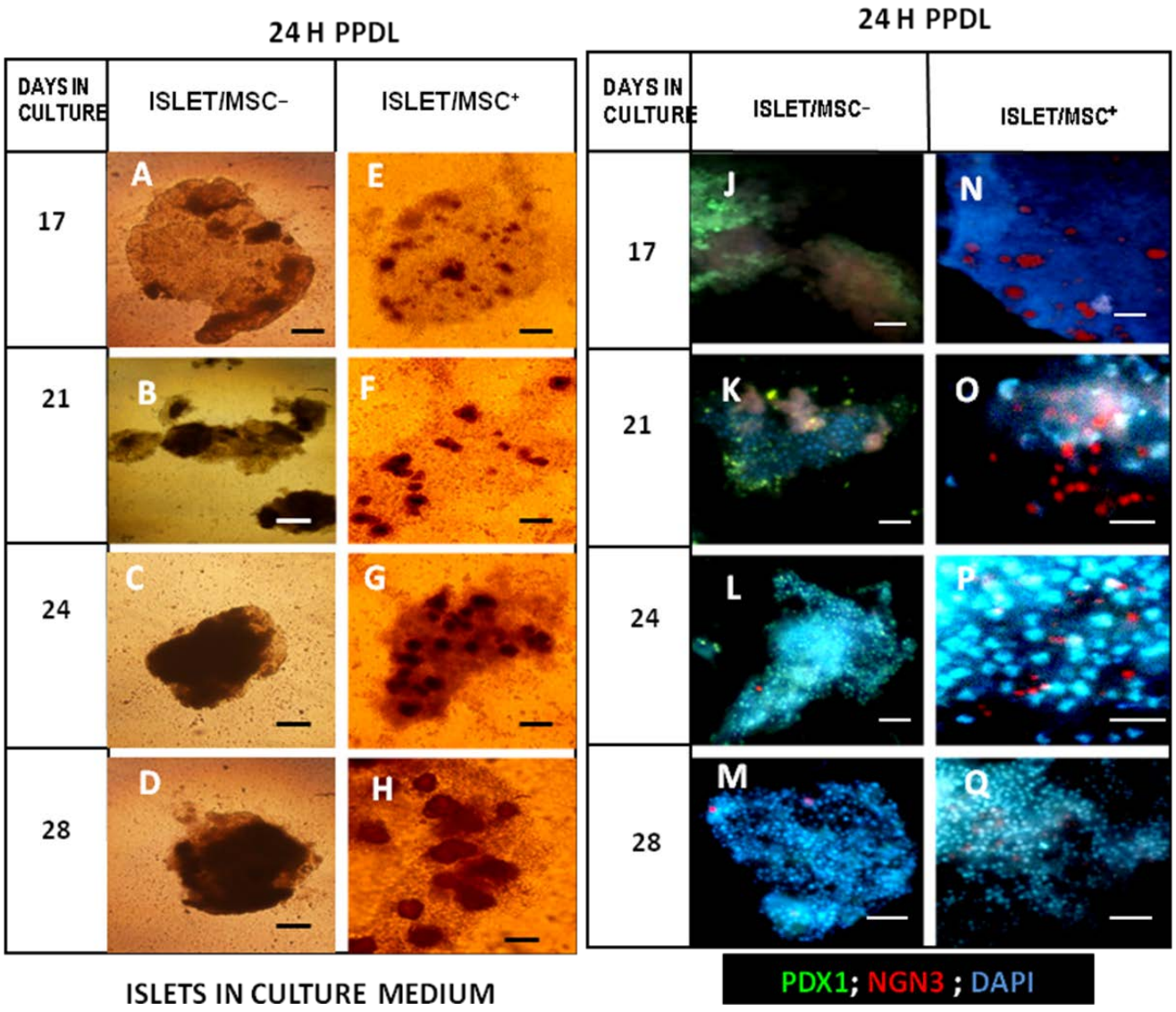


Figure 3.12. Further development of islets cultured by direct configuration with or without MSCs. Islets and MSCs isolated from pancreas 24 h PPDL were further cultured with or without MSCs for different periods. A-H: Representative pictures in culture medium after 17 (A and E, 21 (B and F), 24 (C and G) and 28 (D and H) days of culture. In A, B, C and D no further expansion was observed and islet cells began to form few islet-like spheroid aggregates, which were more visible on day 17. In contrast, in E-H, islet cells continued to expand reaching up 80%confluence by day 21, which later formed numerous new islet-like aggregates by day 28. J-Q: Immunocytochemistry analysis of islets cells cultured with (M-P) or without (I-L) MSCs for 17 (I and M), 21 (J and N), 24 (K and O) and 28 (L and P) days of culture. Islet cells development was evaluated by anti-Pdx1 (green) and anti-Ngn3 (orange/red). Nuclei were counter-stained in blue with DAPI. (n = 10 animals/group were analysed), Bar = 100µm.

With MSCs, few new small islet-like cell aggregates began to form within the expanding islet cells after 17 days and this process continued up to day 28 of culture, such that by end of the fourth week, numerous but small islet-like clusters were formed (Fig. 3.12 E-H).

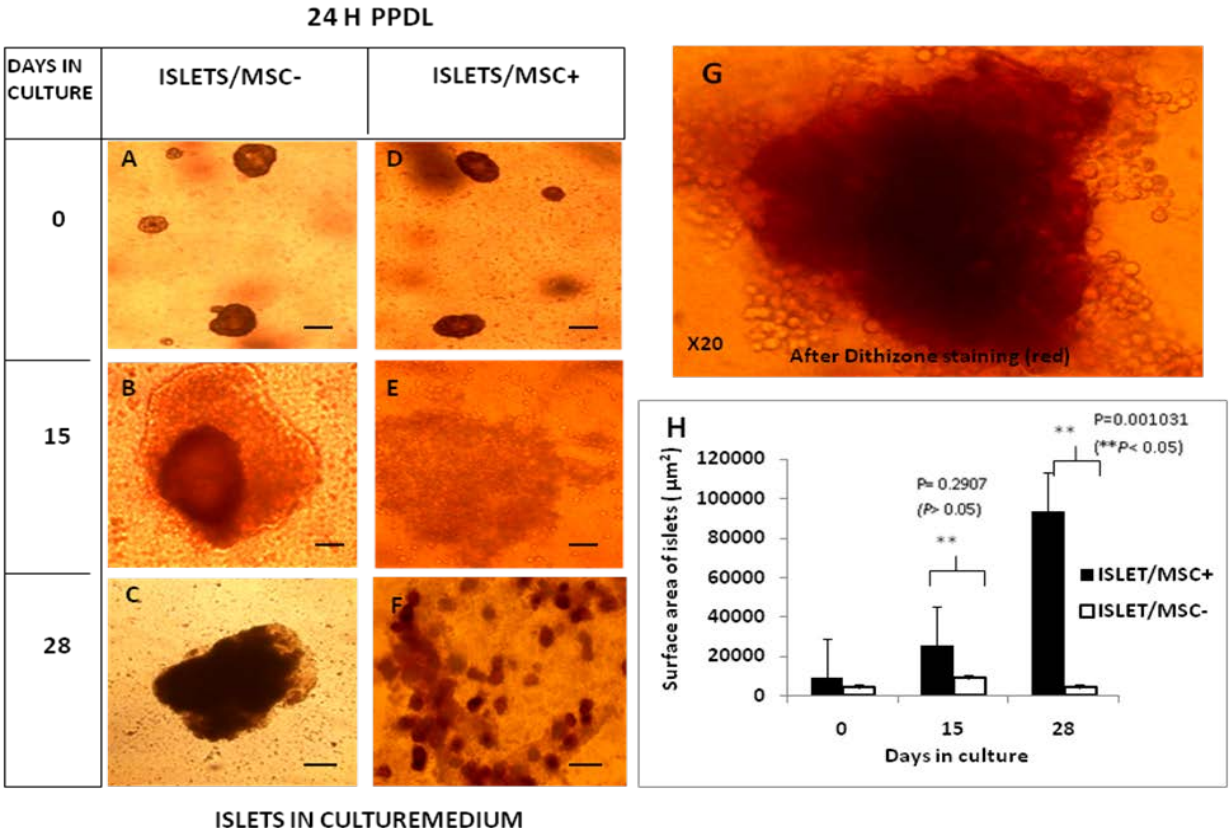


Figure 3.13. Effect of MSCs on the growth size of the islets in culture. Islets isolated 24 h PPDL were grown for 28 days with or without MSCs. A-F: Representative photographs of islets in culture medium before, after 15 days and 28 days in culture. The pictures were taken at the same magnification. Upper Panels: Islets before culture. Middle and lower Panels: Islets cultured for 15 and 28 days with or without MSCs respectively. G: Islets after dithizone staining which specifically binds to zinc found in insulin granules of beta cells to mark the boundary of islets on mesenchymal cells. H: Quantification of the total size of the islets before, after 15 and 28 days in culture in the absence or presence of MSCs. The surfaces of the islets after dithizone staining were marked and measured and areas of all islets were added up. Thirty (30) to 40 islets were analysed in each condition. Data of mean values in μm^2 ± SE are shown in the graph. $^{**}P < 0.05$ (n = 10 animals/group).

To determine the rate and extent of islet growth during the entire period of the experiment, the islets samples were stained with dithizone at three time points: before culturing, on day 15 and on day 28, to demarcate the boundaries of beta cells as shown in Fig. 3.13. Although beta cells were seen to have changed their morphology and phenotypes (de-differentiated) in cultures with MSCs, expansion boundaries of islets were morphologically remarkable by low levels of dithizone staining that allowed us to quantify the absolute area occupied by islets that developed after 15 and 28 says of culture with or without MSCs (Fig. 3.13 A-F, G).

Quantitatively, in islets cultured alone, their sizes marginally increased during the first 15 days of culture (Fig. 3.13 G) In the third and fourth week, their sizes did not increase any further from day 15 to day 28 (Fig. 3.13 B, C and G) and islets failed to reach confluence even after passaging every 6 days. Results also showed that during the third and fourth week, islet cells were either quiescent or dead in cultures without MSCs. Although apoptosis (TUNNEL assay) was not performed to confirm this, DAPI staining, however, showed intact nuclei of islet cells (Fig. 3.12 L, M).

In the presence of MSCs, islet sizes continued to increase such that by day 28 (Fig. 3.13 H) there was significant difference in the size of islets (p = 0.001031) in cultures with MSCs compared to cultures without MSCs (Fig. 3.13 [C compared with F], H). The final number of new islet-like clusters (appeared as dark spots) that developed from individual islets seeded in week one was significantly higher in cultures with MSCs compared to those formed from islets cultured without MSCs (Fig 3.12 [D compare with H], Fig. 3.13 (C compare with F**)** though direct quantification was not done.

Following Pdx1 was downregulation, Ngn3 was highly activated in cultures with MSCs at the end of second week and the fate of $Ngn3^+$ cells was further investigated in the third and fourth weeks. Immunocytochemical analysis of Pdx1 and Ngn3 expression in islet-like aggregates formed during third and forth weeks showed that in cultures without MSCs, Pdx1 expression was down regulated at the beginning of third week of culture and the repression of this gene continued to the end of third week (Fig. 3.12 J, K) during which few $Ngn3^+$ cells were transiently detected between day 17 and 21 of culture (Fig. 3.12 J,K). As expected, Ngn3 was rarely detected thereafter. After down regulation of Ngn3 on day 21, Pdx1 continued to be down regulation in the fourth week, such that by day 28, Pdx1 proteins were rarely detected in cultures without MSCs (Fig. 3.12 L, M).

3.1.3.5 Ngn3 expression is maintained in islet cells cultured with mesenchyme

Interestingly, in the presence of MSCs, $Ngn3^+$ cells began to be detected in the second week from day 9 of culture, a time point when Pdx1 expression was repressed (Fig. 3.7 I, J). Ngn3 co-expressed with Pdx1 between day 9 and day 12 (Fig. 3.7 I, J). $Ngn3^+$ cells were continuously detected in the third week. On day 24, significant higher levels of $Ngn3^+$ cells were detected in cultures with MSCs ($p = 0.03702$; $p < 0.05$) when compared with cultures without mesenchyme (Fig. 3.12 [L compare with P], Fig. 3.14 [A compare with D], G). We continued detecting high levels of $Ngn3^+$ cells with few cells co-expressing Ngn3 and Pdx1 in the fourth week up to day 28 of culture (Fig. 3.12 Q and Fig. 3.14 [C compare with F], G) possibly suggesting that high numbers of $Pdx1^+$ cells have differentiated to $Ngn3^+$ cells.

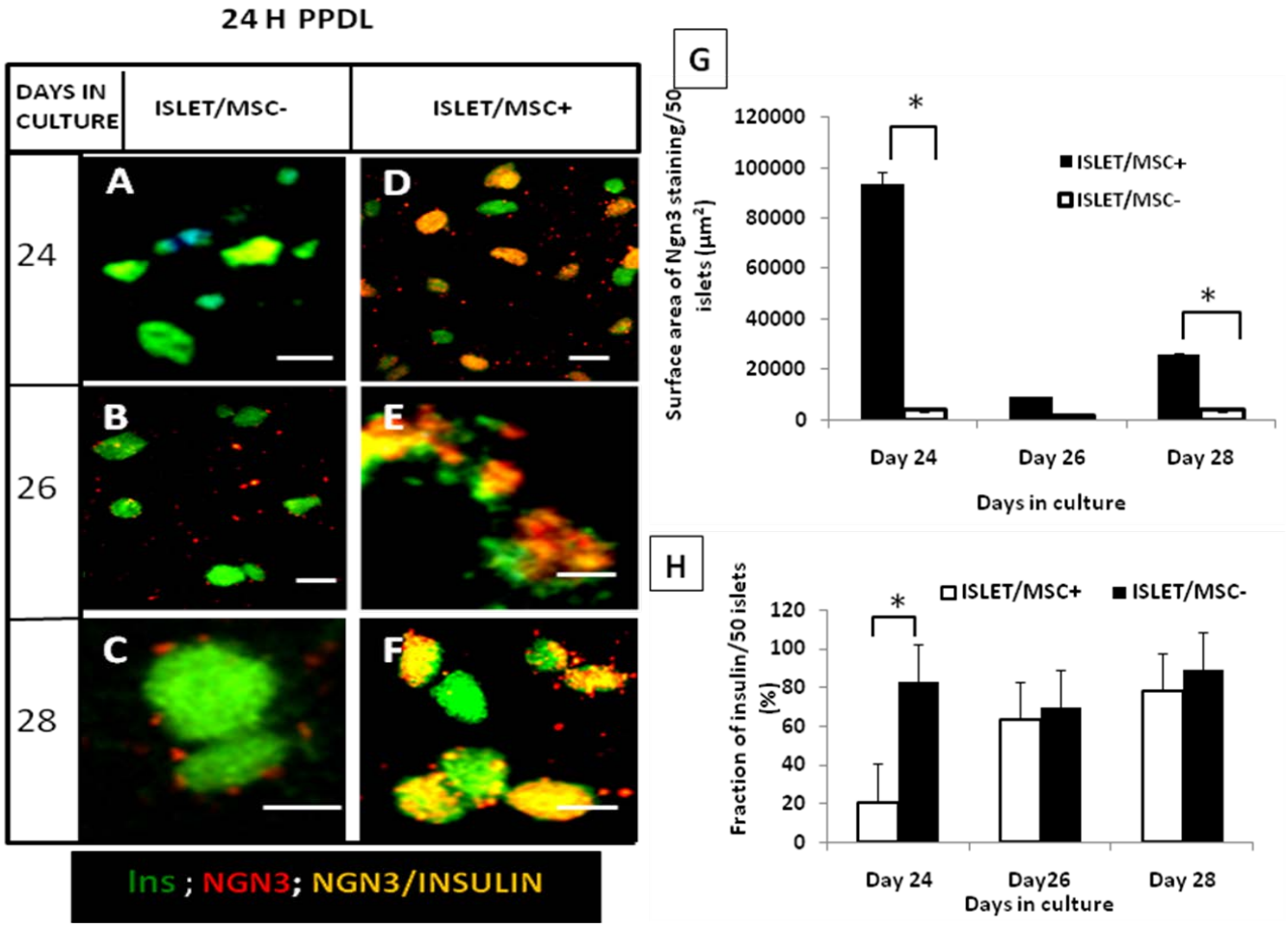


Figure 3.14. Expression of Insulin and Ngn3 during in vitro development of islets cultured by direct contact with or without MSCs. Immunocytochemistry analysis of islets cells isolated 24 h PPDL and cultured without (A-C) or with (D-F) MSCs for 24, 26 and 28 days of culture. Islet cells development was evaluated by anti-insulin (green) and anti-Ngn3 (orange/red). G-H: Graphs show the absolute surface areas occupied by insulin^{+} cells and Ngn3^{+} cells cultured with or without MSCs for 24, 26 or 28 days. For each condition, forty (40) to fifty (50) islets were analyzed. Data are means ± SE. *P < 0.05 (P = 0.03702) (n = 10 animals/group). Scale bar = 100µm

3.1.3.6 Expression of Insulin is lower in islets cultured with MSCs

Since Ngn3 was continuously detected in islets in the fourth week of culture with MSCs, analysis of insulin expression was carried out to assess the maturity and functionality of islet cells in

culture. At the beginning of the fourth week, insulin expression was significantly higher in islets cultured alone than those cultured with MSCs (Fig 3.14 [A-C compare with D-F], **H**). Insulin expression gradually increased in cultures with MSCs towards the end of the seventh week; however, its expression was lower compared to islets cultured without MSCs (Fig. 3.14 H). In addition, in cultures with MSCs, a higher percentage of cells located at the center of islets co-expressed insulin and Ngn3 up to day 28 of culture (Fig. 3.14 F). Single nuclear $Ngn3^{+}$ cells were detected in the periphery of the islets (Fig. 3.14 C, F).

3.1.3.7 Development of islets in indirect co-cultures

To assess whether increased proliferation of $Pdx1^{+}$ cells and maintenance of Ngn3 expression in islet cells were due to their direct cell-to-cell contact with MSCs, rather than to some trophic factors released by mesenchymal cells in culture medium, an indirect co-culture system was set up with islets and mesenchymal cells isolated from 24 h PPDL tail pancreas. Mesenchymal cells were attached as a monolayer at the bottom of the dish while floating islets were physically separated by a Transwell membrane that allowed the sharing of the same culture medium by the two populations of cells. Different from the results obtained with direct co-cultures, islets cells expanded extensively, but without changing their individual cell morphology (Fig. 3.15 [C-D compare with K-L]). Islet cells maintained their round/oval shapes during expansion in indirect co-cultures. After 3 weeks of indirect co-culture, islets continued to expand but failed to form small individual islet-like cell aggregates at the end of the third when compared to islets co-cultured directly on MSC monolayer (Fig. 3.15 Q-T compare with U-X).

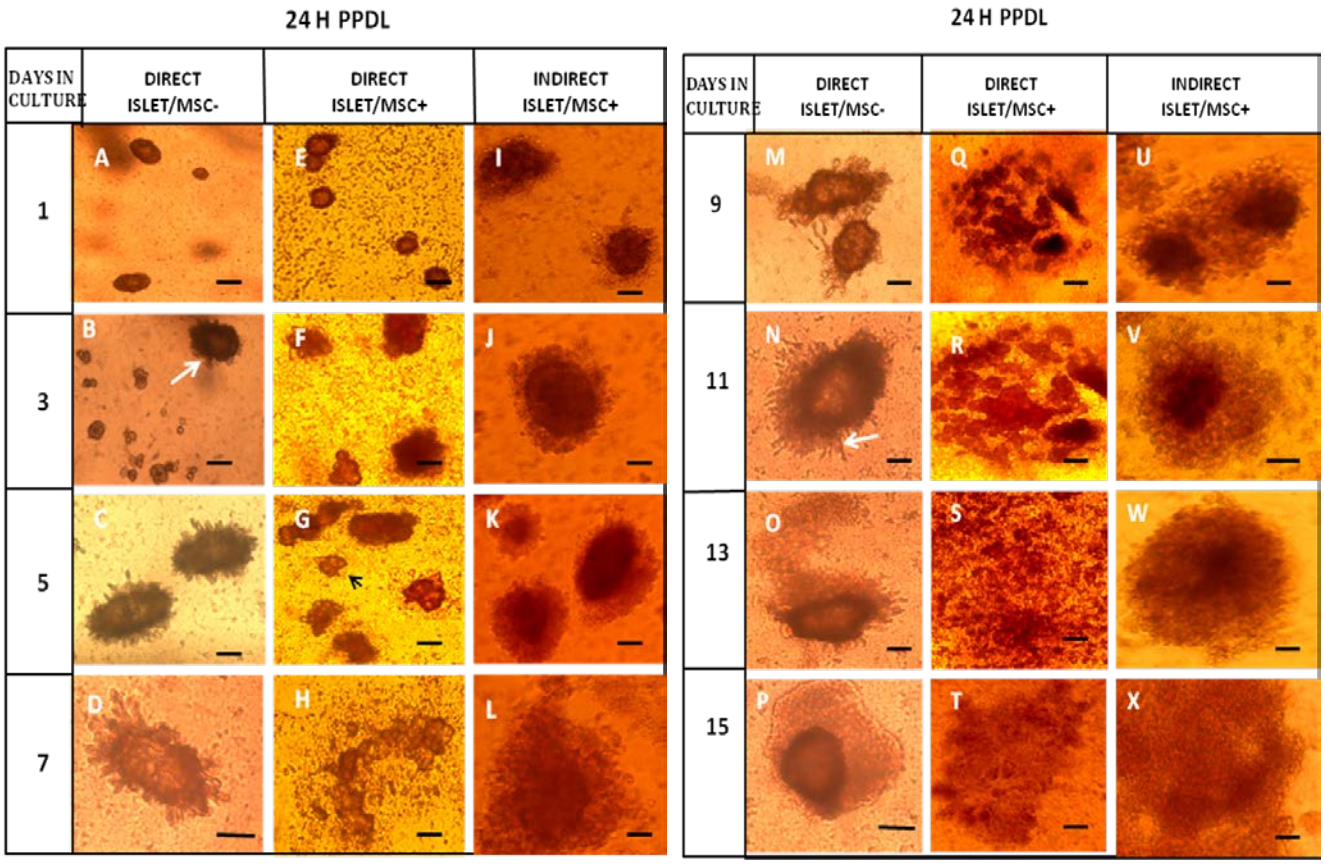


Figure 3.15. Development of islets cultured by indirect configuration. Islets and MSCs were isolated from rat pancreas 24 h post pancreatic duct ligation (PDL) and cultured with or without MSCs for different periods. A-X: Representative pictures of islets in culture medium at different days of culture by direct and indirect configuration. In Q-T, proliferation of islet cells progressively formed islet-like spheroid aggregates while in U-X islet cells continued to proliferate and migrated outward and floated in suspension or at liquid-air interface. Bar = 100µ

However, proliferating islets that grew at the liquid-air interface, later formed more islet-like cell aggregates that spread over culture medium while those that grew on the transmembrane failed to form individual cell aggregates by day 24 of indirect co-culture (Fig.3.16 T, Y), similar to the morphology of islets cultured alone directly (Fig.3.12 C, D). The study did not explore further to understand why there is such a difference in their mode of development due to shortage of antibodies to test this. Further investigations are needed to find the reasons for the difference.

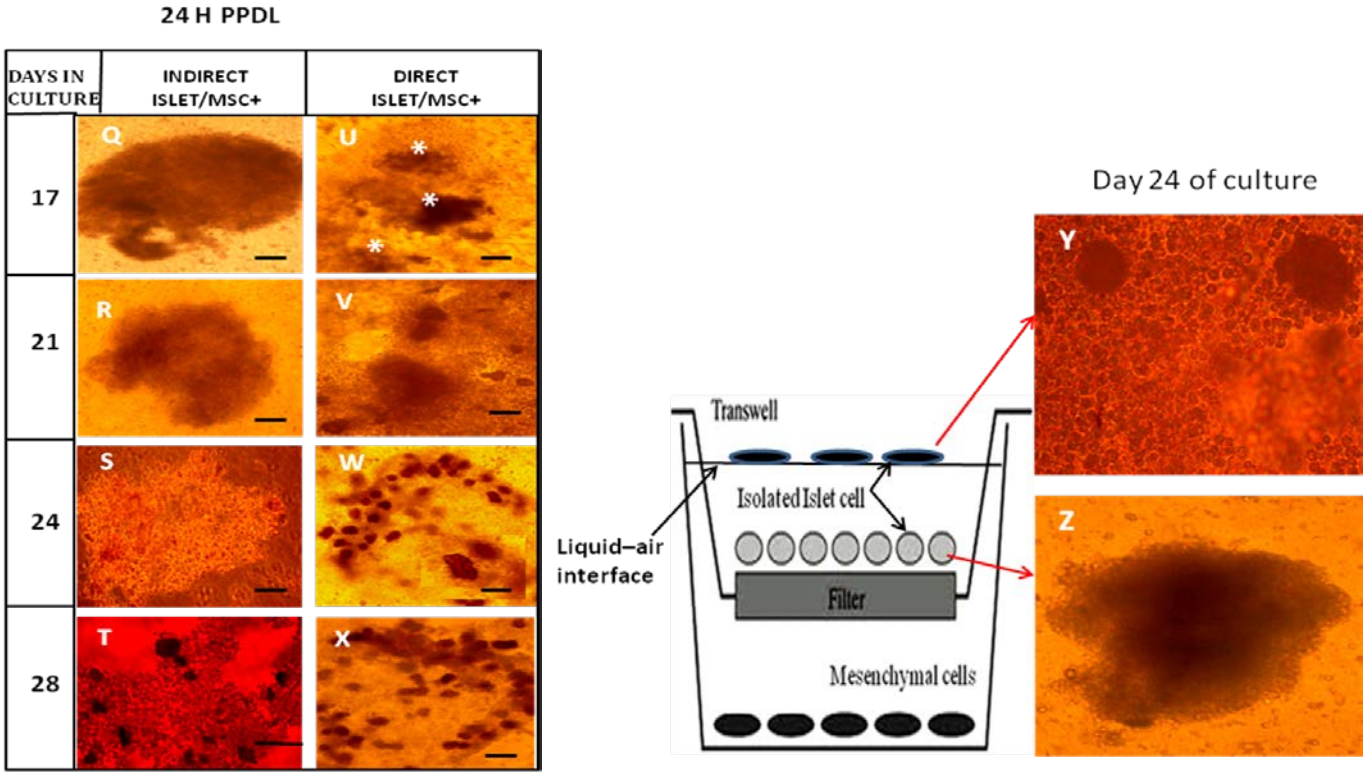


Figure 3.16. Development of islets cultured by indirect configuration. Q-X: Representative pictures of islets in culture medium at different days of culture by direct and indirect configuration. In T and Y, proliferating islet cells that floated at liquid-air interface progressively formed islet-like spheroid aggregates (dark spots) while in Z islet cells continued to proliferate and migrated outward and formed a large cell mass on a filter membrane. Bar = 100µm

3.1.3.8 Pancreatic duct ligation (PDL) has no effect on the developmental potential of islets in culture.

To ascertain whether PDL influences the developmental potential of islets *in vitro*, islets and MSCs were also isolated from the 120 h PPDL tail pancreata and cultured with or without MSCs by direct contact. The choice of post-PDL times of 24 and 120 hours was based on evidence from previous study (Tchokonte-Nana 2011) that defined the time points as landmarks in which similar pancreatic morphological changes and specific gene expressions were observed in the pancreas following pancreatic duct ligation.

Results were compared with cultures of islets with or without MSCs isolated from 24 h PPDL and SOC tail pancreata also cultured by direct contact. With 120 h PPDL samples, analysis of islets development in culture was carried out for only 2 weeks due to scarcity of tissue samples and time limitations.

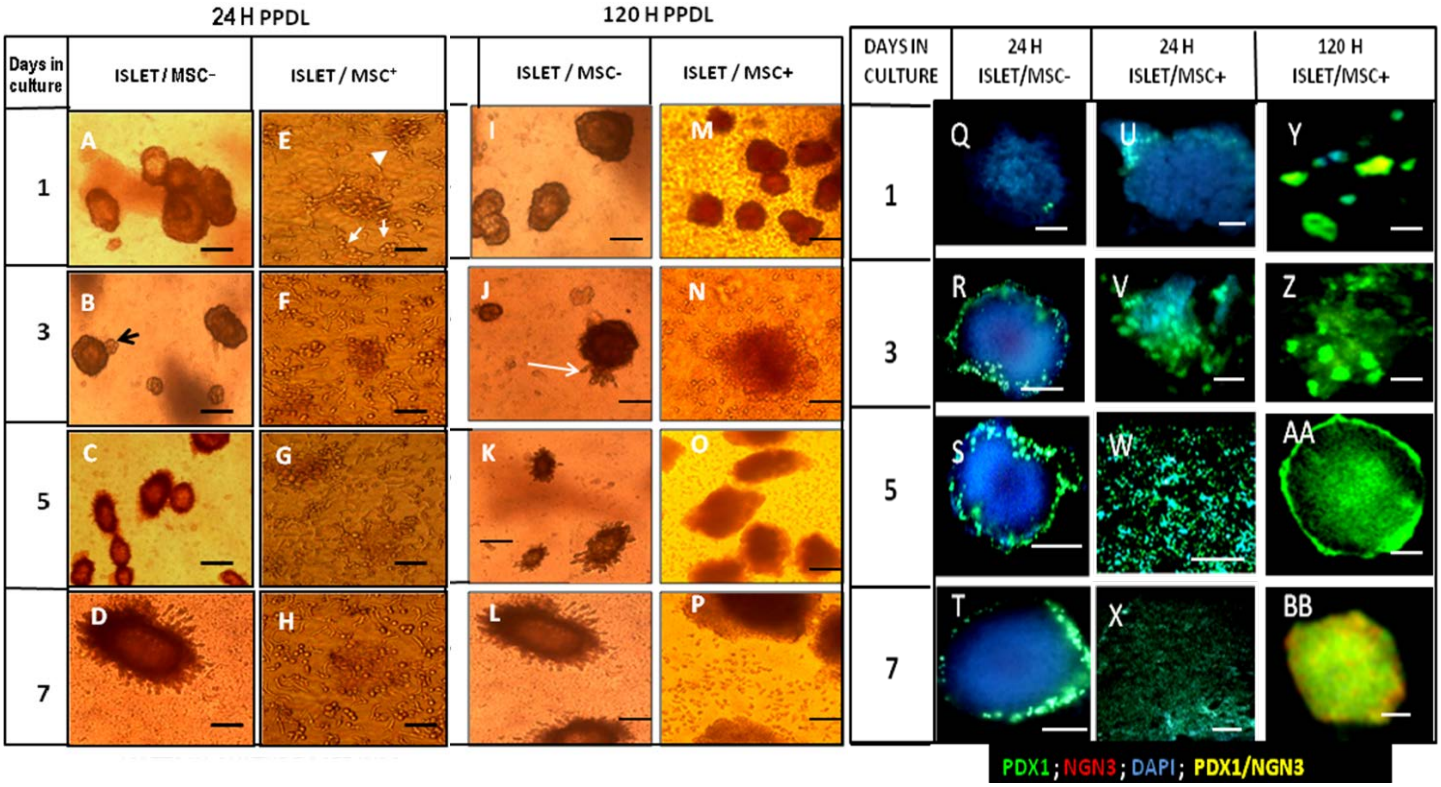


Figure 3.17. Co-expression of Pdx1 and Ngn3 during early development of islets cultured by direct contact with or without MSCs. Islets and mesenchymal cells were isolated from rat tail pancreas 24- and 120 h post pancreatic duct ligation (PDL) and cultured with or without MSCs for different periods. Immunocytochemistry analysis of whole mount islet cells cultured with (U-X) for 24- and (Y-BB) for 120 h PDL, or without (Q-T) MSCs for 24 h PPDL. Islet cells development was evaluated by anti-Pdx1 (green E) and anti-Ngn3 (red). PDX1/NGN3 (orange). Nuclei were counter-stained in blue with DAPI. Bar = 100µm.

3.1.3.9 Comparative development of 24 h- and 120 h PPDL islets in culture

Fig. 3.17 shows morphological and immunocytological analyses of islets isolated from 24- and 120 h PPDL pancreata and cultured with or without MSCs for one week. In cultures without

MSCs, similar morphological changes in islet development were observed between the 24- and 120 h PPDL islets, with peripheral islet cells progressively changing their round outline to elongated shapes as they proliferated and migrated away from the islets (Fig. 3.17 [A-D compare with I-L]). Similarly, in the presence of MSCs, more expansion of islets cells was observed compared to cultures of islets alone (Fig 3.17 A-D compare with E-H versus I-L compare with M-P]). When compared to SOC islets, a similar developmental pattern was observed during the first week of culture with islets expanding more in cultures with MSCs than without MSCs (Fig3.18 A-D compare with E-H versus I-L compare with M-P) in 24- and 120 h post SOC islets respectively.

Immunocytochemical analyses also showed an equivalent Pdx1 expression in cultures of both groups (24- and 120 h PPDL islets) with MSCs compared to cultures of islets only (Fig 3.17 Q-S compared to U-W and Y-AA) in the first 5 days. However, a co-expression of Pdx1 and Ngn3 was detected on day 1 in 120 h PPDL islets with MSCs, which disappeared by day 3, while in 24 h PPDL islets, Ngn3 was rarely detected (Fig. 3.17 U-V and Y-Z). By day 7, Pdx1 was down regulated while Ngn3 was highly activated and co-expressed with Pdx1 in 120 h PPDL islets with MSCs (Fig.3.17 BB). A similar but low level of Ngn3-Pdx1 co-expression was also observed in 24 h PPDL islets with MSCs (Fig.3.17 X) during the same time period.

When compared to SOC islet cultures, Pdx1 was highly expressed in both 24- and 120- SOC islets cultured with MSCs for the first three days (Fig. 3.19 A-B, E-F).

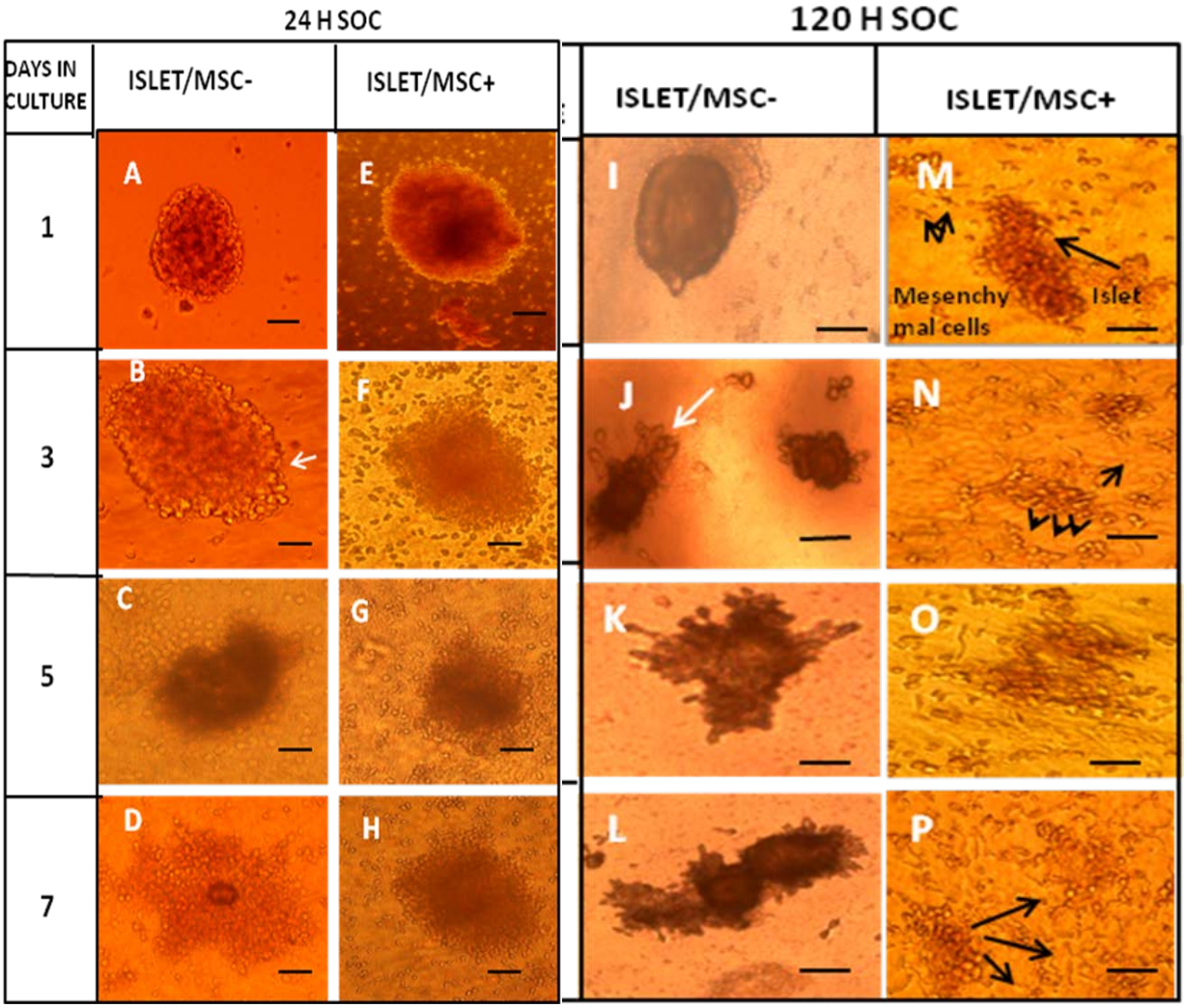


Figure 3.18 Development of islets isolated from sham operated rat pancreata 24- and 120 h post surgery. Islets and mesenchymal cells were isolated from Sham operated control (SOC) rat pancreata 24- and 120 h post operation and cultured with or without MSCs for different periods. Representative pictures of islets in culture medium. Scale bar = 100µm

From day 5 to 7 Pdx1 was down regulated followed by activation of Ngn3. Double positive cells for Pdx1 and Ngn3 were detected on day 5 in both SOC groups cultured with MSCs and by day 7, more Ngn3+ cells were recorded in 120 h SOC cultures than in 24 h SOC cultures (Fig. 3.19 G-H, K-L; S-T W-X). Ngn3 was not detected in islets cultured alone throughout the first week

and the first 3 days in cultures with mesenchyme (Fig. 3.19 M-P) despite SOC islets displaying similar morphological developmental pattern to 24- and 120 h PPDL islets.

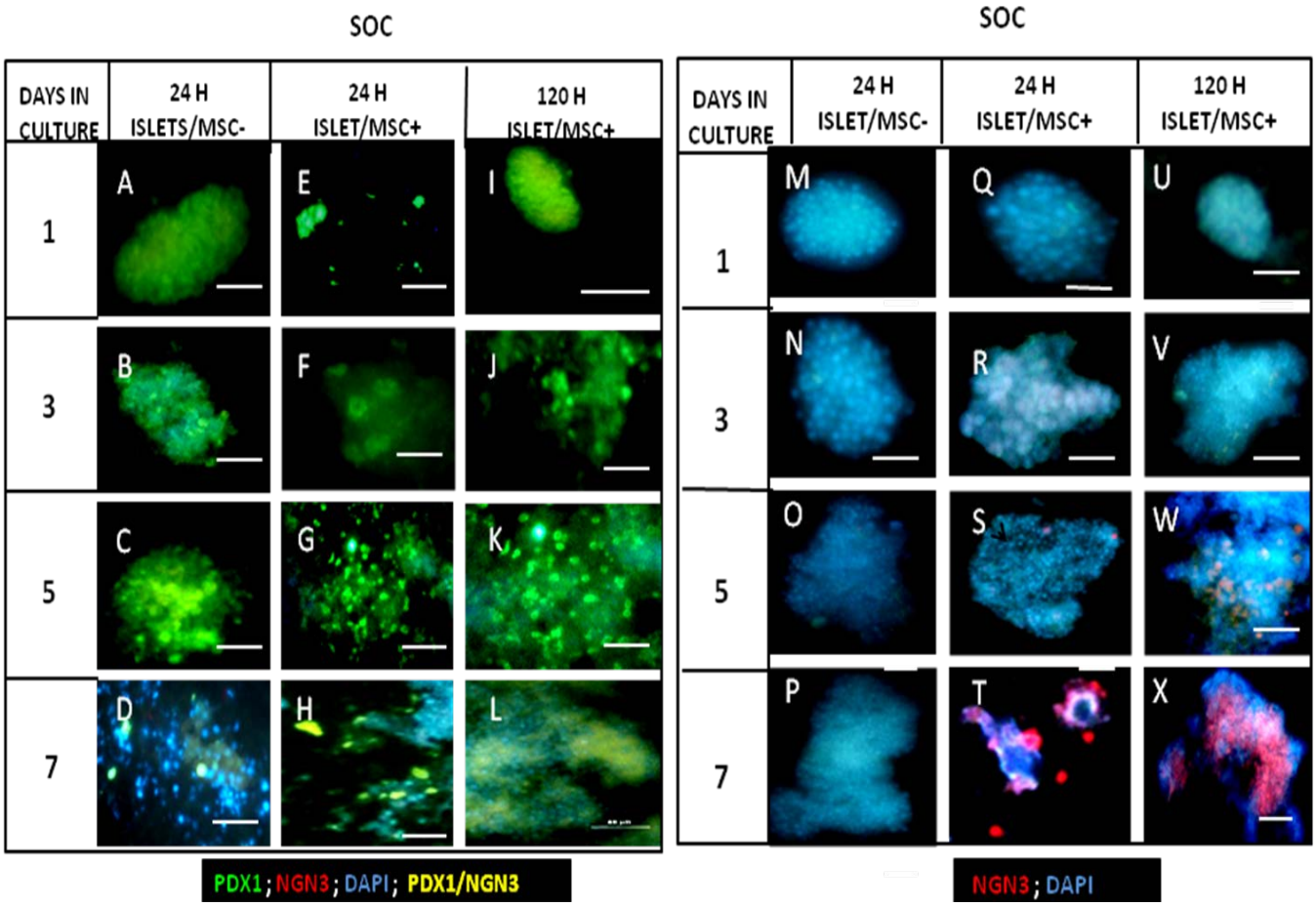


Figure 3.19 Expression of Pdx1 and Ngn3 during early development of SOC islets cultured by direct contact with or without MSCs. Islets and MSCs were isolated from Sham operated control (SOC) rat pancreata 24- and 120 h post operation and cultured with or without mesenchymal cells for different periods. Representative pictures of immunocytochemistry analysis of islets cells cultured without (A-D, M-P) or with (E-H, Q-T) for 24 h SOC; (I-L, U-X) for 120 h SOC for different days of culture. Islet cells development was evaluated by anti-Ngn3 (orange/red) and anti-Pdx1 (green). Nuclei were counter-stained in blue with DAPI. Scale bar = 100µm

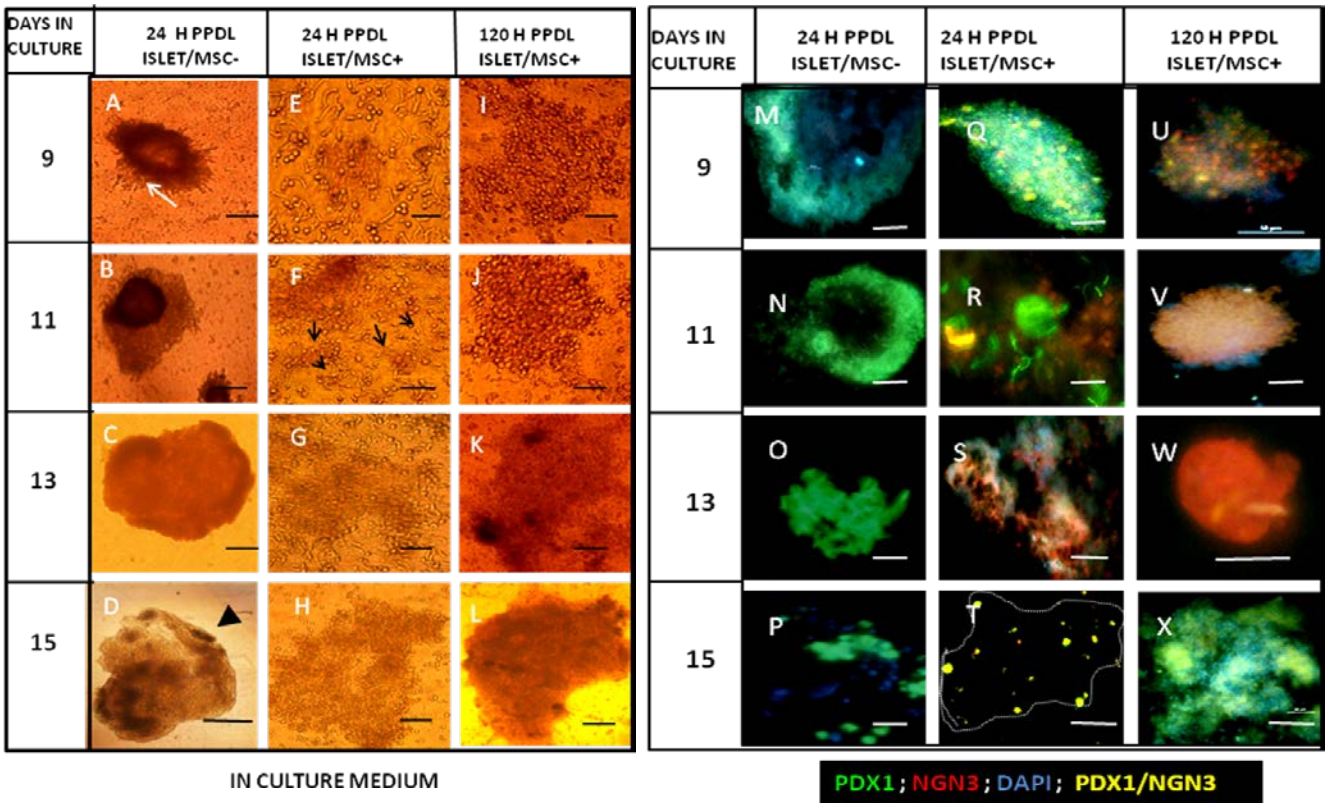


Figure 3.20. Co-expression of Pdx1 and Ngn3 during early development of islets cultured by direct contact with or without MSCs. Islets and MSCs were isolated from rat tail pancreas 24- and 120 h post pancreatic duct ligation (PDL) and cultured with or without MSCs for different periods. Immunocytochemistry analysis of whole mount islets cells cultured with (Q-T) for 24 h PPDL and U-X for 120 h PPDL, or without (M-P) for 24 h PPDL. Islet cells development was evaluated by anti-Pdx1 (green) and anti-Ngn3 (red). PDX1/NGN3 (orange). Nuclei were counter-stained in blue with DAPI. (n = 10 animals/group were analysed) Scale bar = 100µm

In the second week, both 24- and 120 h PPDL islets showed comparable expansion rates in cultures with MSCs (Fig. 3.20 E-H, I-L). A few islet-like cell aggregates (appeared as dark spots) were observed toward the end of second week in 120 h PPDL but not in 24 h PPDL cultures with MSCs (Fig. 3.20 G-H, K-L). Analysis of Ngn3 and Pdx1 in the second week showed that Pdx1 is downregulated in both groups cultured with MSCs, while Ngn3 was highly activated from day 9 up to day 13 of culture with more $Ngn3^{+}$cells in 120 h PPDL islet cultures

than in the 24 h group (Fig. 3.20 Q-S, U-W). After day 13, $Pdx1^+$ cells reappeared and co-expressed with Ngn3 which was getting downregulated by day 15 in both groups (Fig. 3.20 T, X). Comparable high levels of Pdx1-Ngn3 co-expression were detected in islet-like cell clusters that developed within the cultures of islets with MSCs in both 24- and 120 h PPDL islet groups.

To summarise, in our co-cultures of islets and MSCs, expansion of $Pdx1^+$ / $Ngn3^-$ islet cells was observed at early hours when co-cultured with or without MSCs; however, islets co-cultured with MSCs registered a higher number of $Pdx1^+$ cells. Pdx1 expression was down-regulated in these cells at 9 days of culture followed by an up regulation of Ngn3, while Ngn3 expression was maintained in islet cells up to 28 days, after which aggregations of numerous spheroid bodies resulted in islet-like structures.

3.2 Development of PPDL duct fragments in culture

In addition to investigating the effects of MSCs on pre-existing islet cells, it was also tested whether MSCs also stimulate duct epithelial cells to regress to a lesser differentiated phenotype and act as progenitors to form new insulin producing cells in culture. For this reason, assessement of the morphological changes and the expression of Pdx1, Ngn3 and insulin in cultured duct fragments were made.

3.2.1 Morphological changes of ducts in culture.

3.2.1.1 First and second weeks of ducts in culture

Figure 3.21 shows early development of ducts in culture with or without mesenchymal stromal cells (MCSs). As shown in Fig. 3.21 A, in the absence of MSCs, duct epithelial cells proliferated and sealed up the cut ends of the ducts within 3 days of culture (Fig. 3.21 A, B) with some

proliferating cells forming small evaginations (growth areas) on the sides and near the tip of small ducts on day 3 (Fig. 3.21 B).

During the same time period, ducts cultured in the presence of MSCs showed similar morphological changes by sealing the cut ends and developing epithelial evaginations as growth areas (Fig 3.21 D, E). By day 5 of culture, many ducts made point attachments to plastic substrate while the rest of the segments were suspended into the medium in both culture conditions, only few smaller fragments were still lying attached to the bottom of the dish.

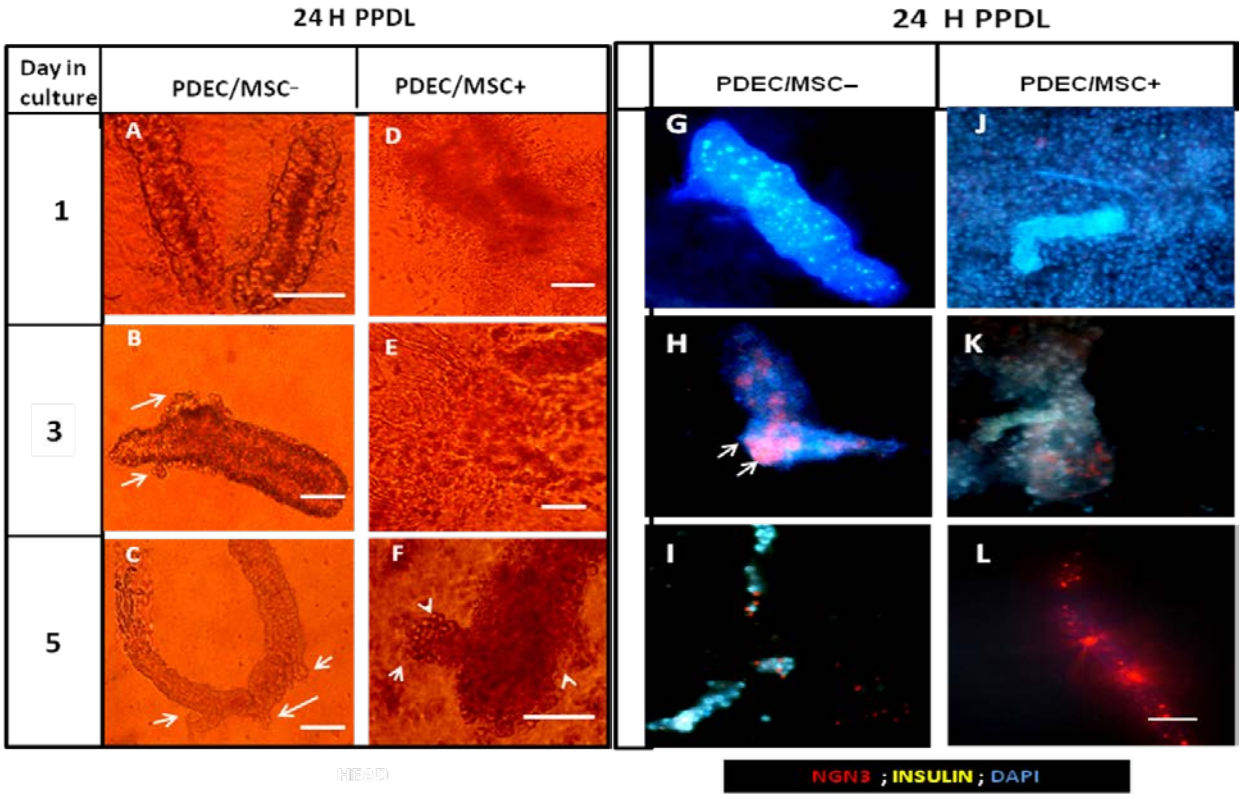


Figure 3.21. Early development of ducts in culture with or without MSCs for different periods. A-F: Representative pictures after 1 (A and D), 3 (B and E), and 5 (C and F) days of culture. In C, terminal duct cells (centroacinar cells) proliferate at the tip duct epithelium. G-L: Immunocytochemistry analysis of duct cells cultured without(G-I) or with (J-L) MSCs for 1 (G and J), 3 (H and K) and 5 (I and L) days of culture. Duct fragment development was evaluated by anti-insulin (yellow) and anti-Ngn3 (orange/red). Nuclei were counter-stained in blue with DAPI. *Arrows i*ndicate growth areas. MSC± = Mesenchymal stromal cells; PDEC = Pancreatic duct epithelial cells, (n = 10 animals/group were analysed), Bar = 100µm

At this time, however, a few noticeable differences in morphology of the ducts were observed between culture conditions. In ducts cultured alone, their cells continued to proliferate and formed more evaginations (growth areas) resulting into ducts becoming thicker and longer (Fig. 3.21 C). Few cuboidal periductal cell outgrowths were observed on the side of the ducts (Fig 3.21 C, arrows) and few squamous, possibly centroacinar cells were seen at the tip (Fig.3.21 C), while in ducts cultured with MSCs, evaginations became more prominent and a few periductal cells began to migrate into suspension (Fig. 3.21 E, F).

3.2.1.2 Expression of Ngn3 in cultured duct cells

Previous *in vitro* studies indicated that MSCs might control the differentiation of epithelial cells in embryonic pancreas (Gittes et al. 1996), For this reason, in both cultures, immunocytochemistry analysis was carried out for the expression of Ngn3, a transcription factor that marks endocrine precursor cells in proliferating duct cells (Gu, Dubauskaite & Melton 2002) as stated in sections 3.1.3.2. As shown in Fig. 3.21 (H, K), nuclear Ngn3+ cells were first detected in duct epithelial cells on day 3 of culture both, with or without MSCs. At this time, more Ngn3 + cells were detected in tips and other zones of cell proliferation (growth areas) in both culture conditions (Fig 3.21 H, K). In cultures without MSCs, however, Ngn3 expression was also detected in scattered cells around ducts on day 5 (Fig 3.21 I) and this expression continued up to day 7, when few cells co-expressing Ngn3 and insulin began to be detected (Fig 3.24 M).

In ducts cultured with MSCs, Ngn3 was highly expressed within the epithelium cells of ductal buds on day 5 (Fig 3.21 L). Ngn3+ cells were clustered in growth areas that were distributed

throughout the duct epithelium, typical for this developmental stage (Fig.3.21 L). Ngn3+ cells were later scattered around ducts on day 7 (Fig.3.24 P). From day 9, Ngn3+ cells were continuously detected in some cell clusters as well as in scattered individual cells up to day 12, with some Ngn3+cells often co-expressing insulin (Fig 3.24 Q, R).

3.2.1.3 Co-expression of Ngn3 with CK7

To find out whether these Ngn3+ cells arise from duct epithelium, a series of double labeling reactions were conducted. Double immunolabeling reactions revealing cytokeratin 7 (CK 7) and Ngn3 on whole mounts of duct fragments of 1- to 5-day old cultures showed that in cultures without MSCs, the ductal epithelium is largely composed of $CK7^{+}$ cells with some few cells located within the ductal epithelium and periductal areas co-expressing CK7 and Ngn3 (Fig. 3.22 D, F). In cultures with MSCs, most epithelial cells in growth areas displayed double immunoreactivity of CK7 and Ngn3 from day 1 and by day 5 more periductal cells in the growth regions of the ducts co-expressed CK7 and the nuclear Ngn3 proteins (Fig. 3.22 J, K and L), providing evidence of the epithelial nature of Ngn3+ cells. At this stage of duct development, we did not detect $CK^{-}/Ngn3^{+}$ cells in duct epithelial or periductal growth areas in most of the slides examined.

In the second week of culture, ducts showed more morphological changes. In cultures without MSCs, more squamous cells proliferated at the tip (Fig. 3.24 G), which later adopted cuboidal shapes by day 9 (Fig. 3.24 H). After day 9, few islet-like cell clusters were formed by periductal cells (Fig. 3.24 H, I). In contrast, with MSCs, more clusters and cell migrations were observed on day 7. These clusters grew and formed islet-like structures within the periductal area (Fig. 3.24 J, K, L). By the end of second week, ducts developed more branches in both culture conditions

(Fig. 3.25), however, in the presence of MSCs, epithelial lining became thicker and more branches developed (Fig. 3.25 O, P)

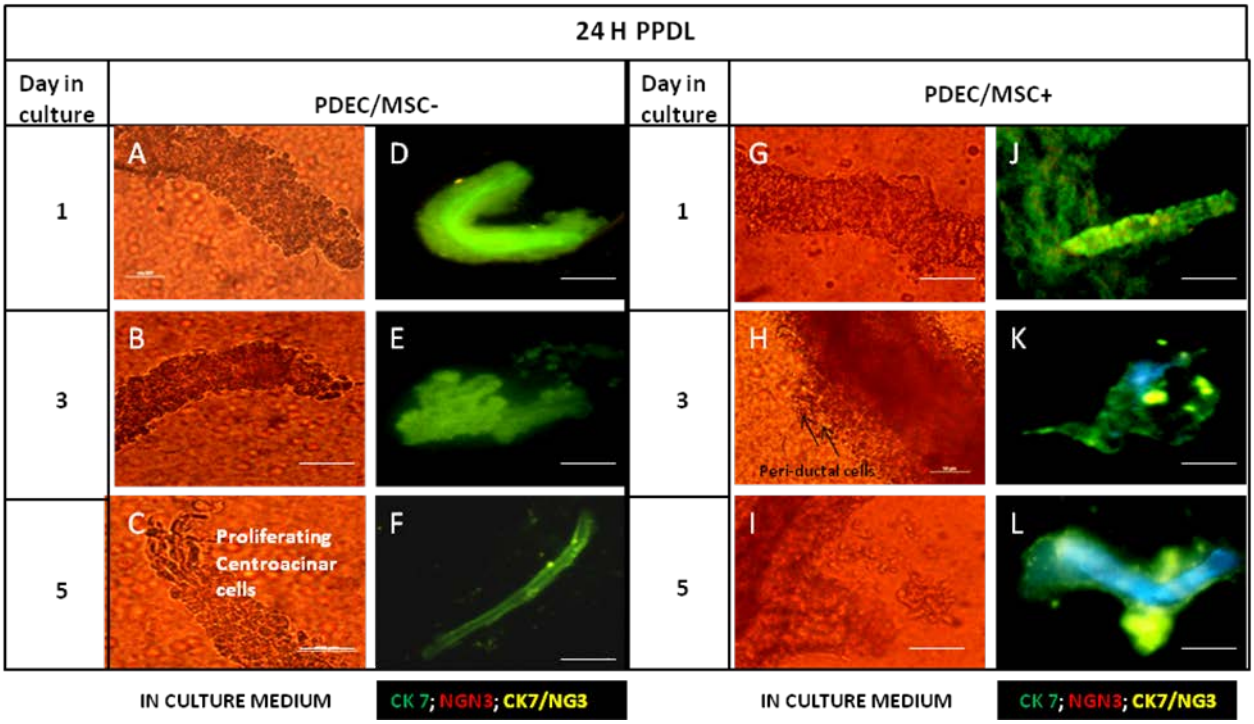


Figure 3.22. Morphological and immunocytochemical characterization of cells of duct fragments cultured without (A-F) or with (G-L) MSCs at different time periods. Immunofluorescent reactions showing the distribution of cytokeratin 7^+ ($CK7^+$) (green), $Ngn3^+$ (red) and double positive CK7+/Ngn3+ (yellow to orange) cells in duct fragments. A-C and G-I: Representative pictures of ducts in culture medium. Bar = 100 µm. MSC± = ± Mesenchymal stromal cells; PDEC = Pancreatic duct epithelial cells, **(n = 10 animals/group were analysed)**.

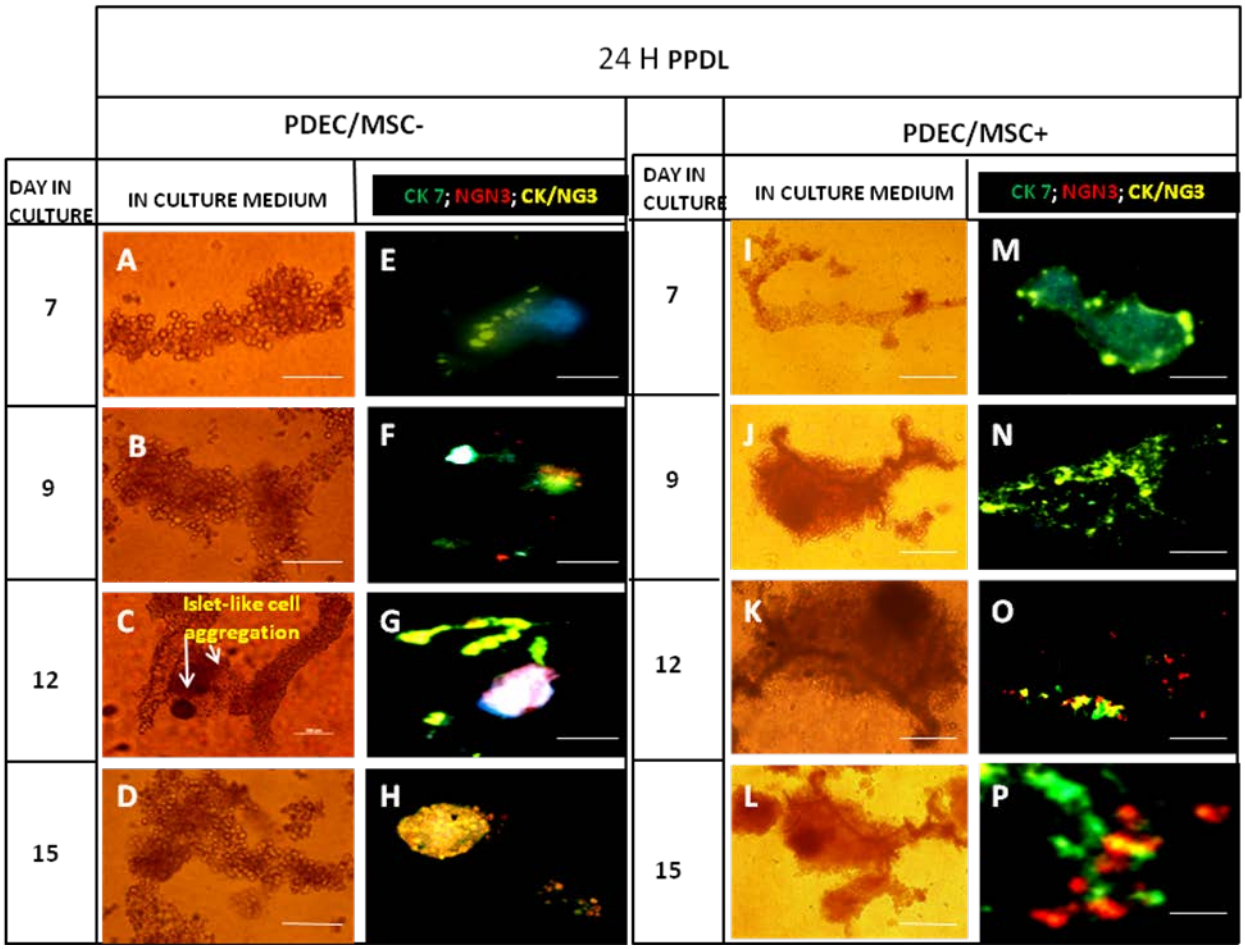


Figure 3.23. Further morphological and immunocytochemical characterization of cells of duct fragments cultured without (A-H) or with (I-P) MSCs at different time periods. Immunofluorescent reactions showing the distribution of cytokeratin 7^+ ($CK7^+$) (green), $Ngn3^+$ (red) and double positive CK7+/Ngn3+ (yellow to orange) cells in duct fragments. A-D and I-L: Representative pictures of ducts in culture medium. (n = 10 animals/group were analysed), Bar = 100 µm; MSC± = ± Mesenchymal stromal cells; PDEC = Pancreatic duct epithelial cells, **(n = 10 animals/group were analysed)**.

Double reactions carried out on whole mount duct fragments in the second week, revealed the continued presence of the double immuno-reactive cells co-expressing CK7/Ngn3 located within the ductal epithelia as well as in growth areas up to day 15 in cultures without MSCs and day 12 in cultures with MSCs (Fig. 3.23 E-H, and M-O, respectively). In cultures with MSCs, single

immuno-reactive cells for Ngn3 and CK7 were respectively present in scattered and ductal epithelial cells on day 15 (Fig. 3.23 P)

3.2.1.4 Expression of insulin in cultured duct epithelial cells

Since Ngn3 was continuously detected in duct epithelial cells in the second week of culture with MSCs, double labeling reactions revealing the expression of insulin and Ngn3 were next carried out to further assess the differentiation potential of Ngn3+ cells within the duct epithelium as well as in cell aggregates located close to ducts.

In ducts cultured alone, insulin expression was first detected in cell aggregates on day 7, with a number of insulin+ cells that transiently co-expressed Ngn3 up to day 9 (Fig.3.24 G, H). However, after day 9, double-positive cells for Ngn3 and insulin were not detected while higher numbers of single-positive insulin expressing cells were detected in cell aggregates cultured alone than those cultured with MSCs up to day 12 (Fig 3.24 [I compare with L**]**), possibly reflecting an early differentiation of Ngn3 expressing cells into insulin-producing cells.

In ducts cultured with MSCs, insulin expression similarly began to be detected from day 9 but its expression was lower compared to ducts-derived cell aggregates cultured alone. In addition, in cultures with MSCs, double-positive cells for insulin and Ngn3 were continuously detected up to day 12 of culture (Fig. 3.24 K, L), a result that could possibly suggest a delayed stage of differentiation into endocrine cells.

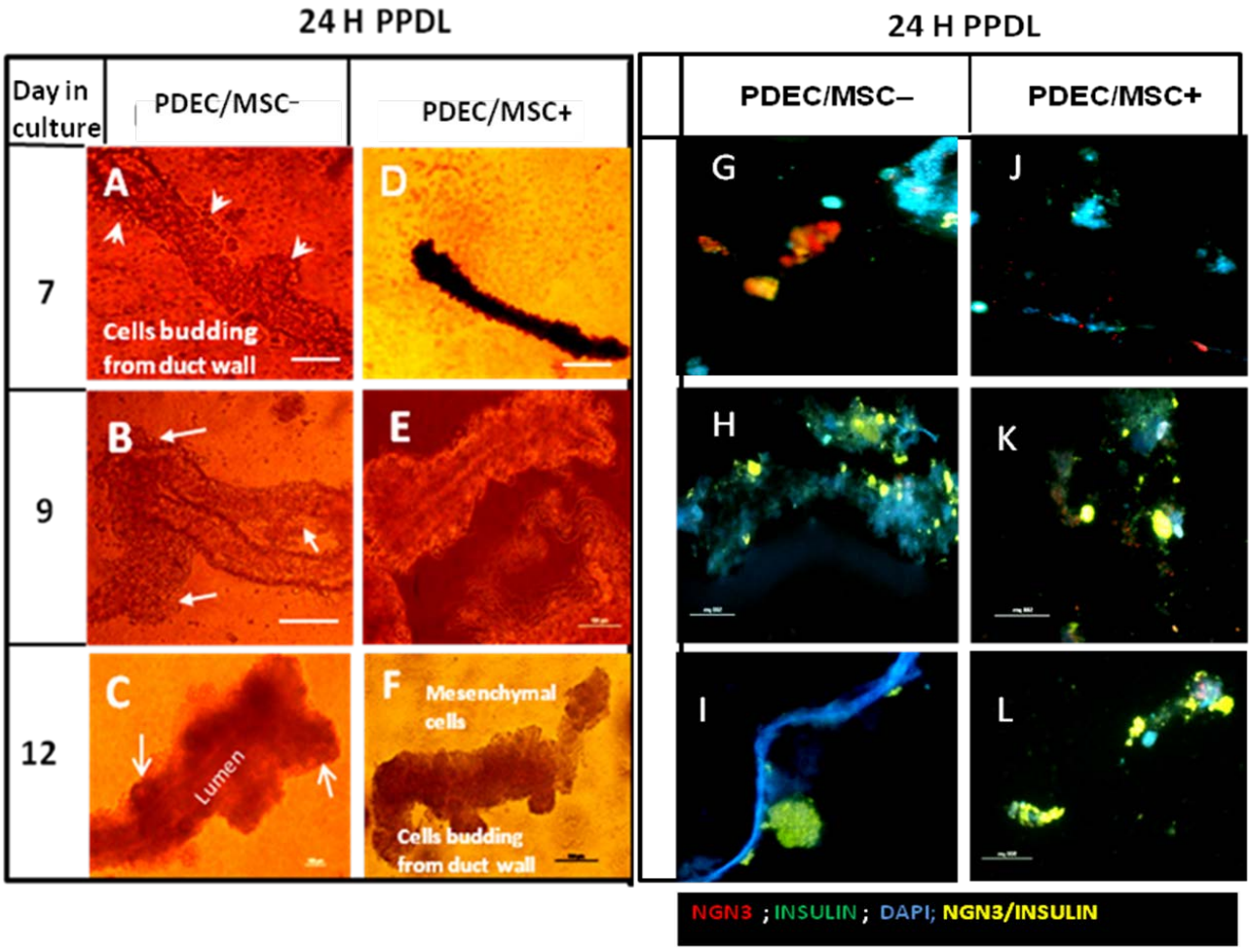


Figure 3.24. Later development and immunocytochemical characterization of pancreatic epithelial duct cells (PEDC) in culture with or without MSCs for different periods. A-F: Representative pictures in culture medium after 7 (A and D), 9 (B and E), and 12 (C and F) days of culture. In C: Cell clusters are formed close to ducts (Arrows). E,F: Cell clusters are formed close to and tip of ducts (Arrows) . G-L**:** Immunocytochemistry analysis of duct cells cultured without (G-I) or with (J-L) MScs for 7 (G and J), 9 (H and K) and 12 (I and L) days of culture. Duct fragments development was evaluated by anti-insulin (green/yellow) and anti-Ngn3 (orange/red). Nuclei were counter-stained in blue with DAPI. Bar = 100µm; MSC± = ± Mesenchymal stromal cells, L = lumen; PDEC = Pancreatic duct epithelial cells; **(n = 10 animals/group were analysed)**.

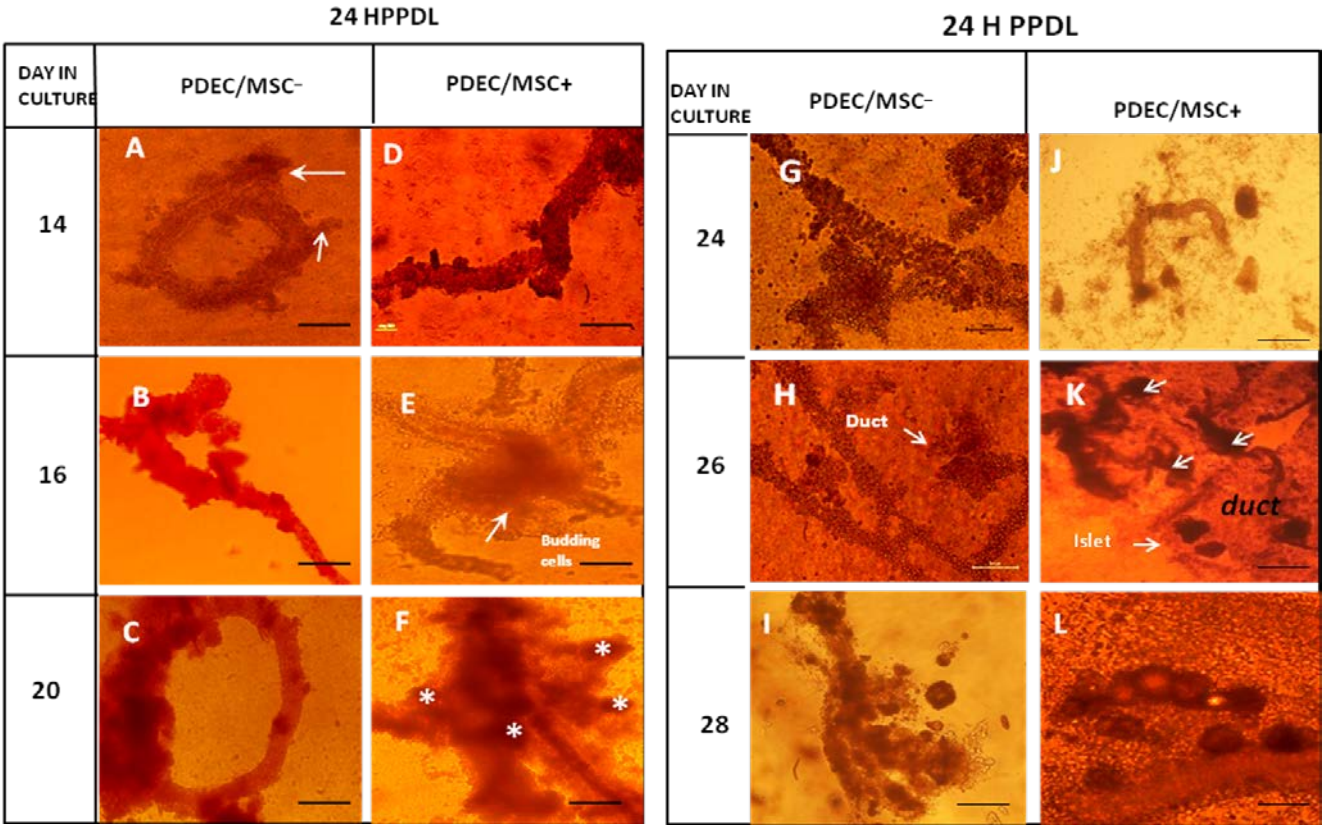


Figure 3.25. Later development of ducts in culture with or without MSCs cells for different periods. A-L Representative pictures taken in culture medium after 14 (A and D) and 16 (B and E) and 20 (C and F) days of culture. In I and L, more cell clusters are visible around ducts (asterisks). Bar = 100µm; MSC± = ± Mesenchymal stromal cells; PDEC = Pancreatic duct epithelial cells; **(n = 10 animals/group were analysed).**

3.2.1.5 Third and fourth weeks of ducts in culture.

In the third week of culture, periductal budding were more visible in cultures without MSCs (Fig. 3.25 A, B, C) while in cultures with MSCs, ducts became highly branched up to day 20 and 22 (Fig .3.25 D-F), with proliferating periductal cells migrating to form a sheet of cells on the sides of ducts that were later aggregating into cells clusters (Fig. 3.25 E,F). This early phase of development ended up with ducts becoming highly branched and interspaced between sheets of proliferating cells.

Observations of later stages in the fourth week showed increase in branching of ducts in both conditions (Fig. 3.25). Within the sheets of migrating cells, aggregations of cells began to form between branches of ducts in both culture conditions (Fig. 3.25). These clusters of cells were more visible on days 24 and 26 in both cultures with or without MSCs (Fig. 3.25). However, cell clusters formed more spheroid bodies closer to ducts in cultures with MSCs than around ducts cultured alone (Fig. 3.25 [compare G & J, and also H & K]) although this was not verified by quantitative data. These cell clusters were morphologically more similar to endocrine islets than exocrine acini, suggesting that these proliferating cells possibly continued to differentiate into endocrine cells. With continued culturing, our morphological data showed spheroid bodies that were attached to ducts and later began to bud off from duct structures (Fig 3.25 I, L).

To assess the fate of Ngn3+ cells, immunocytochemical analysis of later stages of duct development showed that de-differentiated duct Ngn3+ cells could further differentiate into insulin producing cells in both culture conditions. However, in the presence of MSCs, Ngn3 expression was maintained up to two weeks in periductal cells and scattered cells resulting into fewer Ngn3+ cells proceeding to differentiate into insulin producing cells.

3.3 Efficacy of PPDL islets as donor tissue for treatment of STZ-induced diabetic rats

In addition to investigating the effects of MSCs on pre-existing islet and duct epithelial cells, the study also explored whether MSCs can influence the transplantation outcome of islets isolated early PPDL surgery. Streptozotocin-induced diabetic Wistar rats were syngeneically transplanted with islets harvested either 24- or 84 hours (h) post ligation. In a second group, 24 h PPDL islets were syngeneically transplanted either alone or with PPDL pancreas–derived MSCs underneath

the renal capsule. Blood glucose levels were monitored and graft morphology was evaluated by immunohistochemistry. Efficacy of these cells was analysed for a period of 67 days.

3.3.1 Blood glucose levels (BGLs) trends

To assess the efficacy of PPDL-pancreas as a donor tissue in diabetic animals, three groups of rats with fasting blood glucose >11 mMol/L were used as transplant recipients of PPDL islets from syngeneic donors as shown in Fig. 3.26. The results showed that transplantation of normal islets under the renal capsule produced superior glycemic controls compared to those that received 24 h and 84 h PPDL islets (p = 0.0362), as shown in Fig.3.26. The 24 h PPDL islet transplants group also showed more superior outcome than those implanted with 84 h PPDL islets. The mean concentrations of blood glucose of rats with normal islets isografts were significantly lower than that of rats that received islets from both 24- and 84 h PPDL donor groups, at the third and seventh weeks post transplantation (p = 0.0362) (Fig. 3.26). Between fourth and fifth weeks post transplantation, both 24 h PPDL and normal islet grafts showed recovery from diabetes with no significant difference in glycemic levels observed between recipients of normal islets and 24 h PPDL islets (p = 0.537). However, there was a significant difference in glycemic levels between 24- and 84 h PPDL islet implant groups within the same time frame (p = 0.0015).

Considering high graft failure in 24 h PPDL syngeneic islet group between 6 weeks and 9 weeks post transplantation (Fig. 3.26), another experiment evaluated the effect of MSCs on graft survival and function. As shown in Fig. 3.26, the results showed striking early increase in islet function in recipients of islets only compared to recipients of Islet/MSCs co-transplants and this trend continued up to 5 weeks. After 6 weeks, however, diabetes recurrence was observed in

animals transplanted with islets only, whereas in animals that received co-transplants, islet function gradually increased.

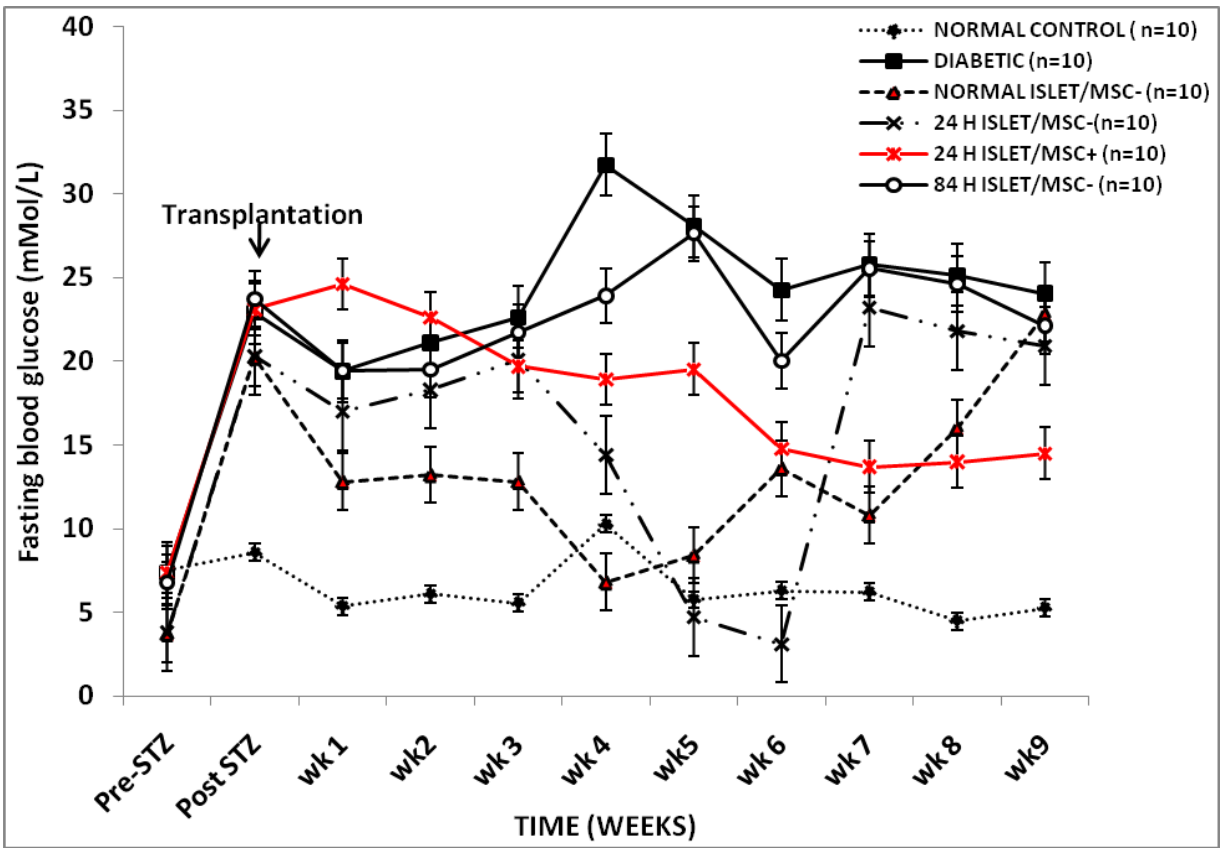


Figure 3.26. Transplantation of early PPDL islets into STZ-induced diabetic rats. Graph shows different patterns of blood glucose levels in various groups of rats , healthy controls (black diamond), STZ –induced diabetic untreated rats (Diabetic-black rectangle), treated with normal islets without MSCs (red triangle), treated with 24 h PPDL islets without MSCs (dotted line with cross), treated with 24 h PPDL islets with MSCs (red line with cross) and treated with 84 h PPDL islets without MSCs (circle) during study time period. Data prresented as means ± SE, $p < 0.05$ ($p = 0.0362$), (n = 10 animals/group)

When analysis of fasting blood glucose as a function of time was performed at the time of diabetes recurrence (5 weeks post transplantation) within each group, a remarkable trend towards an increase in islet function was observed in animals that received islet/MSCs (Fig. 3.26). Mean

values of blood glucose levels for animal recipients that received islets only increased drastically within the same time frame indicating a decrease in islet function in absence of MSCs (Fig. 3.26)

3.3.2 Survival and function of islet grafts

In the 24 h PPDL transplant group that received islets only (Islet/MSC-), 5/10 or (50%) reached average fasting blood glucose of 11.7 mMol/L by 3 weeks compared to 1/10 or (10%) rats that received islets only isolated from syngeneic donors 84 h post ligation (Fig. 3.26 and Fig. 3.27A). By 5 weeks 8/10 or (80%) of the 24 h PPDL transplant group had an average glycemic level of 10.1 mMol/L compared to 2/10 (20%) of the 84 h PPDL recipients. Glycemic levels were monitored for further 7 weeks at which time diabetes recurred in both groups with 8/10 (80%) of 84 h group and 6/10 (60%) of the 24 h group sustaining hyperglycemia after week 9 (Fig 3.26 and Fig. 3.27A). Marginal differences in glycemic levels were observed between groups but not statistically significant (P = 0.287). The curative capacity of 24 h post islets was better than 84 h PPDL islets. The average time to reverse hyperglycemia in 80% of the 24 h PPDL transplant group was 32± 2 days (~4.5 weeks,) whereas only 2 rats (20%) in the 84 h PPDL transplant group attained normoglycemia at 61 ± 2 days (~9 weeks) (p = 0.0011, n = 6) post transplantation (Fig. 3.27 A).

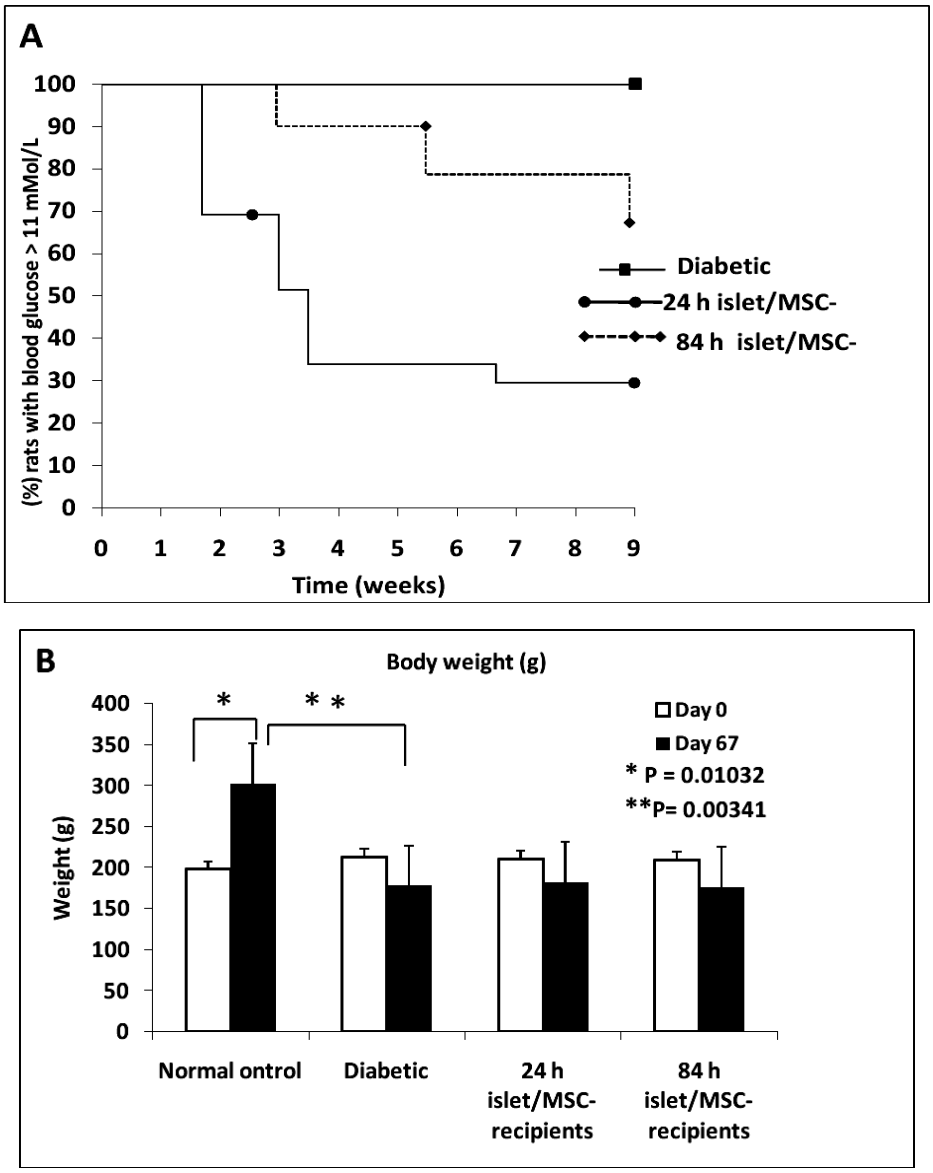


Figure 3.27. Graft survival of syngeneic islets transplanted into STZ induced diabetic rats. Panel A: Graph shows number of rats (%) remaining hyperglycemic (BGL > 11 mMol/L) after STZ (untreated diabetic- [black rectangle]), transplantation with 24- (black circle) and 84 h (black diamond) PPDL islets. Panel B: Graph shows mean body weights of healthy control rats, diabeteic rats treated with 24 h PPDL islets without mesenchyme (Islet/MSC-) and 84 h PPDL islets (Islet/MSC-) groups during 10 week study period (Day 67, black bar) from time of islet transplantation (Day 0, clear bar). Islet/MSC- = recipients of islets without mesenchyme isolated 24- or 84 h post ligation. STZ = Streptozotocin; Data are means ± SE. $^{**}p < 0.05$, t-test; (n = 10 animals/group were analysed).

Next, the proliferative potential of the grafts in both recipients with 24- and 84 h PPDL islets was explored using Ki67 immuno-labeling at 7 weeks post syngeneic transplantation. Marginal but statistically different numbers of Ki67+ cells in the islets were observed between the two groups (351 of 2,562 [13.7%] versus 345 of 1,990 (17.3%]) for the 24 h post-PDL islets vs. 84 h PPDL islet recipient groups respectively, $\rho = 0.0421$ (Fig. 3.29). In addition, no significant differences in weights of rats was observed in either transplant group on day 0 (210 ± 1.0 and 209 ± 0.8 g for 24 h PPDL and 84 h PPDL recipients, respectively, n = 8, p = 0.435) or on day 67 after transplantation (181.4 ± 0.3 and 175.9 ± 0.8, p = 0.71, n=8) (Fig. 3.27 B). Accordingly, immunohistochemical analysis of the pancreas of the isograft recipients showed very few cells that stained positive for insulin indicating no signs of regeneration of beta cells (Fig. 3.30 D). Together, these results therefore indicate an early but transiently longer curative capacity by syngeneic 24 h PPDL islet grafts which lasted up to 5 weeks compared to 84 h PPDL islet grafts that lasted for only two weeks.

3.3.3 Islet graft histomorphological study

Decrease in graft function and recurrence of diabetes in both the 24- and 84 h PPDL recipient groups was observed at 7 weeks and thereafter post transplantation (Fig. 3.26), a result that prompted to histologically examine their grafts. Microscopic examination of hematoxylin and eosin graft sections obtained from 2 animals from each group, 7 weeks post transplantation, showed a strong lymphocyte mononuclear infiltration on the site of transplantation in both 24- and 84 h PPDL grafts as well as in control group transplanted with normal islets (Fig. 3.28 F, G, H, and I). Despite lymphocyte mononuclear filtration in both groups (24- and 84 h PPDL grafts), islet structure was maintained in normal islet control and among 24 h PPDL islets transplanted

groups (Fig.3.28 J, K), whereas in recipients of 84 h PPDL islets, the grafts were mostly destroyed after 7 weeks (Fig. 3.28 I, M).

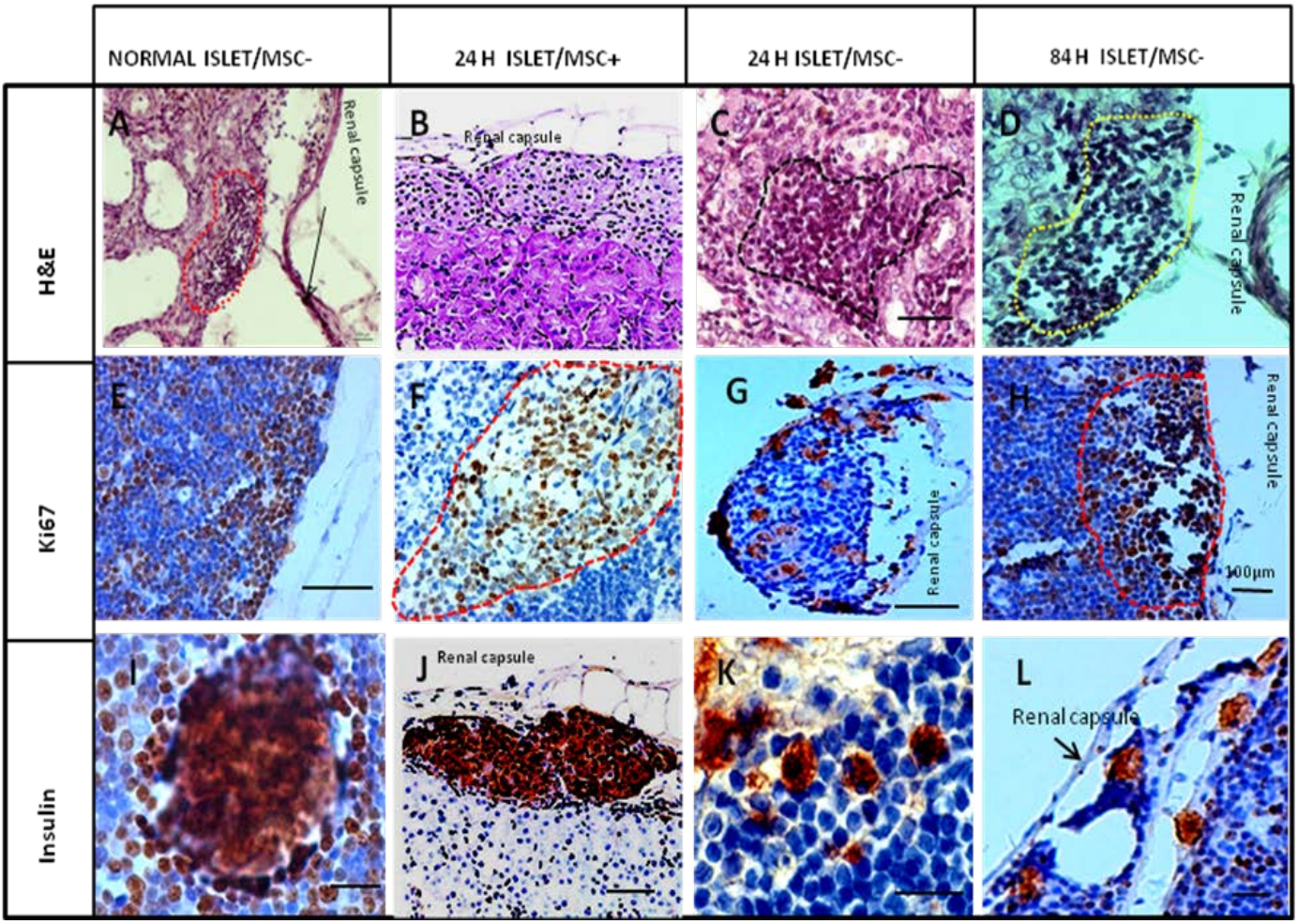


Figure 3.28. Histomorphometric analysis of grafts in recipients of 24- and 84 h PPDL islets at 7 weeks post transplantation. A-D: Hematoxylin and Eosin staining of islet grafts in healthy male rats (A) and STZ-induced diabetic rats treated with 24 h PPDL islets/MSC+ (B), Islets/MSC- (C) and with 84 h PPDL islets/MSC- (D) at 5 weeks post transplantation. Panels E-H: Ki67 staining (brown) in healthy male (E), 24 h PPDL silets/MSC+ (F) islets/MSC- (G) and 84 h PPDL islets/MSC- (H). Panels I-L insulin staining in the same four groups. Bar = 100 µm.

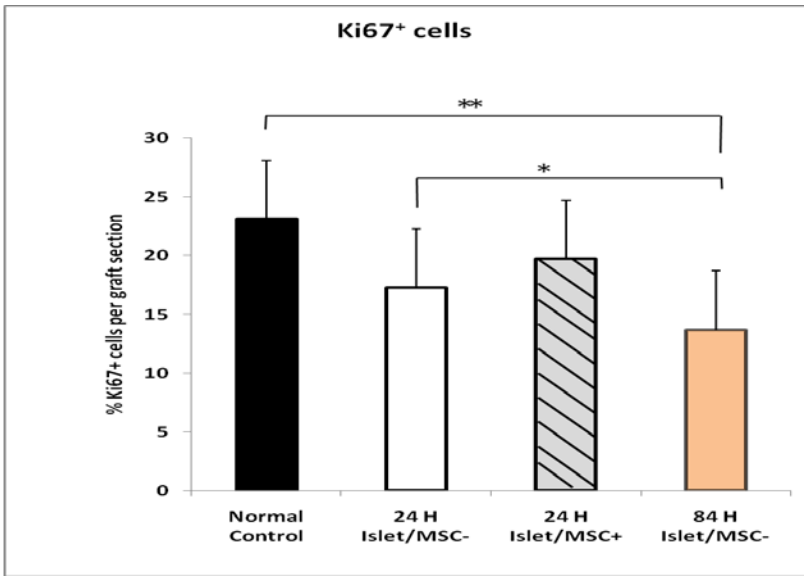


Figure 3.29. Cell proliferation analysis of grafts in recipients of 24- and 84 h PPDL islets at 7 weeks post transplantation. Graph shows percentage of Ki67-positive proliferating cells per graft section. * P = 0.3226, **P= 0.04207 (n = 10 animals/group were analysed).

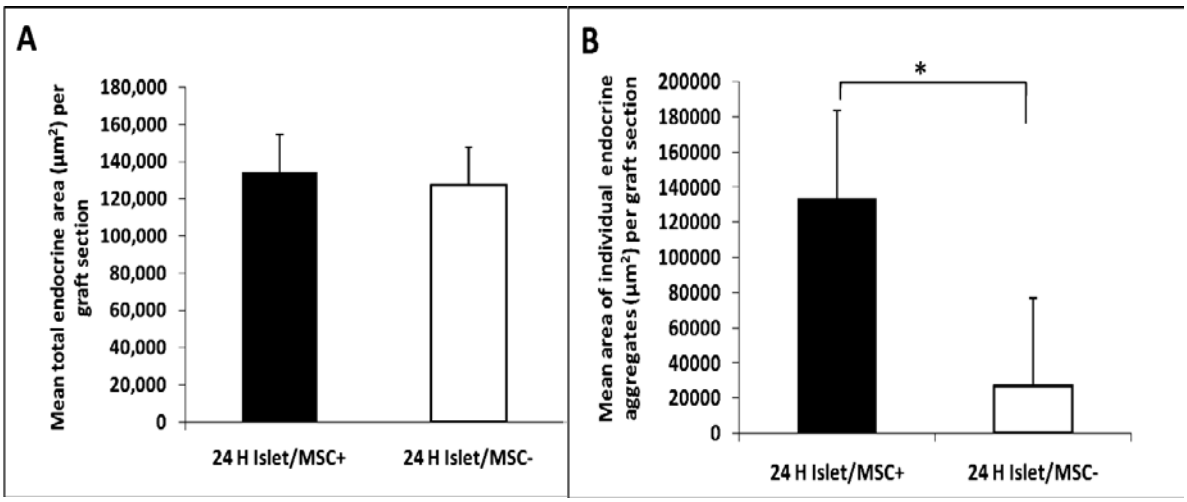


Figure 3.30. Endocrine cell density in isografts. Quantification of mean total endocrine area stained (A) in graft sections (n= 10 animals per transplant group, P = 0.471, student's t-test) and mean individual endocrine aggregate area (B) in graft sections, n= 10 animals per transplant group, *P= 0.0321, student's t-test.

At 9 weeks post transplantation, kidneys with isografts were harvested from recipients of grafts with and without MSCs and immunohistochemically analysed. It was possible to identify the implanted grafts in both groups but they appeared morphologically different as shown in Fig. 3.28 A - D. Isografts of recipients that received 24 h islets and MSCs (Islet/MSC+) showed morphologically intact islets with normal architecture (Fig. 3.28 B, J) whereas isografts of recipients that received islets only displayed scattered islet cell aggregates dispersed over larger sub-capsular areas of the kidney (Fig. 3.28 C, K).

Quantitatively, there were no significant differences in total endocrine area between islets plus MSC grafts and islets-only grafts (287,071 ± 19, 651 vs. 272, 009 ± 31,211, p = 0.471 (Fig. 3.30 A). However, total graft area (endocrine + non endocrine) was significantly higher in grafts with MSCs than in islet-alone grafts (387, 658 ± 29, 818 vs. 290 ± 17,114, p = 0.0321. Quantitatively, no significant differences in total endocrine area (immuno-stained with insulin) between rats co-transplanted with islets and MSCs and those that received islets only (p=0.471) (Fig. 3.30 A) were observed, but morphology of the grafts were different. But data on fusion of islet indicated that the mean area of individual islet aggregates in sections of isografts of islet/MSC+ recipients was 6 times higher than that observed in recipients of islets only (p = 0.00418) (Fig. 3.30 B). In accordance, larger areas of endocrine graft tissue were devoid of beta cells (insulin-negative or empty space) in grafts consisting of islets only (Fig. 3.28 K, L) whereas in islets with MSC co-transplanted grafts, beta cells were distributed throughout the endocrine tissue similar to endogenous islets (Fig. 3.28 J).

3.3.4 Histomorphology of pancreatic tissue in graft recipients

To ensure that changes in glycemic levels in recipients are due to islet graft function and not regeneration of the pancreas after STZ, pancreatic tissues were harvested for immunohistochemical analysis and compared the results with glucose levels in both untreated STZ-induced diabetic control and graft recipient rats. Hematoxylin and eosin sections showed changes in islet architecture with cellular disorganization in pancreata of both untreated STZ diabetic controls and graft recipients compared to normal healthy controls (Fig. 3.31 A compare with B).

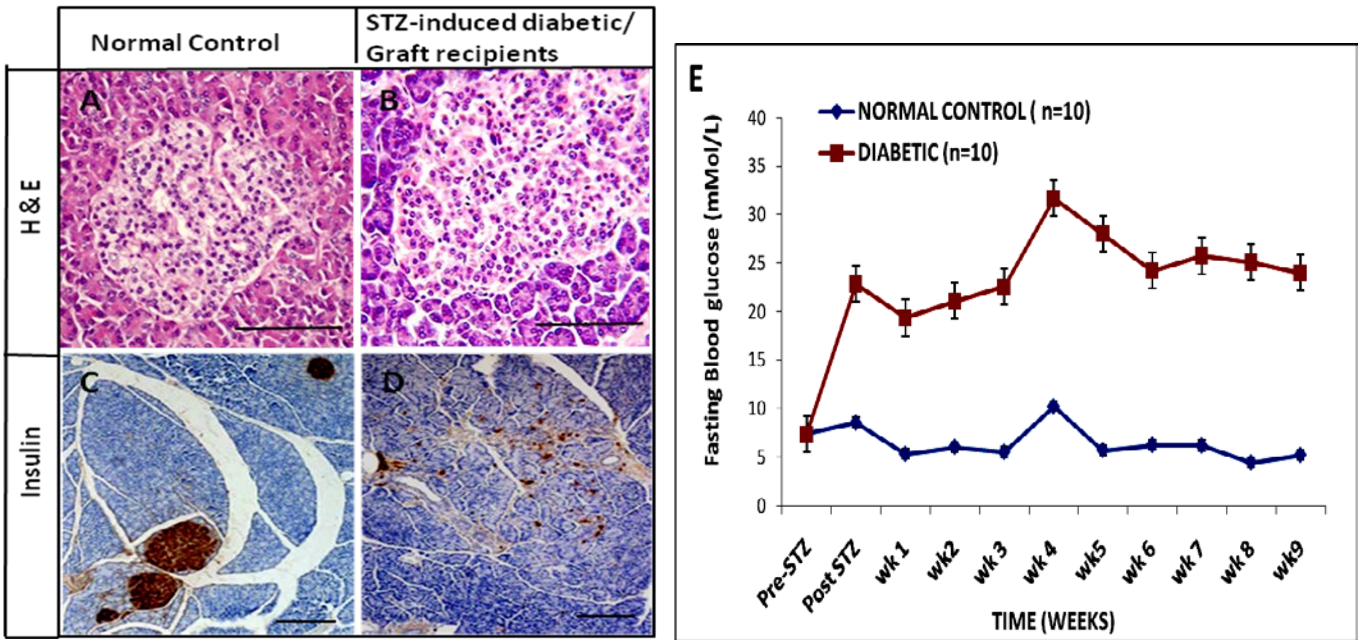


Figure 3.31. Histological analysis of morphology of the pancreas in male control Wistar rats. A-B:Hematoxylin and Eosin staining in healthy male rat (A) and STZ-induced diabetic rat untreated/ treated with islet with or without mesenchyme (B) after 9 weeks. C-D: Insulin staining (brown) in healthy male (C) and STZ induced diabetic rats untreated / treated with islets after 9 weeks. Panel E shows blood glucose levels of control healthy rats (blue diamond) versus STZ-induced diabetic rats (brown rectangle). In panel E STZ-induced diabetic rats maintained hyperglycemia for the entire periodof 9 weeks of study. (n = 10 animals/group were analysed), Bar = 100 µm.

Large areas of the islets in STZ-induced diabetic rats consisted of extracellular tissue predominantly of loose collagen fibers. Immunohistochemical analysis of the islet cellular composition 9 weeks after STZ treatment, showed reduced numbers of beta cells in both untreated diabetic rats and graft recipients compared to normal healthy controls as indicated by insulin staining (Fig. 3.31 C compare with D).

When fasting blood glucose levels were monitored for 9 weeks, however, STZ-treated rats maintained hyperglycemia for the rest of the experimental period in contrast to normal healthy controls (Fig. 3.31 E).

3.3.5 Morphology of donor islets is altered in 84-hr PPDL pancreas

To find out whether the transplantation outcome is affected by the condition of the donor islets, the pancreatic tissue sections prepared from tail pancreata of donor rats 24- and 84 h following ligation and normal control rats were examined by immunohistochemical reactions. Isolated islets from the same animal groups were also examined under inverted microscope. As shown in Fig. 3.32 A, B and C, insulin immunostaining of PPDL pancreatic tissue sections showed altered morphology of islets in tissue sections from 84 h PPDL compared to 24 h PPDL and normal control tissue sections. In the 24 h PPDL pancreata, islets maintained a normal morphology with round or oval outline (Fig. 3.32 B), similar to islet structure from normal health control pancreas (Fig. 3.32 A), while in the 84 h group, irregular shaped islets with rough edges were observed as indicated by irregular distribution of beta cells (by insulin immmunostaining) (Fig. 3.32 C). Similar morphological differences in islet structure were observed in isolated islets from the same animal groups (Fig. 3.32 D, E and F). Islets isolated 24 h PPDL showed normal round shape, similar to islets isolated from normal controls (Fig. 3.32 D & E), while those that were

isolated 84 h following ligation displayed rough edges with loose cells attached to islets (Fig. 3.32 F). It was noted that islets from 84 h group were very sensitive and fragile to collagenase enzyme digestion during isolation, suggesting that PDL induces morphological changes that affect islet integrity as mentioned in section 3.2.1.

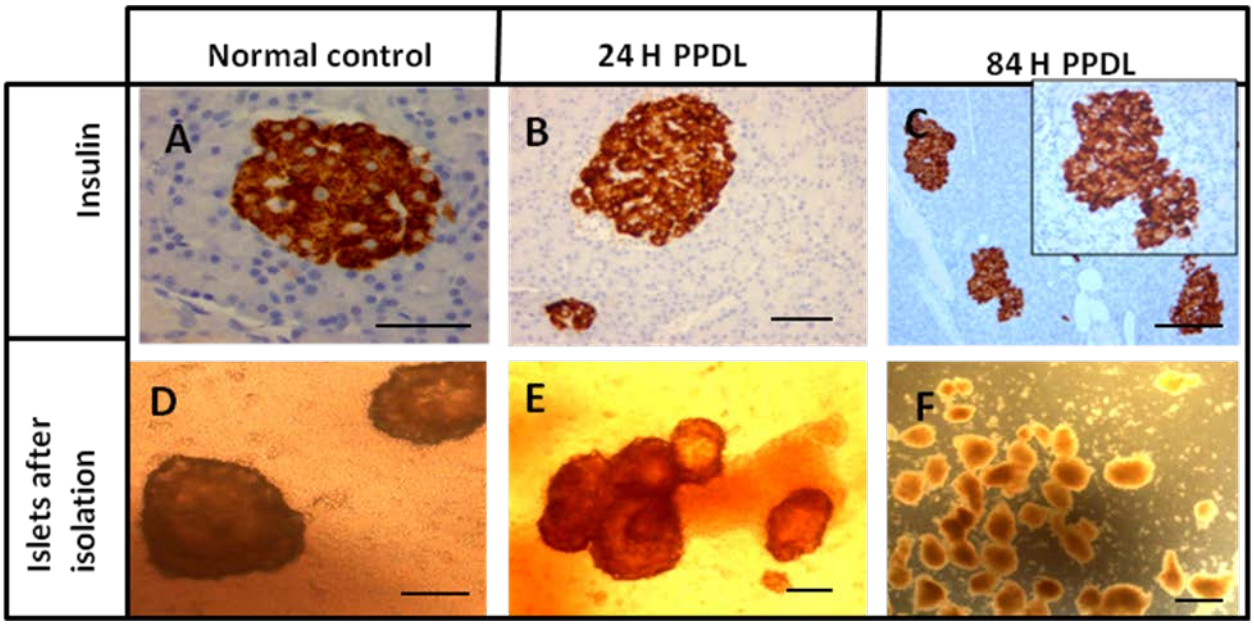


Figure 3.32. Morphology of donor islets. Panels A, B and C show histological pictures of tail pancreatic tissue sections immunostained with insulin and counterstained with hematoxylin obtained from a control rat pancreas A, PPDL tail pancreas harvested 24- (B) and 84 h (C) following ligation. In Panel C, islets show disorganized architecture as indicated by insulin staining. Panels D and E show healthy islets with almost smooth round shape isolated from control rat pancreas (D) and after 24 h post pancreatic duct ligation (PDL)(E). Panel D: Islets isolated 84 h post ligation showing rough edges with cells that are loosely attached. *Bar = 100µm.*

In summary, the results on islet transplantation show that the curative capacity of 24 h post islets was better than 84 h PPDL islets. The average glucose levels were lower throughout the 5 weeks monitoring period in 24 h PPDL transplanted rats. The average time to reverse hyperglycemia in 80% of the 24 h PPDL transplant group was 32± 2 days (~4.5 weeks,) while only 20% in the 84

h PPDL transplant group attained normoglycemia at 61 ± 2 days (~9 weeks) (p = 0.0011, n = 6) post transplantation. Graft survival rate was higher in islets co-transplanted with MSCs compared with islets alone grafts. Islet morphology and distribution of beta cells was normal in islet plus MSCs grafts similar to the endogenous islets in the pancreas of normal controls. In conclusion, transplantation of early harvested PPDL islets had more beneficial effects on short term reversing hyperglycemia. MSCs prolonged graft survival and improved the capacity of early PPDL islets to reverse hyperglycemia and may be applicable to clinical transplantation.

Chapter 4 Discussion and Conclusions

Current efforts to regenerate beta cells in adult pancreas as a strategy for cell replacement therapy for Type 1 and 2 diabetes mellitus could be an alternative solution to islet donor scarcity facing clinical islet transplantation in the treatment of diabetes mellitus.

This study demonstrates that mesenchymal stromal cells (MSCs) isolated from the duct ligated portion of pancreas can induce beta cell neogenesis from ducts and pre-existing islets, and favours the expansion of islet cells *in vitro*. Furthermore, the study showed that the efficiency of islet transplantation is harvest-time-dependent; early PPDL islets produce superior transplantation outcome and more beneficial effects in diabetic animal models. These findings support the previous assumptions by du Toit et al. (du Toit et al. 2013) and can be a new strategy for the design of cellular therapies and islet cell regeneration for type 1 and type 2 diabetes mellitus.

4.1. Mesenchymal stromal cells (MSCs) induce neogenesis and promote islet cells expansion in culture

In this study, an *in vitro* model was developed in which islet cells isolated from the PPDL rat pancreas were allowed to develop with or without MSCs. The morphological findings show that when islets are cultured without MSCs, islet cells undergo a short-term expansion (up to two weeks) and display morphological changes that resemble mesenchymal-like cells before forming new islet-like clusters. The change of the morphology of these islet cells from *in vivo* 3-dimensinal structure to *in vitro* 2-dimensional structure in direct contact culture system resulted into adhesion of islets to plastic substrate, and then migration of cells. Most of the adhered cells were islet cells, although, the presence of intra-islet cells that resembled

fibroblasts cannot be ruled out. These results therefore suggest a possible transient de-differentiation and loss of phenotype during expansion before they re-differentiated into functional islet cells. In previous study (Gershengorn et al. 2004), it was reported that epithelial cells undergo de-differentiation and lose their phenotype in culture. In cultured thyroid cells, for example, epithelial de-differentiation was shown to occur through epithelial-mesenchymal transition (EMT) (Jeon et al. 2015, Yang et al. 2015), which leads to dissociation of cells, alteration of expression of adhesion molecules, switching from E-cadherin to N-cadherin (Fanjul et al. 2010) and this is regulated by Snail 1 (Cano et al. 2000). Recently, a study suggested that EMT generates cells with properties of stem cells (Yang et al. 2015).

Although this study did not examine the dissociation of cells directly, the morphological findings showed changes of cell shapes from polygonal shape to cells with narrow cell bodies and extended cytoplasmic prolongations, features that were similarly observed by Gershengorn and his group (Gershengorn et al. 2004), who postulated that human cultured islet cells undergo EMT upon entrance in their cell cycle. However, long term culture of islets alone (up to 3 to 4 weeks) did not promote expansion but resulted into formation of spheroid bodies that resembled islets suggesting that when islets are cultured alone, their proliferative capacity becomes limited and islet cells quickly undergo re-differentiation and become quiescent. In cultures with mesenchymal, in contrast, islet cells extended the period of expansion as we continued culturing suggesting that, *in vitro*, signals from mesenchymal cells positively regulate the capacity of proliferation of islet cells and determines the final number of mature islets that develop from pre-existing islet cells.

4.1.1 Mesenchymal stromal cells (MSCs) promote expansion of Pdx1$^+$ cells and activate Ngn3 expression in culture

Results from the protein expression analysis in cultures with mesenchymal cells showed activation of Pdx1 in islet cells and these Pdx1+ cells expanded up to 80% confluence by the end of the second week. Pdx1 is a key transcription factor involved in early pancreatic development, differentiation of insulin-producing beta cells and maintenance of mature beta cells phenotypes (Cerf et al. 2005). In mature beta cells, Pdx1 is one of the key genes that regulates the transcription of islet-specific genes, such as insulin, GLUT2 (Waeber et al. 1996), glucokinase (Watada et al. 1996) and Nkx6.1 (Pedersen et al. 2005;Shih et al. 2002). In a study that used a transgene encoding Pdx1 into the liver cells of STZ induced diabetic rats, expression of insulin gene was induced and ameliorated hyperglycemia (Ferber et al. 2000) suggesting that Pdx1 has the capacity to re-program non-pancreatic cells into insulin producing cells. The co-expression of insulin and Pdx1in this study (Fig 3.8) demonstrated that MSC significantly enhanced Pdx1 protein expression in insulin producing beta cells during the first week. Further studies are, however, needed to investigate whether Pdx1 regulated proliferation of beta cells or maintained the identity of mature beta cells in cultures at this stage of development. Nevertheless, Pdx1+ cells significantly expanded in islet/MSCs co-cultures during the first week and this was followed by high levels of Ngn3 expression in the second week (Day 12). Ngn3 is normally downregulated in mature islet cells i*n vivo* (Gu, Dubauskaite & Melton 2002).

When islets were cultured alone, Pdx1 was also activated during the first week and its expression extended up to the second week, but this was followed by low levels of Ngn3 that

transiently expressed for only 2 days. This observation was similar to what occurs during pancreas development in which Ngn3 is expressed transiently, following the down-regulation of Notch1 and Hes-1 (Afelik et al. 2012). The transient expression of Ngn3 and Pdx1 in cultures with islets only (Fig. 3.14 L, K [at 17-21days]) characterizes the temporal heterogeneity of isolated islets whose subpopulations were undergoing different stages of development. Although this observation indicates that some insulin producing beta cells expressed Pdx1 and Ngn3 in islets cultured with or without MSCs, these findings may suggest that secretion of trophic factors by MSCs at the site of islet-MSCs contact and in the culture medium, might have altered or changed the microenvironment that promoted growth, differentiation and survival that resulted into emergence and expansion of Ngn3+ cells. These results, therefore, demonstrate that some cells within the pre-existing islets regress to a lesser differentiated phenotype by expressing Pdx1 followed by Ngn3 - markers of endocrine precursor cells similar to the differentiation process that occurs during embryonic pancreas development.

The mechanism by which MSCs reactivate neogenic pathway and regulate the proliferation and re-differentiation of these mature endocrine cells is not clearly understood. In addition, in the absence of lineage tracing experiments, this study cannot justify whether Ngn3 was evidently expressed in de-differentiated mature islet cells or in activated progenitor cells located within the islets or mesenchymal cells. Although increase in Pdx1+ cells proliferation in cultures with MSCs has been suggested to result into an equivalent increase of the population of Ngn3+ cells, these increases in Ngn3+ cell population might have been produced by the simultaneous conversion of MSCs to Ngn3+ cells and/or quiescence or apoptosis in cultures of islets only. This raises the question of whether MSCs activates

neogenesis from pre-existing islet cells. However, immunocytochemical analysis indicated increased Ngn3 expression in co-cultures of islets and MSCs suggesting that the heterogeneous population (Islet/MSCs) contained stem-cell-like cells with endocrine differentiation potential. Results from studies that used MSCs from bone marrow to differentiate into insulin producing cells are, however, still very controversial (Lecher et al. 2004, Gopurappilly, Bhat & Bhonde 2013). Findings from other *in vitro* and *in vivo* studies demonstrated that islet-derived MSCs can be induced to differentiate into insulin producing cells with phenotypes typical of islet cells (Ianus et al. 2003, Davani et al. 2007, Mutskov et al. 2007). Mesenchymal stromal cells from these studies were found to express transcription factors of epithelial, endocrine and mesenchymal markers (Baertschiger et al. 2008). However, evidence from additional studies, failed to support these findings (Lechner et al. 2004, Choi et al. 2003, Taneera et al. 2006). Although this study did not assess this directly, it is possible that MSCs trophic secretions might have induced expression of endocrine developmental genes in precursor-like cells within the heterogeneous population of MSCs and islet cells. As suggested by other (Finegood, Scaglia & Bonner-Weir 1995), it may be possible that Pdx1 expression maintains the balance between neogenesis and proliferation of mature islet cells, and that this is regulated by signals from the MSCs. This study demonstrate that MSCs activate the expression of transcription factors (Pdx1 and Ngn3) in mature islet cells and promotes proliferation of Pdx1+ cells and maintains Ngn3+ precursor phenotype in proliferating islet cells that ultimately increase beta cell numbers. Mesenchymal cells acted by increasing the proliferation of Pdx1+ cells and extended the period of formation of Ngn3+ precursor-like cells from Pdx1+ islet cells. The number of Ngn3+ cells

was amplified in the presence of mesenchymal cells and the period of re-differentiation of these Ngn3+ islet cells into insulin producing islets was prolonged.

Whether such enhancement was primarily due to direct MSC-to-Islet cell contact or by means of trophic factors secreted by mesenchymal cells needs more investigation. Other studies demonstrated that MSCs differentiate into insulin producing cells only when MSCs are in contact with islets (Scuteri et al. 2014), and that Px1 is involved in the activation of the endocrine pathway. This, in turn, also affected the behavior of pancreatic islet cells. The true direct co-culture system in our study was difficult to achieve, as only few islets were completely attached and surrounded by the mesenchymal cell monolayer, but the rest of the islet cells were just slightly sequestrated over MSCs, thereby hampering their floating state, but still giving a three-dimensional structure with most cells proliferating in suspension. Secondly, since MSCs surround islets in our direct co-culture system, islet-to-MSCs close contacts are likely to occur with peripheral islet cells, for example alpha cells in rat islets, while the direct contact with beta cells that are centrally located might not be common. Whether MSCs contribute to centrally located beta cell mass increase by secretion of growth factors or to other peripherally located endocrine such as α- and δ- cells by cell-to-cell contact, are questions that could not be explained by the direct co-culture model and need further exploration.

To test whether mesenchymal effects on islet cells development are due to MSC-to-Islet cell contact or secretion of growth factors, islets and mesenchyme were cultured in an indirect co-culture system by physically separating them with filter membrane and the results indicated an increase in proliferation of islet cells similar to cultures by direct contact. These results

therefore suggest that some signals from MSCs were secreted in the culture medium and might have interacted and activated directly or indirectly the transcription of genes that maintain islet cells in a precursor state and consequently determine the time of re-differentiation of these cells into mature islet cells. Maintenance of Ngn3+ cells by MSCs may prevent premature differentiation of endocrine cells and allow these cells ample time for expansion thereby increasing the number of re-differentiated islet cells, which is a novel finding in this study.

4.2 Duct fragmentss generate beta cells in culture

Duct fragments and MSCs from the post ligated tail pancreas were cultured under different conditions (with or without MSCs) and sequential changes were observed in their differentiation potency *in vitro* (Fig. 3.20 & Fig. 3.21). This study showed that differentiation of epithelial duct cells into ngn3+cells and later into insulin-producing cells can be promoted *in vitro.*

Morphological and immunocytochemical analyses of cultured ducts showed that when PPDL duct epithelial cells are maintained in MSCs or without MSCs, duct fragments initially developed growth areas, giving rise to cell populations with proliferative capacity. In contrast to duct fragments cultured alone, duct fragments cultured with MSCs resulted into formation of growth areas from day 3 and these cells proliferated rapidly and formed sheet of periductal cells that migrated sideways. At the tips of some small intercalated ducts, a population of proliferating squamous cells, possibly representing centroacinar cells, was also observed. This was followed by rapid branching of ducts before cell clusters began to form closer to ducts within the migrating monolayer, suggesting that these cells are beginning to adopt a

different phenotype. Thereafter, cell clusters formed larger spheroid bodies that were more visible on day 24 and 26. Continued culturing resulted into formation of more spheroid bodies budding off from ducts suggesting that the budded cells are not of duct phenotype but either exocrine or endocrine. The morphological observations, therefore, reveal a possible recapitulation, in adult duct epithelial cells, of sequences of events that occur during pancreas development (i.e. a wave of proliferation of ductal cells, followed by differentiation of these cells).

4.2.1 Ductal and periductal cells in growth areas give rise to Ngn3 positive cells

The ductal origin of islet cells has been advocated by vast literature using animal models of pancreatic injury including PDL (Bonner-Weir et al. 1993; Gu, Sarvetnick 1993; Rosenberg 1995; Rooman, Bouwens 2004; Suarez-Pinzon et al. 2005; Xu et al. 2008a; Inada et al. 2008). However, the hypothesis that new endocrine cells arise from ducts of adult pancreas is debated up to date. In this study, immunocytochemical analysis showed that when duct epithelial cells are cultured in presence of MSCs, some mature duct epithelial cells within the ductal epithelium de-differentiated and lost their phenotype. Duct fragments cultured in the presence of MSCs developed growth areas from day 3. In 1 to 3-day old cultures, insulin+ cells were not detected in growth areas. In 7-day old cultures, the majority of cells in the growth areas revealed some of the features of duct cells expressing CK7 but some activated proteins of endocrine precursors, Ngn3. These Ngn3+ cells were intercalated in the CK7+ duct epithelial cells and that a subset of Ngn3+ cells co-expressed CK7 and Ngn3 in cultures with MSCs. In view of these features, the main population in the growth areas was considered to be de-differentiated duct cells. Moreover, the characteristic periductal location

and the nuclear expression of Ngn3 is an additional argument supporting de-differentiation (β cell neogenesis) process in growth areas of cultured ducts. When these cells began their migration, Ngn3+ cells were detected first in periductal cells and then in scattered cells within a short distance around ducts. Demonstration by double-labeling duct epithelial cells co-expressing CK7 and Ngn3 indicated that some duct cells underwent duct-endocrine transdifferentiation. During the migration of the periductal cells, a progressive disappearance of CK7, an intermediate filament protein of cells arising from ducts, and a subsequent progressive expression of Ngn3, usually a protein expressed in endocrine precursors or neurons, were observed. These cellular architectural and cytoskeletal alterations are features of duct-endocrine transdifferentiation. Duct-endocrine cell transdifferentiation giving rise to Ngn3 has been observed in animal models of pancreatic regeneration (Xu et al. 2008). In this study, similar CK7 and Ngn3 expression patterns were observed in ducts cultured alone, suggesting that the initiation of de-differentiation leading to expression of Ngn3 does not require signals from MSCs. Alternatively, these results might suggest that these duct epithelial cells might have already received instructive signals way before isolation. In addition, in the absence of expreriments tracing the lineage of duct to endocrine cells differentiation in both cultures with or without MSCs, these findings may alternately suggest that the Ficoll-based duct isolation protocol resulted in isolation of impure cells comprising Ngn3+ duct derived cells and pre-existing islet cells. Cell lineage tracing requires labelling of the duct cells and tracking the characteristic changes of the labeled cells and this could not be achieved by the present co-localization studies. Nevertheless, these results agrees with the *in vivo* regeneration results that showed activation of Ngn3+ cells located either within the duct epithelium or close to ducts (Xu et al. 2008a).

Although pancreatic duct epithelial cells have been suggested as a source of new beta cells in a regenerating pancreas following injury by duct ligation (Xu et al., 2008), lineage tracing studies demonstrated that, *in vivo*, PDL-induced progenitors ($Ngn3^{+}$) from ducts do not complete the differentiation program to generate insulin producing beta cells (Solar et al. 2009; Kopp et al. 2011). Several lineage tracing studies showed that, *in vivo*, duct derived Ngn3+ cells contribute to islet cell mass only during embryogenesis and early neonatal stages but not in adult or injury following ligation (Solar et al. 2009; Kopp et al. 2011, Furuyama et al. 2011). The current *in vitro* data, however, showed that de-differentiated duct-derived Ngn3+ cells can further differentiate into insulin producing cells thereby contributing to islet cell mass. The current findings further show that MSCs promote the expansion Ngn3+ cells and maintain their de-differentiated state before they proceed to differentiate into insulin producing cells. In the presence of MSCs, Ngn3 expression was maintained up to 4 weeks in periductal cells and scattered cells with few Ngn3+ cells that subsequently differentiated into insulin producing cells. How mesenchymal cells maintain these Ngn3+ cells require further investigation. In a PDL-induced pancreatic injury, proliferation of MSCs is one of the early regenerating events that occur distal to the ligature and this is followed by the emergence of Ngn3+cells within and next to ducts. Lineage studies did not detect the contribution of these Ngn3+ cells to islet cell mass (Solar et al. 2009; Furuyama et al. 2011; Kopp et al. 2011). One possible explanation for their failure is that the presence of signaling molecules from proliferating MSCs might have promoted and maintained Ngn3 expression in these duct-derived Ngn3+ cells, and only to release few cells at a time to leave the cell cycle and differentiate into insulin producing cells, which were not detected by lineage tracing cre-lox-p-lox systems in these studies. Evidence from this study supports this notion by detecting

Ngn3+ cells for 4 weeks in cultures with MSCs. The molecular mechanisms involved in maintenance of Ngn3+ by MSCs are, however, not clearly understood.

One candidate pathway might be the Notch-Hes1-Ngn3 signaling pathway, which determines exocrine versus endocrine specification through lateral inhibition during embryonic pancreas development. In the developing pancreas, the role of the Notch-Hes1 pathway is to promote precursor cell replication and prevent premature endocrine differentiation (Murtaugh et al. 2003; Hald et al. 2003; Esni et al. 2004). Several studies show that MSC-derived Fgf10 activates Notch signaling which promotes proliferation and inhibits differentiation of endocrine precursors (Jensen & Jensen 2003; Hart, Papadopoulou & Edlund 2003; Miralles et al. 2006). There is also evidence that MSC-derived activin induces Ngn3 through a non-canonical target of activin, p38 mitogen activator protein K (MAPK) (Ogihara et al. 2003), however, this was not tested in our study.

Taken together, the observation of cells co-expressing ductal epithelial and endocrine markers in PPDL pancreatic duct fragment cultures urgues in support of the hypothesis that the observed neogenesis for duct epithelial cells *in vitro* does not only occur in culture, but may also occur *in vivo* during regeneration of tissues or renewal of beta cells in the pancreas.

4.3 Efficacy of PPDL islets

To explore the efficacy of PPDL islets as donor tissue in reverting normoglycemia in STZ-induced diabetic Wistar rats, syngeneic transplantation model was used with minimal islet mass. Two sets of experiments were used to demonstrate the importance the PPDL pancreatic tissue in terms of graft survival and function. In the first experimental set, the results indicated that early isolated syngeneic PPDL islets (24 h following ligation) produced

superior but transient transplantation outcome under the renal capsule compared to islets isolated 84 h PPDL when the donor pancreatic tissue distal to the ligation is at the peak of destruction. In the second set, the findings demonstrated that pancreatic-derived MSCs isolated from PPDL tail pancreas of syngeneic donors 24 h following ligation, prolonged graft survival and function when co-transplanted with islets in diabetic rats.

4.3.1 Efficacy of early and late PPDL islet grafts *in vivo*

The results demonstrated a differential efficacy between islets isolated early or late PPDL in treating chemically induced diabetic rats. In terms of graft survival and function, more beneficial effects were observed when transplanted with 24 h PPDL islets with more than 60% animals recovering from hyperglycemia for 5 weeks compared to only 10% of the animals in the 84 h post group achieving normoglycemia within the same time frame.

Most animals treated with donor islets from late PPDL pancreatic tissue (84 h PPDL) failed to achieve normoglycemia within the same time frame of 5 weeks post transplantation. One possible explanation for low graft survival in the late PPDL tissue could come from the condition of the PPDL donor pancreas. Although endocrine tissue is less affected, a study that chronologically documented the cellular mechanisms in a PPDL pancreas reported that a high peak of exocrine destruction occurs between 72 and 84 h post ligation (Tchokonte-Nana 2011) and this could likely affect the islet microvasculature, the cellular architecture and the composition of connective tissue proteins that bind the islet cells together as revealed in our present study by morphological changes in islet architecture (Fig. 3.32 C &F). This is compounded by enzyme islet isolation, which not only disrupted the external vasculature but also compromises the internal cellular organization and vascular network (Fig. 3.32 F). This

suggests that maintenance of donor islet morphology is a critical parameter for achieving euglycemia in islet transplantation therapy.

However, treatment with early PPDL islets transiently cured diabetic rats but did not permanently prevent the recurrence of diabetes in Wistar rats. In fact, when diabetes recurrence was analyszed at 7 weeks post transplantation, no significant differences in graft survival and function were detected between the 24- and 84 h PPDL grafts. Surprisingly, even grafts with normal islets, displayed a slightly long-term survival and function but did not result in permanently preventing further recurrence of diabetes. These observations are at par with previously reported findings that used a PPDL pancreatic tissue, instead of isolated islets, as a donor tissue harvested 84 h PPDL that only achieved euglycemia in 40% of the animals (Page et al. 2004).

4.3.2. Early PPDL islets and MSCs co-transplantation promotes graft survival and function.

Several studies have reported the beneficial effects of MSCs in different transplantation models and these effects have been proposed to occur by different mechaniscms (Figliuzzi et al. 2009; Ito et al. 2010; Berman et al. 2010; Sordi, Piemonti 2010; Rackham, Jones & King 2013). Considering high graft failure (as reflected by recurrence of hyperglycemia) in both syngeneic recipients of 24- and 84 h PPDL islets between 7 and 9 weeks post transplantation, we next tested whether co-transplantation of MSCs and 24 h PPDL could enhance islet survival and function in our syngeneic transplantation model.

Consistent with the observations of others that reported the beneficial effects of MSCs in islet transplantation (Figliuzzi et al. 2009; Ito et al. 2010; Berman et al. 2010; Sordi, Piemonti

2010; Rackham, Jones & King 2013), the current study demonstrated that co-transplanting pancreatic-derived MSCs with islets under the renal capsule of syngeneic diabetic rats resulted into a gradual but longer term attainment of euglycemia compared to those that received islets without MSCs. It was found that MSCs increased the number and rate of Islet/MSCs isograft recipients achieving normoglycemia by 5 weeks post transplantation. Higher numbers (8/10 or 80%) of animals that received islets + MSCs grafts sustained normoglycemia up to 9 weeks post transplantation without recurrence of diabetes between 7 and 9 weeks post transplantation when compared to (4/10 or 40%) recipients of islet-alone grafts.

Evidence from other studies, however, showed that both human (Lee et al. 2006) and mouse (Xu et al. 2008b) MSCs induced regeneration of endogenous pancreatic islets that eventually ameliorated hyperglycemia in STZ-induced diabetic mice. The histological analysis of the pancreas of MSCs+ islet graft recipients, however, showed no evidence of islet regeneration of endogenous islets by the end of the experiment (Fig. 3.31). These findings are promising and suggest that MSCs may help prolog islet graft survival in diabetic animals. Several reports indicate that MSCs provide a graft microenvironment that limit beta cell damage by secreting several paracrine angiogenic, mitogenic, immuno-regulatory and anti-inflammatory factors (Di Nicola et al. 2002; Le Blanc 2006; Nauta, Fibbe 2007; Abdi et al. 2008; Figliuzzi et al. 2014). MSCs have been shown to reduce autoimmune-mediated islet attack in models of autoimmunity induced diseases including type 1 diabetes (Molano et al. 2001) which may contribute to prevention of post transplantation islet cell loss.

The morphological analysis of islet grafts suggest that MSCs may also play a role in islet graft remodeling that occurs soon after transplantation. In rat recipients of islets and MSCs, graft morphology was maintained similar to *in vivo* islets, unlike the fragmented endocrine cell aggregates that were observed at graft site of animals transplanted with islets only. The morphological findings reaffirm previous transplantation studies that reported similar MSC-induced graft morphological changes (Biarnes et al. 2002; Rackham, Jones & King 2013). These changes may influence graft survival and function through promotion of revascularization. Previous studies reported that revascularization is more efficient in smaller islets than in large amorphous aggregates (Kampf, Mattsson & Carlsson 2006), an effect that was observed in grafts of islets only in our study.

Taken together, our results indicate that a PPDL pancreatic tissue is a potential source of islets for transplantation in diabetic animals. PPDL islets harvested in the early hours of post-surgery produced superior transplantation outcomes compared to islets harvest late PPDL. More beneficial effects were observed when transplanted with 24 h PPDL islets with more than 60% animals recovering from hyperglycemia for 5 weeks. Co-transplantation of MSCs and early PPDL islets significantly increased islet engraftment, survival and function, with 80% of the animals reversing diabetes and may be applicable to clinical transplantation programs.

4.4 Strength and limitations of the study

The strength of this study is based on enzymatic isolation of the two cell populations (islets and mesenchymal cell populations), the development of long-term *in vitro* culture system and the immunocytochemical analysis of the expressions and co-expressions of transcription

factor proteins regulating islet cell development and function. The study has also strongly demonstrated that the PPDL derived MSCs can be included to promote engraftment, prolog islet graft survival and function and provide sustainable adequate insulin levels to ameliorate STZ-induced diabetic animals. The data demonstrate that the islet/MSC-based transplantation therapy is time dependent in treatment of diabetes mellitus.

Firstly, this study was however, limited by not evaluating the contribution of the paracrine factors of MSCs to beta cell development *in vitro* and beta cell survival in grafts.

Secondly, there was no evaluation of the contribution of MSCs to beta cell mass through MSC-beta cell transdifferentiation, though there is some debatable evidence that MSCs can differentiate into insulin producing cells that are able to normalize hyperglycemia after transplantation into diabetic animal models (Hao et al. 2013, Kong et al. 2014).

Thirdly, the use of flow cytometry and Fluorescence activation cell sorter (FACS) to isolate and characterize MSCs, and demonstrate their multipotency, could have been a better tool to ascertain that these cells were true MSCs.

4.5 Conclusions

The study findings demonstrated that pancreatic–derived MSCs promote expansion of Pdx1+ cells, followed by differentiation of Ngn3+ cells into insulin producing cells; MSCs also enhanced aggregation of islet-like structures and engraftment of islet cells, and prolong graft survival and sustain normoglycemia in diabetic animal models. These results suggest that MSCs regulate neogenesis and islet development through cell-to-cell contact and by

secretion of a various growth factors that can promote the expression of Pdx1 and Ngn3 in islet cells and maintain normoglycemia in diabetic animal models.

4.6 Implications and clinical perspectives of the study

The clinical significance of the study relies on the hope that the design of novel syngeneic self-renewing MSCs from the ligated pancreas may help to reactivate regeneration of beta cells in patients with type 1 diabetes. MSCs secrete several factors that favour the stimulation, regeneration and repair of various injured or defective tissues (Chapel et al. 2003, Chapel 2012).

The information provided in this thesis, indicates that duct epithelial and pre-existing islet cells have the capacity to respond to signals (factors) from the MSCs. Based on these findings and other published data, it can be postulated that in an injured pancreas following duct ligation, inductive signals for islet regeneration might arise from the mesenchymal tissue surrounding pre-existing islets and proliferating ductules. Identification of the mesenchymal-derived signaling molecules that trigger de-differentiation and re-differentiation in pre-existing islets and duct progenitors in a regenerating pancreas will be an important step towards generating islet cells for transplantation or endogenously generating beta cells as a therapy for diabetes. Fgf10 was previously shown to promote the proliferation of Pdx1+ cells (Bhushan et al. 2001); and a recent study demonstrated that survivin is required for the expansion of beta-cell mass after PDL (Wu et al. 2012) and appears to promote proliferation of pre-existing beta cells. The *in vitro* protocols that have been developed to expand human islets for transplantation, however, only drives human embryonic stem cells (hESC) to generate pancreatic progenitor cells, but do not proceed to

generate differentiated insulin-producing beta cells. The current findings demonstrate that MSCs provide essential factors for both the expansion and differentiation of Pdx1 expressing islet cells and Ngn3+ precursor cells into insulin-producing cells. The study, therefore, proposes that *in-vitro* strategies to attain increased islet mass for transplantation should include the use of extracellular factors from mesenchyme that stimulate the expression of relevant transcription factors (Pdx1 and Ngn3). This will help obtain a high enough proportion of de-differentiated precursor-like islet cells that ultimately re-differentiate into large number of beta cells.

4.6.1 Recommendations

Based on the findings of this study, the following are recommendations for further research or application in the analysis of factors involved in adult pancreas regeneration.

First, it is recommended that present findings can be the applied in the development of *in vivo* strategies to induce and promote regeneration of the beta cells in type 1 diabetic patients or the development of *in vitro* strategies in obtaining adequate numbers of beta cells for transplantation and promoting graft survival in recipients.

Second, the findings presented in this report indicate that MSCs and/or MSC-derived factors are continuously required in regulation of islet development in culture; from the onset of Pdx1 expression and expansion, followed by Ngn3 activation up to the end of insulin expression. Therefore, it is recommended that evaluation of MSCs functions *in vivo* is required to determine whether MSCs extert the same effects at different stages of endocrine pancreas regeneration. In addition, further investigations are also needed to determine

whether MSCs functions are affected by hyperglycemia in diabetic patients with problems of wound healing.

Third, lineage tracing analysis of the cells *in vitro*, coupled by RT-PCR analysis would be more informative than snapshot immunocytochemical analysis of the phenotypic changes of pre-existing islet and duct epithelial cells during development.

5.0 References

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Appendix A. Preparation of solutions

Table A1. Preparation of Ficoll (Type 400)

Reagent/vol.	25% stock solution	23% working Solution	20.5% working Solution	11% working solution
Ficoll Type 400	33.35 g	46 mL stock solution	41 mL stock solution	22 mL stock solution
HBSS	Adjust up to 100	4 mL	9 mL	28 mL
Final volume	100 mL	50 mL	50 mL	50 mL

Table A2. Phosphate-buffered saline (PBS)

Reagent	Amount to add (1X)	Final conc. (1X)	Amount to add (10X stock)	Final conc. (10X)
NaCl	8 g	137 mM	80 g	M 1.37
KCl	0.2 g	2.7 mM	2 g	27 mM
Na_2HPO_4	1.44 g	10 mM	14.4 g	100 mM
KH_2PO_4	0.24 g	1.8 mM	2.4 g	18 mM
dH_2O	800 mL		800 mL	
dH_2O	Adjust to 1000 mL		Adjust to 1000 mL	

Table A3. Preparation of 10X Trypsin-EDTA

Reagents / Vol.	10 mL	50 mL	100 mL	500 mL
Trypsin	0.05 g	0.25 g	0.5 g	2.5 g
0.5 M EDTA	0.1 mL	0.5 mL	1.0 mL	5.0 mL
10X PBS	1.0 mL	5.0 mL	10.0 mL	50.0 mL
Deionized water	Adjust to 10.0 mL	Adjust to 50.0 mL	Adjust to 100 mL	Adjust to 500 mL

Table A4. Primary antibodies used in the study

Antibody	Cat No.	Raised in	Clonality	Working Dilution	source
Anti-Pdx1	ab47267	Rabbit	polyclonal	1:1000	ABCAM
Anti-Insulin	ab7842	Guinea pig	polyclonal	1:800	ABCAM
Anti-Ngn3	AB5684	Rabbit	polyclonal	1:1000	Millipore
Anti-Ki67	Ab9260	Rabbit	monoclonal	1:200	ABCAM
Anti-insulin	HB125	Mouse	monoclonal	Pre-diluted	BioGenex
Anti-CK 7	ab9021	Mouse	monoclonal	1:200	ABCAM

Table A5. Secondary antibodies used in this study

Antibody	Cat No.	Raised in	Clonality	Working dilution	source
Goat anti-Rabbit (IgG)H&L (Alexa fluor 594)	ab150080	Goat	polyclonal	1:800	ABCAM
Goat anti-Rabbit (IgG)H&L (FITC)	ab97050	Goat	polyclonal	1:500	ABCAM
Goat anti-mouse (IgG) H&L (Alexa fluor 594)	Ab150116	Goat	polyclonal	1:400	ABCAM
Goat anti-mouse (IgG) H&L (Alexa fluor 488)	Ab150113	Goat	polyclonal	1:1000	ABCAM

Appendix B. Immunohistochemistry

Immunochromogenic labeling

- Antigen retrieval using citrate buffer (pH 6.0)

Step	Apply	Incubation Time
1	Peroxide Block (Refine kit)	10 minutes
2	PBS Wash Solution	3 x 5 minutes
3	Primary Antibody	24 hrs
4	PBS Wash Solution	3 x 5 minutes
5	Post Primary block (Refine kit)	30 minutes
6	PBS Wash Solution	3 x 5 minutes
7	"Novolink Polymer" (Refine Kit)	45 minutes
8	PBS Wash Solution	3 x 5 minutes
9	Mixed DAB (Diaminobenzidine) (refine Kit)	10 minutes
10	De-ionised water (wash)	5 x 3 minutes
11	Counterstain with Hematoxylin (Refie Kit)	5 minutes
12	De-ionised water (wash)	5 x 3 minutes

Appendix C. Immunomorphological analysis

Table C1. Expression of Pdx1 in cultured islets cultured with or without MSCs

Days in culture		3		5		9		12	
		Pdx1 + Area (µm^2)							
Slide ID	Islet No.	MSC+	MSC-	MSC+	MSC-	MSC+	MSC-	MSC+	MSC-
Pdx124MS	1	3998.75	217.15	166.28	762.84	998.67	6754.82	154.41	274.57
Pdx124MS	2	1552.6	753.05	8225.36	150.78	925.63	5405.32	229.66	922.57
Pdx124MS	3	2343.28	328.96	601.69	419.61	2778.8	5378.23	181.93	360.37
Pdx124MS	4	1357.48	128.36	249.88	759.36	1412.82	8037.78	882.45	1672.74
Pdx124MS	5	3900.71	179.88	284.17	955.53	336.83	1536.58	544.75	368.86
Pdx124MS	6	7212.4	532.89	1501.23	442.49	602.95	8427.22	217.15	625.38
Pdx124MS	7	15846.41	229.66	5458.62	332.96	258.15	1473.71	753.05	2189.11
Pdx124MS	8	244.74	181.93	1525.96	446.96	1998.8	1851.26	828.96	523.04
Pdx124MS	9	1607.44	882.45	543.56	334.43	1417.8	9870.49	728.36	217.72
Pdx124MS	10	1537.86	544.75	2967.66	336.89	1302.62	1684.11	679.88	8011.9
Pdx124MS	11	1283.44	154.41	982.28	324.16	841.61	1071.22	532.89	463.81
Pdx124MS	12	1320.62	250.46	775.49	581.51	608.87	1212.46	792.87	17016.98
Pdx124MS	13	5289.25	2297.9	478.84	404.2	609.43	1617.96	344.59	133.03
Pdx124MS	14	2150.21	757.71	937.97	208.68	852.64	6856.52	587.61	880.99
Pdx124MS	15	4930.77	220.39	9022.63	246.77	796.04	18228.12	1410.14	630.49
Pdx124MS	16	1839.8	418.16	1857.72	329.56	655.87	1455.47	1800.97	740.73
Pdx124MS	17	849.76	136.89	997.35	205.12	1088.78	1994.44	3197.96	403.72
Pdx124MS	18	4991.95	445.77	1027.02	1998.67	1807.81	1786.87	239.97	662.4
Pdx124MS	19	931.59	3197.96	800.53	1925.63	1012.06	10120.91	441.87	718.61
Pdx124MS	20	1657.91	239.97	676.59	2778.8	5037.58	4994.4	819.72	1274.95

Pdx124MS	21	7920.44	441.87	342.47	412.82	1594.27	1675.67	601.01	778.66
Pdx124MS	22	2378.2	819.72	561.61	1336.83	1583.51	927.79	567.36	841.61
Pdx124MS	23	458.09	601.01	645.66	602.95	1066.75	1772.9	183.35	852.64
Pdx124MS	24	1858.09	567.36	603.9	958.15	389.55	3131.16	316.69	1055.87
Pdx124MS	25	1618.01	109.52	505.53	1998.8	1502.75	5665.03	250.46	807.81
Pdx124MS	26	405.86	202.36	2980.15	1417.8	2371.91	3091.99	12297.9	12.06
Pdx124MS	27	1649.11	2672.7	902.61	1302.62	839.06	4018.91	757.71	937.58
Pdx124MS	28	203.86	428.01	12542.44	8841.61	394.81	1571.3	220.39	725.27
Pdx124MS	29	1046.57	247.97	569.55	608.87	725.27	4910.2	418.16	373.68
Pdx124MS	30	1944.16	839.42	887.51	609.43	506.03	2238.11	136.89	2241.48
Pdx124MS	31	741.08	702.36	665.37	4852.64	1568.4	3001.52	445.77	912.62
Pdx124MS	32	288.18	5672.7	576.54	796.04	373.68	1436.18	809.52	9101.98
Pdx124MS	33	31844.76	428.01	1385.54	655.87	371.77	1620.59	202.36	2310.87
Pdx124MS	34	908.81	699.97	2629.5	911.78	570.4	1959.1	2672.7	8224.57
Pdx124MS	35	2185.42	439.42	6165.76	807.81	241.48	1944.97	428.01	7117.06
Pdx124MS	36	115.61	932.77	311.09	3012.06	921.62	1049.66	647.97	22100.66
Pdx124MS	37	1599.27	912.28	3791.22	8037.58	621.39	7743.38	439.42	7131.93
Pdx124MS	38	122.94	701.81	8677.14	821.27	729.98	1502.56	532.77	2211.96
Pdx124MS	39	453.15	751.28	870.79	1883.51	340.87	1100.82	212.28	5218.36
Pdx124MS	40	7192.29	799.43	647.68	658.09	55.82	2201.95	401.81	9201.88
Pdx124MS	41	961.81	815.98	621.88	958.09	664.57	1332.09	451.28	5114.87
Pdx124MS	42	498.85	935.94	1158.64	1682.01	913.06	1540.91	319.43	8313.59
Pdx124MS	43	524.76	998.65	1400.11	487.86	529.93	2810.46	15.98	527.61
Pdx124MS	44	337.44	809.52	1178.56	649.11	230.69	1612.05	235.94	
Pdx124MS	45			586.12	1203.86		1350.35	438.65	
	Mean	**3002.358**	**787.0173**	**2006.40**	**1321.165**	**1010.258**	**3577.056**	**874.9556**	**3121.083**
	ST.DEV	**5292.133**	**981.8982**	**2758.37**	**1789.371**	**855.7655**	**3413.759**	**1864.033**	**4676.291**

Table C2. Expression of Ngn3 in islets cultured with or without MSCs

Days in culture		**1**		**3**		**5**		**7**		**9**		**12**	
		NUMBER OF NGN3$^+$ CELLS											
Slide ID	**ISLET NO.**	**MSC+**	**MSC-**	**MSC+**	**MSC-**	**MSC+**	**MSC-**	**MSC+**	**MSC-**	**MSC+**	**MSC-**	**MSC+**	**MSC-**
Ngn324MS	1	2	3	3	8	11	3	6	6	14	5	8	1
Ngn324MS	2	1	10	4	2	9	7	7	7	44	4	2	2
Ngn324MS	3	0	5	7	4	5	7	7	7	23	7	11	2
Ngn324MS	4	1	11	2	8	11	5	4	4	45	7	13	2
Ngn324MS	5	1	9	6	6	9	6	6	6	32	3	17	1
Ngn324MS	6	4	14	1	5	13	5	10	10	34	1	17	0
Ngn324MS	7	2	6	0	3	7	3	9	9	1	0	14	0
Ngn324MS	8	1	8	8	11	8	10	7	9	51	4	44	2
Ngn324MS	9	0	5	2	9	8	10	8	4	21	7	23	4
Ngn324MS	10	0	9	5	5	2	5	8	5	7	5	16	7
Ngn324MS	11	0	11	5	11	4	11	7	5	6	5	34	2
Ngn324MS	12	3	7	7	9	8	9	7	11	8	6	34	9
Ngn324MS	13	1	7	4	13	6	14	3	5	7	34	1	1
Ngn324MS	14	0	5	3	7	5	6	9	1	16	8	5	1
Ngn324MS	15	0	6	2	8	3	8	4	1	11	2	33	0
Ngn324MS	16	3	5	2	5	5	5	5	1	23	5	12	2
Ngn324MS	17	0	3	4	9	9	9	5	0	10	7	40	3
Ngn324MS	18	3	10	3	4	4	11	11	2	10	6	18	7
Ngn324MS	19	0	8	7	8	8	8	5	0	11	3	2	0
Ngn324MS	20	0	7	6	7	7	7	1	1	21	5	22	1
Ngn324MS	21	1	4	4	4	4	4	1	4	52	4	3	1
Ngn324MS	22	1	5	6	5	5	5	1	4	17	6	14	5
Ngn324MS	23	2	4	8	4	4	4	0	3	13	9	17	2
Ngn324MS	24	0	6	5	6	6	6	2	2	2	2	14	2
Ngn324MS	25	1	8	4	8	8	8	0	4	15	2	44	2
Ngn324MS	26	1	5	9	5	5	5	1	6	14	1	23	4
Ngn324MS	27	0	13	6	2	2	13	4	7	40	1	52	1

Ngn324MS	28	0	4	3	4	4	4	4	6	19	0	41	6
Ngn324MS	29	0	4	5	4	4	4	3	8	19	3	34	8
Ngn324MS	30	3	5	9	5	5	5	2	7	32	3	1	1
Ngn324MS	31	0	3	8	3	3	3	4	7	17	3	30	0
Ngn324MS	32	0	8	9	8	8	8	6	7	20	5	13	2
Ngn324MS	33	4	4	8	4	4	4	7	6	30	4	25	3
Ngn324MS	34	1	5	7	1	1	5	6	4	31	6	20	5
Ngn324MS	35	0	8	9	6	6	8	8	4	27	3	30	6
Ngn324MS	36	0	6	8	8	8	6	7	5	31	4	67	5
Ngn324MS	37	0	5	5	2	2	5	7	2	24	5	43	9
Ngn324MS	38	1	3	8	6	6	3	7	5	21	5	21	2
Ngn324MS	39	2	4	7	4	4	4	12	2	22	3	17	3
Ngn324MS	40	3	2	6	2	2	2	13	6	54	7	17	8
Ngn324MS	41	1	5	7	5	5	5	11	5	13	2	14	7
Ngn324MS	42	0	8	8	3	3	8	9	5	43	4	22	1
Ngn324MS	43	0	3	7	8	8	3	16	6	24	5	23	6
Ngn324MS	44	0	5	9	3	5	5	12	4	34	6	31	4
Ngn324MS	45	0	4	8	4	4	4	7	4	1	2	22	0
Ngn324MS	46	2	9	5	9	3	9	10	2		4	21	2
Ngn324MS	47	1	3	8	3	3	3	11	9		5	56	1
Ngn324MS	48	4	5	7	5	5	5	10	5		4	43	9
Ngn324MS	49	1	2	9	11	2	2	9	2			33	3
Ngn324MS	50	2	5		4	4	5	13	0			37	2
Ngn324MS	51		6		5	5	6	12	2			40	1
Ngn324MS	52		4		12	4	4	7	3			22	6
Ngn324MS	53		3		6	3	3	7	4				
MEAN		**1.06**	**5.981**	**5.8**	**5.87**	**4.756**	**5.981**	**6.755**	**4.604**	**22.4**	**4.83**	**24.2**	**3.15385**
STDEV		**1.236**	**2.735**	**2.4**	**2.82**	**2.612**	**2.735**	**3.679**	**2.634**	**13.9**	**4.77**	**14.8**	**2.68171**

Table C3. Insulin expression in islet cultured 24- 30 days

Days in culture		24		26		28		30	
		Insulin fraction (%)							
SLIDE ID	Islet No.	MSC+	MSC-	MSC+	MSC-	MSC+	MSC-	MSC+	MSC-
INS24PPDL	1	6.96	85.74	23.61	79.07	50.74	74.15	21.60	61.61
INS24PPDL	2	11.05	91.O4	80.45	60.48	81.04	73.32	91.27	44.44
INS24PPDL	3	10.95	83.54	60.4	80.35	83.12	89.67	25.18	91.04
INS24PPDL	4	18.61	94.26	10.98	96.68	52.02	96.03	68.86	89.67
INS24PPDL	5	51.48	89.93	69.49	93.69	92.97	88.02	30.30	74.96
INS24PPDL	6	36.88	99.54	82.3	85.24	95.61	95.49	24.21	78.61
INS24PPDL	7	1.19	99.17	84.63	76.58	77.39	97.71	2.87	77.51
INS24PPDL	8	23.61	88.02	77.39	80.36	47.57	90.08	1.80	85.84
INS24PPDL	9	6.18	95.49	17.41	90.01	87.14	88.55	1.28	75.56
INS24PPDL	10	60.4	97.71	87.38	96.36	77.38	88.22	7.66	66.37
INS24PPDL	11	18.61	90.08	70.66	62.03	77.36	89.51	0.97	68.53
INS24PPDL	12	6.96	79.49	75.48	97.43	88.02	73.96	80.42	89.75
INS24PPDL	13	34.57	83.3	80.99	66.2	70.66	77.32	15.43	60.02
INS24PPDL	14	27.82	81.63	24.85	67.81	75.48	85.02	25.18	86.31
INS24PPDL	15	68.45	78.39	29.87	67.49	80.99	87.39	27.28	42.04
INS24PPDL	16	7.88	67.81	80.45	92.58	24.85	95.15	13.51	74.63
INS24PPDL	17	11.89	82.19	61.71	86.17	127.3	76.82	30.42	61.19
INS24PPDL	18	2.01	92.11	63.67	84.65	92.76	95.06	67.23	98.5
INS24PPDL	19	0.61	88.24	52.02	79.28	59.38	88.28	61.97	45.67
INS24PPDL	20	14.86	61.82	92.36	54.73	80.45	93.19	75.82	90.29
INS24PPDL	21	0.08	78.72	50.74	91.35	53.02	90.72	78.99	7.56
INS24PPDL	22	33.07	60.12	81.04	47.12	78.3	85.37	91.96	10.9
INS24PPDL	23	7.56	92.83	32.8	86.83	58.1	96.3	83.33	92.34
INS24PPDL	24	7.77	76.92	52.02	80.41	55.94	94.38	34.00	96.06
INS24PPDL	25	1.22	99.97	92.97	93.03	80.45	94.68	43.22	89.9
INS24PPDL	26	18.31	99.75	95.61	36.61	65.93	82.82	64.59	95.55
INS24PPDL	27	24.73	72.66	79.43	93.02	83.12	92.58	39.98	88.97
INS24PPDL	28	8.63	74.48	85.64	75.4	68.45	94.47	79.93	88.95
INS24PPDL	29	34.83	77.99	63.79	90.89	87.39	79.94	70.66	50.09
INS24PPDL	30	30.74	61.97	69.21	60.46	81.63	84.62	90.28	88.96
INS24PPDL	31	37.3	75.82	30.87	97.05	91.99	75.06	7.10	75.99
INS24PPDL	32	8.64	94.85	77.27	50.53	88.45	85.71	80.34	77.11
INS24PPDL	33	9.42	84.63	8.29	83.99	73.29	91.84	35.57	94.95
INS24PPDL	34	5.2	78.39	39.83	70.59	87.32	83.34	66.16	90.27
INS24PPDL	35	36.43	64.81	46.29	68.98	80.18	86.69	68.53	77.98

INS24PPDL	36	8.66	99.63	27.77	77.26	84.65	90.73	80.16	61.04
INS24PPDL	37	11.63	71.66	62.6	6.58	92.99	94.55	85.19	88.51
INS24PPDL	38	29.86	75.01	51.39	17.84	79.56	97.3	92.99	86.05
INS24PPDL	39	24.04	76.99	65.76	27.28	85.64	95.37	87.24	93.84
INS24PPDL	40	59.8	74.85	34.45	43.71	90.33	94.26	66.82	91.05
INS24PPDL	41	5.98	87.3	43.53	55.35	69.49	88.16	70.72	85.91
INS24PPDL	42	32.23	82.76	155.12	64.48	82.3	98.54	68.12	92.09
INS24PPDL	43		69.38	67.53	65.92	84.63	88.35	98.39	81.58
INS24PPDL	44		81.45	28.56	15.97	87.39	95.34	63.87	82.31
INS24PPDL	45		71.63	29.35	20.37	63.81	87.93	68.57	85.68
MEAN		**20.41**	**82.26**	**63.03**	**69.29**	**77.26**	**88.71**	**53.11**	**76.36**
STDEV		**17.47**	**11.12**	**28.22**	**24.08**	**16.63**	**6.95**	**30.69**	**20.71**

Table C4. Endocrine density of the isografts

	Total endocrine area of isograft		**Total endocrine area of individual Aggregates**			
	Insulin$^+$ Area (µm^2)		**Insulin $^+$ Area (µm^2)**		**Density index**	
Islet No.	**MSC+**	**MSC-**	**MSC+**	**MSC-**	**MSC+**	**MSC-**
1	4946.18	52575.94	1946.18	2625.38	0.39	0.05
2	777132.4	6756.54	677132.43	4994.4	0.87	0.74
3	242116.1	786972	241116.1	8037.78	1.00	0.01
4	253106.6	9796.84	157106.59	4063.52	0.62	0.41
5	150674.4	30851.39	50694.4	3091.99	0.34	0.10
7	38945.35	5832.57	9945.35	5427.22	0.26	0.93
8	26465.56	346619.3	25956.11	9870.49	0.98	0.03
9	83732.97	33690.1	13732.97	5378.23	0.16	0.16
10	1448.35	31483.44	1448.35	5405.32	1.00	0.17
11	404811.1	76144.4	404813.11	23131.16	1.00	0.30
12	286781.7	7678.28	26785.65	6754.82	0.09	0.88
13	70056.63	14191.39	69056.63	856.52	0.99	0.06
14	137690.5	1976.69	137690.49	228.12	1.00	0.12
15	86976.15	84483.38	36976.15	10120.91	0.43	0.12
16	47681.84	237859.3	7388.84	15665.03	0.15	0.07
17	6578.71	32885.57	6558.71	19063.52	1.00	0.58
18	9846.73	28030.38	9746.11	15091.99	0.99	0.54
19	3518.44	503118.9	4518.48	18427.22	1.28	0.04
Mean	**146250.5**	**127274.8**	**104589.59**	**8790.756667**	**0.70**	**0.29**
STDEV	**196070.7**	**214020.4**	**177873.38**	**6758.803572**	**0.38**	**0.31**

Table C5. Cell proliferation in isografts

	Control			MSC-			MSC+		
Slide No.	**Total**	**Ki67+**	**% Ki67+ cells**	**Total**	**Ki67+**	**% Ki67+ cells**	**Total**	**Ki67+**	**% Ki67+ cells**
1	98	12	12	89	7	8	48	9	19
2	11	7	67	65	11	17	39	9	22
3	27	24	89	77	15	19	75	12	16
4	83	13	16	29	14	48	87	11	12
5	49	13	26	64	14	21	65	12	19
6	80	11	13	48	6	13	90	15	16
7	93	11	12	125	11	9	53	14	26
8	77	5	7	159	11	7	35	16	45
9	67	44	66	33	33	100	90	7	8
10	62	4	6	144	15	10	138	17	12
11	82	34	41	138	9	6	55	11	21
12	148	3	2	100	12	12	101	15	15
13	44	14	32	72	5	7	52	11	20
14	78	12	15	183	11	6	84	7	8
15	129	10	8	111	7	6	97	5	5
16	101	5	5	90	14	15	99	10	10
17	99	17	17	41	23	55	64	19	29
18	98	53	54	37	9	24	74	17	22
19	46	9	20	139	12	8	68	6	9
20	36	24	68	72	7	10	31	11	36
21	47	9	19	44	10	23	38	13	34
22	39	10	25	140	12	9	34	14	41
23	71	26	36	141	17	12	140	6	4
24	84	12	14	44	8	18	80	12	15
25	69	30	44	58	13	23	56	15	27
26	61	11	18	49	13	26	91	21	23
27	49	10	20	80	11	13	46	9	20
28	37	15	40	127	11	9	33	16	50
29	44	18	41	64	12	19	28	7	25
Total	**2009**	**466**	**833**	**2563**	**353**	**553**	**1991**	**347**	**609**
Mean	**69.28**	**16.07**	**28.72**	**88.38**	**12.17**	**19.07**	**68.66**	**11.97**	**21.00**
STDEV	**30.59**	**11.79**	**22.35**	**43.49**	**5.42**	**19.36**	**29.99**	**4.14**	**11.65**

Table C6. Ngn3 expression in islets cultured with or without MSCs

DAYS IN CULTURE		24		26		28	
		Ngn3+ area (µm²)					
SLIDE ID	Islet No.	MSC+	MSC-	MSC+	MSC-	MSC+	MSC-
Ngn324PPDL	1	12863.5	815.74	23.61	6579.07	3107.71	474.15
Ngn324PPDL	2	117065.1	921.O4	80.45	660.48	1292.42	743.32
Ngn324PPDL	3	53257.4	873.54	60.4	4580.35	25758.14	1189.67
Ngn324PPDL	4	32574.5	974.26	1770.98	946.68	22551.71	926.03
Ngn324PPDL	5	49674.3	189.93	8769.49	793.69	30929.07	828.02
Ngn324PPDL	6	26306.9	699.54	4182.3	2285.24	31156.02	195.49
Ngn324PPDL	7	37908.1	939.17	6684.63	4776.58	23226.56	3397.71
Ngn324PPDL	8	52984.9	848.02	2277.39	2110.36	4299.88	930.08
Ngn324PPDL	9	35247.0	945.49	17.41	2110.01	1628.13	858.55
Ngn324PPDL	10	17771.0	917.71	87.38	956.36	3725.23	188.22
Ngn324PPDL	11	51357.1	390.08	70.66	682.03	3618.43	389.51
Ngn324PPDL	12	38533.1	79.49	75.48	1767.43	941.96	1473.96
Ngn324PPDL	13	15467.6	183.3	80.99	6126.2	16243.87	777.32
Ngn324PPDL	14	99986.3	81.63	24.85	67.81	12037.93	85.02
Ngn324PPDL	15	37989.5	378.39	29.87	67.49	11663.13	827.39
Ngn324PPDL	16	21991.6	67.81	80.45	92.58	5528.94	795.15
Ngn324PPDL	17	18826.9	862.19	61.71	86.17	37380.76	756.82
Ngn324PPDL	18	116024.1	912.11	63.67	84.65	3087.86	295.06
Ngn324PPDL	19	11521.6	188.24	52.02	79.28	1883.3	2288.28
Ngn324PPDL	20	10878.0	161.82	92.36	54.73	1203.71	9113.19
Ngn324PPDL	21	16532.1	378.72	50.74	91.35	21581.17	4390.72
Ngn324PPDL	22	247238.5	1260.12	81.04	47.12	6197.02	585.37
Ngn324PPDL	23	29380.0	92.83	32.8	86.83	55117.27	296.3
Ngn324PPDL	24	10849.8	76.92	52.02	80.41	6755.99	914.38
Ngn324PPDL	25	11433.4	99.97	92.97	93.03	93076.14	394.68
Ngn324PPDL	26	50379.64	5099.75	95.61	36.61	7271.13	182.82
Ngn324PPDL	27	545224.6	672.66	79.43	93.02	8509.04	912.58
Ngn324PPDL	28	166525.3	9774.48	85.64	75.4	8929.96	594.47
Ngn324PPDL	29	41134.95	577.99	63.79	90.89	3498.29	779.94
Ngn324PPDL	30	13933.58	6651.97	69.21	60.46	9056.6	874.62
Ngn324PPDL	31	91248.27	7095.82	30.87	97.05	13060.36	975.06
Ngn324PPDL	32	978195.3	994.85	77.27	50.53	17213.6	3815.71
Ngn324PPDL	33	27601.52	784.63	7198.29	83.99	10339.9	4911.84
Ngn324PPDL	34	141161.1	978.39	3449.83	70.59	3036.75	4873.34
Ngn324PPDL	35	11233.06	8764.81	46.29	68.98	5244.07	896.69
Ngn324PPDL	36	467755.7	2399.63	6527.77	77.26	5459.48	3590.73
Ngn324PPDL	37	237041.5	3471.66	6102.6	456.58	116351.2	694.55
Ngn324PPDL	38	39735.39	975.01	951.39	817.84	9327.39	1897.3
Ngn324PPDL	39	15894.96	1176.99	3265.76	527.28	91248.27	955.37
Ngn324PPDL	40	42870.3	1674.85	1134.45	243.71	45195.25	2294.26
Ngn324PPDL	41	122547.6	3867.3	4113.53	565.35	27601.52	3878.16
Ngn324PPDL	42	5367.82	2882.76	2155.12	674.48	41161.1	3398.54

Ngn324PPDL	43	63263.4	969.38	6887.53	65.92	71233.06	5488.35
Ngn324PPDL	44	29663.44	4781.45	28910.56	175.97	4755.74	3195.34
Ngn324PPDL	45	8168.34	3741.63	17629.35	990.37	57041.46	1287.93
Ngn324PPDL	46	67134.09	9912,4	1975.01	2855.8	7399.63	7334.01
Ngn324PPDL	47	90776.09	7769.11	1176.99	1712.9	7471.66	2433.11
Ngn324PPDL	48	99090.01	4768.05	21248.27	809,88		799.02
Ngn324PPDL	49	109775.1	6222.08	1799.09	845.01		4626.45
Ngn324PPDL	50	88875.01		9971.34	1912.99		5713.52
MEAN		**94565.16**	**2123.84**	**5527.03**	**978.67**	**21767.26**	**1990.36**
STDEV		**164692.8**	**2390.68**	**5291.37**	**24.08**	**27117.29**	**2048.84**

Table C7. Surface area of islets (µm²)

	Total size of islets (µm²)					
	0 DAY		15 DAYS		28 DAYS	
ISLET NO.	ISLETS/MSC+	ISLET/MSC-	ISLETS/MSC+	ISLET/MSC-	ISLETS/MSC+	ISLET/MSC-
1	14655.87	3441.87	31156.02	3998.75	50379.64	2831.01
2	8037.58	2819.72	23226.56	3438.16	545224.57	438.16
3	10725.27	1601.01	4299.88	2461.82	166525.33	461.82
4	9373.68	1567.36	1628.13	2178.19	41134.95	178.19
5	4996.62	1183.35	3725.23	6022.9	13933.58	6022.9
6	2664.57	1316.69	3618.43	20737.14	91248.27	876.66
7	3181.93	1250.46	12941.96	4729.14	8195.25	1095.22
8	10828.96	2297.9	16243.87	63472.69	27601.52	8737.14
9	7728.36	5757.71	12037.93	7956.24	41161.1	729.14
10	7679.88	2920.39	11663.13	6493.01	11233.06	3472.69
11	4792.87	4418.16	5528.94	16276.39	344755.74	34956.24
12	8344.59	3136.89	37380.76	8657.89	127041.46	3493.01
13	8468.14	4945.77	3087.86	2321.11	223735.39	16276.39
14	9601.01	909.52	221883.3	1986.05	801894.96	1657.89
15	9071.22	2702.36	19203.71	10891.7	872870.3	6321.11
16	1212.46	21672.7	21581.17	30291.38	111547.56	1986.05
17	1617.96	1428.01	6197.02	14917.1	545367.82	10891.7
18	6856.52	1647.97	55117.27	47804.89	312263.4	1291.38
19	18228.12	1439.42	60755.99	27126.09	2663.44	14917.1
20	1455.47	2632.77	93076.14	7564.91	8168.34	7804.89
21	1994.44	6292.28	7271.13	14954.91	3107.71	7126.09
22	1786.87	8401.81	8509.04	10272.37	1292.42	7564.91
23	10120.91	4551.28	8929.96	7379.74	25758.14	14954.91
24	4994.4	359.43	3498.29	2023.87	22551.71	10272.37
25	1675.67	1315.98	9056.6	31715.45	30929.07	7379.74
26	927.79	9335.94	13060.36	1356.04	31156.02	2023.87
27	1772.9	8438.65	17213.6	15966.35	23226.56	715.45
28	3131.16	6762.84	10339.9	1732.12	4299.88	956.04
29	5665.03	8150.78	3036.75	1393.01	1628.13	966.35
30	3091.99	1419.61	5244.07	1062.46	3725.23	732.12
31	4018.91	759.36	5459.48	844.76	3618.43	393.01
32	1571.3	1055.53	116351.2	26753.62	941.96	562.46
33	11910.2	2442.49	9327.39	13258.7	16243.87	9307.5
34	2238.11	7332.96	91248.27	1501.72	12037.93	753.62
35	14063.52	1446.96	45195.25	119.88	11663.13	3258.7
36	7436.18	1334.43	27601.52	4358.06	5528.94	501.72
37	12620.59	1336.89	41161.1	1515.91	37380.76	119.88

38	108959.1	1324.16	71233.06	1424.44	3087.86	358.06
39	7944.97	2581.51	4755.74	1204.99	11883.3	1515.91
40	9049.66	5404.2	4991.95	619.64	1203.71	924.44
41	6743.38	2108.68	9931.59	1013.84	21581.17	704.99
42	9502.56	8246.77	14657.91	3575.76	6197.02	619.64
43	10174.82	9329.56	7920.44	2831.01	55117.27	913.84
44	7201.95	2205.12	27378.2	1552.6	1755.99	1575.76
45	6332.09	1944.16	5458.09	843.28	23076.14	777.75
46	13543.91	4741.08	1858.09	1357.48	7271.13	505.46
47	2815.46	6288.18	31618.01	1900.71	8509.04	661.53
48	14612.05	5070.12	41005.86	244.74	8929.96	744.84
49	5358.35	2185.42	1649.11	1607.44	3498.29	1930.4
50	9274.57	1320.62	7203.86	1537.86	9056.6	634.11
51	922.57	5289.25	1046.57	1283.44	13060.36	834.21
MEAN	**8842.68**	**3879.73**	**25442.46**	**8755.52**	**93260.07**	**4014.28**
STDEV	**14924.05**	**3618.32**	**38104.85**	**12689.66**	**195174.05**	**6123.03**

Table C8. Co-expression of Pdx1 and insulin in islets cultured with or without MSCs											
		1		3		5		7		9	
		Fraction of Pdx1+/ Insulin+ area (%)									
SLIDE ID	Islet No.	MSC+	MSC-	MSC+	MSC-	MSC+	MSC-	MSC+	MSC-	MSC+	MSC-
PDX1/INS24MS	1	12.5	43.2	9.2	9.2	10.9	12.8	3.0	70.8	1.3	31.8
PDX1/INS24MS	2	15.0	15.0	8.8	8.8	37.5	20.4	4.4	45.2	1.2	19.4
PDX1/INS24MS	3	5.9	2.2	1.1	1.1	2.6	4.4	5.9	95.2	2.9	18.2
PDX1/INS24MS	4	8.7	4.5	27.3	97.3	7.6	97.0	8.7	62.3	0.8	12.8
PDX1/INS24MS	5	10.1	10.8	3.9	3.9	18.2	16.8	1.1	64.8	1.5	39.0
PDX1/INS24MS	6	10.7	2.8	15.9	15.9	42.3	8.2	1.7	0.5	1.0	47.7
PDX1/INS24MS	7	12.5	57.7	6.7	32.5	3.3	16.1	1.5	5.1	2.3	53.4
PDX1/INS24MS	8	15.0	3.7	7.5	24.8	4.8	4.7	1.0	2.4	1.1	50.3
PDX1/INS24MS	9	16.3	4.6	2.1	30.2	8.6	6.7	1.3	2.3	1.4	12.9
PDX1/INS24MS	10	18.0	47.8	2.0	18.4	8.2	6.6	2.0	5.6	1.7	10.9
PDX1/INS24MS	11	36.3	6.6	6.7	61.2	15.8	4.1	1.3	32.5	2.0	17.2
PDX1/INS24MS	12	40.2	32.4	40.0	43.0	5.1	22.4	2.2	24.8	1.6	99.6
PDX1/INS24MS	13	50.4	30.6	3.2	3.2	3.8	1.9	1.4	30.2	1.2	6.3
PDX1/INS24MS	14	52.5	2.8	34.6	2.0	1.9	0.9	52.5	18.4	2.7	3.0
PDX1/INS24MS	15	70.4	57.7	9.9	20.0	1.8	10.7	2.4	7.8	0.6	14.1
PDX1/INS24MS	16	95.2	3.7	4.0	3.2	1.2	20.4	1.2	16.5	2.2	3.3
PDX1/INS24MS	17	76.8	4.6	6.3	6.3	8.0	12.7	2.8	6.2	1.9	8.1
PDX1/INS24MS	18	51.5	6.7	3.6	3.6	9.7	3.7	1.5	3.5	1.4	6.9
PDX1/INS24MS	19	36.1	52.5	18.4	18.4	2.8	16.6	3.1	29.2	2.2	51.0
PDX1/INS24MS	20	14.5	4.7	16.4	6.4	12.90	9.3	0.5	31.4	1.3	13.9
PDX1/INS24MS	21	11.9	9.7	64.4	34.4	5.53	89.1	1.9	3.1	5.7	39.4
PDX1/INS24MS	22	8.2	7.3	75.6	34.7	2.60	76.9	0.2	18.1	0.9	14.7
PDX1/INS24MS	23	7.3	0.5	5.7	5.9	7.63	19.4	1.6	21.9	0.1	17.3
PDX1/INS24MS	24	3.5	19.4	14.2	14.2	2.25	16.5	1.3	20.1	1.5	18.6
PDX1/INS24MS	25	3.3	20.4	11.0	11.0	6.26	19.3	3.3	3.2	1.0	19.4
PDX1/INS24MS	26	2.9	4.4	14.9	24.9	12.32	38.4	2.9	31.3	1.6	19.6
PDX1/INS24MS	27	2.6	0.3	67.5	29.2	4.81	31.3	2.6	11.9	2.7	11.3
PDX1/INS24MS	28	1.3	12.8	24.3	3.4	8.60	11.9	1.3	10.4	0.2	16.1
PDX1/INS24MS	29	1.0	20.4	5.7	16.5	8.24	4.3	1.0	2.0	1.5	19.0
PDX1/INS24MS	30	0.6	4.4	24.3	94.3	3.81	10.2	0.6	1.1	1.5	13.3
PDX1/INS24MS	31	0.5	57.7	33.4	33.4	4.13	35.2	0.5	29.2	1.2	10.4
PDX1/INS24MS	32	2.0	9.7	14.0	24.0	3.76	43.2	2.0	3.4	1.6	12.7
PDX1/INS24MS	33	64.1	4.6	18.9	18.9	1.92	15.0	2.1	16.5	2.6	14.5
PDX1/INS24MS	34	2.0	17.8	4.6	4.6	1.82	2.2	2.0	0.4	0.9	14.8
PDX1/INS24MS	35	12.9	97.0	23.9	1.3	1.17	4.5	1.9	31.2	1.4	11.8
PDX1/INS24MS	36	12.7	16.8	45.0	2.8	1.02	10.8	6.7	2.8	2.1	15.7
PDX1/INS24MS	37	1.6	22.4	5.8	15.8	2.65	2.8	1.6	10.0	1.2	15.4
PDX1/INS24MS	38	7.4	11.9	17.3	19.8	2.83	57.7	1.4	49.9	0.7	16.1

PDX1/INS24MS	39	1.2	0.9	4.6	11.5	9.7	3.7	1.2	64.3	5.4	13.9
PDX1/INS24MS	40	1.8	10.7	6.3	16.3	7.3	4.6	0.8	2.5	1.3	15.0
PDX1/INS24MS	41	1.8	15.2	27.6	27.6	0.5	47.8	1.8	61.7	0.3	14.7
PDX1/INS24MS	42	1.4	16.1	18.1	18.1	0.3	16.6	7.4	2.9	2.0	19.9
PDX1/INS24MS	43	21.2	23.7	24.1	20.1	23.7	32.4	5.2	31.2	0.4	1.0
PDX1/INS24MS	44	1.3	11.19	14.9	44.9	5.3	63.6	5.3	41.2	7.1	12.8
PDX1/INS24MS	45	11.2	10.19	19.3	25.3	6.5	36.7	9.2	2.0	0.3	0.5
PDX1/INS24MS	46	1.3	30.98	35.4	37.4	1.6	62.5	12.3	1.0	3.6	0.6
PDX1/INS24MS	47	24.3	42.04	31.5	33.5	9.5	24.7	7.3	16	13.6	9.5
PDX1/INS24MS	48	40.5	16.5	18.1	22.9	12.4	9.5		44.8	15.0	20.4
PDX1/INS24MS	49	70.6	50.4		36.0		4.4			5	
	MEAN	**19.8**	**19.6**	**18.7**	**22.3**	**7.6**	**22.3**	**3.9**	**22.7**	**2.3**	**19.3**
	SDEV	**23.63**	**20.1**	**17.0**	**20.2**	**8.3**	**23.1**	**7.6**	**22.7**	**2.8**	**17.2**