

**CELLULAR MECHANISMS INVOLVED IN THE RECAPITULATION OF  
ENDOCRINE DEVELOPMENT IN THE DUCT LIGATED PANCREAS**

by

**Venant Tchokonte-Nana**

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Division of Anatomy and Histology  
Department of Biomedical Sciences  
Faculty of Health Sciences

Promoters: Professor Benedict J. Page  
Professor Don F. du Toit  
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## **Abstract**

Diabetes mellitus is amongst the leading causes of morbidity and mortality in the world, affecting young, adult and old people. Beta cell replacement therapy for insulin delivery remains the ultimate remedy for diabetes. However, insufficient donor pancreas and the use of immunosuppressive drugs prevent the wide-spread of this therapy. Other avenues of self generated beta cells within the organ itself need to be explored. Therefore, understanding the chronobiology of cellular mechanisms in the lineage of beta cell induced neogenesis is a valuable tool in improving beta cell replacement in patients with diabetes. The aim of this study was to induce recapitulation of the morpho-genetic sequence of endocrine cells development in the pancreas of rats after the pancreatic duct ligation (PDL) procedure. Serial sections of PDL tissues of the pancreas were obtained from 78 Sprague-Dawley rats and were assessed morphologically. The immunofluorescent tissues were statistically analysed using a computerized morphometry technique. The protein expression indices of Caspase3, Insulin, Pdx1, Ngn3, NeuroD and Pax6 were quantified. The efficiency levels of co-expression of these homeodomain proteins separately with insulin were defined by the ratio of the mean value of insulin expression to the mean value of their respective protein expression. The morphological changes were characterized by the appearance of granulated acinar cells at 6 hours post-PDL and the proliferation of endocrine tissues from 84 hours through to 120 hours. The morpho-immunofluorescent evaluation showed the highest immunoreactivity of Caspase3 and Pdx1 at 6 hours, Ngn3 at 36 hours, Pax6 and insulin at 84 hours while NeuroD expression was at 120 hours. The immunohistofluorescent analysis showed that caspase3 and Pdx1 were the first to be expressed at 6 hours while the insulin and NeuroD expression appeared later at 84 hours and 120 hours, respectively. However, Pax6 expression was continuous across time periods post-PDL, while Ngn3 expression showed a peak at 36 hours. The efficiency (highest and earliest expression) of co-

expression of all these homeodomain proteins with insulin was restricted between 12 hours and 24 hours. The optimal efficiency was at 12 hours by Ngn3 with insulin. A good efficiency was shown for Pdx1 with insulin, NeuroD with insulin and Pax6 with insulin at 12 hours and 24 hours, respectively. A low efficiency was observed for insulin and caspase3 co-expression at 24 hours. This study suggests that for transplantation, PDL tissues harvested at an early time post-PDL (between 12 and 24 hours) could yield a higher success rate; the study also provides evidence for a connection between morphological changes in the PDL pancreas and the protein synthesis necessary for the lineage of endocrine cell development.

## **Opsomming**

Diabetes Mellitus resorteer onder die vernaamste oorsake van morbiditeit en mortaliteit wêreldwyd, en tuister jongmense, volwassenes en bejaardes. Daar bestaan egter 'n wêreldwye tekort aan skenkerorgane met immuun-onderdrukings terapie as ondersteuningsbehandeling. Beta-sel vervangingsterapie, vir die voorsiening van insulien, bly daarom die voorkeur behandeling vir die siekte wat noodsaak dat die wetenskap kyk na alternatiewe behandelingsregimens wat meganismes rondom orgaanregenerasie insluit. Begrip van die chronobiologie van die sellulêre meganismes betrokke rondom beta-sel ontwikkeling mag waardevolle lig werp op die neogenese van beta-selle wat gevolglik daartoe mag lei dat beta-sel vervanging as 'n moontlike behandelingsterapie oorweeg mag word vir pasiënte met suikersiekte. Die oogmerk van hierdie studie is om die rekapitulering van die morfo-genetiese volgorde van die endokriene pankreas na afbinding van die pankreasbuis te bepaal. Pankreasbuis afbinding is op 78 Sprague-Dawley laboratorium rotte onder algemene narkose uitgevoer, die pankreas is na voorafbepaalde tydsvakke verwyder en in histologiese seriesnitte gesny. Snitte is immunositochemies gekleur en morfometries assessee. Die afskeidingsindeks vir selboodskappers vir Caspase3, Insulien, Pdx1, Ngn3, NeuroD en Pax6 is kwantifiseer. Die gelyktydige afskeiding van elk van bogenoemde boodskappers tesame met insulien is omskryf as 'n verhouding tot mekaar en in terme van dié van insulien. Die morfologiese verandering in die weefsel bespeur is gekenmerk deur die verskyn van gegranuleerde asinêre selle ses (6) ure na buisafbinding en die proliferasie van endokriene weefsel vanaf vier-en-tagtig (84) ure deurlopend tot een-honderd-en-twintig (120) ure. Die morfo-immunofluoresserende evaluering toon dat Caspase3 en Pdx1 by 6 uur die hoogste is, die van Ngn3 by 36 ure, Pax6 en insulien by 84 ure en NeuroD by 120 ure. Verder toon die analise dat Caspase3 en Pdx1 rondom 6 ure hul verskyning gemaak het terwyl dié van insulien en NeuroD eers rondom 84 tot 120 uur verskyn het.

Die verskyning van Pax6 het deurlopend regoor al die tydsduurtes verskyn en Ngn3 het rondom 36 uur sy hoogste vlak bereik. Die gelyktydige uitdrukking van homeodomein proteïene tesame met insulien het slegs tussen die tydperke van 12 en 24 ure plaasgevind. Die uitdrukking van Pdx1 met insulien, NeuroD met insulien en Pax6 met insulien het almal tussen 12 en 24 ure plaasgevind. Caspase3 tesame met insulien is slegs by die 24 uur tydsperiode bespeur. Vir die oorplant van pankreas weefsel wat aan buisafbinding onderwerp is suggereer hierdie studie dat die geskikste tyd vir die oes van endokriene weefsel liever vroeër (12 to 24 ure) as later uitgevoer behoort te word. Verder wil dit voorkom of hierdie tydsperiode ook die hoogste seltelling lewer. Die studie lewer waardevolle inligting oor die verwantskap tussen die morfologiese veranderings wat na buisafbinding plaasvind en die proteïen sintese wat sel-opvolgontwikkeling bevorder.

## **Peer reviewed presentations and publications**

- Morphogenetic and clinical perspectives on the pancreatic duct ligation induced islet cells neogenesis: A Review  
In-press, Journal of Advances in Clinical and Experimental Medicine.
- Efficiency of co-expression of transcription factors Pdx1, Ngn3, NeuroD and Pax6 with insulin: A statistical approach  
In-press, International Journal of Diabetes Mellitus
- Gene expression versus morphological changes in PDL pancreas : A chronobiology study of the remodelling of endocrine development in rats  
Islet Society Annual Meeting Proceedings, Stockholm, July 2010.  
[http://www.isletsociety.org/abstract\\_files/217/Islet%20Meeting%20abstract2010.pdf](http://www.isletsociety.org/abstract_files/217/Islet%20Meeting%20abstract2010.pdf)
- Inter-relation between insulin, Pdx1, Ngn3, NeuroD, Pax6 and caspase3 gene expression in PDL rats: Induction of Beta cells neogenesis?  
WSU International Research Conference Proceedings, Eastern Cape, August 2010

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## **Definition of terms**

Amplification:	expanding the response to a low intensity signal
Antibody:	immune system-related protein
Antigen:	substance that stimulates the release of antibodies
Antigenicity:	the capacity to react with antibody/to stimulate production of antibodies
Apoptosis:	programmed cell death
Cell signaling:	complex system of communication that govern cellular activities
Cytodifferentiation:	gradual transformation from an undifferentiated to a fully differentiated cell
Differentiation:	process in which unspecialized cells are modified to achieve a specific state
Gastrulation:	a process by which the three germ cell layers are acquired
Morphogenesis:	differentiation and growth of cells and tissues which result in establishing the form of various organs
Neogenesis:	the process of repair, reproduction, or replacement of lost or injured cells, tissues or organs
Organogenesis:	the formation and differentiation of organs and organ systems during embryonic development
Proliferation:	reproduction and multiplication (growth) of similar cells
Transcription:	the process by which messenger RNA is formed from a DNA template
Transdifferentiation:	process by which a non-stem cell transforms into a different type of cell or when an already differentiated stem cell creates cells outside its already established differentiation path



## Abbreviations

ACTH	adrenocorticotropic hormone
AR	antigen retrieval
ANOVA	analysis of variances
bHLH	basic helix-loop-helix
Brn4	brain-4
CAQIA	computer-assisted quantitative image analysis
CARD	catalyzed replacement deposition
CCK	cholecystokinin
Cfr	conferon
Cy-3	cyanine-3
DAB	diaminobenzidine
DAPI	4,6-diamindino-2-phenylindole
DIF	double-label immunofluorescence
DLL1	notch high delta like-1
DM	diabetes mellitus
DNA	deoxyribonucleic acid
e	embryonic day
EC	enterochromaffin cells
ES	embryonic system cell
FITC	dichloro triazinyl amino fluorescein
Foxa	forhead-box-a
GHS-R	growth hormone secretagogue receptor

GIP	gastric inhibitory peptide
GLUT-2	glucose transporter 2
Hb9/Hlxb9	homeobox gene-9 motor neuron-specific
Hes	hairy and enhancer of split
HH	hedgehog
HIER	heat induced epitote retrieval
HNF	hepatocyte nuclear factor
IDDM	insulin dependent diabetes mellitus
Ig	immunoglobulin
IHC	immunohistochemistry
ISHH	in site hybridized histochemistry
Isl1	transcription factor homeodomain islet 1
MODY	maturity-onset diabetes of young
NeuroD/Beta2	human neurogenic helix-loop-helix protein gene
Ngn3	neurogenin 3
Nkx	homeobox gene
Pax	paired box gene
PBS	phosphate buffered saline
Pbx1	pre-B-cell leukemia homeobox 1
PCR	polymerase chain reaction
PDL	pancreas duct ligation
Pdx1/IPF1/IDX1	pancreatic duodenal homeobox-1/insulin promoter factor-1/islet duodenal homeobox-1
PIFD	parallel-approach immunofluorescence dual labeling

PP/F/D1	protein polypeptide cell
PTF1	pancreatic transcription factor 1
RBP-jk	recombination signal-binding protein-j kappa
RNA	ribonucleic acid
RT	room temperature
Shh	sonic hedgehog
SIF	simple-label immunofluorescence
STF-1/GSF	somatostatin factor-1/glucose sensitive factor
TH	tyrosine hydroxylase
Type 2 Diabetes	non-insulin dependent diabetes mellitus
VIP	vaso active intestinal peptide

## **Chapter 1. Introduction**

### **1.1. Literature Review**

#### **1.1.1. Historical background of the pancreas**

The name "pancreas" meaning, "all flesh" was attributed to the organ by Ruphos of Ephesus an anatomist / surgeon in the first or second Century AD; the same name was given to the organ four hundred years earlier when Herophilus, also a Greek anatomist and surgeon (born in 336 BC) described the pancreas for the first time; he is said to be the Father of Scientific Anatomy (Fitzgerald 1980; Howard and Hess 2002).

The pancreas was thought to serve as a protection and support to the large blood vessels lying immediately posterior to it, so Galen (Claudius Galenus 138-201 AD) believed. For being the "Physician to the Gladiators" of Rome, as well as to the Roman Emperor, Galen's incorrect assumption held back enthusiastic scientific investigators until the eighteenth century. Andreas Vesalius (1514-1564) was the first who fairly described the pancreas as "glandulous organ" (Fitzgerald 1980; Singer 1957). However, Thomas Wharton (1610-1673) who was a prosector noted the similarity between the thyroid gland and the pancreas and confirmed the early description by Vesalius (Mettler 1947).

Although the pancreas was described and named, its function and microscopic features remained a mystery. Serious study of the pancreas commenced with the discovery of the pancreatic duct by a German émigré in 1642, Johann George Wirsüng. Wirsüng however was not aware of the function of the duct;

he posed the question, "Is it an artery or a vein, I have never seen blood in it". His colleague later named it "The Duct of Wirsüng" (Howard and Hess 2002). At the same time, Franciscus de le Boe (also called Sylvius) suspected that the duct served as the passage for the secreted juice into the intestine (Busnardo *et al.* 1983). In 1742 Santorini mentioned the accessory duct in its illustration that carried its name (Fitzgerald 1980). Meckel however, is presented as the person who first described a comprehensive embryology of the pancreas in 1806 (Brunschwig 1942).

Claude Bernard (1813-1875) first described the function of the pancreas in digestion, and is considered as the "father of experimental medicine in the artificial production of disease by means of chemical and physical manipulation" (Garrison 1929). In 1852, D. Moyses first described the histology of the pancreas in his thesis; although crudely drawn, the pancreas depicted the structure of exocrine acini. Laguesse first called the "islet of Langerhans" island of Langerhans in 1893, following the first description of the pancreatic islet by Paul Langerhans ("Junior") in collaboration with Professor Rudolph Virchow in 1869. The description by Langerhans was the first good histological description of the endocrine pancreas (Whipple 1960).

All these discoveries led to a fascinating era in the history of the pancreas in the 19<sup>th</sup> Century. Frederick Grant Banting (1891-1941) and Charles Herbert Best (1899-1978) discovered insulin, the first islet cell hormone to be described. In 1921 and later again in 1958 Frederick Sanger (b. 1918) of England, determined the molecular structure of insulin (Howard and Hess 2002).

A disturbance in the islet microanatomy as well as any disruption in the beta cell mass balance may impair pancreatic islet function (Bernard *et al.* 1999) leading to the pathogenesis of diabetes. The disease known as diabetes mellitus (DM), however (a name given by Aretaeus who lived in Asia Minor (ca A.D. 81-150) described overabundant urine (polyuria), unquenchable thirst (polydypsia), a sweet-tasting urine (glucosuria), weight loss or even death, as the clinical symptoms of DM.

Allen O. Whipple (1881-1963) is known as the "Father of Pancreatic Surgery". The first human pancreatic transplant of the modern era was performed by a surgical team led by Dr. Kelly on December 17, 1966 at the University of Minneapolis. The patient who was a 28-year-old female with uncontrolled diabetes and renal failure received a cadaveric kidney and pancreas. The grafts functioned for almost two months (Howard and Hess 2002; Kelly 1967).

## **1.1.2. The Morphology of the Pancreas**

### **1.1.2.1. The human pancreas**

The pancreas is a lobulated gland, similar in structure to the salivary glands, though softer and less compactly arranged than those organs; it extends nearly transversely across the posterior abdominal wall from the curvature of the duodenum to the spleen, posterior to the stomach (Fig. 1) (Gray 1995).

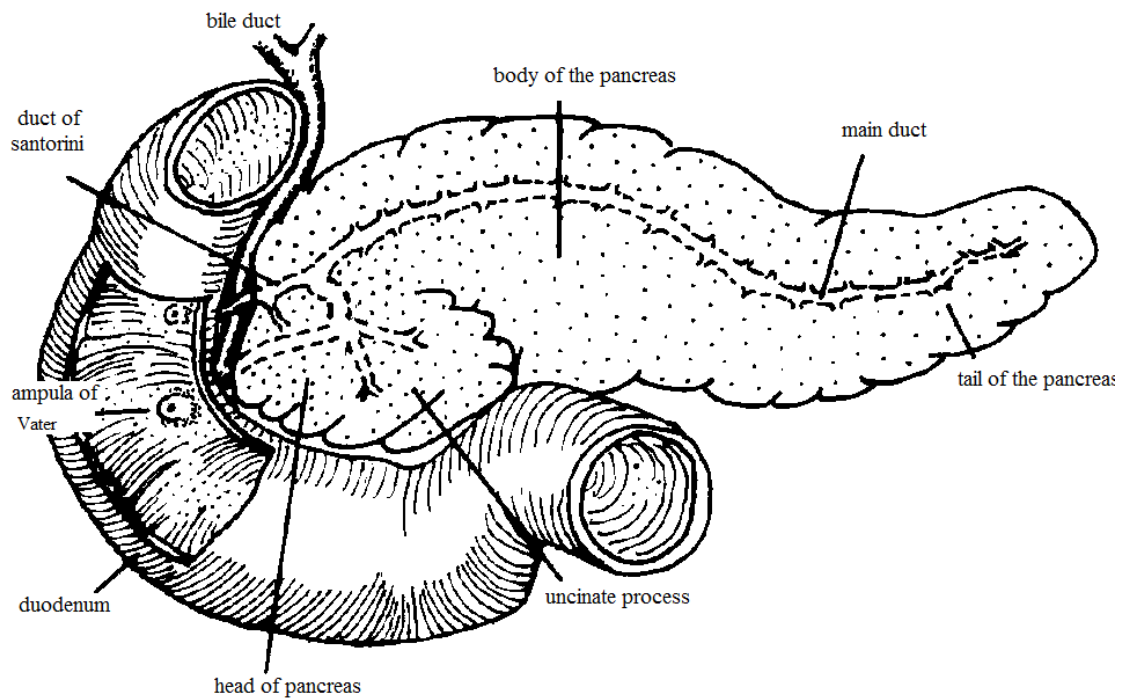
In humans, the pancreas measures between 12.5-25 cm in length and weighs between 60-150 grams (Gray 1995; Slack 1995). The pancreas consists of the head, neck, tail and uncinate processes. The large head joins the body (major part of the gland) on its

right; through a constricted neck. The head of the pancreas is flattened anteroposteriorly with small portions embedded in the wall of the descending part of the duodenum forming the uncinata processes of the pancreas. Both are in close contact with the abdominal aorta anteriorly. A groove formed by the anterior boundary between head and neck gives room to the gastroduodenal artery.

On the right hand side of the head posteriorly as well as the left hand side of the head, the union of the superior mesenteric and splenic veins is contained in a deep incisure where forming the origin of the portal vein. The neck of the pancreas forms a forward, upward curve to the left from the head, joining with the body. The body has three obliquely set surfaces namely, anterior, posterior and inferior. The posterior surface of the body is devoid of peritoneum, is in contact with the aorta and is where begin the superior mesenteric artery, the left crus of diaphragm, the left suprarenal gland, the left kidney and renal veins. The splenic vein runs from the left to the right; forming a partition between it and the above mentioned structures. The tail of the pancreas and the splenic vessels form the content of the space between the two layers of the splenorenal (lienorenal) ligament (Gray 1995).

The lobes of the pancreas are made of lobules from each of which small ducts (interlobular ducts) emerge. The interlobular ducts give rise to intralobular ducts which in turn become intercalated ducts as they enter the substance of the lobules where they lie between the secretory units and the intralobular ducts. Intercalated ducts open into the lumen of acinar cells as centroacinar ducts. Most interlobular ducts join the main pancreatic duct (duct of Wirsüng) at right angle forming a Herring-bone configuration. The main pancreatic duct extends from the tail of the

gland to the body and the neck, and increasing in size as many other ductules join along its path. The main pancreatic duct lies nearer the posterior surface than the anterior surface of the gland. The main pancreatic duct is whitish in colour and it is found posterior to the head of pancreas; it first curves downwards then to the right just before it joins the common bile duct on its right side. The main pancreatic duct enters obliquely into the wall of the descending part of the duodenum, where it unites with the common bile duct in a short dilated hepatopancreatic ampulla (ampulla of Vater) to open on the major duodenal papilla, which lies posteromedial to the duodenum. There is an accessory pancreatic duct (duct of Santorini) which is formed by fusion of small ducts from the lower and left portion of the head of the pancreas; it communicates with the main duct, and drains the lower part of the head of the pancreas, it enters the duodenum at the minor duodenal papilla (Gray 1995).

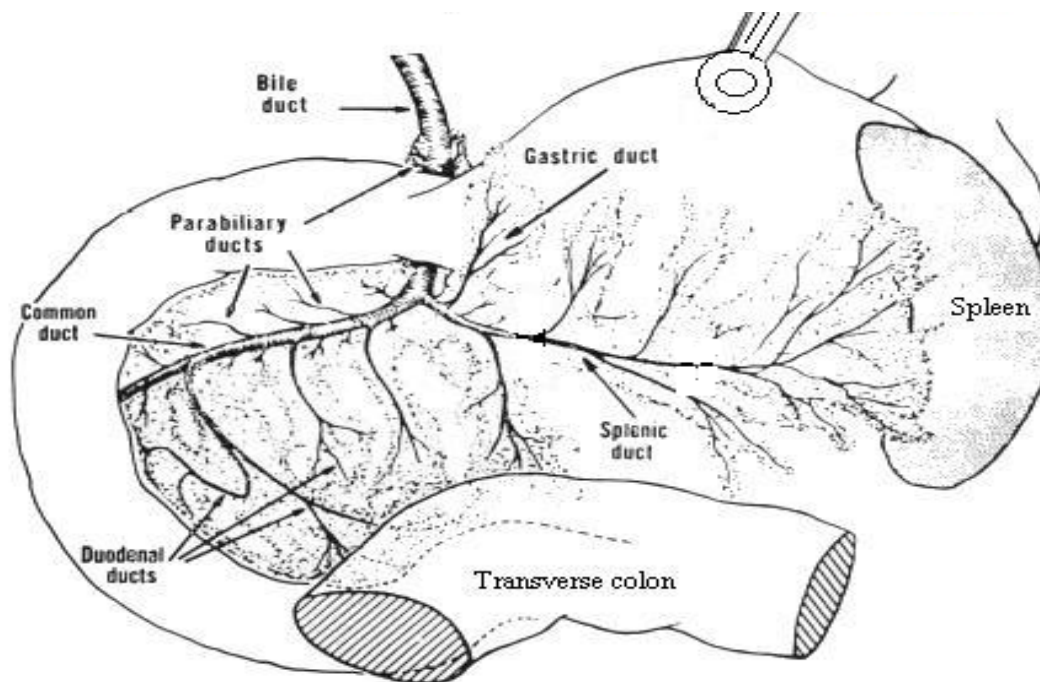


**Figure 1.** The human pancreas as seen in the abdomen (Modified from Slack 1995).



### 1.1.2.2. The rodent pancreas

In rodents the pancreas is diffuse and the shape cannot be well determined (Slack 1995). It weights between 550 mg (at 100 g-body weight) to 1 g (at 300 g-body weight) (Richards *et al.* 1964). The pancreas is found in the craniodorsal part of the abdominal cavity in the rat and could be divided into two parts. The first part is made of the body and right lobe; this is embedded in the mesoduodenum and the beginning of the mesojejunum. A second part, which is a branched flattened left lobe, is partially fused to the ascending colon and blankets the superior mesenteric-portal vein. It then runs along the dorsal aspect of the stomach, embedded in the dorsal part of the greater omentum, and along the lineal artery toward the intestinal surface of the spleen (Hebel and Stromberg 1986).



**Figure 2.** The rodent's pancreas showing a diffuse pattern (Page *et al.* 2000).

Although there are numerous ducts present in rodents, their course differs between rats and mice. However, there is no accessory duct present in the rat (Hebel and Stromberg 1986). At least two large pancreatic ducts are formed from fusion of fifteen to forty excretory ducts. The centroacinar cells form the terminal part of the ductal cells (Ekholm *et al.* 1962). The largest duct (splenic duct) always originates from the left lobe. It opens into the common bile duct at the junction of the hepatic duct. The remaining duct enters the common bile duct just before the latter enters the duodenum (Sun 1987). Sometimes small ducts open directly into the duodenum (Hebel 1969; Richards *et al.* 1964). The hepatic duct is covered along its length by pancreatic tissue (Richards *et al.* 1964).

### **1.1.2.3. The arterial supply and innervations to the pancreas**

The arterial supply to the pancreas comes from two sources, the celiac trunk which forms the superior series of supply and, the superior mesenteric artery that gives off branches to form the inferior series of supply (Gray 1995). The anastomotic arcades between superior and inferior pancreatic arteries create a rich vascular supply around the pancreas. In addition to these arcades, there is a free arterial plexus around and within the gland; the part within the gland and which is situated in the connective tissue between pancreatic lobules is termed the interlobular plexus. From this plexus, intralobular vessels pass to the gland parenchyma. A work by Wharton that was later confirmed by Fujita and co-worker showed that, each pancreatic lobule is supplied by a single intralobular artery that further divides and ends among the cells of the islets of Langerhans (Keynes and Keith 1981).

Ross *et al.* (2003) indicated that, in humans the capillaries first perfuse the alpha and delta cells, peripherally, before the blood reaches the beta cells, centrally. The insular vascular bed is essentially sinusoidal and gives off efferent sinusoids which pass into the exocrine components of the lobules and collectively form an interacinar capillary plexus. This portal-like circulation (portal system of Fujita) provides the exocrine pancreas with islets secretion that directly influences the exocrine function. In mice one to three afferent arterioles arise from arterial rami to supply each islet, before which they may supply the acini (Bunnag *et al.* 1963).

The pancreas receives both sympathetic (adrenergic) and parasympathetic (cholinergic) innervations via the vagus and the splanchnic nerves respectively, with the major innervations for secretory stimulation occurring via the vagus nerves. The fibers of these nerves reach the pancreas through periarterial plexuses, but some fibers may reach the pancreas directly through independent fibers not associated with arteries (Keynes and Keith 1981).

#### **1.1.2.4. Pancreas development and cytodifferentiation**

The pancreas develops from the fusion of two distinct buds, the ventral and dorsal pancreatic buds; the two buds emerge as evaginations of the embryonic gut endoderm about embryonic day 8.5-9 (Wessells and Cohen 1967). Events that occur in the ventral and dorsal pancreatic domains in early development are independent (Yoshitomi and Zaret 2004). Serial reciprocal inductions of the endoderm and adjacent mesoderm determine the cell fate in both ventral and dorsal buds tissue types (Grapin-Botton and Melton 2000). However, the formation and differentiation of all

pancreatic cell types (organogenesis) continue in postnatal life until three months of age (McEvoy 1981).

Two phases of organogenesis have been identified, namely morphogenesis, which is defined as the multicellular structure characteristic of the specific organ, and secondly cytodifferentiation which is the expression of the organ specific, differentiated cellular phenotype (Gittes and Rutter 1992).

The end of gastrulation corresponds to embryonic day 25 in humans (Liu and Potter 1962), day 9.5 (e9.5) in mouse (Slack 1995), and day 10 (e10) in the rat (Altman and Dittmer 1962). At this stage there are three germ layers present, namely endoderm, ectoderm and mesoderm. The endodermal germ layer will give rise to the digestive tract and the associated organs, including the pancreas. Pancreatic morphology is apparent with the evagination of the dorsal pancreatic bud in the early stage of development. In the rat, the notochord loses its connection with the endoderm at e11 and this is the time when the foregut and hindgut become visible. The dorsal pancreatic bud arises as an endodermal evagination from the cluster of cells in the caudal part of the foregut at e12. Soon after (e13), the dorsal pancreas will enlarge and the ventral pancreatic bud will develop from the hepatic duct. As the duodenum and stomach start to rotate, the dorsal and ventral pancreases begin to fuse at e14 meanwhile the dorsal pancreatic duct (of Santorini) degenerates. By e15, the pancreatic anlagen have fused to become one organ with the ventral duct as the main pancreatic duct (Hebel and Stromberg 1986). The beginning of the first foetal stage and the end of the metamorphosing embryo is marked by e16 (Altman and Dittmer

1962). The developing pancreatic epithelium continues growth and proliferation throughout foetal life (Slack 1995).

The main duct elongates, secondary ducts form, which branch off, elongate and from which further ducts form and branch off. They develop acini at their terminal ends, forming the centroacinar duct, and from the walls of the smaller branches, cell clumps bud - these are presumptive islets. The islets increase in size through islet cell proliferation and through the merging of cell clumps that are close together. On e21, the pancreatic islets are separated from the tissue of exocrine pancreas (Hebel and Stromberg 1986).

#### **1.1.2.5. Induction of endocrine development**

One of the first steps required for pancreatic development is an inductive interaction between the endoderm and mesoderm that directs a cluster of endodermal cells close to the mid-foregut junction toward a pancreatic fate (Deutsch *et al.* 2001; Grapin-Botton *et al.* 2000; Hebrok *et al.* 1998; Kim *et al.* 1997; Lammert *et al.* 2001; Wells and Melton 2000). In the early rodent embryo, the notochord is embedded in the endoderm, and there is close connection between both tissue types in the neural plate. At about the 13-20 somite stage (e9 in mouse and e10.5 in rat), the notochord separates from the endoderm, and the dorsal aorta lies between the gut and the notochord (Slack 1995). This interaction sets up a pre-pattern in the endoderm for the pancreas forming regions, although pancreas-specific genes are not turned on at this stage.

A homeobox containing gene in the *Antennapedia*/Ftz class (Hex) (Crompton *et al.* 1992) controls the proliferation rate, and thus the positioning, of the leading edge of endoderm cells that grow beyond the cardiogenic mesoderm, during gut tube closure. Ventral pancreas specification is thus dictated (Bort *et al.* 2004). Aortic endothelial cells induce the crucial pancreatic transcription factor Ptf1a (an exocrine subunit of pancreatic transcription factor 1 - PTF1) in the dorsal pancreatic endoderm; whereas the vitelline veins, which are normally adjacent to the emerging ventral pancreatic bud, are unnecessary for ventral Ptf1a induction or for ventral pancreatic bud initiation (Yoshitomi and Zaret 2004).

Subsequent inductive interactions occur between the notochord and the endodermal epithelium. These permissive inductions allow the pancreatic buds to emerge and continue development. At about e14 in mouse, the first pancreatic-specific genes are expressed, including the homeogene Pdx1 (Guz *et al.* 1995). When the epithelial sheet folds up to make a tube, the two lateral regions fuse to form the site where the ventral bud will emerge. The middle region forms the dorsal pancreatic bud. The two pancreatic buds require interactions with adjacent mesenchyme for further pancreatic growth and differentiation. Henceforth, promotion of pancreatic bud development is induced by signalling from the embryonic blood vessel cells, a derivative of the mesoderm (Lammert *et al.* 2001). However, signals from the notochord are important for development of the dorsal pancreas, whereas signals from the adjacent endothelial cells seem to be necessary for the initiation of both dorsal and ventral pancreatic development. The dorsal and ventral pancreatic buds start to develop precisely where endoderm previously contacted the endothelium (Lammert *et al.* 2001, 2003; Yoshitomi and Zaret 2004).

Thomas and co-workers (1998) have identified Hex expression in the anterior endoderm cells at e7.0 of mouse gestation and subsequently in the ventral-lateral foregut that gives rise to the ventral pancreas and the liver. While, Deutsch and colleagues (2001) reported that fibroblast growth factors from the cardiac mesoderm were responsible for inducing local expression of hedgehog (hh) family signalling molecules in the adjacent gut endoderm that is inhibitory to pancreas but permissive to liver development. However, later ectopic expression of sonic hedgehog (Shh) in the mouse pancreas has been shown to prevent a proper pancreatic morphogenesis (Apelqvist *et al.* 1997; Dilorio *et al.* 2002; Hebrok *et al.* 2000; Kim *et al.* 1997). Specification of the liver and the ventral pancreas occurs simultaneously from the same group of ventral foregut cells that express the homeobox gene Prox 1 (Burke and Oliver 2002); while development of the liver is dependent on its close proximity to the cardiogenic mesoderm (Deutsch *et al.* 2001; Lammert *et al.* 2001, 2003). As development continues, the stomach and the duodenum rotate anti-clockwise causing the two buds to come together to merge forming one organ; meanwhile exocrine and endocrine cytodifferentiation proceeds.

A mature mammalian pancreas is an exocrine and endocrine organ. The exocrine component comprises approximately 99% of the total pancreatic mass while the endocrine component constitutes about 1%. Islets are more numerous in the tail. The exocrine component (ductal and acinar) synthesizes and secretes enzymes (amylase, carboxypeptidase, lipase, etc.) into the duodenum that are essential for digestion in the intestine. Insulin, glucagon, pancreatic polypeptide and somatostatin are hormones synthesized and secreted into the blood by the endocrine component of the pancreas

(islets of Langerhans) to regulate glucose, lipid, and protein metabolism in the body (Ross *et al.* 2003).

### **1.1.3. Microscopic Anatomy of the islets of Langerhans**

The islets of Langerhans are clusters of cells found dispersed within the exocrine pancreas. The pancreas is composed of two structurally distinct components in intimate association with each other. The main mass (exocrine pancreas) is a lobulated, branched acinar gland found throughout the organ; within the exocrine pancreas, the islets of Langerhans constitute the endocrine pancreas responsible for maintaining glucose homeostasis. The islets of Langerhans are small spheroid clusters of cells scattered throughout the organ in cell groupings with a combined mass of approximately 1-1.5 grams, but their distribution is not uniform in the pancreas in all mammals (Massa *et al.* 1997). A most recent islet study clearly distinguishes human islet architecture from that of rodents (Steiner *et al.* 2010).

The human pancreas contains approximately one million islets of Langerhans whilst several hundred are detectable in rodents (Hughes 1956; Slack 1995). The islets constitute about 1 to 2% of the volume of the pancreas but are most numerous in the tail. Individual islets of Langerhans may contain only a few cells or many hundreds of cells. Cells within individual islets are polygonal in shape and are arranged in short, irregular cords that are profusely invested with a network of fenestrated capillaries and a rich autonomic innervation (Ross *et al.* 2003).



Immunohistochemical studies demonstrated that the islets of Langerhans are composed of four cell types designated alpha (or A or A2), beta (or B), delta (or D or A1), and PP (or D1 or F) (protein polypeptide) cells, producing glucagon, insulin, somatostatin, and pancreatic polypeptide respectively (Alumets *et al.* 1983). Although islet composition differs in dorsal and ventral part of the pancreas, diverging reports on the distribution in the cell-type from different regions of the pancreas are documented. Cabrera *et al.* (2006) reported a relatively even distribution in the proportion of endocrine cells, while Brissova *et al.* (2005) observed that more beta and alpha cells are found in the body, neck and tail; and PP-cells are found in the head (Stefan *et al.* 1982). Insulin secretion and proinsulin biosynthesis induced by glucose stimulation are mostly present in the dorsal islets (Baetens *et al.* 1979; Trimble *et al.* 1982). In rodents, beta cells constitute approximately 60-80% of the islet cells and generally form the core of the islet. The surrounding layer of endocrine cells include alpha cells (15-20%), delta-cells (<10%) and PP-cells (<1%) (Cabrera *et al.* 2006; Kim *et al.* 2009; Quesada *et al.* 2008), whereas, the adult human islets constitute about 50% beta cells, 40% alpha cells, 10% delta cells and fewer PP-cells (Cabrera *et al.* 2006; Brissova *et al.* 2005; Miller *et al.* 2009).

A fifth peptide hormone, namely ghrelin, produced by epsilon cells has been identified in the human islet. These ghrelin-producing epsilon cells are thought to regulate the food intake and energy balance, and stimulate the increased secretion of growth hormone (Slack 1995). However, the location of these cells within the endocrine compartment remains questionable (Date *et al.* 2002; Volante *et al.* 2002; Wierup *et al.* 2002; Wierup and Sunder 2005). A number of studies were carried out to trace the origin of certain hormone producing cells within the islet; so common

precursor cells that co-express the various islet hormones are thought to give rise to pancreatic endocrine cells (Teitelman *et al.* 1987). These common islet progenitor cells express peptide YY (Upchurch *et al.* 1994) or neuropeptide Y (Teitelman *et al.* 1993). Teitelman *et al.* (1987) reported that embryonic cells found in duct epithelium express tyrosine hydroxylase (TH). Herrera (2000) however, through various studies using transgenic cell marking analysis, supported a previous work by Jensen *et al.* (2000a), where it was suggested that cells expressing both insulin and glucagon are classified as a subgroup of  $\alpha$ -cells that could sometimes express insulin; but both  $\alpha$ -producing cells and  $\beta$ -producing cells have different embryonic origin (Herrera 2000; Jensen *et al.* 2000a).

Endocrine cell differentiation at early stage of pancreatic development can be detected using specific markers. Somatostatin mRNA is expressed in the mouse gut endoderm as early as e7.5-8.5. The expression of glucagon and insulin mRNA are detected at e8.5-9 preceding the PP mRNA expression (Gittes and Rutter 1992); however, immunoreactivity for either insulin or glucagon revealed scattered endocrine cells and can be seen at e9.5 (Gittes and Rutter 1992; Schwitzgebel *et al.* 2000) while immunoreactivity for PP is first detected much later at e18. Amylase immunoreactivity can be seen at e10.5-12 after the transcription of amylase and carboxypeptidase is detected 2-4 days earlier (Gittes and Rutter 1992; Herrera *et al.* 1991). The early endocrine cells are seen in association with the pancreatic ducts, but as they mature, the islets of Langerhans together with beta cells become first detectable at around e18-19. It is however evident that endocrine cells transdifferentiate from ductal epithelium to fully differentiate into insulin producing cells (Bonner-Weir *et al.* 2000).

Although numerous markers used to identify pancreatic endocrine multipotent precursors have been reported, the most viable is cytokeratin (CK). Cytokeratin is specifically for the pancreatic ductal epithelial cells expression (Bouwens *et al.* 1994). Zulewski *et al.* (2001) postulated that nestin, which is used as a marker for neural stem cells, could also be used to identify pancreatic islet stem cells. Findings by Selander and Edlund (2002) found nestin to be expressed in the mesenchymal cells of the developing pancreas, but not in the pancreatic epithelial cells which is believed to represent the pancreatic progenitor cell pool. Ghrelin is another new marker, which was initially isolated from the rat stomach as a ligand for the growth hormone secretagogue receptor (GHS-R). It was later found to be expressed in the human pancreatic islets throughout life (Weirup *et al.* 2002), but does not express any of the hormones present in the islet cells (e.g. insulin, glucagon, somatostatin or PP) and thus constitutes a novel set of islet cell types (Wierup and Sundler 2005).

#### **1.1.4. Beta cell mass and pathogenesis of diabetes**

Beta-cell mass is the total number of islet cells, including the newly formed islet cells that arise from pre-existing ductal cells or other precursors cells (neogenesis), and, the islet cells formed by replication (proliferation) and programmed cell death (apoptosis) of existing islet cells (Vinik *et al.* 2004). The balance between neogenesis, proliferation and apoptosis is critical to sustain the integrity of beta cell mass (Finegood *et al.* 1995).

#### **1.1.4.1. Beta cell neogenesis and apoptosis**

Neogenesis is the natural regeneration or formation of new cells in response to a total or partial loss of a tissue. Apoptosis however, is a pathway of programmed cell death (Kerr 1993) during which the body disposes of damaged, unwanted, or unneeded cells; this results in cells shrinking and leading to the fragmentation of their DNA. For the survival of living species, most cells in the body are in continuous turnover, with the average life span depending on the cell type. In the pancreas, beta cell neogenesis and replication occurs significantly during foetal life and continues at a reduced rate at the neonatal stage of life (Bouwens *et al.* 1994). An increase in apoptotic index in postnatal beta cells has been reported in humans (Kassem *et al.* 2000), pig (Bock *et al.* 2003) and rodents (Scaglia *et al.* 1997); this shows a direct link between cell death and regeneration or formation of new cells. Newly formed beta cells take about 30-40 days to reach their mature and full functional potential (Samikannu and Linn 2008). A reduction in beta cell replication due for instance to aging, was implicated in glucose intolerance (Swenne 1983); hence indicating the correlation that may exist between beta cell pathogenesis and the etiology of diabetes.

#### **1.1.4.2. Beta cell pathogenesis and the etiology of diabetes**

The significance of the beta cell mass in the pathophysiology of the pancreas has been the focus of extensive research (Akirav *et al.* 2008; Bouwens and Rooman 2005; Donath and Halban 2004; Matveyenko and Butler 2008; Weir and Bonner-weir 2004). The mass of beta cells is an important tool for the regulatory mechanisms where changes can result in a partial or complete deficiency in insulin secretion. However,

changes in beta cell function and insulin synthesis are a direct consequence of the balance between the degeneration and the proliferation of these cells which are critical for the pathophysiology of the pancreas (Rhodes 2005). Beta cell mass loss in the pancreas results from an autoimmune disorder induced by T-cells that destroy beta cells and cause a lack of insulin production, thereby suppressing signaling responses that trigger cellular uptake of glucose and glucose metabolism in cells; this situation leads to a type 1 diabetes also known as insulin dependent diabetes mellitus (IDDM). An increase in beta cell mass however could be a response to insulin resistance in body tissues, whereby insulin may be present but there is a lack of insulin-stimulation signaling for a proper cellular uptake of glucose leads to a decrease in glucose metabolism: a typical case of type 2 diabetes known as non-insulin dependent diabetes mellitus (NIDDM). Environment factors have also been suggested to play a significant role in the pathogenesis of diabetes mellitus, but the mechanism is not yet clearly defined (Lowe 1998).

Diabetes is a disease of glucose metabolism characterized by a high fasting plasma glucose levels. The disease Diabetes Mellitus (DM) was described in 1862 by Ebers who found the first description in an Egyptian papyrus in the Tomb of Thebes (Turkenburg 1996). Diabetes in Greek means “pipe-like”, illustrating the passage of nutrients through the system without being utilized by the body; whereas Mellitus is a Latin word for “honey” or “sweet”, as opposed to “Diabetes Insipidus” which is a disease caused by a dysfunctional pituitary gland leading to a large volume of sugar free urine. Although these distinguishing factors of the two type of diabetes were known just less than a century ago (Bach 1994; Tisch and McDevitt 1996), diabetes in itself is one of the oldest diseases (3000 - 1500 B.C) which remains amongst the

leading causes of death in the world, affecting young (juvenile diabetes), adult (maturity diabetes) and old age (late onset diabetes) individuals (Rotter *et al.* 1990). Although the type 1 and type 2 diabetes mellitus have the same phenotype (fasting and postprandial hyperglycemia), they account for almost 100% of cases of diabetes (10% and 90%, respectively) and, affect about 5% of the population worldwide; but both do not share a common etiology (Lowe 1998). Irrespective of the age of the affected individuals, the life threatening complications of the disease remain the same. In South Africa, the prevalence of diabetes differs within population groups and it varies from one geographic area to another. A survey by King *et al.* (1998) suggested that the world prevalence of diabetes in adults will increase by 35%, while the number of people with diabetes will increase by 122% from 1995 to 2025.

For these reasons, the future of a potential therapy for Diabetes mellitus (DM) resides in the understanding of the morpho-genetics of islet cell neogenesis, which remains the ultimate hope for modifications in the treatment of diabetes in general and transplantation in particular. In this regard, the first attempt of islet transplantation was made in 1893, when sheep pancreatic extracts were transplanted into a young human patient who improved for 24 hours only (Williams 1894). A decade later, with the discovery of insulin by Banting and Best in 1921, insulin remained the only treatment for diabetes until Ballinger and Lacy (1972) successfully treated a diabetic rat by transplanting islet isografts. Although many other subsequent transplants followed in the 1970s and 1980s in rats (Amamoo *et al.* 1975; Kemp *et al.* 1973; Lacy *et al.* 1979) and in humans (Largiader *et al.* 1980; Najarian *et al.* 1980; Sutherland *et al.* 1980), there were numerous setbacks related to the use of immunosuppressive agents. A breakthrough came in 2000, when Shapiro (2000) reported a 100% success

rate with seven human transplants. Despite remarkable progress, the overall world success rate toward the treatment of diabetes by islet cells transplantation remains very low at 10% (Robertson 2004) due to challenges such as insufficient donor organs, the necessity of using immunosuppressive agents (Du Toit *et al.* 1998a, 1998b; Muller *et al.* 1998) and the lack of innovative technical/clinical knowledge needed during the transplant process that is acquired from collaboration between researchers (Shapiro *et al.* 2003). It is therefore relevant to explore other avenues of self generated cells within the organ itself which could be a way of overcoming the challenges of IDDM.

#### **1.1.5. Pancreatic duct ligation procedure - PDL**

The ligation of the pancreatic duct has been an ongoing experimental procedure for many years; it was initially aimed at treating a disease, viz. the pancreatitis; however, a common opinion emerged then, that ligation of the pancreatic duct induced a considerable level of pancreatic atrophy (Auer and Kleiner 1918). This opinion corroborated with a work from a prominent researcher of the 19<sup>th</sup> century Banting, who made the same observation while trying to isolate a pancreatic secretion using the duct ligation procedure (Bliss 1982). Despite the fact that many authors of that time observed atrophy of the pancreas following duct ligation, there was serious controversy as to the reason or the origin of a remarkable increase in mass of the survived islet (Inada *et al.* 2008; Solar *et al.* 2009; Vincent 2007; Wang *et al.* 1995; Xu *et al.* 2008).

Subsequent studies on the pancreas have revealed that transcription factors may be involved in the proliferation of the islets after pancreatic duct ligation (Kritzik *et al.* 1999; Scoggins *et al.* 2000; Sharma *et al.* 1999; Song *et al.* 1999; Solar *et al.* 2009; Wang *et al.* 1995). Page *et al.* (2004) reported a similarity between the formation of normal fetal pancreas tissue and the newly-formed beta cells following PDL; this is thought to be due to the plausible stem cell capacity of the adult pancreas (Bouwens *et al.* 1998, Githens *et al.* 1988; Muller *et al.* 2000). Also, atrophy of the pancreatic cells following duct ligation were noted as a direct consequence to the acinar cell death (Abe *et al.* 1995; Scoggins *et al.* 2000; Page *et al.* 2000; Yasuda *et al.* 1999), which triggered islet proliferation and neogenesis from duct-like epithelial cells (Bouwens 1998; Githens 1988).

A recent study on the cell lineage in duct ligated pancreas revealed that an increase in beta cell mass does not have any contribution from the pre-existing ductal epithelial cells. Insulin-producing beta cells develop from pancreatic exocrine duct cells only during embryogenesis but not at postnatal life (Solar *et al.* 2009). These conflicting evidences warrant an assessment of other lineage-selective transcription factors for endocrine development in PDL tissues.

#### **1.1.6. Transcription factors involved in endocrine development**

Transcription factors are involved both in determining early cellular development and differentiation of progenitor cells, and later in maintaining the pancreatic cell phenotype (Stoffers *et al.* 1997). Several of these factors have been implicated in pancreas development (Kim and McDonald 2002; Sander and German 1997; Servitja



and Ferrer 2004) during which they are recognized to be critical regulators of gene expression (Jensen 2004; Peshavaria and Stein 1997). These include transcription factors of the homeodomain family (Pdx1, Hb9, Pbx1, HNF1 $\beta$ , HNF6, Pax4, Pax6, Nkx2.2, Nkx6.1, Isl1, HNF1 $\alpha$ , HNF4 $\alpha$ , and Brn4) (Ahlgren *et al.* 1996, 1997; Gannon *et al.* 2000; Harrison *et al.* 1999; Jacquemin *et al.* 2000; Jonsson *et al.* 1994; Li *et al.* 1999; Offield *et al.* 1996; Sosa-Pineda *et al.* 1997; Solar *et al.* 2009; St-Onge *et al.* 1997), the basic helix-loop-helix (bHLH) family (Ngn3, Beta2/NeuroD, Hes1, p48) (Gradwohl *et al.* 2000; Ishibashi *et al.* 1995; Jensen *et al.* 2000b; Krapp *et al.* 1998; Naya *et al.* 1995, 1997) and the forkhead/winged helix family (Foxa2/HNF3 $\beta$ , Foxa1/HNF3 $\alpha$ ) (Ang and Rossant 1994; Kaestner *et al.* 1999; Weinstein *et al.* 1994).

A number of homeodomain factors, such as Pdx1 (Ahlgren *et al.* 1996; Jonsson *et al.* 1994), Pax4 (Sosa-Pineda *et al.* 1997), Pax6 (Sander *et al.* 1997; St-Onge *et al.* 1997), Nkx2.2 (Sussel *et al.* 1998), Nkx6.1 (Sander *et al.* 2000), and an additional bHLH transcription factor such as NeuroD (Naya *et al.* 1997) are necessary for differentiation and maintenance of mature and differentiated cells. Whilst bHLH transcription factor Ngn3 is both necessary and sufficient (Apelqvist *et al.* 1999; Gradwohl *et al.* 2000) in driving undifferentiated progenitor cells to an endocrine fate; Ngn3 expression is ceased before islet cells are fully differentiated (Jensen *et al.* 2000a; Schwitzgebel *et al.* 2000). However, Pdx1 expression in mature differentiated cells is specific to insulin-secreting cells (Guz *et al.* 1995).

### **1.1.6.1. Pancreatic duodenal homeobox gene-1 (Pdx1)**

Pancreatic duodenal homeobox gene-1 (Pdx1) is a homeodomain transcription factor with a key regulatory function both in pancreas development and the differentiation of progenitor cells to become adult beta cells. It is also called insulin promoter factor-1 (Ipfl) (Ohlsson *et al.* 1993), islet duodenum homeobox-1 (IDX-1), somatostatin transactivating factor-1 (STF-1) (Leonard *et al.* 1993), or glucose sensitive factor (GSF) (Marshak *et al.* 1996). Pancreatic duodenal homeobox gene-1 belongs to a “ParaHox” gene cluster expressed in the lateral endoderm domain at somites 7 to 9 in the vertebrate axis that contributes to the development of the pancreas. Pancreatic duodenal homeobox gene-1 is a pancreas specific homeoprotein. Because of this, it has been found to be a beta and gamma-cell-specific regulatory factor for the expression of the insulin and somatostatin genes. Pancreatic duodenal homeobox gene-1 also regulates the expression of other islet-specific genes like Glut-2 (Waeber *et al.* 1996), islet amyloid polypeptide (Watada *et al.* 1996a), and glucokinase (Watada *et al.* 1996b). In the developing pancreas, Pdx1 is first detected at e8.5 in the ventral gut endoderm in cells later forming the ventral pancreatic bud. At e9.5 Pdx1 is expressed in both ventral and dorsal pancreatic buds (Guz *et al.* 1995; Offield *et al.* 1996). From e11.5 to e13.5, Pdx1 expression is seen throughout developing pancreatic epithelium. But at the time when exocrine pancreas begins to form (e14-e15), the islets mature into hormone producing-cells and, Pdx1 expression becomes restricted to endocrine compartment (e16.5-e18.5), and in dispersed endocrine cells of the duodenal wall (Guz *et al.* 1995; Jonsson *et al.* 1994; Offield *et al.* 1996). Later in the adult pancreas Pdx1 acts as a master regulator of insulin gene expression (Ohlsson *et al.* 1993; Stoffers *et al.* 1997b). Subpopulations of somatostatin producing and

pancreatic polypeptide producing cells also express Pdx1, but Pdx1 expression is seen only in few glucagon-producing cells (Guz *et al.* 1995; Miller *et al.* 1994; Ohlsson *et al.* 1993).

Targeted disruption of Pdx1 gene (Jonsson *et al.* 1994; Offield *et al.* 1996) and homozygous *Ipfl* mutations (Stoffers *et al.* 1997b) result in agenesis of the pancreas. Mice with an inactivating mutation in Pdx1 are viable, but pancreatic development is arrested at very early stage and animals die within days after birth (Jonsson *et al.* 1994). In early pancreas development, a few insulin-expressing cells are detected in Pdx1 null mice, suggesting that a population of insulin positive, Pdx1-negative cells arise separately from the mature Pdx1 expressing beta cells of the developed pancreas (Ahlgren *et al.* 1996). However, as Stoffers *et al.* (1997a) pointed out “the expression of Pdx1 in gut endoderm is essential for the pancreatic program to continue and all pancreatic tissues subsequently differentiate from Pdx1-positive precursors” found in this germ tissue. Notably, a child homozygous for an inactivating mutation in Pdx1 will be born without a pancreas (Stoffers *et al.* 1997a), thereby underscoring the role of the Pdx1 transcription factor in the development of the mouse as well as the human pancreas (Habener *et al.* 2005).

The rat, mouse and human Pdx1 genes are localized respectively on chromosomes 12 (Yokoi *et al.* 1997), 5 (Sharma *et al.* 1996) and 13 (Inoue *et al.* 1996; Stoffel *et al.* 1995). The coding region of the Pdx1 gene has two exons, the first encodes for the NH<sub>2</sub>-terminal region of the gene, and the second encodes for the homeodomain and COOH-terminal domain (Melloul *et al.* 2002). The activation of Pdx1 is contained within the NH<sub>2</sub>-terminal region, however its homeodomain is involved in DNA

binding; both NH<sub>2</sub>-terminal region and homeodomain are involved in protein-protein interactions (Ashara *et al.* 1999; Qui *et al.* 2002).

#### **1.1.6.2. Neurogenin3 (Ngn3)**

Neurogenin3 (Ngn3) is a proendocrine factor belonging to the basic helix-loop-helix family bHLH. Neurogenin3 is considered as a marker of islet precursor cells, and has been shown to be essential for the development of all endocrine cell lineages of the pancreas (Gradwohl *et al.* 2000; Schwitzgebel, 2001). Pancreatic endocrine cells develop from the precursor cells expressing Pdx1 and the bHLH-family transcription factor Ngn3. In the mouse, Ngn3 expression is first observed at e9.5, and the number of Ngn3 expressing cells increases until e15.5 exactly when islet cell differentiation is at its peak and diminishes greatly thereafter with little or no detection of Ngn3 in the adult pancreas. Co-expression of Ngn3 with islet hormones (insulin, glucagon, somatostatin, pancreatic polypeptide) cannot be detected at this adult stage, although all four-islet cell types develop from Ngn3 expressing cells that are found adjacent to ductal cells (Gradwohl *et al.* 2000).

In mice deficient for Ngn3, all islet cell types are absent in every stage of development (Gradwohl *et al.* 2000); but exocrine tissues and ductal tissues develop normally. It is evident that expression of Ngn3 is a functional marker of an islet cell precursor population in the developing pancreas. Since Ngn3 is both sufficient and necessary to initiate differentiation of islet cells during development, it may be concluded that the endocrine fate of cells is strictly controlled by the activity of specific transcription factors that regulate the cis-acting elements within the promoter region of the Ngn3 gene. Hepatocyte nuclear factor 6 (HNF6), HNF3 $\beta$  / FOXA2 and

HNF1 $\alpha$  bind to the Ngn3 promoter, acting as its activators (Lee *et al.* 2001); while Ngn3 on the other hand acts as an upstream regulator for the transcription factors Pax6, Pax4, Beta2/NeuroD, Nkx6.1, Nkx2.2 and Is11 (Gu *et al.* 2002; Gu *et al.* 2003, Hermans *et al.* 2002; Smith *et al.* 2003), and simultaneously represses its own promoter (Smith *et al.* 2004).

Mice homozygous for a null HNF1 $\alpha$  gene have smaller islets and secrete little insulin (Potonglio *et al.* 1998). Mice embryos missing HNF6 expression present a marked reduction in endocrine differentiation, with critically reduced levels of Ngn3 expression (Jacquemin *et al.* 2000). A lack of foregut formation will be observed in FOXA2 / HNF3 $\beta$  null mice (Ang and Rossant 1994; Weinstein *et al.* 1994) as well. The expression of the HNF factors is therefore considered to be involved in a cooperative mechanism in the cell-type-restricted activation of Ngn3 expression, but are however not sufficient for Ngn3 expression (Jacquemin *et al.* 2000).

Neurogenin3 promoter has binding sites for HES1, which is a transcriptional repressor of bHLH genes and it is thus believed to inhibit Ngn3 expression through the Notch signaling pathway (Jensen *et al.*, 2000b). Overexpression of Ngn3 and an absence of HES1 show a similar pancreatic phenotype. Lateral inhibition of Ngn3 expression via the Notch-pathway is necessary to allow for the expansion of epithelial cells before differentiation. However, premature overexpression of Ngn3 blocks the Notch-pathway, which leads to a poorly branched ductal epithelium, blockage of exocrine development and acceleration in islet cell differentiation. The evidence therefore suggests that Notch signalling pathway contributes in regulating the balance between

progenitor cell differentiation and proliferation during the pancreas development (Apelqvist *et al.* 1999; Jensen *et al.* 2000b; Lammert *et al.* 2001).

### **1.1.6.3. Human neurogenic helix-loop-helix protein gene (NeuroD / Beta2)**

Human neurogenic helix-loop-helix protein gene (NeuroD / Beta2), which is a bHLH factor and an important activator of insulin gene transcription, is also required in order to generate a normal mass of pancreatic  $\beta$ - and  $\alpha$ -cells. The activation of NeuroD expression in cells that co-express Ngn3 and Pdx1 is a very early step in the pancreatic endocrine differentiation (Mutoh *et al.* 1997). NeuroD is activated by Ngn3, although these two factors are expressed in different cells. It has been demonstrated that NeuroD-positive cells arise from cells that express Ngn3. NeuroD expression is detected at e9.5, co-localizing with early glucagon expressing cells (Jensen *et al.* 2000a; Naya *et al.* 1997).

Null mice for NeuroD gene die 3-5 days after birth due to severe hyperglycemia; the islets' beta cell count is reduced by 75%, numbers of alpha and delta cells are also reduced and irregular in shape (Jensen *et al.* 2000a; Naya *et al.* 1997). The Notch pathway antagonizes NeuroD and bHLH proteins Ngn3. As Jensen and co-workers (2000b) reported "Activation of Notch receptors leads to activation of Hairy and Enhancer-of-split (HES) -type proteins, which in turn act as transcriptional repressors of bHLH genes. Mice lacking Notch ligand Delta like-1 (Dll1) or the DNA-binding protein RBP-jk (activator of HES1), have accelerated differentiation of pancreatic endocrine cells and subsequently severe pancreatic hypoplasia due to premature differentiation of pancreatic stem cells into endocrine cells" (Apelqvist *et al.* 1999; Jensen *et al.* 2000b; Lammert *et al.* 2001).

#### **1.1.6.4. Paired box gene 6 (Pax6)**

Paired box gene 6 (Pax6) belong to the Pax multigene family of transcription factors that contribute to the regulation of pancreatic endocrine cell differentiation (Sussel *et al.* 1998). The paired box gene-6 expression is mainly detected in the eye, the central nervous system, the nose and the endocrine pancreas (Turque *et al.* 1994; Walther and Gruss 1991). Sharing similar structure with Pax4 in their corresponding homeodomain (Dohrmann *et al.* 2000; Mansouri *et al.* 1996), Pax6 is also expressed in both the ventral and dorsal developing pancreas; but its expression is detected as early at e9.5 in the mouse and expressing throughout the pancreas development until the endocrine cells are fully formed (Dohrmann *et al.* 2000; Sander *et al.* 1997; Sosa-Pineda *et al.* 1997; St-Onge *et al.* 1997).

A mouse lacking Pax6 gene does not survive after birth; the pancreas of the mutant contains very few fully differentiated  $\beta$ -,  $\delta$ - and PP-cells within a malformed islet (St-Onge *et al.* 1997). Alpha-cells however are completely absent in this pancreas; but the development of the exocrine cells tends to be normal. Mice lacking both Pax4 and Pax6 do not develop any endocrine cells; these findings suggest that both Pax4 and Pax6 are necessary for the regulation of the final steps in the pancreatic endocrine cells differentiation (Dohrmann *et al.* 2000; Sosa-Pineda *et al.* 1997; St-Onge *et al.* 1997; Sussel *et al.* 1998).

Advances in research in recent years have shed significant light on how transcription factors regulate endocrine pancreas development (Ackermann and Gannon 2007; Boucher *et al.* 2009; Brun and Gauthier 2008; Gasa *et al.* 2008; Serafimidis *et al.*

2008; Wang *et al.* 2009; Zertal-Zidani *et al.* 2007). Although recent evidence has shown that newly formed beta cells following PDL do not originate from the pre-existing ductal epithelium (Solar *et al.* 2009). The cellular mechanisms involved in the recapitulation of these newly formed beta cells remain unknown.

### **1.1.7. Problem statement**

The transplantation of whole pancreas has improved the lives of large numbers of diabetic patients in the developed world, but the burden of immunosuppressive drugs impacts on the quality of life. The transplantation of allogeneic foetal tissue is a proven alternative (Muller *et al.* 1998, 2000, 2001, 2002, du Toit *et al.* 1998) although it is also impacted upon by immune suppressive agents. The transplantation of adult or foetal islets of Langerhans is a promising therapeutic option for the treatment of diabetes mellitus (DM), but the low availability of human donor pancreas and the lack of suitable donor tissue remain a major obstacle (Shapiro *et al.* 2000). Duct ligated pancreas transplantation has been shown to have the same efficacy as foetal tissue (Page *et al.* 2000, 2004); as the model involved the transplantation of syngeneic tissue, immune-suppressive agents were unnecessary. However, the lineage of endocrine cell development in the pancreatic duct ligation (PDL) model is poorly understood, and is in part one of the questions that this thesis attempts to answer. Pancreatic stem cells residing within the ductal epithelium have been used to generate islet-like clusters *in vitro* which has partially reverted DM in animal models (Ramiya *et al.* 2000). Hence, understanding the processes of cellular mechanism in the lineage of endocrine cells in the duct ligated induced neogenesis, will be a valuable tool in improving beta cell replacement in patients with diabetes, thereby alleviating the



burden of Diabetes Mellitus. This present study therefore was initiated to address this challenge.

### **1.1.8. Aim and objectives**

The aim of this thesis was to establish the morpho-genetic sequence of endocrine cell development after a duct ligation procedure, using a range of immunohistofluorescent labelling and computerized morphometry techniques.

The following objectives were defined to achieve this aim:

1. to describe the morphological changes of the rat pancreas during various time periods pre- and post-PDL;
2. to determine the expression pattern and variations of Insulin, Pdx1, NeuroD, Ngn3, Pax6 and caspase3 in the remodelling of the rat pancreas between time periods pre- and post-pancreatic duct ligation;
3. to assess potential correlations between the expression of insulin, Pdx1, NeuroD, Pax6 and caspase3;
4. to investigate the time-related profile and efficiency of co-expression of Pdx1, NeuroD, Ngn3, Pax6 and caspase3 with insulin.

## **Chapter 2. Materials and Methods**

### **2.1. Ethical issues**

Ethics approval was obtained from the University of Stellenbosch Ethics Committee reference number P04/01/001. This study complies with the recommendations of the Declaration and the guiding principles laid down by Animal Welfare Organization and the Society for the Prevention of Cruelty to Animals (SPCA).

### **2.2. Laboratory animals**

The difference in the anatomy, physiology, cell and molecular biology of the pancreas in humans and rodents as described in the literature have very little implication to this study, because of the similarities in the morphological events that take place when the pancreas of both the human and rodent undergo duct ligation. Rats (*Rattus norvegicus*) were used in preference to mice (*Mus musculus*) because of the advantage of being larger in size which gives easy access to the organ under study. Also, these animals are readily available as inbred lines.

Seventy-eight male, randomly selected healthy Sprague Dawley rats were obtained from the Central Animal Unit of the Faculty of Health Sciences, University of Stellenbosch. The rats were weighed and put into thirteen (13) groups of six animals each, corresponding to the time periods post-PDL of 6, 12, 24, 36, 48, 60, 72, 84, 96, 108 and 120 hours, and time periods pre-PDL of 0 and 5 hours at which animals were

killed. While animals in both groups pre-PDL 0 hour and 5 hours did not undergo duct ligation, animals in group pre-PDL 5 hours had the abdomen opened and closed only (sham operation) (Table 1).

**Table 1.** Animals in groups of various time periods pre- and post-PDL showing the number of animals killed

<b>Procedures</b>	<b>Groups time periods (hr)</b>	<b>Total animals (n)</b>
<b>Pre-PDL</b>	0	6
	5	6
<b>Post-PDL</b>	6	6
	12	6
	24	6
	36	6
	48	6
	60	6
	72	6
	84	6
	96	6
	108	6
	120	6
	<b>78</b>	

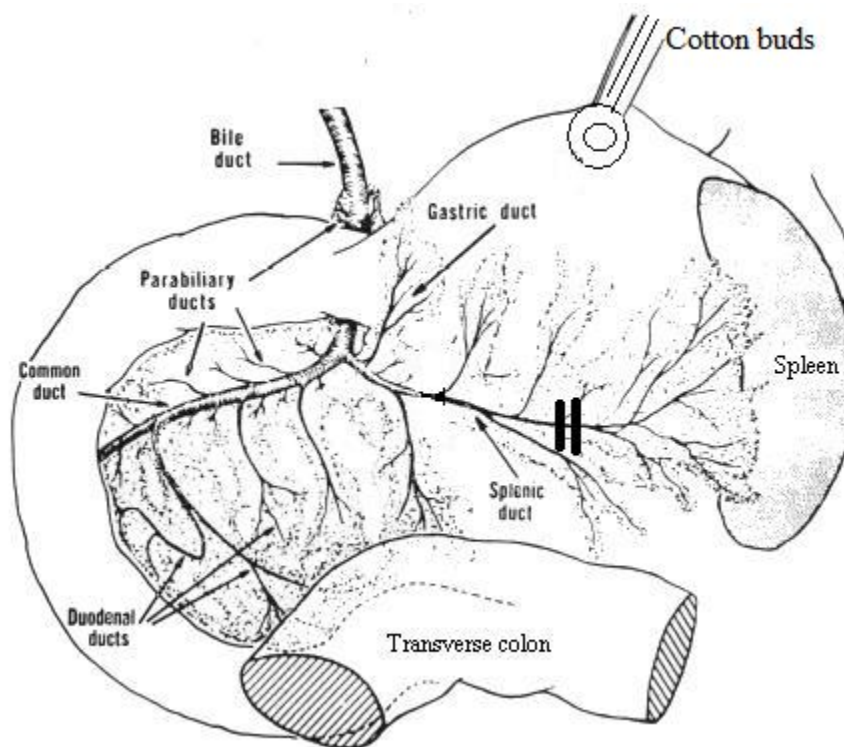
### **2.3. Pancreatic duct ligation**

A fully equipped microsurgery laboratory at the Department of Anatomy and Histology was used for the PDL surgical procedure. One night prior to PDL, rats were housed in clean cages and in a thermally controlled environment with free access to water but no food.

On the day of the surgical procedure, induction of anaesthesia was achieved by 5% halothane vaporized in O<sub>2</sub> such that there was no spontaneous movement and no withdrawal responses to tail or foot pinch. Abdominal hair was shaved using a surgical blade, one centimetre on either side of the linea alba to avoid excessive heat loss. Great care was taken not to abrade or cut the skin. The shaved portion of the abdominal surface was then cleaned with betadine antiseptic solution containing providone-iodine at 10 mg/ml (Adcock Ingram Pharmaceuticals, Industria, Johannesburg, RSA).

The animals were placed on their back (dorsal recumbency); the hind limbs were maintained on the surgical table by means of a paper tape with a minimum tension to avoid muscle strains. A mid-line laparotomy incision starting from the tip of the xiphoid process to about one centimetre above the pelvic symphysis was made to obtain access to the abdominal cavity. The stomach and the duodenum were drawn out and reflected cranially to expose the pancreas. The topographical position of the pancreas was noted, and cotton buds were used to prise the pancreas away from the surrounding tissue. A Zeiss OPMI-1 operating microscope equipped with a zoom and a focus adjustment (Carl Zeiss, AG, Oberkochen, Germany) aided in identifying the

colourless pancreatic duct. The pancreatic duct was traced distally towards the splenic lobe of the pancreas. At about 1/3 proximal to the tail end of the pancreas (splenic lobe), a tight single suture occluding the duct was made using a resorbable suture material (5/0 sterile white braided silicone treated polyester, USP, Davis and Geck, Isando, South Africa) soaked in saline solution (Figure 3). Care was taken to avoid damage to the underlying blood vessels.



**Figure 3.** The pancreas of the laboratory rat is prised away to expose the point of ligation (double bold line) in the splenic lobe.

After ligation, 5ml of warm saline solution was introduced into the abdominal cavity to prevent post-PDL dehydration. The laparotomy incision was closed in two separate layers of a continuous (blanket) stitching using 5/0 sterile white braided silicone treated polyester (Davis and Geck, Isando, South Africa); first the peritoneum together with the abdominal muscles were sutured and the skin followed thereafter,

the whole procedure taking approximately 10 minutes. A subcutaneous injection of 2.5mg of Amoxil (Smith-Kline Beecham Pharmaceuticals, Midrand, RSA) and 0.5mg streptomycin (Novo-Strep 5g/15 ml, Novo Nordisk (Pty) Ltd, Johannesburg, RSA) was administered as a single subcutaneous dose at the scruff of the neck to guard against infection; the wound was then swabbed with an antiseptic rub (Beige Pharmaceuticals Pty. Ltd., Edenvale, South Africa) to minimize scratching. Animals were returned to clean laboratory rodent cages under a 60 watt lamp necessary for rapid recovery and to counter hypothermia, and were housed in a thermally controlled environment with free access to water and standard rat chow (Epol, Midrand, RSA) until they were killed. The effects of the post procedural treatment regimens, which might impact on the recapitulation process of endocrine cells development were not considered and remain an unknown parameter in this study.

## **2.4. Histological study of the PDL Tissue**

### **2.4.1. Removal and processing**

On the day of tissue collection corresponding to time periods pre- and post-PDL, animals were anaesthetised as described earlier; placed in a dorsal recumbence, the hind limbs were maintained on the surgical table by means of a paper tape, and a mid-line laparotomy incision was made along the previous incision to gain access to the abdominal cavity. The stomach and the duodenum were drawn out and reflected cranially to expose the pancreas. A portion from the 1/3 proximal to the tail end of the pancreas was surgically isolated and removed after the surrounding blood vessels had been ligated. The animals were then euthanized by introducing 200mg/kg sodium pentobarbitone into the abdominal cavity and the carcasses disposed of by incineration.

The isolated portions of the pancreas were excised (5x5mm) (PDL tissues) and placed in labelled tubes containing Bouin's fluids (Appendix 1) for tissue preservation. Approximately 6-18 hours later, PDL fixed-tissues of the pancreas were placed in labelled plastic cassettes and were processed through standard histology routine (Appendix 2). The PDL tissues were then embedded in paraffin wax (at 55°C) and the resulting tissue blocks were kept at room temperature (20-25°C) until sectioning took place.

#### **2.4.2. Sectioning and H&E staining**

Sectioning and staining procedures took place at the Walter Sisulu University, within the facility of the Department of Anatomy, Histology and Embryology, with the approval of the Faculty of Health Ethics and Biosafety research committee reference number 0016-04.

##### **2.4.2.1. Sectioning**

One night prior to sectioning, PDL tissue blocks were placed on the cold plate; 12 serial sections at 5µm thick were obtained from each block using a motorized rotary microtome equipped with a section transfer system-STS (Microm International HM 355S). The water bath of the STS was filled with de-ionised water and was replenished between blocks of different groups to avoid contamination. Tissue sections were then mounted from warm de-ionised water (at 40°C) onto frosted microscope slides.

Slides were labelled as follows: the groups representing time periods pre- and post-PDL were prefixed 1 to 13. Each one of the 6 animals in each group were identified by the radicals A, B, C, D, E and F; the 12 serial sections representing the staining procedures used in the study were attributed the suffixes 1 to 12. Therefore, a slide labelled for instance 3A8 means the 8<sup>th</sup> serial section (or double staining procedure, insulin and caspase3) from tissue block A (1<sup>st</sup> animal) in group 3 (6 hours-time post-PDL) (Appendix 3).

A total number of 936 tissues slides were labelled, and sections were allowed to air dry overnight at room temperature (20-25°C) to favour firm adhesion to the glass slides in order to prevent tissue loss during subsequent incubations and washes. Labelled slides (n=78) bearing the same suffixes (serial section number) were put in the same slide box. Twelve labelled microscope slide boxes in total were constituted, and the boxes were labelled according to the staining procedure to which the sections were exposed.

#### **2.4.2.2. H&E staining**

Haematoxylin and eosin (H&E) staining procedure (Appendix 4) was applied to sections in slide box 12 containing the 12<sup>th</sup> serial sections obtained from all groups involved in the study. The stained slides were viewed under the light microscope mainly to validate the morphological changes that occurred in the PDL tissues of the pancreas in various periods of time after PDL.



## **2.5. Immunohistochemical study of the PDL tissue**

### **2.5.1. Immunofluorescence procedure and deparaffinization**

Two immunofluorescent staining procedures were used in the study. The simple-label immunofluorescent (SIF) staining was used to determine the expression of individual primary antibodies, while double-label immunofluorescent (DIF) staining was used to investigate the relationship between two antigens in the same tissues immunofluorescent staining procedures.

The method controls for the immunofluorescent staining of PDL tissues were not considered in this study. It is inappropriate for a chronobiology study and would have been very expensive to use a control for each period of times.

A plastic rack carrying tissue slides were placed into an autostainer machine with a built-in oven at 65°C. Slides were kept in an oven overnight to clear water from sections and were allowed to cool down for 30 minutes prior to treatment. Sections were deparaffinised and rehydrated (Appendix 5); sections were not allowed to dry until the in-situ antigenic determinants were exposed.

#### **2.5.2.2. Detection system**

The antigen retrieval techniques and fluorescence labeling were used as the detection system for immunofluorescent staining.

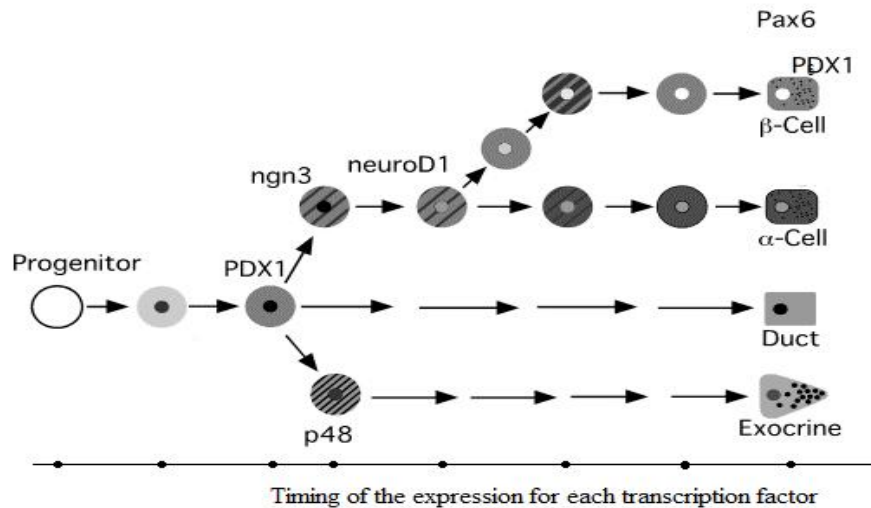
#### **2.5.2.2.1. Antigen retrieval**

To retrieve the antigens in tissues, an electric autoclave at 1000 watt was used. Plastic racks carrying deparaffinised tissue sections were immersed into the autoclave container filled with 100ml pre-treatment solution of 10mM citrate buffer (pH 6.0); the autoclave was switched on until temperature reached 100°C in about 5 minutes. Slides were maintained in boiling pre-treatment solution for further 15 minutes, when the container was taken off the autoclave and slides were allowed to cool in the pre-treatment solution for 20 minutes at room temperature before immunostaining took place.

#### **2.5.2.2.2. Immunochemical markers**

The choice for the markers to label the homeodomain proteins (Pdx1, Ngn3, NeuroD and Pax6) involved in endocrine pancreas development was based on the hierarchy in the time related expressions of the transcription factors (Figure 4), and the availability of these markers on the market by the time the study was initiated.

The markers used as primary antibodies were the markers for endocrine pancreas development genes and cellular apoptosis (Caspase-3) both polyclonal and raised in rabbit, and the monoclonal marker for Beta cells (insulin) raised in mouse (Table 2).



**Figure 4.** A single step in endocrine differentiation in the mouse pancreas showing the stage-related timing of expressions of some transcription factors (Modified from Schwitzgebel *et al.* 2000).

**Table 2.** Primary antibodies and dilutions used in the study

Antibody	Clone N0.	Raised in	Format <sup>a</sup>	Dilution	Sources <sup>b</sup>
Insulin	Lv1384150	Mouse	AF	1:50	Chemicon
Pdx1	0607035666	Rabbit	PurI	1:200	Chemicon
Ngn3	Ab5684	Rabbit	PurI	1:200	Chemicon
NeuroD1	Ab15580	Rabbit	PurI	1:25	Chemicon
Pax6	Lv1375908	Rabbit	PurI	1:500	Chemicon
Caspase3	Lv2359461	Rabbit	PurI	1:10	Chemicon

<sup>a</sup>PurI, Purified Immunoglobulin; AF, Ascite Fluid.

<sup>b</sup>Chemicon International Inc., Belerica, USA.

However, Dichloro Triazinyl Amino Fluorescein (FITC) and Cyanine-3 (Cy3) were used as secondary antibodies; both have a dual-label fluorescent incorporated compound and were goat anti-Rabbit IgG and goat anti-mouse IgG respectively (Table 3).

**Table 3.** Secondary antibodies and dilutions used in the study

Antibody	Label	Form	Format	Dilution	Source <sup>c</sup>
FITC/DTAF	Fluor	Dual	Lyophilized	1:50	Chemicon
Cy3	Fluor	Dual	Lyophilized	1:200	Chemicon

<sup>c</sup>Chemicon International Inc., Bellerica, USA.

The markers (primary antibodies and secondary antibodies) were purchased from CHEMICON International Inc, (Bellerica, USA).

A fluorescent nuclear dye 4',6-diamidino-2-phenylindole (DAPI) with an antifade solution was used as counterstain to labelled cell nuclei. Microscope glass coverslips (Chance Proper Ltd, Smethwich, Warley; England) were used as coverslips to all sections and, a mixture of Distyrene, a plasticizer and xylene (DPX) was used to fix the edges of the coverslips in place.

### **2.5.3. Immunofluorescent labeling**

Sections were rinsed in 500mL de-ionized water for 5 minutes and immediately placed in oxidation blocking solution (PBS + 2% (v/v) hydrogen peroxidise) once for 5 minutes; this helped quench the endogenous peroxidase by blocking the non-specific antigen sites found in tissues. A diamond pen was used to draw a circle around the sections to help maintain fluid during subsequent incubations. Sections were rinsed in PBS and were stained immediately.

### **2.5.3.1. Simple-label immunofluorescence**

The immunostaining protocol was identical across individual antibodies as modified and adapted from their individual data sheets and was applied as follows: The sections were immersed for 3 minutes in working buffer1 (PBS + 2% v/v normal goat serum). Excess buffer was tapped off from slides, caution was taken not to touch sections or allow them to dry. The primary antibodies were quickly applied on sections using their respective working dilutions as shown in Table 2 above, and the sections were incubated with 10 $\mu$ l of the primary antibodies for 30 minutes at room temperature. The sections were next rinsed for 3 minutes in working buffer1; excess buffer was tapped off the slides and subsequently incubated with 10 $\mu$ l of FITC for 30 minutes at room temperature. After a wash for 3 minutes with 0.05% Tween20 in PBS, the sections were finally rinsed in distilled water for 3 minutes; sections were counterstained and coverslipped.

#### **2.5.3.1.1. Double-label immunofluorescence with PIFD approach in a one-step incubation**

A parallel approach of immunofluorescence dual labeling (PIFD), was preferably applied to study the relationship between the expression of anti-Pdx1, anti-Ngn3, anti-NeuroD, anti-Pax6 and anti-Caspase-3 with that of anti-insulin; because their primary antibodies and that of insulin are derived from different species, rabbit and mouse respectively.

Sections were immersed in a working buffer<sup>1</sup> for 3 minutes and sections were incubated for 30 minutes with 10µl of a mixture of the two primary antibodies (raised from different species, mouse and rabbit) solution at appropriate respective working dilutions. Sections were rinsed in working buffer<sup>1</sup> for 3 minutes and subsequently incubated with 10µl in a mixture of two secondary antibodies FITC and Cy3 at appropriate dilution as shown in Table 3 above for 30 minutes. Sections were washed in 0.05% Tween20 in PBS for 3 minutes and were finally rinsed in distilled water for 3 minutes; sections were counterstained and cover slipped as described earlier. After staining was completed, slides were stored in the dark at 4°C until viewed.

## **2.6. Computer-Assisted Quantitative Image Analysis (CAQIA)**

### **2.6.1. Slide viewing and image capturing**

After immunofluorescent staining, labelled tissue slides (n=858) were grouped according to their prefixes following the time periods pre- and post-PDL, and were put (n=78) in their respective slide boxes. Slide boxes were viewed under a Zeiss Axio imager A1 microscope (series number 3517001133) equipped with a Fluorescent HBO 100 (Carl Zeiss Vision GmbH, Zeppelinstrasse 4, 85399 München-Hallbergmoor, Germany) and a Carl Zeiss black and white camera. The system is computerized and driven by MECER premium Pentium 4 HT hardware systems which are operated by Windows XP 2007.

To minimized photo bleaching during image acquisition, tissue slides were subjected to blue light for less than 10s; exposure time for image capture was always less than 1s. Immunofluorescent images were captured at 20x magnification using an A-Plan

lens [numerical aperture (NA), 0.25; Zeiss], and the general morphological aspect of the immunofluorescence images of the pancreas was evaluated to determine if the immunofluorescent procedure had any impact on the morphology of tissue samples; the captured images were then exposed to quantitative image analysis.

### **2.6.2. Quantification of protein expression in PDL pancreas**

The protein expression profiles in tissues were determined by the fluorescence emissions obtained from positive labelled cells; the resulting images were acquired by the Zeiss colour camera with the same exposure time and processed identically under a multidimensional image acquisition module of Axiovision 4.8 (Imaging System, Carl Zeiss; München, Germany) driven by a computerized system. The FITC-labelled cells were excited at 490 nm and emitted green (at 520 nm), while Cy3-labelled cells were excited at 456 nm and emitted red (562 nm); all counterstained DAPI-labelled nuclei were excited at 365 nm and emitted blue (463 nm). The captured images accurately represent the visual impression of the observer.

The digital images were segmented and the features of the positive labelled cells were programmed using the automeasurment software of Axiovision 4.8 and MTB2004 configuration (both Carl Zeiss Vision GmbH). All the images to analyse were given a name (image name) and the features of measurements were set as Regions and Field features as described below:

- The Regions consisted of each labelled cell (Count) and the area of the individual labelled cell (Area)

- The Field feature consisted of the total number of labelled cells (Total count) expressed n, the total area of labelled cell (Total Sum) expressed in  $\mu\text{m}^2$  and the expression index.

The number of fluorescent labelled cells for the same antigen were counted and their cell surface areas measured by automation. Data were recorded per time pre- and post-PDL for each immunofluorescence procedure and saved automatically in files (Appendix 6).

## **2.7. Definitions**

The landmarks in targeting the expression profile of homeodomain proteins involved in the remodelling of the pancreas after surgical duct ligation were determined by the morphological changes observed under H&E slides of the PDL tissues of the pancreas during various time periods post-pancreatic ligation (PDL). These landmarks were used in the analysis of expression patterns of transcription factors following the quantification of protein expression at each period of time pre- and post-PDL. The resulted expression of individual homeodomain protein across the time periods pre- and post-PDL and their co-expression with insulin, defined the lineage of endocrine cell development in the duct ligation pancreas in the laboratory rat.

The expression index, which is essential in quantifying the protein expression in all the tissue sections in each group, was calculated using the following formula:

$$\text{Expression index} = \text{total number of count} / \text{total area}$$



Dual gene expression (Co-expression) of different homeodomain proteins with insulin was expressed as a ratio of the expression index of insulin per the expression index of each one of the proteins across the time periods pre- and post-PDL. Co-expression of insulin and other homeodomain proteins was defined by a curve parallel to the curve of insulin expression index across the time periods pre- and post-PDL.

The efficiency of co-expression was arbitrarily defined by the value of mean ratio (score without unit) of insulin expression divided by each expression of the other homeodomain proteins, occurring within the time interval of 12 hours cycle. Levels of ratio of co-expression were said to be optimal or high ( $>25$ ), average or medium (20-25), and weak or low ( $\leq 20$ ).

## **2.8. Statistical analysis and Immunohistofluorescence evaluation**

Continuous variables were expressed as mean  $\pm$  Standard Deviation (SD) or Standard Error of Mean (SEM). To analyse individual homeodomain protein expression and the efficiency of co-expression of proteins, analysis of variances (one-way ANOVA) was used to compare the means of protein expression indices across the different time periods pre- and post-PDL. Simple coefficient of correlation “r” was calculated to assess potential associations between the different protein expression indices in the study. P-values less than 0.05 were considered statistically significant; no post hoc test was done. Data were exported from MS Excel (Microsoft Inc, USA) to the Statistical Package for Social Sciences (SPSS) version 15 for Window (SPSS Inc, Chicago, IL, USA) for statistical analysis.

## **Chapter 3. Results**

### **3.1. Histological assessment of post-PDL tissues**

The microscopic studies of the post-PDL tissues showed some morphological changes across the time periods post-PDL. Photomicrographs were grouped in a chronological order and the changes observed were similar between certain time periods and led to the time periods post-PDL being grouped together and were considered landmarks as follows: Landmark I: 6, 12 and 24 hour; Landmark II: 36, 48 and 60 hours; Landmark III: 72 hours; Landmark IV: 84, 96, 108 and 120 hours (Figure 5).

The islets of Langerhans appeared unaffected in the first 24 hours post-PDL; but there was evidence of generalised granulation of acinar cells having various shapes from 6 hours through to 24 hours post-PDL. A progressive separation between the acinar cells and their basement membrane was observed at 12 hours. A complete fragmentation of acinar cells was seen at 24 hours. Some fibroblastic cells were seen undergoing mitosis in the underlying connective tissue through this first landmark (Figure 5I).

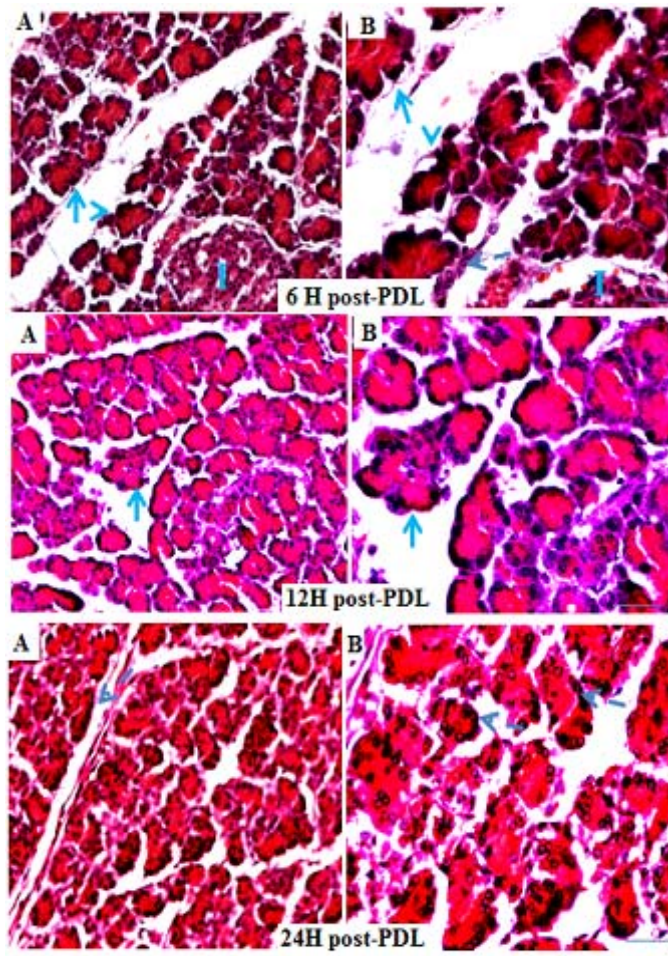
Between 36 and 60 hours, there was a distinct crescent-like clumping appearance of the acinar cells that became progressively atrophied and shapeless. However, there was infiltration of macrophages in the pancreatic tissue (Figure 5II).

Abundant levels of acinar deletion occurred at higher rate at 72 hours post-PDL, with evidence of acinar cell necrosis. The islet cells were detached from the underlying

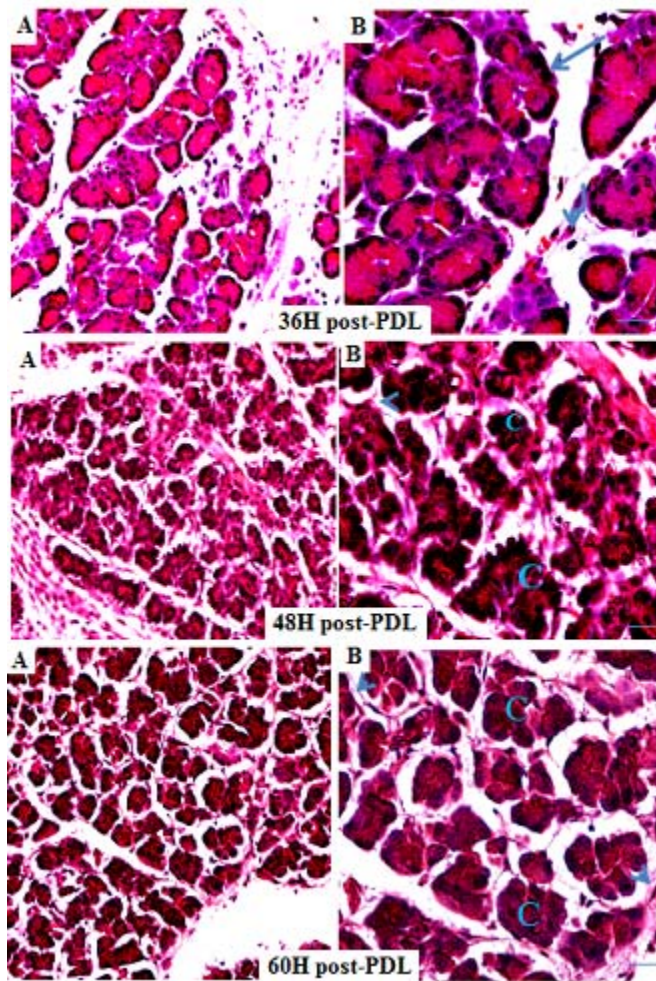
connective tissues with a considerable amount of binucleated macrophages observed within them; there were few visible surrounding necrotic cells (Figure 5III).

At 84 hours, some necrotic acinar cells were still visible; concurrently there was an ongoing acinar cell replacement. However, the islet cells remained unaffected up to this hour. There were some ductal epithelial cells that have begun to bud off and this continued over the following 96 to 120 hours. A glandular formation was apparent at 96 hours with numerous infiltrated mononuclear macrophages. There was evidence of cellular regeneration through to 120 hours with the presence of clearly observable ducts, fully formed acini and islet cells. The diameter of the ducts was clearly delineated with somewhat low cuboidal acinar cells, the presumptive centroacinar ducts. There was evidence of glandular secretion. A rich supply of capillary networks was seen in the vicinity of islets of Langerhans. Many intercalated ducts showed out pocketing of their lumens at 120 hours contributing to the endocrine cell formation (Figure 5IV).

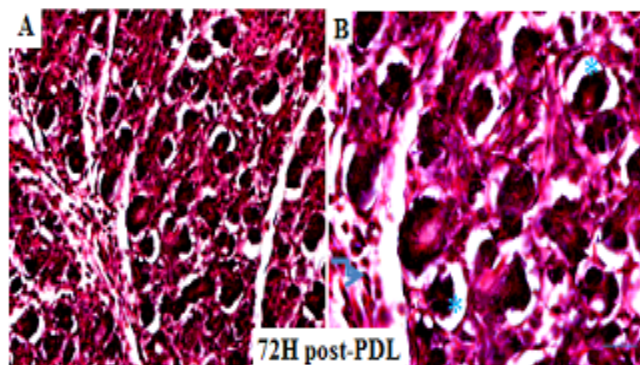
-I-



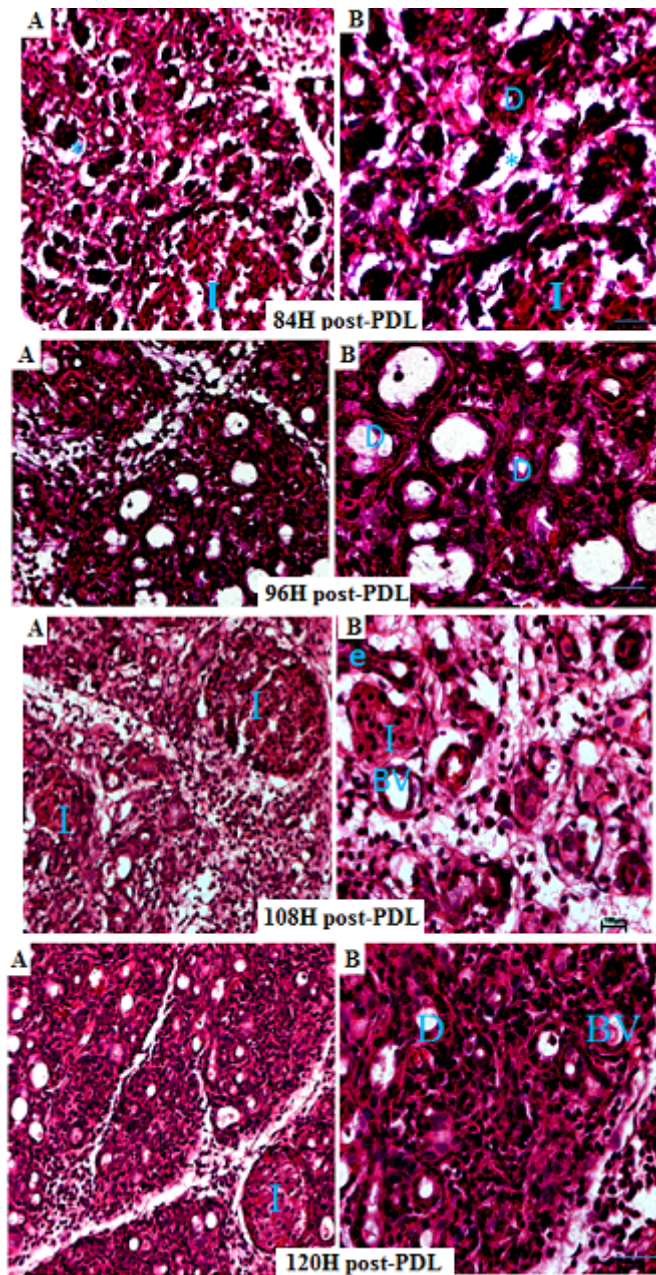
-II-



-III-



-IV-



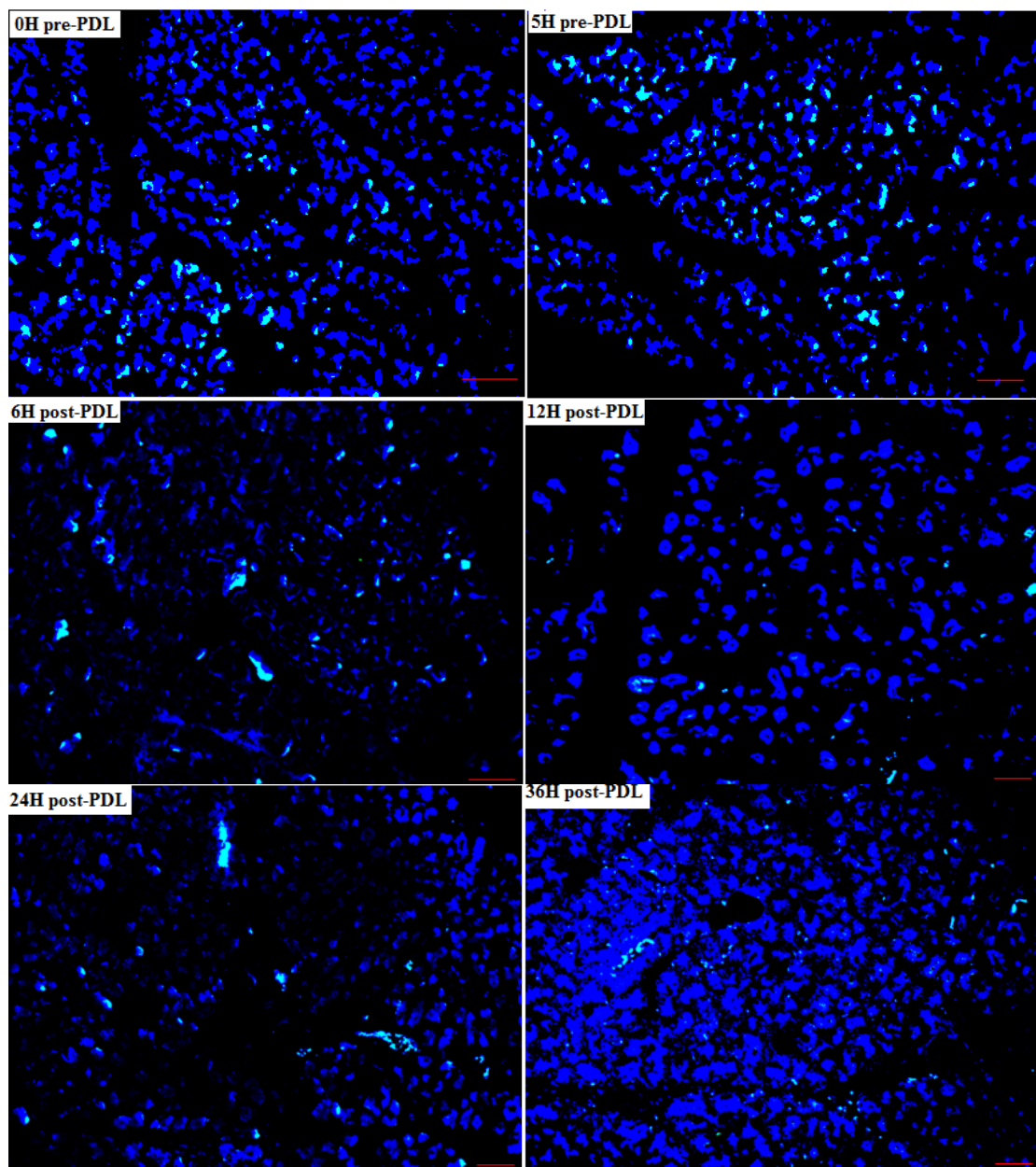
**Figure 5.** Photomicrographs of morphological changes at various landmarks for time periods post-PDL.

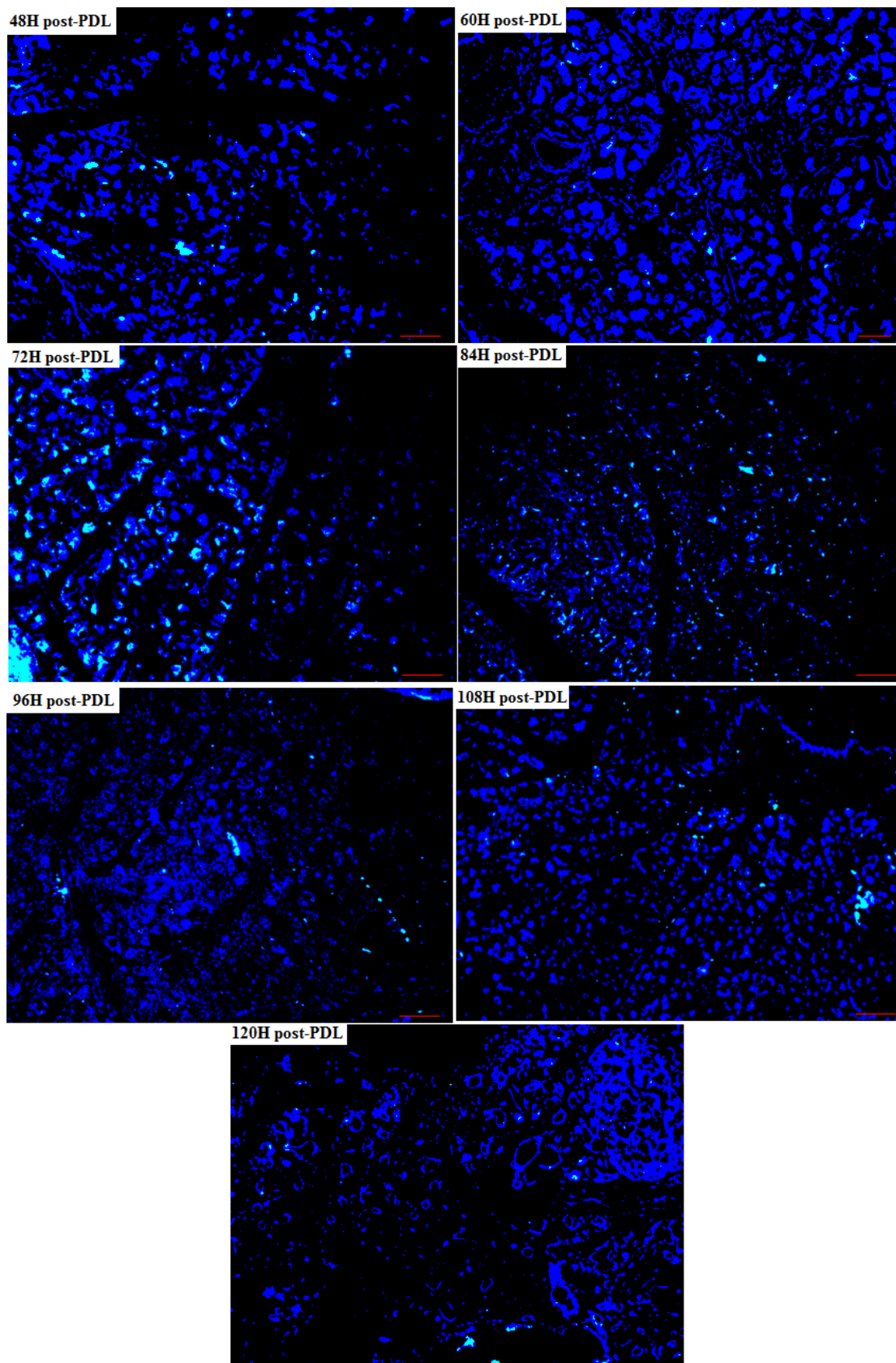
A) x200, bar: 10 $\mu$ m; B) x400, bar: 5 $\mu$ m.

*Arrow*: granulated cells; *Arrow head*: basement membrane; *dash line*: fibroblastic cells. *Letter C*: clump of acinar cells; *Broken arrow*: infiltrated macrophages; *Star*: acinar deletion; *BV*: blood vessel; *D*: duct; *e*: duct evagination.

### 3.2. Morpho-immunofluorescent evaluation of PDL tissues

Figure 6 illustrates the fluorescent reflection of the time-related expression of Pdx1 across the time periods pre- and post-PDL. The fluorescent images were used to analyse the protein expression of single homeodomain protein as well as the dual expression of individual homeodomain proteins with insulin.



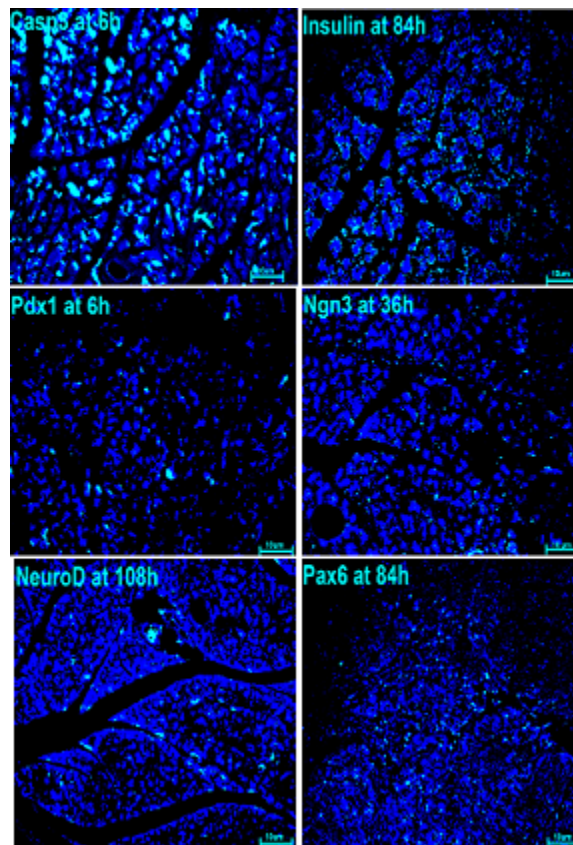


**Figure 6.** Expression of Pdx1 at time periods pre-and post-PDL (x200).



Following the landmarks as determined by the morphological changes observed in H&E micrographs, the immunofluorescent studies revealed high expression of some proteins within a specific landmark as represented in Figure 7.

In the first landmark interval of 6-24H, the expression of Caspase3 was prominent at 6 H, and similarly for Pdx1 expression. Ngn3 expression was noted at 36 hours for the second landmark of 36-60H. For the fourth landmark of 84-120H, however, Pax6 expression as well as insulin expression was observed at 84H and NeuroD expression was seen at 120H. There was no protein expression in the third landmark of 72H.



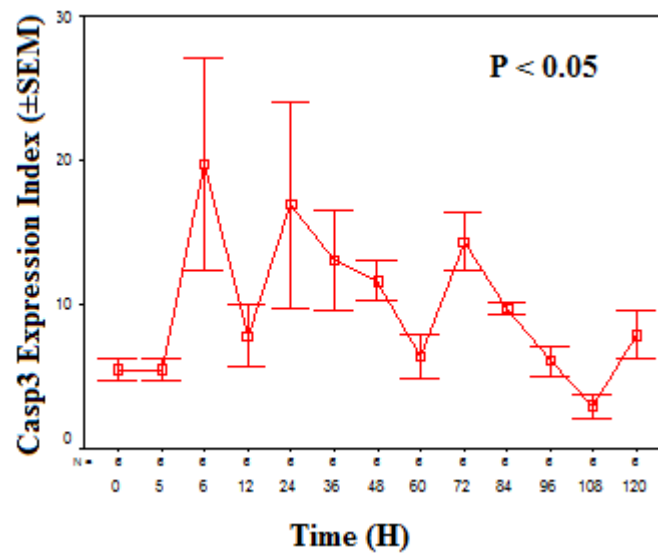
**Figure 7.** Immunofluorescent of the PDL pancreas showing the highest expression of different homeodomain proteins at various time post-PDL (x200).

### 3.3. Immunohistofluorescent analysis of protein expression

#### 3.3.1. Single expression of protein in PDL tissues

##### 3.3.1.1. Expression of Caspase-3

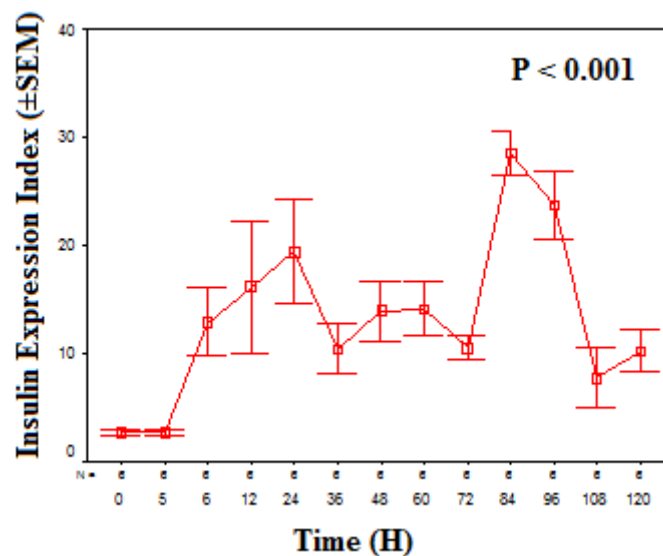
The caspase3 expression was defined by three modes at 6 hours, 24 hours and 72 hours. The highest expression was observed at 6 hours, with higher variability of the expression at both the 1<sup>st</sup> and the 2<sup>nd</sup> mode. The nadir of the curve was observed at 108 hours (Figure 8), lower than that observed at 0 and 5 hours pre-PDL. Caspase3 expression varied significantly across the period of times pre- and post-PDL (ANOVA;  $P=0.013$ ). However, the expression of caspase3 had a significant and positive correlation with that of Ngn3 (section 3.2.1.4;  $r=0.285$ ;  $P=0.011$ ).



**Figure 8.** Caspase3 expression across the period of times pre- and post-PDL.

### 3.3.1.2. Expression of Insulin

Insulin expression varied significantly (ANOVA;  $P < 0.0001$ ) across the different periods of time pre- and post-PDL. The highest mean of the expression index was observed at 84 hours, while the lowest index was observed at 120 hours post-PDL. The latter expression was higher than the mean expression index at the time periods 0 and 5 hours pre-PDL (Figure 9). However, the expression of insulin had a significant and positive correlation with both Ngn3 ( $r=0.245$ ;  $P=0.031$ ) and Pax6 ( $r=0.301$ ;  $P=0.007$ ).

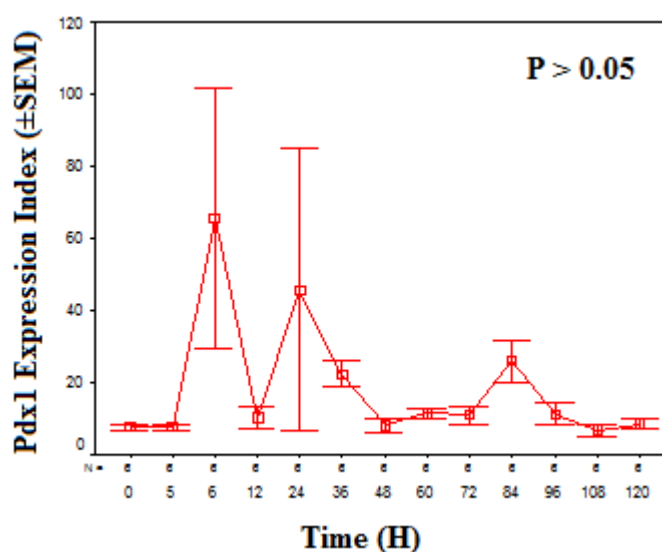


**Figure 9.** Insulin expression across the period of times pre- and post-PDL.

### 3.3.1.3. Expression of Pdx1

The mean expression index of Pdx1 was unequally distributed, but without significant difference (ANOVA;  $P=0.177$ ) between the various time periods of the experiment (Figure 10). The highest expression index was concurrent with 6 hours post-PDL,

while the lowest mean of the expression index was at 108 hours post-PDL and were similar to those of time periods pre-PDL 0 and 5 hours, respectively. However, there was high variability of Pdx1 expression between 6 hours and 24 hours. Furthermore, there was no correlation with any of the proteins expression in the study:  $r=-0.025$  and  $P=0.831$  with Caspase3,  $r=0.116$  and  $P=0.311$  with Insulin,  $r=-0.076$  and  $P=0.510$  with Ngn3,  $r=-0.031$  and  $P=0.785$  with NeuroD and  $r=0.114$  and  $P=0.322$  with Pax6.

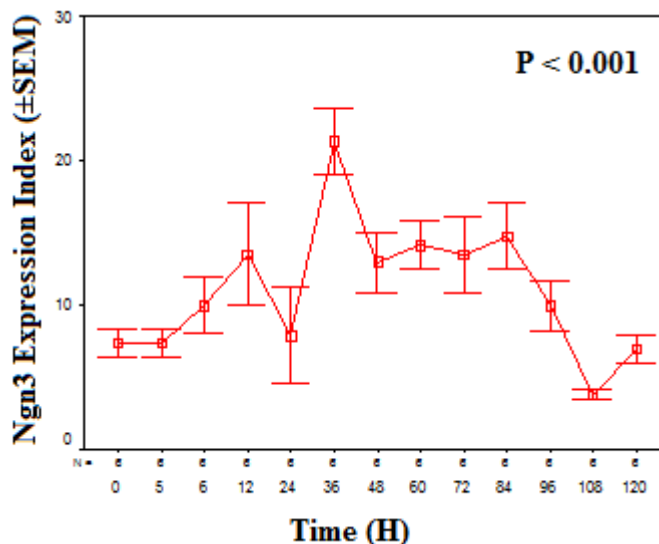


**Figure 10.** Pdx1 expression across the period of times pre- and post-PDL.

#### 3.2.1.4. Expression of Ngn3

The Figure 11 depicts the curve of Ngn3 expression with anabase part between 0 and 36 hours and catabase part between 48 and 120 hours. There was a significant difference (ANOVA;  $P < 0.0001$ ) between the mean expression indexes across the different periods of time pre- and post-PDL. The peak of the curve was observed at 36 hours, while the nadir of the curve was at 108 hours post-PDL. Furthermore, there

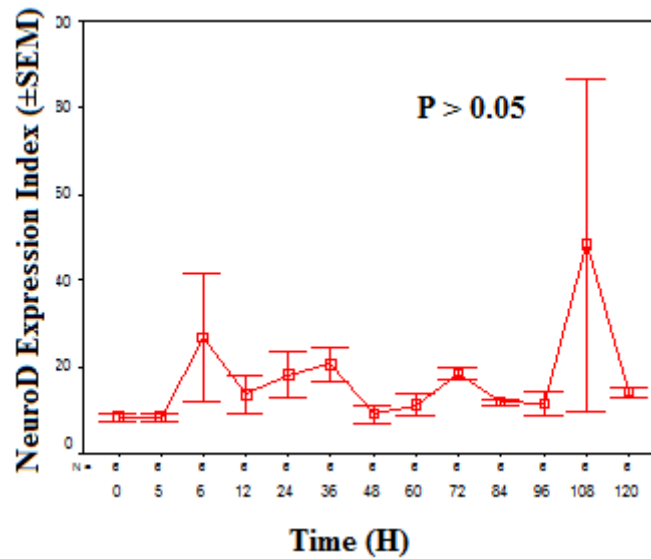
was a significant and positive correlation between the mean index of the Ngn3 ( $r=0.245$ ;  $P=0.031$ ) expression and insulin and between Ngn3 ( $r=0.285$ ;  $P=0.011$ ) and Caspase3 expression.



**Figure 11.** Ngn3 expression across the period of times pre- and post-PDL.

### 3.3.1.5. Expression of NeuroD

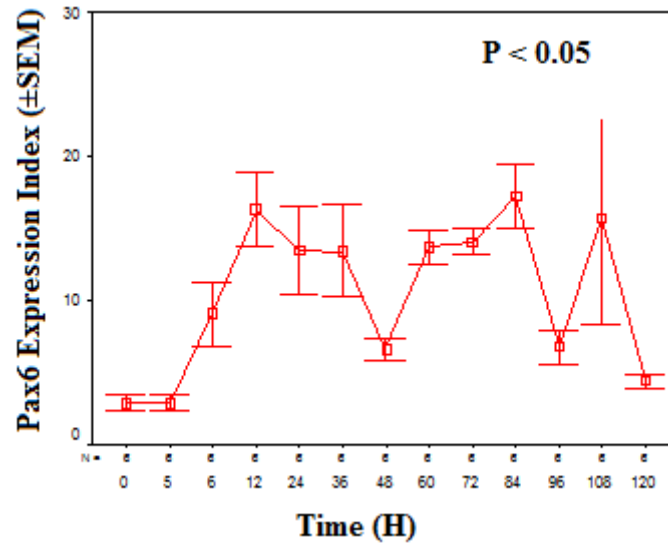
The variations of the NeuroD expression across the different periods of time pre- and post-PDL are shown in Figure 12. The highest (spike) of the NeuroD expression was observed at 108 hours and presented with a higher variability as compared to the rest of the time periods which had a more intermediate mean index between them. The lowest mean of expression indexes was at 48 hours post PDL. However, there was no significant difference (ANOVA;  $P=0.588$ ) between the mean indexes across the time periods in the study. There was also no correlation between NeuroD expression and other protein expression in the study:  $r=0.007$  and  $P=0.955$  with caspase3,  $r=-0.062$  and  $P=0.442$  with insulin,  $r=-0.031$  and  $P=0.785$  with Pdx1,  $r=-0.056$  and  $P=0.623$  with Ngn3 and,  $r=-0.058$  and  $P=0.614$  with Pax6.



**Figure 12.** NeuroD expression across the period of times pre- and post-PDL.

### 3.3.1.6. Expression of Pax6

The curve of the mean expression index of Pax6 expression was defined by three modes, observed at 12 hours, 84 hours and 108 hours (Figure 13). There was a significant difference (ANOVA;  $P \leq 0.0001$ ) of protein expression between all the time periods pre- and post-PDL, with the highest mean index at 84 hours. The lowest mean index was at the time periods pre-PDL 0 and 5 hours. The highest variability of Pax6 expression was observed at 108 hours, while significant and positive correlation was seen between Pax6 ( $r=0.301$ ;  $P=0.007$ ) and insulin expressions.

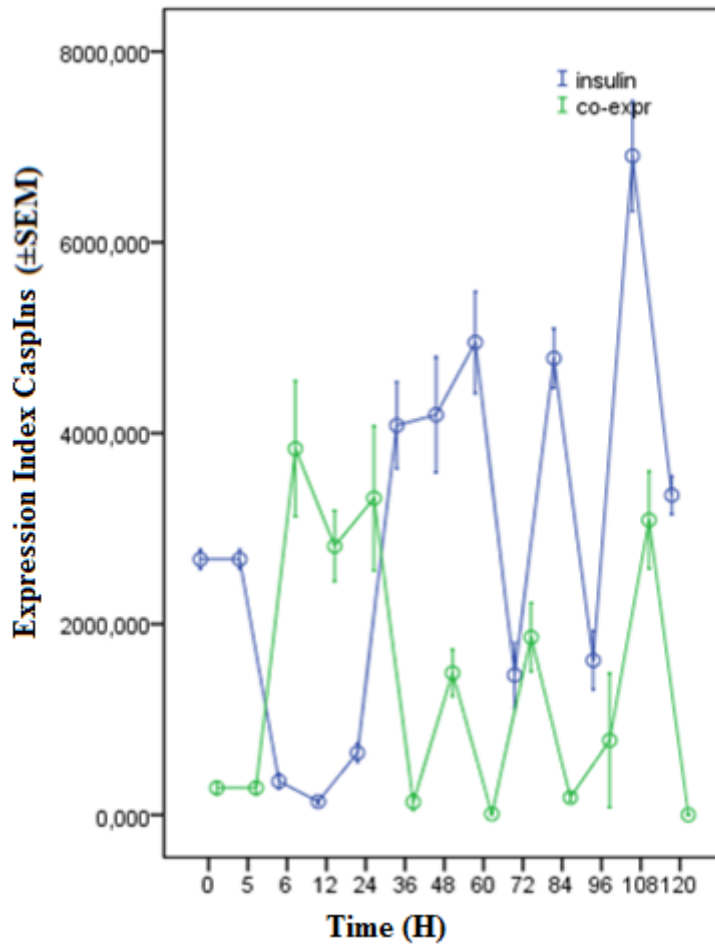


**Figure 13.** Pax6 expression across the period of times pre- and post-PDL.

### 3.3.2. Dual expression of proteins in PDL

#### 3.3.2.1. Co-expression of insulin with Caspase3

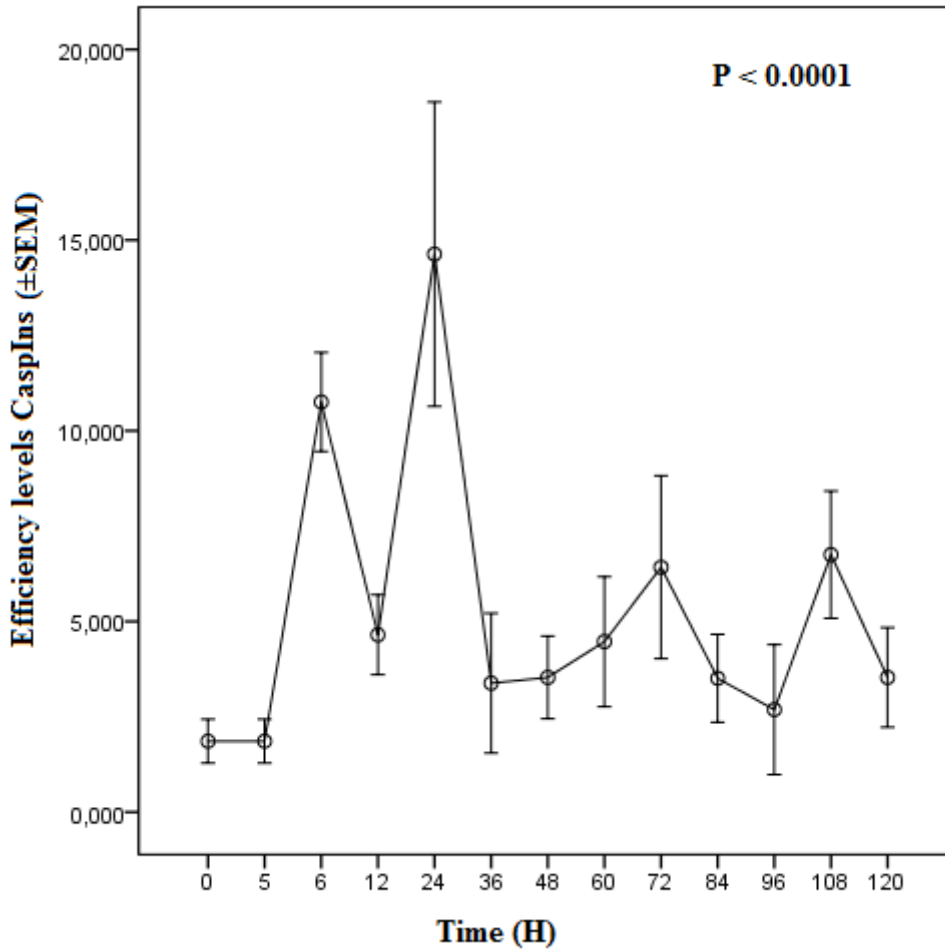
Figure 14 shows the mean expression index of insulin and that of the co-expression (insulin-caspase3) as covariate. The highest mean expression index of the co-expression occurred between 6 and 24 hours post-PDL, whereas the highest expression index of insulin occurred later at 108 hours post-PDL. Co-expression index was low at time periods pre-PDL 0 and 5 hours as well as at time periods post-PDL 36, 60, 84 and 120 hours.



**Figure 14.** Dual expression index across the period of times pre- and post-PDL. Insulin expression (blue); Co-expression (green)

The efficiency curve in Figure 15 represents the co-expression index of insulin and caspase3. It shows four modes at 6 hours, 24 hours, 72 hours and 108 hours post-PDL. There was a statistically significant difference (ANOVA;  $P \leq 0.0001$ ) of mean values between all the time periods pre- and post-PDL. The highest mean level of efficiency was at 24 hours post-PDL; however, the variability of efficiency was very high at that same time. The time periods pre-PDL 0 and 5 hours had the lowest efficiency mean value.

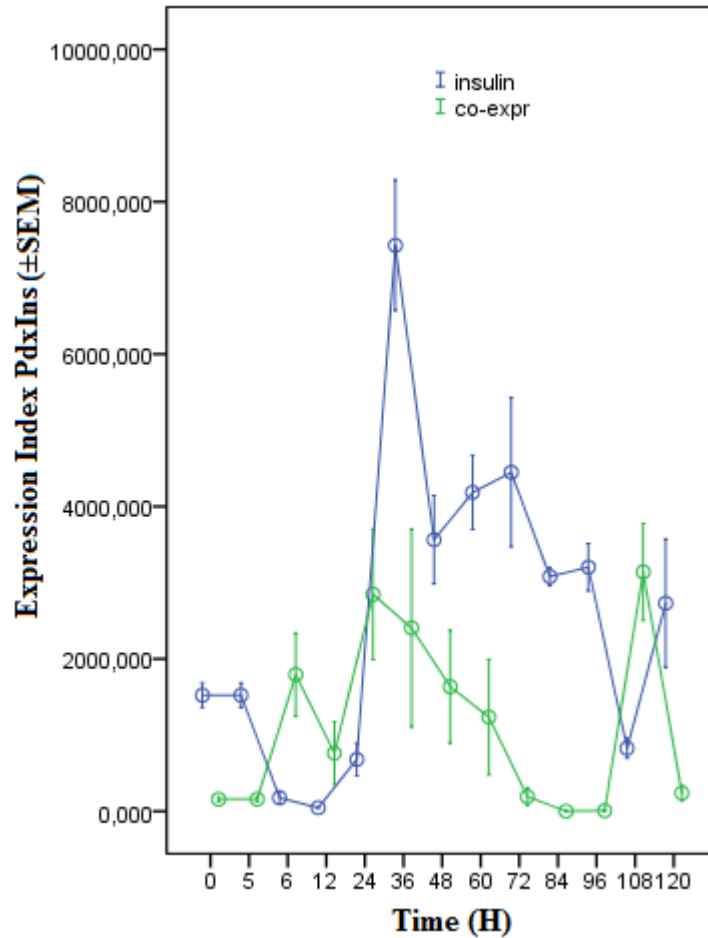




**Figure 15.** Efficiency levels of Caspase3/Insulin co-expression across the period of times pre- and post-PDL.

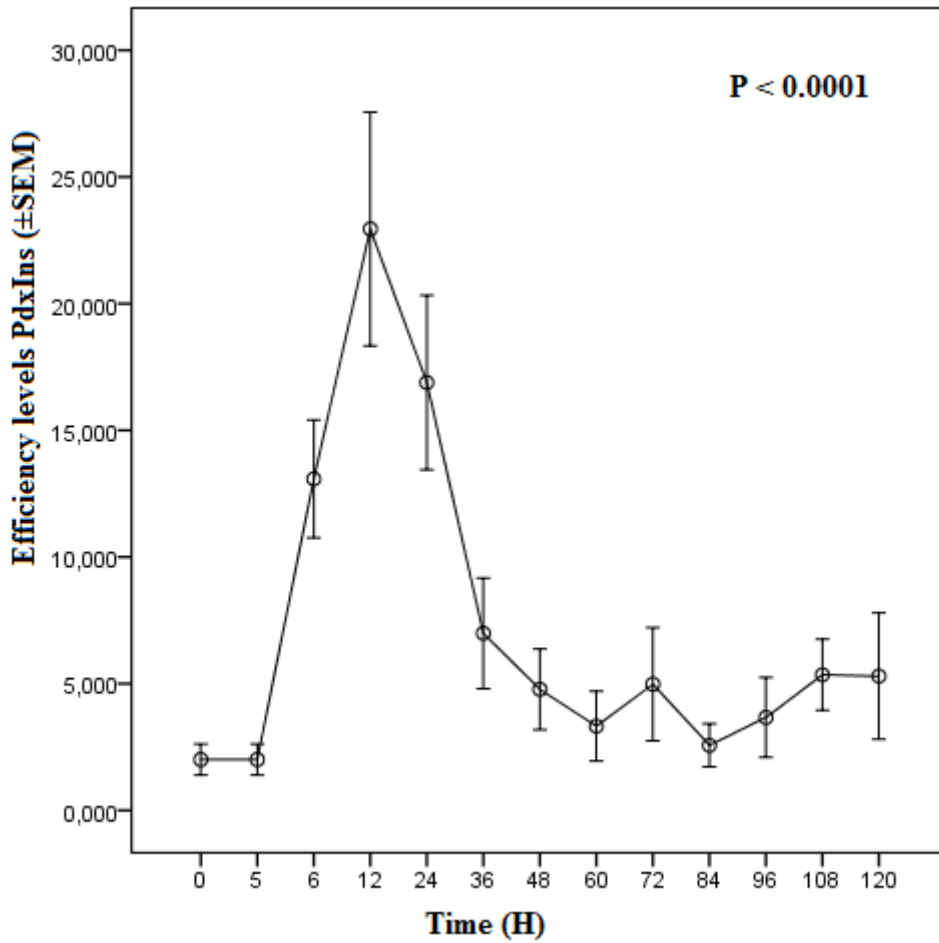
### 3.3.2.2. Co-expression of Insulin with Pdx1

The highest mean expression index of insulin was observed at 36 hours post-PDL, while the highest peaks of the co-expression index of insulin and Pdx1 were successively observed at 24 hours and 108 hours post-PDL (Figure 16). The mean co-expression was low at time periods 0 and 5 hours similar to that observed at 72 and 96 hours post-PDL.



**Figure 16.** Dual expression index across the period of times pre- and post-PDL. Insulin (blue); Co-expression (green).

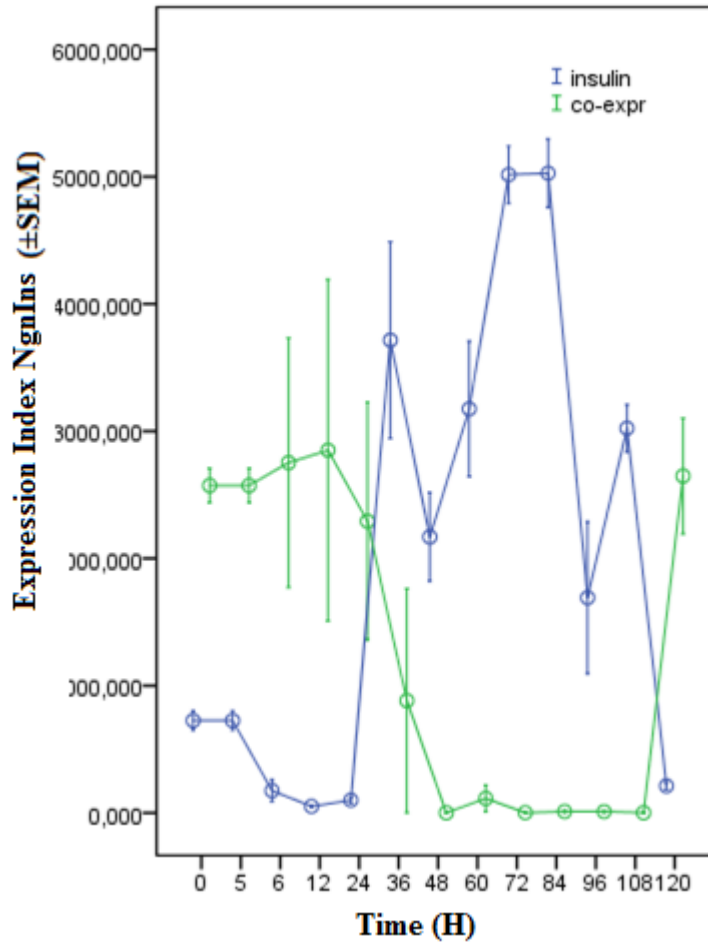
The efficiency level of co-expression of insulin and Pdx1 was characterized by a unimodal curve with a peak at 36 hours (Figure 17). This peak was defined by higher variability. There was a significant difference (ANOVA;  $P \leq 0.0001$ ) between all the times periods pre- and post-PDL. However, the time periods pre-PDL 0 and 5 hours had the lowest levels of efficiency.



**Figure 17.** Efficiency levels of Pdx1/Insulin co-expression across the period of times pre- and post-PDL.

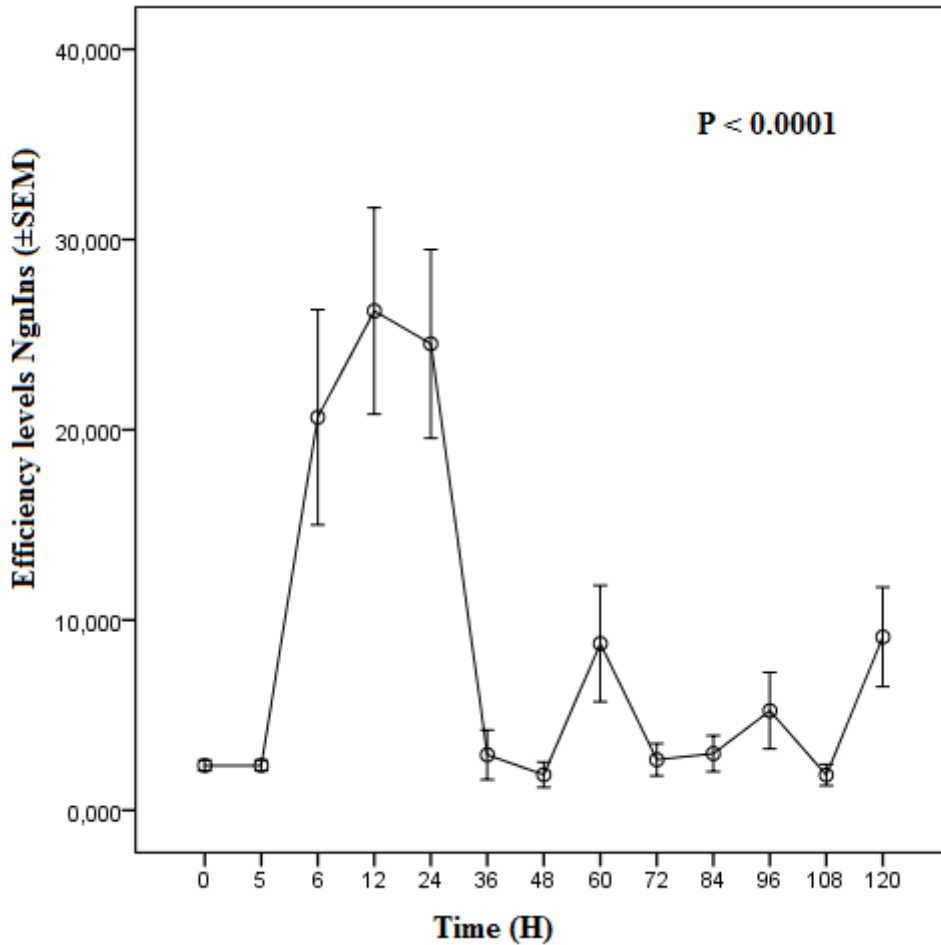
### 3.3.2.3. Co-expression of Insulin with Ngn3

The highest mean levels of expression index of insulin occurred between 72 hours and 84 hours post-PDL, whereas the highest peaks of co-expression of insulin and Ngn3 were successively observed at 12 hours and 120 hours post-PDL. The co-expression index at time periods pre-PDL 0 and 5 hours was higher than most of the time periods post-PDL (Figure 18).



**Figure 18.** Dual expression index across the period of times pre- and post-PDL. Insulin (blue); Co-expression (green).

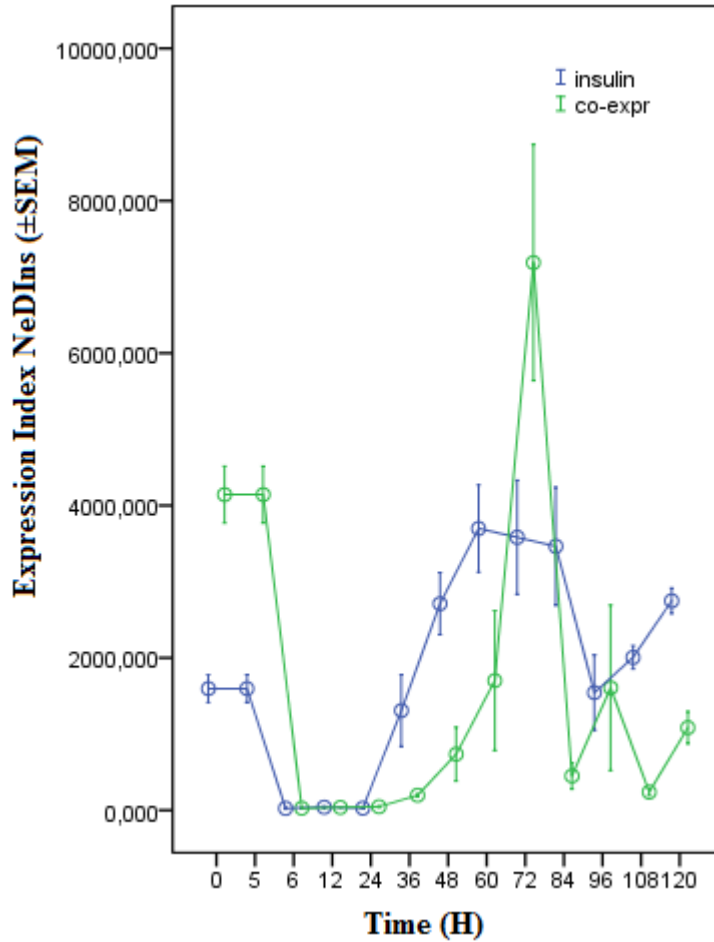
Figure 19 depicts the mean efficiency levels of Insulin and Ngn3 co-expression. The highest level of efficiency was concurrent at 12 hours. A significant difference (ANOVA;  $P \leq 0.0001$ ) was observed between the time periods pre- and post-PDL. Low efficiency levels observed at time periods 0 and 5 hours was similar to that observed at time periods 48 and 108 hours post-PDL.



**Figure 19.** Efficiency levels of Ngn3/Insulin co-expression across the period of times pre- and post-PDL.

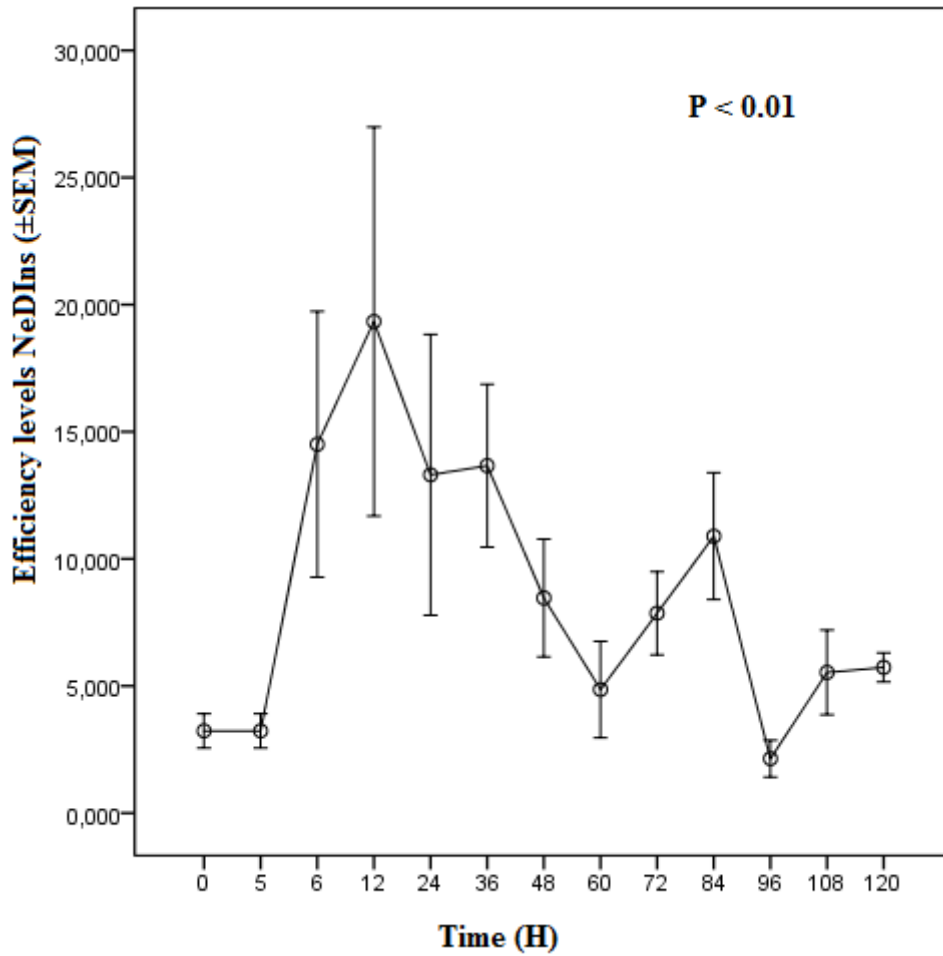
#### 3.3.2.4. Co-expression of Insulin with NeuroD

The curve of co-expression index of insulin and NeuroD was dominant in comparison with that of Insulin expression at the time periods 0 and 5 hours pre-PDL, 6 hours and later between 60 and 84 hours post-PDL with an overshoot observed at 72 hours. This contrasted with the plateau mode of insulin expression between 60 hours and 84 hours post-PDL. The co-expression and insulin expression were similarly low between 6 and 24 hours post-PDL (Figure 20).



**Figure 20.** Dual expression index across the period of times pre- and post-PDL. Insulin (blue); Co-expression (green).

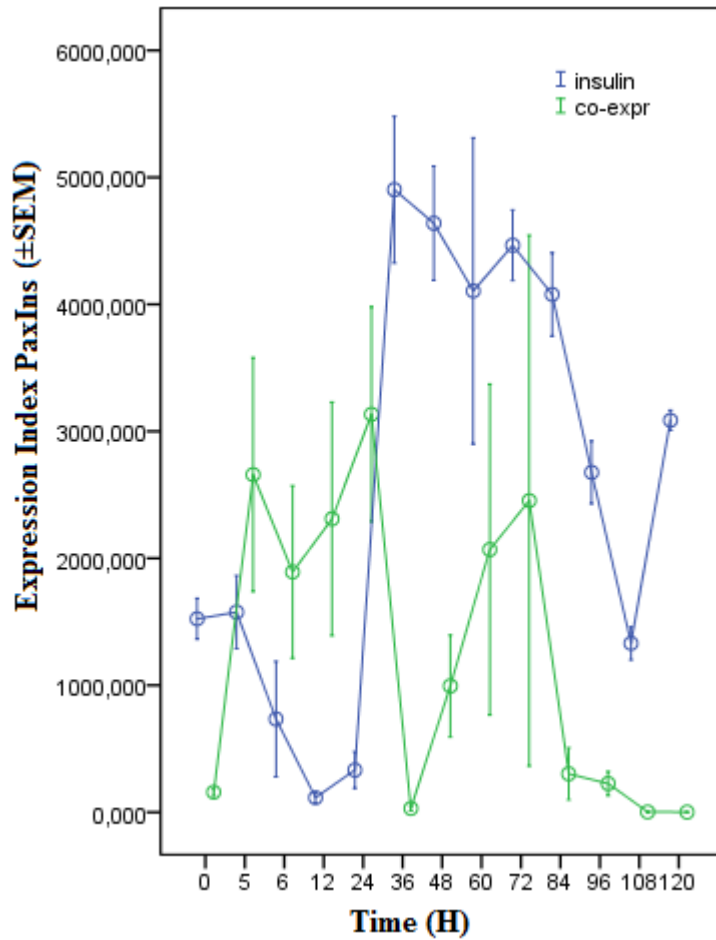
However, the mean efficiency levels of insulin and NeuroD co-expression reached a highest peak at 12 hours (Figure 21). A higher variability of efficiency was observed at 6-24 hours. There was a significant difference (ANOVA;  $P=0.007$ ) of efficiency mean values between the time periods pre- and post-PDL, with the lowest mean value observed at 0 and 5 hours pre-PDL and 96 hours post-PDL



**Figure 21.** Efficiency levels of NeuroD/Insulin across the period of times pre- and post-PDL. Insulin (blue); Co-expression (green).

### 3.3.2.5. Co-expression of Insulin with Pax6

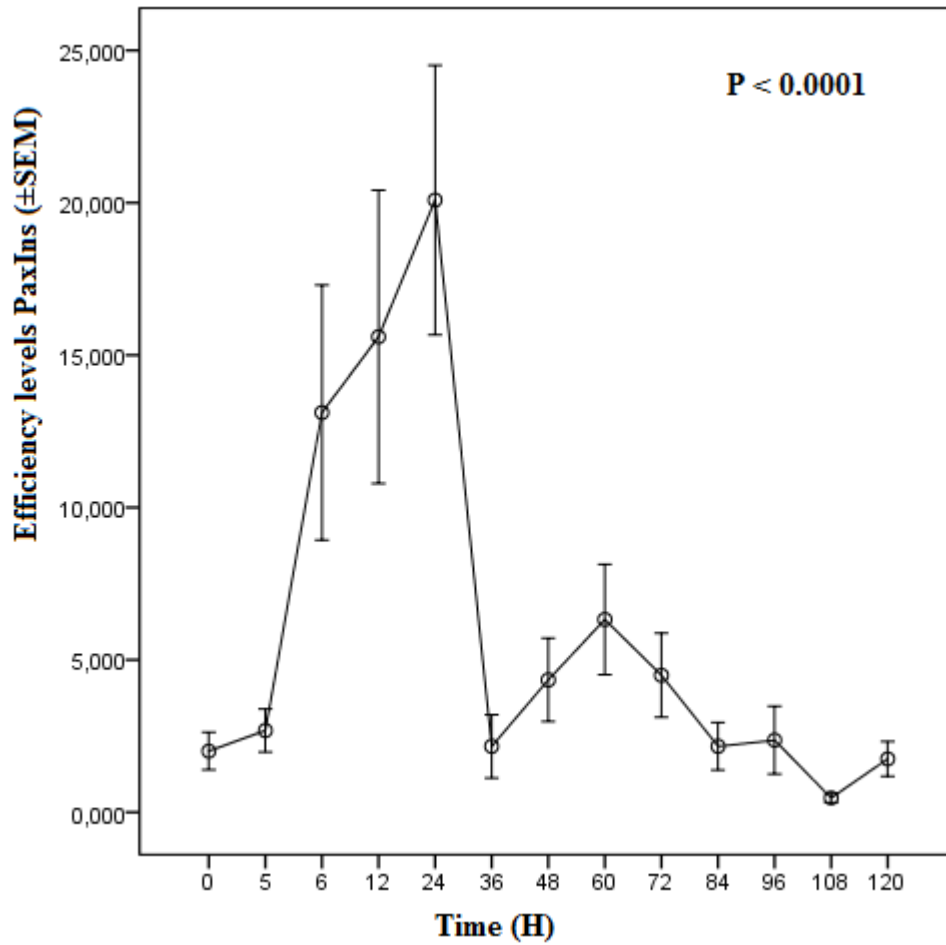
The highest mean of the co-expression index of insulin and Pax6 was observed at 12 hours post-PDL, whereas the expression of insulin was totally dominant over that of the co-expression at time periods pre-PDL 0 and 5 hours, with a plateau mode between 36 hours and 84 hours (Figure 22).



**Figure 22.** Dual expression index across the period of times pre- and post-PDL. Insulin (blue); Co-expression (green).

The mean efficiency levels of insulin and Pax6 co-expression reached a highest peak at 24 hours (Figure 23). There was a significant difference (ANOVA;  $P \leq 0.0001$ ) of mean values between the time periods pre- and post-PDL. The levels of efficiency at time periods pre-PDL were higher than that observed at 108 hours post-PDL.





**Figure 23.** Efficiency levels of Pax6/Insulin co-expression across the period of times pre- and post-PDL

### 3.3.3. Comparison of efficiency levels in protein co-expression

Table 4 compares the levels of efficiency for the various proteins co-expressions with insulin. There was a significant difference of efficiency levels across the genes transcription factors involved in the study (ANOVA;  $P \leq 0.05$ ).

**Table 4.** Different levels of efficiency of protein co-expressed with insulin

<b>Co-expressed proteins</b>	<b>Means <math>\pm</math>SD of ratio</b>	<b>Times concurrent with higher ratio</b>
Ins/Casp3	15 $\pm$ 2.5	24 hours
Ins/Pdx1	24 $\pm$ 11.9	12 hours
Ins/Ngn3	27 $\pm$ 18.8	12 hours
Ins/NeuroD	20 $\pm$ 26.5	12 hours
Ins/Pax6	20 $\pm$ 16.7	24 hours

The optimal or high efficiency of co-expression was observed for insulin and Ngn3 co-expression, while an average or medium efficiency was for the co-expression of insulin with Pdx1, insulin with NeuroD and insulin with Pax6. Weak or low efficiency was observed for Insulin and caspase3 co-expression.

## **Chapter 4. Discussion and Conclusion**

The present study demonstrated that there is a clear and significant relationship between morphological changes and protein synthesis in the remodelling of endocrine development in duct ligated pancreas. Furthermore, the expression of endocrine developmental transcription factors with regard to the insulin expression revealed that islet transplantation for an optimal result should occur earlier than the morphological manifestation of new beta cell formation. These findings are critical in islet transplantation, an important tool for the treatment of diabetes mellitus.

### **4.1. Relationship between morphology and protein expression in PDL pancreas**

The results obtained from the morphological and the morphometric studies, using the cellular changes and the expression of proteins respectively, in the PDL pancreas during the time periods pre- and post-PDL satisfied the classification of events into four landmarks as described earlier in the study. The relationship between the expression of these proteins, and the cellular and observable changes that take place during the PDL were statistically established.

#### **4.1.1. Cellular changes in PDL pancreas**

The first landmark in this study covers the time periods 6 to 24 hours post-PDL. This period was characterized by a progressive granulation of acinar cells and subsequent acinar cell death (apoptosis). This event which is thought to be due to the effect of

duct ligation procedure is well documented (Abe *et al.* 1995; Scoggins *et al.* 2000; Page *et al.* 2000; Yasuda *et al.* 1999). Another important element of the event shown in this study is that the islets of Langerhans remain unaffected at this stage, a fact which has rarely been reported (Page *et al.* 2000). Interestingly, the observation of fibroblast mitotic cells at this early hour post-PDL, suggests that mesenchymal replication precedes endocrine formation (Puri *et al.* 2010). This latest evidence emphasizes a plausible implication of extracellular signaling pathways in the cell fate specification.

The programmed cell death that takes place in the first landmark is an ongoing process (Kerr 1993) sanctioned by cellular atrophy as was observed a decade ago by Auer and Kleiner (1918). This observation is in line with the second landmark of 36 and 60 hours post-PDL. The data showed a high degree of necrotic appearance of the acinar cells which were remarkably atrophied and shapeless. The increase in the number of infiltrated mononuclear macrophages within the ductal epithelium and the adjoining acinar is a characteristic feature of cellular death (Walker 1987).

The increase in the activity of the macrophages will therefore lead to a higher rate of acinar deletion (Abe *et al.* 1995; Edstrom *et al.* 1968) observed in the third landmark at 72 hours post-PDL of this study. This event was designated landmark of total pancreatic acinar cell death, characterized by the increase in numbers of infiltrated macrophages. The presence of these macrophages indicates the occurrence of a complex process of cellular degeneration and regeneration (Yamaguchi *et al.* 1993).

Between 84 to 120 hours post-PDL, which corresponds to the fourth landmark, the acinar cells were progressively replaced by a rapid glandular remodeling of the pancreas. Walker (1987) had noted that the acinar cell deletion occurs without disruption of the underlying basement membrane, allowing the remodeling of the endocrine pancreas to take place. Page *et al* (2000) reported a similar observation from 84 hours post-PDL with evidence of ductal compartment evagination (Bouwen *et al.* 1994; Larsson 1998; Page *et al.* 2000) forming ovoid and/ or kidney shaped islets of Langerhans. This endocrine remodeling is a direct consequence of the increase in the beta cell mass (Scaglia *et al.* 1997; Walker 1987). Although the morphological changes showed evidence of neogenesis from duct (Wang *et al.* 1995), Solar *et al* (2009) postulated that, the duct cells do not contribute in the formation of new islets cell following PDL , and that the pancreatic exocrine duct cells give rise to beta cells during embryogenesis only. Studies on cell proliferation kinetics (Teta *et al.* 2007) and lineage tracing (Dor *et al.* 2004), support this opinion and further suggested that most of the newly formed beta cells originate from the pre-existing beta cells. This evidence warrant a study that will establish the level of similarity between PDL tissues and foetal tissues as they show the same efficacy as a promising therapeutic option for the treatment of DM (Page *et al.* 2000).

#### **4.1.2. Evaluation of transcription factors involved in PDL pancreas**

The morpho-immunofluorescent evaluation of caspase3, Pdx1, Ngn3, NeuroD and Pax6 was successively demonstrated. This will help in tracing the lineage of beta cells in PDL pancreas.

#### **4.1.2.1. Caspase3 evaluation**

The immunohistofluorescent analysis of caspase3 expression showed a pulsatile profile at 6 hours, 24 hours and 72 hours post-PDL. This result contrasted with its morphological and morpho-immunological description, in which caspase3 reaction was observed only at 6 hours post-PDL. However, the three modes of caspase3 expression curve suggested that apoptosis is an ongoing process of programmed cell death (Kerr 1993). The amount of caspase3 expression, significantly and positively correlated to that of Ngn3 which is a proendocrine factor and marker for islet cell precursors. Caspase3 was therefore the first gene to be expressed at 6 hours post-PDL, presumably because of its role in apoptosis (Bock *et al.* 2003; Kassem *et al.* 2000; Scaglia *et al.* 1997). This suggests that apoptosis is in part involved in triggering cell proliferation (Scoggins *et al.* 2000) in PDL pancreas.

#### **4.1.2.2. Pdx1 evaluation**

The profile of Pdx1 expression was similar to that of caspase3 expression with its highest expression occurring also at 6 hours post-PDL as observed for caspase3, and also concurred with its immunofluorescent evaluation. However, Pdx1 expression did not correlate with any other expression in the study. In mature differentiated cells, Pdx1 expression is specific to insulin-secreting cells and it is the first gene to be expressed in the endocrine lineage (Guz *et al.* 1995). These findings emphasize the importance of Pdx1 expression in maintaining the balance between neogenesis, proliferation and apoptosis which is critical to sustain the integrity of beta cell mass (Finegood *et al.* 1995). Disruption of Pdx1 gene results in agenesis of the pancreas,

underscoring the critical implication of Pdx1 transcription factor in the development of the human and rodent pancreas (Habener *et al.* 2005; Jonsson *et al.* 1994; Offield *et al.* 1996; Stoffers *et al.* 1997b). The fact that Pdx1 expression had not correlated with any of the endocrine developmental transcription factors in this study, might be an indication of the presence of other progenitors such as duct and acinar cells (Gu *et al.* 2002).

#### **4.1.2.3. Ngn3 evaluation**

The study showed a positive correlation between Ngn3 expression and caspase3 expression as well as insulin expression. The highest expression of Ngn3 was observed at 36 hours post-PDL, concurrent with the immunofluorescent evaluation. The expression of Ngn3 revealed an islet cell precursor population (Schwitzgebel *et al.* 2000) where islet expressing cells increase greatly when islet differentiation is at its peak (Gradwohl *et al.* 2000). This is evident with the observation of a positive correlation between the insulin expression and Ngn3 expression in the study; although there is no evidence that Ngn3 drives insulin-producing beta cells. In addition, the correlation between caspase3 and Ngn3 supports the opinion by Scoggins *et al.* (2000) that there is a direct link between acinar cell apoptosis and duct-like epithelial cell proliferation. Interestingly, as duct-like cells differentiate, they downregulate Ngn3, bud-off the epithelium and form aggregation of proto-islet structures (Pictet and Rutter 1972; Schwitzgebel *et al.* 2000).

#### **4.1.2.4. NeuroD evaluation**

The expression of NeuroD occurred later in the time post-PDL: its highest expression being observed at 108 hours post-PDL, while its reaction in morpho-immunofluorescent evaluation was described at 120 hours post-PDL. Expression of NeuroD did not correlate with any other expression in the study. This data contradicts Mutoh *et al* (1997) who observed a co-expression of NeuroD with Ngn3 and Pdx1 during pancreatic beta cell development. Although NeuroD expression in the study did not correlate with other transcription factor and, the fact that its expression occurs during recapitulation of foetal neogenesis (Lee *et al.* 2006), might highlight a plausible similarity between PDL tissue and foetal tissue. Furthermore, the fact that NeuroD expression occurred later than Ngn3 expression supports previous reports, that indicated the involvement of NeuroD-positive cells in the differentiation of cells expressing Ngn3 (Jensen *et al.* 2000a; Mutoh *et al.* 1997; Naya *et al.* 1997; Schwitzgebel *et al.* 2000) and Pdx1 (Mutoh *et al.* 1997).

#### **4.1.2.5. Pax6 evaluation**

Pax6 gene is expressed early and occurred throughout the pancreas development until the endocrine cells are fully formed (Dohrmann *et al.* 2000; Sander *et al.* 1997; Sosa-Pineda *et al.* 1997; St-Onge *et al.* 1997). This finding was concurrent with the immunofluorescent evaluation that showed immunoreaction at 84 hours post-PDL, and with the Pax6 expression observed throughout the time period post-PDL. In addition there was a positive correlation between Pax6 and insulin expression with their highest expression common at 84 hours post-PDL, while the early expression of



Pax6 occurred at 12 hours post-PDL. Inactivation of Pax6 gene leads to incomplete and malformed islets (St-Onge *et al.* 1997); but the exocrine component will tend to be normal (Dohrmann *et al.* 2000; Sander *et al.* 1997; Sosa-Pineda *et al.* 1997). However, co-expression of Pax6 with Pax4 is necessary for endocrine cell differentiation. These findings showed the importance of transcription factor Pax4 in the maturation of endocrine cells (Dohrmann *et al.* 2000; Sander *et al.* 1997; Sosa-Pineda *et al.* 1997; St-Onge *et al.* 1997). Although Pax4 was not included in this study, it is important to note that Pax4 is expressed exclusively during embryogenesis and strongly but transiently in beta cells precursors (Wang *et al.* 2004). This information further gives room for speculation on the origin of beta cells in PDL pancreas as having the same efficacy as foetal tissue (Page *et al.* 2000).

The immunofluorescent evaluation and the morphological assessment of the PDL pancreas revealed that there was no homeodomain protein expressed at 72 hours post-PDL. At this period, a morphological assessment showed an increased in acinar deletion rate (Abe *et al.* 1995; Edstrom *et al.* 1968; Page *et al.* 2000) due to an increased in mononuclear phagocytic activities (Walker 1995). Furthermore, when data based on the expression by time and morphological changes are considered, an infradian rhythm of 72 hours post-PDL is observed. Further studies extending up to 216 hours post-PDL are perhaps needed to confirm these findings.

The differences in the timing of expression of different transcription factors as revealed by the immunofluorescent analysis of protein expression and the pattern of cellular changes suggest that some of the protein expression may have overlapped (Sommers *et al.* 1996b; Huang *et al.* 2000); suggesting that reactivation of the

program of embryonic differentiation in adult pancreatic ducts (Pierreux *et al.* 2006). However, there is convincing evidence that there is a relationship between cellular changes and protein synthesis in the remodelling of PDL pancreas. This is therefore a unique finding not previously reported.

#### **4.2. Insulin expression and its variations when co-expressed with other transcription factors**

The highest expression of insulin in this study was observed at 84 hours post-PDL; the same time was observed in the immunofluorescent evaluation. There was a significant and positive correlation between Ngn3, Caspase3 and insulin expression. This is logical as insulin expression requires a mature state of beta cells (Schwitzgebel *et al.* 2000). Previously, it was revealed that early insulin-expressing cells have low insulin levels (Pictet and Rutter 1972) and these cells do not co-express transcription factor Pdx1 (Oster *et al.* 1998; Pang *et al.* 1994). This is demonstrated by the low levels of insulin expression in the first mode of the insulin expression curve and, the non correlation between insulin and Pdx1 expression in the study.

When insulin was expressed at the same time with the other assessed products, the efficiency of co-expression index was observed between insulin and Ngn3 (optimal or high efficiency); insulin with Pdx1, insulin with NeuroD and insulin with Pax6 (good or medium efficiency) and insulin with caspase3 (low or fair efficiency). Indeed the highest efficiency of co-expression of insulin with Pdx1, Ngn3 and NeuroD occurred at 12 hours post-PDL. These findings confirmed the dependency of the islet of Langerhans development on the sequential cascade of transcription factors activation

phases (Schwitzgebel 2001). The highest efficiency of co-expression of insulin with Pax6 occurred differently at 24 hours, and probably may be due to the extent and span of Pax6 function throughout the endocrine development until the endocrine cells reach maturity (Dohrmann *et al.* 2000; Sander *et al.* 1997; Sosa-Pineda *et al.* 1997; St-Onge *et al.* 1997).

Furthermore, the cellular and morphological changes observed in the pancreas during differentiation process may also be dependent on sequential alteration in transcription factors (Jensen 2004; Kim and McDonald 2002; Lyttle *et al.* 2008; Peshavaria and Stein 1997; Sander and German 1997; Servitja and Ferrer 2004; Stoffers *et al.* 1997). The highest co-expression of Ngn3, Pdx1 and NeuroD with insulin at early stage of endocrine cell lineage in the PDL pancreas is in agreement with many studies (Collombat *et al.* 2006; Gradwohl *et al.* 2000; Johannasen *et al.* 2007; Naya *et al.* 1995; Schwitzgebel 2001). However, this study further enlightens previous suggestion that duct ligated pancreatic tissues obtained at 84 hours post-PDL and used for transplantation yielded 50% success rate (Page *et al.* 2000). We therefore propose an early transplantation using 12 to 24 hours post-PDL harvested pancreatic tissues. This suggestion has not been reported in the literature.

#### **4.3. Recapitulation of endocrine development in PDL pancreas**

The present study was conducted to describe the cellular mechanism involved in the recapitulation of endocrine development in PDL pancreas. The qualitative evaluation of the endocrine developmental transcription factors across the time periods post-PDL from 6 to 120 hours helped in mapping out the pattern of cellular neogenesis in the

PDL model of laboratory animals. It is therefore necessary to highlight the elements of embryonic development in comparison with data from this study.

#### **4.3.1. Comparison between embryonic development and development in PDL pancreas**

The transcription factors evaluation data in this study corroborate and expand many of the previous investigations on foetal development, which suggest that the molecular events of homeodomain protein expression precede the visible developing pancreas (Hebrok *et al.* 1998; Gu *et al.* 2003; Lyttle *et al.* 2008; Murtaugh 2007; Schwitzgebel *et al.* 2001; Solar *et al.* 2009). During foetal pancreas development, the transcription factor Pdx1 is the first gene to be expressed in the region destined to become the future pancreas (Hebrok *et al.* 1998; Lammert *et al.* 2001; Ohlsson *et al.* 1993). Similarly in the PDL pancreas, Pdx1 was the first to be expressed. This was observed at the first landmark of morphological changes and was characterized by the presence of mitotic fibroblastic cells (mesenchyme) within a healthy basement membrane. As development continues, the pancreatic epithelium proliferates, expand and branch towards the adjacent mesenchyme whose signals dictate the fate of cells toward an endocrine or an exocrine pancreas (Guz *et al.* 1995; Miralles *et al.* 1998a, 1998b). Hence, all Pdx1-positive cells destined to become endocrine pancreas also express the transcription factor Ngn3 (Gradwohl *et al.* 2000; Gu *et al.* 2002). In contrast with the embryonic development, Pdx1 expression in PDL pancreas did not correlate with Ngn3 expression. However, both Pdx1 and Ngn3 co-expression with insulin were positive at the first landmark. During the embryonic development of the pancreas, beta cell specific genes are regulated by NeuroD as it acts downstream of Ngn3 and,

together with Pdx1 (Naya *et al.* 1995). This observation is in line with the events in the PDL pancreas, although there was no correlation of expression between NeuroD and Ngn3 in the study. Nevertheless, NeuroD co-expression with insulin was observed at the third landmark, establishing a distinct similarity between the events that took place in the PDL model to that of the embryonic development. Other similarities include the mesenchymal cells within the healthy acinar basement membrane and the early presence of Pdx1-expressing cells. Consequently, these findings show that PDL tissues might have the same efficacy as foetal tissue in terms of beta cell transplantation (Page *et al.* 2000).

#### **4.3.2. Origin of the newly formed beta cells in PDL pancreas**

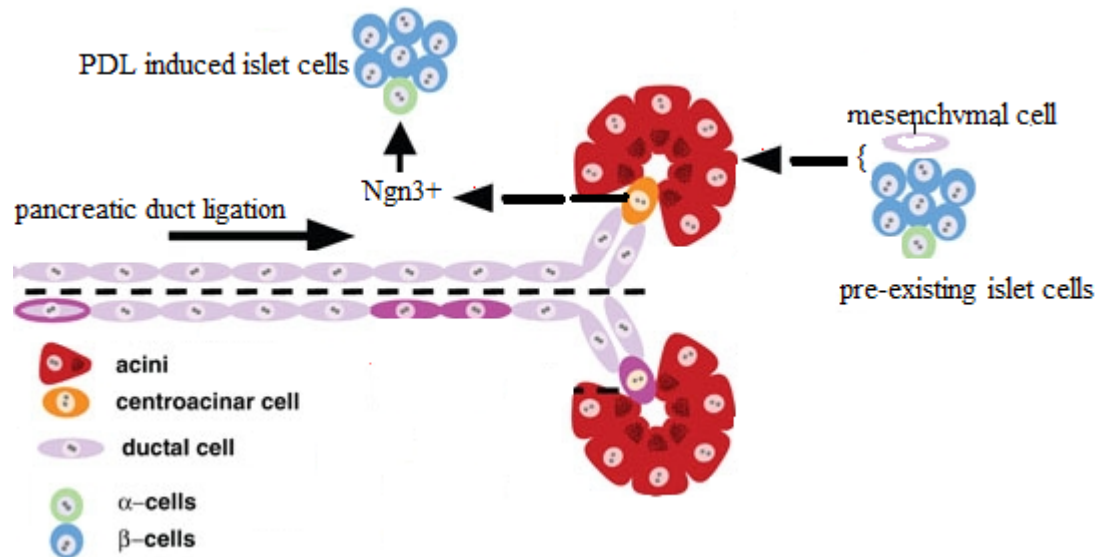
The histological data in this study showed evidence of mesenchymal cell division at the first landmark. The pre-existing islet cells were not also affected at this early stage and remained unaffected through to the fourth landmark, exhibiting a highly vascularised network of supply to the islets. Also, the fourth landmark was characterized by the budding of cells from the ductal epithelium, a histological evidence of duct-induced neogenesis (Wang *et al.* 1995). The presumptive centroacinar cells observed in this study have been shown to have a precursor capacity for new cells from adult cells following activation of the Notch pathway (Miyamoto *et al.*, 2003).

In the PDL model of laboratory rats, the beta cell mass increased by two-fold in a short period of time (Wang *et al.* 1995). Proliferation of the pre-existing beta cells does not account for this observable rapid beta cell mass increase (Wang *et al.* 1995).

Bouwens and Rooman (2005) have described this increase as a result of physiological condition of high insulin demand. However, absence of transient hyperglycemia in PDL model of rats rules out a plausible physiological reaction in this model of laboratory rats (Wang *et al.* 1995; Xu *et al.* 2008). Based on this evidence, the PDL model exhibits production of new beta cells rather than its replacement (Murtaugh and Kopinke 2008). The question on the origin of newly formed beta cells in PDL pancreas has to be supported by its evaluation of transcription factors involved in endocrine development.

The fact that Pdx1 expression did correlate with the expression of Ngn3 might be an indication that the Pdx1-expressing cells did not give rise directly to the islet cells, but rather to the duct or acinar cells. Convincing evidence from rodent studies of normal foetal pancreatic development (Bonner-Weir and Sharma 2002; Bouwens and Blay 1996; Gu *et al.* 2002; Slack 1995; Solar *et al.* 2009), as well as from few human studies (Castaing *et al.* 2005; Piper *et al.* 2004; Sarkar *et al.* 2008), have shown neogenesis of islet cells to derive from duct or acinar cells. However, duct cells after PDL have no evidence of neogenesis of beta cell after PDL (Solar *et al.* 2009; Xu *et al.* 2008). Recent study has demonstrated transdifferentiation of acinar cells into beta cells *in vitro* (Baeyens *et al.* 2010). Therefore, it is most likely that the newly formed beta cells in this study are derived from the centroacinar cells, since the Ngn3 expression was observed at the second landmark and, had peaked at 36 hours post-PDL. So far, the precise lineage of acinar or centroacinar cells has yet been elucidated. There is a possible cell-to-cell interaction between pre-existing mesenchymal cells and the pre-existing islet cells that might induce the “facultative

stem cell” capacity of the pre-existing beta cells as suggested in the case of the liver (Michalopoulos 2007) (Figure 26). This combination of mesenchymal cells with pre-existing beta cells warrants future investigation.



**Figure 24.** Scheme indicating the possible source of beta cells in PDL model.

#### 4.4. Implications of the study

The present data on beta cell development in PDL pancreas and, the co-expression of insulin with caspase3, Pdx1, Ngn3, NeuroD and Pax6 will have implications in the pathophysiology of the pancreas such as diabetes mellitus management.

#### 4.5. Strength and limitations of the study

To carry out the sectioning and staining of the PDL tissues in the present study, a complete laboratory was set up from scratch in the department of Anatomy, Histology

and Embryology at the Walter Sisulu University with limited funding and without any outside assistance.

Despite its strength from rigorous methodology, this study had some limitations to some degree:

The first limitation was the poor standard of equipments used which could not digitally separate the fluorescent colour images to reflect dual expression of proteins, and the inadequate technical support received for this work that required high technical manoeuvre offered elsewhere.

The second limitation was the lack of optimization and method controls which could have helped in excluding possible false positive results of expression. This might probably explain the higher variability (systematic error or bias in measurement) of expression at critical times.

The third limitation was the fact that the study did not assess Nk2.2, Pax4 and Nk6.1 transcription factors which are specific for the lineage of beta cell development. Indeed Ngn3 may not represent a sustained stem cell population or a transient state through endogenous progenitor cells process. Potential genetic alterations of these cells were not considered.

The strength of the study is based on the use of the expression pattern of transcription factors and the definition of the time-related efficiency of the expression of each protein profile according to the sequence of cellular lineage after PDL. This approach helped to categorize the present result in morphological changes as landmarks to each



target protein expression. Also, its strength resides in the use of PDL tissues preserved in Bouin's fluid for immunohistofluorescent study not reported in the literature.

#### **4.6. Conclusion**

This study provides strong evidence of connection between morphological changes in the PDL pancreas and the protein synthesis necessary for the lineage of endocrine cell development. Furthermore, these findings indicate that the optimal efficiency of co-expression of insulin and other assessed homeodomain proteins occur between 12 hours and 24 hours after PDL.

#### **4.7. Recommendations and perspectives of the study**

From this study, we recommend the following for further research in the lineage analysis of endocrine development in the pancreas:

- i) to apply the present results in the development of an animal model that will produce PDL islets for transplantation under the renal capsule (Du Toit *et al.* 1998) and by injection in the liver segment (Keymeulen *et al.* 1998);
- ii) to perform islet transplantation between 12 hours and 24 hours after PDL, as later transplantation at 84 hours post-PDL showed 50% failure (Page *et al.* 2004);
- iii) to develop translational studies to apply the present information on beta cell neogenesis as a potential therapy for diabetes mellitus in humans.

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## **Appendix**

### **Appendix 1**

#### Bouin's fluid preparation

- Picric Acid (standard aqueous solution) 75ml
- Formalin (40% aqueous Formaldehyde) 20ml
- Glacial Acetic Acid 5ml

### **Appendix 2**

#### Tissue processing protocol:

##### Materials:

1. Tissue processor
2. 70%, 80%, 95%, 99% ethanol
3. Paraffin wax (Paraplast; melting point 58°C)
4. Xylenes, Reagent Grade (Sigma)
5. Embedding moulds

##### Method:

1. Fix tissue overnight in Bouin's at room temperature
2. Place the tissue in the tissue processor and process with the times and temperatures described in below.

3. Embed the specimen in paraffin and block.

<b>Station</b>	<b>solution</b>	<b>Concentration</b>	<b>Time(min)</b>	<b>Temp (°C)</b>
1	Ethanol	70	30	40
2	Ethanol	80	30	40
3	Ethanol	95	45	40
4	Ethanol	95	45	40
5	Ethanol	100	45	40
6	Ethanol	100	45	40
7	Xylenes	100	45	40
8	Xylenes	100	45	58
9	Paraffin		30	58
10	Paraffin		30	58
11	Paraffin		30	58
12	Paraffin		30	58

### Appendix 3

**Table 1:** Slides in groups corresponding to the types of staining procedures used in the study for groups 0 and 5 hours pre-PDL, 6 and 12 hours post-PDL

	Sec	1	2	3	4	5	6	7	8	9	10	11	12
<b>GROUPS</b>	<b>Blocks</b>	<b>Insulin</b>	<b>Pdx1</b>	<b>Ngn3</b>	<b>NeuroD</b>	<b>Pax6</b>	<b>Casp3</b>	<b>In/Pdx1</b>	<b>In/Ngn3</b>	<b>In/NeurD</b>	<b>In/Pax6</b>	<b>In/Casp3</b>	<b>H&amp;E</b>
<b>0 hour</b>	1A	1A1	1A2	1A3	1A4	1A5	1A6	1A7	1A8	1A9	1A10	1A11	1A12
	1B	1B1	1B2	1B3	1B4	1B5	1B6	1B7	1B8	1B9	1B10	1B11	1B12
	1C	1C1	1C2	1C3	1C4	1C5	1C6	1C7	1C8	1C9	1C10	1C11	1C12
	1D	1D1	1D2	1D3	1D4	1D5	1D6	1D7	1D8	1D9	1D10	1D11	1D12
	1E	1E1	1E2	1E3	1E4	1E5	1E6	1E7	1E8	1E9	1E10	1E11	1E12
	1F	1F1	1F2	1F3	1F4	1F5	1F6	1F7	1F8	1F9	1F10	1F11	1F12
<b>5 hours</b>	2A	2A1	2A2	2A3	2A4	2A5	2A6	2A7	2A8	2A9	2A10	2A11	2A12
	2B	2B1	2B2	2B3	2B4	2B5	2B6	2B7	2B8	2B9	2B10	2B11	2B12
	2C	2C1	2C2	2C3	2C4	2C5	2C6	2C7	2C8	2C9	2C10	2C11	2C12
	2D	2D1	2D2	2D3	2D4	2D5	2D6	2D7	2D8	2D9	2D10	2D11	2D12
	2E	2E1	2E2	2E3	2E4	2E5	2E6	2E7	2E8	2E9	2E10	2E11	2E12
	2F	2F1	2F2	2F3	2F4	2F5	2F6	2F7	2F8	2F9	2F10	2F11	2F12
<b>6 hours</b>	3A	3A1	3A2	3A3	3A4	3A5	3A6	3A7	3A8	3A9	3A10	3A11	3A12
	3B	3B1	3B2	3B3	3B4	3B5	3B6	3B7	3B8	3B9	3B10	3B11	3B12
	3C	3C1	3C2	3C3	3C4	3C5	3C6	3C7	3C8	3C9	3C10	3C11	3C12
	3D	3D1	3D2	3D3	3D4	3D5	3D6	3D7	3D8	3D9	3D10	3D11	3D12
	3E	3E1	3E2	3E3	3E4	3E5	3E6	3E7	3E8	3E9	3E10	3E11	3E12
	3F	3F1	3F2	3F3	3F4	3F5	3F6	3F7	3F8	3F9	3F10	3F11	3F12
<b>12 hours</b>	4A	4A1	4A2	4A3	4A4	4A5	4A6	4A7	4A8	4A9	4A10	4A11	4A12
	4B	4B1	4B2	4B3	4B4	4B5	4B6	4B7	4B8	4B9	4B10	4B11	4B12
	4C	4C1	4C2	4C3	4C4	4C5	4C6	4C7	4C8	4C9	4C10	4C11	4C12
	4D	4D1	4D2	4D3	4D4	4D5	4D6	4D7	4D8	4D9	4D10	4D11	4D12
	4E	4E1	4E2	4E3	4E4	4E5	4E6	4E7	4E8	4E9	4E10	4E11	4E12
	4F	4F1	4F2	4F3	4F4	4F5	4F6	4F7	4F8	4F9	4F10	4F11	4F12



**Table 2:** Slides in groups corresponding to the types of staining procedures used in the study for groups 24 hours, 36 hours, 48 hours and 60 hours post-PDL

	Sec	1	2	3	4	5	6	7	8	9	10	11	12	
<b>GROUPS</b>	<b>Blocks</b>	<b>Insulin</b>	<b>Pdx1</b>	<b>Ngn3</b>	<b>NeuroD</b>	<b>Pax6</b>	<b>Casp3</b>	<b>In/Pdx1</b>	<b>In/Ngn3</b>	<b>In/NeuroD</b>	<b>In/Pax6</b>	<b>In/Casp3</b>	<b>H&amp;E</b>	
	<b>24 hours</b>	5A	5A1	5A2	5A3	5A4	5A5	5A6	5A7	5A8	5A9	5A10	5A11	5A12
		5B	5B1	5B2	5B3	5B4	5B5	5B6	5B7	5B8	5B9	5B10	5B11	5B12
		5C	5C1	5C2	5C3	5C4	5C5	5C6	5C7	5C8	5C9	5C10	5C11	5C12
		5D	5D1	5D2	5D3	5D4	5D5	5D6	5D7	5D8	5D9	5D10	5D11	5D12
		5E	5E1	5E2	5E3	5E4	5E5	5E6	5E7	5E8	5E9	5E10	5E11	5E12
		5F	5F1	5F2	5F3	5F4	5F5	5F6	5F7	5F8	5F9	5F10	5F11	5F12
	<b>36 hours</b>	6A	6A1	6A2	6A3	6A4	6A5	6A6	6A7	6A8	6A9	6A10	6A11	6A12
		6B	6B1	6B2	6B3	6B4	6B5	6B6	6B7	6B8	6B9	6B10	6B11	6B12
		6C	6C1	6C2	6C3	6C4	6C5	6C6	6C7	6C8	6C9	6C10	6C11	6C12
		6D	6D1	6D2	6D3	6D4	6D5	6D6	6D7	6D8	6D9	6D10	6D11	6D12
		6E	6E1	6E2	6E3	6E4	6E5	6E6	6E7	6E8	6E9	6E10	6E11	6E12
		6F	6F1	6F2	6F3	6F4	6F5	6F6	6F7	6F8	6F9	6F10	6F11	6F12
<b>48 hours</b>	7A	7A1	7A2	7A3	7A4	7A5	7A6	7A7	7A8	7A9	7A10	7A11	7A12	
	7B	7B1	7B2	7B3	7B4	7B5	7B6	7B7	7B8	7B9	7B10	7B11	7B12	
	7C	7C1	7C2	7C3	7C4	7C5	7C6	7C7	7C8	7C9	7C10	7C11	7C12	
	7D	7D1	7D2	7D3	7D4	7D5	7D6	7D7	7D8	7D9	7D10	7D11	7D12	
	7E	7E1	7E2	7E3	7E4	7E5	7E6	7E7	7E8	7E9	7E10	7E11	7E12	
	7F	7F1	7F2	7F3	7F4	7F5	7F6	7F7	7F8	7F9	7F10	7F11	7F12	
<b>60 hours</b>	8A	8A1	8A2	8A3	8A4	8A5	8A6	8A7	8A8	8A9	8A10	8A11	8A12	
	8B	8B1	8B2	8B3	8B4	8B5	8B6	8B7	8B8	8B9	8B10	8B11	8B12	
	8C	8C1	8C2	8C3	8C4	8C5	8C6	8C7	8C8	8C9	8C10	8C11	8C12	
	8D	8D1	8D2	8D3	8D4	8D5	8D6	8D7	8D8	8D9	8D10	8D11	8D12	
	8E	8E1	8E2	8E3	8E4	8E5	8E6	8E7	8E8	8E9	8E10	8E11	8E12	
	8F	8F1	8F2	8F3	8F4	8F5	8F6	8F7	8F8	8F9	8F10	8F11	8F12	

**Table 3:** Slides in groups corresponding to the types of staining procedures used in the study for groups 72 hours, 84 hours, 96 hours and 120 hours post-PDL

	Sec	1	2	3	4	5	6	7	8	9	10	11	12	
<b>GROUPS</b>	<b>Blocks</b>	<b>Insulin</b>	<b>Pdx1</b>	<b>Ngn3</b>	<b>NeuroD</b>	<b>Pax6</b>	<b>Casp3</b>	<b>In/Pdx1</b>	<b>In/Ngn3</b>	<b>In/NeurD</b>	<b>In/Pax6</b>	<b>In/Casp3</b>	<b>H&amp;E</b>	
	<b>72 hours</b>	9A	9A1	9A2	9A3	9A4	9A5	9A6	9A7	9A8	9A9	9A10	9A11	9A12
		9B	9B1	9B2	9B3	9B4	9B5	9B6	9B7	9B8	9B9	9B10	9B11	9B12
		9C	9C1	9C2	9C3	9C4	9C5	9C6	9C7	9C8	9C9	9C10	9C11	9C12
		9D	9D1	9D2	9D3	9D4	9D5	9D6	9D7	9D8	9D9	9D10	9D11	9D12
		9E	9E1	9E2	9E3	9E4	9E5	9E6	9E7	9E8	9E9	9E10	9E11	9E12
		9F	9F1	9F2	9F3	9F4	9F5	9F6	9F7	9F8	9F9	9F10	9F11	9F12
	<b>84 hours</b>	10A	10A1	10A2	10A3	10A4	10A5	10A6	10A7	10A8	10A9	10A10	10A11	10A12
		10B	10B1	10B2	10B3	10B4	10B5	10B6	10B7	10B8	10B9	10B10	10B11	10B12
		10C	10C1	10C2	10C3	10C4	10C5	10C6	10C7	10C8	10C9	10C10	10C11	10C12
		10D	10D1	10D2	10D3	10D4	10D5	10D6	10D7	10D8	10D9	10D10	10D11	10D12
		10E	10E1	10E2	10E3	10E4	10E5	10E6	10E7	10E8	10E9	10E10	10E11	10E12
		10F	10F1	10F2	10F3	10F4	10F5	10F6	10F7	10F8	10F9	10F10	10F11	10F12
<b>96 hours</b>	11A	11A1	11A2	11A3	11A4	11A5	11A6	11A7	11A8	11A9	11A10	11A11	11A12	
	11B	11B1	11B2	11B3	11B4	11B5	11B6	11B7	11B8	11B9	11B10	11B11	11B12	
	11C	11C1	11C2	11C3	11C4	11C5	11C6	11C7	11C8	11C9	11C10	11C11	11C12	
	11D	11D1	11D2	11D3	11D4	11D5	11D6	11D7	11D8	11D9	11D10	11D11	11D12	
	11E	11E1	11E2	11E3	11E4	11E5	11E6	11E7	11E8	11E9	11E10	11E11	11E12	
	11F	11F1	11F2	11F3	11F4	11F5	11F6	11F7	11F8	11F9	11F10	11F11	11F12	
<b>108 hours</b>	12A	12A1	12A2	12A3	12A4	12A5	12A6	12A7	12A8	12A9	12A10	12A11	12A12	
	12B	12B1	12B2	12B3	12B4	12B5	12B6	12B7	12B8	12B9	12B10	12B11	12B12	
	12C	12C1	12C2	12C3	12C4	12C5	12C6	12C7	12C8	12C9	12C10	12C11	12C12	
	12D	12D1	12D2	12D3	12D4	12D5	12D6	12D7	12D8	12D9	12D10	12D11	12D12	
	12E	12E1	12E2	12E3	12E4	12E5	12E6	12E7	12E8	12E9	12E10	12E11	12E12	
	12F	12F1	12F2	12F3	12F4	12F5	12F6	12F7	12F8	12F9	12F10	12F11	12F12	
<b>120 hours</b>	13A	13A1	13A2	13A3	13A4	13A5	13A6	13A7	13A8	13A9	13A10	13A11	13A12	
	13B	13B1	13B2	13B3	13B4	13B5	13B6	13B7	13B8	13B9	13B10	13B11	13B12	
	13C	13C1	13C2	13C3	13C4	13C5	13C6	13C7	13C8	13C9	13C10	13C11	13C12	
	13D	13D1	13D2	13D3	13D4	13D5	13D6	13D7	13D8	13D9	13D10	13D11	13D12	
	13E	13E1	13E2	13E3	13E4	13E5	13E6	13E7	13E8	13E9	13E10	13E11	13E12	
	13F	13F1	13F2	13F3	13F4	13F5	13F6	13F7	13F8	13F9	13F10	13F11	13F12	

## **Appendix 4**

### Haematoxylin and Eosin staining protocol:

#### Materials:

1. Autostainer
2. xylenes
3. plastic rack
4. 99% and 95% ethanol
5. 1% acid alcohol
6. Harris haematoxylin and eosin solutions
7. 0.2% ammonia
8. Glass slides
9. Cover slipper
10. DPX mounting solution

#### Method:

1. Place the tissue in the autostainer with the times programmed as described below

2. Mount cover slip on glass slides using DPX.

Station	Solution	Concentration	Time
1	Xylene		10 min
2	Xylene		10 min
3	Ethanol	99%	5 min
4	Ethanol	99%	5 min
5	Ethanol	95%	2 min
6	Ethanol	70%	2 min
7	Distilled water		5 sec
8	Haematoxylin		8 min
9	Running water		5 min
10	Ethanol	1% acid alcohol	30 sec
11	Running water		1min
12	Ammonia	0.20%	45 sec
13	Running water		5 min
14	Ethanol	95%	10 dips
15	Eosin		45 sec
16	Ethanol	95%	5 min
17	Ethanol	95%	5 min
18	Xylene		5 min
19	Xylene		5 min

## Appendix 5

### Deparaffinization and Rehydration Protocol:

Materials:

1. Autostainer with incorporated oven
2. xylene
3. 99%, 95% and 80% ethanol
4. plastic rack

Method:

1. Place rack in autostainer with oven on at 65°C overnight
2. Proceed with deparaffinization as shown below
3. Replacement of xylene and alcohol bath solutions after every 156 slides processed.

<b>Station</b>	<b>Solution</b>	<b>Concentration</b>	<b>Time</b>
1	In oven	At 65°C	12 hrs
2	Allow to cool		1 hr
3	Xylene		5 min
4	Xylene		5 min
5	Tap off		
6	Ethanol	99%	3 min
7	Ethanol	99%	3 min
8	Ethanol	95%	3 min
9	Ethanol	95%	3 min
10	Ethanol	80%	3 min

## Appendix 6

**Table 4:** Record of the measurements of single gene expressions indicating the counts, the areas and the expression index per period of times pre- and post-PDL

PstPDL	InsCount	InsArea	InsIndex	PdxCount	PdxArea	PdxIndex	NgnCount	NgnArea	NgnIndex
0	28.000	10.156	2.757	153.000	21.878	6.993	350.000	45.814	7.640
0	28.000	10.156	2.757	47.000	6.478	7.255	326.000	53.942	6.044
0	52.000	13.297	3.911	50.000	4.338	11.525	183.000	19.605	9.334
0	66.000	35.712	1.848	14.000	2.082	6.725	929.000	204.106	4.552
0	227.000	88.743	2.558	137.000	21.770	6.293	595.000	105.132	5.660
0	17.000	7.717	2.203	198.000	36.534	5.420	497.000	46.528	10.682
5	66.000	35.712	1.848	14.000	2.082	6.725	350.000	45.814	7.640
5	227.000	88.743	2.558	137.000	21.770	6.293	326.000	53.942	6.044
5	52.000	13.297	3.911	50.000	4.338	11.525	595.000	105.132	5.660
5	28.000	10.156	2.757	153.000	21.878	6.993	497.000	46.528	10.682
5	28.000	10.156	2.757	47.000	6.478	7.255	183.000	19.605	9.334
5	17.000	7.717	2.203	198.000	36.534	5.420	929.000	204.106	4.552
6	2.000	0.141	14.156	4.000	0.549	7.292	4.000	0.220	18.162
6	2.000	0.141	14.156	5.000	0.370	13.520	12.000	1.367	8.777
6	1.000	0.046	21.877	1.000	0.017	60.162	1.000	0.170	5.869
6	1.000	0.299	3.342	6.000	0.519	11.551	7.000	1.184	5.911
6	6.000	1.500	4.000	1.000	0.017	60.162	2.000	0.154	13.008
6	16.000	0.819	19.545	1.000	0.004	240.649	35.000	4.367	8.014
12	1.000	0.253	3.945	2.000	0.220	9.081	5.000	0.877	5.703
12	1.000	0.253	3.945	3.000	0.295	10.168	9.000	0.332	27.073
12	5.000	0.125	40.108	5.000	0.939	5.324	5.000	0.283	17.695
12	4.000	0.137	29.170	2.000	0.087	22.919	3.000	0.175	17.189
12	3.000	0.345	8.698	3.000	1.101	2.724	3.000	0.395	7.599
12	5.000	0.440	11.351	6.000	0.594	10.097	6.000	1.068	5.618
24	2.000	0.141	14.156	8.000	1.517	5.275	6.000	1.421	4.222
24	2.000	0.141	14.156	7.000	1.646	4.254	9.000	1.824	4.934
24	8.000	0.199	40.108	2.000	0.220	9.081	2.000	3.495	0.572
24	11.000	0.420	26.209	2.000	0.652	3.066	4.000	0.204	19.645
24	5.000	0.366	13.673	1.000	0.004	240.649	1.000	0.881	1.135
24	2.000	0.249	8.022	7.000	0.586	11.947	8.000	0.486	16.455
36	254.000	31.993	7.939	266.000	19.343	13.751	535.000	24.505	21.833
36	1.000	0.253	3.945	1282.000	42.552	30.128	951.000	39.905	23.832
36	773.000	38.762	19.942	621.000	28.290	21.951	731.000	31.685	23.071
36	219.000	18.330	11.948	793.000	34.548	22.953	1 349.000	54.407	24.795
36	180.000	15.778	11.408	216.000	18.126	11.917	1 439.000	59.456	24.203
36	86.000	12.175	7.063	600.000	17.910	33.501	172.000	17.104	10.056
48	1.000	0.253	3.945	56.000	12.641	4.430	371.000	29.836	12.435
48	254.000	31.993	7.939	142.000	31.261	4.542	187.000	31.652	5.908

48	862.000	48.344	17.830	311.000	82.119	3.787	145.000	17.947	8.079
48	812.000	39.659	20.474	233.000	39.344	5.922	642.000	35.101	18.290
48	1568.00	77.972	20.110	539.000	31.037	17.366	1 065.000	59.601	17.869
48	1200.00	87.659	13.689	235.000	20.162	11.656	692.000	44.945	15.397
60	388.000	31.880	12.170	76.000	12.819	5.928	852.000	51.723	16.473
60	2293.00	149.707	15.317	628.000	47.359	13.260	404.000	30.119	13.414
60	160.000	15.998	10.001	503.000	35.504	14.167	641.000	45.182	14.187
60	124.000	17.756	6.984	584.000	52.977	11.024	305.000	30.098	10.134
60	885.000	35.109	25.207	700.000	48.897	14.316	231.000	23.703	9.746
60	474.000	31.303	15.142	257.000	26.632	9.650	1 782.000	85.045	20.954
72	2293.00	149.707	15.317	672.000	40.910	16.426	132.000	17.149	7.697
72	388.000	31.880	12.170	686.000	41.966	16.347	824.000	38.616	21.338
72	283.000	27.368	10.341	423.000	45.327	9.332	125.000	13.680	9.138
72	167.000	19.169	8.712	10.000	11.236	0.890	112.000	16.514	6.782
72	281.000	33.135	8.480	477.000	36.776	12.971	623.000	34.424	18.098
72	158.000	19.788	7.985	292.000	31.905	9.152	667.000	37.490	17.791
84	1059.00	34.623	30.587	966.000	46.291	20.868	569.000	27.767	20.492
84	1989.00	57.511	34.585	3497.000	63.620	54.967	776.000	37.540	20.671
84	2315.00	72.387	31.981	1026.000	50.904	20.156	962.000	52.915	18.180
84	2481.00	86.449	28.699	1013.000	46.375	21.844	150.000	14.901	10.066
84	2752.00	113.526	24.241	947.000	47.147	20.086	222.000	21.010	10.566
84	2071.00	98.184	21.093	725.000	39.722	18.252	378.000	45.190	8.365
96	1989.00	57.511	34.585	456.000	27.521	16.569	322.000	23.162	13.902
96	1059.00	34.623	30.587	17.000	8.743	1.944	366.000	22.847	16.020
96	166.000	11.178	14.850	1062.000	50.808	20.902	96.000	17.640	5.442
96	548.000	21.405	25.602	22.000	9.017	2.440	375.000	49.952	7.507
96	337.000	18.280	18.436	97.000	11.311	8.576	86.000	14.228	6.044
96	401.000	21.845	18.357	85.000	5.369	15.832	829.000	78.903	10.507
108	230.000	11.594	19.838	85.000	14.802	5.743	1 085.000	227.509	4.769
108	237.000	25.111	9.438	50.000	9.736	5.135	1 140.000	264.019	4.318
108	220.000	31.814	6.915	20.000	10.384	1.926	846.000	235.962	3.585
108	48.000	12.878	3.727	789.000	57.457	13.732	407.000	112.591	3.615
108	7.000	8.477	0.826	87.000	12.329	7.056	431.000	154.316	2.793
108	98.000	17.814	5.501	150.000	21.180	7.082	279.000	82.556	3.380
120	237.000	25.111	9.438	273.000	19.460	14.029	91.000	12.042	7.557
120	230.000	11.594	19.838	65.000	11.764	5.525	82.000	10.135	8.091
120	187.000	23.333	8.015	81.000	7.999	10.126	123.000	21.812	5.639
120	102.000	12.022	8.485	87.000	13.784	6.312	87.000	13.887	6.265
120	72.000	12.541	5.741	265.000	26.009	10.189	62.000	6.084	10.191
120	111.000	11.452	9.692	268.000	53.667	4.994	295.000	81.691	3.611

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PstPDL	NeDCount	NeDArea	NeDIndex	PaxCount	PaxArea	PaxIndex	CaspCount	CaspArea	CaspIndex
0	351.000	35.413	9.912	110.000	61.820	1.779	386.000	85.269	4.527
0	1580.000	278.052	5.682	40.000	7.201	5.555	27.000	6.341	4.258
0	1 205.000	161.642	7.455	214.000	94.532	2.264	64.000	11.245	5.692
0	281.000	36.293	7.742	112.000	44.642	2.509	167.000	18.454	9.049
0	272.000	37.083	7.335	104.000	38.720	2.686	77.000	19.410	3.967
0	146.000	12.608	11.580	561.000	241.25	2.325	280.000	54.141	5.172
5	351.000	35.413	9.912	104.000	38.720	2.686	77.000	19.410	3.967
5	1 580.000	278.052	5.682	40.000	7.201	5.555	27.000	6.341	4.258
5	272.000	37.083	7.335	214.000	94.532	2.264	64.000	11.245	5.692
5	146.000	12.608	11.580	112.000	44.642	2.509	167.000	18.454	9.049
5	1 205.000	161.642	7.455	110.000	61.820	1.779	386.000	85.269	4.527
5	281.000	36.293	7.742	561.000	241.256	2.325	280.000	54.141	5.172
6	11.000	0.109	100.678	3.000	0.503	5.967	1.000	0.058	17.189
6	2.000	0.382	5.232	2.000	1.296	1.543	51.000	0.964	52.901
6	1.000	0.054	18.511	13.000	1.122	11.587	1.000	0.382	2.616
6	6.000	1.085	5.532	10.000	0.569	17.566	13.000	0.515	25.229
6	7.000	0.648	10.798	14.000	1.961	7.138	3.000	0.486	6.170
6	22.000	1.060	20.762	5.000	0.465	10.743	9.000	0.627	14.343
12	3.000	0.661	4.541	4.000	0.474	8.444	2.000	0.798	2.507
12	7.000	0.399	17.547	3.000	0.199	15.041	5.000	0.877	5.703
12	3.000	0.270	11.107	9.000	0.881	10.216	6.000	0.494	12.134
12	1.000	0.066	15.041	4.000	0.233	17.189	2.000	0.125	16.043
12	10.000	0.324	30.852	5.000	0.199	25.068	3.000	0.432	6.942
12	2.000	1.267	1.578	1.000	0.046	21.877	8.000	2.427	3.297
24	8.000	0.744	10.755	6.000	0.287	20.926	5.000	0.341	14.674
24	7.000	0.175	40.108	4.000	0.657	6.092	5.000	0.677	7.382
24	20.000	1.130	17.695	17.000	4.168	4.079	1.000	0.357	2.798
24	7.000	1.172	5.974	14.000	0.665	21.057	9.000	0.183	49.224
24	5.000	0.661	7.568	5.000	0.274	18.231	4.000	0.997	4.011
24	4.000	0.150	26.739	23.000	2.202	10.443	3.000	0.129	23.289
36	2 208.000	110.447	19.991	252.000	22.855	11.026	768.000	36.738	20.905
36	473.000	35.321	13.391	67.000	16.618	4.032	648.000	29.333	22.091
36	239.000	17.902	13.351	123.000	10.206	12.052	258.000	19.460	13.258
36	211.000	18.787	11.231	<b>1269.00</b>	51.797	24.499	46.000	11.540	3.986
36	1 473.000	44.180	33.341	<b>1049.00</b>	49.508	21.189	771.000	48.440	15.917
36	2 137.000	67.758	31.539	81.000	10.522	7.698	21.000	9.333	2.250
48	178.000	19.385	9.182	120.000	22.942	5.231	777.000	46.150	16.836
48	576.000	37.204	15.482	272.000	33.568	8.103	858.000	59.614	14.393
48	402.000	44.060	9.124	194.000	25.302	7.667	597.000	53.713	11.115
48	286.000	23.424	12.210	337.000	38.508	8.751	265.000	33.347	7.947
48	80.000	18.201	4.395	68.000	13.231	5.139	263.000	29.994	8.768
48	70.000	19.593	3.573	49.000	11.265	4.350	473.000	44.052	10.737
60	418.000	35.882	11.649	379.000	43.142	8.785	411.000	36.717	11.194
60	312.000	36.264	8.603	570.000	47.729	11.942	123.000	18.296	6.723
60	494.000	28.398	17.395	767.000	50.430	15.209	62.000	15.338	4.042



60	812.000	44.945	18.067	744.000	46.117	16.133	265.000	25.045	10.581
60	237.000	36.447	6.503	964.000	59.842	16.109	33.000	13.871	2.379
60	262.000	57.025	4.594	569.000	41.504	13.709	58.000	17.224	3.367
72	872.000	47.409	18.393	560.000	38.330	14.610	529.000	31.743	16.665
72	628.000	41.205	15.241	283.000	18.978	14.912	1 535.000	79.103	19.405
72	1 039.000	62.884	16.522	465.000	28.008	16.603	296.000	28.390	10.426
72	1 044.000	41.982	24.868	498.000	32.716	15.222	662.000	38.616	17.143
72	530.000	30.725	17.250	547.000	43.757	12.501	74.000	11.922	6.207
72	1 206.000	61.999	19.452	352.000	33.588	10.480	652.000	40.387	16.144
84	341.000	28.377	12.017	629.000	42.564	14.778	438.000	42.934	10.202
84	390.000	34.648	11.256	775.000	38.072	20.356	274.000	31.735	8.634
84	536.000	48.340	11.088	93.000	10.800	8.611	281.000	32.209	8.724
84	281.000	31.286	8.982	1061.00	42.896	24.734	334.000	32.109	10.402
84	457.000	37.307	12.250	826.000	50.617	16.319	164.000	18.188	9.017
84	465.000	31.232	14.888	2393.00	128.19	18.668	635.000	57.258	11.090
96	23.000	11.843	1.942	125.000	12.587	9.931	33.000	11.926	2.767
96	56.000	11.033	5.076	122.000	15.799	7.722	34.000	9.553	3.559
96	392.000	20.432	19.185	117.000	18.168	6.440	82.000	11.132	7.366
96	504.000	32.699	15.413	13.000	9.350	1.390	115.000	12.321	9.334
96	307.000	20.465	15.001	164.000	22.207	7.385	71.000	11.016	6.445
96	441.000	35.836	12.306	178.000	23.067	7.717	69.000	10.164	6.789
108	55.000	6.075	9.053	5.000	0.096	52.315	0.000	0.000	0.000
108	195.000	17.282	11.283	365.000	23.836	15.313	49.000	14.656	3.343
108	178.000	15.309	11.627	130.000	16.069	8.090	36.000	15.363	2.343
108	51.000	9.092	5.609	173.000	26.985	6.411	84.000	36.751	2.286
108	45.000	3.835	11.733	603.000	97.943	6.157	867.000	247.680	3.500
108	1.000	0.004	240.649	376.000	64.463	5.833	236.000	37.370	6.315
120	211.000	19.730	10.694	812.000	141.542	5.737	80.000	12.570	6.364
120	75.000	4.870	15.400	805.000	156.58	5.141	30.000	5.061	5.927
120	21.000	1.321	15.892	1357.00	501.96	2.703	24.000	1.737	13.817
120	97.000	7.081	13.699	1296.00	317.82	4.078	24.000	9.416	2.549
120	121.000	6.765	17.886	1304.00	370.24	3.522	61.000	8.639	7.061
120	511.000	45.548	11.219	1284.00	257.66	4.983	45.000	3.968	11.339

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**Table 6:** Record of measurements of the co-expression of genes indicating the counts, the areas and the expression index per period of times pre- and post-PDL

<b>PstPDL</b>	<b>PdxInsCnt</b>	<b>PdxInsArea</b>	<b>PdxInsIndex</b>	<b>NgnInsCnt</b>	<b>NgnInsArea</b>	<b>NgnInsIndex</b>
0	1 683.000	369.143	4.559	383.000	94.685	4.045
0	1 277.000	276.863	4.612	675.000	266.940	2.529
0	1 500.000	325.590	4.607	721.000	207.875	3.468
0	1 408.000	441.518	3.189	828.000	461.156	1.795
0	2 208.000	551.716	4.002	835.000	248.370	3.362
0	1 065.000	360.570	2.954	915.000	311.835	2.934
0	190.000	5 959.647	0.032	2 226.000	1 504.425	1.480
0	217.000	5 930.941	0.037	2 267.000	2 079.524	1.090
0	226.000	5 949.669	0.038	2 450.000	2 282.699	1.073
0	152.000	5 954.922	0.026	2 554.000	1 314.012	1.944
0	19.000	5 989.071	0.003	2 934.000	1 769.309	1.658
0	146.000	5 961.882	0.024	3 009.000	1 065.675	2.824
5	1 065.000	360.570	2.954	915.000	311.835	2.934
5	2 208.000	551.716	4.002	835.000	248.370	3.362
5	1 408.000	441.518	3.189	828.000	461.156	1.795
5	1 500.000	325.590	4.607	721.000	207.875	3.468
5	1 277.000	276.863	4.612	675.000	266.940	2.529
5	1 683.000	369.143	4.559	383.000	94.685	4.045
5	146.000	5 961.882	0.024	3 009.000	1 065.675	2.824
5	19.000	5 989.071	0.003	2 934.000	1 769.309	1.658
5	152.000	5 954.922	0.026	2 554.000	1 314.012	1.944
5	226.000	5 949.669	0.038	2 450.000	2 282.699	1.073
5	217.000	5 930.941	0.037	2 267.000	2 079.524	1.090
5	190.000	5 959.647	0.032	2 226.000	1 504.425	1.480
6	357.000	71.926	4.963	106.000	15.861	6.683
6	22.000	1.442	15.257	30.000	5.074	5.913
6	107.000	24.239	4.414	152.000	44.006	3.454
6	69.000	6.009	11.483	14.000	0.594	23.560
6	16.000	1.679	9.531	590.000	76.252	7.737
6	488.000	66.167	7.375	157.000	19.032	8.249
6	1 263.000	39.664	31.843	6 011.000	260.450	23.079
6	6.000	0.561	10.696	1 258.000	28.490	44.156
6	802.000	90.476	8.864	3 095.000	1 368.576	2.261
6	2 816.000	151.910	18.537	5 153.000	169.259	30.445
6	3 573.000	152.238	23.470	622.000	24.762	25.119
6	2 299.000	217.852	10.553	378.000	5.622	67.232
12	16.000	7.550	2.119	71.000	4.953	14.334
12	90.000	7.796	11.545	71.000	4.899	14.492
12	44.000	2.913	15.105	57.000	5.273	10.809
12	16.000	1.513	10.578	16.000	1.006	15.911
12	56.000	4.962	11.287	31.000	3.245	9.552

12	52.000	3.565	14.585	61.000	4.908	12.430
12	571.000	36.534	15.629	507.000	8.111	62.505
12	382.000	14.868	25.693	4 727.000	102.307	46.204
12	217.000	5.144	42.182	173.000	3.395	50.958
12	111.000	2.098	52.895	2 247.000	65.660	34.222
12	502.000	11.299	44.430	817.000	23.241	35.153
12	2 795.000	95.392	29.300	8 632.000	1 028.089	8.396
24	389.000	45.884	8.478	40.000	8.411	4.756
24	1 554.000	229.147	6.782	124.000	9.699	12.785
24	731.000	101.367	7.211	89.000	6.832	13.028
24	982.000	113.534	8.649	210.000	23.532	8.924
24	262.000	29.848	8.778	51.000	4.608	11.067
24	164.000	12.670	12.944	88.000	14.731	5.974
24	2 051.000	62.963	32.575	26.000	0.698	37.243
24	1 190.000	49.362	24.107	2 211.000	50.817	43.509
24	1 337.000	46.200	28.939	6 624.000	255.251	25.951
24	1 640.000	39.111	41.932	840.000	16.626	50.524
24	4 539.000	331.985	13.672	1 931.000	40.291	47.926
24	6 325.000	739.474	8.553	2 124.000	65.224	32.565
36	6 575.000	837.846	7.848	5 627.000	1 204.849	4.670
36	7 260.000	492.642	14.737	4 611.000	755.456	6.104
36	10 428.000	883.119	11.808	3 837.000	1 547.808	2.479
36	7 091.000	974.044	7.280	87.000	5.547	15.683
36	4 279.000	178.704	23.945	3 833.000	1 953.299	1.962
36	8 934.000	1 763.982	5.065	4 298.000	1 430.604	3.004
36	16.000	5 991.884	0.003	1.000	5 998.437	0.000
36	8 250.000	687.315	12.003	1.000	5 998.437	0.000
36	2 961.000	5 067.327	0.584	1.000	5 998.437	0.000
36	305.000	5 951.897	0.051	5 289.000	5 043.583	1.049
36	1.000	5 998.437	0.000	1.000	5 998.437	0.000
36	2 901.000	5 423.867	0.535	1.000	5 998.437	0.000
48	2 528.000	236.938	10.669	1 798.000	549.397	3.273
48	5 051.000	467.780	10.798	2 768.000	972.315	2.847
48	5 236.000	321.983	16.262	1 883.000	772.074	2.439
48	2 183.000	471.063	4.634	761.000	102.435	7.429
48	2 242.000	479.145	4.679	2 958.000	777.987	3.802
48	4 141.000	495.817	8.352	2 847.000	1 092.332	2.606
48	1.000	5 998.354	0.000	1.000	5 998.437	0.000
48	145.000	5 977.706	0.024	1.000	5 998.437	0.000
48	4 348.000	4 590.808	0.947	1.000	5 998.437	0.000
48	983.000	5 801.620	0.169	1.000	5 998.437	0.000
48	888.000	5 780.452	0.154	1.000	5 998.437	0.000
48	3 443.000	5 097.641	0.675	1.000	5 998.437	0.000
48	1 859.000	899.741	2.066	3 332.000	585.034	5.695
60	4 114.000	771.471	5.333	3 957.000	401.136	9.864
60	4 348.000	820.721	5.298	3 581.000	176.772	20.258

60	4 783.000	1 167.042	4.098	1 788.000	66.749	26.787
60	4 965.000	1 037.435	4.786	4 902.000	237.379	20.651
60	5 042.000	300.017	16.806	1 493.000	68.693	21.734
60	916.000	5 876.147	0.156	1.000	5 998.080	0.000
60	710.000	5 811.044	0.122	14.000	5 993.887	0.002
60	20.000	5 989.553	0.003	1.000	5 998.437	0.000
60	515.000	5 841.562	0.088	28.000	5 987.621	0.005
60	4 963.000	4 577.137	1.084	1.000	5 998.437	0.000
60	290.000	5 926.806	0.049	635.000	5 848.705	0.109
60	7 911.000	2 742.160	2.885	5 499.000	869.269	6.326
72	5 683.000	3 216.485	1.767	4 931.000	666.734	7.396
72	5 853.000	3 154.008	1.856	5 532.000	1 222.650	4.525
72	3 159.000	179.282	17.620	4 268.000	1 307.379	3.265
72	2 011.000	108.265	18.575	4 473.000	989.149	4.522
72	2 077.000	123.296	16.846	5 391.000	923.223	5.839
72	1.000	5 998.437	0.000	5.000	5 995.205	0.001
72	1.000	5 998.437	0.000	1.000	5 997.906	0.000
72	1.000	5 998.437	0.000	1.000	5 998.425	0.000
72	251.000	5 926.615	0.042	1.000	5 998.425	0.000
72	715.000	5 795.474	0.123	1.000	5 998.433	0.000
72	182.000	5 966.316	0.031	1.000	5 997.947	0.000
72	3 028.000	756.632	4.002	5 843.000	1 301.047	4.491
72	3 364.000	1 028.201	3.272	4 703.000	843.663	5.574
84	2 529.000	300.080	8.428	5 372.000	665.691	8.070
84	3 077.000	616.814	4.989	4 290.000	592.098	7.245
84	3 191.000	592.834	5.383	4 380.000	691.600	6.333
84	3 290.000	693.050	4.747	5 580.000	1 382.015	4.038
84	1.000	5 998.437	0.000	61.000	5 985.335	0.010
84	1.000	5 998.437	0.000	1.000	5 998.176	0.000
84	1.000	5 998.396	0.000	1.000	5 998.334	0.000
84	1.000	5 998.437	0.000	1.000	5 998.437	0.000
84	1.000	5 998.437	0.000	1.000	5 998.437	0.000
84	1.000	5 998.437	0.000	1.000	5 998.026	0.000
84	3 270.000	239.739	13.640	3 077.000	1 337.718	2.300
84	2 406.000	3 111.112	0.773	160.000	10.401	15.383
96	2 298.000	163.109	14.089	155.000	10.542	14.703
96	3 257.000	2 308.130	1.411	1 323.000	83.553	15.834
96	4 288.000	525.420	8.161	1 754.000	145.253	12.075
96	3 703.000	624.290	5.932	3 682.000	1 487.492	2.475
96	1.000	5 998.437	0.000	1.000	5 998.437	0.000
96	1.000	5 998.437	0.000	56.000	5 977.328	0.009
96	43.000	5 983.844	0.007	1.000	5 998.429	0.000
96	1.000	5 998.421	0.000	1.000	5 998.437	0.000
96	1.000	5 998.421	0.000	1.000	5 998.437	0.000
96	1.000	5 998.437	0.000	1.000	5 998.437	0.000
96	1 184.000	108.590	10.903	2 685.000	747.465	3.592

96	1 107.000	106.932	10.352	2 676.000	690.083	3.878
96	937.000	90.227	10.385	2 574.000	795.298	3.237
108	642.000	82.248	7.806	3 417.000	998.361	3.423
108	313.000	42.643	7.340	3 677.000	904.017	4.067
108	778.000	63.707	12.212	3 110.000	769.497	4.042
108	1 034.000	4 779.897	0.216	1.000	5 998.437	0.000
108	1 348.000	4 794.773	0.281	1.000	5 998.437	0.000
108	3 503.000	3 971.521	0.882	1.000	5 998.437	0.000
108	4 092.000	3 531.624	1.159	1.000	5 998.437	0.000
108	4 213.000	3 204.555	1.315	1.000	5 998.429	0.000
108	4 652.000	3 404.880	1.366	1.000	5 998.437	0.000
108	2 976.000	668.221	4.454	327.000	25.681	12.733
108	5 109.000	1 863.583	2.741	203.000	13.372	15.181
108	5 033.000	1 107.637	4.544	218.000	10.480	20.802
120	2 271.000	403.213	5.632	121.000	6.615	18.291
120	291.000	14.556	19.991	251.000	12.853	19.529
120	699.000	26.886	25.999	154.000	8.194	18.793
120	52.000	5 979.327	0.009	1 883.000	4 522.227	0.416
120	6.000	5 994.822	0.001	2 253.000	4 374.776	0.515
120	380.000	5 792.636	0.066	1 362.000	4 872.787	0.280
120	679.000	5 633.828	0.121	4 478.000	3 549.256	1.262
120	150.000	5 936.709	0.025	2 577.000	4 391.710	0.587
120	162.000	5 923.723	0.027	3 335.000	3 675.306	0.907

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PstPDL	NeDInsCnt	NeDInsArea	NeDInsIndex	PaxInsCnt	PaxInsArea	PaxInsIndex
0	1 034.000	340.516	3.037	1 683.000	369.143	4.559
0	2 190.000	467.049	4.689	1 277.000	276.863	4.612
0	1 972.000	375.663	5.249	1 500.000	325.590	4.607
0	1 367.000	207.904	6.575	1 408.000	441.518	3.189
0	1 744.000	233.630	7.465	2 208.000	551.716	4.002
0	1 271.000	301.484	4.216	1 065.000	360.570	2.954
0	2 810.000	3 432.297	0.819	190.000	5 959.647	0.032
0	4 050.000	3 679.615	1.101	217.000	5 930.941	0.037
0	3 896.000	3 283.188	1.187	226.000	5 949.669	0.038
0	5 379.000	2 916.638	1.844	152.000	5 954.922	0.026
0	4 951.000	3 412.920	1.451	19.000	5 989.071	0.003
0	3 770.000	3 242.319	1.163	146.000	5 961.882	0.024
5	1 034.000	340.516	3.037	2 156.000	808.986	2.665
5	2 190.000	467.049	4.689	1 838.000	700.085	2.625
5	1 972.000	375.663	5.249	1 371.000	292.064	4.694
5	1 367.000	207.904	6.575	2 309.000	318.596	7.247
5	1 744.000	233.630	7.465	1 420.000	258.451	5.494
5	1 271.000	301.484	4.216	363.000	69.840	5.198
5	2 810.000	3 432.297	0.819	1.000	5 998.437	0.000
5	4 050.000	3 679.615	1.101	1.000	5 998.437	0.000
5	3 896.000	3 283.188	1.187	3 616.000	4 814.391	0.751
5	5 379.000	2 916.638	1.844	3 590.000	4 773.884	0.752
5	4 951.000	3 412.920	1.451	3 011.000	4 994.720	0.603
5	3 770.000	3 242.319	1.163	5 733.000	2 653.421	2.161
6	6.000	0.536	11.193	2 953.000	1 007.449	2.931
6	45.000	8.581	5.244	418.000	33.750	12.385
6	8.000	0.116	68.757	21.000	0.798	26.321
6	69.000	3.395	20.324	695.000	71.012	9.787
6	16.000	0.706	22.649	196.000	22.755	8.613
6	5.000	0.723	6.915	122.000	43.998	2.773
6	28.000	7.833	3.575	4 023.000	2 922.564	1.377
6	28.000	3.428	8.167	119.000	6.458	18.428
6	17.000	2.373	7.165	148.000	3.025	48.923
6	31.000	5.390	5.752	1 108.000	5 627.200	0.197
6	23.000	4.367	5.266	2 731.000	110.825	24.642
6	27.000	2.975	9.075	3 225.000	3 158.824	1.021
12	54.000	7.251	7.447	19.000	4.218	4.505
12	5.000	0.058	85.946	53.000	15.816	3.351
12	76.000	10.817	7.026	179.000	9.416	19.010
12	6.000	0.170	35.217	13.000	2.975	4.369
12	85.000	14.124	6.018	187.000	27.264	6.859
12	18.000	0.316	56.996	239.000	17.095	13.980
12	66.000	13.289	4.966	2 396.000	51.232	46.767
12	26.000	3.096	8.399	242.000	70.509	3.432
12	32.000	8.755	3.655	19.000	1.064	17.861

12	23.000	4.721	4.872	4 727.000	562.299	8.407
12	35.000	5.223	6.701	1 207.000	23.287	51.832
12	43.000	8.951	4.804	5 274.000	769.942	6.850
24	50.000	10.235	4.885	157.000	11.340	13.845
24	67.000	5.381	12.451	119.000	10.172	11.698
24	3.000	0.141	21.234	241.000	20.918	11.521
24	18.000	1.230	14.634	1 039.000	90.958	11.423
24	19.000	2.020	9.408	147.000	30.085	4.886
24	8.000	0.112	71.304	293.000	67.044	4.370
24	35.000	13.904	2.517	6 508.000	1 382.954	4.706
24	48.000	5.606	8.563	1 599.000	41.612	38.426
24	26.000	5.855	4.441	2 812.000	76.846	36.593
24	92.000	21.949	4.192	4 158.000	163.566	25.421
24	58.000	13.592	4.267	3 167.000	113.422	27.922
24	18.000	10.098	1.783	558.000	11.099	50.274
36	535.000	21.961	24.361	3 554.000	2 285.038	1.555
36	3 335.000	240.595	13.861	5 146.000	2 629.664	1.957
36	371.000	12.840	28.893	3 414.000	280.308	12.179
36	456.000	16.418	27.774	4 209.000	2 545.883	1.653
36	1 886.000	78.475	24.033	6 420.000	2 274.297	2.823
36	1 268.000	54.129	23.426	6 676.000	1 156.866	5.771
36	200.000	48.485	4.125	1.000	5 997.723	0.000
36	285.000	68.668	4.150	73.000	5 973.301	0.012
36	194.000	67.659	2.867	105.000	5 980.253	0.018
36	177.000	63.241	2.799	1.000	5 998.437	0.000
36	163.000	42.389	3.845	1.000	5 998.433	0.000
36	160.000	41.637	3.843	1.000	5 998.176	0.000
48	2 527.000	159.294	15.864	3 717.000	417.990	8.893
48	1 067.000	60.162	17.735	3 953.000	1 123.236	3.519
48	3 831.000	654.155	5.856	5 905.000	775.477	7.615
48	2 444.000	220.179	11.100	6 083.000	658.772	9.234
48	3 664.000	1 292.491	2.835	4 546.000	391.989	11.597
48	2 733.000	1 131.871	2.415	3 628.000	356.423	10.179
48	1.000	5 997.972	0.000	188.000	5 952.092	0.032
48	365.000	61.716	5.914	1.000	5 998.392	0.000
48	1 982.000	105.564	18.775	1 785.000	5 602.812	0.319
48	1.000	5 998.371	0.000	2 151.000	5 414.538	0.397
48	406.000	5 934.215	0.068	1 701.000	5 689.960	0.299
48	1 660.000	79.136	20.977	140.000	5 976.547	0.023
48	2 730.000	1 069.976	2.551	7 954.000	666.177	11.940
60	2 707.000	1 322.061	2.048	7 530.000	884.328	8.515
60	2 300.000	1 561.363	1.473	1 911.000	124.521	15.347
60	4 284.000	226.882	18.882	880.000	66.470	13.239
60	4 137.000	229.080	18.059	3 344.000	244.513	13.676
60	6 027.000	1 173.105	5.138	3 013.000	311.998	9.657
60	46.000	5 989.906	0.008	1.000	5 998.047	0.000

60	4 728.000	4 661.633	1.014	1.000	5 998.039	0.000
60	4 421.000	5 096.594	0.867	7 362.000	2 980.079	2.470
60	108.000	29.300	3.686	4 716.000	4 725.078	0.998
60	98.000	21.916	4.472	24.000	5 980.623	0.004
60	811.000	5 904.080	0.137	308.000	5 943.636	0.052
60	1 238.000	55.911	22.142	3 455.000	520.970	6.632
72	6 015.000	989.888	6.076	4 077.000	776.807	5.248
72	3 471.000	696.586	4.983	4 297.000	1 780.641	2.413
72	4 908.000	457.421	10.730	5 414.000	387.747	13.963
72	1 749.000	227.202	7.698	4 646.000	439.739	10.565
72	4 113.000	296.361	13.878	4 902.000	648.633	7.557
72	6 489.000	898.295	7.224	8.000	5 995.982	0.001
72	11 122.000	2 235.984	4.974	1.000	5 998.359	0.000
72	9 739.000	1 257.070	7.747	150.000	5 965.763	0.025
72	9 331.000	2 119.831	4.402	12 868.000	1 777.665	7.239
72	5 842.000	1 329.008	4.396	1 106.000	5 734.364	0.193
72	609.000	5 797.793	0.105	593.000	5 842.588	0.101
72	4 935.000	320.844	15.381	3 999.000	617.924	6.472
72	5 155.000	422.544	12.200	3 135.000	1 820.940	1.722
84	5 005.000	433.627	11.542	5 416.000	1 058.727	5.116
84	443.000	12.495	35.453	3 826.000	1 734.515	2.206
84	2 528.000	358.264	7.056	3 559.000	492.675	7.224
84	2 720.000	1 164.541	2.336	4 534.000	1 549.774	2.926
84	374.000	44.139	8.473	151.000	5 967.421	0.025
84	421.000	45.203	9.314	54.000	5 980.902	0.009
84	307.000	38.263	8.023	147.000	5 959.655	0.025
84	121.000	28.627	4.227	138.000	5 968.265	0.023
84	197.000	45.294	4.349	1 318.000	5 556.055	0.237
84	1 281.000	103.682	12.355	10.000	5 995.512	0.002
84	1 678.000	4 345.697	0.386	2 267.000	2 056.174	1.103
84	396.000	57.395	6.900	2 611.000	2 700.078	0.967
96	14.000	6.640	2.108	2 034.000	3 038.550	0.669
96	1 429.000	265.665	5.379	2 898.000	262.336	11.047
96	3 095.000	1 277.199	2.423	2 489.000	300.101	8.294
96	2 650.000	462.316	5.732	3 761.000	618.938	6.077
96	1.000	5 998.437	0.000	268.000	5 925.522	0.045
96	6 309.000	3 004.359	2.100	91.000	5 955.724	0.015
96	3 331.000	5 114.400	0.651	2.000	5 998.030	0.000
96	1.000	5 998.437	0.000	468.000	5 810.006	0.081
96	1.000	5 998.437	0.000	524.000	5 841.400	0.090
96	1.000	5 998.437	0.000	1.000	5 998.030	0.000
96	1 698.000	163.408	10.391	1 666.000	1 884.564	0.884
96	2 665.000	270.979	9.835	1 534.000	2 148.470	0.714
96	1 959.000	182.390	10.741	1 538.000	1 140.007	1.349
108	1 799.000	152.521	11.795	1 361.000	1 088.447	1.250
108	2 201.000	196.576	11.197	982.000	1 347.072	0.729



108	1 716.000	140.557	12.209	899.000	1 311.294	0.686
108	273.000	5 912.649	0.046	1.000	5 998.413	0.000
108	62.000	5 978.778	0.010	2.000	5 997.174	0.000
108	241.000	5 894.972	0.041	3.000	5 995.824	0.001
108	269.000	5 905.024	0.046	6.000	5 997.120	0.001
108	408.000	5 887.641	0.069	4.000	5 997.577	0.001
108	180.000	5 934.635	0.030	1.000	5 998.421	0.000
108	2 337.000	261.231	8.946	3 203.000	662.632	4.834
108	2 375.000	284.979	8.334	3 076.000	919.637	3.345
108	3 322.000	831.824	3.994	2 965.000	1 331.427	2.227
120	3 166.000	721.473	4.388	3 106.000	1 032.058	3.010
120	2 662.000	699.744	3.804	3 367.000	686.883	4.902
120	2 627.000	909.556	2.888	2 804.000	1 032.768	2.715
120	1 398.000	170.464	8.201	1.000	5 998.437	0.000
120	365.000	56.900	6.415	1.000	5 998.437	0.000
120	1 616.000	265.241	6.093	1.000	5 998.437	0.000
120	612.000	97.445	6.280	1.000	5 998.437	0.000
120	979.000	195.708	5.002	1.000	5 998.437	0.000
120	1 534.000	346.596	4.426	1.000	5 998.437	0.000

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<b>PstPDL</b>	<b>CaspInsCnt</b>	<b>CaspInsArea</b>	<b>CaspInsIndex</b>
0	3 024.000	688.807	4.390
0	2 888.000	576.287	5.011
0	2 696.000	861.586	3.129
0	2 627.000	680.276	3.862
0	2 535.000	754.937	3.358
0	2 299.000	1 006.793	2.283
0	511.000	5 862.592	0.087
0	406.000	5 848.115	0.069
0	310.000	5 893.268	0.053
0	201.000	5 937.245	0.034
0	165.000	5 935.196	0.028
0	102.000	5 967.056	0.017
5	3 024.000	688.807	4.390
5	2 888.000	576.287	5.011
5	2 627.000	680.276	3.862
5	2 535.000	754.937	3.358
5	2 696.000	861.586	3.129
5	2 299.000	1 006.793	2.283
5	102.000	5 967.056	0.017
5	201.000	5 937.245	0.034
5	165.000	5 935.196	0.028
5	511.000	5 862.592	0.087
5	406.000	5 848.115	0.069
5	310.000	5 893.268	0.053
6	190.000	16.850	11.276
6	294.000	28.165	10.438
6	180.000	11.565	15.565
6	271.000	14.411	18.805
6	643.000	43.661	14.727
6	533.000	37.274	14.299
6	3 609.000	539.931	6.684
6	1 409.000	208.985	6.742
6	4 892.000	798.685	6.125
6	5 800.000	1 440.831	4.025
6	2 217.000	188.224	11.779
6	5 098.000	594.093	8.581
12	245.000	31.552	7.765
12	65.000	9.150	7.104
12	76.000	8.689	8.747
12	163.000	28.984	5.624
12	84.000	12.495	6.722
12	206.000	18.438	11.173
12	2 837.000	1 299.829	2.183
12	2 325.000	1 553.584	1.497
12	3 501.000	2 922.144	1.198

12	2 776.000	2 782.102	0.998
12	4 043.000	1 659.664	2.436
12	1 422.000	4 101.009	0.347
24	361.000	24.218	14.906
24	455.000	38.367	11.859
24	498.000	44.687	11.144
24	934.000	82.169	11.367
24	730.000	61.064	11.955
24	932.000	90.002	10.355
24	3 844.000	645.811	5.952
24	5 575.000	1 353.787	4.118
24	2 619.000	140.773	18.604
24	470.000	8.311	56.553
24	2 468.000	184.148	13.402
24	4 938.000	919.097	5.373
36	5 035.000	1 572.861	3.201
36	4 262.000	2 297.912	1.855
36	2 631.000	118.280	22.244
36	3 895.000	753.191	5.171
36	3 095.000	2 238.066	1.383
36	5 574.000	848.130	6.572
36	1.000	5 998.437	0.000
36	1.000	5 998.437	0.000
36	3.000	5 995.396	0.001
36	302.000	5 925.003	0.051
36	7.000	5 997.299	0.001
36	498.000	5 835.316	0.085
48	3 235.000	300.961	10.749
48	5 940.000	812.157	7.314
48	6 076.000	801.590	7.580
48	4 072.000	701.340	5.806
48	2 539.000	602.246	4.216
48	3 295.000	646.156	5.099
48	2 270.000	5 057.379	0.449
48	1 845.000	5 652.137	0.326
48	842.000	5 772.702	0.146
48	860.000	5 711.996	0.151
48	1 195.000	5 683.647	0.210
48	1 909.000	5 468.579	0.349
48	4 338.000	601.523	7.212
60	3 996.000	282.964	14.122
60	6 029.000	1 703.645	3.539
60	3 301.000	198.343	16.643
60	6 760.000	824.627	8.198
60	5 285.000	1 344.595	3.931
60	2.000	5 996.007	0.000

60	1.000	5 998.437	0.000
60	1.000	5 998.433	0.000
60	62.000	5 981.994	0.010
60	1.000	5 998.417	0.000
60	1.000	5 998.437	0.000
60	1 026.000	38.994	26.311
72	865.000	62.244	13.897
72	1 242.000	83.316	14.907
72	2 727.000	329.193	8.284
72	2 292.000	642.075	3.570
72	638.000	79.718	8.003
72	2 024.000	5 572.627	0.363
72	2 965.000	5 263.650	0.563
72	1 390.000	5 579.695	0.249
72	372.000	5 893.343	0.063
72	2 186.000	5 305.429	0.412
72	2 229.000	5 121.539	0.435
72	3 787.000	370.124	10.232
72	5 241.000	744.947	7.035
84	4 594.000	748.936	6.134
84	5 052.000	1 047.828	4.821
84	5 888.000	616.133	9.556
84	4 148.000	1 001.237	4.143
84	198.000	5 927.081	0.033
84	228.000	5 952.719	0.038
84	10.000	5 995.059	0.002
84	204.000	5 931.955	0.034
84	182.000	5 934.739	0.031
84	273.000	5 920.901	0.046
84	1 367.000	2 081.543	0.657
84	2 000.000	1 224.957	1.633
96	1 710.000	1 958.194	0.873
96	2 620.000	1 428.880	1.834
96	1 694.000	318.563	5.318
96	327.000	15.708	20.818
96	1.000	5 998.437	0.000
96	1.000	5 998.437	0.000
96	1.000	5 998.437	0.000
96	1.000	5 998.437	0.000
96	400.000	5 937.860	0.067
96	4 282.000	4 145.447	1.033
96	5 621.000	500.334	11.235
96	5 544.000	446.430	12.419
96	6 779.000	515.638	13.147
108	9 081.000	646.256	14.052
108	8 123.000	734.554	11.058

