The Growth and Differentiation of Fetal Pancreatic Progenitor Cells: The Novel Roles of PDZ-Domain-Containing 2 and Angiotensin II

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Four years are merely a moment of an eye blink. It is just like yesterday when I did not even know how to use a pipetteman, until now I conclude everything already by writing up this doctoral dissertation. There has been a lot going through, but every piece of memory still vividly pops up. I am thankful for having such a valuable experience, for the good moments and the bad.

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List of Publications/ Academic Activities

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Ma, M.T., **Leung, K.K.**, Tsang, K.S. and Leung, P.S. (2010). Reduced immunogenicity of the pancreatic progenitor cells derived from the first trimester human fetal pancreas. *Cell Res* (in submission)

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Leung, K.K., Suen, P.M., Lau, T.K., Ko, W.H., Yao, K.M. and Leung, P.S. (2009). PDZ-domain containing-2 (PDZD2) drives the maturity of human fetal pancreatic progenitor-derived islet-like cell clusters with functional responsiveness against membrane depolarization. *Stem Cells Dev* 18:979-990.

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International Conferences:

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Poster Presentation

Leung, K.K. and Leung, P.S. Regulated expressions and functional implications of a local renin-angiotensin system in human fetal pancreatic progenitor cells. The *7th Annual Meeting of the International Society for Stem Cell Research.* 8-11 July, 2009. Barcelona, Spain.

Leung, K.K., Ma, M.T. and Leung, P.S. Involvement of the renin-angiotensin system in the growth and differentiation of human pancreatic progenitor cells. *Gordon Research Conferences – Angiotensin.* 24-29 February, 2008. Ventura, CA, United States.

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Academic Activities:

1. A selected participant in the 2008 HKSAR Government and Croucher Foundation co-funded Joint School Summer Teaching Laboratory (JUSTL) Programme in the Marine Science Laboratory (MBL) in Woods Hole, M.A., U.S.A.

   Duration: 8 weeks (6-June-2008 to 4-August-2008)

   Coordinators: Professor Andrew L. MILLER (Hong Kong University of Science and Technology)
   Professor Robert Baker (New York University)

   Mentor: Professor Karen CRAWFORD (St. Mary's College of Maryland)

2. An invited chairperson of the oral presentation session in the Physiology Symposium 2009, jointly organized by the Chinese University of Hong Kong, the University of Hong Kong, the National Taiwan Normal University, the National Cheng Kung University and the Kaoshiung Medical University. *29-30 May, 2009*, hosted in the University of Hong Kong, Hong Kong.

Abstract

Fetal pancreatic tissues can be a promising source for pancreatic progenitor cells (PPCs). In this regard, we have successfully isolated and characterized a population of fetal PPCs from first trimester human fetal pancreas using a previously established basic protocol. Upon exposure to a cocktail of conventional growth factors, these PPCs are amenable to differentiate into insulin-secreting islet-like cell clusters (ICCs); however, these ICCs have yet to exert additional efforts to direct to glucose-responsive cells. To address this issue, we have proposed two novel morphogenic factors in the present study, namely PDZ-domain-containing 2 (PDZD2) and angiotensin II (Ang II), a physiologically active peptide of the renin-angiotensin system (RAS), that potentially promote the differentiation and maturation of PPCs/ICCs.

PDZD2 and its secreted form (sPDZD2) have been found to express in our fetal PPCs. We first evaluated the potential role of sPDZD2 in stimulating PPC differentiation and established an optimal concentration for such stimulation. We found that 10^{-9} M sPDZD2 promoted PPC differentiation, as evidenced by the up-regulation of the pancreatic endocrine markers and C-peptide content in the ICCs. It enhanced their expression of the L-type voltage-gated calcium ion channel ($Ca_v1.2$) and conferred an ability to secrete insulin in response to membrane depolarization. Yet these ICCs remained

glucose-unresponsive because of the minimal expression of *GLUT-2*. We thus attempted to study another potential morphogenic candidate, Ang II.

Local RASs have been reported to regulate the differentiation of tissue progenitor cells. It has yet to be confirmed whether such systems exist and govern the PPC development. To address this issue, we herein provided evidence that expression of RAS components was highly regulated throughout PPC differentiation. Locally generated Ang II was found to maintain PPC growth and differentiation via mediation of the Ang II type 1 and type 2 (AT_1 and AT_2) receptors. We found that the AT_2, but not AT_1, receptor was a key mediator of Ang II-induced upregulation of β-cell transcription factors. Transplantation of AT_2 receptor-depleted ICCs into immune-privileged diabetic mice failed to ameliorate hyperglycemia, implying that AT_2 receptors are indispensable during ICC maturation *in vivo*.

To further test whether a functional RAS is present and if so, whether it regulates islet development *in vivo,* we employed a mouse embryo model at different embryonic days and reported a stronger AT_2 receptor expression during the 2nd developmental transition of pancreas development. AT_2 receptor blockade from e8.0 resulted in abnormalities in fetal pancreatic development. Neonates from these mother mice displayed destructed pancreas/islet architecture, a hampered ability in glucose-stimulated insulin-secretion

possibly attributed to a decreased ratio of β-cell to α-cell, and an impaired glucose tolerance at 4-wk old.

In light of these findings, we conclude that we have discovered two novel mechanisms, the PDZD2 and Ang II/AT_2 receptor signaling pathways, in the regulation of the development of PPCs/ICCs, thus implying their novel roles during islet development *in vivo*. The present study provides a "proof-of-principle" that a local RAS is critically involved in governing islet cell development. This work may contribute to devising protocols for maturation of pancreatic progenitors for clinical islet transplantation.

論文摘要

胚胎胰臟是一個胰臟祖細胞(pancreatic progenitor cells; PPCs)的可靠來源。由此，我們根據先前確立的方法，從人類胚胎胰臟中成功分離了 PPCs 並描述了它們的特性。利用一些生長因子的混合物，可令 PPCs 分化爲釋出胰島素的類小島細胞球(islet-like cell clusters; ICCs)。但是，這些 ICCs 還需要額外的分化才能成爲對葡萄糖有反應的細胞。正因如此，我們在這項研究提出兩個可能促進 PPCs/ICCs 分化和成熟的嶄新分化因子，PDZ 結構域蛋白(PDZD2)和腎素-血管緊張素系統(renin-angiotensin system; RAS)中的一個生理具有活躍的縮氨酸：血管緊張素 II (Angiotensin II; Ang II)。

PDZD2 和它的分泌型縮氨酸(sPDZD2) 被發現可以在胎兒 PPCs 上表達。我們目前的研究目的是爲了評估 sPDZD2 在促進 PPCs 分化過程中的潛在作用，並建立一個最優的濃度來促進 PPCs 分化。我們發現 sPDZD2 於 10^{-9} M 的濃度可以促進 PPCs 的分化，這一現象可由 ICCs 中的胰腺內分泌標記信息核糖核酸表達和 C 縮氨酸(C-peptide)含量的上升得以証實。sPDZD2 誘導了 ICCs 的 L 型電壓依賴鈣離子通道($Ca_v1.2$)表達量的增加，促進了 ICCs 在細胞膜退極化下釋放胰島素。但是，這些 ICCs 未能對葡萄糖刺激作出反應，這一點體現於葡萄糖轉運器 2(*GLUT-2*)信息核糖核酸的極微量表達。由此，我們嘗試把研究的焦點放在另外一個因子：Ang II。

有報告指出本體的 RAS 可以調節數種組織的祖細胞分化。但是，這個系統是否在 PPCs 上表達並調節它們的發展則有待確認。針對這個問題，我們在這份報告指

出，在 PPCs 的分化過程中，RAS 的主要構成要素的表達受著高度調節。內源性 Ang II 通過血管緊張素受體一型(AT_1)和二型(AT_2)來維持 PPCs 的生長和分化。我們發現 Ang II 令 β 細胞發育轉錄因子的表達量上升，是通過 AT_2 受體實現，而並非 AT_1 受體。將缺乏 AT_2 受體的 ICCs 移植到免疫豁免的糖尿病小鼠上，動物的高血糖症不能得到改善。這証明了 AT_2 受體對於 ICCs 在體內成熟的過程是不可缺少的。

爲了進一步測試 RAS 是否也調節體內的胰島發育，我們採用了不同胚胎日數(embryonic day; e)的小鼠胚胎模型進行研究。我們發現在胰臟第二期發育轉變的時期，AT_2 受體在胰臟的表達較強。在 e8.0 的懷孕小鼠上運用 AT_2 受體的拮抗會令胎兒的胰島發育不正常，破壞這些初生鼠的胰臟/胰島組織結構，它們的葡萄糖刺激性胰島素釋放的能力也受到阻礙，歸因這些胰島的 β 細胞/α 細胞比例也下降。這些幼子在四周時也顯示了葡萄糖耐量異常。

總括這些研究結果，我們發現了在調節 PPCs/ICCs 發育過程中的兩個嶄新機制：PDZD2 和 AngII/AT_2 受體信號通路，它們在體內的胰島發育中扮演了潛在的角色。這項研究提供了一個原理驗證，證明了本體 RAS 顯著地誘導了胰島細胞的發育。這項課題爲研究分化 PPCs 的方法和實現臨床胰島移植作出了貢獻。

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ABCG2	ATP-binding cassette sub-family G member 2
ACE	Angiotensin-converting enzyme
ADP	Adenosine diphosphate
Arx	Aristaleless related homeobox
AT_1 receptor	Angiotensin II type 1 receptor
AT_2 receptor	Angiotensin II type 2 receptor
ATP	Adenosine triphosphate
Ang II	Angiotensin II
AUC	Area under curve
bFGF	Basic fibroblast growth factor
bHLH	Basic helix-loop-helix
BrdU	5-bromo-2-deoxyuridine
BSA	Bovine serum albumin
Ca_v channel	Voltage-gated calcium ion channel
CCK	Cholecystokinin-pancreozymin
CEL	Carboxyester lipase
CK 19	Cytokeratin 19
C_T	Cycle threshold
DAPI	4'6'-Diamidino-2-phenylindole
EGF	Epidermal growth factor
EIA	Enzyme immunoassay
ELISA	Enzyme-linked immunosorbent assay
EMT	Epithelial-to-mesenchymal transition
ERK	Extracellular signal-regulated kinase
ESC	Embryonic stem cell
FACS	Fluorescent-activated cell sorting
FBS	Fetal bovine serum
GCK	Glucokinase
GLP-1	Glucagon-like peptide-1
Glut-2	Glucose transporter 2
GSIS	Glucose-stimulated insulin secretion
HBSS	Hanks' balanced salt solution
HGF	Hepatocyte growth factor
H&E	Hematoxylin and eosin

HLA	Human leukocyte antigen
HRP	Horseradish peroxidase
HSC	Hematopoietic stem cell
IAPP	Islet amyloid polypeptide
ICC	Islet-like cell cluster
IDDM	Insulin-dependent diabetes mellitus
IFG	Impaired fasting glucose
IFN	Interferon
IGT	Impaired glucose tolerance
IPGTT	Intraperitoneal glucose tolerance test
iPSC	Induced pluripotent stem cell
Isl-1	Islet 1
Klf	Krüppel-like factor
KRBB	Krebs-Ringer bicarbonate buffer
LOS	Losartan
Maf	V-maf musculoaponeurotic fibrosarcoma oncogene homolog
MEF	Mouse embryonic fibroblast
MHC	Major histocompatibility complex
MODY	Maturity-onset diabetes in youth
MOI	Multiplicities of infection
MSC	Mesenchymal stem cell
MTT	(3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
Ngn3	Neurogenin 3
NIDDM	Non-insulin-dependent diabetes mellitus
Oct 4	Octamer-4
Pax 4	Paired-box gene 4
PBS	Phosphate buffered saline
PC	Pro-hormone convertase
PCR	Polymerase chain reaction
PD	PD 123,319
Pdx-1	Pancreatic and duodenal homeobox gene-1
PDZD2	PDZ-domain-containing 2
PECAM	Platelet endothelial cell adhesion molecule
PP	Pancreatic polypeptide
PPC	Pancreatic progenitor cell
Ptf1a	Pancreas transcription factor 1 subunit alpha

RAS	Renin-angiotensin system
RISC	RNA-induced silencing complex
RNAi	RNA interference
ROS	Reactive oxygen species
RT	Reverse transcription
Shh	Sonic hedgehog
shRNA	Short hairpin RNA
siRNA	Small-interfering RNA
Sox	Sex determining region Y (SRY)-box
sPDZD2	Secreted PDZ-domain-containing 2
STZ	Streptozotocin
T1DM	Type 1 diabetes mellitus
T2DM	Type 2 diabetes mellitus
TGF	Transforming growth factor
TNF	Tumor necrosis factor
VEGF	Vascular endothelial growth factor
VNTR	Variable number tandem repeat
vWF	von Willebrand Factor

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Chapter I

General Introduction

I. 1 The Pancreas

I. 1.1 Anatomy of the Pancreas

The human pancreas is an elongated and tapered organ, light tan in colour, visually appeared as a lobulated gland with an irregularly prismatic shape. It is situated transversely across the posterior wall of the abdomen, with the anterior surface covered by peritoneum where it is surrounded by blood vessels, nerves, as well as lymphatic vessels and nodes. The organ measures approximately 12.5 – 15 cm in length, 2 – 3.5 cm in thickness, 3 – 5 cm from superior to inferior border, and weighs around 60 – 100 g (Bockman, 1998). Unlike other abdominal organs, the surfaces of the pancreas are encased in a poorly defined fibrous capsule.

The pancreas is divided into four regions designated as the head, neck, body and tail, with most of the pancreatic parenchyma lying in the head and the uncinate process. Figure I. 1.1 depicts the anatomical position and the basic pancreatic structure. The head of the pancreas, which lies within the concave surface of the duodenum, represents the thickest portion of the gland. It prolongates to the uncinate process that curves behind the superior mesenteric vessels. These vessels separate the ucinate process from the neck of the pancreas and form the pancreatic notch. The neck springs from the front of the head, narrows behind the pylorus, and forms the border between the head and the body of the

pancreas. Most part of the body lies between the gastric antrum, and is thinner than the head while the tail attenuates as extended to the splenic hilum (Bockman, 1998).

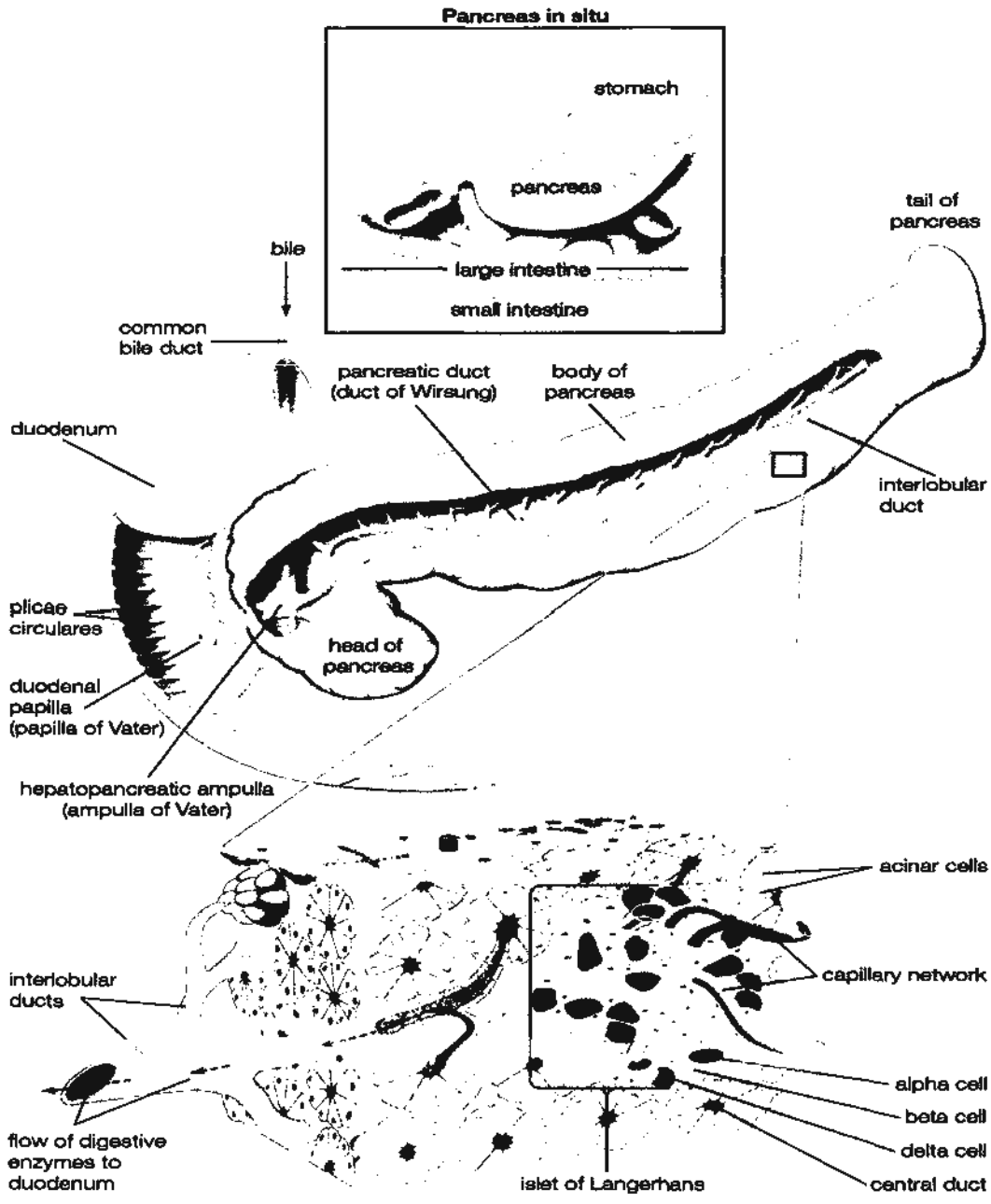


Figure I. 1.1. Anatomical position of the pancreas. Lower insert: the microscopic view of the pancreatic architecture showing its typical acinar and islet structure. (Figure extracted from Pancreas. (2010). In Encyclopædia Britannica. Retrieved May 18, 2010, from Encyclopædia Britannica Online:

http://www.britannica.com/EBchecked/topic/440971/pancreas

I. 1.2 Structure and Function of the Exocrine Pancreas

Histological examination of the pancreas reveals its lobulated structures which are defined by the connective tissues surrounding them. Blood vessels and nerves travel within these interlobular tissues, gaining proximity of the surrounding parenchymal cells. The bulk of the pancreas is composed of exocrine tissues and their associated ductal cells organized in lobules, which comprise between 75 – 90 % and around 5 % of the gland-cell mass, respectively. The exocrine tissues are structurally analogous to grape-like clusters termed as acinar cells, with the terminal portion of the duct system extended into the acini and flattened, forming the nonacinar cells called centroacinar cells. Acinar cells are recognized by the zymogen granules in their cytoplasm where synthesis of different digestive enzymes is found. These enzymes are discharged within the pancreatic juice through a network of ducts that joins the main pancreatic duct, the duct of Wirsung, and ends by emptying into the duodenum at the major papilla. This main duct serves as the backbone of the whole panacreas. The accessory duct, the duct of Santorini, anastomoses with the pancreatic main duct and ends by emptying into the duodenum at the minor papilla (Klimstra, 1997).

The enzymes secreted along with the pancreatic juice are to help digestion of food by breaking down carbohydrates, proteins and fats. The enzymes are usually synthesized and travelled through the ducts in the form of inactive precursors, and they are activated as

they reach the duodenal lumen so as to prevent autodigestion of the secretory cells. Some nonproteolytic enzymes like lipase or amylase are secreted already in full active form. Apart from digestive enzyme production, another major role of the exocrine pancreas is the secretion of bicarbonate ions which are alkaline to neutralize gastric acidic chime entering into the duodenum. Secretion of digestive enzymes and bicarbonate ions normally increases after food intake. In fact, previous reports have shown the adaptive response of pancreatic enzyme secretion against dietary change (Sabb et al., 1986). These secretions are mainly regulated by major neurohormonal pathways such as cholecystokinin (CCK) and secretin, as well as by acetylcholine.

I. 1.3 Composition and Function of the Endocrine Pancreas

Small clusters of cells, called the Islet of Langerhans or pancreatic islets, were embedded within the exocrine architecture and are considered to be evenly distributed within the whole pancreas. They comprise approximately 20 % of the pancreatic mass in newborn human and drop to around 1 – 2 % in adulthood, and each of them consists of around 1000 endocrine cells and a dense capillary network, allowing a rapid access of pancreatic hormones to the circulation (Gepts and Pipeleers, 1976). The blood flow into the islets is markedly higher than that reaching the exocrine compartment. Characterization of the endocrine components reveals five endocrine cell types, namely the α-cells that produce the hormone glucagon, β-cells that produce insulin, δ-cells that produce somatostatin, PP cells that produce pancreatic polypeptide, and ε-cells, a more recently defined islet cell type that produces ghrelin (Kojima et al., 2001). Figure I. 1.2 shows the typical islet structures from different animal species and Table I. 1.1 summarizes the characterization of these major endocrine cell types within an islet. Some minor hormones like substance P, peptide YY or vasoactive intestinal polypeptide, are also identified in an islet (Fahrenkrug and Emson, 1982; Tatemoto, 1982; de Giorgio et al., 1992). Among these endocrine cells, β-cells are the most abundant cell type that constitutes around 75 % of an islet, centrally located and are surrounded by a ring of α- and δ-cells. Yet compositions and distributions

of these endocrine cells within an islet might vary among different species (Cabrera et al., 2006). These cells serve as a paracrine feedback system that regulates the hormone secretion profile of each other. Secretion of these hormones is controlled by food nutrients as well as nerve signals of both sympathetic and parasympathetic fibers that directly innervate the islets.

Among the major pancreatic endocrine hormones, insulin is the major regulator of anabolic processes and is involved in maintaining glucose homeostatis. The change of blood glucose after food intake is responded quickly by insulin release, which exerts actions on target organs including the liver, adipose tissues and muscles. Major actions of insulin include the stimulation of glucose oxidation and storage by glycogen synthesis. It also simultaneously restrains glucose production and output into circulation. Failure to attain an appropriate insulin secretory response upon stimulation may lead to diabetes mellitus, which will be furthered discussed in Chapter I. 3 (pp.35-49). Glucagon, as an antagonistic hormone of insulin, or catabolic hormone, also involves in regulating blood glucose levels together with insulin in an opposite action manner. Table I. 1.2 summarizes the major actions of glucagon and insulin in flow of fuels. Somatostatin, also known as the growth hormone-inhibiting hormone, possesses an inhibitory effect on different hormone release, including insulin.

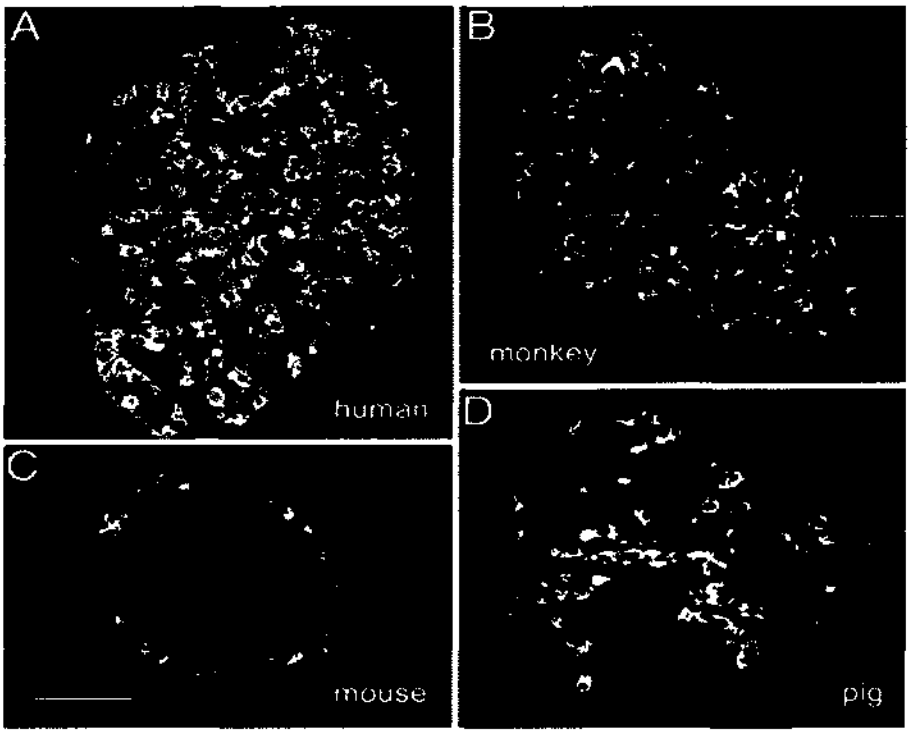


Figure I. 1.2. Localization of different endocrine cell compositions within an islet isolated from different species including (*A*) human, (*B*) monkey, (*C*) mouse and (*D*) pig. Red: insulin; Green: glucagon; Blue: somatostatin. Note the interspecies difference of the distributions and abundance of each cell type. Scale bar: 50 μm. (Figure extracted from Cabrera et al., 2006).

Cell type	Hormone produced	Percentage of an islet	Distribution	Approximate size and description of secretory granules
α-cell	Glucagon	20 - 25	More abundant in the tail and body	Dense core, 200 – 300 nm
β-cell	Insulin	60 - 70	Evenly distributed	Crystalloid, 250 – 400 nm
δ-cell	Somatostatin	10 - 15	Evenly distributed	Low density, 150 – 400 nm
pp-cell	Pancreatic polypeptide	5 - 10	More abundant in uncinate process	Elongated, 100 – 200 nm

Table I. 1.1. Major endocrine cell types of an islet. (Information extracted from Merrel et al., 1988; Smith, 1994).

Target organs	Biological actions	Hormones	
		Insulin	Glucagon
Liver	Glycogen synthesis	+	
	Glycolysis	+	
	Glycogenolysis	-	+
	Gluconeogenesis	-	+
	Ketogenesis	-	+
Muscle	Glucose uptake	+	Minimal action
	Amino acid uptake	+	
	Proteolysis	-	
Adipose tissue	Glucose uptake	+	Minimal action
	Free fatty acid uptake	+	
	Lipolysis	-	

Table I. 1.2. Major actions of glucagon and insulin on overall flow of fuels. + denotes stimulation and - denotes inhibition. (Extracted and modified from Nussey and Whitehead, 2001)

I. 1.4 Mechanisms for Insulin Secretion

Insulin is secreted from pancreatic β-cell in response to nutrients, notably glucose challenge. Figure I. 1.3 depicts the putative mechanism of a glucose-stimulated insulin secretion (GSIS) in a β-cell. Extracellular glucose enters a β-cell through a specific glucose transporter (Glut-2), a transmembrane carrier protein that is concentrated in the microvilli of the canaliculi between β-cells. Glucose is first phosphorylated by glucokinase into glucose-6-phosphate. The glucose-6-phosphate is then employed as a metabolic fuel for glycolysis and oxidized. This contributes to a rapid rise of the amount of intracellular adenosine triphosphate (ATP). An increment of ATP/ adenosine diphosphate (ADP) ratio causes a closure of the ATP-dependent potassium (K^+) channels on the plasma membrane. This suppresses K^+ efflux from the β-cell, and in turn triggers depolarization of cell membrane. The depolarization leads to an opening of the L-type voltage-gated calcium (Ca^{2+}) channels and an increase in Ca^{2+} influx into the cytoplasm. This surge in intracellular Ca^{2+} concentrations elicits movements of insulin-containing secretory vesicles along the microtubules, followed with their fusion with cell membrane and exocytosis of insulin (Ashcroft, 1976).

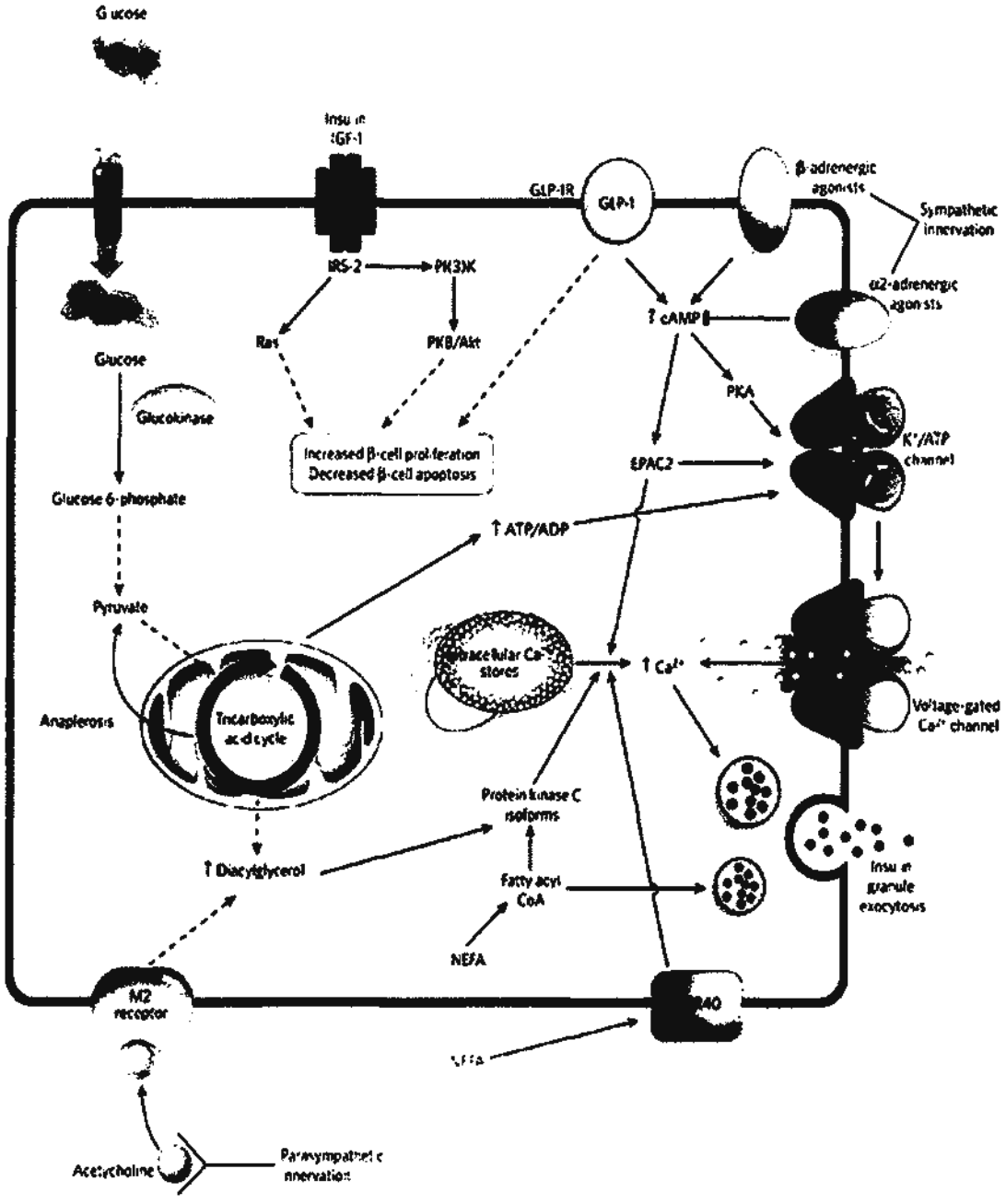


Figure I. 1.3. Mechanisms of a GSIS within a β-cell. Note that nutrient molecules other than glucose like fatty acids can also trigger insulin release. The incretin, glucagon-like peptide-1 (GLP-1), can potentiate GSIS through its own GLP-1 receptor-induced signaling after binding (Figure extracted and modified from Kahn et al., 2006).

I. 2 Developmental Biology of the Pancreas

I. 2.1 Embryology of the Human Pancreas

Similar to the fetal pancreas organogenesis in mouse, the human pancreas originates from two separate outgrowths, designated as the dorsal and ventral buds, from the foregut endoderm directly posterior to the stomach. The dorsal bud arises from evagination of the dorsal side of the primitive duodenum at around 3.75^{th} week of gestation while the ventral bud arises from the base of the hepatic diverticulum at around 4.5^{th} week of gestation. After undergoing the rotation of the ventral bud to the right of and then behind the developing duodenal loop, the dorsal and ventral buds come into contact with one another and fusion of the two buds occurs at the end of 6^{th} week of gestation. The ventral bud gives rise to the head and uncinate process of the pancreas while the dorsal bud forms the remaining portion of the organ. Meanwhile, the ventral bud duct also fuses with the distal portion of the dorsal bud duct and thus forms the subsequent duct of Wirsung, the main pancreatic duct which runs through the entire pancreas. The promixal portion of the dorsal bud duct becomes the future duct of Santorini, the accessory duct (Skandalakis, et al., 1998; Gittes, 2009). During the fusion of the two pancreatic buds at 6^{th}-7^{th} week of gestation, the pancreatic architecture is observed with tubular structures surrounded by dense mesenchymal tissues next to the duodenal structure. The mesenchymal layer

probably provides signals to the invading epithelium that regulates the balanced development of the future endocrine and exocrine portions of the pancreas (Piper et al., 2004). The dual origin of the organ indeed accounts for the regional differences in the islet cell distribution in adult pancreas.

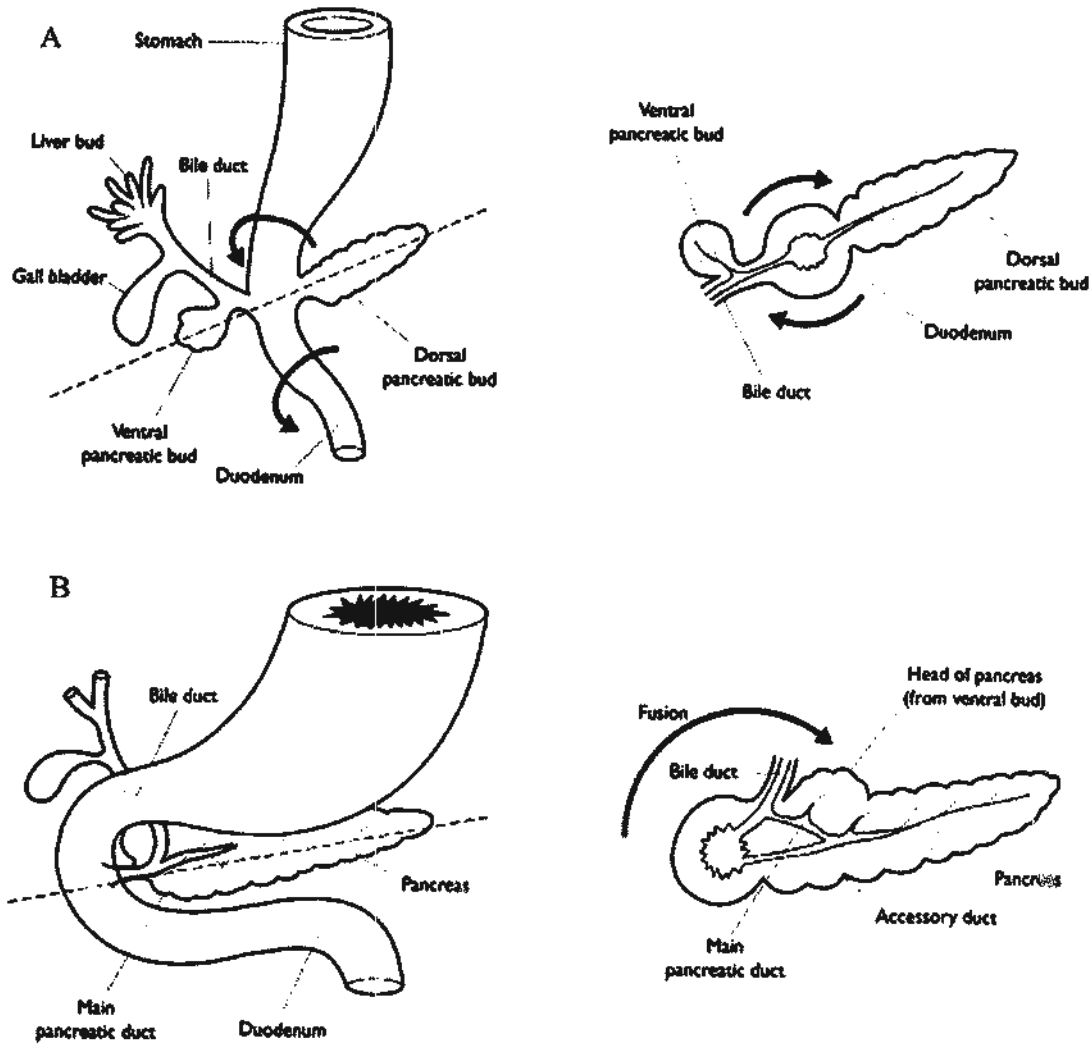


Figure I. 2.1. Schematic drawings of the development of the human pancreas at (*A*) 6th week of gestation when the gut rotation occurs, and (*B*) and the fused dorsal and ventral pancreatic buds at 8th week of gestation. Blue arrows indicate the rotation of the primitive duodenum that causes movement of the ventral bud towards the dorsal bud. Dotted lines indicate the level of the corresponding diagrammatic transverse sections shown on the right. (Extracted from Nussey and Whitehead, 2001)

I. 2.2 Specification of Early Pancreas Development

The specification of the early developing pancreas is regulated by a series of transcription factor expression and signaling processes. During the early stage of development when the two pancreatic buds emerged as dorsal and ventral evaginations from the primitive gut tube, molecular signals from the adjacent notochord including the transforming growth factor (TGF)β family member activin-βB and fibroblast growth factor (FGF)2 are essential to initiate the pancreatic developmental programme by their suppression of Sonic hedgehog (Shh). The exclusion of Shh to initiate pancreas development might involve interspecies difference (dilorio et al., 2002). In rodents and human, regulation of Shh signals determines the bipotential choice between liver and pancreas development from the common precursor cells (Kemp et al., 2003).

Molecular signals from the pancreatic mesenchyme including hepatocyte growth factor (HGF) and epidermal growth factor (EGF) families are critical to the proliferating and invading epithelium. Following the formation of two pancreatic buds, two transcription factors namely the *pancreatic and duodenal homeobox gene-1* (*Pdx-1*) and basic helix-loop-helix (bHLH) *pancreas transcription factor 1 subunit alpha* (*Ptf1a*)/ *p48* regulate the early steps of development. *Pdx-1* is regarded as a master gene in such a way that its temporal and spatial regulated expression throughout the whole pancreas

development critically contributes to the commitment of endoderm to a pancreatic phenotype. Inactivation of this gene often results in severe loss of β-cell phenotype and pancreatic agenesis (Ahlgren et al., 1998; Oliver-Krasinski et al., 2009). Another transcription factor, *Ptf1a*, is originally thought to be exclusively required for exocrine cell differentiation (Zecchin et al., 2004). Yet a more recent study using a recombination-based lineage tracing has identified robust *Ptf1a* expression in early bud stage in uncommitted pancreatic progenitors. The ventral pancreatic bud is found to be completely lacking in *Ptf1a* null mice, and meanwhile these cells turns to intestinal cell fate (Kawaguchi et al., 2002). In addition, recent data have suggested *Ptf1a* as an early activator of *Pdx-1* in governing both acinar and endocrine progenitor cell development (Wiebe et al., 2007).

Another regulatory transcription factor immediately downstream of *Pdx-1*, *neurogenin 3* (*Ngn3*), together with the delta/Notch signaling in the early developing pancreas has also been proposed to determine the cell fate. *Ngn3* is a recognized transcription factor that is required for the specification of a common precursor for all endocrine cell type (Gradwohl et al., 2000). Expression of *Ngn3* leads to the expression of the extracellular ligand delta which in turn binds to the Notch receptor on the adjacent cell. This delta/Notch binding induces expression of another bHLH factor, *hes*, which inhibits expression of *Ngn3* and prevents the cell from entering the endocrine cell fate. This

mechanism is known as lateral inhibition which has also been reported to function in neural development (Artavanis-Tsakonas et al., 1999). The existence of such lateral inhibition mechanism highlights the importance of cell-cell signaling between epithelial neighbors in selection of pro-endocrine precursors. Of note, Notch signaling has recently been shown to regulate exocrine differentiation by directly regulating *Ptf1a* activity (Ghosh and Leach 2006). Figure I. 2.2 generalizes the regulatory mechanisms of early pancreas specification.

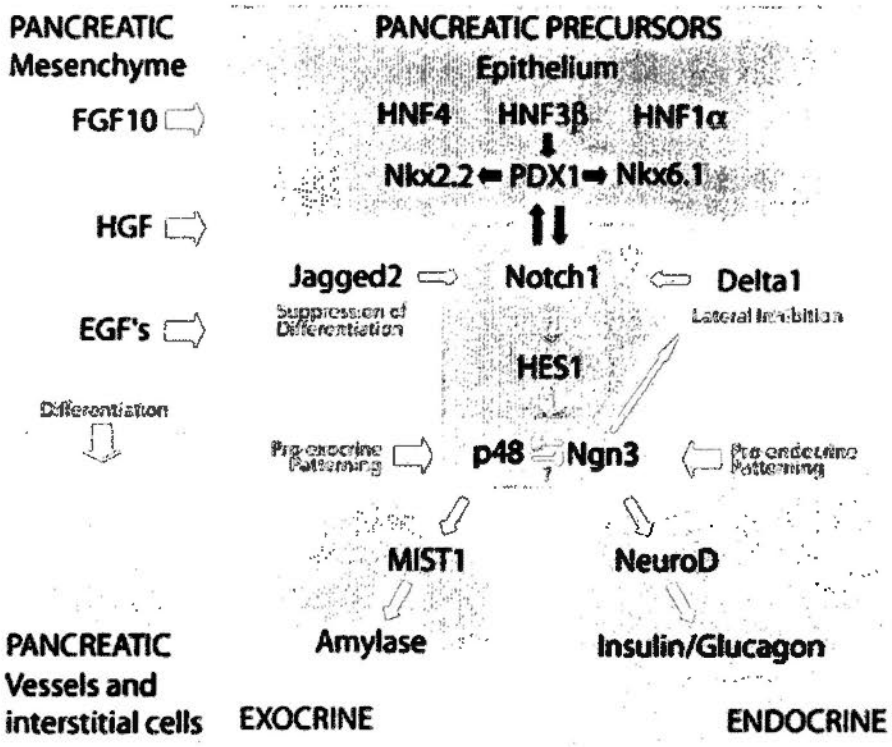


Figure I. 2.2. Schematic presentation of the regulatory mechanisms in a developing pancreas. Note the importance of delta/Notch signaling in regulating both early exocrine and endocrine specification. (Extracted from Jensen, 2004)

I. 2.3 Hierarchy of Transcription Factors Regulating β-cell Development

In the past decade, a growing body of evidence has elaborated upon the transcription dynamics and signaling pathways involved in the development of the different pancreatic endocrine cell types (Habener et al., 2005; Ackermann and Gannon, 2007; Murtaugh, 2007; Scharfmann et al., 2008). This valuable information has been garnered mainly from loss-of-function studies in animal models, and thus the hierarchy of events that occurs in a developing pancreas is being elucidated. Figure I. 2.3 summarizes a consensus of transcription factor expression requirement in the developing pancreas. Concomitant with the establishment of endocrine fate is the activation of a LIM homeodomain transcription factor, *Islet 1*(*Isl-1*). *Isl-1* is expressed early and exclusively in early dorsal not the ventral pancreatic mesenchyme (Ahlgren et al., 1997). A definite role of *Isl-1* in pancreatic development is elusive, and still remains unclear whether it is involved in the selection process of endocrine precursor cells. Yet it is noteworthy that *Isl-1*-deficient mice exhibit a complete dorsal pancreas agenesis and a disrupted endocrine cell formation. This information lends further support to a recent finding that *Isl-1* was actually involved in the terminal differentiation and maintenance of islet cell mass (Du et al., 2009).

Upon initiation of the endocrine precursor marker, *Ngn3*, the temporally regulated expression of a series of transcription factors determine the progenitor cell fate towards

different endocrine cell types. Downstream of *Ngn3* is the expression of the bHLH transcription factor *NeuroD/ Beta2*. *NeuroD* is expressed in differentiated endocrine cells, and earlier study has revealed its activated expression by *Ngn3* (Huang et al., 2000). *NeuroD* was also found to be important to promote *Insulin* gene expression by regulating its enhancer activity. In *NeuroD*-deficient mice, formation of islets could still be observed, but a significant β-cell apoptosis was noted prior to birth, resulting in a markedly decrease in amount of β-cells arranged in irregular islet aggregates (Naya et al., 1995).

Paired-box gene 4 (*Pax4*), a member of the *Pax*-gene family, is found to be critical for β-cell development. It is expressed early in both dorsal and ventral pancreas, restricted to the β-cell of the neonate pancreas and is minimally expressed in adult. Homozygous disruption of *Pax4* leads to a complete absence of both β- and δ-cells, with an elevated but disorganized α-cells (Sosa-Pineda et al., 1997; Wang et al., 2004). Since *Pax4* contributes to all endocrine lineages, it might be suggested to mark endocrine progenitors like *Ngn3*. The importance of *Pax4* in β-cell development is further supported by a recent study, elegantly showing that ectopic expression of *Pax4* is sufficiently enough to convert endocrine progenitor cells and even mature α-cells into the β-cell fate (Collombat et al., 2009).

Further downstream is the expression of a NK type of homeodomain transcription

factor *Nkx 2.2*. Its expression is restricted to α-, β- and PP-cells (Sussel et al., 1998). *Nkx 2.2*-null mice lack β-cells but have a large number of hormone-negative arrested precursor cells. Other than its function as a transcriptional activator for β-cell maturation, *Nkx 2.2* has also been shown to function in part as a transcriptional repressor for β-cell formation during development (Doyle et al., 2007). Another NK type of homeodomain transcription factor that is also a regulator for β-cell differentiation is *Nkx 6.1*. *Nkx 6.1* lies downstream of *Nkx 2.2*, and is different from *Nkx 2.2* in a way that its expression is found exclusively in developing β-cells and is maintained after birth. *Nkx 6.1*-null mice exhibit a selective suppression on the amount of β-cells but a normal development of other endocrine cell types (Rudnick et al., 1994; Sander et al., 2000). More recent data have revealed the role of *Nkx 6.1* as a transcriptional repressor on glucagon expression and regulated insulin release in mature β-cells (Schisler et al., 2005).

High level of *Pdx-1* expression is noted again in differentiated β-cells and its expression is gradually lost in differentiated exocrine cells (Oster et al., 1998). This makes *Pdx-1* as a recognized marker for designating mature β-cells. Its sustained expression, together with a member of another class of transcription factor, V-maf musculoaponeurotic fibrosarcoma oncogene homolog (*MafA*), in β-cells is critical to maintain their survival and insulin secretory functions (Brissova et al., 2005; Shao et al., 2009).

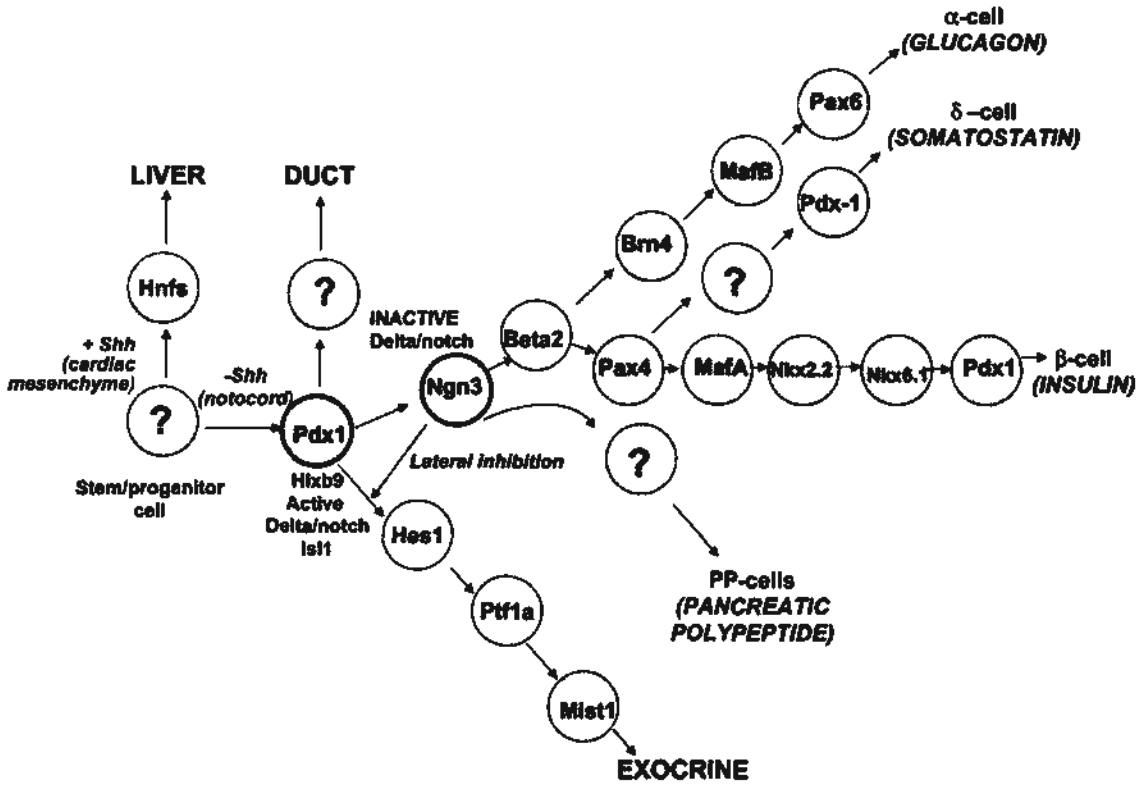


Figure I. 2.3. The hierarchy of transcription factor expression during the developing of progenitor cells toward different pancreatic endocrine cell types. The different classes of transcription factors are designated by different colours: pink-homeodomain genes; blue-bHLH; green-Maf genes; orange-Hnfs; gray-unidentified factors. (Extracted from Habener et al., 2005).

I. 2.4 The Ontogeny of Endocrine Cells in Human Fetal Pancreas

The sequentially development of the endocrine cells in early trimester human fetal pancreas has been thoroughly described in a number of reports in recent years (Stefan et al., 1983; Bouwens et al., 1997; Polak et al., 2000; Piper et al., 2004; Lyttle et al., 2008; Phan-Hug et al., 2008; Sarkar et al., 2008; Andralojc et al., 2009; Jeon et al., 2009). Based on the observations as reported in these studies, here the ontogenetic and morphogenetic pattern of the major endocrine cells during human pancreas development at 6^{th}-23^{rd} weeks of gestation is briefly summarized as follow:

6^{th} week of gestation

Pdx-1 is already detected early around 3.75^{th} week of gestation in the pancreatic buds from the outgrowth of primitive duodenum. At 6^{th} week of gestation, Pdx-1 is present both in the duodenal structures and the pancreatic epithelial cell nuclei. Its expression persists during the early fetal period while the initial Pdx-1 expression is also found with a cytoplasmic localization (Phan-Hug et al., 2008; Sarkar et al., 2008). Ngn3 expression is found at this stage and persists until 22^{nd} week of gestation. This contrasts with the down-regulation of the gene as observed in mice during the later stages of development, suggesting its function other than islet neogenesis in human pancreatic development such

as islet innervation (Bouwens et al., 1997; Polak et al., 2000; Amella et al., 2008). Ki67, a marker of cellular proliferation, is noted in almost half of the pancreatic epithelial cells. They are found mainly in the periphery along with the central endocrine differentiation (Piper et al., 2004).

7th week of gestation

The first endocrine cells are observed around 7th-8th weeks of gestation (Bouwens et al., 1997). Unlike the pancreatic development in rodents, insulin expression precedes that of glucagon in human. The first insulin cells do not express Pdx-1, reflecting their Pdx-1-independent developmental processes (Phan-Hug et al., 2008). *PAX4* and *ARX*, markers for β-cells and α-cells, respectively, are undetectable at 7th-8th weeks of gestation. *NKX2.2*, *NKX6.1* and *MAF B* expression are all already present at these stages while *MAF A*, a marker for mature β-cells is not observed till 21st week of gestation (Jeon et al., 2009).

8th week of gestation

Insulin- and glucagon-expressing cells are found to disperse in the epithelial cell tubules, and the former is not localized in close association with CD 34-positive vascular endothelial cells. Somatostatin is detected in isolated epithelial cells but with a much

lower expression level than that of insulin and glucagon (Piper et al., 2004). Ptf1a, a marker for exocrine cell differentiation, starts to express in a large populations of Pdx-1-positive epithelial cells (Phan-Hug, et al., 2008). It is noted with a high level of co-expression of Pdx-1 and cytokeratin (CK)19, a pancreatic ductal cell marker, during the 8^{th}-12^{th} weeks of gestation (Lyttle et al., 2008).

9^{th} week of gestation

The pancreatic tissue at the 9^{th} week of gestation consists mainly of islands of branched epithelial cell tubules surrounded within a much larger volume of mesenchymal tissues. Mesenchymal markers like smooth muscle actin and vimentin are highly expressed (Sarkar et al., 2008). Few isolated insulin cells are observed and small insulin cell clusters are started to form. Most of the glucagon-positive cells co-expressed insulin, though cells only expressed with insulin were also detectable (Jeon et al., 2009). Diffused expressions of CK19 are noted in all epithelial clusters, while low signals of the fetal acinar cell marker, carboxyester lipase (CEL), are detected (Sarkar et al., 2008). For the pancreatic transcription factors, *PAX4* became prominent from 9^{th} week of gestation onwards. Ngn3 expression is observed with a notable increase especially in the Pdx-1-positive, insulin-positive and glucagon-positive cell populations (Lyttle et al., 2008). These cells

were scattered within epithelial clusters, and they are either expressed with beta-catenin or pancreatic hormones. This indicates that beta-catenin might be required for the development of the exocrine but not the endocrine pancreas (Sarkar et al., 2008), as reported also elsewhere (Murtaugh et al., 2005). The number of Pdx-1 positive cells is also increased with further branching between 9^{th} and 13^{th} weeks of gestation.

10^{th} week of gestation

All four islet hormones including glucagon, insulin, somatostatin and pancreatic polypeptide are detected. A more than 10-fold increase of *INSULIN* mRNA expression is noted, and the insulin-positive cells remained more prevalent than those of other pancreatic hormones. Small clusters peripherally localized with endocrine cells are observed near the duct-like structures. These clusters are also marked with strong beta-catenin expression. Many of the larger aggregates have been already in contact with multiple CD34-positive cells (Piper et al., 2004). All endocrine cells are expressed with Nkx2.2 but the expression of Nkx6.1 is confined to insulin-positive cells only, indicating that it is a β-cell-specific transcription factor. Both of them do not co-localize in amylase-expressing cells (Lyttle et al., 2008).

11th week of gestation

A marked transition of the exocrine genes was observed in the 11th week of gestation. The CEL was expressed in the beta-catenin-positive epithelium, and its expressions as well as that of other exocrine markers like elastase or Aristaleless related homeobox (Arx) are dramatically increased (Sarkar et al., 2008; Jeon et al., 2009). CK19 expression is found in the luminal side within the epithelial clusters and carbonic anhydrase, the ductal cell functional markers, also started to increase in its expression. Ghrelin expression is first noted at this stage in some cells (Andralojc et al., 2009). The expression levels of some pro-hormone convertases like PCSK1 (PC1/3) which cleaves pro-insulin, and PCSK2, also increase here starting from the 11th week of gestation (Jeon et al., 2009). The expression of mesenchymal markers like smooth muscle actin or vimentin decline here till 15th week of gestation (Sarkar et al., 2008).

12th week of gestation

Some Ptf1a-expressing cell aggregates are observed at a distance from the insulin-positive epithelial cell clusters, representing the future acinar cell population. These cells also express with carboxypeptidase A in their cytoplasm (Phan-Hug et al., 2008). The insulin-expressing clusters are maximally around 20 cells in diameter. Glucose

transporter-2 (GLUT-2) and the secretory marker, chromogranin A, are stronger expressed in the periphery of these clusters where glucagon is present. PC1/3 and islet amyloid polypeptide (IAPP) which is co-secreted with insulin in mature β-cells, are expressed centrally within the clusters in insulin-expressing cells, implying the capability of fetal pancreatic cells to process and secrete insulin (Piper et al., 2004).

13th week of gestation

The size of the insulin-expressing clusters next to the epithelial structures continues to increase and the large primitive islets expressing all four pancreatic hormones can be observed. Cytoplasmic and nuclear detection of Pdx-1 are detected within an islet, but its expression also remained in the nuclei of non-endocrine epithelial cells, visible at a distance of the duct-like structures (Piper et al., 2004; Phan-Hug et al., 2008).

14th week of gestation

The islet clusters become very prominent with the core expressing insulin which is surrounded by glucagon-positive cells. They reach a maximum average diameter (Jeon et al., 2009). The developing CD34-positive blood vessels start to penetrate into the islet structures (Piper et al., 2004). Pdx-1 expression is co-localized with insulin-expressing

cells and not with glucagon expressing cells, indicating that the loss of Pdx-1 expression might be critical for α-cell development. Co-localization of CK19 and Pdx-1 is high at early developmental stages but is profoundly decreased here at the 14th week of gestation (Lyttle et al., 2008).

15th week of gestation

Starting from the 15th week of gestation, the fetal pancreatic development proceeds to gradual growth without any critical alterations of the constituent cell types. The majority of the endocrine cells express a single hormone, though the cells co-expressing insulin and glucagon is also noticeable. These cells, on the other hand, are not Ki67-positive, reflecting that they are non-proliferative (Sarkar et al., 2008). A peak expression of CEL is observed in both luminal and peripheral areas of the whole epithelium. The expression of the endocrine cell phenotypic markers like *ISL-1* or *NEURO D* is originally low while an increase starts from this stage. The former is expressed both in differentiated endocrine cells and early pancreatic mesenchymal cells (Jeon et al., 2009).

16th-17th weeks of gestation

Typical acinar structures are formed at this stage from PTF1A-positive cells and a

significant peak in α-cell mass was observed later after the 17th week of gestation (Stefan et al., 1983). The size of insulin-positive and glucagon-positive areas is similar to that observed in human adult islets. Somatostatin-expressing cells are interspersed with the glucagon-expressing cells at the periphery of the islet clusters (Jeon et al., 2009). Ghrelin-expressing cells start to aggregate in clusters, and few of them exist as single cells in the pancreatic mesenchyme (Andralojc et al., 2009). Expression of the *IAPP* mRNA is profoundly increased after these gestational weeks and that of *NGN3* started to decrease (Jeon et al., 2009).

18th-20th weeks of gestation

The architecture of the organ at these gestational weeks generally resembles that observed in adult human pancreas in terms of its Pdx-1-positive epithelium and islet clusters. An extensive capillary network is noted in the islet clusters. Proliferation of glucagon-expressing cells reaches a peak (Sarkar et al., 2008). A dramatic decline of Ngn3 expression is observed in Pdx-1-postive, insulin-positive and glucagon-positive cells. Cells that are maintained with Ngn3 expression remain at the edge of the islets during and after the 20th week of gestation (Lyttle et al., 2008).

21st-23rd weeks of gestation

The growth of epithelial tissues is increased to more than two-third to that of mesenchymal tissues. The typical amylase-positive acinar structures are able to be observed where Pdx-1 was not expressed. *INSULIN* mRNA expression continues to increase, but there are still few proliferative endocrine cells observed, indicating that the differentiation of a putative precursor population was the predominant means to generate early endocrine cells over the second trimester (Sarkar et al., 2008). Ghrelin-expressing ε-cells are mainly localized to the peripheral rim of the islets (Andralojc et al., 2009). Pdx-1 is exclusively expressed in β-cells as well as in few ductal cells. Expression of *GLUT-2* remains barely detectable, while that of *GLUCOKINASE* (*GCK*) is already strongly expressed (Jeon et al., 2009).

I. 2.5 Post-natal Maturation of the Endocrine Pancreas

The early postnatal period is also a critical window for development of pancreatic islets and their functional maturation. Though related studies in human are limited, data from animal studies revealed the existence of postnatal structural and functional modifications in both the endocrine and exocrine pancreas (de Assis et al., 2003; Aguayo-Mazzucato et al., 2006). It was found that not only the α- and β-cell mass was increased during the first month of post-natal period, but also the glucose-responsiveness of islets was enhanced in those retrieved from neonate pancreas at later days (Aguayo-Mazzucato et al., 2006). In the same study, periods of hyperglycemia was noted in early postnatal weeks. Glucose can be an important signaling factor that attributes to such restructuring of islets and β-cell functional maturation (Schuit et al., 2002). The change in the islet compositions exhibited regional differences within the pancreas and it might possibly reflect the characteristic of a dual origin during the embryological development of the pancreas. Earlier study has revealed an active proliferation and apoptosis in neonatal islets, and this possibly contributed to the remodeling of the endocrine pancreas early postnatally (Scaglia et al., 1997). Yet it should be well noted that the proliferation rate of β-cell would be progressively decreased and thus contributed to a very low β-cell turnover in aged individuals (Teta et al., 2005).

I. 3 Diabetes Mellitus

I. 3.1 Classification and Diagnostic Criteria of Diabetes Mellitus

Diabetes mellitus is a heterogenous group of metabolic disorder defined as the condition of increased circulating blood glucose levels, with a disturbed metabolism of food molecules including fats, carbohydrates and proteins resulting from defects in insulin secretion and/or insulin actions. This hyperglycemic condition often associated with a variety of complications including neuropathy that leads to foot ulcers or amputations, retinopathy that leads to loss of visions, nephropathy that leads to kidney failure and erectile dysfunction that leads to sexual disorder. Classification of the major types of diabetes was somehow based on their conventional treatment paradigms. They include type 1 diabetes mellitus (T1DM) or the former insulin-dependent diabetes mellitus (IDDM) which is often attributed to an insufficient insulin supply, and type 2 diabetes mellitus (T2DM) or the former non-insulin-dependent diabetes mellitus (NIDDM) which is often attributed to an insufficient response of peripheral tissues to insulin actions. There are also other types of diabetes designated as gestational diabetes mellitus and 'other specific types' of diabetes mellitus (or secondary diabetes mellitus) by the World Health Organization (WHO) group (Alberti and Zimmet, 1998). Table I. 3.1 summarizes the etiologic classification of diabetes mellitus. A more common type of diabetes fallen into

the category of 'other specific types' is a person with genetic defects of β-cell functions. This type of diabetes is formerly known as maturity-onset diabetes in youth (MODY).

The WHO Expert Committee has tried to establish a set of criteria for clinical diagnosis of diabetes mellitus. Table I. 3.2 lists the blood glucose values for such diagnosis on patients. A clinical diagnosis of diabetes mellitus can be concluded only when more than one plasma/blood glucose test on the same individual results with a value that falls within the diabetic range is noted. An intermediate group of individuals exists whose glucose levels are too high to be considered normal but do not yet fall into the diabetic range. These subjects are defined to have impaired glucose tolerance (IGT) or impaired fasting glucose (IFG) if they have their glucose levels fallen within the ranges listed in Table I. 3.2. Note that these subjects are often euglycemic or have near normal glycated hemoglobin levels. They are referred as having a 'pre-diabetes' condition which means they are at high risk for development into real diabetes.

Type 1 *(beta-cell destruction, usually leading to absolute insulin deficiency)*
Autoimmune
Idiopathic
Type 2 *(may range from predominantly insulin resistance with relative insulin deficiency to a predominantly secretory defect with or without insulin resistance)*
Other specific types
Genetic defects of beta-cell function
Genetic defects in insulin action
Diseases of the exocrine pancreas
Endocrinopathies
Drug- or chemical-induced
Infections
Uncommon forms of immune-mediated diabetes
Other genetic syndromes sometimes associated with diabetes
Gestational diabetes

Table I. 3.1 Etiologic classification of Diabetes Mellitus. (Extracted from Alberti and Zimmet, 1998)

	Glucose concentrations (mM [mg/dl])	
	Whole blood (venous)	Plasma (venous)
Impaired glucose tolerance (IGT)		
Fasting concentration* (if measured)	< 6.1 [< 110]	< 7.0 [< 126]
and		
2-h post-glucose load^	≥ 6.7 [≥ 120] and < 10.0 [< 180]	≥ 7.8 [≥ 140] and < 11.1 [< 200]
Impaired fasting glycemia (IFG)		
Fasting*	≥ 5.6 [≥ 100] and < 6.1 [< 110]	≥ 6.1 [≥ 110] and < 7.0 [< 126]
2-h (if measured)^	< 6.7 [< 120]	< 7.8 [< 140]
Diabetes Mellitus		
Fasting*	≥ 6.1 [≥ 110]	≥ 7.0 [≥ 126]
or		
2-h post glucose load^	≥ 10.0 [≥ 180]	≥ 11.1 [≥ 200]
or both		

Table I. 3.2. Values for clinical diagnosis of diabetes mellitus and other categories of hyperglycemia. (Extracted and modified from Alberti and Zimmet, 1998)

**Fasting is defined as no calorie intake for as least 8 h*

^ This test requires a glucose load containing the equivalent of 75 g anhydrous glucose dissolved in water

I. 3.2 Etiology of Type 1 Diabetes Mellitus

Type 1 diabetes mellitus (T1DM) is the result of β-cell destruction that leads to a severe loss of insulin production and thus an absolute insulin deficiency. It is an autoimmune disease resulting from a diverse interaction of both environmental (viral infections or nutritional factors) and genetic (family history or racial differences) factors (Dorman et al., 2003). It has been suggested that multiple genes are involved in the susceptibility of the disease including the human leukocyte antigen (HLA) genotype and the *Insulin* gene itself. Specific alleles of the major histocompatibility complex (MHC) that encode the class II antigens DQ and DR have been reported as some of the major elements of HLA-linked susceptibility of disease (Strominger, 1986; She, 1996). Particular polymorphisms on HLA-DQ might influence the interactions between class II molecules on antigen-presenting cells with other T-cell components, which in turn affect the specificity of immune response against foreign or self antigens (Sanjeevi et al., 1995). Regarding the *Insulin* gene, a polymorphic locus consisting of a variable number tandem repeat (VNTR) region has been identified adjacent to the coding sequence of the insulin gene. This *Insulin* VNTR has been suggested to genetically regulate *Insulin* expression and thus its polymorphism might also contribute to susceptibility of disease (Kennedy et al., 1995).

T1DM patients usually present acutely with a dramatic history of polydipsia, polyuria

and severe weight loss. Unless insulin is administered, a delay in diagnosis poses risks of severe metabolic disorder including diabetic ketoacidosis, cerebral edema and death. T1DM is often characterized by infiltration of mononuclear cells within the islets. Its onset involves the presence of the immune system cells, primarily CD8+ T-cells, CD4+ T-cells and macrophages in the islets. β-cell death is often a result of the attack by these immune components or other cytokine-mediated apoptosis including tumor necrosis factor (TNF) or interferon (IFN)-γ (Augstein et al., 1998). Interactions of β-cells and immune cells often lead to the induction and amplification of insulitis, causing a progressive and selective β-cell destruction (Elzirik et al., 2009). Figure I. 3.1 illustrates the mechanisms between β-cells and immune cells in T1DM. Since the presence of multiple islet cell autoantibodies can often be detected long before the clinical onset of T1DM (Greenbaum et al., 1999), a series of anti-islet antibodies reacting against insulin, islet cell antigens (ICAs), an islet enzyme glutamic acid decarboxylase (GAD)65 or 67, heat shock protein 65 (hsp 65) or the protein tyrosine phosphatase-related islet antigen 2 (IA-2) could be used for predictive testing of T1DM and for therapeutic intervention (Roep, 1996; Verge et al., 1996). Yet some T1DM patients do not have these autoantibodies but still require insulin supplementation for survival and diabetic ketoacidosis is episodic. These subjects are said to suffer from an idiopathic form of T1DM.

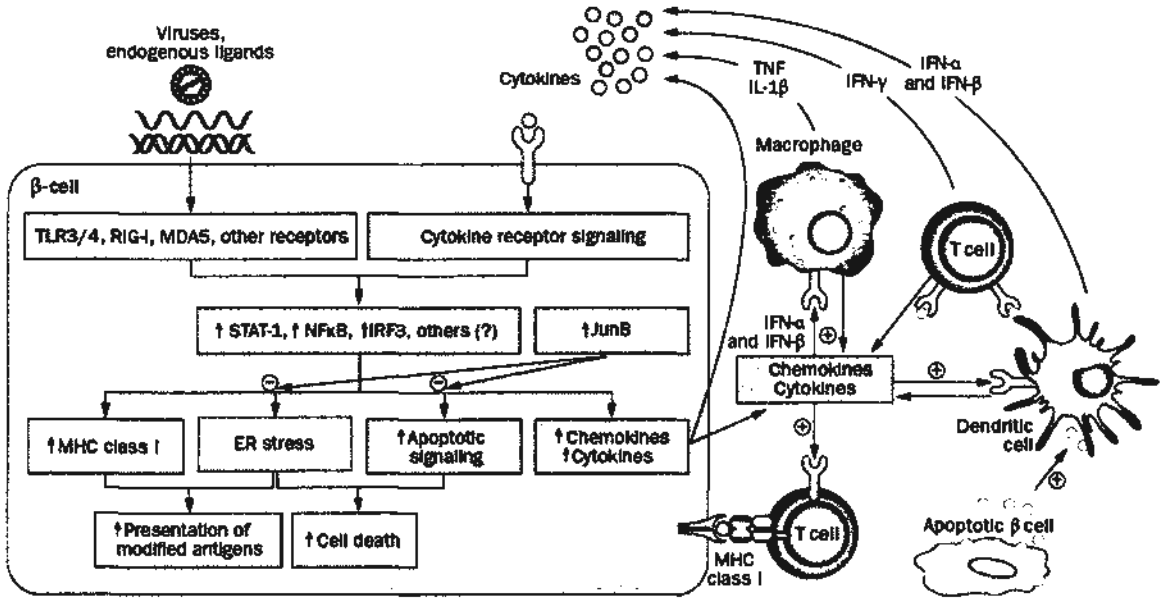


Figure I. 3.1. Interactions of β-cells with the immune cells in T1DM that leads to insulitis. Recognition of endogenous and exogenous ligands results in an activation of key transcription factors including NFκB or STATs. This recruits and activates immune cells, which in turn elevates the pro-inflammatory cytokines IFNs or TNF in β-cells, and causes β-cell death. (Extracted from Eizirik et al., 2009)

I. 3.3 Management of Type 1 Diabetes Mellitus

A direct remedial strategy for insulin insufficiency in T1DM is the administration of exogenous insulin. Short-acting (regular) or modified insulin which is rapid-acting is injected into patients either at multiple daily doses or in the form of continuous subcutaneous infusion. The latter is achieved by an insulin infusion device, which allows a continuous basal insulin delivery supplemented with postprandial elevation in insulin levels. This permits great lifestyle flexibility and facilitates achievement of glycemic control. Insulin dosage should be adjusted according to changes in blood glucose levels, amount of food intake and physical activities like exercises (Kalergis et al., 2000; Franc et al., 2009). Yet it represents a disadvantage to this unphysiological insulin supplementation therapy in terms of the need for frequent monitoring of blood glucose, which often creates an intense psychological impact to patients due to interference of daily activities and pain in injections. Patients might even start to discontinue from prescribed regular insulin supplements (Peyrot et al., 2010). An inappropriate insulin regimen might increase the risk for severe hyperglycaemic or hypoglycaemic episodes. These often associate to an impaired brain adaptation to fluctuated blood glucose level.

Apart from injecting insulin, attempts have been sought for preserving β-cell mass by applying systemic immune suppression to cope with the autoimmune attack in islets of

T1DM patients. Chronic immune suppressants like cyclosporine or prednisone were employed in early immunologic approaches. Yet they are often required with continual treatment which exerts profound side effects with decreased efficacy over time (Silverstein et al., 1988). In this regard, many of the current strategies apply immunomodulation on β-cell autoimmunity through eliminating self-reactivity. Infusion of non-Fc-receptor-binding anti-CD3 monoclonal antibodies, for example, has been reported to preserve insulin secretion for a long period (Herold et al., 2009), however, long term efficacy was not guaranteed due to the varied auto-aggressive T-cell repertoire and adverse events till appear disregarded with increased dosage of antibodies applied.

In order to free the burden of multiple insulin injections or diet adjustment, β-cell replacement by transplantation of pancreas, a readily vascularised organ, or isolated pancreatic islets can provide hope for achieving insulin independence on T1DM patients. Pancreas transplant is sometimes performed simultaneously with kidney transplant in diabetic patients with advanced kidney nephropathy. Yet this invasive and costly therapy often poses high surgical risk and limitations for the recipients. Meanwhile the requirement of strong immunosuppressants after surgery has made this treatment method less commonly applied in the current clinical settings. Transplantation of isolated islets, however, might represent a simpler and less invasive β-cell replacement therapy for such

patients. Recent advances in modifying protocols for islet transplants have made it a more attractive option than other therapeutic means for T1DM treatment.

I. 3.4 Islet Transplantation

A prominent success of islet transplantation has been dated back to 2000 when the Edmonton's team reported a method of islet transplantation on T1DM patients and an average of 1-year euglycemia with insulin independence could be maintained (Shapiro et al., 2000). Though immunosuppression was still required, the success of such trial has been attributed to improved islet isolation techniques, the avoided use of glucocorticoids which might exert toxicity to the islet graft and induce insulin-resistance, a large number of infused islets (averaged 800,000 islet equivalents/patient) as well as better post-transplant oral nutrients supplement and antiviral medications to reduce graft loss.

Several hurdles are to be overcome which include, but not limited to, promoting functional performance of the transplanted islet graft, enhancing graft survival by preventing graft rejection by the host immune system but at the same time minimizing excessive use of immunosuppressants, and most importantly the highly limited availability of human islets from cadaveric donors. Recent advances in biomedical research have brought solutions into these issues. Various pre-transplantation biological manipulations of the islets have been suggested to optimize protocols for feasible islet grafting. Gene delivery of the vascular endothelial growth factor (VEGF) or hepatocyte growth factor (HGF), for example, in isolated human islets can promote revascularization and graft

function in transplanted diabetic animal models (Lopez-Talavera et al., 2004; Narang et al., 2004); results from glucose tolerance tests also revealed better performance in ameliorating hyperglycemia in animals transplanted with VEGF-transfected islets (Chae et al., 2005).

Site of islet transplantation is a major issue that critically affect the graft survival and function. Unlike other whole organ transplantations, islets are not transplanted homotopically into the highly sensitive pancreas, but instead are transplanted into other heterotopic graft sites. Clinical islet transplantation is normally performed through infusion of the isolated islets via the hepatic portal vein into the liver. Yet studies using transplanted mouse models have revealed profound functional impairment in islet grafts retrieved from the liver. These retrieved islets were found to have markedly depleted expression of several β-cell phenotypic factors as well as low insulin release in response to a glucose challenge. These observations have drawn the attention to the notion of implantation site-dependent functional performance of the transplanted islet grafts (Mattsson et al., 2004; Lau et al., 2007). Since the loss of islet graft function might be partly attributable to the necessity for multiple islet donors to cure each T1DM patient, attentions have been given to locate an optimal anatomical site for islet transplantation (van der Windt et al., 2008). New islet transplantation sites, such as the renal capsule,

spleen, or the omental pouch, have been reported in animal studies (Merani et al., 2008). The route of each transplantation method offers particular benefits. In the clinical setting, subcutaneous transplantation maximizes patient safety.

While human donor islets are highly limited, recent advances have also suggested utilization of islets from xenogenic donors (Cozzi and Bosio, 2008; Schuurman and Pierson, 2008). Porcine islets have received substantial attention, particularly since they respond to glucose challenge in the same physiological ranges as do human islets (Dufrane and Gianello, 2008). Porcine islets may also perform better than islets from other species, such as the rat, in terms of achieving hyperglycemia reversal (Davalli et al., 1995).

Minimization of acute rejection of grafted tissues is probably the most critical issue in achieving successful clinical transplantation. This issue is being addressed by studies of immuno-isolation of transplanted islets by microencapsulation. Figure I. 3.2 illustrates an encapsulated human islet. Encapsulation of islets with biomaterials can provide a physical barrier while allowing the passage of glucose and insulin and, at the same time, preventing the entry of large molecules like antibodies (Teramura et al., 2007; Campos-Lisbôa et al., 2008). This strategy is designed to greatly maintain islet graft survival in the host, thus facilitating xenogenic-based therapeutic options. Typical protocols make use of the alginate-based materials for microencapsulation, titrated against a cation solution (usually

calcium or barium) for gelification and beads formation. Biocompatibility of the encapsulation materials is the major concern for success, and in this context, alginate is a suitable choice for not interfering with the celluar functions of the transplanted islets (Fritschy et al., 1991). Recent findings have even suggested that immunosupression might not be necessary to maintain long-term normoglycemia in diabetic animals given xenogenic islet transplantations (Meyer et al., 2008). These data provide preliminary support to the possibility that immunosuppressive drugs may be minimized after islet transplantation in patients with diabetes.

While provided with the impetus for continued research in improving islet transplantation, a promising solution is to seek alternative source for generating these physiologically transplantable islets to cope with the rising needs for diabetic patients. In this context, a stem cell-based approach might represent exciting potentials to overcome such limitation. Strategies for achieving an unlimited propagation of a putative pancreatic stem cell and the possibility of devising a complete differentiation protocol for generating glucose-responsive insulin-secreting cells warrant much investigation.

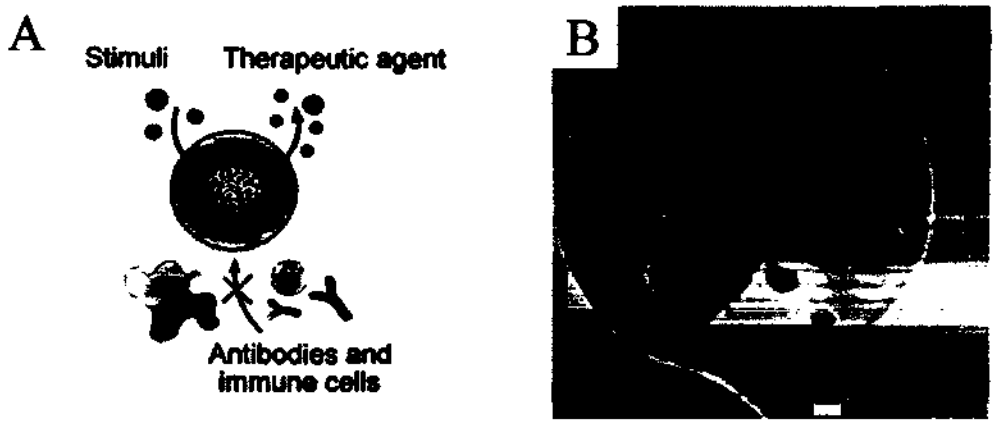


Figure I. 3.2. Encapsulation of isolated human islets. (*A*) Schematic presentation of an encapsulated islet showing the free diffusion of nutrients, oxygen and stimuli across the membrane whereas immune components are excluded. (Extracted from Orive et al., 2003) (*B*) The microscopic view of an encapsulated human islet. Scale bar: 150 μm. (Extracted from Barnett et al., 2007)

I. 4 Stem Cells

I. 4.1 Definition of Stem Cells

A remarkable discovery in stem cell biology has revolutionized the medical interventions, making it possible to translate the notion of regenerative medicine into clinical settings. Stem cells are defined by their self-renewal ability through mitotic cell division and their differentiation capacity into lineage-committed cells upon stimulation by suitable biochemical or biophysical cues. Though a lot of ethical and political issues arise from recruiting stem cells into scientific research and clinical application, the stem cell topic has still captured a profound public attention due to their ability to regenerate damaged tissues/organs and a possibility to cure life-threatening diseases. Stem cells can be categorized into several levels according to their different potencies to differentiate (Blau et al., 2001; Müller et al., 2008). They are the totiopotent stem cells which refer to the fertilized egg that can give rise to all tissue types including the extraembryonic tissues; the pluripotent stem cells include the inner cell mass of the blastocyst of an early embryo that has the potential to differentiate into any cells of the three germ layers: ectoderm, mesoderm and endoderm; multipotent stem cells or better known as progenitor cells that refer to those capable of differentiating into limited cell lineages; oligopotent progenitor cells which only possess the potential to differentiate into few cell types, as well as the

unipotent precursor cells that can exclusively differentiate into one cell type. Traditional belief on stem cells is their identity as a relatively homogenous population of similar stem cells in which maturation results in their irreversible loss of differentiation capacity. Yet modern concepts of stem cells emphasize their functional identity as engaged by multiple cell types and thus their ability to differentiate or trans-differentiate by means of de-differentiation and re-differentiation into a variety of target cell types (Blau et al., 2001). Figure I. 4.1 illustrates these concepts of defining stem cells. Depending on the differentiation capacity of the isolated stem/progenitor cells, they can be reprogrammed and developed into various target cell types under suitable stimulations. Figure I. 4.2 summarizes the various pathways that direct differentiation of these cells.

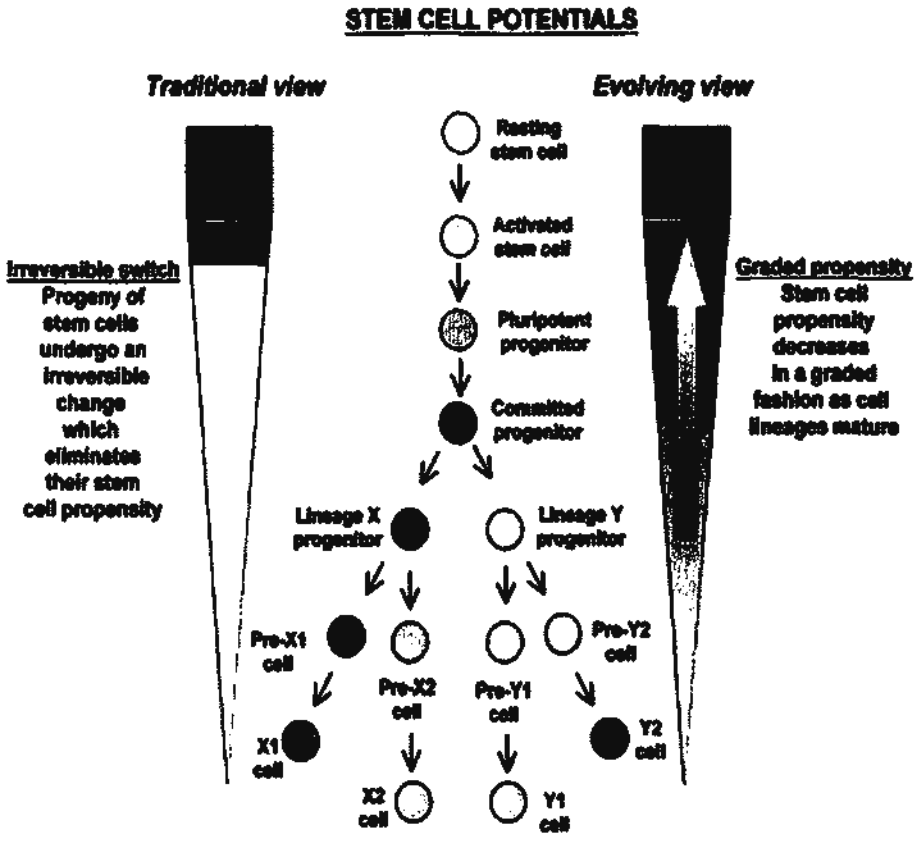


Figure I. 4.1. The comparisons of two conceptual views of stem cells. The stem cell potential is indicated by the colour intensity in the lateral triangular diagrams. Traditional view regards differentiation of stem cells as irreversible while evolving concepts emphasize the graded propensity as stem cells differentiate and maturate. (Extracted from Blau et al., 2001)

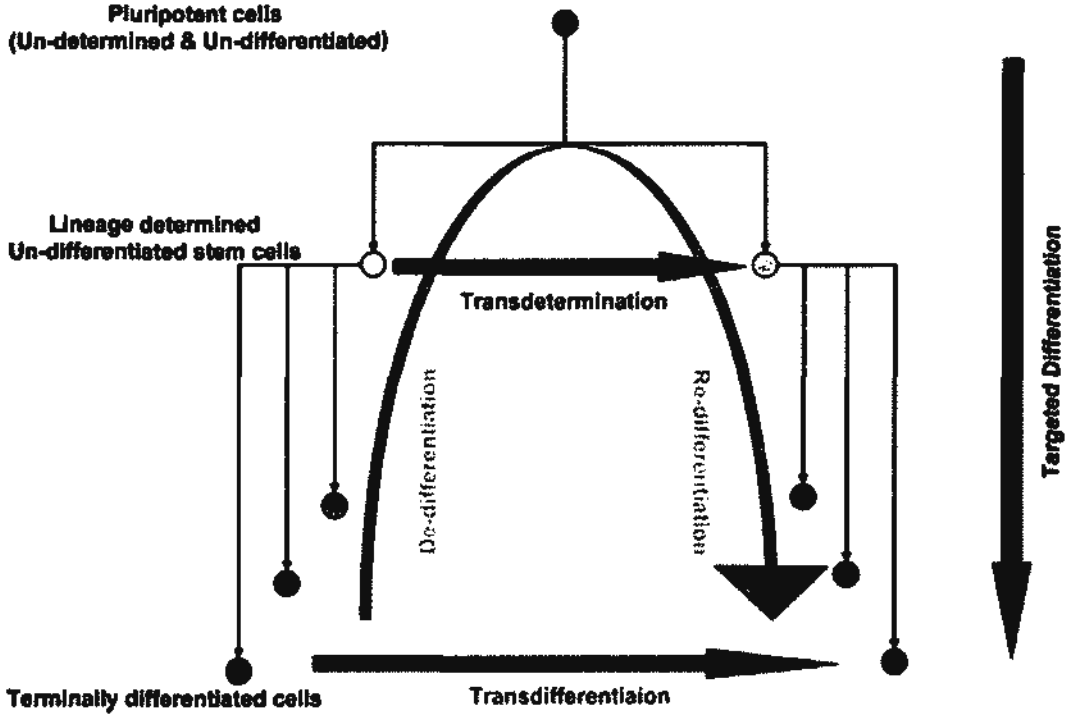


Figure I. 4.2. Various pathways of reprogramming cell development by directed differentiation. Lineage determination by targeted differentiation of pluripotent ESCs or iPSCs is represented by the green arrow; Lineage switching between the lineage committed progenitor cells by transdetermination is represented by the horizontal blue arrow; Lineage switching between terminally differentiated cells can occur either via dedifferentiation and redifferentiation with an intervening progenitor state (orange arrow) or via direct transdifferentiation (red arrow). (Extracted from Yechoor and Chan, in press)

I. 4.2 Embryonic Stem Cells

Embryonic stem cells (ESCs) are commonly derived from the undifferentiated inner cell mass of either a mouse or human blastocyst (termed as mouse ESCs or human ESCs). Profound attention has been given to the ESC application due to their pluripotency into developing into any definite cell types. Conventional maintenance of an ESC culture requires a feeder layer derived from mouse embryonic fibroblasts (MEF) to maintain its undifferentiated state. This technical limitation often hinders their large-scale propagation for broader application as well as generates a potential risk for cross-contamination with the pathogenic factors from the feeder layer derived from animal origin. More recent studies have formulated methods for feeder-free growth of undifferentiated mouse or human ESCs (William et al., 1988; Xu et al., 2001; Yamane et al., 2005). Generation for a chemically-define medium for maintaining ESC fate provide a platform for a clearer phenotypic and genetic characterization of the cells (Yao et al., 2006). In this regard, a thorough gene profiling in ESCs has been described (Ramalho-Santos, et al., 2002; Zhou et al., 2007). Of note, *Octamer-4* (*Oct 4*), a commonly used marker for undifferentiated cell, is one of the most important regulator in maintaining self-renewal in ESCs. Yet the fact that ESC culture undergoes senescence and spontaneous differentiation creates obstacles for their extended use (Heng et al., 2006). Upon more information can be

garnered regarding the complex network that regulates the cell fate of ESC, a better manipulation of ESCs in translating into regenerative therapy can be achieved. Issues on their risk of development into teratoma due to uncontrolled growth, the avoidance of immune rejection and the associated ethical controversies should also be addressed.

I. 4.3 Mesenchymal Stem Cells

Mesenchymal stem cells (MSCs) refer to the multipotent stem cells that can be differentiated to a multiple cells types. They possess a morphological feature with a small cell body that bears several long and thin cell processes. Discovery of MSCs has to be dated back to the late nineteenth century when the detection of a stromal precursor population giving rise to mesodermal cells was noted in bone marrow. Their ability to adhere to cell culture plates has given ease to distinguish them from the residual marrow cell components and hence a straightforward isolation method (Prockop, 1997). Advances in MSC-related studies revealed their isolation from other tissue types including umbilical cord blood, adipose tissues, placental tissues or liver (Zuk et al., 2001; Götherström et al., 2003; Romanov et al., 2003; Battula et al., 2008). Till now no unique MSC markers have been characterized, but immunophenotyping of a MSC population generally reveals a set of CD marker expression profile which helps to identify them. Table I. 4.1 summarizes some common phenotype tests for identifying MSCs. Though MSCs derived from different tissue sources might differ in the pattern of CD marker expression, a typical MSC population should possess the ability to differentiate into at least three cell types, namely the adipocytes, osteocytes and chondrocytes (Ruiz and Chen, 2008).

Information for a potential translation of MSCs into clinical therapy has been

garnered from some recent studies that transplanted MSCs were able to exert therapeutic effects in injured model (Amado et al., 2005), as well as their ability to secrete specific soluble factors to exert an immunomodulatory effect on co-transplanted cells by suppressing the host immune attack (Li et al., in press). Upon identification of such favourable factors secreted by MSCs, a combination therapy coupled with MSC transplantation might represent an exciting approach that is potentially able to conquer complex diseases.

Phenotype test	Known MSC phenotype		Methods
Colony formation	Formation of fibroblastic colonies after isolation		Colony forming units-fibroblastic (CFU-F) assay
Immunophenotype	CD marker-positive	CD 29; CD 44; CD 71; CD 73; CD 90; CD 105; CD 106; CD 120a; CD 166; Stro-1; ICAM-1 and MHC I	Fluorescent-activated cell sorting (FACS)
	CD marker-negative	CD 11; CD 14; CD 18; CD 31; CD 34; CD 40; CD 45; CD 56; CD 80; CD 86 and MHC II	
In vitro multipotency	Ability to differentiate into adipocytes, osteocytes and chondrocytes		Differentiation via defined culture media

Table I. 4.1. Some common phenotype tests for MSCs. (Extracted and modified from Parekkadan and Milwid, in press)

I. 4.4 Comparisons between Fetal and Adult Stem Cells

Stem cells derived from the tissue itself might represent a better source over the ESCs in terms of their multipotency that leads to a more controlled development into target cell lineages. Of note, fetal tissues should be enriched of tissue progenitors. Isolation of fetal stem cells has been reported from a variety of fetal tissues in addition to fetal blood and bone marrow. They include the fetal kidney, lung, liver and the pancreas. They might exhibit advantages over their adult counterparts. This is attributed to their lower immunogenicity and their more primitive nature which allows a greater multi-lineage capacity. The paucity of molecular markers designating a specific stem cell type has also contributed variability and inconsistence in distinguishing stem cell niches from adult tissues (O'Donoghue and Fisk, 2004).

The potential for translating fetal MSCs into transplantation therapy has been suggested because of their minimal activation of lymphocyte proliferation (Götherström et al., 2004). This provides supplemental findings to an earlier report suggesting a lower expression of MHC molecules in fetal hematopoietic stem cells (HSCs) relative to their adult counterparts (Rebel et al., 1996). Of note, fetal MSCs were recognized with an absence of human leukocyte antigen (HLA) class II antigens. In addition to this immune privilege of fetal MSCs, their efficiency in propagation in culture was found to be much

higher than the adult MSCs (Campagnoli et al., 2002). The diminished self-renewal ability in older HSCs, likely attributed to their lower telomerase activities, as well as their weaker homing capacity after transplantation together support again the privilege of applying fetal stem cells over those derived from adult tissues (Verfaillie et al., 2002). The ethical issues, however, regarding the collection of aborted fetal tissues and the related religious controversies should be well addressed.

I. 4.5 Induced Pluripotent Stem Cells

Knowledge in stem cell biology has been nourished by a breakthrough discovery of induced pluripotent stem cells (iPSCs). It was first discovered by Yamanaka's team in 2006 when they successfully demonstrated the iPSCs generation from mouse embryonic and adult fibroblasts (Takahashi and Yamanaka, 2006). These iPSCs were derived by retrovirus transfection with four key pluripotency genes, namely *Oct 4*, *sex determining region Y (SRY)-box (Sox) 2*, *c-Myc* and *krüppel-like factor (Klf) 4* (Figure I. 4.3). One year later the same research team has repeated this reprogramming experiment on human adult fibroblast by transfecting the same defined transcription factors. The resulting iPSCs could be differentiated into three germ layers, behaving exactly like ESC development (Takahashi et al., 2007). One of the four transcription factors, *c-Myc*, is a protooncogene that can lead to tumor growth, and thus was eliminated in more recent iPSC protocols. Deletion of such factor was also reported to get higher differentiation efficiency into particular target tissue cell type (Martinez-Fernandez et al., 2010). Noteworthy, the essence of this iPSC technology is the generation of patient-specific iPSCs which provides an immunologically matched cell population for huge therapeutic potentials. These cells should contain the patients' genotype responsible for the diseases which might help in remodeling their initiation and progression profiles (Maehr et al., 2009).

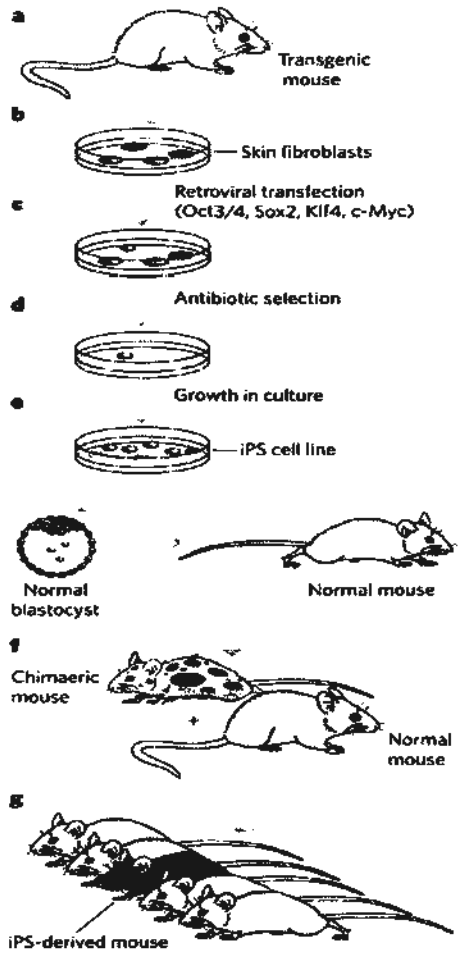


Figure I. 4.3. Generation of iPSCs from adult skin fibroblasts. (*A*) Mice that carried a drug-selectable marker were generated. Antibiotics resistance was linked to expression of pluripotency markers like *Oct 4*. (*B*) Skin fibroblasts were then isolated from these genetically modified mice and (*C*) transfected with four transcription factors: *Oct 4*, *Sox 2*, *Klf 4* and *c-Myc*. (*D*) The transfected cells were subjected to appropriate drug selection. (*E*) Colonies that resembled ESCs were isolated and expanded into stable iPSC line. When injected into blastocysts of normal mice, iPSCs could contribute to all body cell types of including the germ line. (*F*) When the chimeric animals resulting from these blastocysts were crossed with normal mice, this led to (*G*) the birth of live offspring carrying the genetic content of an iPSC. (Extracted from Rossant, 2007)

I. 5 Pancreatic Progenitor Cells

I. 5.1 Sources of Pancreatic Progenitor Cells

Tremendous effort has been made in the search for a reliable source of pancreatic progenitor cells (PPCs) that would be profoundly expandable in laboratory manipulations and possess a high differentiation potentiality into functional and transplantable islets. Potential sources for such hormone-producing cells include ESCs, adult and fetal pancreas-derived stem cells, MSCs, and those trans-differentiated from different adult tissues like the liver, spleen and bone marrow (Figure I. 5.1). None of them is currently considered to be a perfect source for clinical use. Yet it is appropriate to pursue multiple possibilities as each cell type may have its pros and cons and each will thus contribute to research progress.

Among the various pancreatic progenitors, the potential of pluripotent ESCs that are able to differentiate into pancreatic cells is being studied extensively. There have been a number of reported differentiation protocols instructing the generation of hormone-producing cells from ESCs (Shim et al., 2004; León-Quinto et al., 2004; D'Amour et al., 2006; Jiang et al., 2007). These protocols are generally designed according to the instructive signals that direct pancreatic organogenesis and β-cell differentiation during embryonic development. Figure I. 5.2 illustrates the comparison of pancreas organogenesis and the stepwise ESC differentiation process. Formation of the definitive

endoderm lineage is the critical first step in achieving the derivation of pancreatic epithelium. The ESCs can then be sequentially developed through stages of foregut endoderm, pancreatic endocrine/exocrine precursors, and finally hormone-secreting endocrine/exocrine cells (Baetge, 2008). A profile of transcription factor expression at these different stages of development has been proposed, which help to monitor the differentiation process (Figure I. 5.3). The functional performance of the differentiated cells depends on the protocol employed.

Fetal pancreas represents another promising source of tissue for *de novo* development of islets. Though progenitors are abundant in the fetal pancreas (Huang and Tang, 2003; Suen et al., 2008), unresolved issues related to accurate isolation of these cells remain. Basic science researchers need to search for improved markers designating PPC subpopulations within the fetal pancreas. Details will be discussed in section I. 5.2, pp. 69-72. Studies attempting to characterize the pancreas-derived PPCs have revealed a similar phenotypic expression of markers in MSCs (Gallo et al., 2007). Adult human pancreas also contains pancreatic MSCs, though their origin is unclear (Davani et al., 2007). These MSCs, after transplantation into diabetic animals, can adopt a β-cell lineage within the pancreatic microenvironment (Chang et al., 2009).

Generation of β-cells from the trans-differentiation of other adult cells provides an

intriguing alternative approach (Tateishi et al., 2008). With suitable genetic engineering, cells can regain differentiation plasticity, a process known as de-differentiation and re-differentiation. This notion has been demonstrated to be feasible in adult liver cells, for example, which undergo a stable transfection with *Pdx-1*. These *Pdx-1* gene-transfected cells can follow the pancreatic lineage and develop into insulin-secreting cells *in vitro* (Ber et al., 2003; Zalzman et al., 2005). Nevertheless, the issue of stem cell plasticity has yet to be investigated and in particular, a possible presence of artefacts and other false positive factors in cell culture should be noted. Some resolution of this issue is coming from *in vivo* experiments showing the existence of *in vivo* reprogramming of pancreatic exocrine cells into β-cells (Zhou et al., 2008). The trans-differentiation of acinar cells into β-cells has also been reported in culture (Minami and Seino, 2008). The capacity of adult cells to be convertible into pluripotent stem cells, as recently described as iPSCs, provides additional support for the possibility of cell reprogramming (Stadtfeld et al., 2008). This iPSC technology also represents another source for PPCs by reprogramming of somatic cells into pancreatic lineages (Maehr et al., 2009).

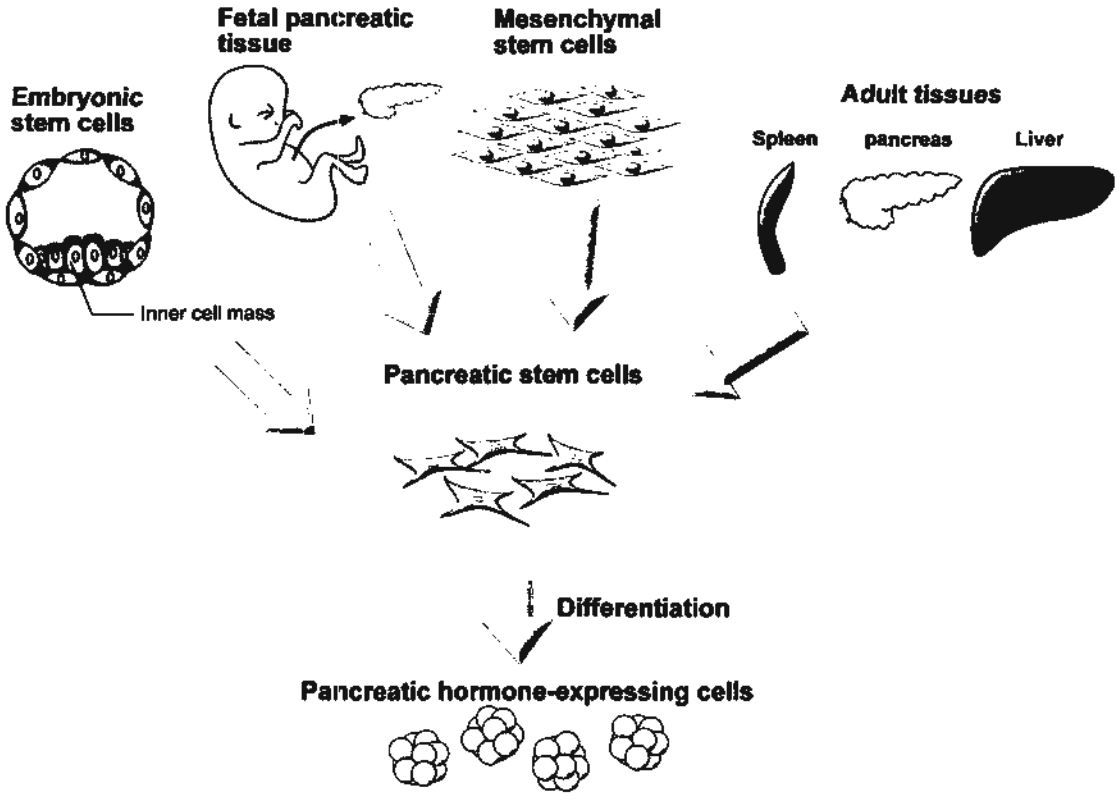


Figure I. 5.1. Sources of PPCs. Formulation of a full differentiation protocol for the development of PPCs into functional endocrine cells should be critical. (Extracted from Leung, in press)

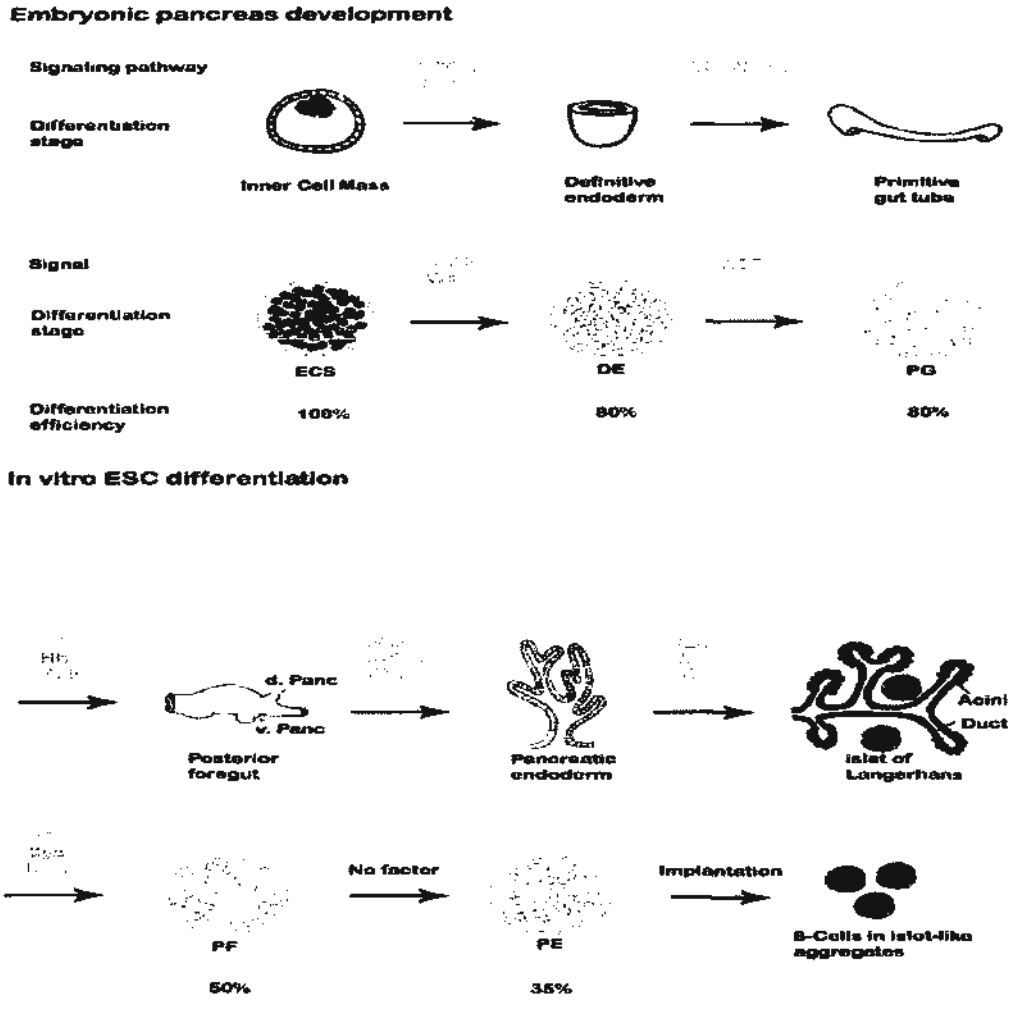


Figure I. 5.2. Comparison of mouse pancreas organogenesis and the stepwise differentiation processes of hESCs into pancreatic β-cells. (Extracted from Guo and Hebrok, 2009)

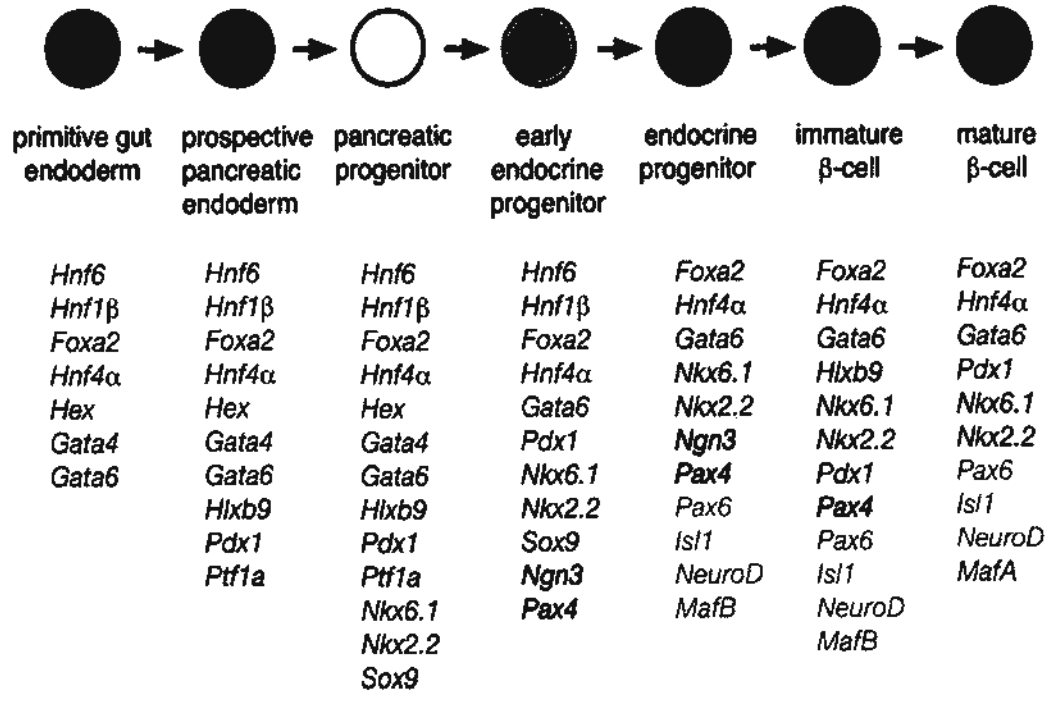


Figure I. 5.3. Repertoire of transcription factor expression at each stage of differentiation from endodermal derivatives to β-cells. Expression of factors at each stage are colour coded as follow: purple – gut endoderm; blue – pancreatic endoderm progenitor; black – early endocrine progenitor; orange – endocrine progenitor; red – β-cells. (Extracted from Oliver-Krasinski and Stoffers, 2008)

I. 5.2 Isolation of PPCs from the Pancreas

Efforts have been put on isolating organ-specific progenitor cells at least partly due to their lineage committed identity. Figure I. 5.4 illustrates the different strategies for obtaining β-cells from the pancreas. The fetal pancreas, which is enriched with undifferentiated mesenchymal precursors, can be a promising source for PPCs. Yet the problem rests on designating specific and reliable molecular markers for identifying such PPCs. This topic remains controversial, with the fact that many different cell types share a common lineage of markers. Nestin, an intermediate filament protein that denotes neuroepitheial precursors, was once believed to identify also the PPC population in the developing pancreas. Earlier reports have shown that isolated nestin-positive islet-derived progenitors could be differentiated to bearing phenotypic markers as a pancreatic islet (Huang and Tang, 2003; Ueno et al., 2005). Yet given the existence of the enteric nervous system that innervates the pancreas, it would not be reasonable for such neural stem cell marker, nestin, alone to identify β-cell progenitors unless they are assumed to be derived exclusively from neural precursors. This idea was further rejected when studies showed that selection of fetal pancreatic cells based purely on nestin expression could not generate fully functional β-cells after differentiation *in vitro* or *in vivo* (Humphrey, et al, 2003).

Attention has been given to a transcription factor, Ngn3, which has become a

promising marker for islet progenitors. Lineage tracing experiments have proven that Ngn3-postive progenitors could give rise to all four types of endocrine cells within an islet. These Ngn3-positve cells are unipotent progenitors, which means that each of them develops into a single type of endocrine cell (Desgraz and Herrera, 2009). Other pancreatic cells like the duct cells would be developed from another progenitor population without Ngn3 expression (Gu et al., 2002). Of note, Ngn3 also marked the residing endogenous progenitors in adult pancreas. They could be activated during pancreatic injuries and contributed to the neogenesis of β-cells (Xu et al, 2008). This progenitor population might possibly reside in the pancreatic ductal epithelium (Bonner-Weir et al, 2004). Recent studies suggested also another reliable cell surface marker, CD 133 (prominin-1), to identify the PPC population. CD 133 is a transmembrane glycoprotein that was originally identified in neuroepithelial and haematopoietic precursors (Shmelkov et al, 2005). Until recently, it has been found to be expressed in the multipotent PPCs near the pancreatic ductal region (Oshima, et al, 2007). Protocols for isolating PPCs from pancreatic tissues based on these markers have been reported (Sugiyama et al., 2007, Hori et al., 2008, Koblas et al., 2008).

Identification of such PPC markers can help devising protocols to isolate and characterize these PPC populations from the fetal pancreas. Different methodologies have

been suggested to isolate and expand them. Our laboratory has previously reported an isolation protocol for such PPCs from early trimester human fetal pancreas. Upon harvesting the fetal islet aggregates from digesting the pancreas, addition of two growth factors, the basic fibroblast growth factor (bFGF) and the epidermal growth factor (EGF), could initiate an outgrowth of a monolayer of PPC population from the aggregates (Suen, et al, 2008). The use of these growth factors is critical to maintain their propagation in culture and keep their undifferentiated state. Their use has also been reported elsewhere in similar protocols (Huang and Tang, 2003; Zhang et al, 2005b). Though a certain degree of residual hormone-producing cells might contribute to the PPC population after digestion of the human fetal pancreas, these cells have recently been proven with their capacity to proliferate *in vitro* and to generate mesenchymal populations. These mesenchymal cells could represent a precursor population to be differentiated along the endocrine lineage (Joglekar et al, 2009). This property should be different to that observed in mouse islet β-cells where the latter exhibits minimal proliferation and transition *in vitro* (Atouf et al, 2007).

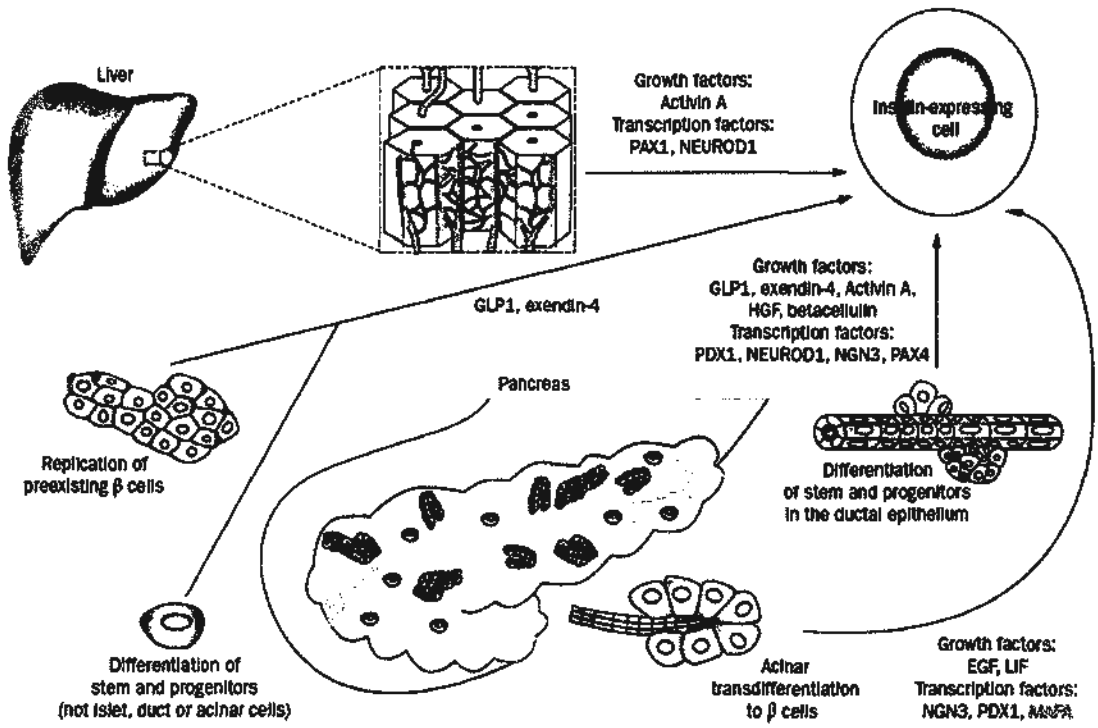


Figure I. 5.4. Different strategies for obtaining insulin-expressing β-cells from the pancreas or liver. Neogenesis of β-cells can be obtained either via replication of pre-existing β cells, transdifferentiation of acinar tissues, differentiation of progenitors within the ductal epithelium or differentiation of the putative PPCs. The liver represents another source of embryologically-related tissue that can be trans-differentiated into β-cells. (Extracted from Aguayo-Mazzucato and Bonner-Weir, 2010)

I. 5.3 Characterization of the Identity of Fetal PPCs

Previous reports have indeed suggested that cells derived from human pancreatic islets possessed a similar expression profile of MSC phenotypic markers, in which they were amenable to differentiate into adipocytes, osteocytes and chondrocytes (Gallo et al, 2007). The PPC population derived from early trimester human fetal pancreas also appeared to be positive for those typical MSC markers including CD44, CD90 and CD 13 (Hu et al, 2003). It is the reason why the islet-derived precursors are also termed as pancreatic MSCs, or otherwise, the mesenchymal stromal cells (Davani et al, 2007). Meanwhile, characterization of the human fetus-derived PPCs revealed an expression of vimentin, a mensenchymal cell marker (Suen et al, 2008; Joglekar et al, 2009). It could not be ruled out the possibility that this vimentin expression reflected the mesenchymal components originally resided within the fetal pancreas. They might also represent the occurrence of an epithelial-to-mesenchymal transition (EMT) from the residual epithelial cells as proposed earlier. This EMT theory postulates that epithelial cells can phenotypically be de-differentiated into highly proliferative mesenchymal cell population, followed by a re-differentiation into β-like cells (Moss and Rhodes, 2007). Yet this theory still remains controversial (Chase et al, 2007; Morton et al, 2007).

A further characterization that our laboratory has performed previously on human

fetal PPCs is the co-expression of nestin with other stem cell markers: c-kit and ATP-binding cassette sub-family G member 2 (ABCG2). Expressions of the respective endocrine and ductal cell markers, insulin and CK 19, were minimally detected in such PPC population (Suen et al., 2008). These observations consolidated the stem cell nature of the cells.

I. 5.4 *In vitro* Differentiation of PPCs by Novel Morphogenic Factors

To devise a complete differentiation protocol for PPCs, it basically rests on the provision of accurate morphogenic signals that direct their sequential development towards into endocrine lineages. A raft of extrinsic factors including hormones, cytokines, growth factors, nutrients, extracellular matrix components or even pharmacological agents that regulate specific cellular signalling pathways have been suggested to help nourishing their differentiation (Soria, 2001; Roche et al., 2006). Some commonly employed growth factors include hepatocyte growth factor (HGF), glucagon-like peptide 1 (GLP-1) or its analogue exendin-4, betacellulin and nicotinamide were all together recruited in our previously reported differentiation cocktail to differentiate the human fetal PPCs into insulin-secreting islet-like clusters (ICCs) (Suen et al., 2008). The essence to formulate different differentiation protocols is to generate functionally mature and glucose responsive ICCs which are suitable for islet transplantation for patients with diabetes. It is in this context that several novel morphogenic factors including GLP-1/exendin 4 (Suen et al., 2006), PDZ-domain-containing 2 (PDZD2) (Suen et al., 2008), as well as vitamins A and D (Ng et al., in press) have been previously studied on their potential regulation on PPC development and ICC differentiation/ maturation. Their demonstrated effects might often depend on the different combinations of selected growth factors and their optimal

concentrations as well as their duration of exposure. PDZD2 which our laboratory has previously proven to inhibit PPC differentiation at a particular concentration (Suen et al., 2008), could exert opposite effect at another concentration (For detail: see Chapter III). Clues for selecting possible morphogenic candidates for regulating PPC development might be garnered by some previously discovered mechanisms that regulate the development of other stem cells/ tissue progenitors, or even from some known regulatory systems that function locally in the pancreas. The renin-angiotensin system, for example, is one of such categories that has been identified in the pancreas, and at the same time suggested to regulate the growth and differentiation of several types of stem cells and organogenesis, notably the kidney. It warrants exploration of its potential regulation in PPC development. (For detail: see Chapter IV-VI). It is thus critical to decipher the underlying mechanisms played by the factors of interest so that their phenotypic alterations on PPCs/ICCs as well as their transplantable functions can be thoroughly depicted.

I. 6 Aims of Study

In light of the aforementioned background on pancreas development and its relevance to stem cell biology, we therefore hypothesize that the human fetal PPCs are amenable to proliferate and differentiate under stimulation by novel morphogenic factors at appropriate conditions. This approach will provide extensive, as yet unavailable information, on the potential of these functional ICCs for clinical islet transplantation.

In order to address these issues, we have set sequential and original objectives for the current study:

- To study the potential concentration-dependent effects of PDZD2 on PPC differentiation and functional maturation of the ICCs;
- To characterize the expression profile of RAS in the PPC/ICC culture;
- To evaluate the effects and underlying mechanisms of angiotensin II, a physiologically active peptide of the RAS, on the growth and differentiation of PPCs/ICCs and their potential for islet transplantation
- To further explore the effects of manipulating RAS components during mouse pregnancy on the fetal endocrine cell development

Chapter II

General Materials and Methods

II. 1 Culture of Human Fetal Pancreatic Progenitor Cells

II. 1.1 Human Fetal Tissue Procurement

Human fetal pancreata were provided by the Department of Obstetrics and Gynecology, Prince of Wales Hospital, The Chinese University of Hong Kong (CUHK). Tissue was obtained by dilation and extraction after termination of pregnancy between the 9^{th} and 15^{th} weeks of gestation. Maternal consent and ethical approval from the Clinical Research Ethics Committee (CREC-2005.461) were obtained prior to the procedures.

II. 1.2 Tissue Processing and Culture of Human Fetal Pancreatic Progenitor Cells

Isolation of pancreatic progenitor cells (PPCs) was performed as previously reported (Suen et al., 2008; Leung et al., 2009; Ng et al., in press) (Figure II. 1.1). Different experiments were done using preparations derived from a single independent fetal pancreas. Briefly, each fetal pancreas was minced and digested with 3 mg/ml collagenase P (Roche, Mannheim, Germany). Digested cell clusters were resuspended in modified RPMI 1640 media supplemented with 10% fetal bovine serum (FBS), 1 mM sodium pyruvate, 1% penicillin and streptomycin, 10 mM HEPES buffer (Invitrogen, Carlsbad, CA, USA) and 71.5 μM β-mercaptoethanol (Sigma Aldrich, St. Louis, MO, USA) in 60 mm non-treated suspension culture dishes (Corning Incorporated, NY, USA), incubated at

37°C with 5% CO_2/95% humidified atmosphere. Rounded, non-adherent cell clusters were formed within 48-h incubation. PPC outgrowth from the cell clusters was induced by further addition with 20 ng/ml each of basic fibroblast growth factor (bFGF) (Sigma-Aldrich), and epidermal growth factor (EGF) (Invitrogen) in the medium. A monolayer of PPCs migrated out from the cell clusters within 3-4 d, and the cells were harvested with 0.05% trypsin-EDTA (Invitrogen) and replated upon reaching confluence.

II. 1.3 *In vitro* Differentiation of PPCs into Insulin-secreting Islet-like Cell Clusters

Differentiation of PPCs into islet-like cell clusters (ICCs) were performed as previously reported (Suen et al., 2008; Leung et al., 2009) (Figure II. 1.1). Only PPCs derived from fetal pancreata from between the 9^{th} and 13^{th} weeks of gestation and in passages 3 to 8 were used. Briefly, 4 confluent T-75 tissue culture flasks of PPCs were required to derive ICCs on 3 non-treated suspension culture dishes (Corning Incorporated). PPC differentiation was initiated in a serum-free DMEM/F12 media containing 1x B-27, 0.05% bovine serum albumin (BSA) (Invitrogen), and a cocktail of growth factors: 10 ng/ml human hepatocyte-derived growth factor (HGF) (R&D Systems, Minneapolis, MN, USA) 10 nM Exendin-4, 500 ρM betacellulin and 10 mM nicotinamide (Sigma-Aldrich). Differentiation lasted for 7 days and then ICCs were handpicked by micro-pipettes under

an inverted microscope (Leica Microsystems, Wetzlar, Germany) for use in different experiments. The media was replaced at least every other day.

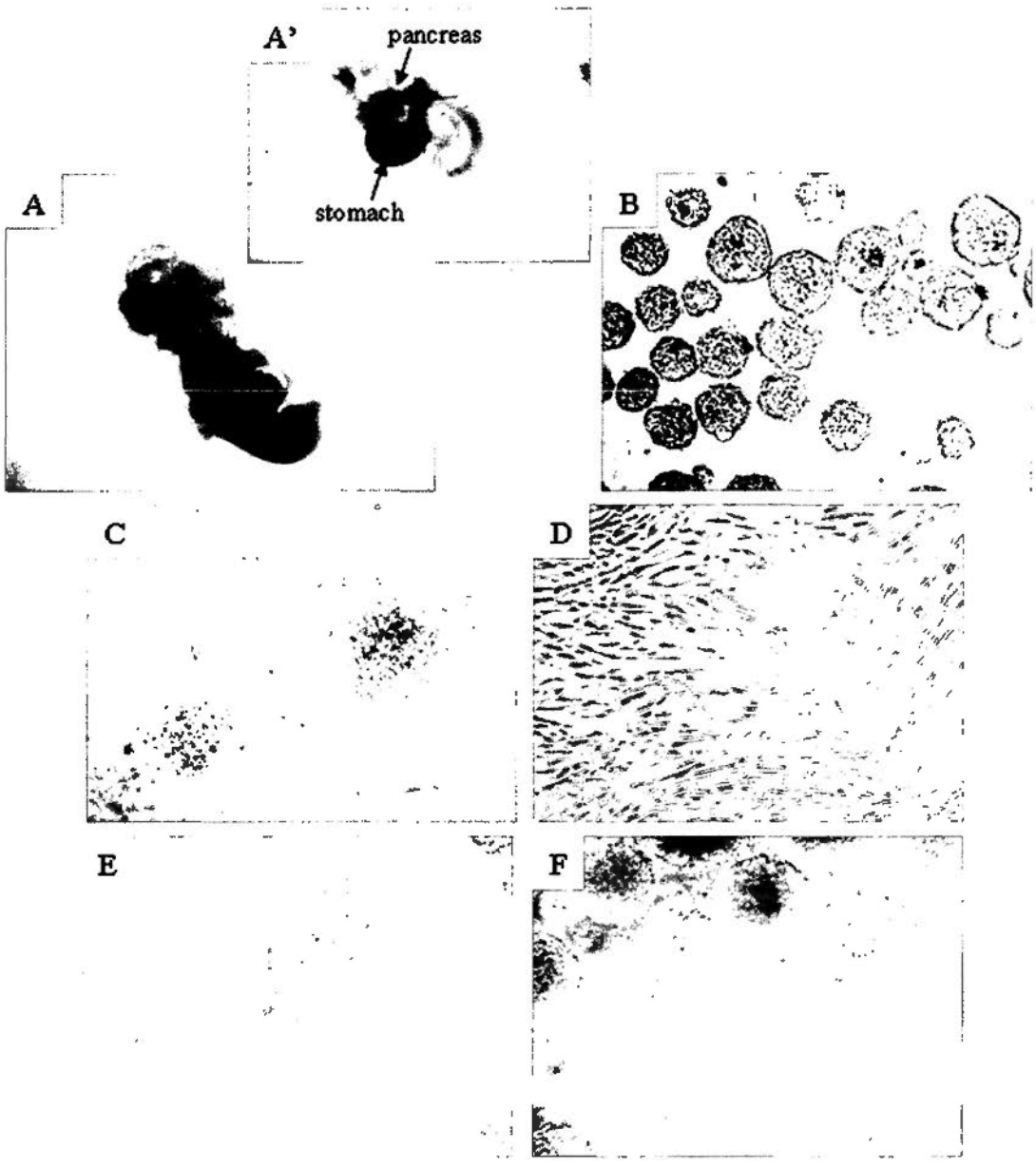


Figure II. 1.1. Representative microscopic images showing the PPC/ICC culture. (*A*) A human fetus collected from STOP, (insert *A'*) showing the dissected pancreas tissue. (*B*) Digestion of fetal pancreas harvests islet aggregates. (*C*) Addition of bFGF and EGF initiate outgrowth of cells from these aggregates, and (*D*) a monolayer of cells termed PPCs are formed. (*E*) Upon differentiation is initiated in serum-free condition supplemented with a cocktail of morphogenic factors, PPCs start to aggregate themselves within the first 24 h, and (*F*) differentiated ICCs are formed.

II. 2 Experimental Mouse Models

II. 2.1 An Immune-privileged Mouse Model for Human ICC Transplantation

BALB/c nude mice were supplied by the Laboratory Animal Services Centre of the Chinese University of Hong Kong (CUHK). The animals were housed under pathogen-free conditions in specifically designed air-controlled rooms with a 12-h light/dark cycle and fed with food and sterile water *ad libitum*. The care and use of animals were in compliance with the institutional guidelines, and the experimental procedures were approved by the Animal Experimentation Ethics Committee of the CUHK (Ref. 08/032/MIS).

II. 2.2 Induction of Diabetes

Young adult (8-10 wk-old) male nude mice were used for induction of diabetes. Freshly prepared streptozotocin (STZ; Sigma-Aldrich, St. Louis, MO, USA), dissolved in chilled 0.05 M citrate buffer (pH 4.5), was intraperitoneally injected at multiple doses of 75 mg/kg body weight for 5 consecutive days (Kroon et al., 2008). Only STZ-treated mice that reached blood glucose levels >16.7 mM (~300 mg/dl) for at least 7 consecutive days were used in the transplantation studies.

II. 2.3 Time-pregnant Mouse Model and Harvest of Mouse Embryos

Time-pregnant ICR mice were supplied by the Laboratory Animal Services Centre of the CUHK. The animals were housed with a 12-h light/dark cycle and fed with food and tap water *ad libitum*. The care and use of animals were in compliance with the institutional guidelines, and the experimental procedures were approved by the Animal Experimentation Ethics Committee of the CUHK (Ref. 08/032/MIS). ICR mouse embryos were harvested at different embryonic days (e15.5 - e17.5; embryonic stages are counted from the day that vaginal plugs are evident and recorded as e0.5). Dissection of embryos and neonatal mice was performed in ice-cold phosphate buffered saline (PBS, pH7.4; Invitrogen) under a dissection microscope (Leica Microsystems). Microscopic images depicting the dissection of mouse embryos and neonate pancreas are shown in Figure II. 2.1.

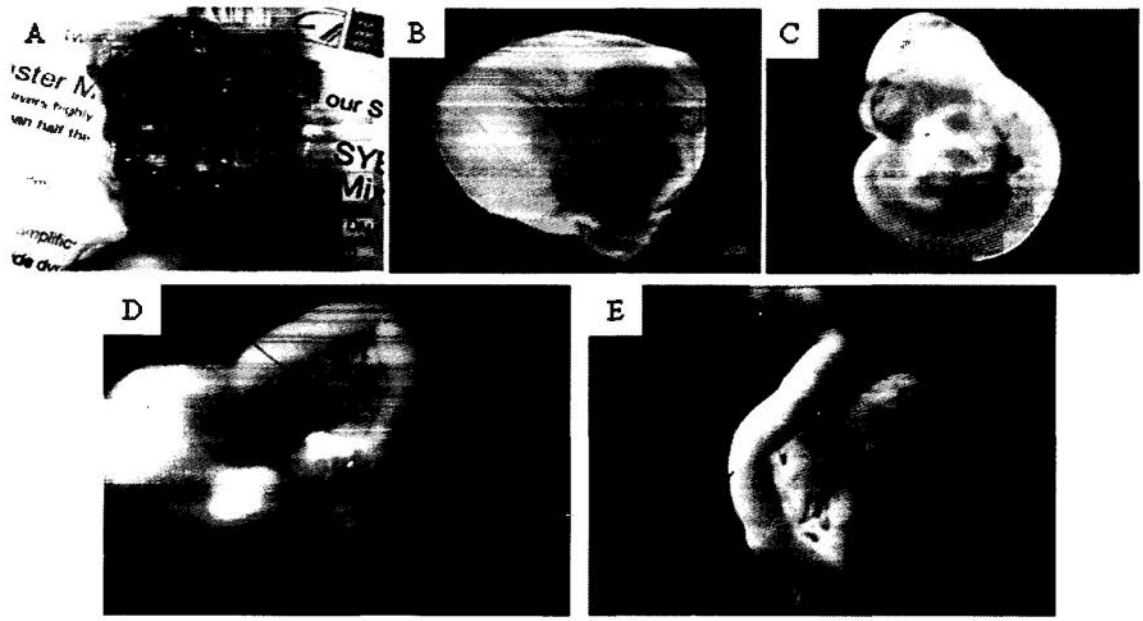


Figure II. 2.1. Representative images showing the procedures for harvesting mouse embryos and dissection of neonatal pancreas. (*A*) The embryo-containing uterus was exposed. (*B*) Dissection of e13.5 embryo under microscope showing the yolk sac and (*C*) after removal of the extraembryonic membranes. (*D and E*) Microscopic images showing the dissection of neonate mouse pancreas with its associated digestive organs.

II. 3 Molecular Studies

II. 3.1 Immunocytochemical/ Immunohistochemical staining

Immunocytohemistry of PPCs and ICCs, as well as immunohistochemistry of pancreata, kidneys and mouse embryos were performed according to our previously reported protocols with minor modifications (Suen et al., 2006; Suen et al., 2008; Cheng et al., 2008; Leung et al., 2009). Briefly PPC monolayers grown on coverslips or handpicked ICCs were fixed with 4% (wt/vol) paraformaldehyde (Sigma-Aldrich) in phosphate-buffered saline (PBS; Invitrogen). Fixed pancreata, kidneys or embryos were first dehydrated in sucrose gradients (10%, 20% and 30% sucrose in PBS, each step overnight). Fixed ICCs and tissues were then embedded in Tissue-Tek OCT compound (Optimal Cutting Temperature compound; Sakura Finetek, Torrance, CA, USA), followed with cryosectioning at 5 μm by a Leica CM1100 Benchtop Cryostat (Leica Microsystems). For immunoperoxidase labeling, air-dried sections were first quenched with 3% (vol/vol) hydrogen peroxide in PBS for 10 min to remove endogenous peroxidase activity, and then subjected to three washes in PBS. Sections were permeabilized with 0.1% Tritox-X in PBS for 10 min, and blocked with 4% (vol/vol) normal donkey serum (Sigma) and 2% (vol/vol) bovine serum albumin (BSA; Invitrogen) at room temperature for 1 h. Slides were incubated overnight at 4°C with appropriate primary antibodies (Brief details of all

antibodies used for immunostaining are listed in Table II. 3.1). After three washes in PBS supplemented with 0.1% Tween-20 (Thermo Scientific, Fremont, CA, USA), bound primary antibodies were detected using fluorophore-labeled or horseradish peroxidase-conjugated secondary antibodies (Table II. 3.1). For immunofluorescent staining, 4'6'-Diamidino-2-phenylindole (DAPI; 1:1000; Invitrogen) was applied together with the secondary antibodies. Immunoperoxidase staining was developed using a diaminobenzidine chromogen kit (Dako Denmark A/S, Glostrup, Denmark) according to the manual. These slides were further counterstained with hematoxylin for 1 min, dehydrated and mounted with Vectashield® mounting medium (Vector Laboratories Incorporation, Burlingame, CA, USA). In all cases, omission of primary antibodies was used as a negative control for detection of non-specific antibody binding or endogenous peroxidase activity. Imaging was done using a fluorescent/light microscope equipped with a DC 200 digital camera (Leica Microsystems).

Antibody	Species	Stock concentration	Dilution	Source
Primary antibodies				
Polyclonal anti-$Ca_v1.2$	Rabbit	0.78 mg/ml	1:100	Chemicon International Inc.
Polyclonal anti-AT_1 receptor	Rabbit	200 µg/ml	1:50	Santa Cruz Biotechnology
Polyclonal anti-AT_2 receptor	Rabbit	2.6 mg/ml	1:400	Abcam
Monoclonal anti-Ngn3	Mouse	500 µg/ml	1:100	Abcam
Polyclonal anti-Insulin	Guinea pig	1.5 mg/ml	1:100	Zymed Laboratories
Monoclonal anti-Glucagon	Mouse	11.8 mg/ml	1:1000	Abcam
Polyclonal anti-Glut-2	Rabbit	200 µg/ml	1:50	Santa Cruz Biotechnology
Polyclonal anti-Pdx-1	Rabbit	N/A	1:200	Millipore
Monoclonal anti-Ang II	Mouse	1 mg/ml	1:50	GenWay Biotech, Inc.
Monoclonal anti-Nkx 6.1 (F65A2)	Mouse	N/A	1:20	Developmental Studies Hybridoma Bank
Monoclonal anti-Proinsulin (GS-9A8)	Mouse	N/A	1:50	Developmental Studies Hybridoma Bank
Secondary antibodies (fluorophore-labeled)				
Alexa Fluor 488 anti-mouse IgG	Goat	2 mg/ml	1:400	Invitrogen
Alexa Fluor 350 anti-rabbit IgG	Goat	2 mg/ml	1:400	Invitrogen
Alexa Fluor 488 anti-guinea pig IgG	Goat	2 mg/ml	1:400	Invitrogen
Secondary antibodies (HRP-labeled)				
Anti-rabbit IgG	Donkey	N/A	1:200	Amersham Biosciences
Polyclonal anti-guinea pig IgG	Rabbit	2 mg/ml	1:250	Abcam

Table II. 3.1. Details of the primary and secondary antibodies used for immunofluorecent staining/ immunohistochemistry.

II. 3.2 Protein Extraction and Quantification

Total proteins were extracted from cell lysates of PPCs or groups of ~300 ICCs using CytoBuster® Protein Extraction reagent (Novagen, Darmstadt, Germany). The mixtures were incubated for 10-15 min at room temperature, followed by a centrifugation at 16,000 g for 15 min at 4 °C. The supernatant was collected for protein quantification and stored at -80 °C before use. Protein content of lysates was quantified by a Bio-Rad protein assay kit (Bio-Rad Laboratories Incorporation, Munich, Germany) according to the manufacturer's protocol. Briefly, the diluted protein samples were incubated with the assay reagent for 8 min at room temperature and their absorbance was then measured at wavelength 595 nm with a spectrophotometer (Version 1.3, KCjunior, Bio-tek Instrument Inc., VT, USA). A relative quantification of protein concentrations was obtained by an interpolation with the 0.1 - 0.5 μg/μl BSA standard curve.

II. 3.3 Western Blot Analysis

Western blot procedures were performed as previously reported with minor modifications (Suen et al., 2008; Leung et al., 2009; Ng et al., in press). Briefly, total protein (20 μg/lane) was applied for electrophoresis on a 12% (wt/vol) SDS-polyacrylamide gels for 1.5 h at 110 V and the resultant bands were electrotransferred to polyvinylidene fluoride transfer

membranes (GE Osmonics Labstore, Minnetonka, MN, USA) by a semi-dry transfer system (Bio-Rad Laboratories Incorporation) for 1 h at 17V. The blotted protein was blocked with 5% (wt/vol) nonfat dry milk in PBS (Invitrogen) and 0.1% (vol/vol) Tween-20 for 1 h at room temperature. The membranes were then sequentially incubated in suitable primary antibodies overnight at 4°C (Brief details of all antibodies used for Western blot analysis are listed in Table II. 3.2), followed by an incubation with appropriate horseradish peroxidase (HRP)-labeled secondary antibodies for 1 h at room temperature (Table II. 3.2). To verify equal loading of samples, the membranes were subsequently incubated with mouse monoclonal anti-β-actin serum (1:8000 dilution) (Abcam, Cambridge, UK), followed by the HRP-labeled sheep anti-mouse IgG antibody (1:2500 dilution) (Amersham Biosciences). All antibodies were diluted in 1% BSA (wt/vol) supplemented with 0.5% (vol/vol) Tween-20 (Sigma-Aldrich). Positive bands were visualized using enhanced chemiluminescent (ECL) plus western blot detection reagents and autoradiography film (Amersham Biosciences, Buckinghamshire, UK). The chemiluminescent intensity of the protein bands was quantified using an image analyzer (Molecular Dynamic Imaging Quant, Sunnyvale, CA, USA).

Antibody	Species	Stock concentration	Dilution	Source
Primary antibodies				
Polyclonal anti-PDZD2	Rabbit	N/A	1:8000	Gift from Prof. Yao, K.M.
Polyclonal anti-AT_1 receptor	Rabbit	200 μg/ml	1:1600	Santa Cruz Biotechnology
Polyclonal anti-AT_2 receptor	Rabbit	2.6 mg/ml	1:1000	Abcam
Monoclonal anti-phospho AKT	Mouse	100 μg/ml	1:2800	Millipore
Monoclonal anti-AKT	Mouse	1 μg/μl	1:1000	Millipore
Monoclonal anti-phospho ERK 1/2	Rabbit	0.36 μg/μl	1:2500	Millipore
Monoclonal anti-ERK	Rabbit	0.36 μg/μl	1:400	Millipore
Monoclonal anti-β-Actin	Mouse	3.10 mg/ml	1:8000	Abcam
Secondary antibodies (HRP-labeled)				
Polyclonal anti-rabbit IgG	Donkey	N/A	1:2600	Amersham Biosciences
Polyclonal anti-mouse IgG	Sheep	N/A	1:2500	Amersham Biosciences

Table II. 3.2. Details of the primary and secondary antibodies used for Western blotting experiments.

II. 3.4 RNA Isolation and Expression Analysis

Total RNA of cultured cells or homogenized human fetal tissues was extracted using the TRIzol® reagent (Invitrogen) according to the manufacturer's instructions with minor modifications. Briefly, 1 ml of TRIzol® regent was added to each cell or homogenized tissue sample, followed by a vigorous votex. Samples were incubated for 10 min at room temperature to allow complete dissociation of nucleoprotein complexes. 200 µl of chloroform was added to each sample, followed by another vigorous vortex for 15 s. The mixture was then incubated for 5 min at room temperature, followed by a centrifugation at 12,000 g for 15 min at 4°C. The RNA-containing upper aqueous layer was collected and 1 ml of isopropanol was added to each sample, incubated overnight at -80°C for RNA precipitation. The samples were then centrifuged at 12,000 g for 20 min at 4°C. The supernatant was discarded and the RNA pellet was washed with 1 ml of ice-cold 75% (vol/vol) ethanol, followed by a centrifugation at 7,500 g for 20 min at 4°C. The supernatant was discarded and the RNA pellet was air dried at room temperature. The pellet was then reconstituted in UltraPure™ DNAse/RNAse-free deionized water (Invitrogen). Quantification of the RNA samples was performed using a UV-spectrophotometer and calculated from the absorbance value (Optical density: O.D.) at wavelength 260 nm × 40 × dilution factors. The purity of RNA was assessed by

comparing the ratio of $O.D._{260}$ to $O.D._{280}$. The RNA obtained was considered pure if the values fell within 1.6 to 2.0 as done previously (Suen et al., 2008; Leung et al., 2009; Ng et al., in press).

II. 3.5 Reverse Transcription

First strand cDNA was reverse-transcribed using SuperScript™ III Reverse Transcriptase (Invitrogen) according to the manufacturer's protocol. Briefly, 2-3 μg total RNA was added with 1 μl each of 0.5 μg/μl olido$(dT)_{12-18}$ and 10 mM dNTP mix to a reaction volume of 13 μl. This reaction mix was denatured for 5 min at 65°C, followed by a chill on ice for 1 min. 4 μl 5× first-strand buffer and 1 μl each of 0.1 M DTT, RNaseOUT™ and 200 units/μl SuperScript™ III Reverse Transcriptase were added to the reaction mix and it was then incubated for 60 min at 50°C. The reaction was inactivated by heating for 15 min at 70°C and the first-strand cNDA obtained could be recruited directly for the subsequent polymerase chain reaction for specific gene analyses.

II. 3.6 Design of Primers for Polymerase Chain Reaction (PCR) and Real-time PCR

PCR primers for different genes were designed from human DNA sequences using an online programme Primer 3 (version 0.4.0) (Rozen and Skaletsky, 2000). Procedures were

done according to the description by the software. Briefly, primers for each gene target were selected containing minimal internal structures including hairpins and possibility for primer-dimer formations. The primers were designed with an optimal size of 20 base pair (b.p.) ± 3 b.p. of each complimentary primer), an optimal melting temperature of 60°C ± 3°C and an optimal GC content of 50% ± 20%). The suggested primers which generated a product size ranging from 50 – 200 b.p. were picked for real-time PCR. Each primer was tested by conventional PCR (procedures described in section II. 3.7) using positive cDNA templates, and only those which generated a single band with a correct product size would be recruited for subsequent experiments. A cDNA dilution curve with at least 10 serial 2-fold dilutions was obtained from appropriate real-time PCR experiments (procedures described in section II. 3.8) and the PCR efficiency for each primer was evaluated.

II. 3.7 Polymerase Chain Reaction (PCR)

PCR was performed in a 25 µl reaction volume with 5 µl each of diluted cDNA template, 1X PCR buffer, 1.5 mM $MgCl_2$, 0.2 mM dNTP mix, 0.3 µM each of forward and backward primers (Details of the sequences of all primers used for PCR are summarized in Table II. 3.3), and 0.5 U *Taq* DNA polymerase (Invitrogen). The reaction was initiated at 94°C for 3 min for denaturation, followed by 30-35 cycle repeats of 94°C, 30 s; 59-62°C,

30s for primer annealing depending on genes of interest; 72°C, 30 s for extension. The reaction was further incubated at 72°C for 30 s for a final extension. The amplified PCR products were then analyzed on a 2% (wt/vol) agarose gel (Cambrex Corporation, Charles City, IA, USA) in 1× Tris-acetate-EDTA buffer pre-stained with ethidium bromide. Separation of DNA was performed by gel electrophoresis for 15 min at 100 V and the DNA bands were visualized using a camera equipped with an UV illuminator (FluroChem 8000 Advanced Fluorescence, Alpha Innotech Corporation, CA, USA).

II. 3.8 Quantitative Real-time PCR

Real-time PCR analyses were performed using an i-Cycler Thermal Cycler (version 3.1) (Bio-Rad Laboratories Incorporation) as previously reported (Suen et al., 2008; Leung et al., 2009; Ng et al., in press). SYBR green that preferentially binds to double-stranded DNA causing fluorescent emission was employed in the current study. Briefly, reactions were performed in triplicate in 25 μl reactions with 5 μl of diluted cDNA templates, 1× SYBR Green PCR master mix (Bio-Rad Laboratories Incorporation) and 0.3 μM each primer (Invitrogen) with the following parameters: 40 cycles of 94°C for 15 s, 59-62°C for 1 min depending on genes of interest, and 72°C for 30 s (Details of the sequences of all primers used for real-time PCR are summarized in Table II. 3.3). Melt-curve data through

temperature gradients (+ 0.5°C for every 10 s) was analyzed at the end of each real-time PCR experiment to guarantee the specificity of each amplified target PCR product. Amplification data was collected using an i-Cycler Detector and then analyzed using the Sequence Detection System software. Transcript levels relative to those of β-actin were calculated using the $2^{-\Delta\Delta CT}$ statistical method as described in section II. 3.9.

II. 3.9 The Comparative Cycle Threshold (C_T) Method for Calculation.

The fold changes of the target mRNA expressions were assessed using the $2^{-\Delta\Delta CT}$ statistical method as previously described (Lau et al., 2004; Suen et al., 2008). C_T values were recorded based on the PCR cycle numbers during which fluorescent emission increases beyond a threshold level, and therefore, such C_T values are correlated to the input target mRNA levels, and in other words, a larger quantity of input mRNA contributes to a lower C_T value because of less PCR cycles required for fluorescent signal to reach the threshold. The ΔC_T values were calculated by the subtraction of the C_T value of *β-ACTIN* from each target C_T. The $\Delta\Delta C_T$ values were then calculated by the subtraction of the ΔC_T values of control samples from the ΔC_T values of treated samples. Expression level of each target gene relative to control ΔC_T values were calculated using the $2^{-\Delta\Delta CT}$ equation.

Human Gene	Forward primer	Reverse primer	Annealing temperature (°C)	Product size (b.p.)
ISL-1	GATCAAATGCGCCAAGTGCAG	CAGCGGAAACACTCGATGTGA	62	93
NGN3	TGTGGGTGCTAAGGGTAAGG	GGGAGAAGCAGAAGGAACAA	60	99
NEURO D	TCCAAAATCGAGACTCTGCGC	GCAAAGCGTCTGAACGAAGGA	62	108
NKX 2.2	TCTCCTTGGAGTGGCAGATTC	AAACACGGCGTAGAGTTCAGC	59	90
NKX 6.1	GACGGGAAGAGAAAACACACG	ACTCTCTGTCATCCCCAACGA	59	138
PDX-1	ACTCCACCTTGGGACCTGTTT	TTAAGGTACTCGGCCCAGCTT	62	117
INSULIN	CAGCCTTTGTGAACCAACACC	GGTCTTGGGTGTGTAGAAGAAGC	59	95
GLUT-2	AGCTTTGCAGTTGGTGGAAT	CCCATCAAGAGAGCTCCAAC	59	122
GLUCOKINASE	CTCCATGGGGAAGTGCTC	CACCGAAAAACTGAGGGAAG	60	129
AT_1 RECEPTOR	ACTTTGCCACTATGGGCTGT	GAAACTGACGCTGGCTGAAG	60	93
AT_2 RECEPTOR	ACAGGATAACCCGTGACCAA	AGCATCCAGGAAGGTCAGAA	60	101
RENIN	GAAGAGGCTGTTTGATTATGTCG	CCAGTGTGCACAGCTTTTTACTA	60	149
ANGIOTENSINOGEN	GAGTGACATCCAGGACAACTTCT	ATAAGATCCTTGCAGCACCAGT	60	205
ACE	AGCCCTCTCAGTGTCTACGC	CTCCTTGGTGATGCTTCCAT	60	187
ACE2	TTGTTGGGACTCTGCCATTT	GAGATGCGGGGTCACAGTAT	60	171
(PRO)RENIN RECEPTOR*	AGATGACATGTACAGTCTTTATGGTGG	TGCTGGGTTCTTCGCTTGT	60	127
β-ACTIN	TGTCCACCTTCCAGCAGATGT	CGGACTCGTCATACTCCTGCTT	62	51
β-ACTIN (for PCR)	TGGCACCACACCTTCTACAATGAGC	GCACAGCTTCTCCTTAATGTCACGC	60	396

Table II. 3.3. Sequence of specific PCR/ real-time PCR primers and expected product size of different target genes.

* The primer sequences of *(PRO)RENIN RECEPTOR* was extracted from Kaneshiro Y et al., 2006.

II. 4 Islet Isolation and the *in vitro* Functional Assessments of Islets/ICCs

II. 4.1 Isolation and Culture of Islets from Neonatal Mice

Islet isolation from mouse pancreata was performed according to previously reported protocols with minor modifications (Chu et al., 2006; Suen et al., 2006; Cheng et al., 2008). Briefly, time-pregnant ICR mice were sacrificed by cervical dislocation. Dissected pancreata from 5-6 neonatal mice within the same treatment group were pooled together for islet isolation. They were finely minced and digested in 3 mg/ml collagenase P (Roche Molecular Biochemicals, Mannheim, Germany) in 5 ml Hanks' balanced salt solution (HBSS; Sigma Chemical, Poole, Dorset, UK) for 5 min at 37°C with vigorous shaking. The digested tissues were washed in ice-cold HBSS to terminate the digestion before centrifugation for 5 min at 1600× rpm twice. The supernatant was removed and the pellet was resuspened in HBSS. The islets were handpicked under microscope and were resuspended in RPMI 1640 culture medium supplemented with 10% fetal bovine serum, 1% penicillin and streptomycin, 1 mM sodium pyruvate and 10 mM HEPES buffer (Invitrogen). The isolated islets were cultured for 2-3 days before subsequent functional assessments. Fresh medium was changed every day.

II. 4.2 C-peptide Extraction from ICCs and Analyses

Assay of C-peptide content was included for detection of true insulin biosynthesis in ICCs. Extraction of C-peptide from ICCs was performed as recently reported (Suen et al., 2008; Leung et al., 2009). Briefly, ~300 ICCs from each experimental group were handpicked and sonicated twice at 25 W for 10 s in acid-ethanol (0.18 mmol HCl in 95% [vol/vol] ethanol). C-peptide was extracted overnight in the solution at 4°C. The samples were then centrifuged for 2 min at 10,000 g and the supernatants were stored at -80°C. Concentration of the C-peptide content was assessed using a human ultrasensitive C-peptide ELISA kit (Mercodia, Uppsala, Sweden).

II. 4.3 Glucose/KCl-Stimulated Insulin Secretion

Procedures for the measurement of glucose- and KCl-stimulated insulin secretions were described previously (Lau et al., 2004; Chu et al., 2006; Leung et al., 2009). Procedures are schematically shown in Figure II. 4.1. Approximately 400-500 ICCs or 20 neonatal mouse islets were collected for each group of experiments, which were done in triplicate. The ICCs/islets were first washed thoroughly three times with PBS and then transferred to Pyrex® Spot Plates (Fisher Scientific, Pittsburgh, PA, USA) containing 200 µl Krebs-Ringer bicarbonate buffer (KRBB; 120 mM NaCl, 4.7 mM KCl, 1.2 mM KH_2PO_4, 1.8 mM $CaCl_2$ and 25 mM $NaHCO_3$; pH 7.4) per well supplemented with 10 mM HEPES

and 0.1% (vol/vol) BSA. The ICCs/islets were incubated in 5% CO_2/95% humidified air at 37°C for 1 h with an addition of 2.5 mM glucose to the buffer to determine the basal level of insulin release. The buffer was then collected and replaced with fresh KRBB buffer containing 25.5 mM glucose or 20 mM KCl and the ICCs/islets were then incubated for an additional hour to determine the stimulated level of insulin secretion. The buffer was collected after the two incubation periods for measurement of insulin release using a human insulin ELISA kit (Mercodia).

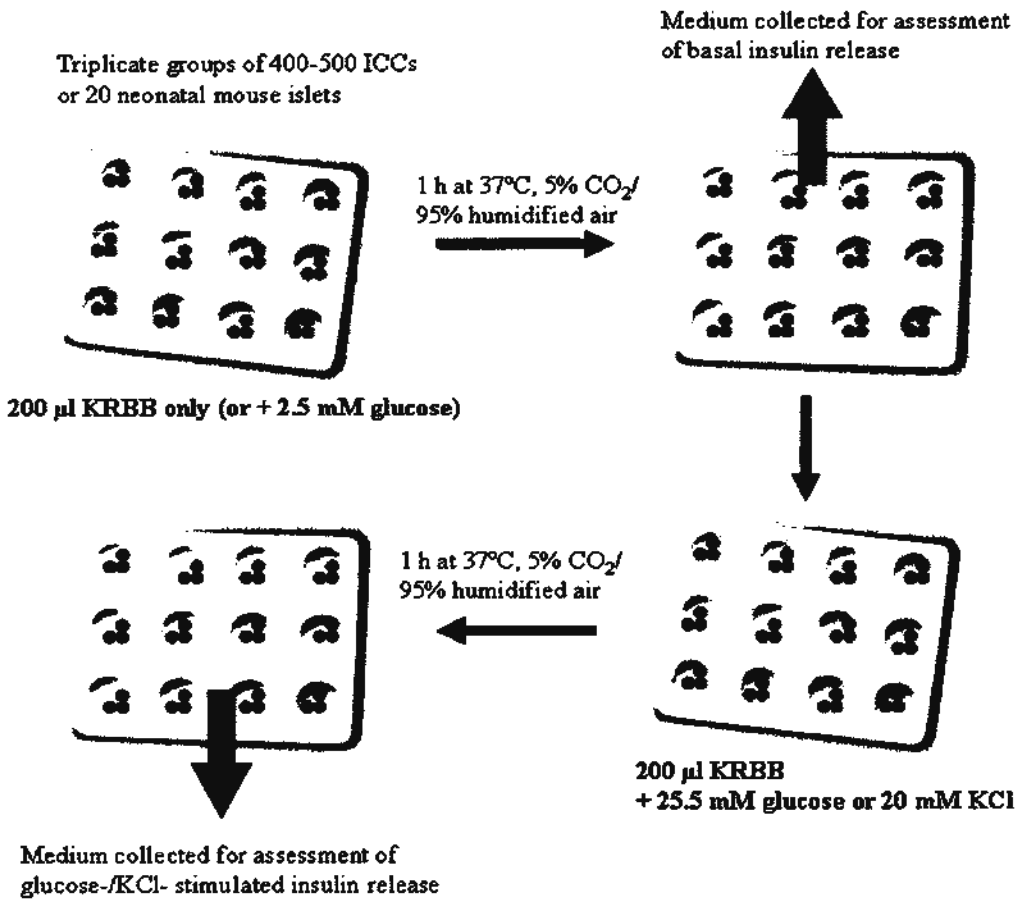


Figure II. 4.1. Procedures for assessment of glucose-/ KCl-stimulated insulin release of ICCs or neonatal mouse islets.

II. 5 ICC Transplantation and the *in vivo* Functional Assessment

II. 5.1 Procedures of ICC Transplantation

A previously described islet transplantation procedure has been employed (Lau et al., 2004; Suen et al., 2006) (Figure II. 5.1). Briefly, mice were anaesthetized with an intraperitoneal injection of 1% (vol/vol) ketamine supplemented with 0.1% (vol/vol) xylazine. A small incision was made on the left renal capsule. Approximately 1500 ICCs were handpicked, washed once in 0.9% (wt/vol) saline and packed using a positive displacement pipette. The tip of the pipette was introduced between the renal parenchyma and capsule followed by a gentle ejection of the ICCs. The ICC graft-bearing kidneys were harvested 10 d after transplantation in some mice for immunohistochemical analyses.

II. 5.2 Assessment of Blood Glucose and Glucose-tolerance Test

Body weight and blood glucose level were monitored once a week in the transplanted mice. Glucose tolerance tests were performed on the sham and transplanted mice after a 5-h fasting period; 1.5 g/kg body weight glucose dissolved in saline was intraperitoneally injected and blood was withdrawn from tail snip at t = 0, 15, 30, 60 and 120 min after glucose administration. Blood glucose level was assessed using a handheld glucometer (Bayer Corporation, Emeryville, CA, USA).

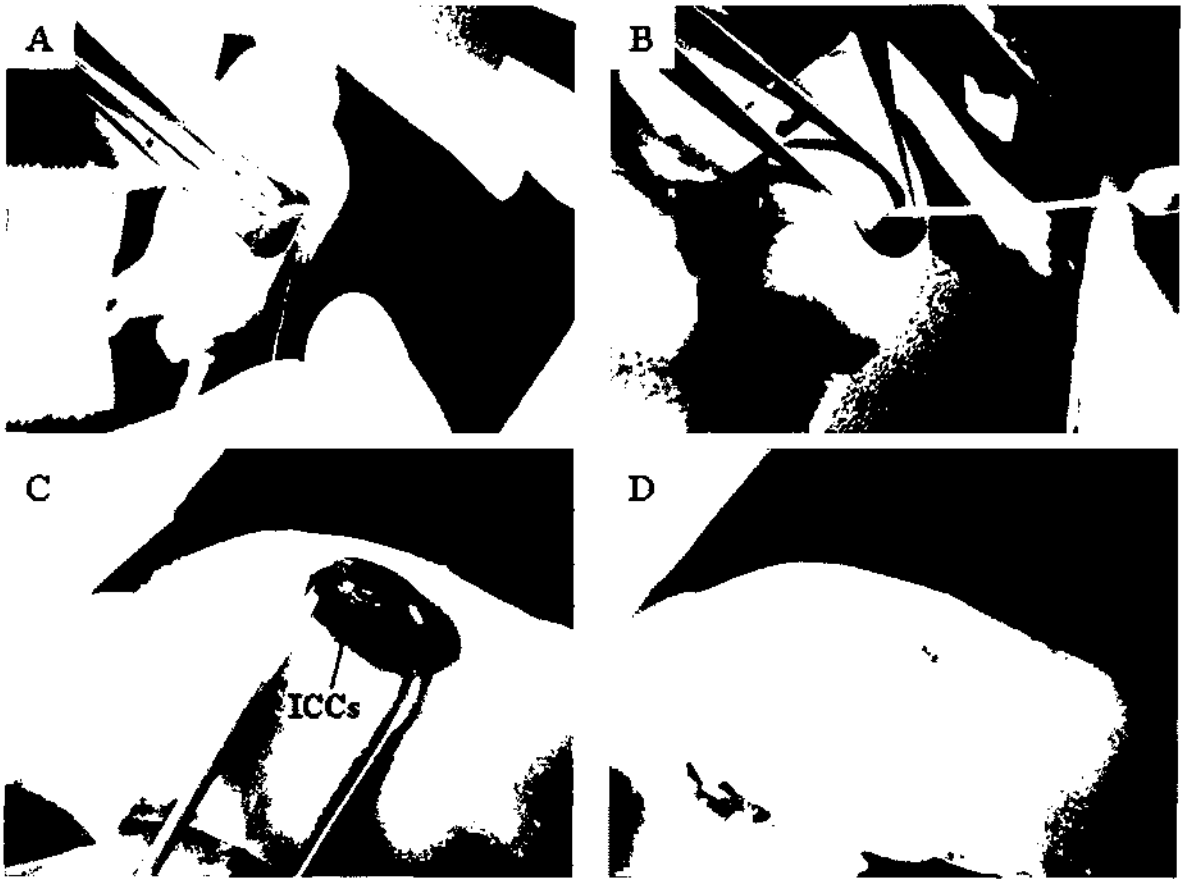


Figure II. 5.1. The ICC transplantation procedures on BALB/c nude mouse. (*A*) A small incision was made on the left renal capsule of an anesthetized mouse. (*B*) The ICCs were gently ejected in the space between the renal parenchyma and capsule. (*C*) The ICC graft resided and was visible within the renal capsule, followed by (*D*) suturing of the skin to end the operation.

II. 6 Statistical Data Analysis

Data are expressed as mean ± S.E.M. for all groups. Multiple comparisons between groups were performed using ANOVAs followed by Tukey's *post hoc* tests. When comparisons were made between two groups, probabilities (p value) of chance differences between experimental groups were calculated using Student's independent two-tailed t tests. Differences were considered statistically significant at $p < 0.05$. Graphics and statistical analyses were done using GraphPad Prism 5 (GraphPad Software, San Diego, CA, USA). For real-time PCR, relative expression was normalized to *β-ACTIN* and calculated using the comparative C_T method, with fold change defined as $2^{-\Delta\Delta C_T}$ as described in section II. 3.9.

Chapter III

PDZ-domain-Containing 2 (PDZD2) Drives the Maturity of Human Fetal Pancreatic Progenitor-derived Islet-like Cell Clusters with Functional Responsiveness against Membrane Depolarization

[The content of this chapter has been published in *Stem Cells and Development* 18(7):979-990]

III. 1 Introduction

The success of a stem cell-based approach to cure diabetes relies on successful isolation of pancreatic progenitor cells (PPCs) and establishment of a complete differentiation protocol to direct their full maturation into transplantable islets. In this regard, we previously characterized a population of PPCs from early trimester human pancreas. Previously available protocols were based on growth factors such as activin A, hepatocyte-derived growth factors (HGF) and nicotinamide, we modified those protocols to develop a cocktail of differentiation factors for PPC differentiation (Suen et al., 2008). Among many morphogens that potentially regulate PPC development, the secreted PDZ-domain-containing 2 (sPDZD2) protein is one candidate that might govern the differentiation process. sPDZD2 is a secreted peptide generated by the post-translationally cleavage at the C-termius of PDZD2 by a caspase-dependent mechanism which shows extensive homology to interleukin-16, an atypical cytokine for growth and differentiation (Yeung et al., 2003). Being one of the most modular protein-interaction domains, PDZ domains have been identified in diverse cell signal-processing machinery (Harris and Lim, 2001; Kurakin et al., 2007). As such, PDZD2, a multi-PDZ-domain protein, may also function in signal processing. The protein was originally found to be ubiquitously expressed in many tissues including the pancreas (Yeung et al., 2003), so it is plausible to

have it found in our PPCs (Suen et al., 2008).

The role of PDZ-domain-containing proteins in β-cell development has received limited attention. We previously reported a concentration-dependent mitogenic effect of sPDZD2 in an INS-1E β-cell line (Ma et al., 2006) and our isolated PPCs (Suen et al., 2008). This provided initial insight into the potential role of sPDZD2 in the optimization of β-cell development. In fact, the whole pancreas develops under a hierarchy of transcription factor expression. One such factor, the *PDX-1* gene, is critical for development of the pancreas and for maintenance of the β-cell phenotype (Soria, 2001; Kemp et al., 2003; Jensen, 2004). A PDZ-domain coactivator, Bridge-1, was reported to directly interact with the *PDX-1* gene and increase the transcriptional activation of the *insulin* gene (Stanojevic et al., 2005), providing further support to the notion that PDZ-domain-containing proteins promote β-cell renewal and restore insulin secretion.

The normal glucose-stimulated insulin secretory process is maintained by normal expression and functioning of several indispensable components, including the glucose transporter 2 (Glut-2), Glucokinase (GCK) and the voltage-gated calcium ion (Ca_v) channels. Several studies revealed that PDZ-domain-containing proteins play a role in regulating channel or transporter activities (Zhang et al., 2005a; Watanabe et al., 2006; Fenster et al., 2007). Specifically, the proteins could be involved in the direct regulation of

L-type $Ca_v1.3$ channels through relief of an autoinhibitory domain (Calin-Jageman et al., 2007). All of these mechanistic components are crucial to governing normal β-cell secretory function. Therefore, it is important to understand whether sPDZD2, a secreted PDZ-domain-containing factor, plays a role in regulating the components that ultimately drive the maturation of the differentiated ICCs.

Morphogens direct differentiation or organogenesis at optimal expression levels, so an optimal condition for sPDZD2 regulation of PPC development likely exists. In this study, we define a novel role for sPDZD2 in promoting PPC differentiation and also demonstrate the degree to which sPDZD2 drives the maturity of the differentiated ICCs.

III. 2 Materials and Methods

III. 2.1 Human Fetal Tissue Procurement and Tissue Processing

Procurement of human fetal tissues and the procedures for subsequent tissue digestion were described in sections II. 1.1 and II. 1.2 (pp. 79-80) in details.

III. 2.2 Culture of Human Fetal Pancreatic Progenitor Cells (PPCs) and their Differentiation into Islet-like Cell Clusters (ICCs)

The protocols for PPC culture, as well as their differentiation to ICCs were described in section II. 1.3. (pp. 80-81). In some experiments, recombinant sPDZD2 (gift from Dr. Shannon WN Au, CUHK) was added during the differentiation process. PANC-1 cells, which were used as negative controls in some experiments, were grown in DMEM supplemented with 10% FBS and 1% penicillin and streptomycin (Invitrogen, Carlsband, CA, USA).

III. 2.3 Blockade of Endogenous sPDZD2 Production by Caspase-3 Inhibition

Full length PDZD2 protein can be proteolytically cleaved at its C-terminus by caspase-3 to generate a 37 kDa peptide, sPDZD2 (Yeung et al., 2003). Caspase inhibitors have been reported to inhibit endogenous production of sPDZD2 in prostate cancer cell lines (Tam et

al., 2006). A similar protocol was applied in our PPC system. Briefly, PPCs were incubated with 20 μM caspase-3-specific peptide inhibitor, Z-DEVD-FMK, 20 μM negative control peptide, Z-FA-FMK (R&D Systems, Minneapolis, MN, USA), or vehicle (1% BSA in PBS) for 48 h. For PPC differentiation experiments, PPCs were incubated with these inhibitors 48 h prior to harvesting so that differentiation could be processed under low levels of endogenous sPDZD2. The inhibitors were continually added along with other growth factors during the differentiation period. Medium was replaced every other day. After treatment, PPCs/ICCs were harvested for Western blot and real-time PCR.

III. 2.4 Western Blot

Extraction and quantification of total proteins from PPC/ICC lysates were described in section II. 3.2 (pp. 89).Western blot procedures were performed according to section II. 3.3 (pp. 89-91). The types and dilutions of all antibodies used are listed in Table II. 3.2 (pp. 91).

III. 2.5 RNA Expression Analysis

Total RNA of cultured PPCs, ICCs, PANC-1 or homogenized human fetal liver was extracted using the TRIzol® reagent (Invitrogen) according to the manufacturer's

instructions. Procedures for RNA isolation, cDNA preparations by reverse transcriptase reactions and the subsequent analyses by PCR or real-time PCR were described in sections II. 3.4 – II. 3.9 (pp. 92-96) in details. The sequences of the primers used are listed in Table II. 3.3 (pp. 97).

III. 2.6 Immunofluorescent Staining

Immunocytohemistry of ICCs were performed according to section II. 3.1 (pp. 86-88). The types and dilutions of all antibodies used are listed in Table II. 3.1 (pp. 88). At least 5 ICCs per group harvested from each differentiation, and ICCs derived from at least 5 different human fetal pancreata were randomly chosen for analysis. As a result, at least 25 ICCs per group were analyzed. Images were scanned separately at different laser wavelengths and captured as overlapping images.

III. 2.7 C-peptide Content Analysis and Glucose-/KCl-stimulated Insulin Secretion

Assessment of C-peptide content of the ICCs and their glucose-/KCl-stimulated insulin secretion was performed according to section II. 4.2 and II. 4.3 (pp. 98-100).

III. 2.8 Measurement of the Ca^{2+} influx in ICCs by Calcium Imaging Experiments

Harvested ICCs were plated in 1% (wt/vol) gelatin-coated cover slips and were allowed to attach to the slips overnight at 37°C. Procedures for calcium imaging experiments were modified and performed as previously reported (Weinhaus et al., 2003; Lai et al., 2005; Trus et al., 2007). Briefly, the attached ICCs were loaded with 1.5 μM of the fluorescent intracellular dye fura-2 AM (Invitrogen) in Krebs-Ringer bicarbonate buffer (KRBB; pH 7.4) plus 0.02% (wt/vol) Pluronic F-127 solution and 2.5 mM probenecid (Sigma-Aldrich) at 37°C for 45 min in the dark. In some experiments, the L-type Ca_v-channel blocker, nifedipine (5 μM) (Sigma-Aldrich), was added in addition to the dye during the incubation period. The fura-2 loaded ICCs were washed with KRBB and the entire cover slip was then transferred to a closed perfusion chamber with a flow rate of approximately 2 ml/min, as regulated by a manual control casette pump. The chamber was mounted on an Olympus inverted microscope (Olympus, Center Valley, PA) equipped with a ×20 water immersion objective that allowed the entire ICC to be captured within the field of view. The ICCs were first perfused with KRBB with or without 2.5 mM glucose and were then perfused with KRBB containing 25 mM glucose or 20 mM KCl, respectively, to investigate the induction of calcium influx in ICCs. In the L-type Ca_v-channel blocker experiment, 5 μM nifedipine (Sigma-Aldrich) was added to the perfusing solution. All solution was warmed to 37°C before initiating perfusion. The ICCs were irradiated alternately with light at 340

nm and 380 nm using a monochromator (Polychrome IV, TILL Photonics GmBH, Germany) and the fluorescence images were captured by a cooled CCD camera (Quantix, Photometrics, USA). Fura-2 ratios were calculated as 340/380 nm and the change of intracellular calcium concentration in ICCs was reflected by the change of fura-2 ratios. All data analyses were performed with MetaFluor (Series 7.5). Similar to the immunostaining experiments, at least 5 ICCs per group were harvested from each differentiation and ICCs derived from at least 5 different human fetal pancreata were randomly chosen. Therefore at least 25 ICCs per group were analyzed.

III. 2.9 Statistical Data Analysis

Detailed procedures for statistical data analysis were described in section II. 6 (pp. 104).

III. 3 Results

III. 3.1 sPDZD2 Induced Up-regulation of the Critical β-cell Transcription Factors and C-peptide Content of ICCs

Recombinant sPDZD2 exerted a dose-dependent up-regulation of the genes *PDX-1*, *NGN3*, *NEURO D*, *ISL-1*, *NKX 2.2*, *NKX 6.1* and *INSULIN* mRNA. At 10^{-9} M sPDZD2 the genes were upregulated around 2-fold (*NGN 3, NEURO D, NKX 2.2*, *NKX 6.1* and *INSULIN*) to 3-fold (*PDX-1* and *ISL-1*) relative to the control, and were mostly suppressed when lower or higher concentrations were used (Figure III. 3.1). Moreover, 10^{-9} M sPDZD2 also enhanced the C-peptide content of the differentiated ICCs to almost 1.5-fold relative to the control (Figure III. 3.2).

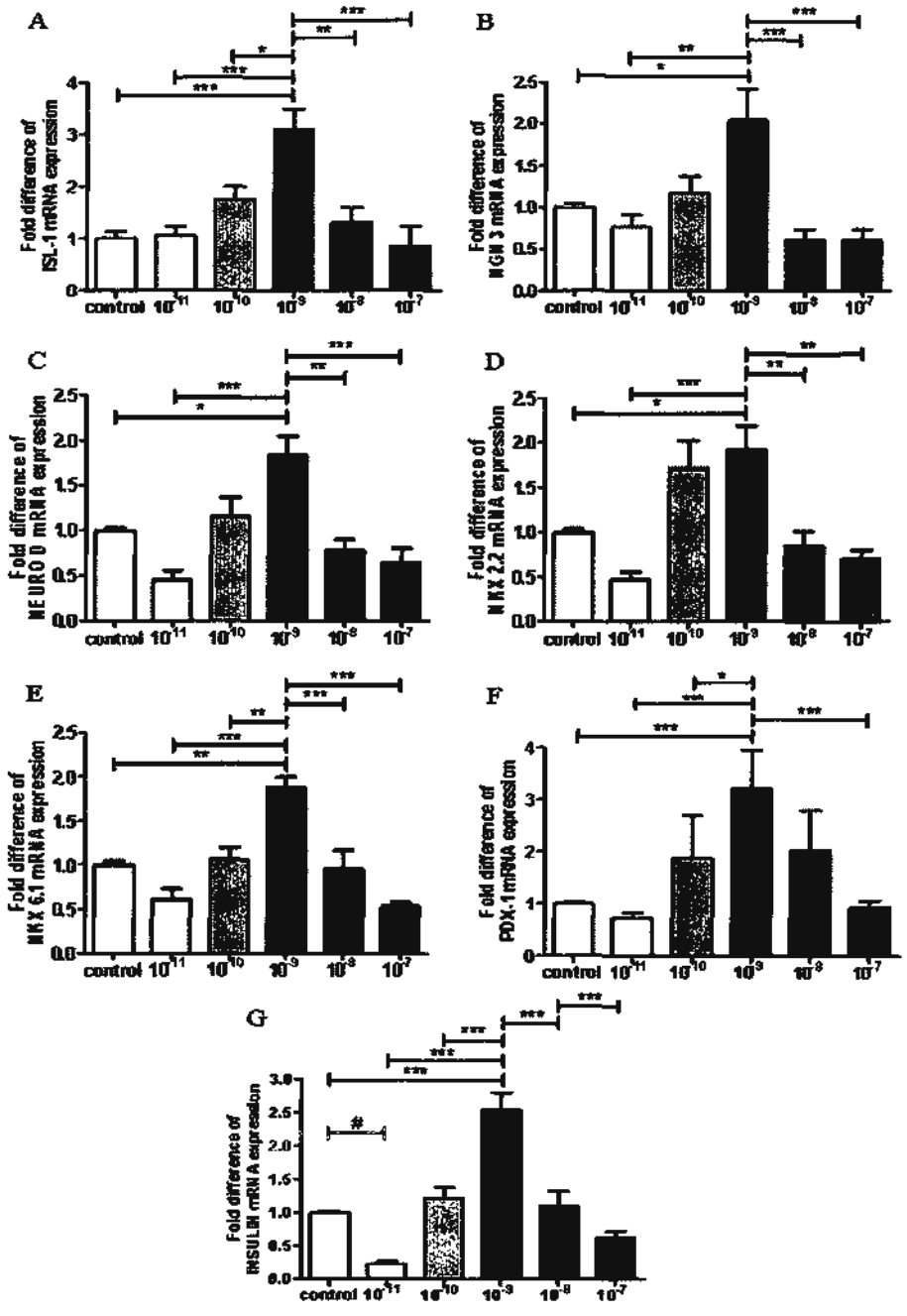


Figure III. 3.1. Effect of recombinant sPDZD2 on expression of the β-cell phenotypic factors in differentiated ICCs. Genes including (*A*) *ISL-1*, (*B*) *NGN3*, (*C*) *NEURO D*, (*D*) *Nkx2.2*, (*E*) *NKX6.1*, (*F*) *PDX-1* and (*G*) *INSULIN* were up-regulated around 2 to 3-fold after treatment with 10^{-9} M sPDZD2, while their expression levels were suppressed at both higher and lower levels of sPDZD2 treatment. All data are expressed as means ± S.E.M. n = 4 in each group. * $p < 0.05$, ** $p < 0.01$ vs control ICCs.

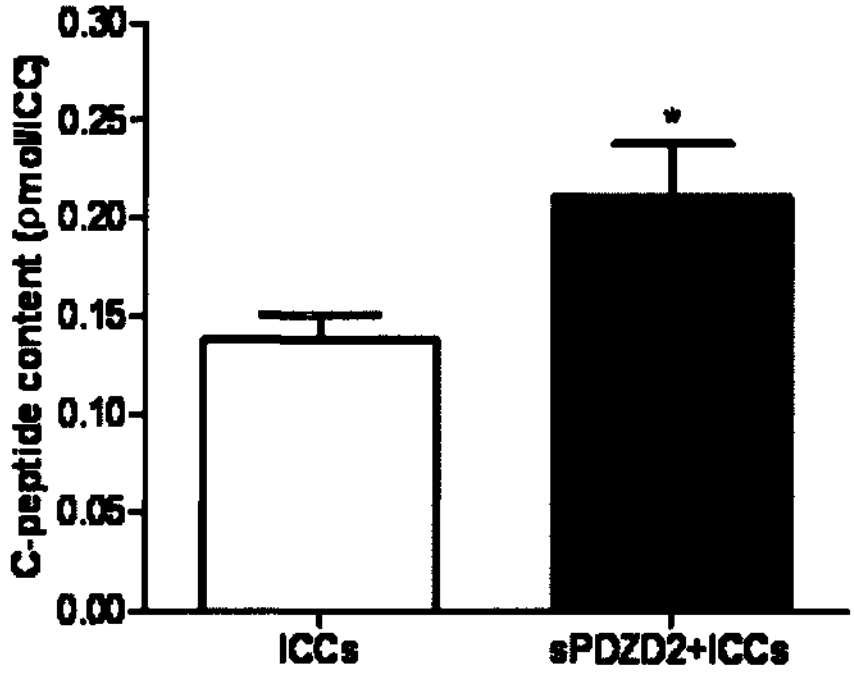


Figure III. 3.2. The C-peptide content of the ICCs was enhanced by 10^{-9} M sPDZD2 treatment. All data are expressed as means ± S.E.M. n = 5 in each group. * $p < 0.05$ vs control ICCs.

III. 3.2 Blockade of Endogenous sPDZD2 Production Suppressed the Expression of β-cell Transcription Factors in ICCs

Incubation with 20 μM specific caspase-3 peptide inhibitor Z-DEVD-FMK successfully suppressed approximately 44% and 33% of the endogenous production of sPDZD2 in our PPC and ICC systems, respectively (Figure. III. 3.3). This partial sPDZD2 blockade caused a nearly 0.6-fold decrease in the expression levels of *PDX-1*, *ISL-1*, and *INSULIN* mRNA in the differentiated ICCs. The negative control, Z-FA-FMK, did not produce a similar response (Figure. III. 3.4).

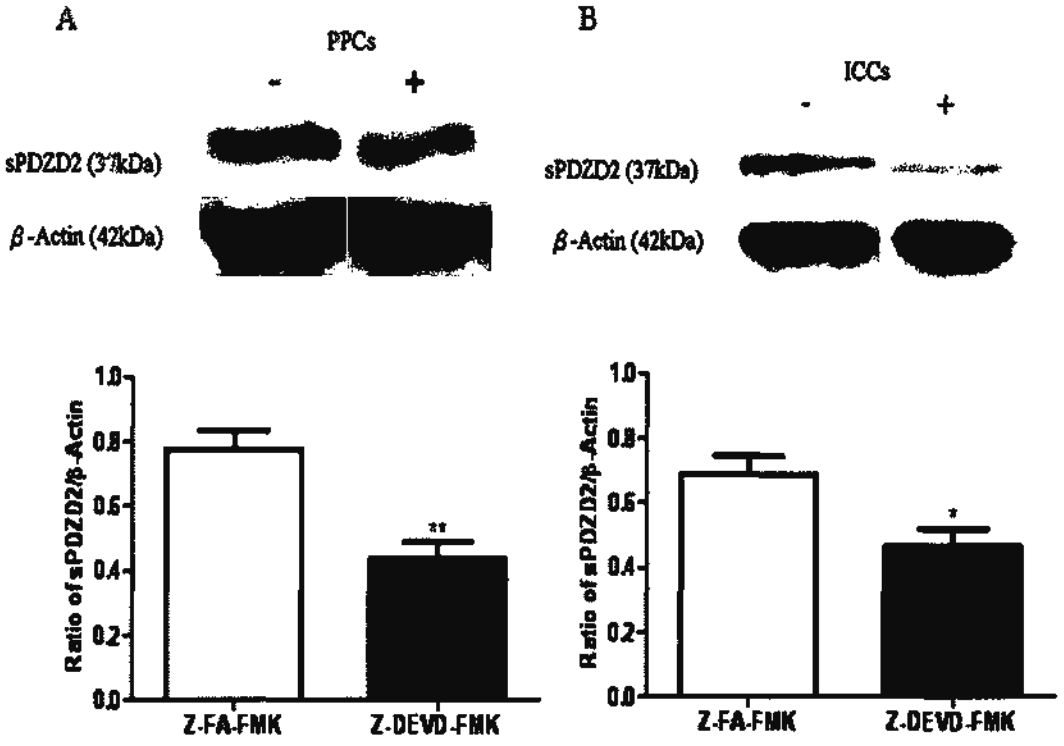


Figure III. 3.3. Effects of Z-DEVD-FMK, a specific caspase-3 peptide inhibitor, and Z-FA-FMK, a negative control peptide, on the endogenous production of sPDZD2 in PPCs/ICCs. 48-hour treatment of 20 μM Z-DEVD-FMK in PPCs and its concomitant incubation in the PPC differentiation cocktail significantly suppressed both the endogenous production of sPDZD2 in (*A*) PPCs and (*B*) the differentiated ICCs. All data are expressed as means ± S.E.M. n = 4 in each group. * $p < 0.05$, ** $p < 0.01$ vs the negative control peptide, Z-FA-FMK.

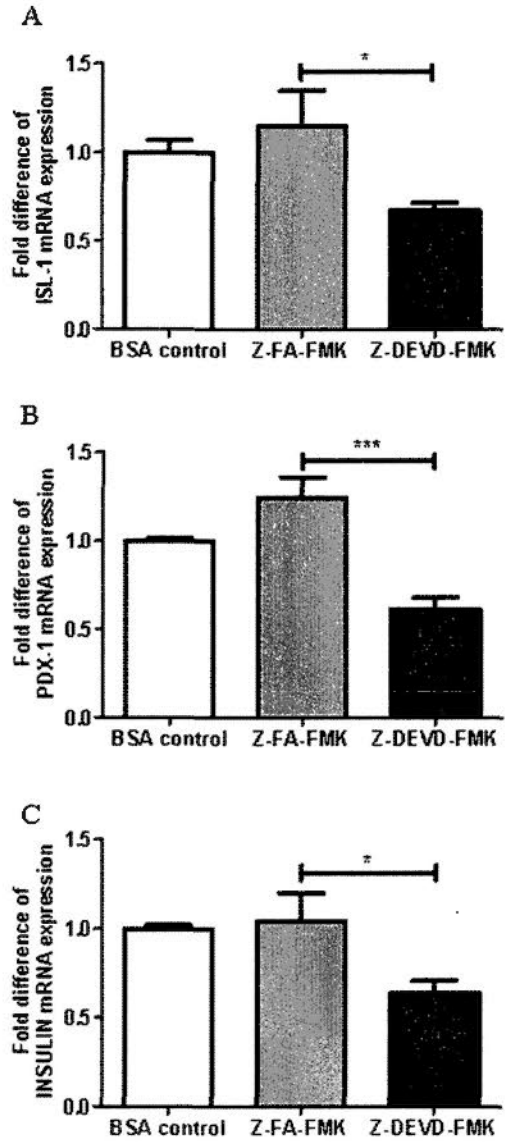


Figure III. 3.4. Effects of Z-DEVD-FMK,and Z-FA-FMK on the expression levels of critical β-cell phenotypic factors in ICCs. The expression levels of (*A*) *PDX-1*, (*B*) *ISL-1*, and (*C*) *INSULIN* mRNA in the differentiated ICCs dropped significantly to around 0.6-fold with blockade of endogenous sPDZD2 production. BSA treatment was applied as vehicle control of the peptide inhibitors. All data are expressed as means ± S.E.M. n = 6 in each group. * $p < 0.05$, *** $p < 0.001$ vs the negative control peptide, Z-FA-FMK.

III. 3.3 Minimal Expression of Glut-2 Contributes to the Glucose-unresponsiveness of ICCs

Glut-2 transporter and glucokinase (GCK) are both indispensable for the regulation of insulin in response to glucose challenge. *GLUT-2* mRNA was not detected in PPCs or the differentiated ICCs. *GCK* mRNA was also not detected in PPCs, but it was strongly expressed in the differentiated ICCs (Figure III. 3.5*A*). Quantitative real-time PCR revealed that sPDZD2 treatment could neither induce *GLUT-2* expression in ICCs (data not shown), nor further increase *GCK* expression (Figure III. 3.5*B*). The absence of Glut-2 might be in part responsible for the glucose-unresponsiveness of ICCs, as evidenced by glucose-stimulated insulin secretion (GSIS) analysis (Figure III. 3.6). We found that sPDZD2 treatment significantly enhanced (by 2-fold) the basal level of insulin secretion, but neither sPDZD2-treated ICCs nor non-treated ICCs secreted more insulin in response to 25.5 mM glucose stimulation (Figure III. 3.6).

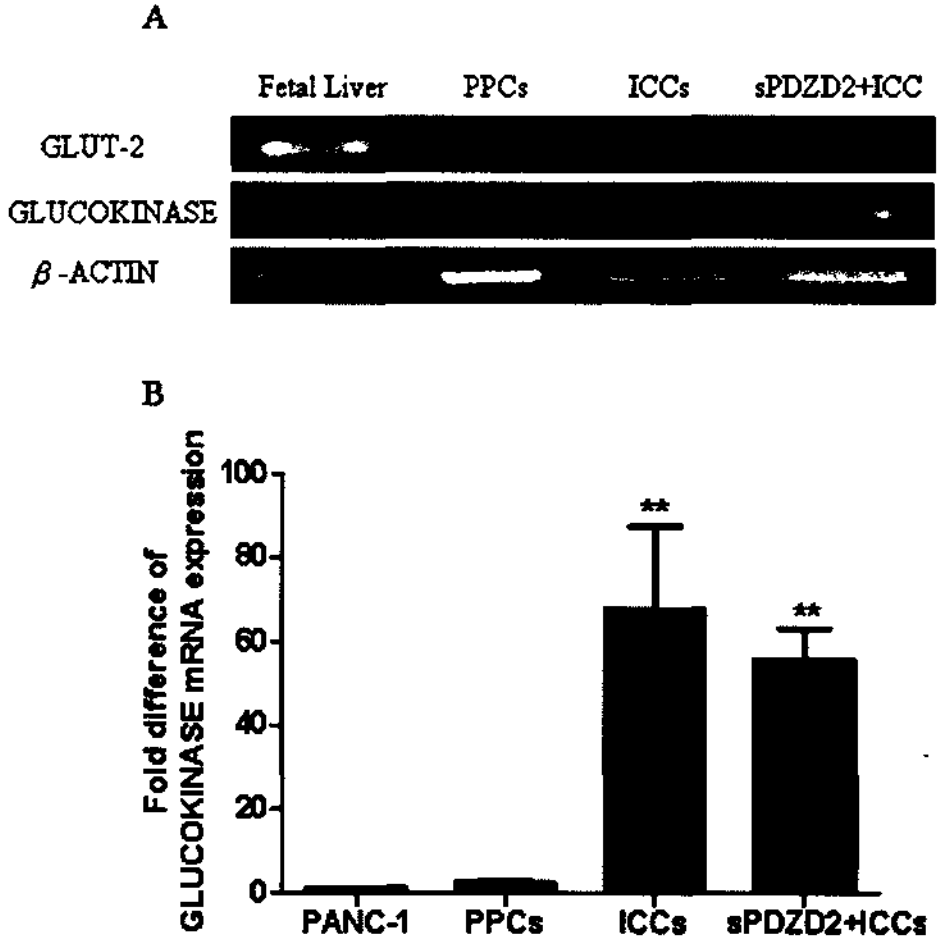


Figure III. 3.5. Effect of sPDZD2 on the expression levels of *GLUT-2* and *GCK* mRNA in ICCs. (*A*) *GLUT-2* mRNA was not detected in either PPCs or ICCs, while *GCK* mRNA was detected in ICCs but not in PPCs. Human fetal liver, which expresses *GLUT-2* and *GCK*, was used as positive control. (*B*) Results from real-time PCR showed that sPDZD2 could neither induce *GLUT-2* mRNA expressions (data not shown) nor alter *GCK* mRNA expression levels in ICCs. PANC-1, a cell line that lacks expressions of *GLUT-2* and *GCK*, was used as negative control. All data are expressed as means ± S.E.M. n = 6 in each group. ** $p < 0.01$ vs. PANC-1.

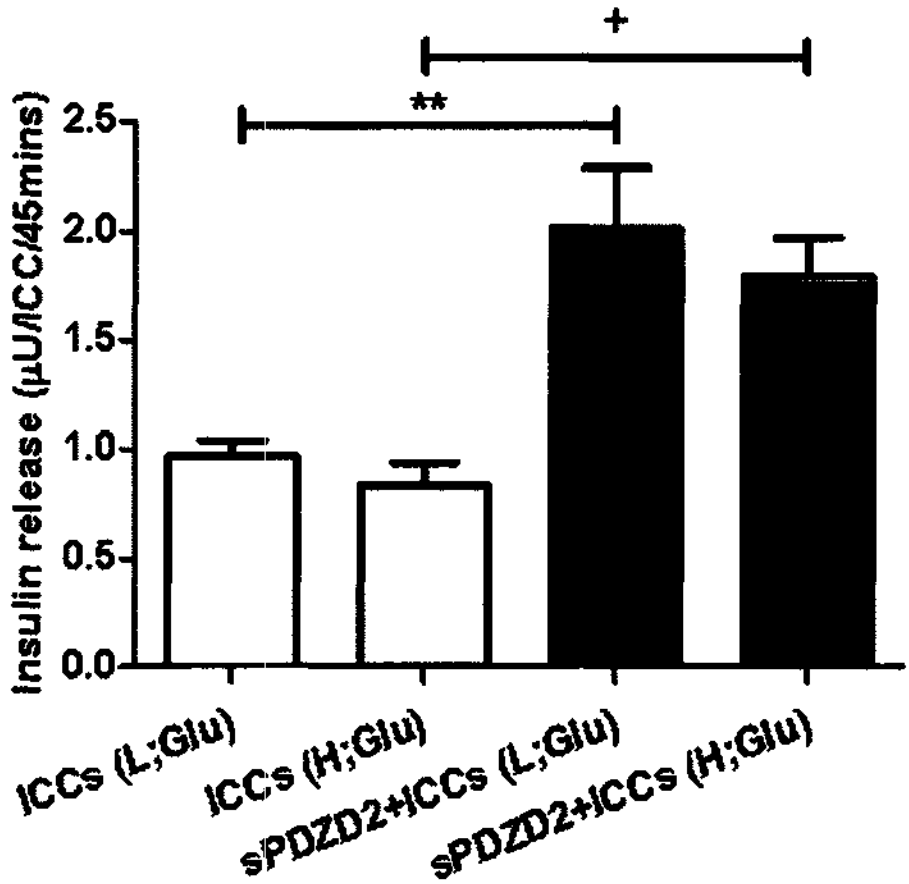


Figure III. 3.6. Effect of sPDZD2 on GSIS profile of ICCs. Insulin release of ICCs in 2.5 mM glucose (L) and 25.5 mM glucose (H) was measured using a human insulin ELISA kit. The 10^{-9} M sPDZD2 treatment enhanced the basal level of insulin release of ICCs to approximately 2-fold, but additional insulin was not secreted in response to higher glucose challenge. All data are expressed as means ± S.E.M. n = 4 per group; ** $p < 0.01$ *vs.* ICCs exposed to 2.5 mM glucose; + $p < 0.05$ *vs.* ICCs exposed to 25.5 mM glucose.

III. 3.4 sPDZD2 Triggered an Increased Expression of L-type $Ca_v1.2$ in ICCs and Conferred an Insulin-secreting Ability in Response to KCl Stimulation

Immunocytochemistry showed that the $Ca_v1.2$ channels were localized to the cell membrane of the ICCs and cells with positive $Ca_v1.2$ staining were scattered throughout the ICCs. The immunoreactivity of $Ca_v1.2$ was more intense in the sPDZD2-treated ICCs (Figure III. 3.7). Thereafter, we studied the effect of KCl on the ability of ICCs to secrete insulin. ICCs without sPDZD2 treatment remained unresponsive to 20 mM KCl challenge. In contrast, basal levels of insulin secretion were enhanced after sPDZD2 treatment, consistent with the GSIS results. The sPDZD2-treated ICCs responded to 20 mM KCl stimulation and secreted insulin to greater than 1.6-fold relative to the control (Figure III. 3.8).

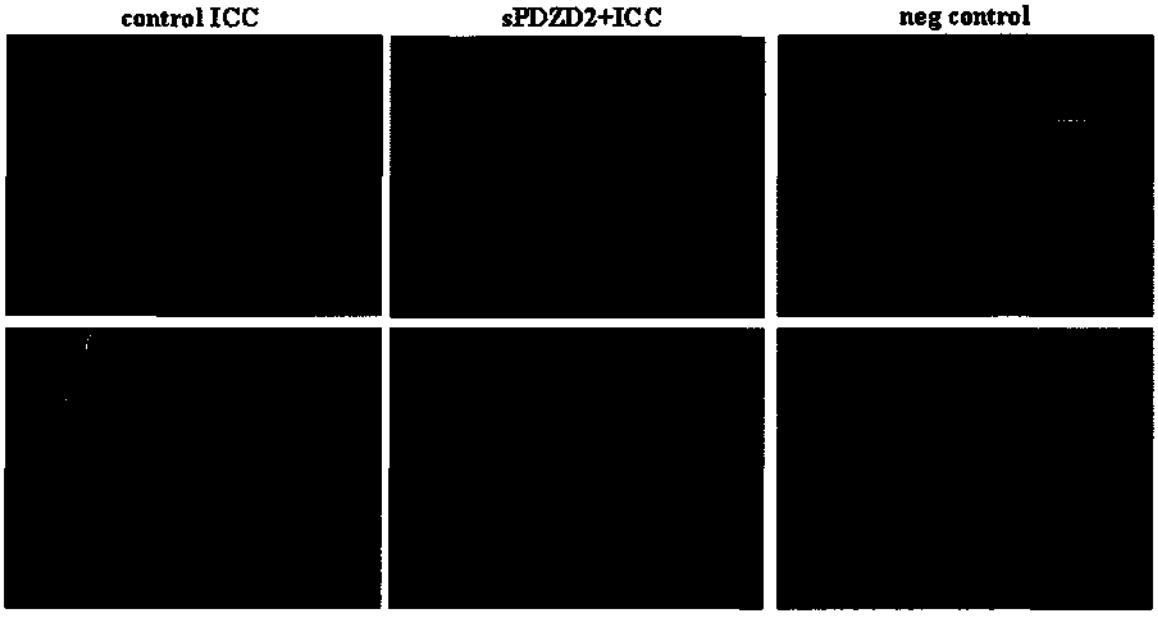


Figure III. 3.7. Expression of L-type $Ca_v1.2$ in ICCs. Cryosectioned ICCs were immunostained with $Ca_v1.2$ antibody (red) followed by appropriate secondary antibody conjugated with fluorochromes. Intensity of staining was enhanced in sPDZD2-treated ICCs compared with those without sPDZD2 treatment (upper panel). Overlapping images of $Ca_v1.2$ and DAPI (blue) show the localization of $Ca_v1.2$ to the cell membrane of the cells within an ICC (lower panel). Negative control was done by omission of the $Ca_v1.2$ primary antibody. Original magnification 400 ×; Scale bar 100 μm.

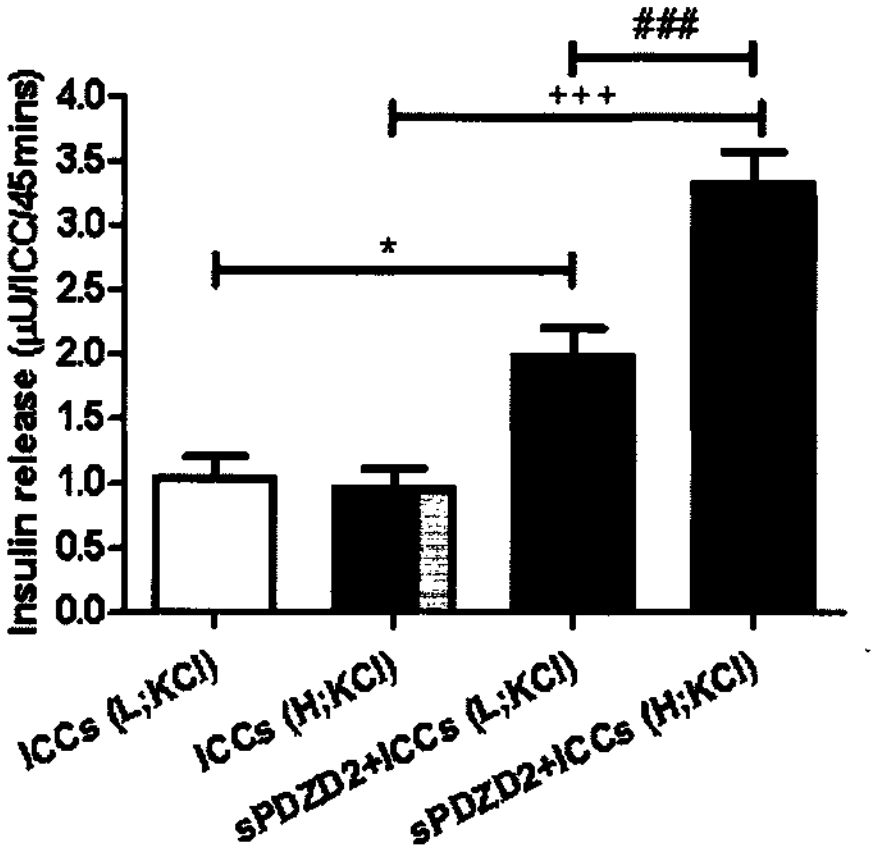


Figure III. 3.8. The insulin secretion of ICCs in response to KCl-induced membrane depolarization. Insulin release in KRBB (L) and 20 mM KCl (H) was measured using a human insulin ELISA kit. 10^{-9} M sPDZD2 treatment enhanced the basal level of insulin release by approximately 2-fold. Only sPDZD2-treated ICCs were responsive to high KCl stimulation and secreted insulin to over 1.6-fold relative to their basal level. All data are expressed as means ± S.E.M. n = 6 in each group; * $p < 0.05$ *vs.* ICCs exposed to KRBB; +++ $p < 0.001$ *vs.* ICCs exposed to 20 mM KCl; ### $p < 0.001$ *vs.* sPDZD2-treated ICCs exposed to KRBB.

III. 3.5 KCl Stimulation Triggered an Elevated Ca^{2+} Ion Influx only in sPDZD2-treated ICCs

Perfusion of ICCs or sPDZD2-treated ICCs with 25.5 mM glucose failed to elicit an increase in $[Ca^{2+}]_i$ (Figure III. 3.9). However, perfusion with 20 mM KCl induced Ca^{2+} ion influx in sPDZD2-treated ICCs, but not in the non-treated ICCs. Fura-2 fluorescence (340/380 nm ratio) in sPDZD2-treated ICCs increased to over 6-fold relative to control (Figure III. 3.10 *A and B*). This is due to the increase in the concentration of extracellular K^+ which led to membrane depolarization that triggered Ca^{2+} influx via Ca_v channels (Gilon and Henquin, 1992; Leclerc and Rutter, 2004). We found in the L-type Ca_v-channel blocker experiment that pre-incubation with nifedipine could almost completely abolish the KCl-induced Ca^{2+} influx in sPDZD2-treated ICCs (Figure III. 3.10 *C and D*).

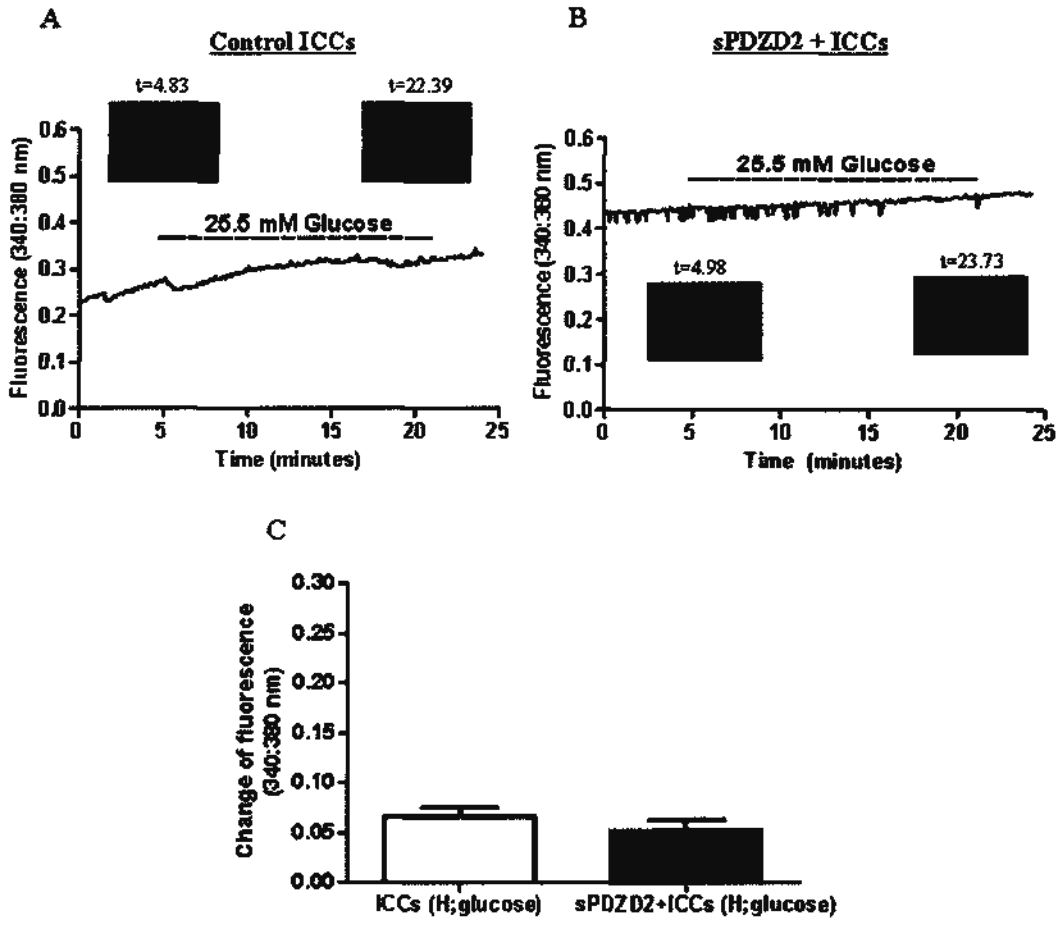


Figure III. 3.9. Effects of glucose on the calcium influx of a single (*A*) control and (*B*) sPDZD2-treated ICC. (*A-C*) Fura-2 imaging of ICCs exposed to 25.5 mM glucose detected no change in fluorescence (340/380 nm ratio), indicating their unresponsiveness to high glucose stimulation. The addition of 25.5 mM glucose is indicated by the black bars. Representative images with their respective time during the trace are shown below each trace. All data are expressed as means ± S.E.M. n = 5 in each group.

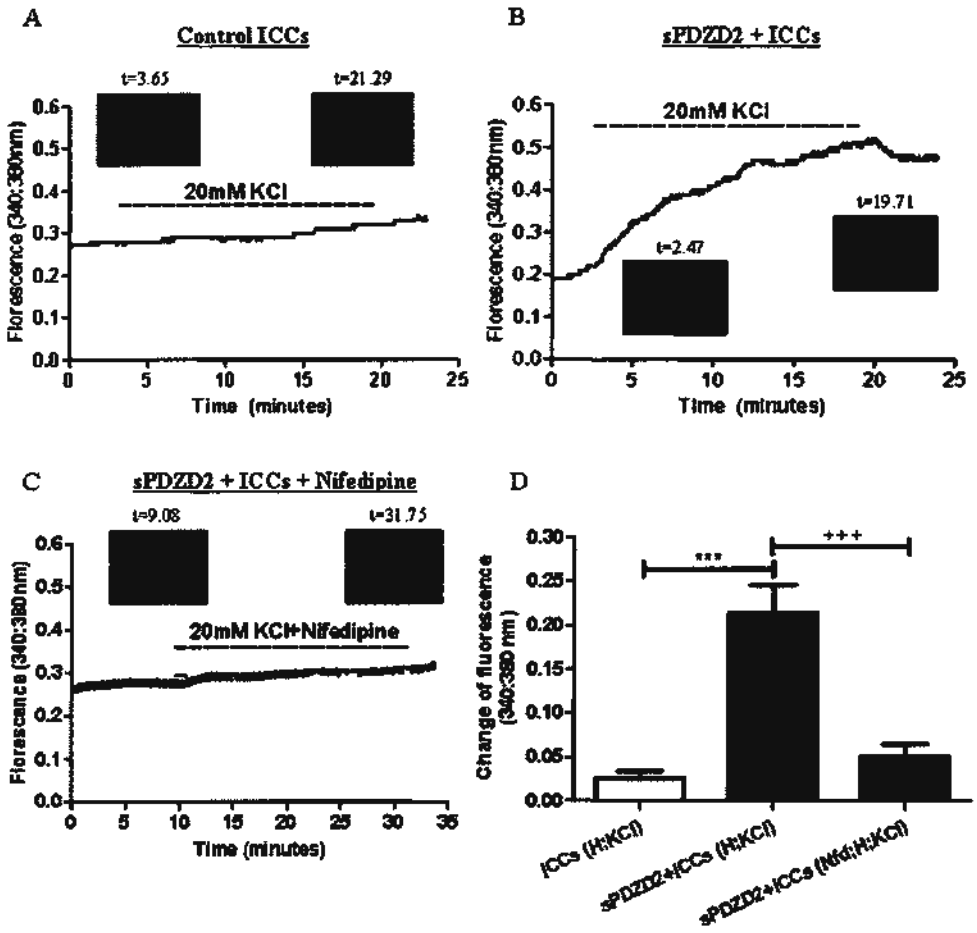


Figure III. 3.10. Effects of the KCl-induced membrane depolarization on the calcium influx of a single ICC. Fura-2 imaging detected an apparent increase of fluorescence (340/380 nm ratio) in response to 20 mM KCl in (*B*) sPDZD2-treated ICCs but not in (*A*) control ICCs, indicating sPDZD2 confers responsiveness against membrane depolarization. (*C and D*) The increase in fluorescence was markedly abolished by pre-treatment of the ICCs with specific L-type Ca_v channel blocker, nifedipine (5 μM), indicating the presence of L-type Ca_v channels that govern the calcium influx process in the ICCs. The addition of 20 mM KCl with or without 5 μM nifedipine is indicated by the black bars. Representative images with their respective time during the trace are shown above/below each trace. All data are expressed as means ± S.E.M. n = 5 per group; *** $p < 0.001$ *vs.* control ICCs exposed to KRBB; +++ $p < 0.001$ *vs.* sPDZD2-treated ICCs exposed to 20 mM KCl.

III. 3.6 sPDZD2 Exhibited a Temporal Expression Profile in PPCs from Different Gestational Weeks

Expression of sPDZD2 peaked in PPCs derived from human fetal pancreata at 10^{th} and 13^{th} weeks, and was reduced by about 2.5-fold in pancreata at 11^{th} and 15^{th} gestational weeks (Figure III. 3.11).

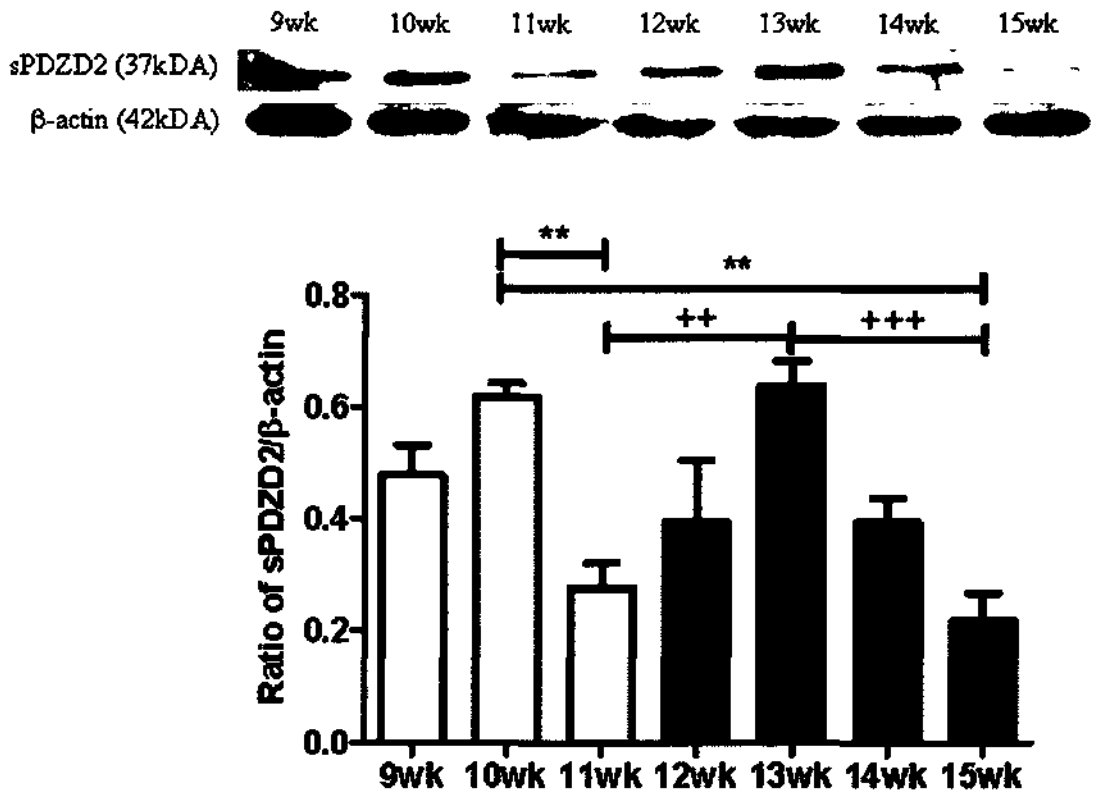


Figure III. 3.11. The temporal expression profile of sPDZD2 in PPCs derived from human fetus of 9 to 15 gestational weeks. The endogenous expression levels of sPDZD2 were self-regulated in different weeks; peaking in 10 and 13 gestation week PCCs, and dropping by 2.5-fold in PCCs derived from 11 and 15 week fetal pancreata. All data are expressed as means ± S.E.M. n = 4 per group ** $p < 0.01$ *vs.* 10^{th} week PPCs; ++ $p < 0.01$, +++ $p < 0.001$ *vs.* 13^{th} week PPCs.

III. 4 Discussion

Using our recently reported protocol for differentiation of the human pancreatic progenitors (Suen et al., 2008), we demonstrated that sPDZD2 has an important role in promoting differentiation into insulin-secreting ICCs. In this study, PPCs isolated from early-trimester human fetal pancreas consistently exhibited enhanced expression of key β-cell phenotypic factors after differentiation in the presence of sPDZD2. Most importantly, treatment with sPDZD2 drove the maturation of the differentiated ICCs by inducing their Ca_v channel expression, thus conferring an ability to secrete insulin in response to membrane depolarization. In this way, these *ex vivo* derived fetal ICCs intends to function towards normal adult islets.

The PDZ domain family, one of the most abundant domain families found in the human genome, is widely recognized to have a role in organizing diverse cell signaling assemblies (Harris and Lim, 2001; Lander et al., 2001). PDZD2, which contains multiple copies of the PDZ domain, might manage organization or scaffolding within a cell. Within an islet, exclusive expression of PDZD2/sPDZD2 in β-cells has been reported previously as well as recently by our laboratory (Ma et al., 2006; Suen et al., 2008). In this study, we provided evidence that sPDZD2 at a concentration of 10^{-9} M promotes PPC differentiation, while in our earlier study we reported that 10^{-12} M sPDZD2 had a mitogenic effect on

PPCs and inhibited PPC differentiation (Suen et al., 2008). Other studies also showed biphasic morphogens, such as TGF-β, can both inhibit and stimulate cell development at different concentrations (Battegay et al., 1990; Pepper et al., 1993). Another PDZ-domain coactivator, Bridge-1, has also been found to promote insulin gene transcription by directly modulating *PDX-1* functions (Thomas et al., 1999; Stanojevic et al., 2005) and yet its overexpression could suppress insulin gene expression and reduce β-cell mass (Volinic et al., 2006). Based on this, it is reasonable to suggest that sPDZD2 exerts a dose-dependent, biphasic pattern of regulation on PPC development. The indispensable role of sPDZD2 in PPC differentiation was highlighted when endogenous production of sPDZD2 in PPC/ICCs was blocked by means of a specific caspase-3 peptide inhibitor. This blockade strategy, used previously to describe different prostate cancer cells (Tam et al., 2006), only partially suppressed endogenous sPDZD2 production. This suggests that other mechanisms, in addition to the recently proposed proteolytic cleavage by caspase-3, may be acting to generate sPDZD2 from the intact PDZD2 molecule (Yeung et al., 2003). It is worth noting that the partial blockade could have resulted in lower optimal expression levels of the morphogenic regulator, as reflected in the down-regulation of *PDX-1*, *ISL-1* and *INSULIN* mRNA. On the other hand, we cannot rule out the possibility that these observations could also be the result of an increased PDZD2 expression after such

treatment of caspase-3 peptide inhibitor as reported elsewhere (Tam et al., 2006).

The mechanisms involved in GSIS have been described in detail elsewhere (Ashcroft et al., 1976; Kahn et al., 2006). Glut-2 transporter first moves glucose into a β-cell, where it is then degraded by GCK. Normal fetal islets poorly express *GLUT-2* mRNA and protein but abundantly express *GCK* mRNA and protein (García-Flores et al., 2002). Even in neonatal β-cells, Glut-2 expression was predominantly in the cytoplasm, which contributes to the low response to extra-cellular glucose stimulation (Navarro-Tableros et al., 2007). Our differentiated ICCs showed similar responses and sPDZD2 treatment did not alter the expression levels of *GLUT-2* and *GCK* in the ICCs. It has been proposed that either incubation with high glucose concentrations or hyperglycemic conditions in diabetic individuals could significantly induce Glut-2 and GCK expression in fetal islets (Tu et al., 1999; García-Flores et al., 2002). Hence, further evaluation is necessary to optimize the differentiation protocol for pancreatic progenitors (Roche et al., 2006).

Besides Glut-2 and GCK, the expression and normal functioning of Ca_v channels, which govern the influx of Ca^{2+} and insulin exocytosis from β-cells, can also elicit GSIS. Ca_v channels are hetromeric protein complexes formed by different subunits. One of these, the α1 subunit, forms the ion-conducting pore, and its type designates the classification of Ca_v channels (Yang and Berggren, 2005). Expression of the α1 subunits, notably α1C

($Ca_v1.2$) and α1D ($Ca_v1.3$), was lower in neonatal β-cells compared to adult β-cells (Navarro-Tableros et al., 2007). $Ca_v1.2$ is one of the predominant types of Ca_v channels mediating insulin secretion in normal islets or β-cells (Yang and Berggren, 2005). Data from this study revealed a low expression of $Ca_v1.2$ in the differentiated ICCs, as well as suppressed channel activity in response to membrane depolarization. Interestingly, sPDZD2 treatment promoted the expression of $Ca_v1.2$ in our ICCs, triggering calcium ion influx under KCl stimulation and conferring an ability to secrete insulin in response to membrane depolarization. Since the multi-domain features of PDZ-containing proteins like PDZD2 often interact with multiple binding partners simultaneously and thereby assemble larger protein complexes (Hung and Sheng, 2002), we cannot rule out the possibility that the organization around a PDZ-based scaffold within the cells might have resulted in the increased $Ca_v1.2$ expressions and conferred activated channel activity to the ICCs. Notably, $Ca_v1.2$ contains VSXL, a PDZ-domain-binding carboxyl-terminal motif that has been shown to associate with other PDZ proteins (Kurschner and Yuzaki, 1999). PDZ proteins themselves might also be involved in direct facilitation of the Ca_v channels (Calin-Jageman et al., 2007), which may be important in coupling L-type Ca^{2+} channel activity.

Although sPDZD2 induced an up-regulated expression of $Ca_v1.2$ in our ICCs, the

expression remained scattered across the whole cluster. This is out of our expectation that their expression should be localized solely on insulin-secreting β-cells. This phenomenon might be attributed to the expression of more than one endocrine hormone within the cells of an ICC (Suen et al., 2008), indicative of a partially mature islet (Polak et al., 2000). Sustained expressions of the proendocrine precursors like *NGN3* in our differentiated ICCs may also imply their further differentiation capacity into fully mature islets (Gasa et al., 2004) and the possibility to have more cells turned into an endocrine cell fate (White et al., 2008). This is an unavoidable limitation for various up-to-date differentiation protocols for full maturation of pancreatic progenitors, and yields cells that co-express insulin together with other pancreatic hormones, such as glucagon and somatostatin (Segev et al., 2004; Jiang et al., 2007). Ideally, differentiation protocols yield physiologically transplantable ICCs. It is reasonable to expect our sPDZD2-treated ICCs, like other progenitor-derived cells in the pancreatic lineages, could be subjected to further functional maturation after transplantation *in vivo* (Brolén et al., 2005, Kroon et al., 2008). The addition of sPDZD2 to our differentiation protocol is a step toward promoting maturation of early gestation-derived ICCs, creating the possibility that they will become functional islets that could treat diabetic individuals.

Taken these findings together with our previous study, it is plausible to propose the

bi-phasic morphogenic property of sPDZD2 on the PPC development. Here, we provided additional evidence for the regulated endogenous expression of sPDZD2 in the PPC system. sPDZD2 expression peaked only in PPCs derived from 10^{th} and 13^{th} week fetal pancreata, suggestive of a specific role of sPDZD2 in islet development. A previous report looked at β-cell differentiation during early human pancreatic development and found a significant increase in hormone-expressing endocrine cells in 9^{th}-10^{th} week of gestation (Piper et al., 2004). It is of interest that the number of cells co-expressing more than one endocrine hormone, such as insulin, glucagon, somatostatin and pancreatic polypeptide (PP), dropped dramatically during the 9^{th}-10^{th} gestational week, suggesting that this is a crucial stage in islet differentiation (Polak et al., 2000). By the 13^{th} week of development, the endocrine cells have aggregated into large primitive islet structures. PC1/3, a marker for mature β-cell function that cleaves pro-insulin and islet amyloid polypeptide, was co-expressed at this gestational age, implying that first trimester β-cells are capable of processing and secreting insulin (Piper et al., 2004). A well-defined endocrine pancreas that contains small clusters of cells producing the three hormones and PP could be noted by the 14^{th} week (Lyttle et al., 2008). Increased islet size in the pancreatic body was observed during the second and third trimesters only (Piper et al., 2004). We cannot rule out the possibility that sPDZD2 might work as an intrinsic factor to exert its

concentration-dependent regulation during the developmental process.

In summary, the use of sPDZD2 as a novel factor in regulating PPC development might assist in designing new protocols for maturation of pancreatic progenitors. First, the present study highlights sPDZD2 as a bi-phasic morphogenic factor regulating the developmental process of islet progenitors. Second, we proposed an optimal sPDZD2 concentration for promoting PCC growth or differentiation, generating transplantable islets that are prone to exhibit full functional maturation in diabetic individuals. This information can improve our further understanding of pancreas development and lead to development of a better differentiation protocol for stem cell-based therapy of diabetes.

Chapter IV

Characterization of a Local Renin-Angiotensin System in the Human Fetal Pancreatic Progenitor Cells and Islet-like Cell Clusters

[Some content of this chapter have been modified and submitted to *Stem Cells*]

IV. 1 Introduction

The renin-angiotensin system (RAS) has been well known as a circulating or hormonal system that critically regulates fluid homeostasis and blood pressure (Taquini and Taquini, 1961; Haber, 1969; Peach, 1977). This classical RAS consists of several key components including the liver-derived precursor angiotensinogen, the renal tissue-derived renin and the pulmonary-bound angiotensin-converting enzyme (ACE) that sequentially generate angiotensin I and angiotensin II (Ang II), respectively, the latter being the physiologically active component of the RAS. A cascade of alternative enzymatic actions produce a number of bioactive peptides from these components and each of them mediates functions on its specific cellular receptors. Most, but not all, of the biological functions are mediated by the angiotensin II type 1 (AT_1) and type 2 (AT_2) receptors through binding of their ligands, Ang II. Figure IV. 1.1 depicts the skeleton of the RAS.

In addition to this circulating RAS, the existence of local renin-angiotensin systems (RASs) in diverse tissues and organs has been increasingly recognized over the past decade. With this recognition, there has been great interest in knowing how such local RASs may be involved in the regulation of diverse physiological functions (Karlsson et al., 1998; Mckinley et al., 2003; Lubel et al., 2008). In this regard, the identification of a local RAS in the adult pancreas has been previously reported (Lau et al., 2004; Leung, 2007a).

This pancreatic RAS has been shown to be critically involved in the regulation of normal exocrine and endocrine functions in the pancreas, where activation of the RAS components has also been suggested to contribute to the etiology of diverse pathophysiological conditions of the pancreas (Ip et al., 2003; Chu et al., 2006; Chan et al., 2007). Such conditions could potentially be ameliorated by inhibition of local pancreatic RAS (Leung, 2007b). However, development of such clinical applications of local pancreatic RAS blockade will require elucidation of when and why ruinous RAS components are present in the pancreas.

The RAS regulates development of different types of stem/progenitor cells. Table IV. 1.1 summarizes the previous findings for the involvement of RAS components in different stem/progenitor cell and tissue/organ development. The growth and differentiation of human embryonic stem cells (ESCs) or mesechymal stem cells (MSCs), as well as the progenitors of fetal kidney, skin, or haematopoietic tissues were found to express and be regulated by major RAS components, including the precursor peptide angiotensiogen, renin, ACE, and the active peptide Ang II, as well as AT_1 and AT_2 receptors (Matsusaka et al., 2002; Kato et al., 2005; Savary et al., 2005; Liu et al., 2007; Song et al., 2010). In fact, RAS components are expressed early in diverse developing tissues of human embryos, although the specific cell types responsible have not been thoroughly defined. The

presence of Ang II in particular suggested its potential regulation of organogenesis (Schütz et al., 1996). The fetal pancreas should be enriched with progenitor cells (Bonner-Weir et al., 2004; Sugiyama et al., 2007). However, it remains to be clarified whether a RAS is present in such progenitors and if so whether it regulates their development toward an endocrine lineage. Since a regulated expression of the RAS components has been reported during cell differentiation (Saiki et al., 2008; Fowler et al., 2009), we also speculated the possibility of detecting a temporal expression profile of the RAS components during the differentiation of pancreatic progenitors into the endocrine lineage.

To address this issue, we performed a series of experiments using our previously described pancreatic progenitor cells (PPCs) isolated from early trimester human fetal pancreas (Suen et al., 2008; Leung et al., 2009; Ng et al., in press). Upon stimulation with a morphogenic cocktail, these PPCs are amenable to differentiate into hormone-secreting islet-like cell clusters (ICCs), and thus follow an endocrine lineage cell fate. This culturing system is an excellent platform for assessing potential RAS-mediated regulatory activities in PPC growth and differentiation. To monitor the progress of endocrine development, we assessed the expression of a hierarchy of β-cell transcription factors in a developing pancreas (Kemp et al., 2003; Habener et al., 2005; Oliver-Krasinski and Stoffers, 2008).

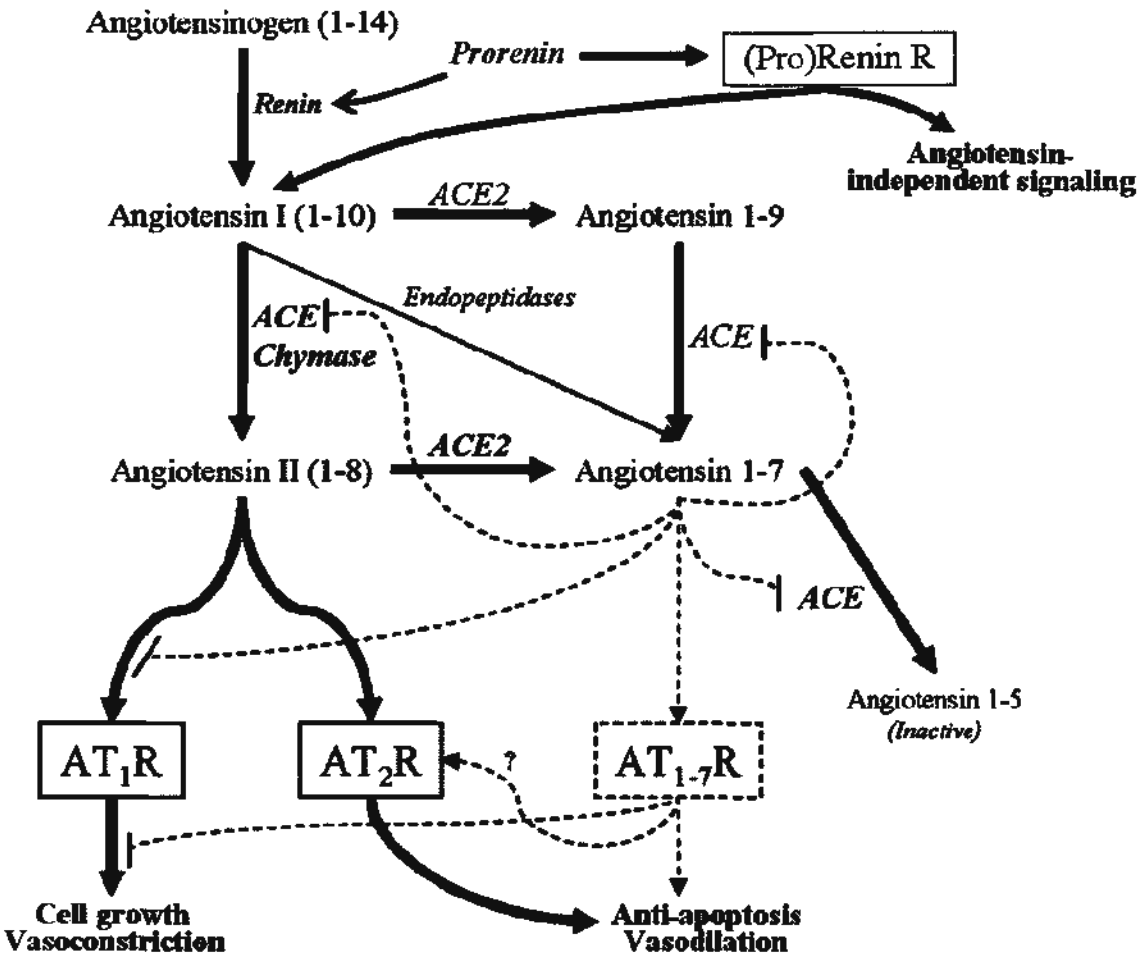


Figure IV. 1.1. An outline of the RAS showing the typical Ang II pathway and the recently identified angiotensin$_{(1-7)}$ pathway. Possible actions mediated by Ang$_{(1-7)}$ are denoted by dash lines. Arrows indicate stimulation while lines ended with bars indicate inhibition. (Figure and information extracted and modified from Katovich et al., 2005; Leung, 2006; Jan Danser et al., 2007)

	Types of cells/ tissues	Origins	Ang II-mediated actions	Major RAS components involved					References
				AT1R	AT2R	Renin	Angiotensiogen	ACE	
Stem/ progenitor cells	ESCs	Mouse	Glucose uptake and proliferation	✓					Han et al., 2005; Kim and Han, 2008
		Human	Differentiation into epithelial cells	✓					Huang et al., 2007
	MSCs	Human	Adipogenic differentiation	✓	✓	✓			Matsushita et al., 2006
	3T3-L1 preadipocytes	Mouse	Adipogenic differentiation	✓		✓	✓		Saiki et al., 2008; Fowler et al., 2009
Tissues/ Organs	Vascular tissue	Mouse; Chick	Erythropoiesis	✓				✓	Kato et al., 2005; Savary et al., 2005
		Rat	Senescence in endothelial progenitors	✓					Kobayashi et al., 2006
	Kidney	Mouse; Rat	Renal organogenesis	✓	✓				Sánchez et al., 2008
	Skin	Human	Epidermal tissue development		✓				Liu et al., 2007
		Human	Wound healing		✓				Steckelings et al., 2005

Table IV. 1.1. A summary of the recent studies showing the RAS involvement in stem/progenitor cell and tissue/organ development. (Extracted and modified from Leung, in press)

IV. 2 Materials and Methods

IV. 2.1 Human Fetal Tissue Procurement and Tissue Processing

Procurement of human fetal tissues and the procedures for subsequent tissue digestion were described in sections II. 1.1 and II. 1.2 (pp.79-80) in details.

IV. 2.2 Culture of Human Fetal Pancreatic Progenitor Cells (PPCs) and their Differentiation into Islet-like Cell Clusters (ICCs)

The protocols for PPC culture, as well as their differentiation to ICCs were described in section II. 1.3 (pp. 80-81) with slight modifications. First, differentiation of PPCs (passages number <10) harvested from four confluent T-75 tissue culture flasks was performed on an ultra-low attachment 6-well plate (Corning, New York, USA) with the medium changed at least every other day to allow for a better three-dimensional aggregation of PPCs into ICCs. Second, the differentiation period lasted for 8 d before ICCs were harvested. These modifications were made empirically based on morphological observations and expression levels of endocrine cell markers measured using real-time PCR.

IV. 2.3 RNA Expression Analysis

Total RNA of cultured PPCs, ICCs or homogenized human fetal liver was extracted using the TRIzol® reagent (Invitrogen, Carlsband, CA, USA) according to the manufacturer's instructions. Procedures for RNA isolation, cDNA preparations by reverse transcriptase reactions and the subsequent analyses by PCR or real-time PCR were described in sections II. 3.4 – II. 3.9 (pp. 92-96) in details. The sequences of the primers used are listed in Table II. 3.3 (pp. 97).

IV. 2.4 Immunofluorescent Staining/ Immunohistochemistry

Immunocytohemistry of PPCs and ICCs were performed according to section II. 3.1 (pp. 86-88). The types and dilutions of all antibodies used are listed in Table II. 3.1 (pp. 88).

IV. 2.5 Western Blot

Extraction and quantification of total proteins from PPC/ICC lysates were described in section II. 3.2 (pp. 89). Western blot procedures were performed according to section II. 3.3 (pp. 89-91). Types and dilutions of antibodies used are listed in Table II. 3.2 (pp. 91).

IV. 2.6 Immunophenotyping of PPCs by Flow Cytometry

The employed procedures for flow cytometry have been reported elsewhere (Jiang et al.,

2007). PPC cultures were washed twice in PBS (Invitrogen) and enzymatically segregated using 0.05% trypsin/EDTA (Invitrogen) for 5 min at 37°C. Cells were then washed and adjusted to ~ 1 x 10^7 cell/ml in PBS supplemented with 1% BSA (Sigma-Aldrich, St. Louis, MO, USA). Cell suspension aliquots (100 µl) were stained with 20 µl FITC- and phycoerythrin (PE)-conjugated monoclonal antibodies from Beckman (Beckman Coulter, Brea, CA, USA), unless specified otherwise, for 20 min at 4°C in the dark. Specifically, the conjugated antibodies used were CD3-PE, CD16-FITC, CD19-FITC, CD33-FITC, CD34-PE, CD38-FITC, CD45-FITC, 10 µl CD133-PE (Miltenyi Biotec, Bergisch Gladbach, Germany), HLA-DR-FITC, 10 µl CD29-Rhodamin, CD44-FITC, CD90-PE, CD105-PE (Serotec, Oxford, UK) and CD166-PE. Background level of non-specific antibody uptake was evaluated by staining in parallel with isotype-matched IgG1-FITC, IgG1-PE and IgG2b-PE. Cells were processed using the ImmunoPrep reagent kit in the Q-Prep Immunology Workstation (Immuno-Tech, Beckman Coulter) and then analyzed with the Coulter Epic XL MCL flow cytometer (Beckman Coulter).

IV. 2.7 Measurement of Ang II and Prorenin Production

Differentiation of PPCs was performed on ultra-low attachment 6-well plates with the medium changed every day. Medium and ICC lysates were collected each day during the

8-d differentiation period for quantification of Ang II production using an Ang II EIA kit (SPI-Bio, Montigny-le-Bretonneux, France). For preparation of ICC lysates, ~300 ICCs were washed twice in ice-cold PBS (Invitrogen). They were then sonicated twice at 25 W for 10 s in 250 μl lysis buffer containing 0.44 mM 1,10-phenanthroline, 25 mM EDTA, 0.12 mM pepstatin A (Sigma-Aldrich) and 1 mM p-hydroxymercuribenzoic acid (Fluka, Buchs, Switzerland) in PBS (Invitrogen) adjusted to pH 7.4. The ICC lysates were then centrifuged at 3000 *g* for 20 min at 4°C and the supernatant was collected. Ang II was extracted from the lysates using Strata® solid phase extraction (SPE) methods (Phenomenex, Torrance, CA, USA) as suggested by the Ang II EIA kit. Briefly, the phenyl cartridges were pre-washed with 1 ml methanol, followed by another wash with 1 ml of ddH_2O. Each sample of ICC lysate was then passed through an individual cartridge, followed by another wash in 1 ml ddH_2O. The absorbed angiotensin peptides were then eluted with 0.5 ml methanol and the eluents were then dried by vacuum centrifugation to evaporate the methanol. A 200-μl aliquot of EIA buffer was then added to each of dried sample to resuspend the pellet. The samples were then centrifuged at 3000 *g* for 10 min at 4°C and the supernatant was collected to submit to an Ang II assay as suggested by the manufacturer's protocol. Quantification of human prorenin in the culture medium was performed using a human prorenin ELISA assay kit (Molecular Innovations, Novi, MI,

USA) according to the manufacturer's protocol.

IV. 2.8 Differentiation of Cultured Human Embryonic Stem Cells (hESCs) into Hormone-producing Cells

Culture of hESCs was performed according to the detailed online Basic National Stem Cell Bank (NSCB) hESC Culture Protocols given by the WiCell® Research Institute (WiCell Research Institute, Madison, WI, USA). Undifferentiated H1 hESCs (gift from Dr. KS Tsang, CUHK) were maintained on mouse embryonic fibroblast feeder layers in DMEM/F12 (Invitrogen) supplemented with 20% (vol/vol) Knockout serum replacement, 1 mM glutamax, 1 mM non-essential amino-acids, 4 ng/ml bFGF (Invitrogen), and 0.55 mM β-mercaptoethanol (Sigma-Aldrich). Differentiation was performed in 24-well plates in triplicate, strictly following D'Amour et al.'s five-stage differentiation protocol (D'Amour et al., 2006) (Figure IV. 2.1). The differentiating hESCs were harvested at the end of each stage (day 4 for Stage I, day 8 for stage II, day 12 for stage III, day 15 for stage IV, and day 18 for stage V) for subsequent real-time PCR analyses.

IV. 2.9 Statistical Data Analysis

Detailed procedures for statistical data analysis were described in section II. 6 (pp. 104).

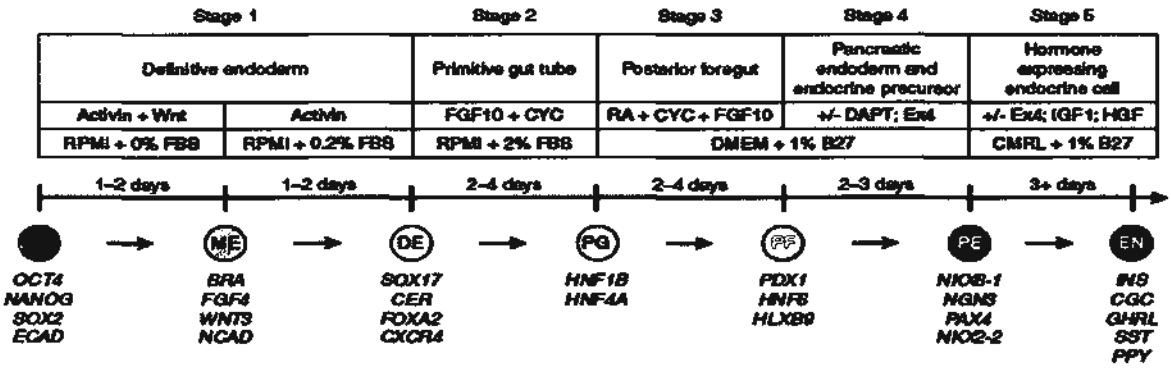


Figure IV. 2.1. The stepwise differentiation procedures of hESCs into pancreatic hormone-producing cells. Some key transcription factor characteristics of each cell population are listed. (Extracted from D'Amour et al., 2006)

CYC - KAAD-cyclopamine; RA - all-trans retinoic acid; DAPT - γ-secretase inhibitor; Ex4 - exendin-4.

ES – hESCs; ME - mesendoderm; DE - definitive endoderm; PG - primitive gut tube; PF - posterior foregut endoderm; PE - pancreatic endoderm and endocrine precursor; EN - hormone-expressing endocrine cells.

IV. 3 Results

IV. 3.1 Expression of a Local RAS in PPCs/ICCs.

Based on earlier studies showing the presence of RAS components in stem/progenitor cell cultures (Han et al., 2005; Kobayashi et al., 2006; Matsushita et al., 2006), we characterized expression of the major RAS components in our previously reported fetal-derived PPCs/ICCs in this study. Detection by reverse transcription-polymerase chain reaction (RT-PCR) revealed expression of AT_1 and AT_2 *RECEPTORS*, *ANGIOTENSINOGEN*, *RENIN*, *ACE*, *ACE2*, and *(PRO)RENIN RECEPTOR* in both PPCs and their differentiated ICCs (Figure IV. 3.1). Interestingly, while the RAS precursor *ANGIOTENSINOGEN* and *RENIN* were detected selectively in differentiated ICCs, they were only minimally present in undifferentiated PPCs (Figure IV. 3.1). This pattern of expression suggests development of an incomplete local RAS into a fully functional RAS during the PPC differentiation process. Detection of neurogenin 3 (Ngn3) confirmed the endocrine nature of progenitor cells (Desgraz and Herrera, 2009). Of note, AT_1 receptor expression was localized to the cell membrane and nuclei of Ngn3-positive PPCs while AT_2 receptor expression was confined to the cell nuclei (Figure IV. 3.2), suggesting that their ligand, Ang II, may mediate signaling in regulating the intracrine system.

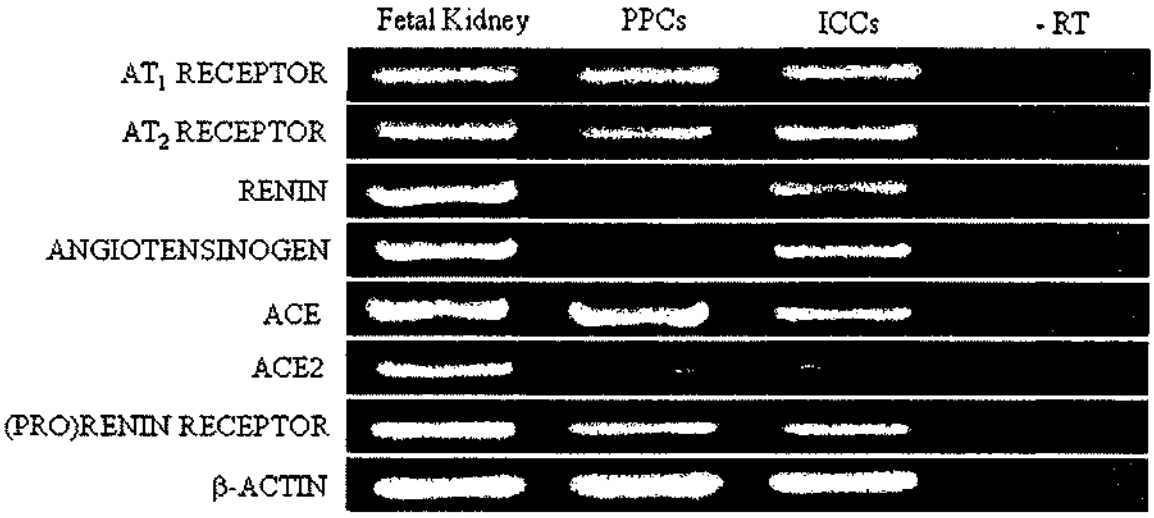


Figure IV. 3.1. Expression of major RAS components in PPC/ICC culture. The mRNA expressions of *AT_1 RECEPTOR*, *AT_2 RECEPTOR*, *RENIN*, *ANGIOTENSINOGEN*, *ACE*, *ACE2* and *(PRO)RENIN RECEPTOR* in PPCs and ICCs. Human fetal kidney was used as a positive control.

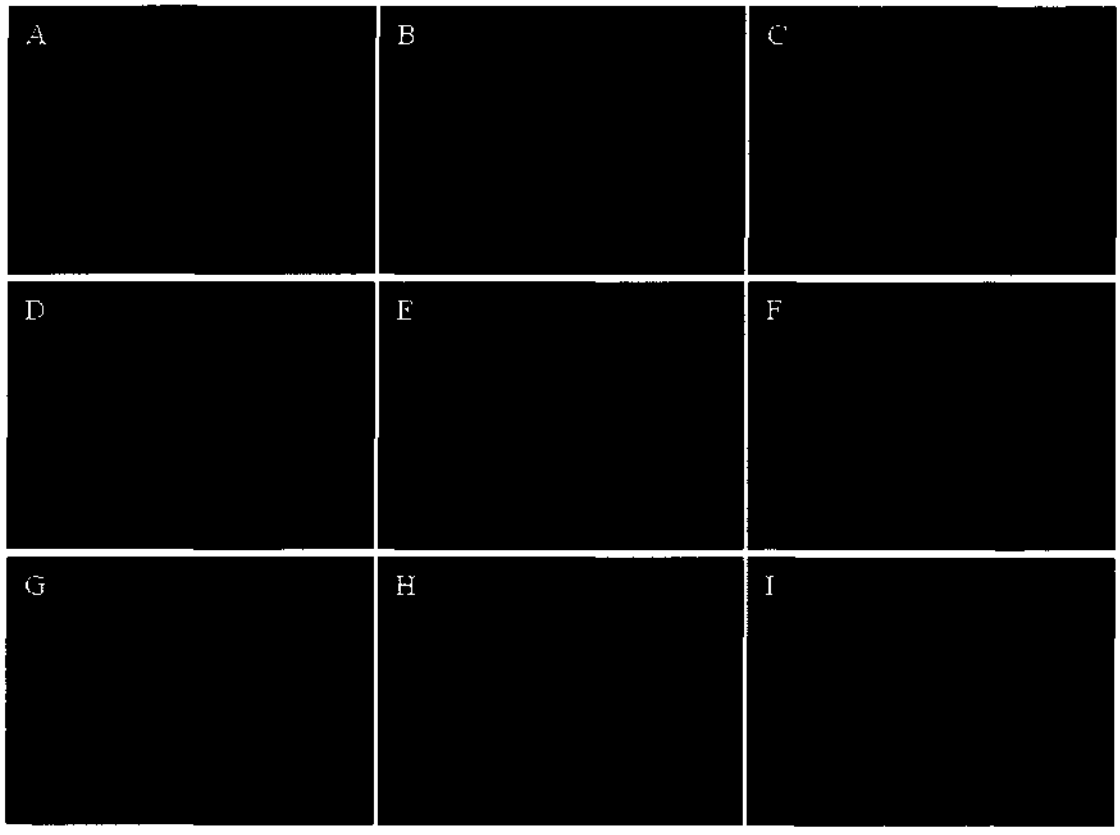


Figure IV. 3.2. Immunofluorescent staining of (*A*) AT_1 receptor, (*D*) AT_2 receptor and (*B, E*) Ngn3 in the PPC culture. (*G-I*) Negative controls were produced by omitting primary antibodies. Original magnification: 630×; Scale bar: 40 μm.

IV. 3.2 Expression of RAS Components is Highly Regulated during PPC Differentiation into ICCs.

To elucidate potential involvement of a local RAS in directing PPC differentiation towards an endocrine lineage, we first investigated whether RAS component expression is regulated during PPC differentiation. Immunocytochemistry revealed endogenous secretion of Ang II in differentiated ICCs (Figure IV. 3.3 *A and B*), as well as intense co-localization of Ang II with cells positive for the mature β-cell phenotypic marker pancreatic and duodenal homeobox (Pdx)-1 (Melloul et al., 2002), within ICCs (Figure IV. 3.3 *C-H*). We then examined the temporal expression profile of other RAS components serving as precursors for Ang II generation during the 8-d PPC differentiation period. Real-time PCR data showed that the mRNA expression levels of *ANGIOTENSINOGEN*, *RENIN* and *ACE* were temporally regulated (Figure IV. 3.4 *A-C*). Additionally, an ELISA detected endogenous production of prorenin with a gradual drop in secretion near the end of the differentiation period (Figure IV. 3.5). Given the high level of Ang II precursors that were detected at the start of differentiation, we expected that a considerable quantity of endogenous Ang II would be detected late in the differentiation process. We thus assessed the amount of both secretory Ang II in the culture media and intracellular Ang II using an Ang II EIA kit. As expected, the level of both secretory and intracellular Ang II was

minimal in the early stage of differentiation, but increased as differentiation proceeded (Figure IV. 3.6). The amount of cellular Ang II, in particular, was noted to be profoundly increased in the late stages of differentiation (~8-fold on day 8), consistent with intracellular production of Ang II as reported elsewhere (Singh et al., 2008). The detection of intracellular Ang II supports the view that Ang II may mediate intracrine actions and further implies that it engages in regulation of the expression of the various transcription factors that drive the ICC differentiation process.

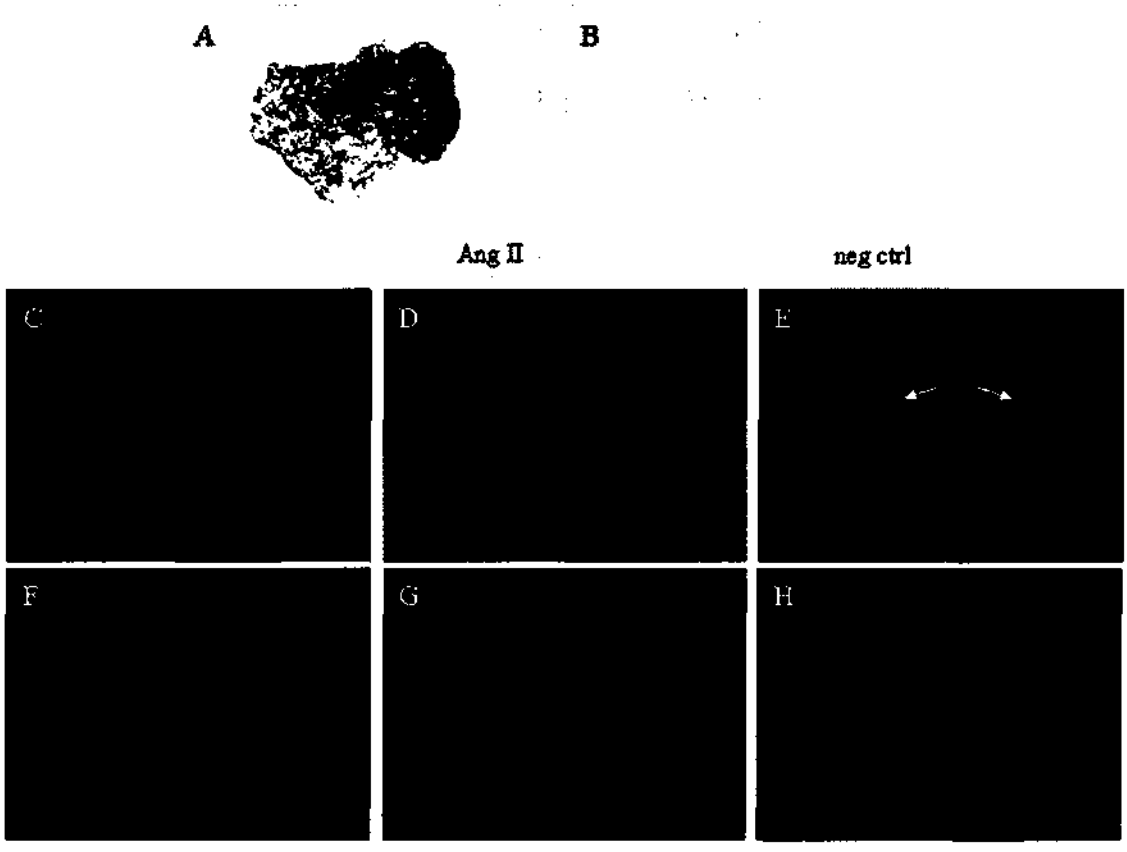


Figure IV. 3.3. Localization of (*A,C*) Ang II and (*D*) Pdx-1 in the differentiated ICCs. Arrows indicated cells co-localized with Ang II and Pdx-1. (*B, F-H*) Negative controls were produced by omitting of primary antibodies. Original magnification: 630×; Scale bar: 40 μm.

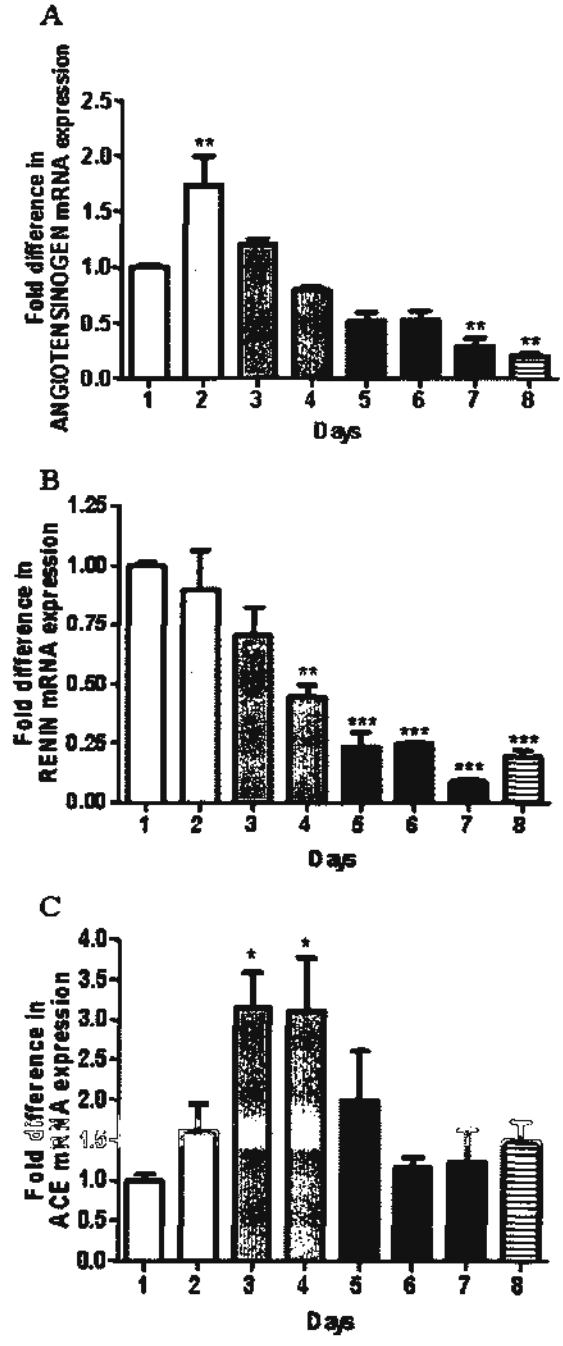


Figure IV. 3.4. The mRNA expression profile of (*A*) *ANGIOTENSINOGEN*, (*B*) *RENIN* and (*C*) *ACE* during the 8-d PPC differentiation period. All data are expressed as means ± S.E.M. n = 5 in each group. * $p<0.05$, ** $p<0.01$, *** $p<0.001$ vs day 1 ICCs.

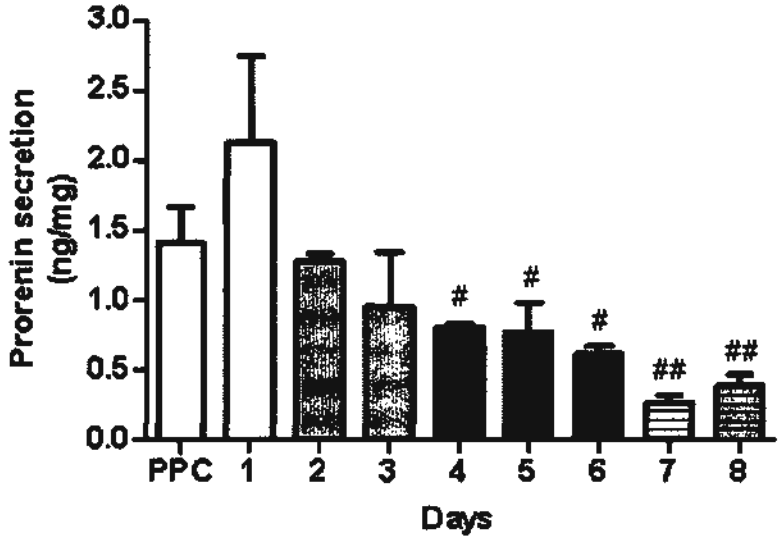


Figure IV. 3.5. Secretion of prorenin during PPC differentiation into ICCs. All data are expressed as means ± S.E.M. n = 6 in each group. # $p<0.05$; ## $p< 0.01$ vs day 1 ICCs.

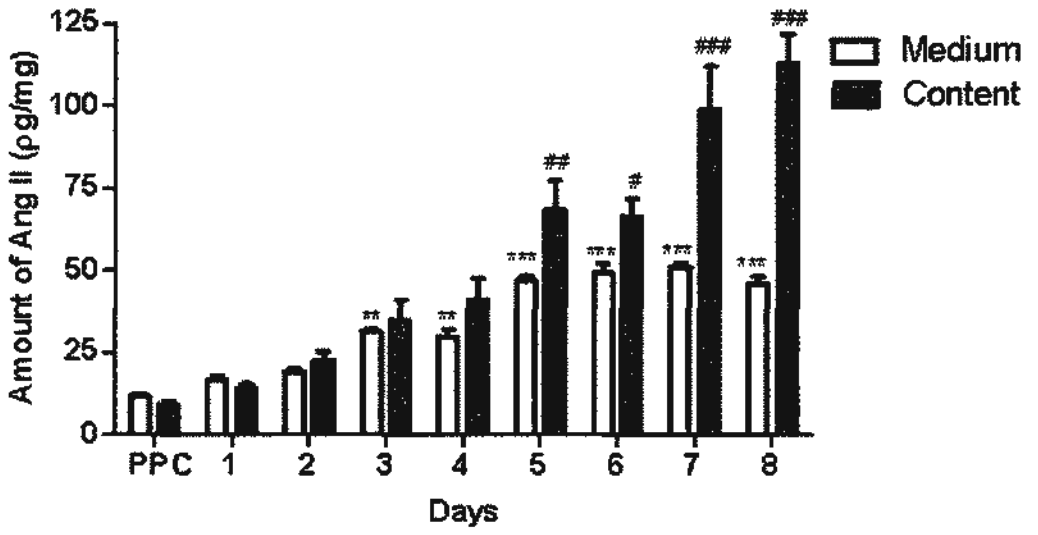


Figure IV. 3.6. Quantification of the amount of Ang II in the culture medium and cell contents during PPC differentiation into ICCs. All data are expressed as means ± S.E.M. ** $p<0.01$; *** $p<0.001$; # $p<0.05$; ## $p<0.01$; ### $p<0.001$ vs PPCs. n = 6 each group.

In order to correlate the changes of expression of these RAS components in PPC/ICC development, we examined in detail the expression profile of transcriptional regulators of β-cell development during the 8-d PPC differentiation period. *NGN3*, which encodes a functional marker for endocrine cell precursors (Desgraz and Herrera, 2009), was found to be profoundly upregulated throughout the differentiation period (Figure IV. 3.7 *B and I*), and thus attested PPC development toward an endocrine lineage. Of particular interest is the significant upregulation of transcripts for mature β-cell markers: *NKX6.1*, *PDX-1* and *INSULIN*. The sustained high expression levels of *PDX-1* and *INSULIN* on day 3 of differentiation (Figure IV. 3.7 *F and G*) were concomitant with an upregulation of endogenous Ang II production (Figure IV. 3.6) and thus suggestive of a role for Ang II in the expression of these mature β-cell phenotypic markers.

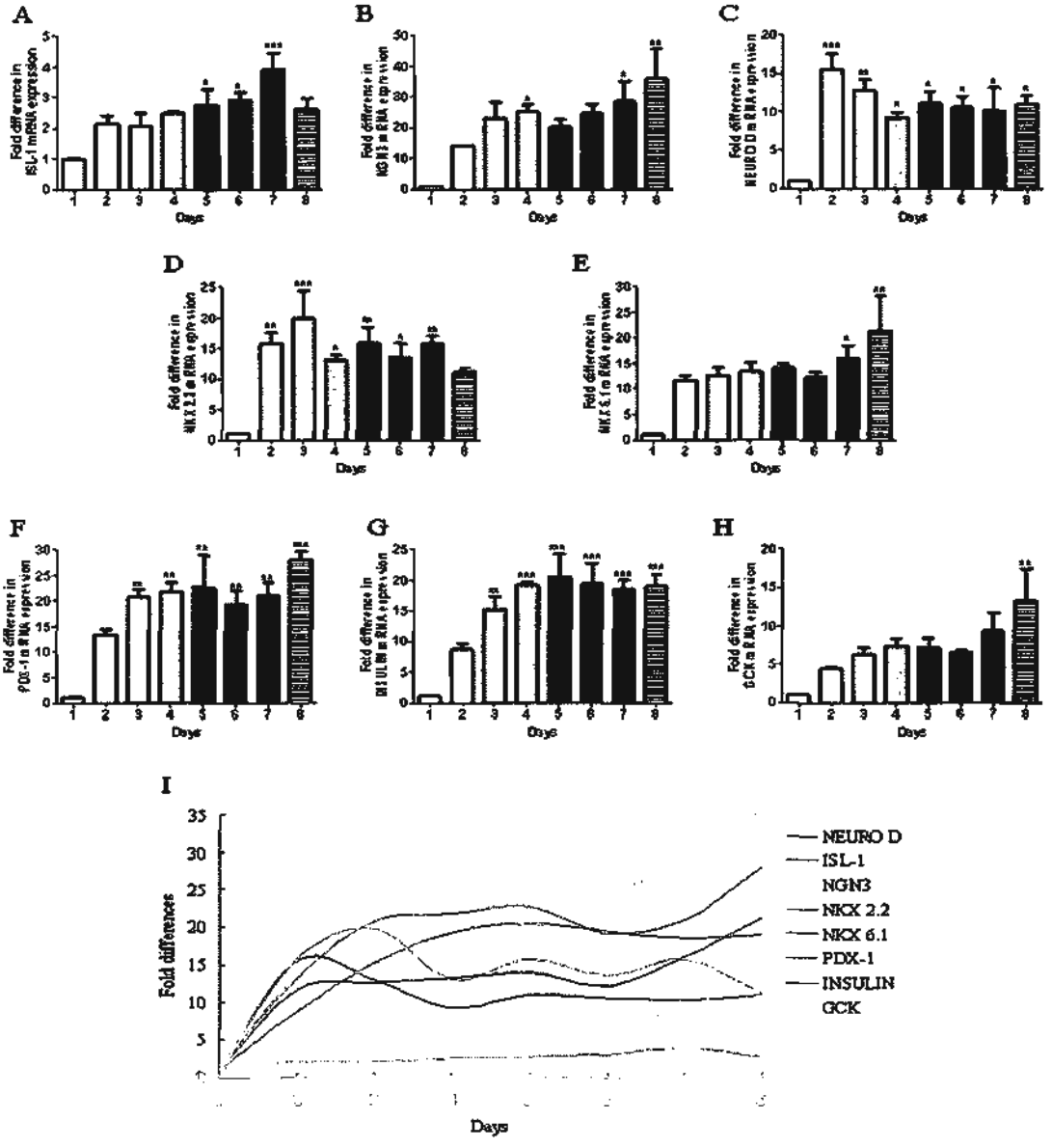


Figure IV. 3.7. Characterization of the expression profile of β-cell transcription factors during PPC differentiation. Changes in mRNA expression levels of the following major β-cell transcription factors during the 8-d PPC differentiation period: (*A*) *ISL-1*, (*B*) *NGN3*, (*C*) *NEURO D*, (*D*) *NKX 2.2*, (*E*) *NKX 6.1*, (*F*) *PDX-1*, (*G*) *INSULIN* and (*H*) *GLUCOKINASE*. (*I*) Summary of fold changes for these β-cell transcription factors during the differentiation period. All data are expressed as means ± S.E.M. n = 6 in each group. $*p < 0.05$; $**p < 0.01$; $***p < 0.001$ *vs.* day 1 ICCs.

We next sought to find out whether similar changes of expression occur in the two major receptors of the RAS, AT_1 and AT_2, during PPC differentiation. Interestingly, we observed intense upregulation of *AT_2 RECEPTOR* mRNA expression in cultures on day 2 till the end on day 8 with approximately 150 folds higher than that derived from pre-differentiation cultures. Meanwhile *AT_1 RECEPTOR* mRNA remained relatively constant throughout the entire differentiation period (Figure IV. 3.8). These data suggest that the AT_2, but not the AT_1, receptor may play an important role in regulating PPC differentiation.

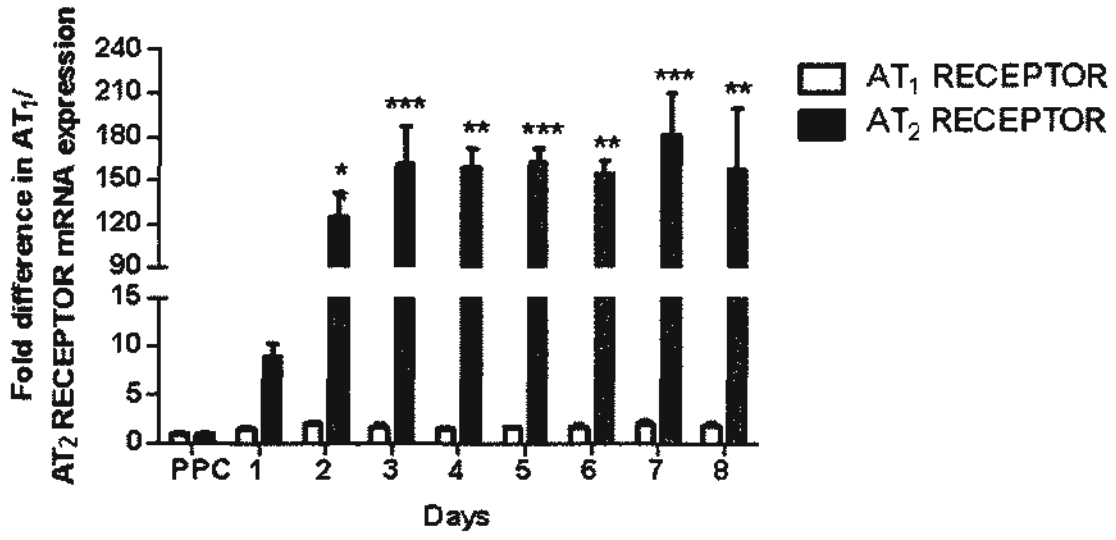


Figure IV. 3.8. mRNA expression profile of *AT_1 RECEPTOR* and *AT_2 RECEPTOR* during PPC differentiation into ICCs. All data are expressed as means ± S.E.M. * $p<0.05$; ** $p<0.01$; *** $p<0.001$ vs PPCs. n = 5 in each group.

IV. 3.3 Evidence for a Regulated Local RAS Expression during Differentiation of hESCS into Pancreatic Hormone-expressing Cells.

We subsequently examined whether RAS components are present and display differential expression in human embryonic stem cells (hESCs) in culture. We followed D'Amour et al.'s (2006) five-stage protocol to induce production of hormone-expressing endocrine cells from hESCs H1. *PDX-1* and *NGN3* mRNA levels were studied to evaluate the differentiation into the endocrine lineage. RT-PCR detection revealed the expressions of *RENIN*, *ANGIOTENSINOGEN*, AT_1 *RECEPTOR* and AT_2 *RECEPTOR* in the undifferentiated hESCs (Figure IV. 3.9 *A*). An upregulation of gene expressions of all these components was noted as the differentiation commenced and proceeded, and their upregulated expression was maintained throughout the whole development (Figure IV. 3.9 *B and C*). The magnitude of the increase in AT_2 *RECEPTOR* mRNA expression was markedly greater than that of the AT_1 *RECEPTOR*. It is noteworthy that AT_2 *RECEPTOR* expression was profoundly upregulated during stage 3 of the development, being similar to the gene pattern observed in the posterior foregut (D'Amour et al., 2006). This upregulated expression was maintained until hormone-expressing endocrine cells were formed in the final stage of development (~8000-fold *vs.* undifferentiated H1 hESCs). (Figure IV. 3.9 *B*). Interestingly, *RENIN* and *ANGIOTENSINOGEN* transcripts were

markedly elevated during stage 5, the period during which endocrine cells were present and hormone production could be detected (Figure IV. 3.9 *C*). We can hardly rule out that an enhanced local production of Ang II is attributable to a likely up-regulation of RAS precursors, which in turn regulates the maturation of these hormone-producing cells.

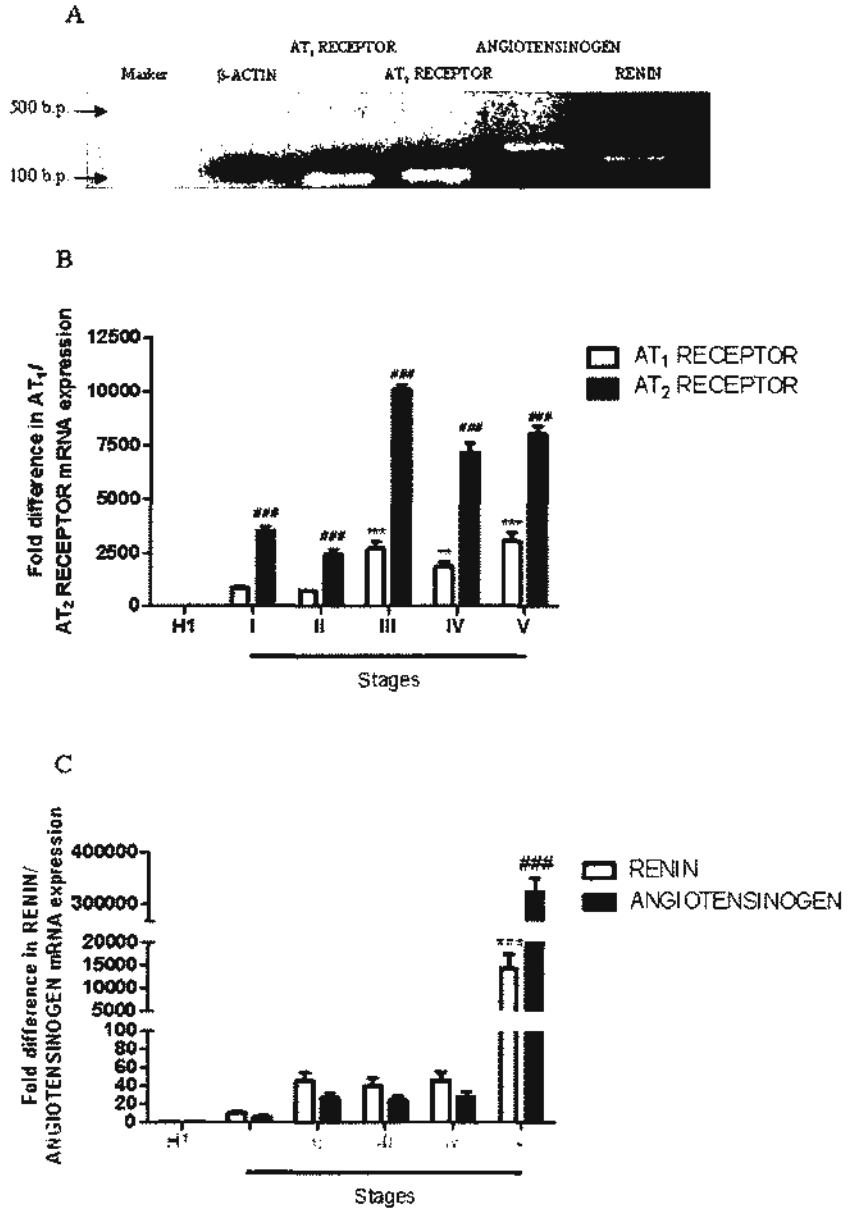


Figure IV. 3.9. Characterization of the expression profiles of major RAS components during differentiation of H1 hESCs into hormone-producing cells. (*A*) RT-PCRs showing the presence of *AT_1 RECEPTOR*, *AT_2 RECEPTOR*, *ANGIOTENSINOGEN* and *RENIN* in undifferentiated H1 hESCs. (*B* and *C*) Changes in mRNA expression levels of these RAS components during the five-stage period during which they differentiate into endocrine cells. All data are expressed as means ± S.E.M. n = 3 in each group. $^{**}p < 0.01$; $^{***}p < 0.001$; $\#\#\#p < 0.001$ *vs.* undifferentiated H1 hESCs.

IV. 3.4 Co-localization of AT_2 receptor, but Not AT_1, with Mature β-cell Markers in Differentiated ICCs.

We next evaluated whether AT_1 and AT_2 receptors are potentially involved in regulating PPC differentiation into ICCs. Immunocytochemistry showed that mature β-cell markers namely Nkx 6.1, proinsulin and insulin, were localized to the differentiated ICCs (Figure IV. 3.10 and 3.11). The presence of proinsulin in ICCs provides evidence of their ability to synthesize insulin. In line with readouts of real-time RT-PCR analyses, ICCs showed a more intense immunoreactivity for the AT_2 receptor than for the AT_1 receptor (Figure IV. 3.10 and 3.11). Interestingly, we detected cells within ICCs expressing Nkx6.1, pro-insulin or insulin with an intense co-localization of AT_2 receptor expression (Figure IV. 3.11), but no detectable expression of AT_1 receptor (Figure IV. 3.10). This observed pattern of expression is consistent with the possibility that the AT_2 receptor in particular may play an important role in the differentiation of pancreatic progenitors toward a β-cell lineage.

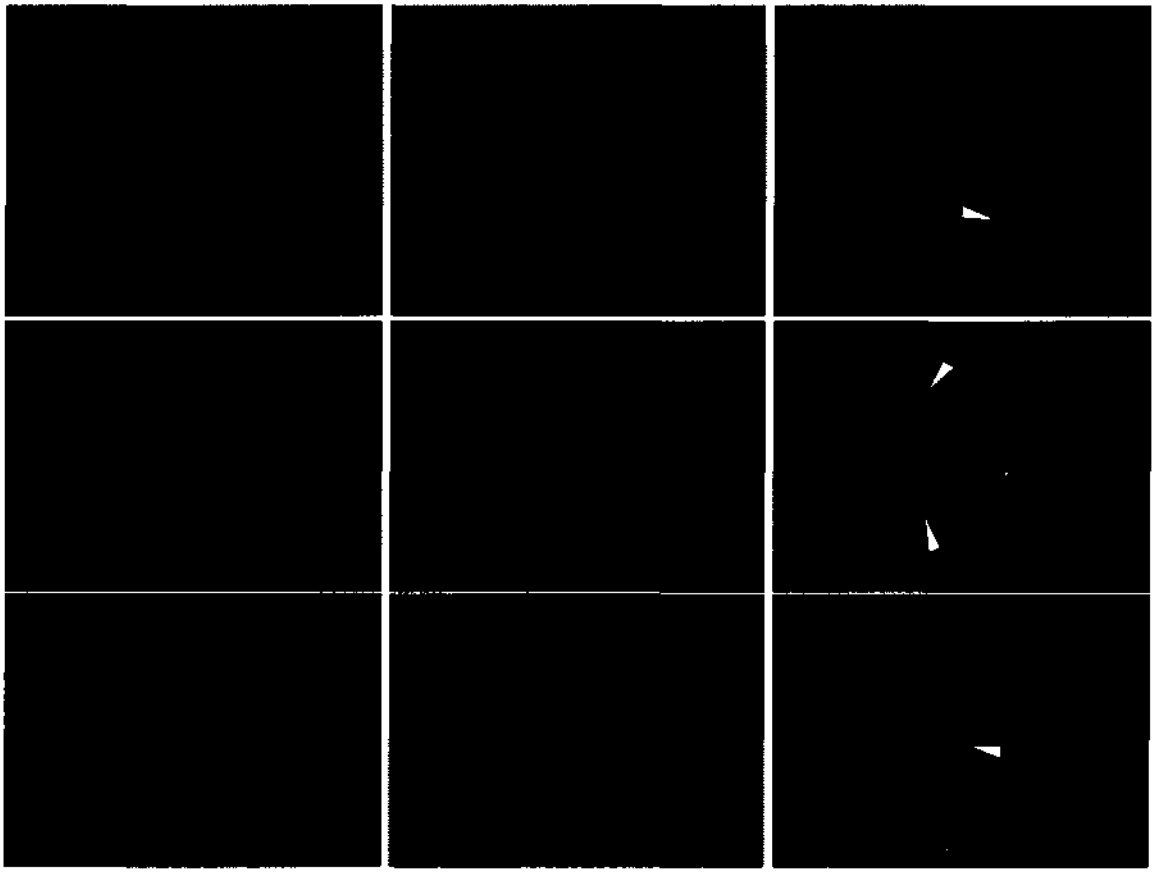


Figure IV. 3.10. Localization of AT_1 receptors with mature β-cell markers (Nkx 6.1, proinsulin and insulin) in differentiated ICCs. Arrowhead: cells expressing β-cell markers only. Original magnification: 630×; Scale bar: 40 μm.

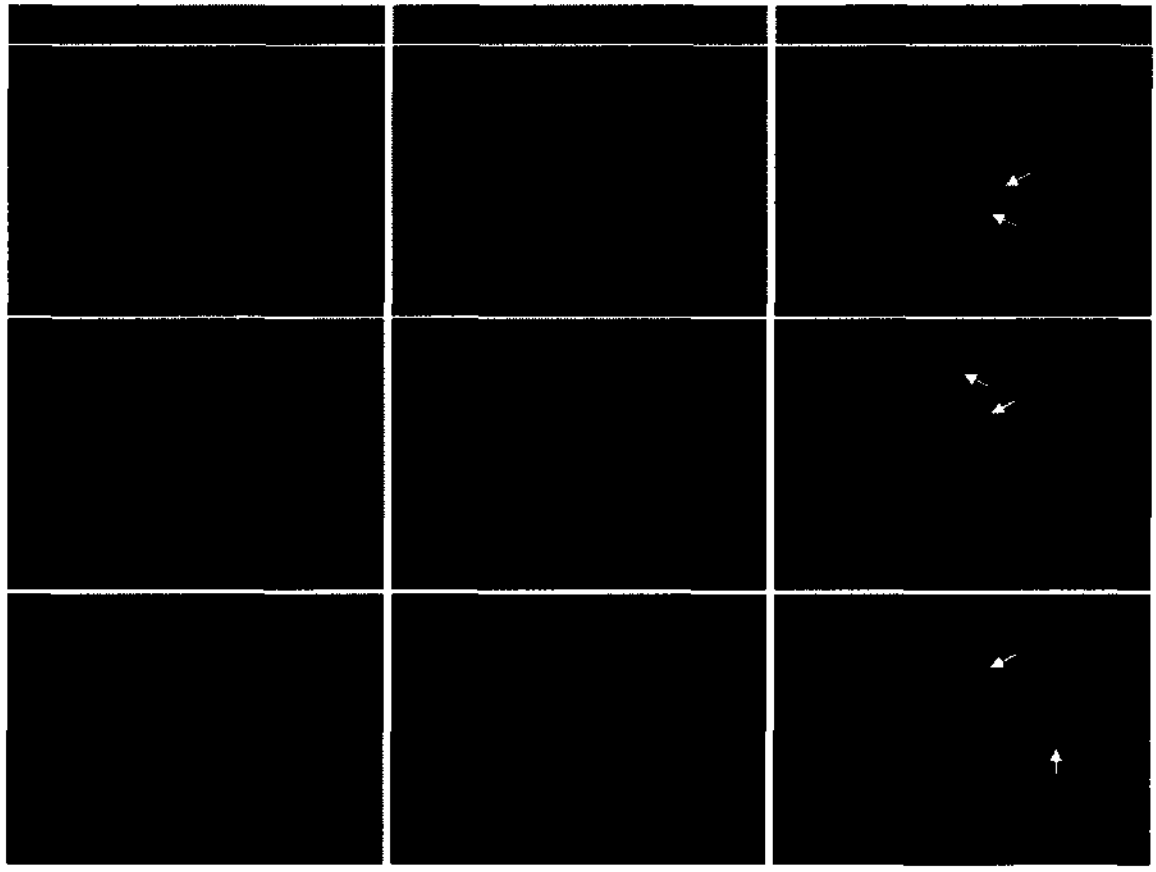


Figure IV. 3.11. Localization of AT_2 receptors with mature β-cell markers (Nkx 6.1, proinsulin and insulin) in differentiated ICCs. Arrow: cells co-expressing β-cell markers and AT_2 receptors. Original magnification: 630×; Scale bar: 40 μm.

IV. 3.5 Expression of AT_1 and AT_2 receptors is Temporally Regulated in PPCs Derived from Different Gestational Weeks of Human Fetus.

We next sought to investigate whether such regulated expressions of the RAS components could be observed across different gestational weeks of PPCs. Detection by Western blot revealed a temporally regulated expression of both AT_1 and AT_2 receptors in PPCs from the 9^{th}-15^{th} gestational weeks, with peak expression levels being observed at gestational weeks 12 to 13 (Figure IV. 3.12 *A and B*). This possibly reflected the involvement of both receptors in regulating particular developmental events of the pancreatic progenitors at these gestational periods.

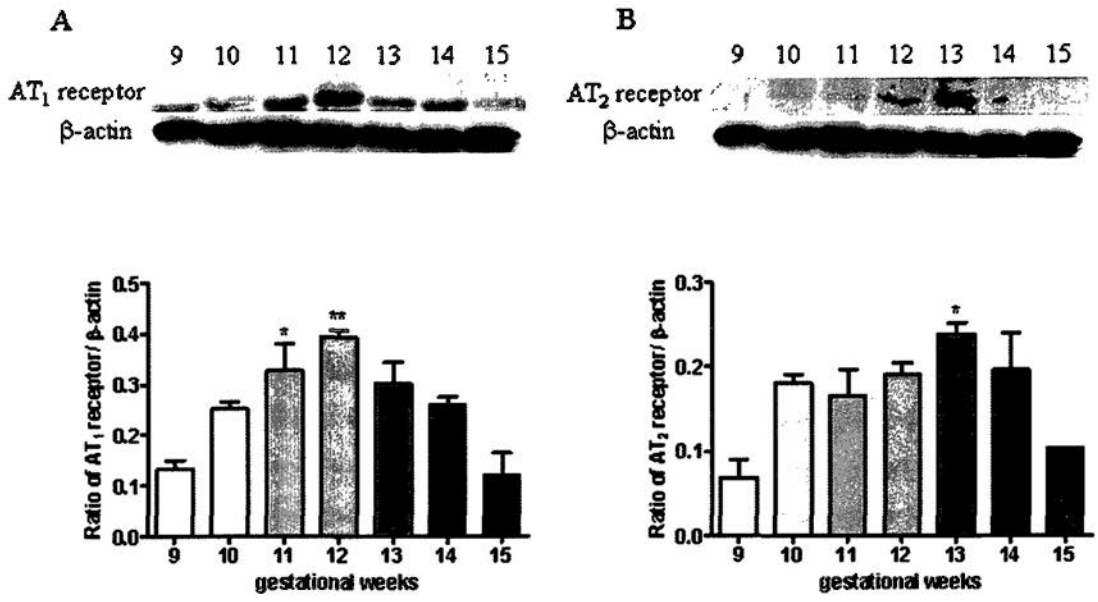


Figure IV. 3.12. Temporal expression profiles of (*A*) AT_1 receptor and (*B*) AT_2 receptor in PPCs derived from fetuses (9-15 wks gestational age). All data are expressed as means ± S.E.M. n = 4 in each group. $*p < 0.05$; $**p < 0.01$ vs. 9^{th}-week PPCs.

IV. 4 Discussion

We have previously established a population of PPCs derived from human fetal pancreas using a basic protocol developed for differentiating PPCs into ICCs; such differentiation process is subject to stimulation by morphogenic factors (Suen et al., 2008; Leung et al., 2009; Ng et al., in press). To further elucidate the nature of our human fetal pancreas derived PPCs as multipotent progenitors, we carried out a series of flow cytometry analyses in this study. PPCs derived from human fetal pancreata (9-14 wks gestational age) were enriched with surface markers expressed by mesenchymal stem cells (MSCs) including CD29, CD44, CD73, CD90, CD105 and CD166. We observed no significant differences in the expression patterns of these components across different gestational weeks of PPCs. The absence of markers designating cells as immune-related cells confirmed that the culture should be void of contamination from haemic components (Table IV. 4.1). These data confirm the MSC nature of our PPCs, which possess the multipotentiality to differentiate into hormone-expressing cells both *in vitro* and *in vivo* as reported elsewhere (Davani et al., 2007; Gallo et al., 2007).

Surface marker	Cell type	Amount positive (%)
CD29 (Integrin beta-1)	MSC	100
CD44	MSC	100
CD73 (5'nucleotidase, ecto)	MSC	100
CD90 (Thy-1)	MSC	91.97 ± 5.34
CD105 (Endoglin)	MSC	84.75 ± 0.85
CD166 (Activated leukocute cell adhesion molecule)	MSC	100
CD3	T-cell	0
CD16 (Fc fragment of IgG)	NK cell	0
CD19	B-cell	0
CD33	Myeloid cell	0
CD38 (Cyclic ADP ribose hydrolase)	Myeloid cell	0
CD45 (Protein tyrosine phosphatase receptor type C)	Haemic cell	0
HLA-DR	MHC	0
CD34	HSC	2.12 ± 0.82
CD133 (Prominin 1)	HSC	0

Table IV. 4.1. Characterization of the expressions of different surface markers of the human fetal PPCs from 9^{th}-14^{th} gestational weeks. (n = 5)

HSC: Hematopoietic stem cell; MHC: Major histocompatibility complex; MSC: Mesenchymal stem cell; NK: Natural killer

Here we have presented evidence indicating that a local RAS is expressed in a temporally regulated manner during the development of pancreatic progenitors toward a β-cell lineage. Of particular interest during PPC differentiation was the marked transcriptional upregulation of *NGN3*, a well established islet progenitor marker (Gradwohl et al., 2000; Desgraz et al., 2009), in the differentiated ICCs (~36-fold *vs.* day 1). This result confirms the efficacy of our morphogenic cocktail in profoundly directing differentiation of the non-endocrine population of PPCs toward an endocrine cell fate.

Although it was once thought that Ngn3 expression ceases in mature β-cells (White et al., 2008), recent data support the importance of sustained Ngn3 expression both in promoting endocrine maturation and maintaining islet function (Wang et al., 2009). One notable finding in the present report is the significant upregulation of AT_2 receptor during the PPC differentiation. This highlights the possibility of a critical role of AT_2 receptor in maintaining normal endocrine development. Of note, we noticed similar observations, in addition to the regulated expression of other RAS components, in a differentiating hESC culture towards the pancreatic hormone-producing cells. The upregulated AT_2 receptor expressions were detected even at earlier stages of development before endocrine progenitors appeared. These observations, which were acquired from a pure stem cell culture, provided excellent supportive evidence for AT_2 receptor in pancreatic endocrine

cell development. Furthermore, our observation that AT_2, but not AT_1, receptors co-localize with mature β-cell markers in the ICCs suggests that the AT_2 receptor itself may be a hitherto unrecognized marker for β-cell progenitors, similar to ACE, which has also been suggested to identify a stem cell niche of hematopoietic tissues (Jokubaitis et al., 2008).

Though RAS governance of PPC development during pancreas organogenesis *in vivo* has not been confirmed, we did obtain fundamental observations of the expression profiles of AT_1 and AT_2 receptors in PPCs across gestational weeks. The peak expression levels of the two receptors were noted on the PPCs from 12^{th}-13^{th} gestational weeks, the time period during which endocrine cells aggregate into primitive islet structures is initiated in the developing human pancreas (Piper et al., 2004). These RAS components could function locally to regulate islet formation processes.

Taken together all these observations in the present study, we concluded that major components of a local RAS is temporally expressed and regulated during the development of pancreatic progenitors into the β-cell lineage, and that the endogenously generated Ang II might potentially regulate such differentiation and maturation processes. The effects of Ang II on the transcriptional dynamics of different endocrine cell phenotypic factors and their possible mechanisms, particularly by AT_2 receptor, warrant further investigations.

Chapter V

Involvement of Angiotensin II/ Angiotensin II Type 2 Receptor in the Development of Human Fetal Pancreatic Progenitor Cells into Islet-like Cell Clusters and their Potential for Transplantation

[Some content of this chapter have been modified and submitted to *Stem Cells*]

V. 1 Introduction

The correlation of RAS with the development of tissue progenitors has been supported by some recent findings that RAS antagonism in pregnant animals could inhibit fetal RAS and cause fetal anomalies (Grove et al., 1995; Hård et al., 2008; Sánchez et al., 2008). Moreover, clinical reports have attributed developmental toxicity during human pregnancy to RAS blockade (Serreau et al, 2005; Gersak et al., 2009). These phenomena could be explained by a disturbance of the developmental program of progenitor cells residing in specific organs. Whether a RAS functions locally that governs the differentiation process of those progenitors into their target cell types warrants further investigations.

Angiotensin II (Ang II), the active peptide of the RAS, is a key component that mediates most of the RAS-induced physiological functions through binding with Ang II type 1 (AT_1) and type 2 (AT_2) receptors. Direct evidence for the Ang II-induced proliferation, differentiation or even nutrient metabolism of stem/progenitor cells has been reported elsewhere (Rodriguez-Pallares et al., 2004; Han et al., 2005; Kim and Han, 2008; Kim et al., 2008; Kim et al., 2010). These Ang II-induced effects could be mediated by different intermediate signaling molecules that are important for the cell survival and normal development (Schmitz and Berk, 1997; Zhang et al., 2004; Shi et al., 2009).

Based on the findings in chapter IV that a complete local RAS is expressed in the

human fetal pancreatic progenitor cells (PPCs), and is temporally regulated during the differentiation of PPCs into islet-like cell clusters (ICCs), we attempted to study the potential role Ang II in regulating the growth and differentiation of PPCs. Changes in the endocrine cell phenotypic factor expression (Oliver-Krasinski and Stoffers, 2008) will be monitored through manipulations of the specific RAS components. Functional performance of the differentiated ICCs will also be assessed. Using RAS blockers or lentivirus-mediated knockdown, we were able to demonstrate that Ang II, generated by the local RAS, indispensably mediated this transcription factor expression via AT_2 receptor signaling pathway and thus directed PPC differentiation toward an endocrine lineage.

The present work provides a novel perspective of the pancreatic RAS, in which some of its specific components may act as a mediator in PPC development, and thus provides insight into an undiscovered mechanism for regulating islet neogenesis. Data in this study will be informative in the development of novel protocols for promoting the functional maturation of PPC-derived islets for transplantation into diabetic patients. Given the modest regenerative capacity of the pancreas (Bouwens, 2006; Nir et al., 2007), pharmacological manipulations of the pancreatic RAS components that promote PPC differentiation would also be valuable for triggering β-cell renewal in diabetic patients.

V. 2 Materials and Methods

V. 2.1 Human Fetal Tissue Procurement and Tissue Processing

Procurement of human fetal tissues and the procedures for subsequent tissue digestion were described in detail in sections II. 1.1 and II. 1.2 (pp. 79-80).

V. 2.2 Culture of Human Fetal Pancreatic Progenitor Cells (PPCs) and their Differentiation into Islet-like Cell Clusters (ICCs)

The protocols for PPC culture, as well as their differentiation to ICCs were described in section II. 1.3 (pp. 80-81) with some modifications as described in section IV. 2.2 (pp. 144).

V. 2.3 Animals and Induction of Diabetes

BALB/c nude mice were obtained from the Laboratory Animal Services Centre of the CUHK. Husbandry of the animals has been described in Section II. 2.1 (pp. 83). Young adult (8-10 wk-old) male nude mice were used for induction of diabetes. Freshly prepared STZ (Sigma-Aldrich, St. Louis, MO, USA), dissolved in chilled 0.05 M citrate buffer (pH 4.5), was intraperitoneally injected at a multiple doses of 75 mg/kg body weight for 5 consecutive days (Kroon et al., 2008). Only STZ-treated mice that reached blood glucose

levels >16.7 mM (~300 mg/dl) for at least 7 consecutive days were used in the transplantation studies.

V. 2.4 Lentiviral Transduction and Analysis of PPCs/ICCs

A knockdown of AT_2 receptor by lentiviral delivery of short hairpin RNAs (shRNAs) was performed in the PPCs/ICCs. The mechanism of RNA interference (RNAi) in a mammalian cell is illustrated in Figure V. 2.1. Briefly, PPCs were plated at 2.5 x 10^4 cells/well in 24-well plates, in triplicate, and cultured overnight with full RPMI medium supplemented with 20 ng/ml each of basic fibroblast growth factor (bFGF; Sigma-Aldrich) and epidermal growth factor (EGF; Invitrogen, Carlsband, CA, USA). Upon reaching a cell confluency of ~70%, the PPCs were first transduced by incubation with MISSION® shRNA control lentiviral particles (pLKO.1-puro) expressing TurboGFP (Sigma-Aldrich) for 18-20 h at MOIs of 0.5, 1, 2 and 5, supplemented with 8 μg/ml hexadimethrine bromide (Polybrene®; Sigma-Aldrich) to enhance transduction. The TurboGFP lentiviral particles was incorporated to allow visual confirmation of successful transduction and to optimize MOIs for transduction. An optimal transduction efficiency was noted with an MOI = 2 (~50% PPCs positive for GFP after transduction) (Fig. V. 2.2). Similar transduction procedures were then performed on PPCs with lentiviral particles targeting

AT_2 receptor (sh-AT_2R) or lucifarase (sh-luci) using an MOI = 2 (Detailed insert sequences of the lentiviral particles are listed in Table V. 2.1, pp. 183). The lentivirus-containing medium was replaced and the transduced cells were incubated overnight with fresh, full RPMI medium supplemented with bFGF and EGF. Because the vector contained a puromycin resistance gene, the medium was then replaced again with 0.8 μg/ml puromycin (Sigma-Aldrich) and incubated for 4 d (Optimization of time and dosage for puromycin treatment was obtained by constructing a puromycin kill curve prior to the transduction experiments, detailed procedures see below at section V. 2.5, pp. 184-185), with the medium changed every other day, to allow for selection. The untransduced PPCs were eliminated by this puromycin treatment and the puromycin-resistant clones were then exposed to fresh, full RPMI medium supplemented with bFGF and EGF, expanded, and harvested for assessment of AT_2 receptor-knockdown efficiency by western blotting. The transduced PPCs were expanded for differentiation into ICCs for further analyses.

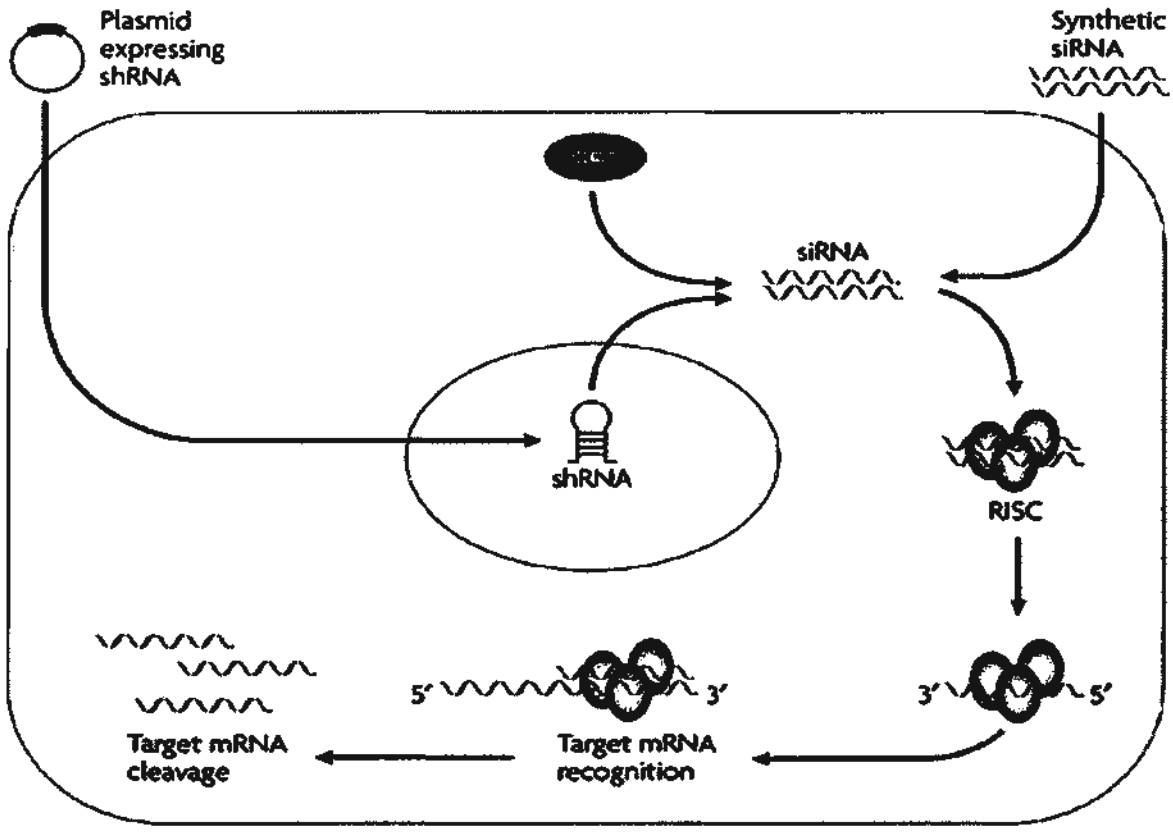


Figure V. 2.1. Mechanisms of RNAi in a mammalian cell. shRNAs that are encoded by plasmids are transcribed by RNA polymerase III. The enzyme, Dicer, initiates the RNAi pathway by cleaving the shRNAs into small-interfering RNAs (siRNAs) duplexes. Alternatively, synthetic siRNAs can also be synthesized chemically and introduced directly into the cell by transfection reagents or electroporation. siRNAs are then incorporated into the RNA-induced silencing complex (RISC), a multiprotein endoribonuclease. A helicase within RISC unwinds duplex siRNA allowing its antisense strand to bind mRNA with a high sequence complementarity. An RNase within RISC degrades the target mRNA by cleavage, contributing to a silenced gene expression and reduced protein production. (Extracted from Iorns et al., 2007).

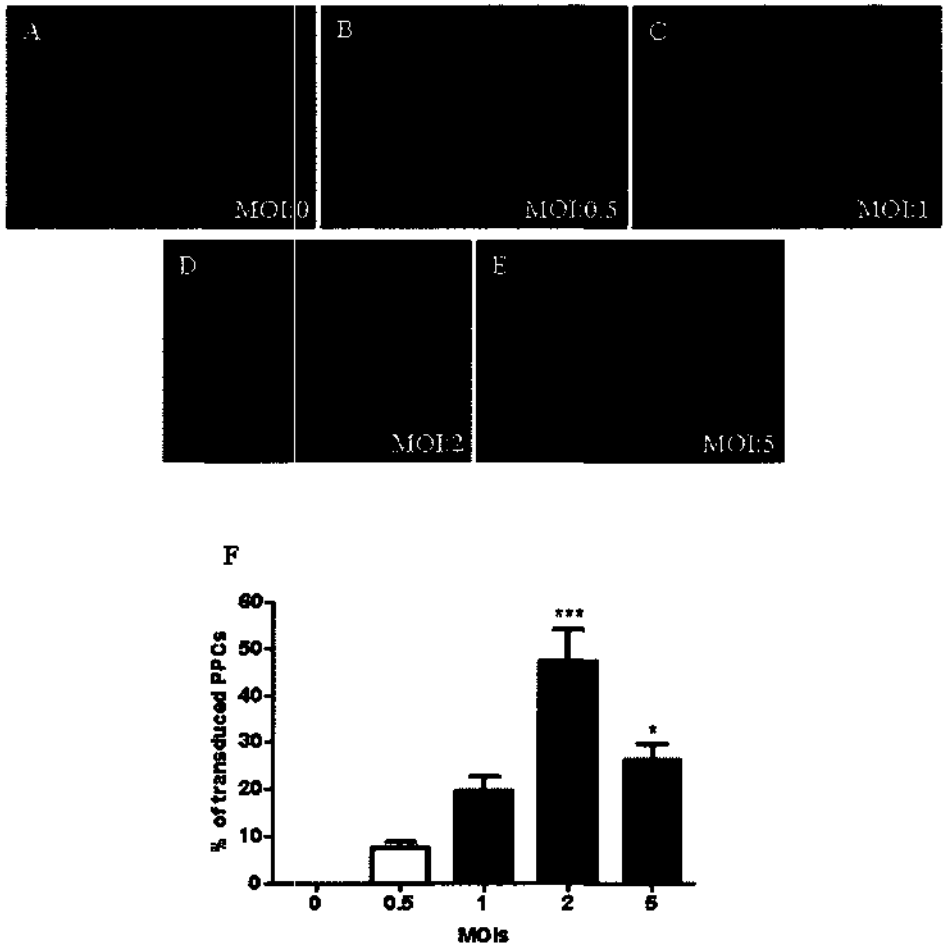


Fig. V. 2.2. Optimization of MOIs for lentiviral transduction of PPCs. (*A-E*) Fluorescent images showing the GFP signal of the PPCs transduced with lentiviral vectors expressing TurboGFP with different MOIs. (*F*) Percentage of transduced PPCs before puromycin selections evaluated by counting the number of GFP-positive cells within a single microscopic view. n = 10 in each group. Original magnification 400×; Scale bar: 40 μm. $*p < 0.05$; $***p < 0.001$ *vs.* MOI = 0.

Description	Insert	Insert sequence
MISSION® control transduction particles, TurboGFP	TurboGFP	CCGGCGTGATCTTCACCGACAAGATCTCG AGATCTTGTCGGTGAAGATCACGTTTTT
MISSION® luciferase shRNA control transduction particles	shRNA targeting luciferase	CCGGCGCTGAGTACTTCGAAATGTCCTCG AGGACATTTCGAAGTACTCAGCGTTTTT
MISSION® human AT_2 receptor shRNA transduction particles (5 clones)	shRNA targeting AT_2 receptor	
I		CCGGCCACCTGAGAAATATGCCCAACTCG AGTTGGGCATATTTCTCAGGTGGTTTTT
II		CCGGGTATGATTCTATGGAGCTATTCTCGA GAATAGCTCCATAGAATCATACTTTTT
III		CCGGTCTTCCTCTATGGGCAACCTACTCG AGTAGGTTGCCCATAGAGGAAGATTTTT
IV		CCGGGCAGTGTGTTTAGGGTTCCAACTCG AGTTGGAACCCTAAACACACTGCTTTTT
V		CCGGGCTGCGTTAATCCGTTTCTGTCTCG AGACAGAAACGGATTAACGCAGCTTTTT

Table V. 2.1. Details of the insert sequences of the lentiviral shRNA vectors.

V. 2.5 Optimization of Puromycin Treatment on PPCs

Prior to lentiviral transduction experiments, the concentration of puromycin used for selection was determined by generating a puromycin kill curve. 1.6×10^4 cells were plated in triplicate into wells of a 96-well plate. Medium was replaced the next day with fresh medium containing 0, 0.2, 0.4, 0.6, 0.8, 1.0 and 2.0 μg/ml puromycin. Cell viability was assessed each day for 5 d by (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay (Sigma-Aldrich) for mitochondrial activity as previously described (Suen et al., 2008). Briefly, medium was replaced with MTT solution (1 mg/ml in PBS, sterilized by filtration). The MTT solution was aspired after a 4-h incubation at 37°C and the formazan was dissolved by addition of 100 μl DMSO (Sigma-Aldrich). The absorbance was measured at wavelength 540 nm. The puromycin kill curve generated on PPCs is shown as Fig. V. 2.3.

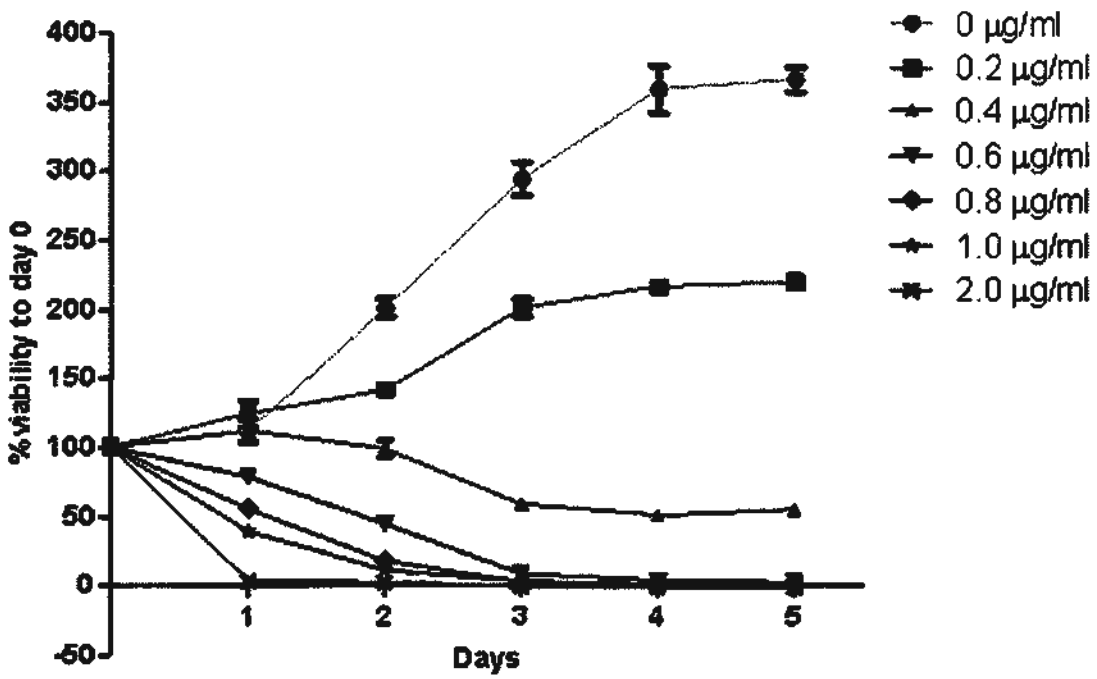


Fig. V. 2.3. A puromycin kill curve on PPCs. Different concentrations of puromycin (0, 0.2, 0.4, 0.6, 0.8, 1.0 and 2.0 µg/ml) were applied on PPCs and their viability was monitored by MTT assay for 5 d. A minimum amount of puromycin (0.8 µg/ml) that caused complete cell death after 4 d was chosen for an optimized time and dosage for the subsequent transduction experiments. All data are expressed as means ±S.E.M. n = 6 in each group.

V. 2.6 Transplantation of Human ICCs and Functional Analysis

Procedures of ICC transplantation and the subsequent functional assessment of the grafts were described in section II. 5.1 and II. 5.2 (pp. 102) in details.

V. 2.7 RNA Expression Analysis

Total RNA of cultured PPCs, ICCs or homogenized human fetal liver was extracted using the TRIzol® reagent (Invitrogen) according to the manufacturer's instructions. Procedures for RNA isolation, cDNA preparations by reverse transcriptase reactions and the subsequent analyses by PCR or real-time PCR were described in sections II. 3.4 – II. 3.9 (pp. 92-96) in details. The sequences of the primers used are listed in Table II. 3.3 (pp. 97).

IV. 2.8 Immunoflourescent Staining/ Immunohistochemistry

Immunocytohemistry of PPCs, ICCs as well as immunohistochemistry of graft-bearing kidneys and pancreata were performed according to section II. 3.1 (pp. 86-88). The types and dilutions of all antibodies used are listed in Table II. 3.1 (pp. 88). For visualization of graft vascularization, a blood vessel staining kit using an alkaline phosphatase system (Chemicon, International, Temecula, CA, USA) was used. The staining procedures for two endothelial cell makers, von Willebrand Factor (vWF; 1:200 dilution) and platelet

endothelial cell adhesion molecule (PECAM-1; 1:200 dilution), were done according to the manufacturer's suggested protocol.

IV. 2.9 Western Blot

Extraction and quantification of total proteins from PPC/ICC lysates were described in section II. 3.2 (pp. 89). Western blot procedures were performed according to section II. 3.3 (pp. 89-91). The types and dilutions of all antibodies used are listed in Table II. 3.2 (pp. 91).

IV. 2.10 Assessment of PPC Proliferation/Apoptosis and Ang II Signaling

For the PPC proliferation assay, cells were plated at 1.5 x 10^4 cells/well in 96-well plates with serum-free RPMI medium overnight. Medium supplemented with 0.5% fetal bovine serum (FBS; Invitrogen) was replaced the next day. Ang II was added at concentrations ranging from 0.1 μM to 100 μM for 3 d. In some experiments, 1 μM or 10 μM losartan or PD 123,319 (Sigma-Aldrich) was applied 15 min before the addition of Ang II. Cell proliferation was assessed by 5-bromo-2'-deoxyuridine (BrdU) uptake as previously described (Suen et al., 2008; Ng et al., in press). The BrdU-incorporation assay (Amersham Biosciences) was performed according to the manufacturer's protocol.

Quantification of PPC apoptosis was performed as previously described (Cheng et al., 2008). A serum-free condition was used to trigger PPC apoptosis, and effects of Ang II, losartan, and PD 123,319 were assessed and analyzed using an *in situ* Cell Death ELISA^{plus} assay (Roche Diagnostics, Mannheim, Germany) according to the manufacturer's protocol. For studying Ang II signaling, PPCs were plated at 2.5 x 10^5 cells/well in 6-well plates. Ang II (1 μM) was applied to PPCs for 0, 5, 15, 30, 45 and 60 min and cells were harvested for western blotting.

IV. 2.11 Measurement of C-peptide Content and Insulin Release

Measurement of C-peptide content of the ICCs and their level of insulin secretion was performed according to section II. 4.2 and II. 4.3 (pp. 98-100).

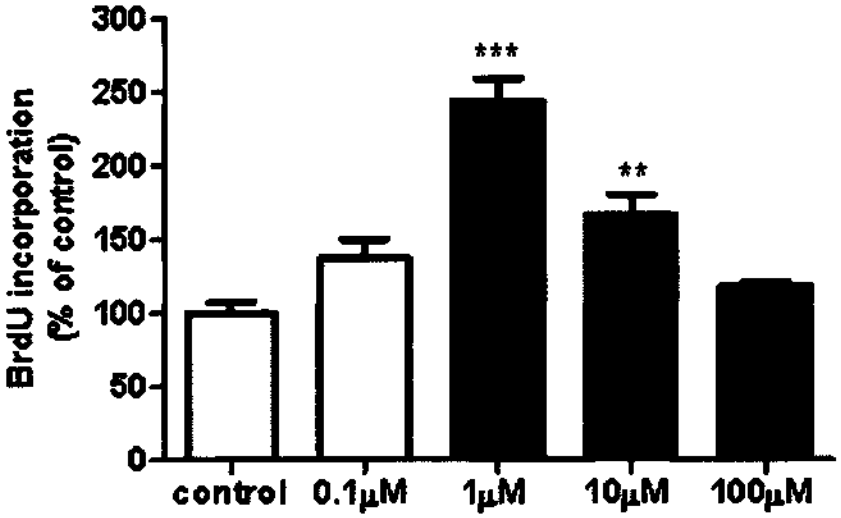
IV. 2.12 Statistical Data Analysis

Detailed procedures for statistical data analysis were described in section II. 6 (pp. 104). For MTT assay, data were presented as the percentage of initial control values for each treatment group (experimental value/initial control value × 100%).

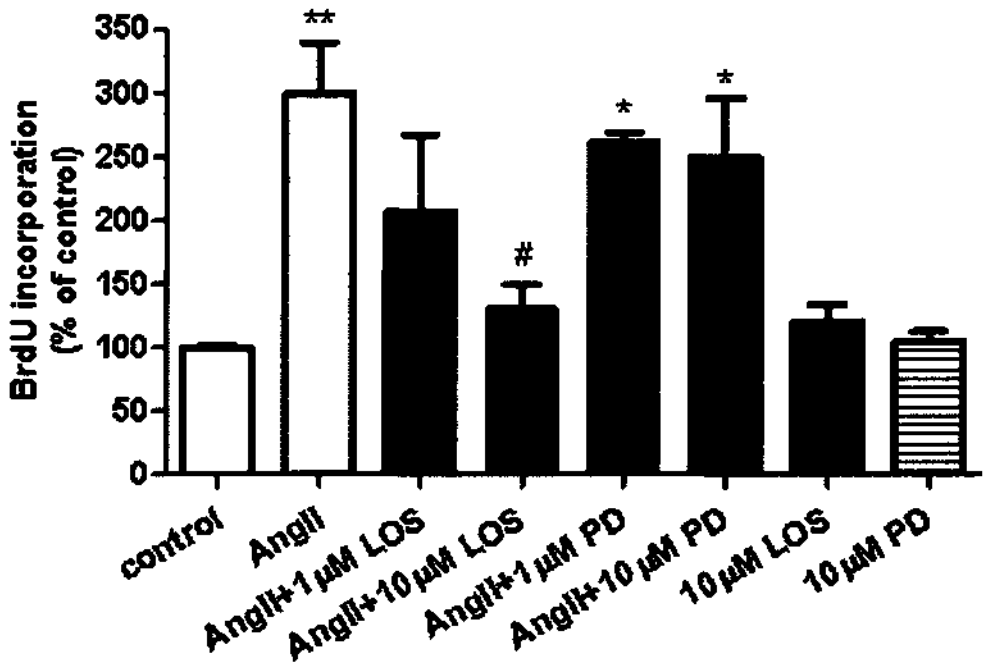
V. 3 Results

V. 3.1 Ang II Exerts a Mitogenic and Anti-apoptotic Effect on PPCs.

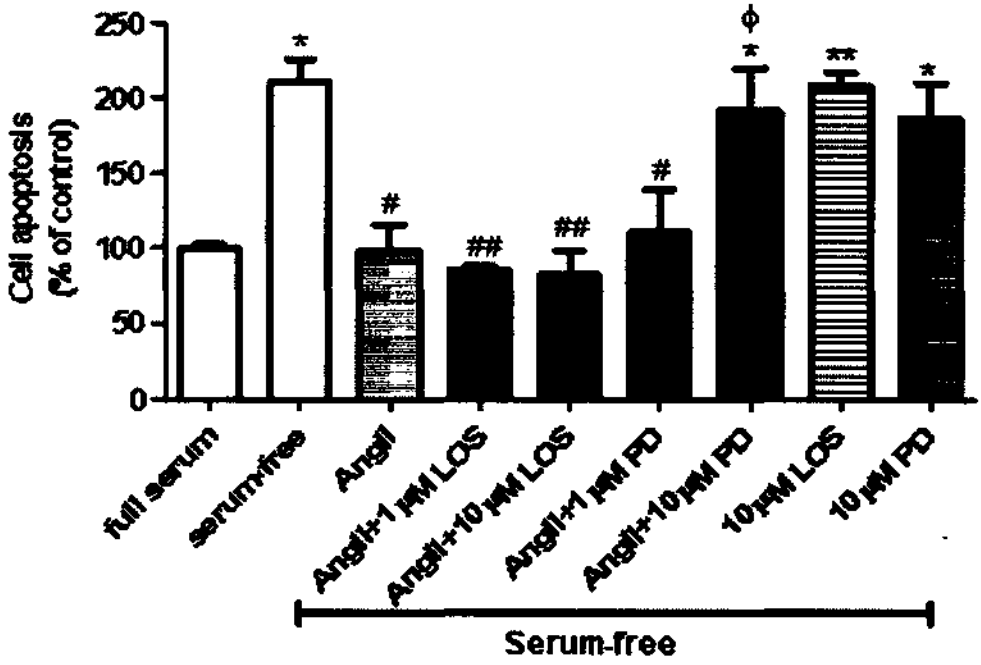
The effects of Ang II on embryonic stem cell proliferation have been reported elsewhere (Kim et al., 2010). We thus assessed the effect of Ang II on the mitogenesis and apoptosis of PPCs. Cell proliferation was evaluated by measuring BrdU incorporation with a quantitative cellular enzyme immunoassay (EIA) while cell apoptosis was assessed by an *in situ* cell death detection enzyme-linked immunosorbent assay (ELISA). Addition of Ang II at concentrations ranging from 0.1 μM to 100 μM exerted a dose-dependent mitogenic effect on PPCs, with a maximal efficacy at 1 μM (~250% of untreated PPCs) (Figure. V. 3.1). This Ang II-induced mitogenic effect could be abolished by pre- or co-treatment with 10 μM losartan, an AT_1 receptor antagonist, but not by 10 μM PD 123,319, an AT_2 receptor antagonist (Figure. V. 3.2). Ang II at 1 μM was also found to exert an anti-apoptotic effect on PPCs, as evidenced by a rescue of a number of apoptotic bodies under serum-free conditions (Figure. V. 3.3). Conversely, this Ang II-induced anti-apoptotic effect was abolished by pre- or co-treatment with 10 μM PD 123,319 (Figure. V. 3.3). In both cases, addition of losartan or PD 123,319 alone posed no observable effects on PPC growth, consistent with an absence of endogenous AT_1 and AT_2 receptor-mediated Ang II regulation of undifferentiated PPC development (Figure. V. 3.2 and V. 3.3).



Figureure V. 3.1. Dose-dependent mitogenic effect of Ang II on PPCs. Control cells received an equivalent volume of dilution buffer alone. Results are presented as a percentage of control values. All data are expressed as means ± S.E.M.; n = 6 in each group. ** $p<0.01$, *** $p<0.001$ vs control.



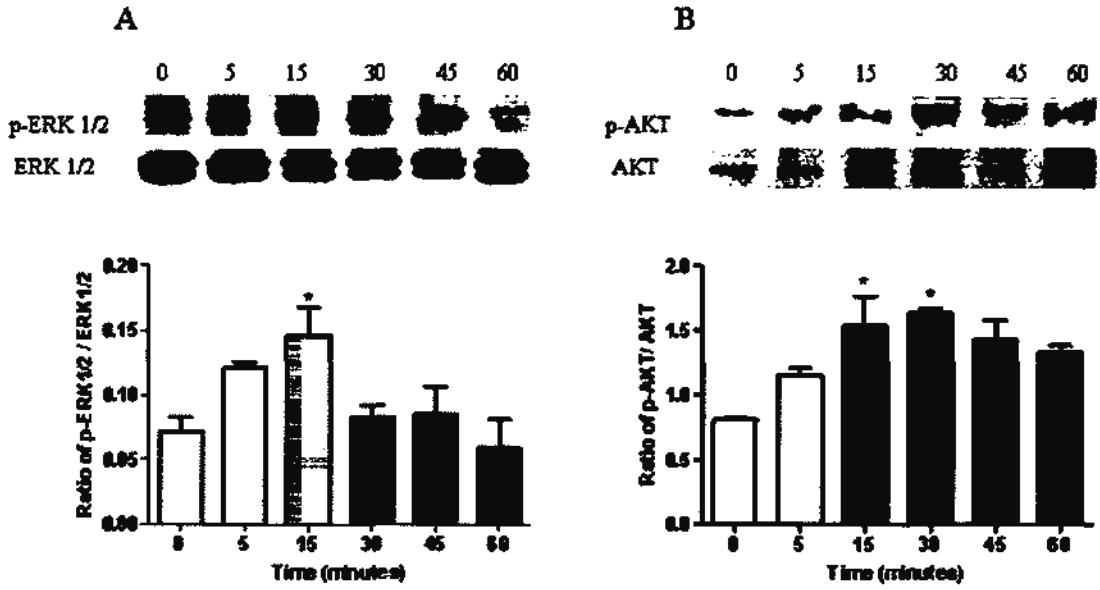
Figureure V. 3.2. Effects of exogenous addition of 1 μM Ang II, losartan (LOS; AT_1 receptor antagonist) or PD 123,319 (PD; AT_2 receptor antagonist) on PPC proliferation. Control cells received an equivalent volume of dilution buffer alone. Results are presented as a percentage of control values. All data are expressed as means ± S.E.M.; n = 6 in each group. * $p<0.05$, ** $p<0.01$ vs control; # $p<0.05$ vs Ang II.



Figureure V. 3.3. Effects of exogenous addition of 1 μM Ang II, LOS or PD on the apoptotic effects of PPCs cultured under serum-free conditions. Control cells received an equivalent volume of dilution buffer alone. Results are presented as a percentage of control values. All data are expressed as means ± S.E.M.; n = 6 in each group. * $p<0.05$, ** $p<0.01$ vs full serum control; # $p<0.05$, ## $p<0.01$ vs serum-free condition; ϕ $p<0.05$ vs Ang II-treated group.

V. 3.2 Ang II is a Signaling Molecule in PPC Development

In order to elucidate the role of Ang II as a signaling molecule in PPC development, we investigated its ability to activate extracellular signal-regulated kinase (ERK)1/2 and AKT in PPCs. Western blot results showed that Ang II at 1 μM could induce phosphorylation of ERK1/2 and AKT, which peaked at 15 min and 30 min, respectively (Figure. V. 3.4). These findings suggest that Ang II-induced mitogenic and anti-apoptotic effects on PPCs may be mediated by these signaling kinases.

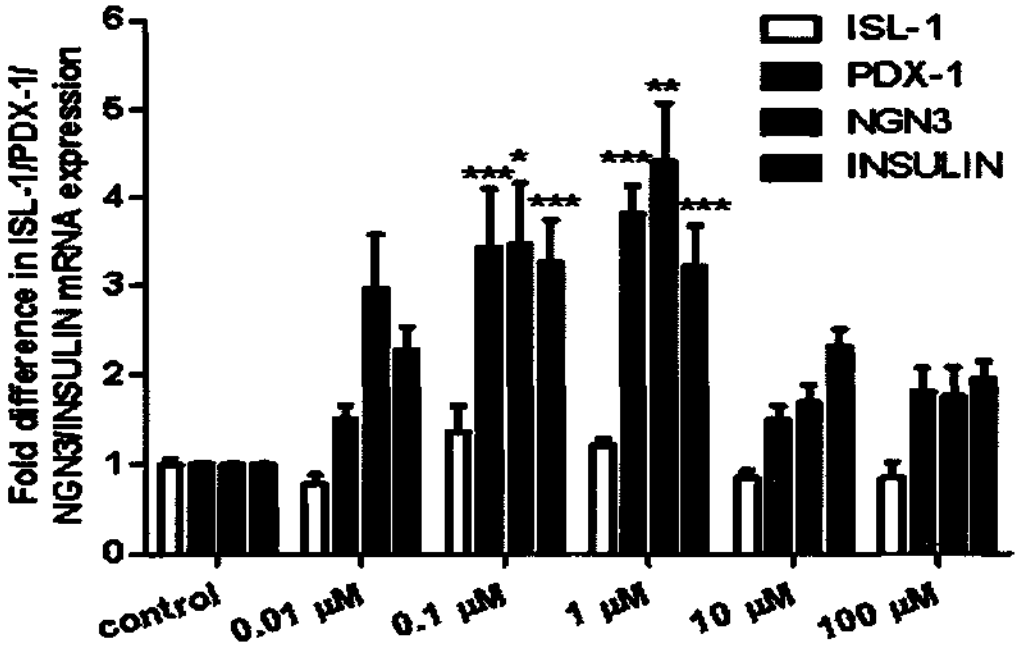


Figureure V. 3.4. Evidence of Ang II acting as a signaling molecule in PPCs. Western blot analyses showed that addition of 1 μM Ang II resulted in (*A*) ERK1/2 and (*B*) AKT phosphorylation. Equal protein loading was confirmed by reprobing with an antibody to ERK1/2 or AKT. All data are expressed as means ± S.E.M. n = 4 in each group. **p* < 0.05 *vs.* t = 0.

V. 3.3 Endogenous Production of Ang II Enhances PPC Differentiation and is AT_2 receptor-mediated

We proceeded to examine the effects of Ang II on PPC differentiation into ICCs. The addition of exogenous Ang II at concentrations ranging from 0.01 μM to 100 μM produced a dose-dependent upregulation of the expression of the endocrine marker *NGN3*; expression of the mature β-cell markers *PDX-1* and *INSULIN* also peaked at 1 μM. The delivery of Ang II did not exert any observable effects on the expression of the early transcription factor *ISL-1* (Figure. V. 3.5). The insulin regulatory function of the *ISL-1* transcription factor has been controversial because of its low expression level in β-cells (Ahlgren et al., 1997). We did not note any evidence of strong regulation of the *ISL-1* gene during the 8-d differentiation (Figure. IV. 3.7 *A and I*). The Ang II-induced upregulation of *PDX-1* and *INSULIN* could be abolished by pre- or co-treatment with PD 123,319 but not losartan, further supporting the notion that PPC differentiation may be promoted by AT_2 receptor activation (Figure. V. 3.6 *A and B*). The addition of losartan to differentiation cultures did not exert any observable effects on differentiated ICCs, whereas applying PD 123,319 alone suppressed the expression of β-cell phenotypic factors (Figure. V. 3.6 *A and B*), suggesting that locally generated Ang II promotes the differentiation of PCCs toward a β-cell lineage through AT_2 receptor signaling. We observed a suppression of C-peptide

production in cultures supplemented with PD 123,319, but not losartan treatment, further substantiating the importance of an Ang II-AT_2 receptor axis in the maturation of ICCs (Figure. V. 3.6 *C*).



Figureure V. 3.5. Dose-dependent effect of Ang II on the expression of β-cell transcription factors of ICCs. Control cells received an equivalent volume of dilution buffer alone. All data are expressed as means ± S.E.M. n = 6 in each group. $*p < 0.05$; $**p < 0.01$; $***p < 0.001$ *vs.* control.

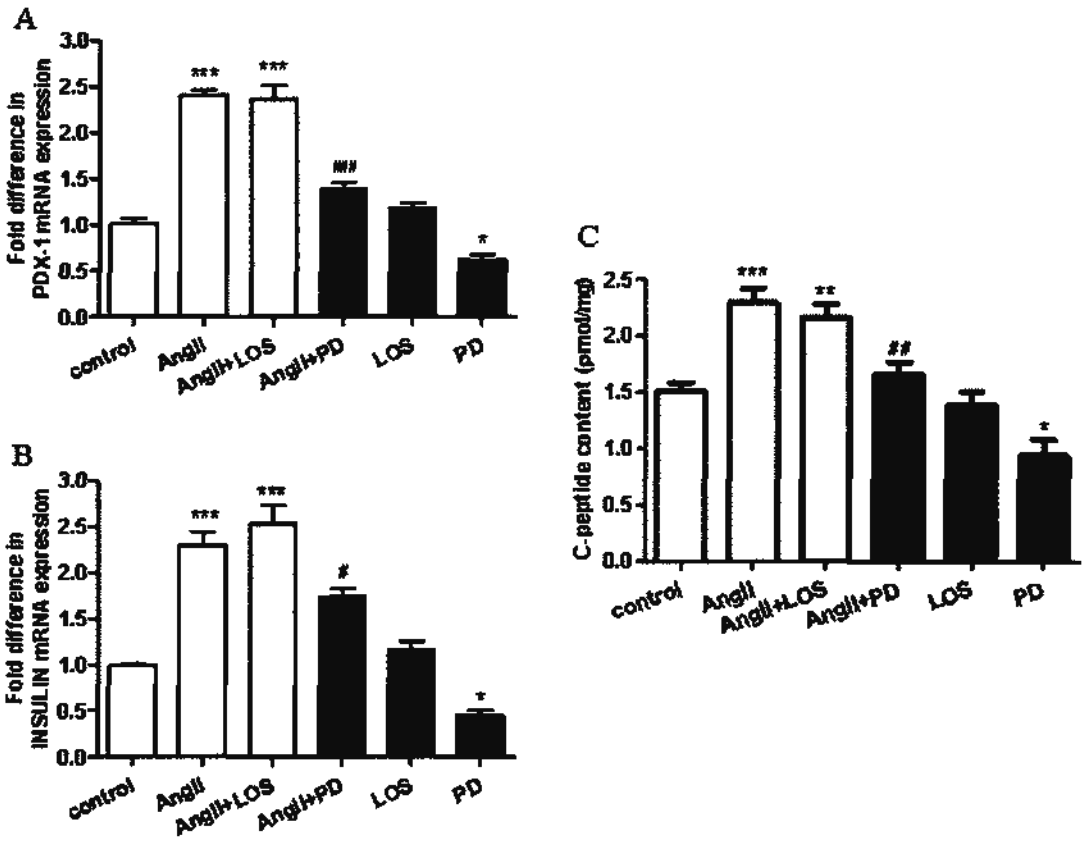


Figure. V. 3.6. Effects of exogenous addition of 1 μM Ang II and 10 μM LOS or 10 μM PD on (*A*) *PDX-1* and (*B*) *INSULIN* mRNA expression of ICCs. n = 5 in each group. (C) C-peptide content of ICCs. n = 4 in each group. Control cells received an equivalent volume of dilution buffer alone. All data are expressed as means ± S.E.M. $*p < 0.05$; $**p < 0.01$; $***p < 0.001$ *vs.* control. $\#p < 0.05$; $\#\# p < 0.01$; $\#\#\# p < 0.001$ *vs.* Ang II-treated group.

V. 3.4 Lentivirus-mediated Knockdown of AT_2 Receptor in PPC/ICC Cultures Suppresses ICC Maturation.

We used lentiviral vectors (pLKO.1-puro) expressing short-hairpin RNAs (shRNAs) to knock down AT_2 receptor in our PPC/ICC culture (sh-AT_2R; GeneBank accession number: NM_000686; Sigma-Aldrich MISSION® shRNA technology; detailed insert sequences in Table V. 2.1). Lentiviral vectors expressing shRNAs against luciferase (sh-luci) were used for negative non-target control experiments, while lentiviral vectors expressing TurboGFP were used to confirm successful transduction (Figure. V. 3.7 *A*) and optimize the multiplicity of infections (MOIs) (details in section V. 2.4). We first compared the transduction efficiency of five different vectors (sh-AT_2R I-V) on PPCs. Western blot experiments demonstrated an approximately 80%-decrease in translation of AT_2 receptor protein by using sh-AT_2R (V) relative to sh-luci (Figure. V. 3.7 *B*). ICCs which differentiated from sh-AT_2R (V)-transduced PPCs displayed an approximately 70%-reduction in protein translation of AT_2 receptor (Figure. V. 3.7 *C*), confirming the reliability of the transduction system. We observed a strong suppression of *PDX-1*, *NGN3*, *NKX 6.1* and *INSULIN* mRNA expression in sh-AT_2R-transduced ICCs compared to expression levels in sh-luci-transduced ICCs (~70% reduction) (Figure. V. 3.8 *A*). This decrease in β-cell phenotypic factor expression was much more pronounced than that

achieved by a pharmacological blockade of AT_2 receptors using PD 123,319 (~40% reduction) (Figure. V. 3.6 *A and B*), likely limited to drug diffusion. We also assessed the amount of basal insulin release from transduced ICCs and found a markedly reduced insulin production in sh-AT_2R-induced ICCs relative to control ICCs (Figure. V. 3.8 *B*).

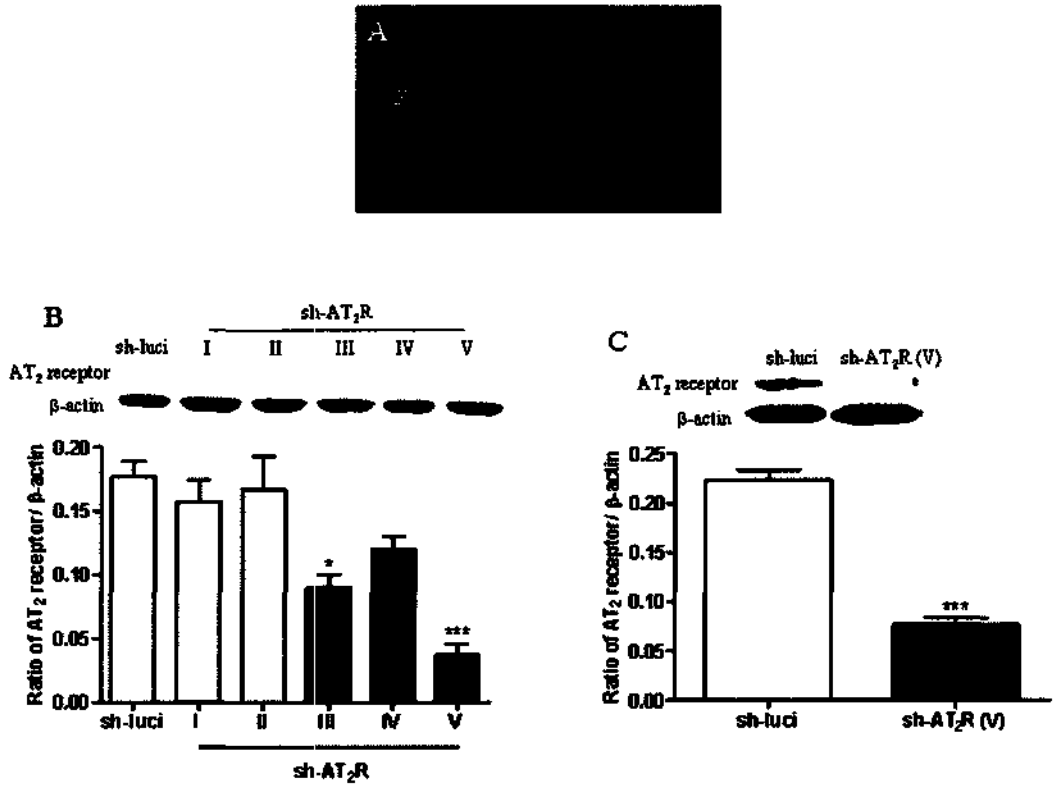


Figure. V. 3.7. A lentivirus-mediated knockdown of AT_2 receptor in PPC/ICC culture. (*A*) A fluorescent image showing the GFP signal of PPCs transduced with lentiviral vectors expressing TurboGFP. Original magnification: 400×; Scale bar: 40 μm. (*B*) Western blot results showing the AT_2 receptor-knockdown efficiency in PPCs transduced with one of five different clones (I-V) of sh-AT_2R. n = 8 in each group (*C*) Assessment of the AT_2 receptor-knockdown efficiency of ICCs derived from sh-AT_2R(V)-transduced PPCs. n = 6 in each group. All data are expressed as means ± S.E.M. $*p < 0.05$; $***p < 0.001$ *vs.* sh-luci ICCs.

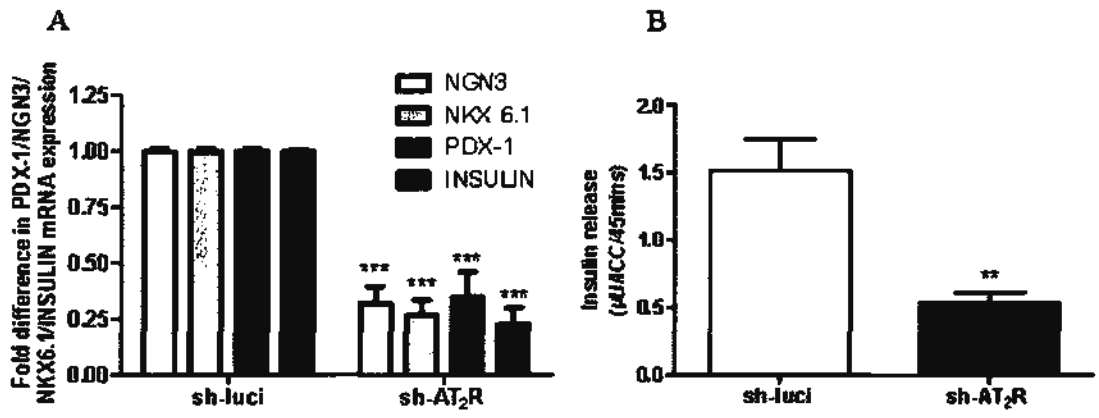


Figure. V. 3.8. Effects of AT_2 receptor-knockdown on (*A*) the expression of β-cell transcription factors (n = 6 in each group) and (*B*) the basal insulin secretion level of ICCs (n = 5 in each group). All data are expressed as means ± S.E.M. $^{**}p < 0.01$; $^{***}p < 0.001$ *vs.* sh-luci ICCs.

V. 3.5 Transplanted sh-AT_2R-transduced ICCs Fail to Ameliorate Hyperglycemia in Diabetic Nude Mice

We endeavored to assess the functionality of transduced ICCs by transplanting them into immune-privileged diabetic mice. Diabetes was induced in these BALB/c nude mice by a multiple intra-peritoneal injections of streptozotocin (STZ) over 5 consecutive days (75 mg/kg/day). Each adult diabetic mouse received an ICC transplant of approximately 1500 sh-luci- or sh-AT_2R-transduced ICCs placed between the real parenchyma and kidney capsule. Immunohistochemistry of kidney sections 10 d post-transplantation confirmed that the sh-luci-transduced ICCs were successfully engrafted to the renal parenchyma (Figure. V. 3.9 *F*). Robust insulin immunoreactivity was detected in the grafts 10 d (Figure. V. 3.9 *A*) after transplantation, confirming the *in vivo* cellular function in the kidney. Interestingly, the sh-luci-transduced ICC grafts also showed a strong Glut-2 immunoreactivity (Figure. V. 3.9 *B*). Based on our previous finding that Glut-2 was not expressed in the differentiation cultures of ICCs (Leung et al., 2009) and other observation that a hyperglycemic microenvironment plays a major role in the development of islet progenitors (Guillemain et al., 2007), we inferred that Glut-2 expression was likely attributable to the microenvironment of the kidney of the diabetic mouse. We also detected a strong expression of AT_2 receptors scattered in the ICC graft, further suggesting their

involvement in the functional maturation of the transplanted ICCs (Figure. V. 3.9 *C*).

As normal functioning of a graft depends on suitable vascularization, we examined the expression of two common endothelial cell markers: platelet endothelial cell adhesion molecule (PECAM-1) and von Willebrand Factor (vWF) 10 d after transplantation when early angiogenesis is evident (Merchant et al., 1997). We were able to detect immunoreactivities of these two molecules in the vicinity of the ICC graft but not in the glomeruli, implying revascularization (Figure. V. 3.9 *D and E*). Immunohistological examination of the normal and STZ-treated pancreata confirmed the presence of an intact islet architecture (Figure. V. 3.9 *G*) and a complete destruction of β-cells after STZ treatment (Figure. V. 3.9 *H*), respectively.

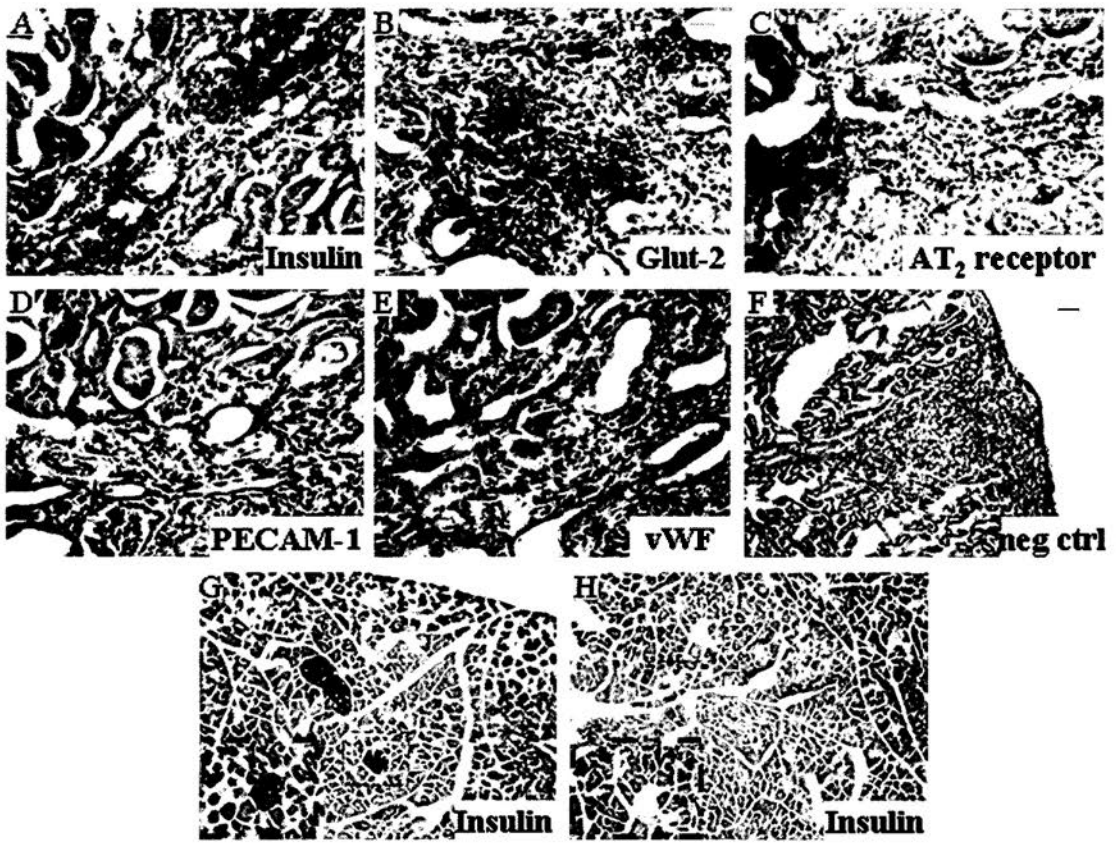


Figure. V. 3.9. Transplantation of sh-luci ICCs into diabetic nude mice. (*A-F*) Sh-luci ICC graft-bearing kidneys were removed 10 d after transplantation and sectioned for histological analyses. Robust immunoreactivity was detected for (*A*) insulin, (*B*) Glut-2, and (*C*) AT_2 receptor, as indicated by arrows. Signs of revascularization were noted by immunoreactivity against (*D*) PECAM-1 and (*E*) vWF near the ICC graft (arrows). (*F*) Negative controls were produced by omitting primary antibodies. The area of the ICC graft is demarcated by a red dotted line. (*G*) Insulin staining in pancreatic sections from normal nude mice without STZ treatment (inset: high magnification image of an intact islet), and (*H*) that from transplanted diabetic nude mice (inset: high magnification image showing complete destruction of β-cells. Original magnifications: *A-E*, *G* and *H*, 630×; *F*, 100×. Scale bars: *A-E*, *G* and *H*, 40 μm; *F*, 100 μm.

We then compared the post-transplant functional performance of the sh-luci- and sh-AT_2R-transduced ICC grafts in terms of the ability to reverse hyperglycemia. Body weight and blood glucose were monitored once a week for 6 wks after transplantation. As expected, the STZ-treated mice exhibited a significant drop in body weight. Mice transplanted with sh-luci-transduced ICCs achieved an average weight gain of 2.6 g, while those transplanted with sh-AT_2R-transduced ICCs exhibited an average weight loss of 0.7 g (Figure. V. 3.10 *A and B*). Similarly, the transplant of sh-luci-transduced ICCs ameliorated hyperglycemia, as evidenced by a gradual drop in blood glucose level to ~12.8 mM, while no hyperglycemia reversal was observed in mice transplanted with sh-AT_2R-transduced ICCs (Figure. V. 3.11 *A and B*). Intraperitoneal glucose tolerance tests (IPGTTs) performed on mice 30 d post-transplantation revealed that mice transplanted with sh-luci-transduced ICCs exhibited an enhanced glucose tolerance, as evidenced by a restoration of blood glucose levels within 120 min (Figure. V. 3.12 *A and B*). Meanwhile, mice transplanted with sh-AT_2R-transduced ICCs showed no improvement in glucose tolerance, reflecting an abnormal secretory function of these ICCs following the glucose challenge (Figure. V. 3.12 *A and C*).

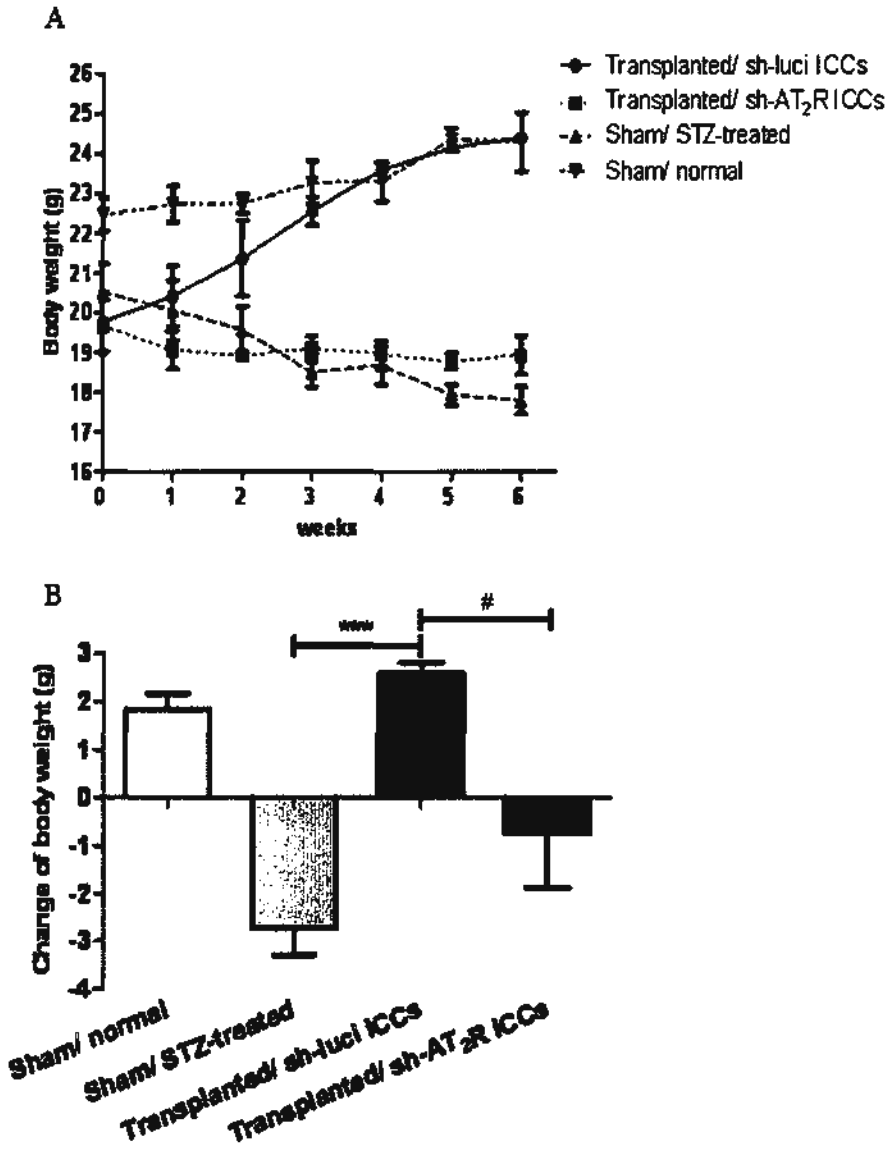


Figure. V. 3.10. Change in body weight of sham normal mice (n = 5), sham diabetic mice (n = 13), sh-luci ICC-transplanted mice (n = 10), and sh-AT_2R ICC-transplanted mice (n = 6). All data are expressed as means ± S.E.M. *** $p<0.001$ sh-luci ICC-transplanted mice *vs.* sham diabetic mice; # $p<0.05$ sh-AT_2R ICC-transplanted mice *vs.* sh-luci ICC-transplanted mice.

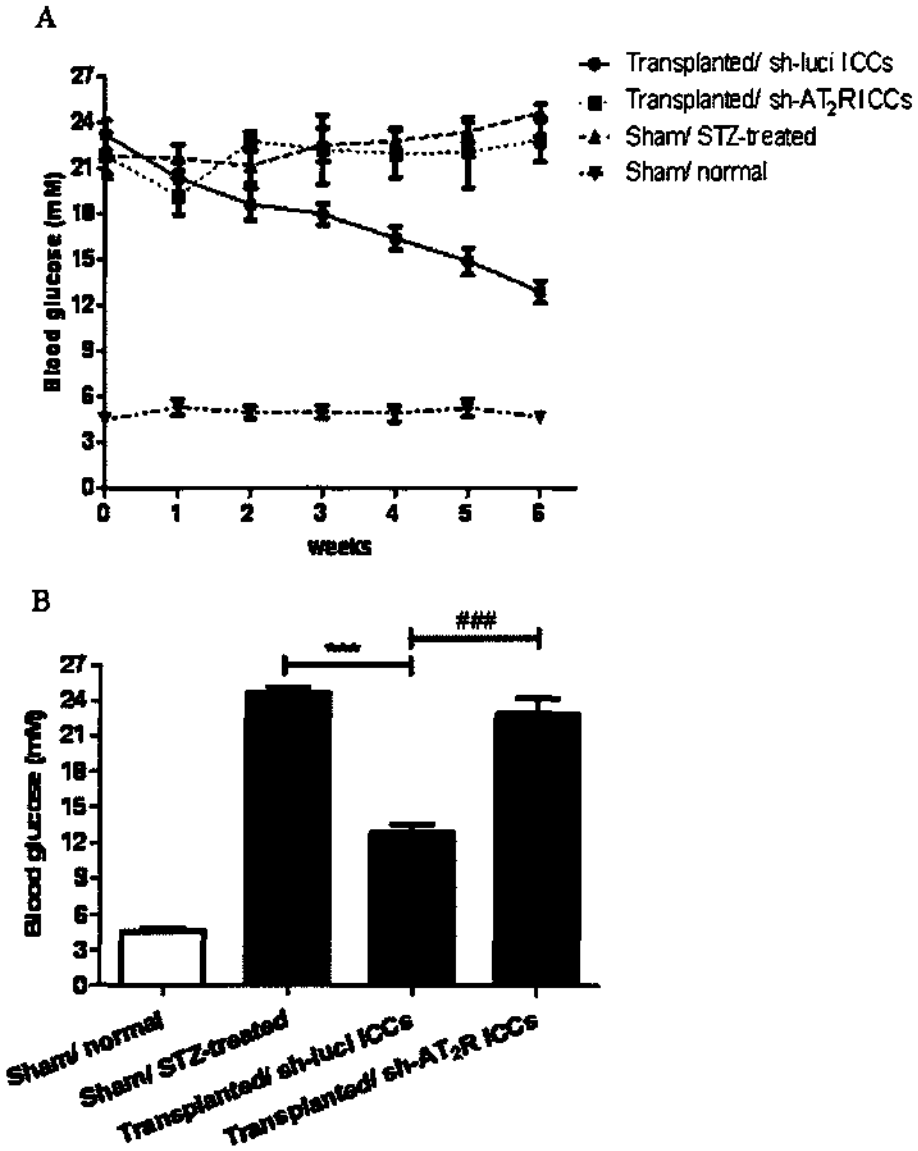


Figure. V. 3.11. (*A*) Change in blood glucose levels of sham normal mice (n = 5), sham diabetic mice (n = 13), sh-luci ICC-transplanted mice (n = 10), and sh-AT_2R ICC-transplanted mice (n = 6). (*B*) Their blood glucose levels at the 6^{th} week post-transplantation were compared. All data are expressed as means ± S.E.M. *** $p<0.001$ sh-luci ICC-transplanted mice *vs.* sham diabetic mice; ### $p<0.001$ sh-AT_2R ICC-transplanted mice *vs.* sh-luci ICC-transplanted mice.

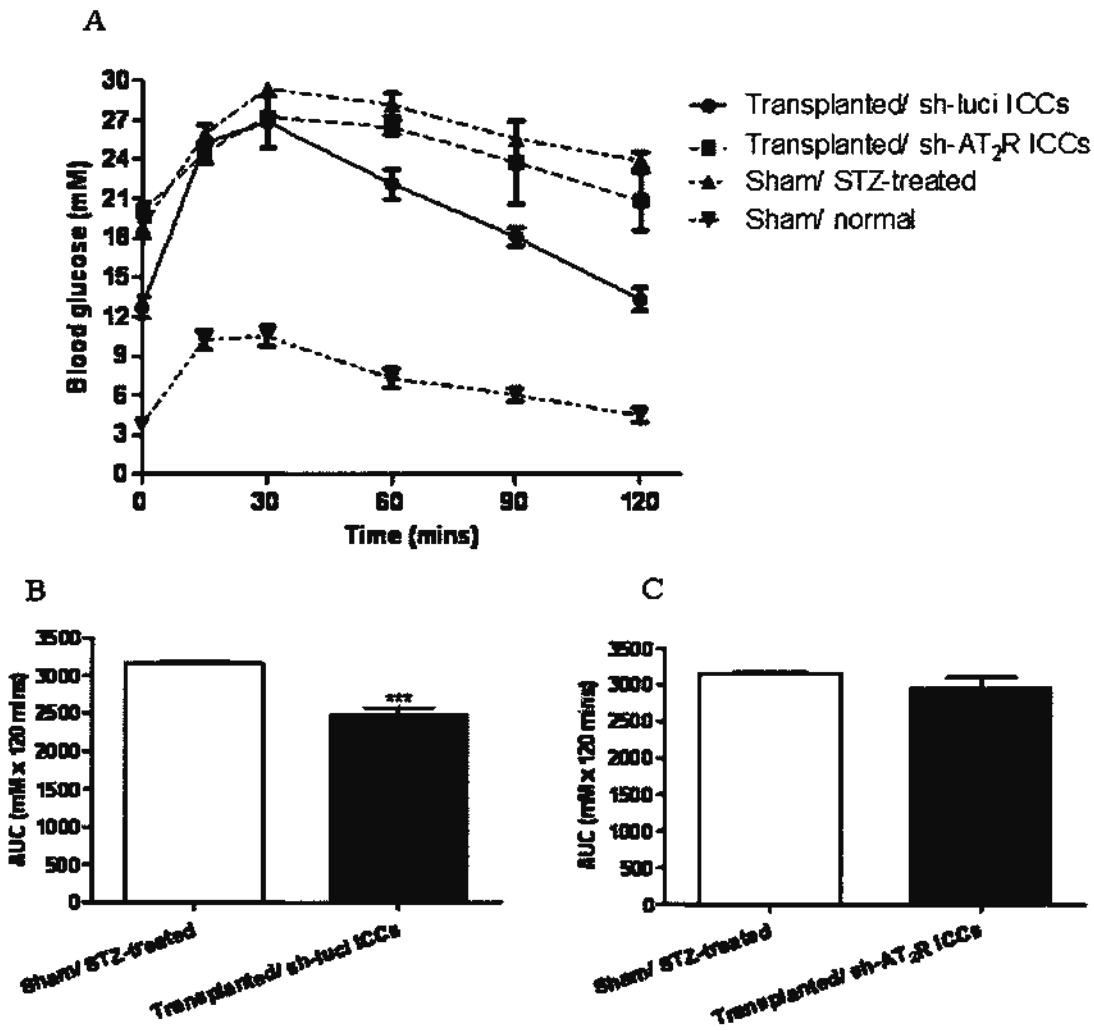


Figure. V. 3.12. IPGTTs were performed 30 d after transplantation on the sham/ transplanted animals. (*A*) Change of blood glucose level was measured immediately before (t = 0 min) and after (t = 15, 30, 60, 90, 120 min) a glucose challenge. (*B* and *C*) The areas under curve (AUC) were compared between sham diabetic mice (n = 8) with sh-luci ICC-tranplants (n = 10) or sh-AT_2R ICC-transplants (n = 6). All data are expressed as means ± S.E.M. *** $p<0.001$ sh-luci ICC-transplanted mice *vs.* sham diabetic mice.

V. 4 Discussion

Here we demonstrated that Ang II is a mitogenic factor for the growth of PPC, and that the endogenously generated Ang II during PPC differentiation into ICCs promotes such development process. Involvement of the Ang II receptors, especially AT_2 receptors, in regulating fetal or adult cell growth has been reported elsewhere. Concerns have been raised regarding whether AT_1 and AT_2 receptor would counter-regulate the Ang-II induced effects. Yet the possible antagonizing effects between the two receptor-induced signaling were excluded in some tissues (Chamoux et al., 1999; D'Amore et al., 2005). Of note, Ang II induced ERK 1/2 and AKT phosphorylation in our PPCs. These signaling mechanisms have been suggested to regulate the differentiation plasticity and reprogramming of pancreatic cells (Elghazi et al., 2009; Saleem et al., 2009). Whether the Ang II-induced growth and differentiation of our PPCs/ICCs is mediated by these kinases warrants further studies. In the present report, the significant suppression of a critical endocrine progenitor marker, *NGN3* (Gradwohl et al., 2000; Desgraz and Herrera, 2009), as well as other mature β-cell phenotypic factors including *PDX-1*, *NKX 6.1* and *INSULIN*, observed in our differentiated sh-AT_2R ICCs is noteworthy. This finding underscores the importance of the Ang II-AT_2 receptor axis in maintaining normal endocrine development, and hence the functional maturation of differentiated ICCs.

Achieving a stem cell-based therapy for diabetes depends on the success of transplantation of stem cell-derived islets into diabetic individuals. The present results provide an initial evidence that transplant of human fetal PPC-derived ICCs is a potential treatment modality for diabetes given the ability of ICCs to reverse hyperglycemia and glucose intolerance in diabetic mice. Although *in vitro* differentiation has not been able to drive a full functional maturation of ICCs (Chapter III, Leung et al., 2009) and exogenous addition of Ang II has also not been shown to be capable of inducing *GLUT-2* mRNA expression, the induction of Glut-2 and the glucose-responsiveness in transplanted animals indirectly reveals the competence of such ICC grafts to differentiate further *in vivo* in a diabetic microenvironment. The fact that blood glucose levels were not completely normalized in the transplanted mice implies that there is further effort or time needed to enable grafts to reach full maturation. To this end, the introduction of the appropriate RAS components through delivery of suitable genes in order to enhance local Ang II production would be an attractive approach for driving functional maturation of ICC grafts. The present results, including the finding that AT_2 receptor knockdown abolishes functional maturation in transplanted ICCs, provide critical information for delineating the mechanisms governing the transplantation potential of ICCs.

As a local RAS in adult pancreas has been identified (Leung, 2007a), the

predominant focus would be the role of upregulated RAS activities in the pathology of hyperglycemia-induced pancreatic cell damage (Lau et al., 2004; Chu et al., 2006; Leung and Leung, 2008). Up to now, there is little attention being paid to study whether such upregulation represents a possible mechanism for promoting β-cell neogenesis and islet regeneration from resident PPCs within the pancreas through *in vivo* cell reprogramming as recently proposed (Xu et al., 2008; Zhou et al., 2008; Puri and Hebrok, 2010). Of note, upregulated RAS activities in the pancreas were found to be closely associated with the generation of reactive oxygen species (ROS), and ROS have been recently proven to be critical for regulating differentiation, functions and recruitment of tissue progenitors (Owusu-Ansah and Banerjee, 2009; Ushio-Fukai and Urao, 2009; Lewandowski et al., 2010). In this regard, AT_2 receptor-mediated ROS production, as reported to exist in endothelial tissues (Pinaud et al., 2007), might warrant further investigation as a possible mechanism for promoting RAS-induced differentiation of PPCs.

Chapter VI

Potential Role of Angiotensin II Type 2 Receptor in Regulating the Pancreatic Endocrine Cell Development of Mouse Embryos

[The content of this chapter will be modified and submitted to *Developmental Dynamics*]

VI. 1 Introduction

The inadequate supply of transplantable pancreatic islets from cadaveric donors has posed a grave limitation of the clinical application to patients with Type 1 (T1DM) and some severe forms of Type 2 (T2DM) diabetes mellitus. The notion for pancreatic regeneration thus provides an insight to an alternative approach to curing those patients. To this end, devising a therapeutic strategy for promoting the *in vivo* neogenesis of islet cells is of paramount importance. This sort of progress will require a thorough understanding of the developmental biology of the endocrine pancreas and, more importantly, deciphering the underlying mechanisms involved in the development of pancreatic islet cells is critical in the formulation process. In chapter IV and V, we have demonstrated existence of a local renin-angiotensin system (RAS) in isolated pancreatic progenitor cells (PPCs) derived from human fetal pancreas; additionally, we have also shown that angiotensin II (Ang II), a physiologically active peptide of the RAS, is critical for the development of these PPCs into islet-like cell clusters (ICCs) and their potential for transplantation. Nevertheless, it remains to be investigated whether such a RAS is present *in vivo* in the developing pancreas and, if so, whether specific components of the RAS regulate the endocrine cell development.

Expression of the RAS components has been found in several types of tissue

progenitor cells (Perlegas et al., 2005; Liu et al., 2007; Contrepas et al., 2007), while blockade of some specific RAS components can suppress their differentiation into target cell types which, in turn, interferes with normal tissue formation and organogenesis during embryonic development (Amsterdam et al., 2004; Zhang et al., 2004). Among them, an obvious evidence is the kidney, where components of the RAS, angiotensin II type 1 (AT_1) and type 2 (AT_2) receptors, in particular, critically governs the normal development of the organ (Matsusaka, et al., 2002). Earlier research findings have revealed a differential gene expression and regulation of these two receptors during fetal kidney development (Robillard et al., 1995; Norwood et al., 1997), with AT_2 receptor being predominant until early postnatal life, suggestive of its role in the development of tissue progenitors (de Gasparo et al., 2000). Recent loss-of-function studies either by pharmacological blockade or gene targeting confirmed the essential role of these receptors in the maintenance of a normal development and prevention of malformations in the kidney (Sánchez et al., 2008; Song et al., 2010). Given the identification of a local functional RAS in the adult endocrine pancreas (Lau et al., 2004; Chu et al., 2006), we thus hypothesized that major RAS components exist differentially in the developing pancreas. Based on the aforementioned observations in the fetal kidney, we also hypothesized that such RAS components regulate the normal development of islet cells.

In order to address this issue, we aimed to investigate the correlation of a possible RAS expression during the endocrine cell development using mouse embryonic pancreata. Details of the gene expression profile and morphological dynamics of a developing mouse pancreas have been previously described (Setty et al., 2008; Gittes, 2009). Different from the human pancreatic development, a developing mouse pancreas exhibits three temporal waves of endocrine differentiation, termed as the first transition (around e9.0-e12.0) which refers to the early development of endocrine cells within the undifferentiated epithelium; the second transition (around e12.5-e15) which refers to the rapid expansion and differentiation of endocrine tissues resulting in the generation of single hormone-expressing cell; the third transition (around e15.5-birth, continues for 2-3 weeks after birth) which refers to the maturation of endocrine cells and a gain of functional secretory responsiveness to nutrient challenges (Habener et al., 2005; Bonal and Herrera, 2008).

Based on this temporal developmental transition, we applied pharmacological blockade of the RAS according to these three time windows during mouse pregnancy and examined its effects on the development of the fetal endocrine pancreas; the functions of these neonate islets were also assessed. Data in this study would elucidate an undiscovered role of the pancreatic local RAS which might act as a critical mediator for islet

development. Additionally, these data might also help in devising novel therapeutic strategies to promote β-cell reprogramming within the pancreas by means of these developmental cues (Kordowich et al., 2010).

VI. 2 Materials and Methods

VI. 2.1 Animals

Time-pregnant ICR mice were obtained from the Laboratory Animal Services Centre of the CUHK. Husbandry of the animals has been described in Section II. 2.3 (pp. 84).

VI. 2.2 Dissection and Handling of Mouse Embryo and Neonatal Pancreata

Dissection of mouse embryos and the subsequent handling processes have been described in Section II. 2.3 (pp. 84). The pancreata dissected from neonates were first blotted dry on tissue paper and weighed before fixation for histological evaluation.

VI. 2.3 Drug Treatment on Pregnant Animals

Protocols for the treatment of pregnant mice with Ang II receptor blockers were modified from a previous report (Sánchez et al., 2008). Briefly, losartan (Merck; 10 mg/kg/day), PD 123,319 (Sigma-Aldrich; 10 mg/kg/day), or vehicle were applied on the ICR mother mice from e8.0-birth through drinking water. In some experiments, the time window for PD 123,319 treatment was subdivided into 2 groups: e8.0-e12.0 or e12.0-birth, as determined by the temporal transitions of mouse pancreatic development (Habener et al., 2005).

VI. 2.4 Immunohistochemistry

Immunostaining of mouse embryo sections underwent antigen retrieval processes. Briefly, air-dried sections were placed in 10 mM citrate buffer (10 mM citric acid and 10 mM trisodium citrate, pH 6.0; Sigma-Aldrich) and microwaved for 15 min at 450 W. The slides were cooled and rinsed 3 times in PBS (Invitrogen, Carlsbad, CA, USA) before proceeding to the staining processes as described in Section II. 3.1 (pp. 86-88). (Types and dilutions of all antibodies are listed in Table II. 3.1, pp. 88). For assessment of the neonate islet β-cell or α-cell area, proportion of area occupied by Alexa Fluor 350 or 480 fluorescence within each islet (63× objective) was measured using Leica Qwin image analysis software (Leica Microsystems, Wetzlar, Germany), as described previously (Chu and Leung, 2007; Cheng et al., 2008). At least 5 islets per experimental group, and islets isolated from at least 4 different mother mice were randomly chosen for analysis. As a result, at least 20 islets per group were analyzed. Images were scanned separately at different laser wavelengths and captured as overlapping images.

VI. 2.5 Hematoxylin and Eosin (H&E) Staining

Slides of the neonate pancreatic sections were stained with hematoxylin for 1 – 2 min to visualize cell nuclei. Any undesirable overstaining was removed by controlled leaching

with 1% acid alcohol, followed by neutralization with the alkaline Scott's tap water. The sections were then stained with 1% eosin for 5 min to visualize cell cytoplasm. The slides were then rinsed in water, followed by de-hydration processes in ethanol gradient (70%, 80%, 95% ethanol each for 1 min; absolute ethanol × 3, each for 1 min; xylene × 3, each for 1 min). Finally the slides were mounted by Entellan® microscopic rapid-mounting media (Merck, Darmstadt, Germany). Histological sections were examined under a light microscope (Leica Microsystems).

VI. 2.6 Islet Isolation from Neonatal Mice

Procedures for islet isolation from neonatal mouse pancreata have been described in Section II. 4.1 (pp. 98).

VI. 2.7 Glucose-stimulated Insulin Secretion

Procedures for the measurement of glucose-stimulated insulin secretion (GSIS) have been described in Section II. 4.3 (pp. 99-100).

VI. 2.8 Glucose Tolerance Test

Briefly, mice from different experimental groups were fasted for 16 h before the glucose

tolerance tests. Procedures were performed as described in Section II. 5.2 (pp. 102).

VI. 2.9 Statistical Data Analyses

Detailed procedures for statistical data analysis have been described in Section II. 6 (pp. 104).

VI. 3 Results

VI. 3.1 Differential Expression of AT_1 and AT_2 Receptors in Late Stages of Mouse Fetal Pancreata and in Neonates

Based on the reported temporal endocrine development in the mouse fetal pancreas, we first harvested the pancreata from e15.5 - e17.5 embryos where primitive islets should be formed through a massive maturation and clustering of endocrine cells (Habener et al., 2005). A differential expression profile of the major RAS receptors, namely AT_1 and AT_2 receptors, was investigated by immunohistochemistry performed on the developing pancreas at these embryonic stages. Scattered insulin-expressing β-cells were found in the e15.5 pancreas, while they were clustered together and the islet structure was observed in e17.5 pancreas (Figure. VI. 3.1 and VI. 3.2, both *A, D, G*). We detected expression of both AT_1 and AT_2 receptors in the pancreas of e15.5-e17.5; however, immunoreactivity of the AT_1 receptor was less intense (Figure. VI. 3.1 *B, E, H*) when compared to that of AT_2 receptor (Figure. VI. 3.2 *B, E, H*) throughout these developmental stages studied. On the other hand, immunoreactivity of the AT_2 receptor was found to become intense as the development proceeds and, in particular, at e17.5, a strong co-localization of AT_2 receptor, but not AT_1 receptor, with insulin-expressing cells was noted (Figure. VI. 3.1*I* and VI. 3.2*I*). It is noteworthy that immunostaining of the day 2 post-natal pancreas (n_2) displayed

a very strong immunoreactivity of AT_2 receptor (Figure. VI. 3.2*K*) which was co-localized to β-cells (Figure. VI. 3.2*L*); yet a less intense staining for AT_1 receptor was noted (Figure. VI. 3.1*K*). Since the endocrine cells undergo additional remodeling and maturation 2-3 weeks after birth (Habener et al., 2005), this observation might represent a correlation between AT_2 receptor and the maturation process of β-cells. In normal adult pancreas, expression of both AT_1 receptor (Figure. VI. 3.1*M*) and AT_2 receptor (Figure. VI. 3.2*M*) were found consistently to be low in islets.

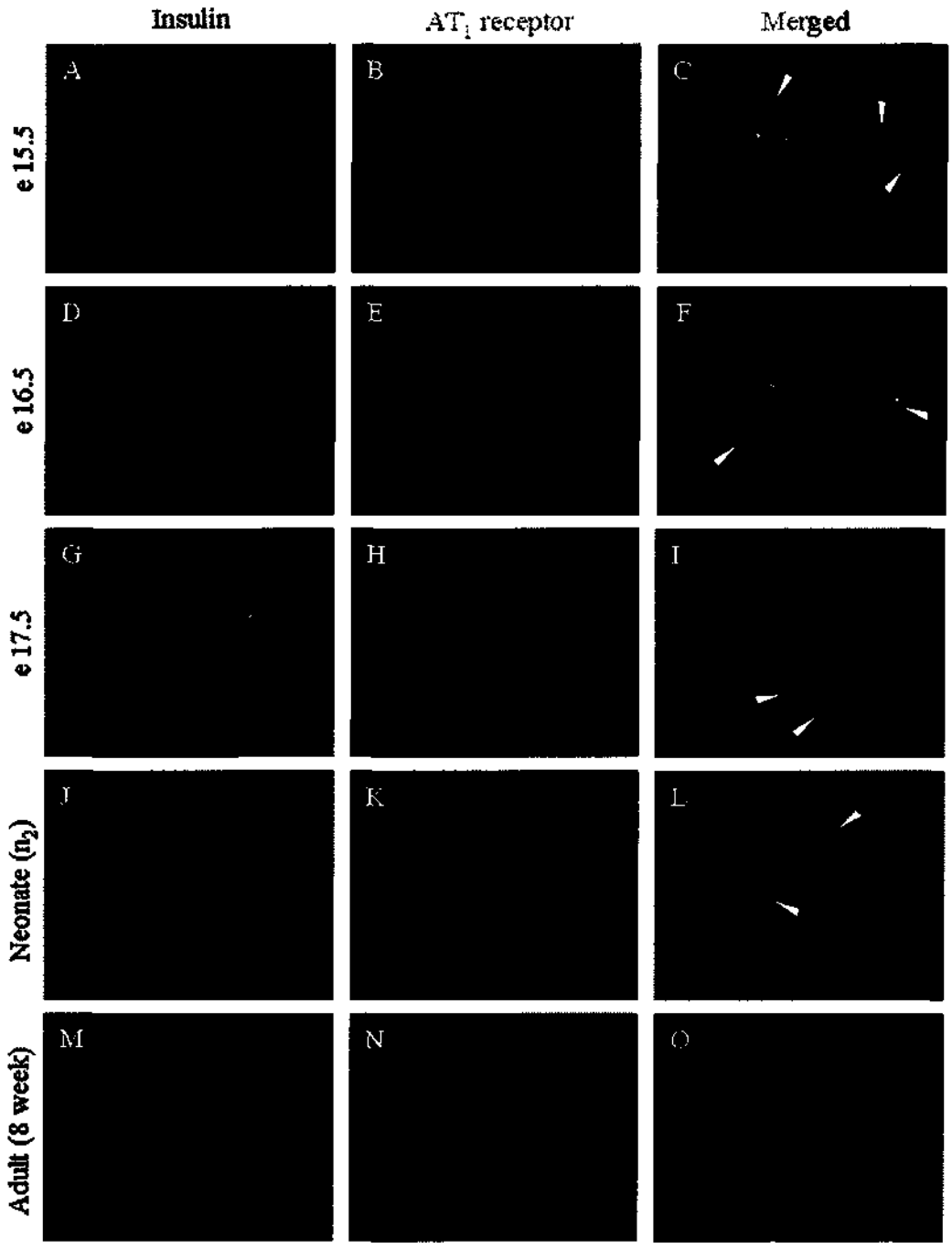


Figure VI. 3.1. Localization of AT_1 receptor and insulin in e15.5, e16.5, e17.5, neonatal and adult mouse pancreata. Arrowheads: cells expressed with insulin only. Original magnification: 630×; Scale bar: 40 μm.

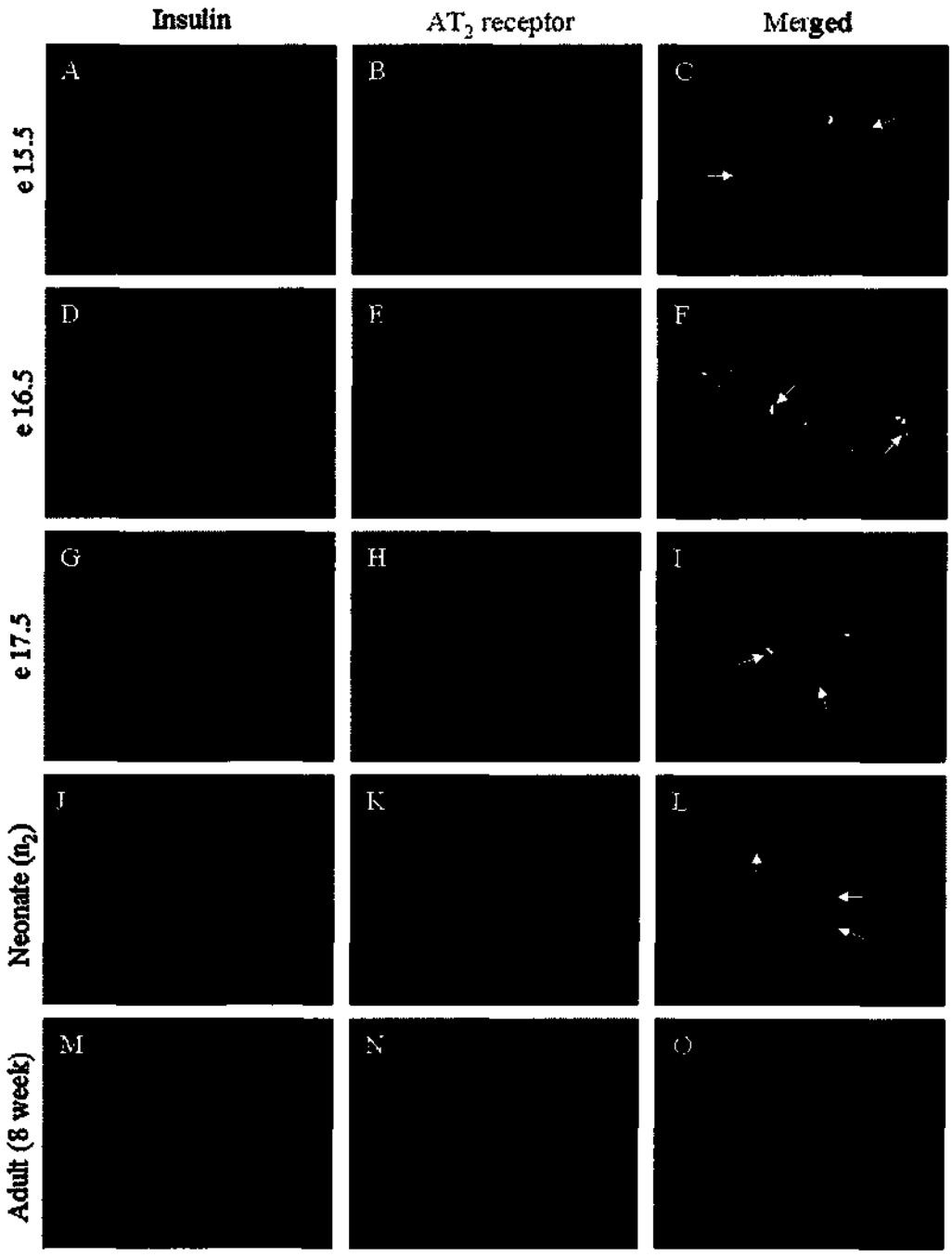


Figure VI. 3.2. Localization of AT_2 receptor and insulin in e15.5, e16.5, e17.5, neonatal and adult mouse pancreata. Arrows: cells co-localized with insulin and AT_2 receptor. Original magnification: 630×; Scale bar: 40 μm.

VI. 3.2 AT_2 Receptor Blockade during Pregnancy Caused Abnormalities in Fetal Pancreatic Development

Next we investigated the effects of Ang II receptor blockers during pregnancy on the pancreas architecture of the neonates. Similar drug treatment modalities on maternal mice and assessment of fetal development have been reported elsewhere (Sánchez et al., 2008; Theys et al., 2009). In the current study, Losartan (LOS; AT_1 receptor blocker), or PD 123,319 (PD; AT_2 receptor antagonist), was applied to pregnant mice via drinking water at a concentration of 10 mg/kg/day from e8.0 to birth. Neonatal pancreata were harvested at n_0 for morphological assessment. Analyses from H&E staining revealed destruction in the architecture of the n_0 pancreas by PD treatment, as evidenced by the loosely-packed acinar structures observed (Figure. VI. 3.3*C*). In contrast, LOS treatment did not exert such observable effect when compared with the control section (Figure. VI. 3.3 *A and B*). Similar observations were also noted on islet morphology, where PD treatment severely caused negative effect on the islet architecture of the neonates (Figure. VI. 3.3*F*). On the other hand, islets appeared to have intact histology in the LOS-treated group compared with those of respective control (Figure. VI. 3.3 *D and E*).

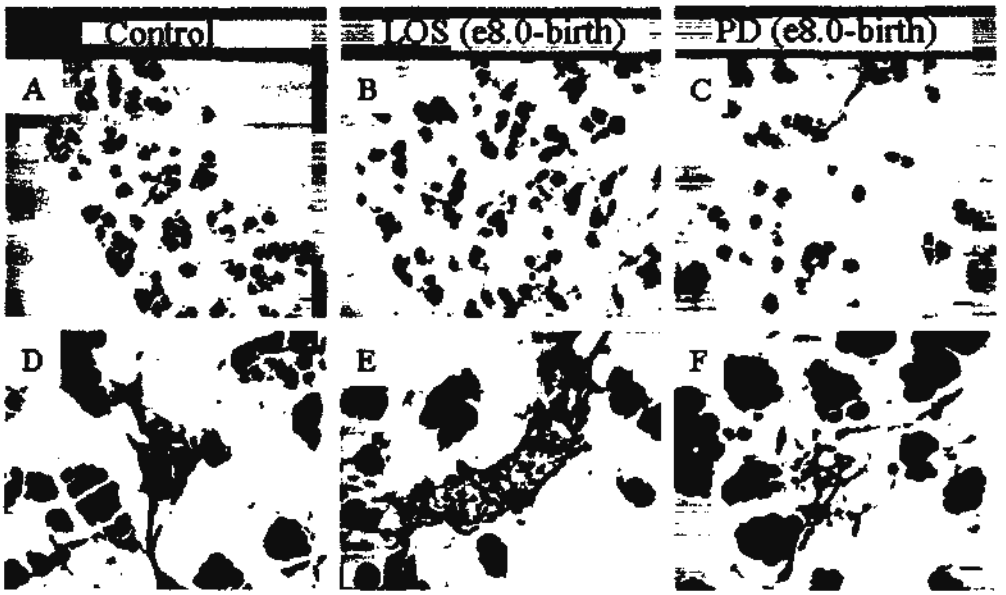


Figure VI. 3.3. Histological examination of n_0 pancreata by H&E staining after LOS or PD treatment on mother mice. (*A-C*) Lower magnification showing the whole pancreas architecture. Original magnification: 200×; Scale bar: 100 μm (*D-F*) Higher magnification showing the islet structure. Original magnification: 630×, Scale bar: 40 μm.

Based on these preliminary observations, we then compared the pancreatic weight of the n_0 pancreas after drug treatment. We applied two additional groups for the different time windows of PD treatment: e8.0-e12.0 and e12.0-birth, which coincide with the first transition and the second/third transitions of mouse pancreatic development, respectively (Habener et al., 2005). The pancreata were blotted dried and their weights among different drug treatment groups were compared. Results revealed a decrease in neonatal pancreatic weight from the PD-treated group but not the LOS-treated group. PD treatment during the first developmental transition did not significantly affect the neonatal pancreatic weight (Figure. VI. 3.4). These observations suggest that a role of AT_2 receptor in regulating the pancreas organogenesis after e12.0 and, in particular, the acinar compartment of which it constitutes the bulk mass of the tissue.

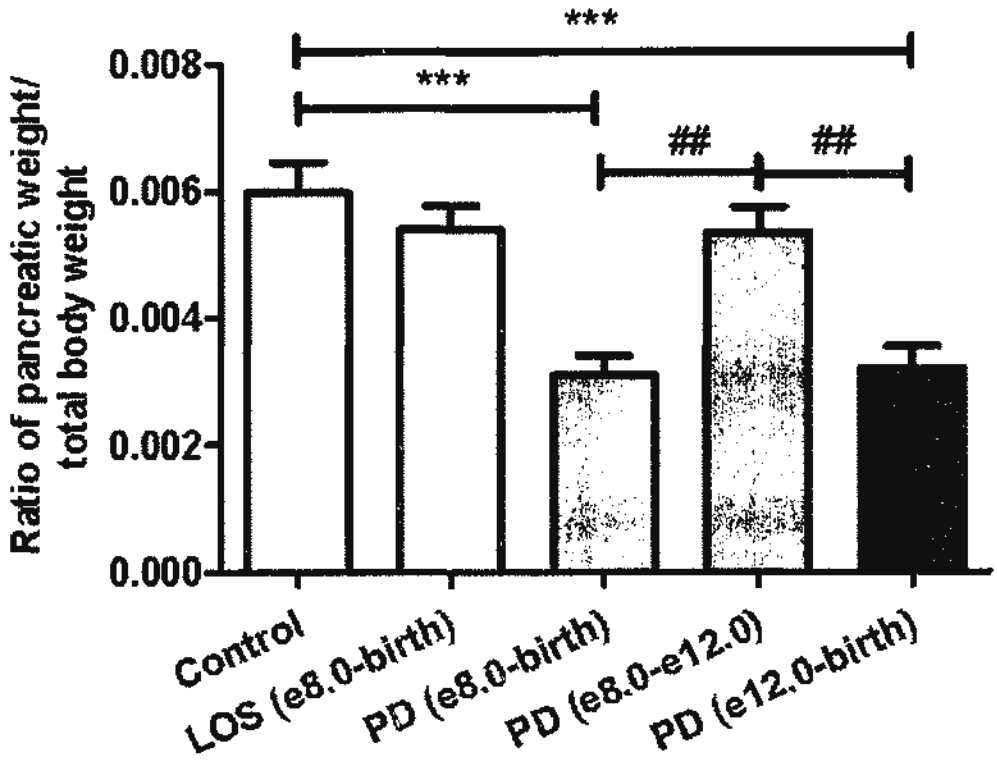


Figure VI. 3.4. Ratio of the weight of n_0 pancreata to total body weight of the neonate. All data are expressed as means ± S.E.M. n = 8 in each group. *** $p<0.001$ vs control; ## $p<0.01$ vs PD treatment at e8.0-e12.0..

VI. 3.3 AT_2 Receptor Blockade during the Late Stage of Fetal Development Impaired Islet Secretory Functions of the Neonates and their Glucose Tolerance.

Next we focused on the effects of these drug treatments on the endocrine functions of the neonates. Islets were isolated from n_4 pancreata and their abilities for glucose-stimulated insulin secretion (GSIS) were assessed. Islets isolated from control group exhibited a normal upregulated insulin release in response to high glucose challenge. LOS treatment and PD treatment at e8.0-e12.0 did not affect this insulin secretory ability. Interestingly, PD treatment group, particularly during the second/third developmental transitions, severely hampered their GSISs (Figure. VI. 3.5). Taken together, the results have demonstrated a critical role for AT_2 receptor in regulating the endocrine cell development during the second/third developmental transitions, and hence critically govern the β-cell functions of the neonates.

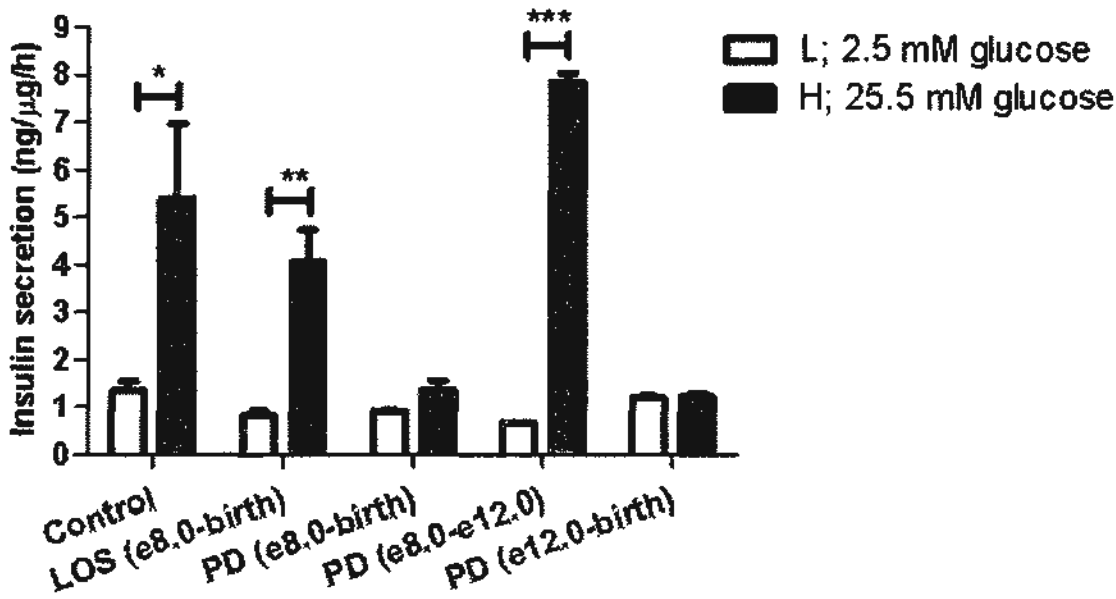


Figure VI. 3.5. Insulin release of n_4 islets in 2.5 mM glucose (L) and 25.5 mM glucose (H). All data are expressed as means ± S.E.M.; n = 6 in each group. * $p<0.05$, ** $p<0.01$, *** $p<0.001$ vs islets exposed to 2.5 mM glucose.

Since maturation of islet cells continues in post-natal period (Habener et al., 2005), we performed further analyses of the endocrine functions of the 1-month old pups after similar drug treatments of their mother mice during pregnancy. No observable differences in the blood glucose levels of the pups were detected among the experimental groups. Yet results from intraperitoneal glucose tolerance tests showed an impaired glucose tolerance in the PD-treated group only during the second/third developmental transitions. The pups in these two groups exhibited a significantly worse glucose tolerance while those from the other groups did not display a distorted alteration in the glucose excursion profile (Figure. VI. 3.6).

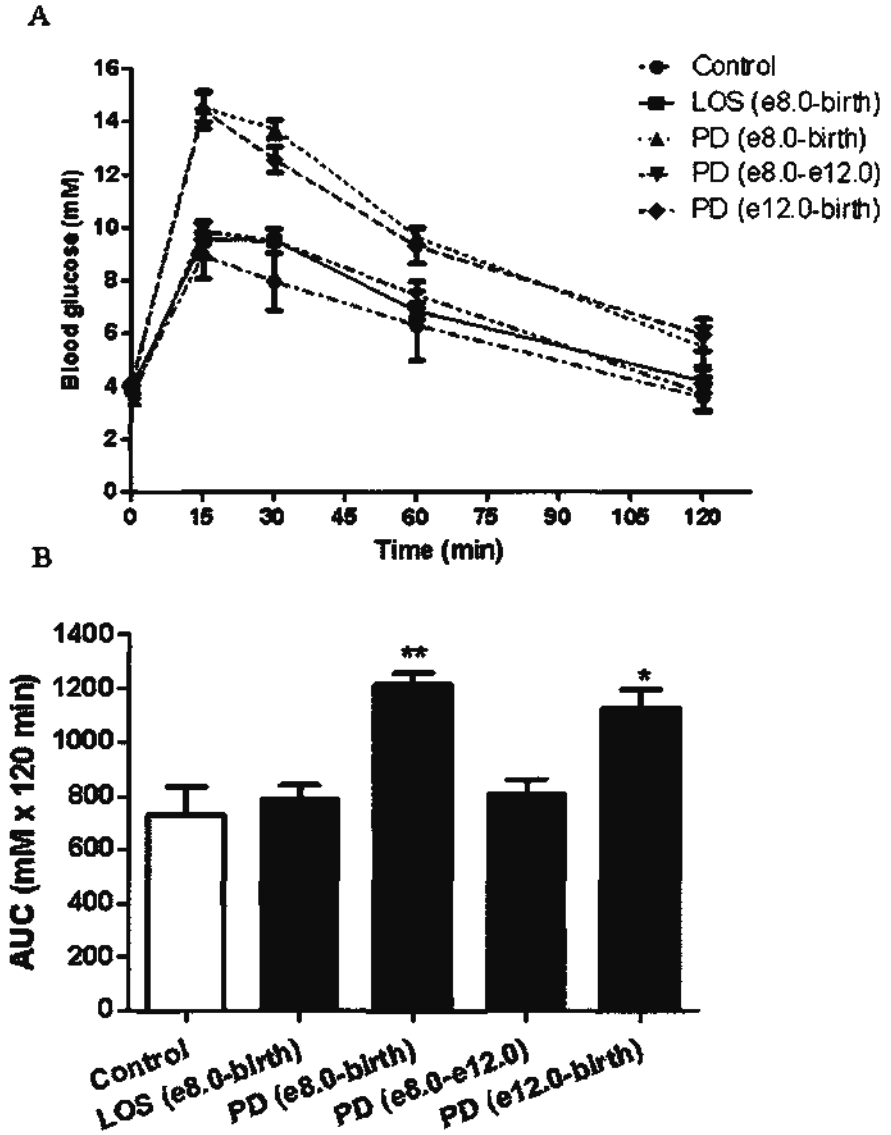


Figure VI. 3.6. IPGTTs were performed on 1-month old pups from each experimental group. (A) Change of blood glucose level was measured immediately before (t = 0 min) and after (t = 15, 30, 60, 90, 120 min) glucose challenge. (*B*) The areas under curve (AUC) were compared between all treatment groups. All data are expressed as means ± S.E.M. n = 5 in each group. * $p<0.05$, ** $p<0.01$ vs control.

VI. 3.4 Evidence for AT_2 Receptor in Governing α-Cell and β-Cell Mass during Islet Development

We further investigated how AT_1 or AT_2 receptor might alter the proportion of both α-cell or β-cell development within an islet. The pancreata of neonatal mice at n_4 were harvested from the control, LOS and PD treatment group, and immunohistochemistry was performed to analyze their total α-cell and β-cell area by glucagon and insulin staining, respectively. Results revealed a dramatic decrease of the stained β-cell area from islets isolated from the PD-treated group (Figure. VI. 3.7*G*) but not the LOS-treated group (Figure. VI. 3.7 *D*; Figure. VI. 3.8*A*). Interestingly, the α-cell area per islet was found to increase in the PD-treated group (Figure. VI. 3.7*H*; Figure. VI. 3.8*B*), with a localization of some α-cells nearer to the centre of the islet. (Figure. VI. 3.7*I*). The calculated β-cell to α-cell ratio of islets isolated from this group was thus found to be significantly lower that those from the others (Figure. VI. 3.8*C*). These data specifically imply the critical role of AT_2 receptor in maintaining normal differentiation of endocrine progenitors into the β-cell linage.

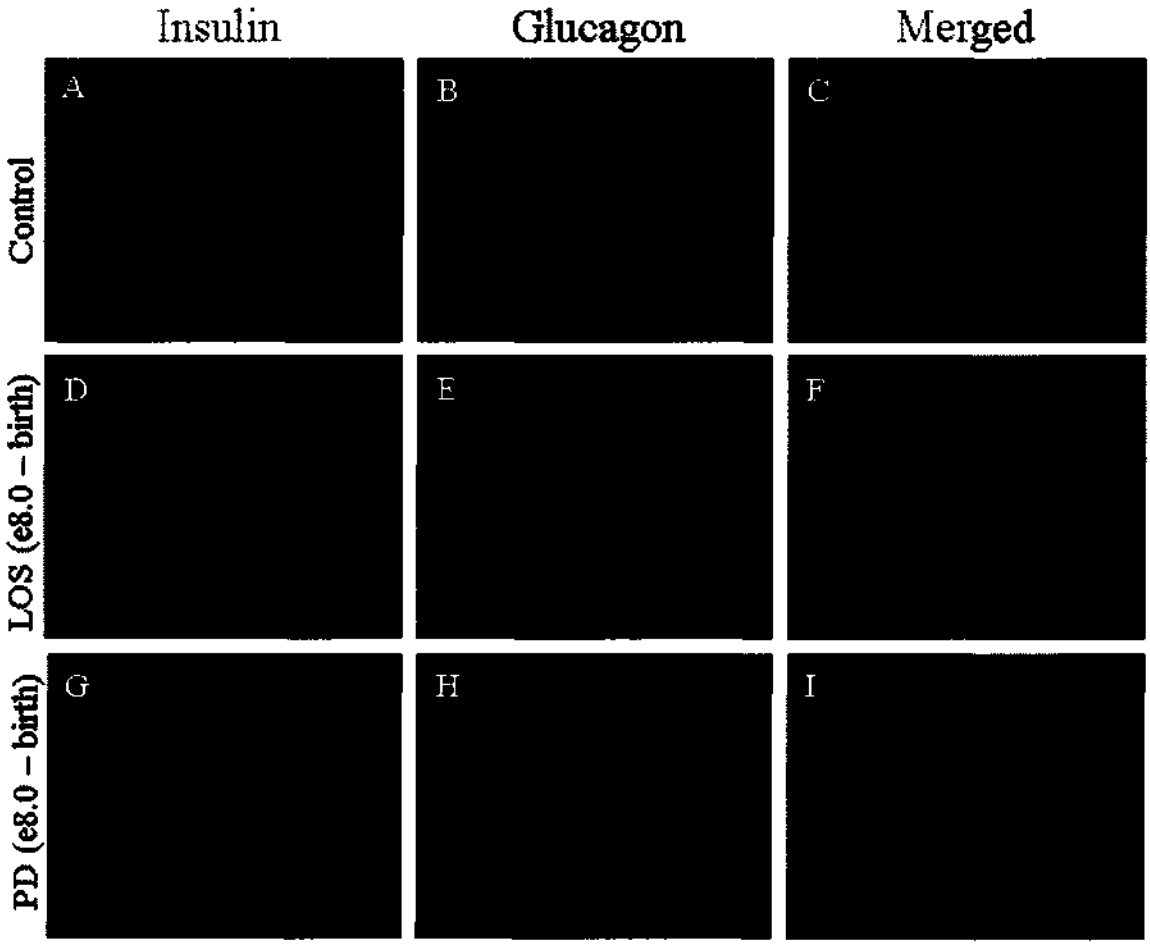


Figure VI. 3.7. Localization of insulin and glucagon in n_4 pancreata after LOS or PD treatment in mother mice during pregnancy. Original magnification: 630×; Scale bar: 40 μm.

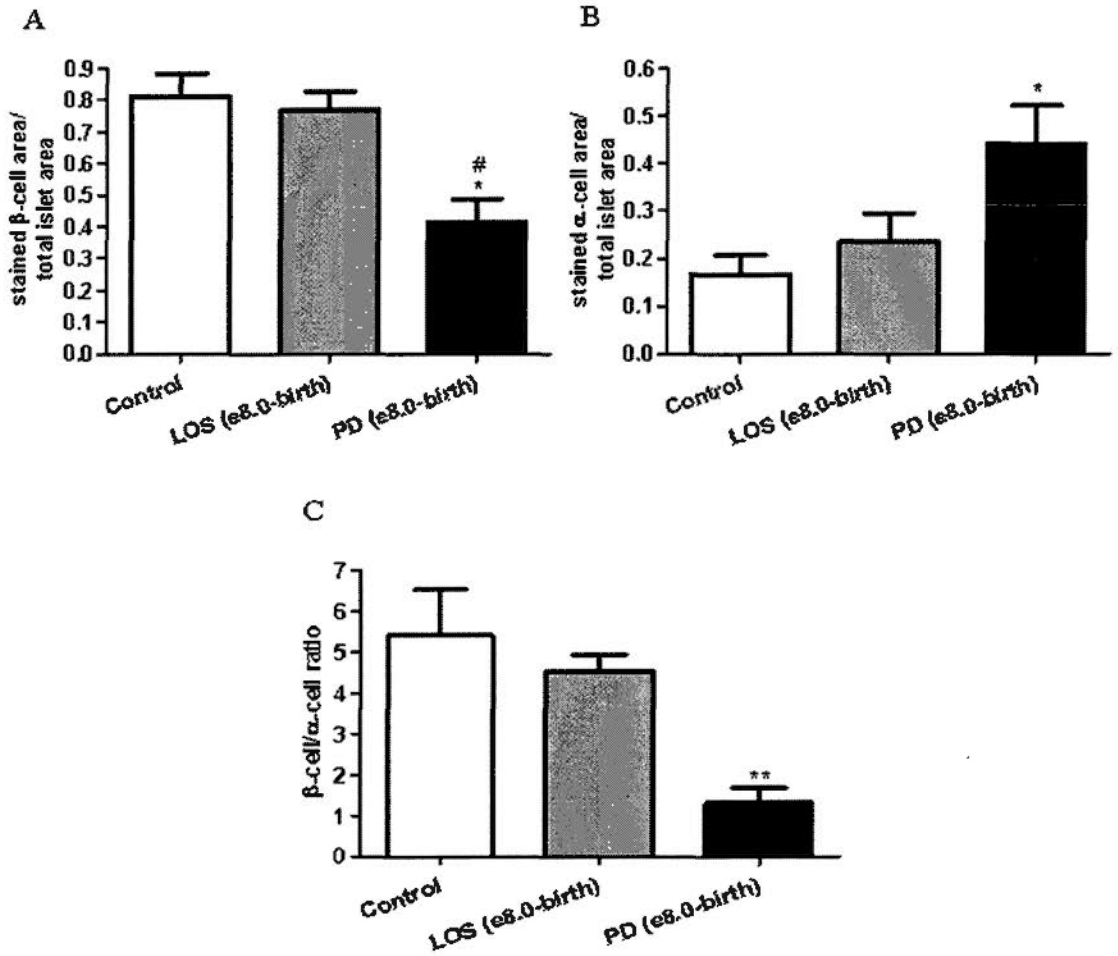


Figure VI. 3.8. Assessment of α-cell and β-cell areas on n_4 pancreata after LOS or PD treatment on mother mice during pregnancy. Stained (*A*) β-cell areas and (*B*) α-cell areas, as well as (*C*) β-cell to α-cell ratio was calculated and compared among different experimental groups. All data are expressed as means ± S.E.M. n = 5 in each group. * $p<0.05$, ** $p<0.01$, # $p<0.05$ vs control.

VI. 4 Discussion

In this study, we provide preliminary evidence that RAS components, notably the AT_1 and AT_2 receptors, are present in a developing mouse pancreas. In chapters IV and V, we have characterized a functional local RAS in a pancreatic progenitor cell (PPC) culture derived from human fetal pancreas, and we have shown that AT_2 receptor is critically involved in their *in vitro* differentiation into insulin-secreting islet-like cell clusters (ICCs). These findings are in line with our present report and they provide informative data for an undiscovered role for AT_2 receptor-mediated *in vivo* development and maturation of β-cells. One notable finding was the suppressed amount of β-cells developed within an islet after AT_2 receptor antagonism while the α-cell mass appeared to be increased. This should have implied a role for AT_2 receptor to function like other pancreatic transcription factors that determine an adoption of either α- or β-cell fates of an endocrine progenitor cell. In this context, *Pax 4* is one of such notable examples that has been recently demonstrated to force an endocrine precursor cell to adopt a β-cell lineage by its ectopic expression (Collombat et al., 2009). The capacity for cellular reprogramming within the pancreas (Zhou et al., 2008; Zhou and Melton, 2008) might also contribute to the findings in our present study, where the decrease in β-cell mass during development might probably be replaced by other components of the endocrine pancreas, α-cells, or even by

ghrelin-expressing ε-cells, as reported elsewhere (Prado et al., 2004).

A differential expression of both AT_1 and AT_2 receptors has been reported in other developing fetal tissues (Norwood et al., 1997; Liu et al., 2007). In neonatal life, such regulated expression still happens in terms of a more abundant expression in the neonates than that in fetal life, followed by a gradual drop of expression levels with aging (Kalinyak et al., 1991). It should be well noted that the postnatal period has also been proposed as a critical window for pancreatic acinar and islet cell maturation (de Assis et al., 2003; Aguayo-Mazzucato et al., 2006), and in this regard, the strong detection of AT_2 receptor observed in our neonatal islets might have implication on its critical involvement in β-cell maturation. Of particular interest is the AT_2 receptor blockade during a specific developmental period during pregnancy where it hampered not only the functional performance of neonatal islets, but also the impaired function as reflected at 1-month old. An inhibited β-cell development in a critical differentiation period during fetal life is thus detrimental to the mature β-cell functions at least in young life.

A correlation of the Ang II receptors to organ development has been focused on the kidney where alterations of either AT_1 or AT_2 receptor would exert severe developmental defects. Blockade of each of these receptors during kidney organogenesis contributes to a different and multiple impairment of the developed kidney architecture, possibly mediated

by different signaling mechanisms (Chen et al., 2008; Sánchez et al., 2008; Song et al., 2010). Our present immunostaining results revealed high levels of expression of AT_2 receptors in the late stage developing pancreas; however, it would be of great interest to characterize the expression of both Ang II receptors during earlier embryonic stages. Expression of AT_1 receptor, in particular, might be noticed in earlier developmental stages. Despite the lack of observable alterations in the differentiated endocrine cells after LOS treatment in e8.0-birth, we cannot exclude its possible regulation in the development of other pancreatic compartments like the ductal cells.

Meanwhile, whether a local RAS including its precursor angiotensinogen or critical enzyme renin could be detected in the developing endocrine pancreas, thus leading to the production of endogenous Ang II warrants further investigations. We cannot rule out the possibility that Ang II might have come from the peripheral developing tissues or in the circulation system, given all major RAS components already detected in very early stages of developing embryos (Schütz et al., 1996). Among these, liver have shown an early expression of *ANGIOTENSINOGEN* mRNA (Schütz et al., 1996), and thus it might possibly contribute to the source of Ang II production that promotes differentiation of pancreatic progenitors. Of note, both the liver and pancreatic progenitors are developed in the vicinity of the definitive endodermal epithelium of the embryonic foregut (Zaret and

Grompe, 2008). It would thus be reasonable to speculate a shared inductive signal for development, which also attributes to the recent findings of the capacity to redirect the developmental route of liver cells towards a pancreatic β-cell phenotype (Ber et al., 2003; Zalzman et al., 2005).

In conclusion, we demonstrated an undiscovered role of AT_2 receptor in regulating the *in vivo* β-cell differentiation from putative pancreatic progenitors. This idea might be extended to the application of triggering β-cell neogenesis in diabetes. Of particular interest is the identification of a down-regulation of AT_2 receptor expression in adult islets exposed to hyperglycemic conditions, as reported in our recent findings (Chu et al., 2010). Recent advances in the *in vivo* gene delivery system that allows the promotion of AT_2 receptor expression in such condition may provide an invaluable tool to rescue the β-cell mass in patients with T1DM or severe form of T2DM.

Chapter VII

General Discussion

The explosion in the prevalence of diabetes mellitus (DM) afflicts not just the Western world but also China (Wong and Wang, 2006). Given the complication of DM, this expanding prevalence carries an attendant medical, social and financial burden. Central to the pathogenesis of diabetes is a state of insulin insufficiency, either absolute (T1DM) or relative (T2DM). The administration of exogenous insulin has become not only the mainstay of treatment in T1DM, but also contributes to the management of T2DM. However, such therapy carries with a substantial social and lifestyle impact. Recent success in islet transplantation shows great promise in these diabetic patients; however, the lack of availability of human donor islets for transplantation hampers the execution of such therapeutic strategies. A new source of such cells must be identified and, in this context, the induction of a stem-cell based therapy offers hope. In this regard, the progenitor cells derived from the pancreas itself might represent a reliable source to achieve the goal.

Both fetal and adult pancreatic tissues are excellent sources of pancreatic progenitor cells (PPCs). These cells are often lineage committed, with a high potential to generate hormone-producing cells through a massive endocrine differentiation *in vivo* (Beattie et al., 1994; Hao et al., 2006). The privileges of applying fetal PPCs over the adult counterpart have been emphasized, regarding not only their multipotentiality to differentiate into the

target cell type, but also for their longer lifespan and reduced immunogenicities (O'Donoghue and Fisk, 2004). Though fetal cells might be suggested with a lower replication potential when compared to the pluripotent ESCs (Inada et al., 2008), a simple genetic modification strategy by introducing telomerase reverse transcriptase was recently reported to greatly enhance expansions of human fetal PPCs *in vitro*, even associated with a gain of additional mesenchymal properties (Cheng et al., 2010). Their potential use as a source of mature islet cells has also been fuelled by the recent demonstration that such cells may be induced to differentiate into islet-like cell clusters (ICCs) (Huang and Tang, 2003; Suen et al., 2008). Devising a complete differentiation protocol that drives a full maturation of such physiologically transplantable ICCs is of utmost importance. It is in this context that we first investigated the capacity of a novel morphogenic factor, PDZ-domain-containing 2 (PDZD2), in directing the functional maturation of our previously reported human fetal PPC-derived ICCs, and second we evaluated a possible regulation by the recently identified pancreatic renin-angiotensin system (RAS) on the growth and development of PPCs. Third we extended such findings on the RAS to a developing pancreas, where understanding the molecular determinants that governs the endocrine cell development will definitely help in manipulating cellular reprogramming within the pancreas. To achieve these objectives, we employed multidisciplinary

approaches embracing cellular and animal studies, genomic and proteomic assays, as well as mechanistic and functional assessment.

VII. 1 PDZD2 as a Novel Morphogenic Factor in PPC Differentiation

The observation that PDZD2, a multi-PDZ-domain protein, that is expressed in fetal pancreatic β-cells suggests its correlation to PPC development. Previous study has confirmed a dose-dependent mitogenic effect of the secreted form of PDZD2, sPDZD2, on the PPC population at a peak concentration of 10^{-12} M, and yet it also suppressed PPC differentiation at this concentration (Suen et al., 2008). Herein we defined a novel role of sPDZD2 that it promoted PPC differentiation at another concentration of 10^{-9} M, evidenced by an upregulation of some critical endocrine transcription factors and an elevated C-peptide content in the differentiated ICCs. Noteworthy, different concentrations of sPDZD2 contributed to multiple functional outcomes on different cell types - sPDZD2 at high doses ($>10^{-9}$ M) decreased the viability of different cancer cells (Tam et al., 2006; Tam et al., 2008) while a lower dose promoted proliferation of a pancreatic β-cell line (Ma et al., 2006). These observations might imply multiple mechanistic pathways involved in sPDZD2 signaling.

The regulation of the functional expressions of ion channels by PDZD2 has recently been supported (Shao et al., 2009). One notable observation was the ability of sPDZD2 to trigger an expression of the $Ca_v1.2$ channels in ICCs and in turn confer on them an insulin-secretory response against membrane depolarization. As such, the cell phenotype

of a sPDZD2-treated ICC functions more like a mature islet, though still remained a distance toward acquiring a glucose-responsiveness. Detailed mechanistic pathways depicting this sPDZD2-induced functional maturation of ICCs warrant further investigations. Meanwhile, we detected a temporally regulated sPDZD2 expression in PPCs derived from different gestational weeks of human fetal pancreas. This phenomenon might represent a role of sPDZD2 in regulating particular developmental event in a developing pancreas. It might also imply the possibility of designating sPDZD2 as a developmentally regulated marker of endocrine cell precursor, as suggested similarly with other molecules that also exhibited temporally regulated expression across gestational weeks (Beattie et al., 1994). These data, taken together with our previous study, reveal the bi-phasic morphogenic property of sPDZD2 on PPC development, which also provide insight to amend the customary growth factors used to differentiate PPCs.

VII. 2 Existence of a Local RAS and its Regulation on the Development and Transplantation Potential of the PPC-derived ICCs

In view of the fact that the ICCs exhibit poor glucose-responsiveness, and sPDZD2 treatment in the previous study was not competent enough to drive their full functional maturation, we therefore endeavored to search for other possible morphogenic factors to promote PPC development. Angiotensin II (Ang II), a physiologically active component of the RAS, was recently proven to regulate the differentiation of several stem cells and tissue progenitors (Rodriguez-Pallares et al., 2004; Kim and Han, 2008; Kim et al., 2010). In the current study, Ang II was found to exert a proliferative and anti-apoptotic effect on PPCs. It also promoted expression of several endocrine and β-cell phenotypic factors in ICCs, notably the marker for endocrine progenitor *NGN3*. This reflected the ability of Ang II to turn the pancreatic precursor population into endocrine cell fates. In contrast to the sPDZD2, which exhibited a concentration-dependent morphogenic effect on PPCs/ICCs, higher concentrations (> 1 μM) of Ang II were not detected with a suppression of PPC differentiation. This reflected that Ang II is not a bi-phasic morphogenic factor in cell development, as suggested for sPDZD2 or others (Yan et al., 2009).

Of note, we provided evidence for a temporally regulated expression of the major RAS components during PPC differentiation into ICCs. Ang II type 2 (AT_2) receptor, in

particular, was found to be profoundly upregulated during differentiation, and meanwhile it was shown to critically mediate the Ang II-induced PPC differentiation using a lentivirus knockdown system. This Ang II-AT_2 receptor axis has therefore proven an undiscovered mechanism in governing endocrine cell development. The activation of ROS pathways by Ang II stimulation might warrant further investigations, given that several reports recently elucidated the mediation of stem cell differentiation by ROS (Owusu-Ansah and Banerjee, 2009; Ushio-Fukai and Urao, 2009).

An important finding of such fetal PPC-derived ICCs is their capacity to reverse hyperglycemia and improve glucose tolerance in diabetic animals. Their *in vivo* maturation under a high glucose environment, reflected by an induction of Glut-2 expression, represents a critical breakthrough for their potential to achieve a successful stem cell-based therapy for diabetic individuals. In parallel to the findings of other similar transplant studies that made use on different stem cell sources, our diabetic mice did not exhibit a full recovery of the diabetic condition (Baetge, 2008). This implies a requisite of additional stimulatory signals for hastening their full maturation. In this context, we demonstrated that AT_2 receptor as a critical mediator of such process. Proper gene deliveries that augment this Ang II-AT_2 receptor mechanistic pathway might potentially represent a novel strategy for promoting the graft maturation *in vivo*.

VII. 3 A Possible Regulation by the RAS Components on the Developing Endocrine Pancreas

Based on the previous findings that components of a RAS regulated the *in vitro* differentiation of PPCs into ICCs, we extended such study by investigating a potential RAS expression in a developing mouse pancreas, and if so, its role in regulating β-cell development. Both AT_1 and AT_2 receptors were found to express in the developing pancreas from e15.5 to e17.5, with an intense co-localization of the latter with the scattered insulin-secreting β-cells. This phenomenon, in keeping with the data in our previous study, reflected the possibility of designating AT_2 receptor as a marker for future β-cell population. In other words, the pattern and amount of AT_2 receptor expression in the progenitors might be important in shaping the Ang II-induced neogenesis of β-cell. This concept has been established by the morphogen-regulated patterning of some invertebrate development which is tightly controlled by the concentration of different morphogens and their receptors (Cadigan, 2002).

Since the post-natal period is also a critical time window for islet cell development (Habener et al., 2005; Aguayo-Mazzucato et al., 2006), a strong expression of AT_2 receptor noted in neonatal pancreata reflected its potential involvement in the functional maturation of β-cells. Blockade of AT_2 receptor in mother mice during pregnancy,

particularly at e12.0-birth during which a massive endocrine differentiation occurs, severely hampers the β-cell secretory functions of the neonate islets. These islets exhibited a reduced β-cell to α-cell ratio, which possibly attributed to their poor glucose-responsiveness. This observation demonstrated the importance of AT_2 receptor signaling on the commitment of α- or β-cell fate of an endocrine progenitor. Of note, the importance of maintaining a normal β-cell mass in sustaining β-cell secretory function has been suggested (Thyssen et al., 2006). The pups of the mother mouse with AT_2 receptor blockade during pregnancy thus exhibited an impaired glucose tolerance at 1-month old. This also implied that the islet cell development during the post-natal time window was inadequate to reverse the distorted β-cell development in fetal life.

VII. 4 Future Studies

In summary, we characterized a novel factor, sPDZD2, as well as an undiscovered regulatory mechanism, RAS, that possess morphogenic properties in the growth and differentiation of PPCs. Some immediate issues that need to be further addressed include but are not limited:

(1) The temporal expression profile of sPDZD2 in the differentiating ICCs and in the developing pancreas. This helps to delineate its mechanistic pathways on regulating a particular signaling target;

(2) A further elaboration of the role of PDZD2 on pancreatic development, especially for the previously identified NLS (nuclear localization signal)-like sequence in the N-terminal portion of PDZD2. This NLS-like sequence might represent a nuclear function of the molecule, and thus potentially serve to modulate transcriptional gene expressions (Thomas et al., 2009);

(3) A novel implication of an upregulation in the islet RAS in diabetes (Lau et al., 2004; Chu et al., 2006). This phenomenon might be due, in part, to the existence of a protective mechanism to trigger differentiation of the residing PPC within the injured pancreas, and in turn achieve β-cell renewal;

(4) The possible mediation of PPC differentiation by the ACE2 pathway of the RAS since

an expression of *ACE2* mRNA in PPCs and ICCs has been detected;

(5) Study of a detailed expression profile of the major RAS components in the developing pancreas from e8.0-birth. The information might help in further elucidating their potential involvement during early pancreas organogenesis;

(6) The possibility of having protein-protein interactions between sPDZD2 and Ang II signaling cascade since they share a common target action on promoting the expression of β-cell phenotypic factors in ICCs. This phenomenon can be possible, as supported by a recent study reporting an overexpression of PDZ-domain-containing proteins that enhanced Ang II-induced protein activations (Ying et al., 2009).

As such, future directions on pancreatic stem cell research will inevitably rests on decoding the complex regulatory mechanisms underlying pancreas organogenesis. The identification of novel morphogenic factors that direct PPC development is definitely essential to facilitate the progressive production of islet cells from progenitors, as well as to catalyze the notion of achieving β-cell regeneration for diabetic patients.

Chapter VIII

Bibliography

[in alphabetical order]

Ackermann, A.M. and Gannon, M. (2007). Molecular regulation of pancreatic beta-cell mass development, maintenance, and expansion. J Mol Endocrinol *38*:193-206.

Aguayo-Mazzucato, C. and Bonner-Weir, S. (2010). Stem cell therapy for type 1 diabetes mellitus. Nat Rev Endocrinol *6*:139-148.

Aguayo-Mazzucato, C., Sanchez-Soto, C., Godinez-Puig, V., Gutiérrez-Ospina, G. and Hiriart, M. (2006). Restructuring of pancreatic islets and insulin secretion in a postnatal critical window. PLoS One *1*:e35.

Ahlgren, U., Jonsson, J., Jonsson, L., Simu, K. and Edlund, H. (1998). beta-cell-specific inactivation of the mouse Ipf1/Pdx1 gene results in loss of the beta-cell phenotype and maturity onset diabetes. Genes Dev *12*:1763-1768.

Ahlgren, U., Pfaff, S.L., Jessell, T.M., Edlund, T. and Edlund, H. (1997). Independent requirement for ISL1 in formation of pancreatic mesenchyme and islet cells. Nature *385*:257-260.

Alberti, K.G. and Zimmet, P.Z. (1998). Definition, diagnosis and classification of diabetes mellitus and its complications. Part 1: diagnosis and classification of diabetes mellitus provisional report of a WHO consultation. Diabet Med *15*:539-553.

Amado, L.C., Saliaris, A.P., Schuleri, K.H., St John, M., Xie, J.S., Cattaneo, S., Durand, D.J., Fitton, T., Kuang, J.Q., Stewart, G., Lehrke, S., Baumgartner, W.W., Martin, B.J., Heldman, A.W. and Hare, J.M. (2005). Cardiac repair with intramyocardial injection of allogeneic mesenchymal stem cells after myocardial infarction. Proc Natl Acad Sci U S A *102*:11474-11479.

Amella, C., Cappello, F., Kahl, P., Fritsch, H., Lozanoff, S. and Sergi, C. (2008). Spatial and temporal dynamics of innervation during the development of fetal human pancreas. Neuroscience *154*:1477-1487.

Amsterdam, A., Nissen, R.M., Sun, Z., Swindell, E.C., Farrington, S. and Hopkins, N. (2004). Identification of 315 genes essential for early zebrafish development. Proc Natl Acad Sci U S A *101*:12792-12797.

Andralojc, K.M., Mercalli, A., Nowak, K.W., Albarello, L., Calcagno, R., Luzi, L.,

Bonifacio, E., Doglioni, C. and Piemonti, L. (2009). Ghrelin-producing epsilon cells in the developing and adult human pancreas. Diabetologia *52*:486-493.

Artavanis-Tsakonas, S., Rand, M.D. and Lake, R.J. (1999). Notch signaling: cell fate control and signal integration in development. Science *284*:770-776.

Ashcroft, S.J. (1976). The control of insulin release by sugars. Ciba Found Symp *41*:117-139.

Atouf, F., Park, C.H., Pechhold, K., Ta, M., Choi, Y. and Lumelsky, N.L. (2007). No evidence for mouse pancreatic beta-cell epithelial-mesenchymal transition in vitro. Diabetes *56*:699-702.

Augstein, P., Stephens, L.A., Allison, J., Elefanty, A.G., Ekberg, M., Kay, T.W. and Harrison, L.C. (1998). Beta-cell apoptosis in an accelerated model of autoimmune diabetes. Mol Med *4*:495-501.

Baetge, E.E. (2008). Production of beta-cells from human embryonic stem cells. Diabetes Obes Metab *10 (Suppl 4)*:186-194.

Barnett, B.P., Arepally, A., Karmarkar, P.V., Qian, D., Gilson, W.D., Walczak, P., Howland, V., Lawler, L., Lauzon, C., Stuber, M., Kraitchman, D.L. and Bulte, J.W. (2007). Magnetic resonance-guided, real-time targeted delivery and imaging of magnetocapsules immunoprotecting pancreatic islet cells. Nat Med *13*:986-991.

Battegay, E.J., Raines, E.W., Seifert, R.A., Bowen-Pope, D.F. and Ross, R. (1990). TGF-β induces bimodal proliferation of connective tissue cells via complex control of an autocrine PDGF loop. Cell *63*:515-524.

Battula, V.L., Treml, S., Abele, H. and Bühring, H.J. (2008). Prospective isolation and characterization of mesenchymal stem cells from human placenta using a frizzled-9-specific monoclonal antibody. Differentiation *76*:326-336.

Beattie, G.M., Levine, F., Mally, M.I., Otonkoski, T., O'Brien, J.S., Salomon, D.R. and Hayek, A. (1994). Acid beta-galactosidase: a developmentally regulated marker of endocrine cell precursors in the human fetal pancreas. J Clin Endocrinol Metab *78*:1232-1240.

Ber, I., Shternhall, K., Perl, S., Ohanuna, Z., Goldberg, I., Barshack, I., Benvenisti-Zarum, L., Meivar-Levy, I. and Ferber, S. (2003). Functional, persistent, and extended liver to pancreas transdifferentiation. J Biol Chem *278*:31950-31957.

Bockman, D.E. (1998). The Pancreas Volume 1. In: Beger, H.G., Warshaw, A.L., Büchler, M.W., Carr-Locke, D.L., Neoptolemos, J.P., Russell, C. and Sarr, M.G., eds. Blackwell Science Ltd. *Chapter 2*: 11-18.

Ber, I., Shternhall, K., Perl, S., Ohanuna, Z., Goldberg, I., Barshack, I., Benvenisti-Zarum, L., Meivar-Levy, I. and Ferber, S. (2003). Functional, persistent, and extended liver to pancreas transdifferentiation. J Biol Chem *278*:31950-31957.

Blau, H.M., Brazelton, T.R. and Weimann, J.M. (2001). The evolving concept of a stem cell: entity or function? Cell *105*:829-841.

Bonal, C. and Herrera, P.L. (2008). Genes controlling pancreas ontogeny. Int J Dev Biol *52*:823-835.

Bonner-Weir, S., Toschi, E., Inada, A., Reitz, P., Fonseca, S.Y., Aye, T. and Sharma, A. (2004). The pancreatic ductal epithelium serves as a potential pool of progenitor cells. Pediatr Diabetes *5 Suppl*:16-22.

Bouwens, L. (2006). Beta cell regeneration. Curr Diabetes Rev *2*:3-9.

Bouwens, L., Lu, W.G., De Krijger, R. (1997). Proliferation and differentiation in the human fetal endocrine pancreas. Diabetologia *40*:398-404.

Brissova, M., Blaha, M., Spear, C., Nicholson, W., Radhika, A., Shiota, M., Charron, M.J., Wright, C.V. and Powers, A.C. (2005). Reduced PDX-1 expression impairs islet response to insulin resistance and worsens glucose homeostasis. Am J Physiol Endocrinol Metab *288*:E704-E714.

Brolén, G.K., Heins, N., Edsbagge, J. and Semb, H. (2005). Signals from the embryonic mouse pancreas induce differentiation of human embryonic stem cells into insulin-producing beta-cell-like cells. Diabetes *54*:2867-2874.

Cabrera, O., Berman, D.M., Kenyon, N.S., Ricordi, C., Berggren, P.O. and Caicedo A. (2006). The unique cytoarchitecture of human pancreatic islets has implications for islet cell function. Proc Natl Acad Sci U S A *103*:2334-2339.

Cadigan, K.M. (2002). Regulating morphogen gradients in the Drosophila wing. Semin Cell Dev Biol *13*:83-90.

Calin-Jageman, I., Yu, K., Hall, R.A., Mei, L. and Lee, A. (2007). Erbin enhances voltage-dependent facilitation of $Ca_v1.3$ Ca^{2+} channels through relief of an autoinhibitory domain in the $Ca_v1.3$ alpha1 subunit. J Neurosci *27*:1374-1385.

Campagnoli, C., Roberts, I.A., Kumar, S., Choolani, M., Bennett, P.R., Letsky, E. and Fisk, N.M. (2002). Expandability of haemopoietic progenitors in first trimester fetal and maternal blood: implications for non-invasive prenatal diagnosis. Prenat Diagn *22*:463-469.

Campos-Lisbôa, A.C., Mares-Guia, T.R., Grazioli, G., Goldberg, A.C. and Sogayar, M.C. (2008). Biodritin microencapsulated human islets of Langerhans and their potential for type 1 diabetes mellitus therapy. Transplant Proc *40*:433-435.

Chae, H.Y., Lee, B.W., Oh, S.H., Ahn, Y.R., Chung, J.H., Min, Y.K., Lee, M.S., Lee, M.K. and Kim, K.W. (2005). Effective glycemic control achieved by transplanting non-viral cationic liposome-mediated VEGF-transfected islets in streptozotocin-induced diabetic mice. Exp Mol Med *37*:513-523.

Chamoux, E., Breault, L., Lehoux, J.G. and Gallo-Payet, N. (1999). Involvement of the angiotensin II type 2 receptor in apoptosis during human fetal adrenal gland development. J Clin Endocrinol Metab *84*:4722-4730.

Chan, Y.C. and Leung, P.S. (2007). Angiotensin II type 1 receptor-dependent nuclear factor-kappaB activation-mediated proinflammatory actions in a rat model of obstructive acute pancreatitis. J Pharmacol Exp Ther *323*:10-18.

Chang, C., Wang, X., Niu, D., Zhang, Z., Zhao, H. and Gong, F. (2009). Mesenchymal stem cells adopt beta-cell fate upon diabetic pancreatic microenvironment. Pancreas *38*:275-281.

Chase, L.G., Ulloa-Montoya, F., Kidder, B.L. and Verfaillie, C.M. (2007). Islet-derived fibroblast-like cells are not derived via epithelial-mesenchymal transition from Pdx-1 or insulin-positive cells. Diabetes *56*:3-7.

Chen, Y.W., Tran, S., Chenier, I., Chan, J.S., Ingelfinger, J.R., Inagami, T. and Zhang, S.L. (2008). Deficiency of intrarenal angiotensin II type 2 receptor impairs paired homeo box-2 and N-myc expression during nephrogenesis. Pediatr Nephrol *23*:1769-1777.

Cheng, K., Follenzi, A., Surana, M., Fleischer, N. and Gupta, S. (2010). Switching of mesodermal and endodermal properties in hTERT-modified and expanded fetal human pancreatic progenitor cells. Stem Cell Res Ther *1*:6.

Cheng, Q., Law, P.K., de Gasparo, M. and Leung, P.S. (2008). Combination of the dipeptidyl peptidase IV inhibitor LAF237 [(S)-1-[(3-hydroxy-1-adamantyl)ammo] acetyl-2-cyanopyrrolidine] with the angiotensin II type 1 receptor antagonist valsartan [N-(1-oxopentyl)-N-[[2'-(1H-tetrazol-5-yl)-[1,1'-biphenyl]-4-yl]methyl]-L-valine] enhances pancreatic islet morphology and function in a mouse model of type 2 diabetes. J Pharmacol Exp Ther *327*:683-691.

Chu, K.Y., Cheng, Q., Chen, C., Au, L.S., Seto, S.W., Tuo, Y., Motin, L., Kwan, Y.W. and Leung, P.S. (2010) Angiotensin II exerts glucose-dependent effects on Kv currents in mouse pancreatic beta-cells via angiotensin II type 2 receptors. Am J Physiol Cell Physiol *298*:C313-C323.

Chu, K.Y., Lau, T., Carlsson, P.O. and Leung, P.S. (2006). Angiotensin II type 1 receptor blockade improves beta-cell function and glucose tolerance in a mouse model of type 2 diabetes. Diabetes *55*:367-374.

Chu, K.Y. and Leung, P.S. (2007). Angiotensin II Type 1 receptor antagonism mediates uncoupling protein 2-driven oxidative stress and ameliorates pancreatic islet beta-cell function in young Type 2 diabetic mice. Antioxid Redox Signal *9*:869-878.

Collombat, P., Xu, X., Ravassard, P., Sosa-Pineda, B., Dussaud, S., Billestrup, N., Madsen, O.D., Serup, P., Heimberg, H. and Mansouri, A. (2009). The ectopic expression of Pax4 in the mouse pancreas converts progenitor cells into alpha and subsequently beta cells. Cell *138*:449-462.

Contrepas, A., Praizovic, N., Duong, van Huyen, J.P., Lelievre-Pegorier, M., Corvol, P. and Nguyen, G. (2007). Expression of (pro)rennin receptor in mouse embryonic and newborn kidney and proliferative effect of soluble (P)RR on mesangial cells. Hypertension *50*:e145.

Cozzi, E. and Bosio, E. (2008) Islet xenotransplantation: current status of preclinical studies in the pig-to-nonhuman primate model. Curr Opin Organ Transplant *13*:155-158.

D'Amore, A., Black, M.J. and Thomas, W.G. (2005). The angiotensin II type 2 receptor causes constitutive growth of cardiomyocytes and does not antagonize angiotensin II type 1 receptor-mediated hypertrophy. Hypertension *46*:1347-1354.

D'Amour, K.A., Bang, A.G., Eliazer, S., Kelly, O.G., Agulnick, A.D., Smart, N.G., Moorman, M.A., Kroon, E., Carpenter, M.K. and Baetge, E.E. (2006). Production of pancreatic hormone-expressing endocrine cells from human embryonic stem cells. Nat Biotechnol *24*:1392-1401.

Davalli, A.M., Ogawa, Y., Scaglia, L., Wu, Y.J., Hollister, J., Bonner-Weir, S. and Weir, G.C. (1995). Function, mass, and replication of porcine and rat islets transplanted into diabetic nude mice. Diabetes *44*:104-111.

Davani, B., Ikonomou, L., Raaka, B.M., Geras-Raaka, E., Morton, R.A., Marcus-Samuels, B. and Gershengorn, M.C. (2007). Human islet-derived precursor cells are mesenchymal stromal cells that differentiate and mature to hormone-expressing cells in vivo. Stem Cells *25*:3215-3222.

de Assis, G.F., Cestari, T.M., Sesso, A. and Taga, R. (2003). Post-natal maturation of acinar cells of the guinea pig pancreas: an ultrastructural morphometric study. Anat Histol Embryol *32*:36-41.

de Gasparo, M., Catt, K.J., Inagami, T., Wright, J.W. and Unger, T. (2000). International union of pharmacology. XXIII. The angiotensin II receptors. Pharmacol Rev *52*:415-472.

de Giorgio, R., Sternini, C., Anderson, K., Brecha, N.C. and Go, V.L. (1992). Tissue distribution and innervation pattern of peptide immunoreactivities in the rat pancreas.

Peptides *13*:91-98.

Desgraz, R. and Herrera, P.L. (2009). Pancreatic neurogenin 3-expressing cells are unipotent islet precursors. Development *136*:3567-3574.

diIorio, P.J., Moss, J.B., Sbrogna, J.L., Karlstrom, R.O. and Moss, L.G. (2002). Sonic hedgehog is required early in pancreatic islet development. Dev Biol *244*:75-84.

Dorman, J.S., La Porte, R.E. and Songer, T.J. (2003). Type 1 Diabetes. Etiology and Treatment. In: Sperling, M.A., eds. Humana Press Inc. *Chatper 1*: 3-22.

Doyle, M.J., Loomis, Z.L. and Sussel, L. (2007). Nkx2.2-repressor activity is sufficient to specify alpha-cells and a small number of beta-cells in the pancreatic islet. Development. *134*:515-523.

Du, A., Hunter, C.S., Murray, J., Noble, D., Cai, C.L., Evans, S.M., Stein, R. and May, C.L. (2009). Islet-1 is required for the maturation, proliferation, and survival of the endocrine pancreas. Diabetes *58*:2059-2069.

Dufrane, D. and Gianello, P. (2008) Pig islet xenotransplantation into non-human primate model. Transplantation **86**:753-760.

Eizirik, D.L., Colli, M.L. and Ortis, F. (2009). The role of inflammation in insulitis and beta-cell loss in type 1 diabetes. Nat Rev Endocrinol *5*:219-226.

Elghazi, L., Weiss, A.J., Barker, D.J., Callaghan, J., Staloch, L., Sandgren, E.P., Gannon, M., Adsay, V.N. and Bernal-Mizrachi, E. (2009). Regulation of pancreas plasticity and malignant transformation by Akt signaling. Gastroenterology *136*:1091-1103.

Fahrenkrug, J. and Emson, P.C. (1982). Vasoactive intestinal polypeptide: functional aspects. Br Med Bull *38*:265-270.

Fenster, C.P., Fenster, S.D., Leahy, H.P., Kurschner, C. and Blundon, J.A. (2007). Modulation of $K_v4.2$ K^+ currents by neuronal interleukin-16, a PDZ domain-containing protein expressed in the hippocampus and cerebellum. Brain Res *1162*:19-31.

Fowler, J.D., Johnson, N.D., Haroldson, T.A., Brintnall, J.A., Herrera, J.E., Katz, S.A. and Bernlohr, D.A. (2009). Regulated renin release from 3T3-L1 adipocytes. Am J Physiol Endocrinol Metab *296*:E1383-E1391.

Franc, S., Dardari, D., Boucherie, B., Riveline, J.P., Biedzinski, M., Petit, C., Requeda, E., Leurent, P., Varroud-Vial, M., Hochberg, G. and Charpentier, G. (2009). Real-life application and validation of flexible intensive insulin-therapy algorithms in type 1 diabetes patients. Diabetes Metab *35*:463-468.

Fritschy, W.M., Wolters, G.H. and van Schilfgaarde, R. (1991). Effect of alginate-polylysine-alginate microencapsulation on in vitro insulin release from rat pancreatic islets. Diabetes *40*:37-43.

Gallo, R., Gambelli, F., Gava, B., Sasdelli, F., Tellone, V., Masini, M., Marchetti, P., Dotta, F. and Sorrentino, V. (2007). Generation and expansion of multipotent mesenchymal progenitor cells from cultured human pancreatic islets. Cell Death Differ *14*:1860-1871.

García-Flores, M., Zueco, J.A., Arenas, J. and Blázquez, E. (2002). Expression of glucose transporter-2, glucokinase and mitochondrial glycerolphosphate dehydrogenase in pancreatic islets during rat ontogenesis. Eur J Biochem *269*:119-127.

Gasa, R., Mrejen, C., Leachman, N., Otten, M., Barnes, M., Wang, J., Chakrabarti, S., Mirmira, R. and German, M. (2004). Proendocrine genes coordinate the pancreatic islet differentiation program *in vitro*. Proc Natl Acad Sci U S A *101*:13245-13250.

Gepts, W. and Pipeleers, D. (1976). The endocrine pancreas. Functional morphology and histopathology. Acta Med Scand Suppl *601*:9-52.

Gersak, K., Cvijic, M. and Cerar, L.K. (2009). Angiotensin II receptor blockers in pregnancy: a report of five cases. Reprod Toxicol *28*:109-112.

Ghosh, B. and Leach, S.D. (2006). Interactions between hairy/enhancer of split-related proteins and the pancreatic transcription factor Ptf1-p48 modulate function of the PTF1 transcriptional complex. Biochem J *393*:679-685.

Gilon, P. and Henquin, J.C. (1992). Influence of membrane potential changes on

cytoplasmic Ca^{2+} concentration in an electrically excitable cell, the insulin-secreting pancreatic β-cell. J Biol Chem *267*:20713-20720.

Gittes, GK. (2009). Developmental biology of the pancreas: a comprehensive review. Dev Biol *326*:4-35.

Götherström, C., Ringdén, O., Tammik, C., Zetterberg, E., Westgren, M. and Le Blanc, K. (2004). Immunologic properties of human fetal mesenchymal stem cells. Am J Obstet Gynecol *190*:239-245.

Götherström, C., Ringdén, O., Westgren, M., Tammik, C. and Le Blanc, K. (2003). Immunomodulatory effects of human foetal liver-derived mesenchymal stem cells. Bone Marrow Transplant *32*:265-272.

Gradwohl, G., Dierich, A., LeMeur, M. and Guillemot, F. (2000). neurogenin3 is required for the development of the four endocrine cell lineages of the pancreas. Proc Natl Acad Sci U S A *97*:1607-1611.

Greenbaum, C.J., Sears, K.L., Kahn, S.E. and Palmer, J.P. (1999). Relationship of beta-cell function and autoantibodies to progression and nonprogression of subclinical type 1 diabetes: follow-up of the Seattle Family Study. Diabetes *48*:170-175.

Grove, K.L., Mayo, R.J., Forsyth, C.S., Frank, A.A. and Speth, R.C. (1995). Fosinopril treatment of pregnant rats: developmental toxicity, fetal angiotensin-converting enzyme inhibition, and fetal angiotensin II receptor regulation. Toxicol Lett *80*:85-95.

Gu, G., Dubauskaite, J. and Melton, D.A. (2002). Direct evidence for the pancreatic lineage: NGN3+ cells are islet progenitors and are distinct from duct progenitors. Development *129*:2447-2457.

Guillemain, G., Filhoulaud, G., Da Silva-Xavier, G., Rutter, G.A. and Scharfmann, R. (2007). Glucose is necessary for embryonic pancreatic endocrine cell differentiation. J Biol Chem *282*:15228-15237

Guo, T. and Hebrok, M. (2009). Stem cells to pancreatic beta-cells: new sources for diabetes cell therapy. Endocr Rev *30*:214-227.

Habener, J.F., Kemp, D.M. and Thomas, M.K. (2005). Minireview: transcriptional regulation in pancreatic development. Endocrinology *146*:1025-1034.

Haber, E. (1969). Recent developments in pathophysiologic studies of the renin-angiotensin system. N Engl J Med *280*:148-155.

Han, H.J., Heo, J.S. and Lee, Y.J. (2005). ANG II increases 2-deoxyglucose uptake in mouse embryonic stem cells. Life Sci *77*:1916-1933.

Hao, E., Tyrberg, B., Itkin-Ansari, P., Lakey, J.R., Geron, I., Monosov, E.Z., Barcova, M., Mercola, M. and Levine, F. (2006). Beta-cell differentiation from nonendocrine epithelial cells of the adult human pancreas. Nat Med *12*:310-316.

Hård, A.L., Wennerholm, U.B., Niklasson, A. and Hellström, A. (2008). Severe ROP in twins after blockage of the renin-angiotensin system during gestation. Acta Paediatr *97*:1142-1144.

Harris, B.Z. and Lim, W.A. (2001). Mechanism and role of PDZ domains in signaling complex assembly. J Cell Sci *114*:3219-3231.

Heng, B.C., Liu, H., Rufaihah, A.J. and Cao, T. (2006). Human embryonic stem cell (hES) colonies display a higher degree of spontaneous differentiation when passaged at lower densities. In Vitro Cell Dev Biol Anim *42*:54-57.

Herold, K.C., Gitelman, S., Greenbaum, C., Puck, J., Hagopian, W., Gottlieb, P., Sayre, P., Bianchine, P., Wong, E., Seyfert-Margolis, V., Bourcier, K., Bluestone, J.A. and Immune Tolerance Network ITN007AI Study Group. (2009). Treatment of patients with new onset Type 1 diabetes with a single course of anti-CD3 mAb Teplizumab preserves insulin production for up to 5 years. Clin Immunol *132*:166-173.

Hori, Y., Fukumoto, M. and Kuroda, Y. (2008). Enrichment of putative pancreatic progenitor cells from mice by sorting for prominin1 (CD133) and platelet-derived growth factor receptor beta. *Stem Cells* 26:2912-1920.

Huang, H. and Tang, X. (2003). Phenotypic determination and characterization of nestin-positive precursors derived from human fetal pancreas. Lab Invest *83*:539-547.

Hu, Y., Liao, L., Wang, Q., Ma, L., Ma, G., Jiang, X. and Zhao, R.C. (2003). Isolation and identification of mesenchymal stem cells from human fetal pancreas. J Lab Clin Med *141*:342-349.

Huang, H.P., Liu, M., El-Hodiri, H.M., Chu, K., Jamrich, M. and Tsai, M.J. (2000). Regulation of the pancreatic islet-specific gene BETA2 (neuroD) by neurogenin 3. Mol Cell Biol. *20*:3292-3307.

Huang, Z., Yu, J., Toselli, P., Bhawan, J., Sudireddy, V., Taylor, L. and Polgar, P. (2007). Angiotensin II type 1 and bradykinin B2 receptors expressed in early stage epithelial cells derived from human embryonic stem cells. J Cell Physiol *211*:816-825.

Humphrey, R.K., Bucay, N., Beattie, G.M., Lopez, A., Messam, C.A., Cirulli, V. and Hayek, A. (2003). Characterization and isolation of promoter-defined nestin-positive cells from the human fetal pancreas. Diabetes *52*:2519-2525.

Hung, A.Y. and Sheng, M. (2002). PDZ domains: structural modules for protein complex assembly. J Biol Chem *277*:5699-5702.

Inada, M., Follenzi, A., Cheng, K., Surana, M., Joseph, B., Benten, D., Bandi, S., Qian, H. and Gupta, S. (2008). Phenotype reversion in fetal human liver epithelial cells identifies the role of an intermediate meso-endodermal stage before hepatic maturation. J Cell Sci *121*:1002-1013.

Iorns, E., Lord, C.J., Turner, N. and Ashworth, A. (2007). Utilizing RNA interference to enhance cancer drug discovery. Nat Rev Drug Discov *6*:556-568.

Ip, S.P., Wong, T.P., Tsai, S.J. and Leung, P.S. (2003). The recovery of some components of the renin angiotensin system in the rat pancreas after chronic exposure to hypoxic condition. J Mol Endocrinol *31*:563-571.

Jan Danser, A.H., Batenburg, W.W. and van Esch, J.H. (2007). Prorenin and the (pro)renin receptor--an update. Nephrol Dial Transplant *22*:1288-1292.

Jensen, J. (2004). Gene regulatory factors in pancreatic development. Dev Dyn *229*:176-200.

Jeon, J, Correa-Medina, M., Ricordi, C., Edlund, H. and Diez, J.A. (2009), Endocrine cell clustering during human pancreas development. J Histochem Cytochem *57*: 811-824.

Jiang, J., Au, M., Lu, K., Eshpeter, A., Korbutt, G., Fisk, G. and Majumdar, A.S. (2007) Generation of insulin-producing islet-like clusters from human embryonic stem cells. Stem Cells *25*:1940-1953.

Joglekar, M.V., Joglekar, V.M., Joglekar, S.V. and Hardikar, A.A. (2009). Human fetal pancreatic insulin-producing cells proliferate in vitro. J Endocrinol *201*:27-36.

Jokubaitis, V.J., Sinka, L., Driessen, R., Whitty, G., Haylock, D.N., Bertoncello, I., Smith, I., Péault, B., Tavian, M. and Simmons, P.J. (2008). Angiotensin-converting enzyme (CD143) marks hematopoietic stem cells in human embryonic, fetal, and adult hematopoietic tissues. Blood *111*:4055-4063.

Kahn, S.E., Hull, R.L. and Utzschneider, K.M. (2006). Mechanisms linking obesity to insulin resistance and type 2 diabetes. Nature *444*:840-846.

Kalergis, M., Pacaud, D., Strychar, I., Meltzer, S., Jones, P.J. and Yale, J.F. (2000). Optimizing insulin delivery: assessment of three strategies in intensive diabetes management. Diabetes Obes Metab *2*:299-305.

Kalinyak, J.E., Hoffman, A.R. and Perlman, A.J. (1991). Ontogeny of angiotensinogen mRNA and angiotensin II receptors in rat brain and liver. J Endocrinol Invest *14*:647-653.

Kaneshiro, Y., Ichihara, A., Takemitsu, T., Sakoda, M., Suzuki, F., Nakagawa, T., Hayashi, M. and Inagami, T. (2006). Increased expression of cyclooxygenase-2 in the renal cortex of human prorenin receptor gene-transgenic rats. Kidney Int *70*:641-646.

Karlsson, C., Lindell, K., Ottosson, M., Sjöström, L., Carlsson, B. and Carlsson, L.M. (1998). Human adipose tissue expresses angiotensinogen and enzymes required for its conversion to angiotensin II. J Clin Endocrinol Metab *83*:3925-3929.

Kato, H., Ishida, J., Imagawa, S., Saito, T., Suzuki, N., Matsuoka, T., Sugaya, T., Tanimoto, K., Yokoo, T., Ohneda, O., Sugiyama, F., Yagami, K., Fujita, T., Yamamoto, M., Nangaku, M. and Fukamizu, A. (2005). Enhanced erythropoiesis mediated by

activation of the renin-angiotensin system via angiotensin II type 1a receptor. FASEB J *19*:2023-2035.

Katovich, M.J., Grobe, J.L., Huentelman, M. and Raizada, M.K. (2005). Angiotensin-converting enzyme 2 as a novel target for gene therapy for hypertension. Exp Physiol *90*:299-305.

Kawaguchi, Y., Cooper, B., Gannon, M., Ray, M., MacDonald, R.J. and Wright, C.V. (2002). The role of the transcriptional regulator Ptf1a in converting intestinal to pancreatic progenitors. Nat Genet *32*:128-134.

Kemp, D.M., Thomas, M.K. and Habener, J.F. (2003). Developmental aspects of the endocrine pancreas. Rev Endocr Metab Disord *4*:5-17.

Kennedy, G.C., German, M.S. and Rutter, W.J. (1995). The minisatellite in the diabetes susceptibility locus IDDM2 regulates insulin transcription. Nat Genet *9*:293-298.

Kim, Y.H. and Han, H.J. (2008). Synergistic effect of high glucose and ANG II on proliferation of mouse embryonic stem cells: involvement of PKC and MAPKs as well as AT1 receptor. J Cell Physiol *215*:374-382.

Kim, Y.H., Ryu, J.M., Lee, Y.J. and Han, H.J. (2010). Fibronectin synthesis by high glucose level mediated proliferation of mouse embryonic stem cells: Involvement of ANG II and TGF-beta1. J Cell Physiol *223*:397-407.

Kim, Y.M., Jeon, E.S., Kim, M.R., Jho, S.K., Ryu, S.W. and Kim, J.H. (2008). Angiotensin II-induced differentiation of adipose tissue-derived mesenchymal stem cells to smooth muscle-like cells. Int J Biochem Cell Biol *40*:2482-2491.

Klimstra, D.S. (1997). Pancreas. Histology for Pathologists. In: Sternberg, S.S., eds. Philadelphia: Lippinocott-Raven Publishers. 613-647.

Kobayashi, K., Imanishi, T. and Akasaka, T. (2006). Endothelial progenitor cell differentiation and senescence in an angiotensin II-infusion rat model. Hypertens Res *29*:449-455.

Koblas, T., Pektorova, L., Zacharovova, K., Berkova, Z., Girman, P., Dovolilova, E.,

Karasova, L. and Saudek, F. (2008). Differentiation of CD133-positive pancreatic cells into insulin-producing islet-like cell clusters. Transplant Proc *40*:415-428.

Kojima, M., Hosoda, H. and Kangawa, K. (2001). Purification and distribution of ghrelin: the natural endogenous ligand for the growth hormone secretagogue receptor. Horm Res *56 Suppl 1*:93-97.

Kordowich, S., Mansouri, A. and Collombat, P. (2010). Reprogramming into pancreatic endocrine cells based on developmental cues. *Mol Cell Endocrinol* 315:11-18.

Kroon, E., Martinson, L.A., Kadoya, K., Bang, A.G., Kelly, O.G., Eliazer, S., Young, H., Richardson, M., Smart, N.G., Cunningham, J., Agulnick, A.D., D'Amour, K.A., Carpenter, M.K. and Baetge, E.E. (2008). Pancreatic endoderm derived from human embryonic stem cells generates glucose-responsive insulin-secreting cells in vivo. Nat Biotechnol *26*:443-452.

Kurakin, A., Swistowski, A., Wu, S.C. and Bredesen, D.E. (2007). The PDZ domain as a complex adaptive system. PLoS ONE *2*:e953.

Kurschner, C. and Yuzaki, M. (1999). Neuronal interleukin-16 (NIL-16): a dual function PDZ domain protein. J Neurosci *19*:7770-7780.

Lai, J.K., Cheng, C.H., Ko, W.H. and Leung, P.S. (2005). Ghrelin system in pancreatic AR42J cells: its ligand stimulation evokes calcium signalling through ghrelin receptors. Int J Biochem Cell Biol *37*:887-900.

Lander, E.S., Linton, L.M., Birren, B., Nusbaum, C., Zody, M.C., Baldwin, J., Devon, K., Dewar, K., Doyle, M., FitzHugh, W., et al. (2001). Initial sequencing and analysis of the human genome. Nature *409*:860-921.

Lau, J., Mattsson, G., Carlsson, C., Nyqvist, D., Köhler, M., Berggren, P.O., Jansson, L. and Carlsson, P.O. (2007). Implantation site-dependent dysfunction of transplanted pancreatic islets. Diabetes *56*:1544-1550.

Lau, T., Carlsson, P.O. and Leung, P.S. (2004). Evidence for a local angiotensin-generating system and dose-dependent inhibition of glucose-stimulated insulin release by angiotensin II in isolated pancreatic islets. Diabetologia *47*:240-248.

Leclerc, I. and Rutter, GA. (2004). AMP-activated protein kinase: a new beta-cell glucose sensor?: Regulation by amino acids and calcium ions. Diabetes *53*:S67-S74.

León-Quinto, T., Jones, J., Skoudy, A., Burcin, M. and Soria, B. (2004). In vitro directed differentiation of mouse embryonic stem cells into insulin-producing cells. Diabetologia *47*:1442-1451.

Leung, K.K. and Leung, P.S. (2008). Effects of hyperglycemia on angiotensin II receptor type 1 expression and insulin secretion in an INS-1E pancreatic beta-cell line. JOP *9*:290-299.

Leung, K.K., Suen, P.M., Lau, T.K., Ko, W.H., Yao, K.M. and Leung, P.S. (2009). PDZ-domain containing-2 (PDZD2) drives the maturity of human fetal pancreatic progenitor-derived islet-like cell clusters with functional responsiveness against membrane depolarization. Stem Cells Dev *18*:979-990.

Leung, P.S. (2006). Importance of the Local Renin-Angiotensin System in Pancreatic Disease. In: Leung, P.S., eds. Proteases in Biology and Diseases- Proteases in Gastrointestinal Tissues. Springer. *Chapter 6*:131-152.

Leung, P.S. (2007a). The physiology of a local renin-angiotensin system in the pancreas. J Physiol *580*:31-37.

Leung, P.S. (2007b). Mechanisms of protective effects induced by blockade of the renin-angiotensin system: novel role of the pancreatic islet angiotensin-generating system in Type 2 diabetes. Diabet Med *24*:110-116.

Leung, P.S. (in press). The renin-angiotensin system: current research progress in the pancreas. Advances in Experimental Medicine and Biology 677. *Springer*.

Lewandowski, D., Barroca, V., Ducongé, F., Bayer, J., Van Nhieu, J.T., Pestourie, C., Fouchet, P., Tavitian, B. and Roméo, P.H. (2010). In vivo cellular imaging pinpoints the role of reactive oxygen species in the early steps of adult hematopoietic reconstitution. Blood *115*:443-452.

Li, F.R., Wang, X.G., Deng, C.Y., Qi, H., Ren, L.L. and Zhou, H.X. (in press). Immune

modulation of co-transplantation mesenchymal stem cells with islet on T and dendritic cells. Clin Exp Immunol *7 May 2010*. Epub ahead of print.

Liu, H.W., Cheng, B., Fu, X.B., Sun, T.Z. and Li, J.F. (2007). Characterization of AT1 and AT2 receptor expression profiles in human skin during fetal life. J Dermatol Sci *46*:221-225.

Lopez-Talavera, J.C., Garcia-Ocaña, A., Sipula, I., Takane, K.K., Cozar-Castellano, I. and Stewart, A.F. (2004). Hepatocyte growth factor gene therapy for pancreatic islets in diabetes: reducing the minimal islet transplant mass required in a glucocorticoid-free rat model of allogeneic portal vein islet transplantation. Endocrinology *145*:467-474.

Lubel, J.S., Herath, C.B., Burrell, L.M. and Angus, P.W. (2008). Liver disease and the renin-angiotensin system: recent discoveries and clinical implications. J Gastroenterol Hepatol *23*:1327-1338.

Lyttle, B.M., Li, J., Krishnamurthy, M., Fellows, F., Wheeler, M.B., Goodyer, C.G. and Wang, R. (2008). Transcription factor expression in the developing human fetal endocrine pancreas. Diabetologia *51*:1169-1180.

Ma, R.Y., Tam, T.S., Suen, A.P., Yeung, P.M., Tsang, S.W., Chung, S.K., Thomas, M.K., Leung, P.S. and Yao, K.M. (2006). Secreted PDZD2 exerts concentration-dependent effects on the proliferation of INS-1E cells. Int J Biochem Cell Biol *38*:1015-1022.

Maehr, R., Chen, S., Snitow, M., Ludwig, T., Yagasaki, L., Goland, R., Leibel, R.L. and Melton, D.A. (2009). Generation of pluripotent stem cells from patients with type 1 diabetes. Proc Natl Acad Sci U S A *106*: 15768-15773.

Martinez-Fernandez, A., Nelson, T.J., Ikeda, Y. and Terzic, A. (2010). c-MYC independent nuclear reprogramming favors cardiogenic potential of induced pluripotent stem cells. J Cardiovasc Transl Res *3*:13-23.

Matsusaka, T., Miyazaki, Y. and Ichikawa, I. (2002). The renin angiotensin system and kidney development. Annu Rev Physiol *64*:551-561.

Matsushita, K., Wu, Y., Okamoto, Y., Pratt, R.E. and Dzau, V.J. (2006). Local renin angiotensin expression regulates human mesenchymal stem cell differentiation to adipocytes.

Hypertension *48*:1095-1102.

Mattsson, G., Jansson, L., Nordin, A., Andersson, A. and Carlsson, P.O. (2004). Evidence of functional impairment of syngeneically transplanted mouse pancreatic islets retrieved from the liver. Diabetes *53*:948-954.

McKinley, M.J., Albiston, A.L., Allen, A.M., Mathai, M.L., May, C.N., McAllen, R.M., Oldfield, B.J., Mendelsohn, F.A. and Chai, S.Y. (2003). The brain renin-angiotensin system: location and physiological roles. Int J Biochem Cell Biol *35*:901-918.

Melloul, D., Marshak, S. and Cerasi, E. (2002). Regulation of pdx-1 gene expression. Diabetes *51 Suppl 3*:S320-S325.

Merani, S., Toso, C., Emamaullee, J. and Shapiro, A.M. (2008). Optimal implantation site for pancreatic islet transplantation. Br J Surg *95*:1449-1461.

Merchant, F.A., Diller, K.R., Aggarwal, S.J. and Bovik, A.C. (1997). Angiogenesis in cultured and cryopreserved pancreatic islet grafts. Transplantation *63*:1652-1660.

Merrel, R.C., Basadonna, G. and Kakizaki, K. (1988). Endocrine pancreas. In: Miller, T.A. and Rowlands, B.J., eds. Physiologic Basics of Modern Surgical Care. St. Louis: CV Mosby. 982-997.

Meyer, T., Höcht, B. and Ulrichs, K. (2008). Xenogeneic islet transplantation of microencapsulated porcine islets for therapy of type I diabetes: long-term normoglycemia in STZ-diabetic rats without immunosuppression. Pediatr Surg Int *24*:1375-1378.

Minami, K. and Seino, S. (2008). Pancreatic acinar-to-beta cell transdifferentiation in vitro. Front Biosci *13*:5824-5837.

Morton, R.A., Geras-Raaka, E., Wilson, L.M., Raaka, B.M. and Gershengorn, M.C. (2007). Endocrine precursor cells from mouse islets are not generated by epithelial-to-mesenchymal transition of mature beta cells. Mol Cell Endocrinol *270*:87-93.

Moss, L.G. and Rhodes, C.J. (2007). Beta-cell regeneration: epithelial mesenchymal

transition pre-EMTpted by lineage tracing? Diabetes *56*:281-282.

Müller, F.J., Laurent, L.C., Kostka, D., Ulitsky, I., Williams, R., Lu, C., Park, I.H., Rao, M.S., Shamir, R., Schwartz, P.H., Schmidt, N.O. and Loring, J.F. (2008). Regulatory networks define phenotypic classes of human stem cell lines. Nature *455*:401-405.

Murtaugh, L.C. (2007). Pancreas and beta-cell development: from the actual to the possible. Development *134*:427-438.

Murtaugh, L.C., Law, A.C., Dor, Y. and Melton, D.A. (2005). Beta-catenin is essential for pancreatic acinar but not islet development. Development *132*:4663-4674.

Narang, A.S., Cheng, K., Henry, J., Zhang, C., Sabek, O., Fraga, D., Kotb, M., Gaber, A.O. and Mahato, R.I. (2004). Vascular endothelial growth factor gene delivery for revascularization in transplanted human islets. Pharm Res *21*:15-25.

Navarro-Tableros, V., Fiordelisio, T., Hernández-Cruz, A. and Hiriart, M. (2007). Physiological development of insulin secretion, calcium channels, and GLUT2 expression of pancreatic rat beta-cells. Am J Physiol Endocrinol Metab *292*:E1018-E1029.

Naya, F.J., Stellrecht. C.M. and Tsai MJ. (1995). Tissue-specific regulation of the insulin gene by a novel basic helix-loop-helix transcription factor. Genes Dev 9:1009-1019.

Ng, K.Y., Ma, M.T., Leung, K.K. and Leung, P.S. (in press). Vitamin D and vitamin A receptor expression and the proliferative effects of lingand activation of these receptors on the development of pancreatic progenitor cells derived from human fetal pancreas. Stem Cell Rev & Rep *31 Mar 2010* Epub ahead of print.

Nir, T., Melton, D.A. and Dor, Y. (2007). Recovery from diabetes in mice by beta cell regeneration. J Clin Invest *117*:2553-2561.

Norwood, V.F., Craig, M.R., Harris, J.M. and Gomez, R.A. (1997). Differential expression of angiotensin II receptors during early renal morphogenesis. Am J Physiol *272*:R662-R668.

Nussey, S.S. and Whitehead, S.A. (2001) Endocrinology: An integrated approach. BIOS

Scientific Publishers Ltd. London: Taylor and Francis. *Chapter 2.*

O'Donoghue, K. and Fisk, N.M. (2004). Stem Cells in Obstetrics and Gynaecology. In: Fisk, N. and Itskovitz-Eldor, J. eds. Best Practice & Research Clinical Obstetrics & Gynaecology. Elsevier. *Chapter 3*: 853-875.

Oliver-Krasinski, J.M., Kasner, M.T., Yang, J., Crutchlow, M.F., Rustgi, A.K., Kaestner, K.H. and Stoffers, D.A. (2009). The diabetes gene Pdx1 regulates the transcriptional network of pancreatic endocrine progenitor cells in mice. J Clin Invest *119*:1888-1898.

Oliver-Krasinski, J.M. and Stoffers, D.A. (2008). On the origin of the beta cell. Genes Dev *22*:1998-2021.

Orive, G., Hernández, R.M., Gascón, A.R., Calafiore, R., Chang, T.M., De Vos, P., Hortelano, G., Hunkeler, D., Lacík, I., Shapiro, A.M. and Pedraz, J.L. (2003). Cell encapsulation: promise and progress. Nat Med *9*:104-107.

Oshima, Y., Suzuki, A., Kawashimo, K., Ishikawa, M., Ohkohchi, N. and Taniguchi, H. (2007). Isolation of mouse pancreatic ductal progenitor cells expressing CD133 and c-Met by flow cytometric cell sorting. Gastroenterology *132*:720-732.

Oster, A., Jensen, J., Serup, P., Galante, P., Madsen, O.D. and Larsson, L.I. (1998). Rat endocrine pancreatic development in relation to two homeobox gene products (Pdx-1 and Nkx 6.1). J Histochem Cytochem *46*:707-715.

Owusu-Ansah, E. and Banerjee, U. (2009). Reactive oxygen species prime Drosophila haematopoietic progenitors for differentiation. *Nature* 461:537-541.

Parekkadan B, Milwid JM. (in press). Mesenchymal Stem Cells as Therapeutics. Annu Rev Biomed Eng *20 April 2010*. Epub ahead of print.

Peach, M.J. (1977). Renin-angiotensin system: biochemistry and mechanisms of action. Physiol Rev *57*:313-370.

Pepper. M.S., Vassalli, J.D., Orci, L. and Montesano, R. (1993). Biphasic effect of transforming growth factor-beta 1 on *in vitro* angiogenesis. Exp Cell Res

204:356-363.

Perlegas, D., Xie, H., Sinha, S., Somlyo, A.V. and Owens, G.K. (2004). ANG II type 2 receptor regulates smooth muscle growth and force generation in late fetal mouse development. Am J Physiol Heart Circ Physiol *288*:H96-H102.

Peyrot, M., Rubin, R.R., Kruger, D.F. and Travis, L.B. (2010). Correlates of insulin injection omission. Diabetes Care *33*:240-245.

Phan-Hug, F., Guimiot, F., Lelièvre, V., Delezoide, A.L., Czernichow, P., Breant, B. and Blondeau, B. (2008). Potential role of glucocorticoid signaling in the formation of pancreatic islets in the human fetus. Pediatr Res *64*:346-351.

Pinaud, F., Bocquet, A., Dumont, O., Retailleau, K., Baufreton, C., Andriantsitohaina, R., Loufrani, L. and Henrion, D. (2007). Paradoxical role of angiotensin II type 2 receptors in resistance arteries of old rats. Hypertension *50*:96-102.

Piper, K., Brickwood, S., Turnpenny, L.W., Cameron, I.T., Ball, S.G., Wilson, D.I. and Hanley, N.A. (2004). Beta cell differentiation during early human pancreas development. J Endocrinol *181*:11-23.

Polak, M., Bouchareb-Banaei, L., Scharfmann, R. and Czernichow, P. (2000). Early pattern of differentiation in the human pancreas. Diabetes *49*:225-232.

Prado, C.L., Pugh-Bernard, A.E., Elghazi, L., Sosa-Pineda, B. and Sussel, L. (2004). Ghrelin cells replace insulin-producing beta cells in two mouse models of pancreas development. Proc Natl Acad Sci U S A *101*:2924-2929.

Prockop, D.J. (1997). Marrow stromal cells as stem cells for nonhematopoietic tissues. Science *276*:71-74.

Puri, S. and Hebrok, M. (2010). Cellular plasticity within the pancreas--lessons learned from development. Dev Cell *18*:342-356.

Ramalho-Santos, M., Yoon, S., Matsuzaki, Y., Mulligan, R.C. and Melton, D.A. (2002). "Stemness": transcriptional profiling of embryonic and adult stem cells. Science *298*:597-600.

Rebel, V.I., Miller, C.L., Thornbury, G.R., Dragowska, W.H., Eaves, C.J. and Lansdorp, P.M. (1996). A comparison of long-term repopulating hematopoietic stem cells in fetal liver and adult bone marrow from the mouse. Exp Hematol *24*:638-648.

Robillard, J.E., Page, W.V., Mathews, M.S., Schutte, B.C., Nuyt, A.M. and Segar, J.L. (1995). Differential gene expression and regulation of renal angiotensin II receptor subtypes (AT1 and AT2) during fetal life in sheep. Pediatr Res *38*:896-904.

Roche, E., Jones, J., Arribas, M.I., Leon-Quinto, T. and Soria, B. (2006). Role of small bioorganic molecules in stem cell differentiation to insulin-producing cells. Bioorg Med Chem *14*:6466-6474.

Rodriguez-Pallares, J., Quiroz, C.R., Parga, J.A., Guerra, M.J. and Labandeira-Garcia, J.L. (2004). Angiotensin II increases differentiation of dopaminergic neurons from mesencephalic precursors via angiotensin type 2 receptors. Eur J Neurosci *20*:1489-1498.

Roep, B.O. (1996). T-cell responses to autoantigens in IDDM. The search for the Holy Grail. Diabetes *45*:1147-1156.

Romanov, Y.A., Svintsitskaya, V.A. and Smirnov, V.N. (2003). Searching for alternative sources of postnatal human mesenchymal stem cells: candidate MSC-like cells from umbilical cord. Stem Cells *21*:105-110.

Rossant, J. (2007). Stem cells: the magic brew. Nature *448*:260-262.

Rozen, S. and Skaletsky, H.J. (2000). Primer3 on the WWW for general users and for biologist programmers. Bioinformatics Methods and Protocols: Methods in Molecular Biology. *Humana Press, Totowa, NJ*: 365-386.

Rudnick, A., Ling, T.Y., Odagiri, H., Rutter, W.J. and German, M.S. (1994). Pancreatic beta cells express a diverse set of homeobox genes. Proc Natl Acad Sci U S A. *91*:12203-12207.

Ruiz, S.A. and Chen, C.S. (2008). Emergence of patterned stem cell differentiation within multicellular structures. Stem Cells *26*:2921-2927.

Sabb, J.E., Godfrey, P.M. and Brannon, P.M. (1986). Adaptive response of rat pancreatic lipase to dietary fat: effects of amount and type of fat. J Nutr *116*:892-899.

Saiki, A., Koide, N., Watanabe, F., Murano, T., Miyashita, Y. and Shirai K. (2008). Suppression of lipoprotein lipase expression in 3T3-L1 cells by inhibition of adipogenic differentiation through activation of the renin-angiotensin system. Metabolism *57*:1093-1100.

Saleem, S., Li, J., Yee, S.P., Fellows, G.F., Goodyer, C.G. and Wang, R. (2009). beta1 integrin/FAK/ERK signalling pathway is essential for human fetal islet cell differentiation and survival. J Pathol *219*:182-192.

Sánchez, S.I., Seltzer, A.M., Fuentes, L.B., Forneris, M.L. and Ciuffo, G.M. (2008). Inhibition of Angiotensin II receptors during pregnancy induces malformations in developing rat kidney. Eur J Pharmacol *588*:114-123.

Sander, M., Sussel, L., Conners, J., Scheel, D., Kalamaras, J., Dela Cruz, F., Schwitzgebel, V., Hayes-Jordan, A. and German, M. (2000). Homeobox gene Nkx6.1 lies downstream of Nkx2.2 in the major pathway of beta-cell formation in the pancreas. Development *127*:5533-5540.

Sanjeevi, C.B., Lybrand, T.P., De Weese, C., Landin-Olsson, M., Kockum, I., Dahlquist, G., Sundkvist, G., Stenger, D. and Lernmark, A. (1995). Polymorphic amino acid variations in HLA-DQ are associated with systematic physical property changes and occurrence of IDDM. Members of the Swedish Childhood Diabetes Study. Diabetes *44*:125-131.

Sarkar, S.A., Kobberup, S., Wong, R., Lopez, A.D., Quayum, N., Still, T., Kutchma, A., Jensen, J.N., Gianani, R., Beattie, G.M., Jensen, J., Hayek, A. and Hutton, J.C. (2008). Global gene expression profiling and histochemical analysis of the developing human fetal pancreas. Diabetologia 51:285-297.

Savary, K., Michaud, A., Favier, J., Larger, E., Corvol, P. and Gasc, J.M. (2005). Role of the renin-angiotensin system in primitive erythropoiesis in the chick embryo. Blood *105*:103-110.

Scaglia, L., Cahill, C.J., Finegood, D.T. and Bonner-Weir, S. (1997). Apoptosis participates in the remodeling of the endocrine pancreas in the neonatal rat. Endocrinology *138*:1736-1741.

Scharfmann, R., Duvillie, B., Stetsyuk, V., Attali, M., Filhoulaud, G. and Guillemain, G. (2008). Beta-cell development: the role of intercellular signals. Diabetes Obes Metab *10 (Suppl) 4*:195-200.

Schmitz, U. and Berk, B.C. (1997). Angiotensin II signal transduction: Stimulation of multiple mitogen-activated protein kinase pathways. Trends Endocrinol Metab *8*:261-266.

Schisler, J.C., Jensen, P.B., Taylor, D.G., Becker, T.C., Knop, F.K., Takekawa, S., German, M., Weir, G.C., Lu, D., Mirmira, R.G. and Newgard, C.B. (2005). The Nkx6.1 homeodomain transcription factor suppresses glucagon expression and regulates glucose-stimulated insulin secretion in islet beta cells. Proc Natl Acad Sci U S A *102*:7297-7302.

Schuit, F., Flamez, D., De Vos, and Pipeleers, D. (2002). Glucose-regulated gene expression maintaining the glucose-responsive state of beta-cells. Diabetes *51 Suppl 3*:S326-S332.

Schütz, S., Le Moullec, J.M., Corvol, P. and Gasc, J.M. (1996). Early expression of all the components of the renin-angiotensin-system in human development. Am J Pathol *149*:2067-2079.

Schuurman, H.J. and Pierson, R.N. 3rd. (2008) Progress towards clinical xenotransplantation. Front Biosci *13*:204-220.

Segev, H., Fishman, B., Ziskind, A., Shulman, M. and Itskovitz-Eldor, J. (2004). Differentiation of human embryonic stem cells into insulin-producing clusters. Stem Cells *22*:265-274

Serreau, R., Luton, D., Macher, M.A., Delezoide, A.L., Garel, C. and Jacqz-Aigrain, E. (2005). Developmental toxicity of the angiotensin II type 1 receptor antagonists during human pregnancy: a report of 10 cases. BJOG *112*:710-712.

Setty, Y., Cohen, I.R., Dor, Y. and Harel, D. (2008). Four-dimensional realistic modeling of pancreatic organogenesis. Proc Natl Acad Sci U S A *105*:20374-20379.

Shao, D., Baker, M.D., Abrahamsen, B., Rugiero, F., Malik-Hall, M., Poon, W.Y., Cheah, K.S., Yao, K.M., Wood, J.N. and Okuse, K. (2009). A multi PDZ-domain protein Pdzd2 contributes to functional expression of sensory neuron-specific sodium channel Na(V)1.8. Mol Cell Neurosci *42*:219-225.

Shao, S., Fang, Z., Yu, X. and Zhang, M. (2009). Transcription factors involved in glucose-stimulated insulin secretion of pancreatic beta cells. Biochem Biophys Res Commun *384*:401-404.

Shapiro, A.M., Lakey, J.R., Ryan, E.A., Korbutt, G.S., Toth, E., Warnock, G.L., Kneteman, N.M. and Rajotte, R.V. (2000). Islet transplantation in seven patients with type 1 diabetes mellitus using a glucocorticoid-free immunosuppressive regimen. N Engl J Med *343*:230-238.

She, J.X. (1996). Susceptibility to type I diabetes: HLA-DQ and DR revisited. Immunol Today *17*:323-329.

Shi, R.Z., Wang, J.C., Huang, S.H., Wang, X.J. and Li, Q.P. (2009). Angiotensin II induces vascular endothelial growth factor synthesis in mesenchymal stem cells. Exp Cell Res *315*:10-15.

Shim, J.H., Kim, S.E., Woo, D.H., Kim, S.K., Oh, C.H., McKay, R. and Kim, J.H. (2004). Directed differentiation of human embryonic stem cells towards a pancreatic cell fate. Diabetologia *50*:1228-1238.

Shmelkov, S.V., St Clair, R., Lyden, D. and Rafii, S. (2005). AC133/CD133/Prominin-1. Int J Biochem Cell Biol *37*:715-719.

Silverstein, J., Maclaren, N., Riley, W., Spillar, R., Radjenovic, D. and Johnson, S. (1988). Immunosuppression with azathioprine and prednisone in recent-onset insulin-dependent diabetes mellitus. N Engl J Med *319*: 599-604.

Singh, V.P., Le, B., Khode, R., Baker, K.M. and Kumar, R. (2008). Intracellular angiotensin II production in diabetic rats is correlated with cardiomyocyte apoptosis,

oxidative stress, and cardiac fibrosis. Diabetes *57*:3297-3306.

Skandalakis, J.E., Skandalakis, L.J. and Colborn, G.L. (1998). The Pancreas Volume 1. In: Beger, H.G., Warshaw, A.L., Büchler, M.W., Carr-Locke, D.L., Neoptolemos, J.P., Russell, C. and Sarr, M.G., eds. Blackwell Science Ltd. *Chapter 4*:27-59.

Smith, F.E. (1994). Islets of Langerhans: Morphology and its implications. In: Bonner-Weir, S., Smith, F.E., Kahn, C.R. and Weir, G.C., eds. Joslin's Diabetes Mellitus. Philadelphia, Lea and Febiger. 15-28.

Song, R., Spera, M., Garrett, C., El-Dahr, S.S. and Yosypiv, I.V. (2010). Angiotensin II AT2 receptor regulates ureteric bud morphogenesis. Am J Physiol Renal Physiol *298*:F807-F817.

Soria, B. (2001). In-vitro differentiation of pancreatic beta-cells. Differentiation *68*:205-219

Sosa-Pineda, B., Chowdhury, K., Torres, M., Oliver, G. and Gruss, P. (1997). The Pax4 gene is essential for differentiation of insulin-producing beta cells in the mammalian pancreas. Nature *386*:399-402.

Stadtfeld, M., Brennand, K. and Hochedlinger, K. (2008). Reprogramming of pancreatic beta cells into induced pluripotent stem cells. Curr Biol *18*:890-904.

Stanojevic, V., Yao, K.M. and Thomas, M.K. (2005). The coactivator Bridge-1 increases transcriptional activation by pancreas duodenum homeobox-1 (PDX-1). Mol Cell Endocrinol *237*:67-74.

Steckelings, U.M., Henz, B.M., Wiehstutz, S., Unger, T. and Artuc, M. (2005). Differential expression of angiotensin receptors in human cutaneous wound healing. Br J Dermatol *153*:887-893.

Stefan, Y., Grasso, S., Perrelet, A. and Orci, L. (1983). A quantitative immunofluorescent study of the endocrine cell populations in the developing human pancreas. Diabetes *32*:293-301.

Strominger, J.L. (1986). Biology of the human histocompatibility leukocyte antigen (HLA)

system and a hypothesis regarding the generation of autoimmune diseases. J Clin Invest *77*:1411-1415.

Suen, P.M., Li, K., Chan, J.C. and Leung, P.S. (2006). In vivo treatment with glucagon-like peptide 1 promotes the graft function of fetal islet-like cell clusters in transplanted mice. Int J Biochem Cell Biol *38*:951-960.

Suen, P.M., Zou, C., Zhang, Y.A., Lau, T.K., Chan, J., Yao, K.M. and Leung, P.S. (2008). PDZ-domain containing-2 (PDZD2) is a novel factor that affects the growth and differentiation of human fetal pancreatic progenitor cells. Int J Biochem Cell Biol *40*:789-803.

Sugiyama, T., Rodriguez, R.T., McLean, G.W. and Kim, S.K. (2007). Conserved markers of fetal pancreatic epithelium permit prospective isolation of islet progenitor cells by FACS. Proc Natl Acad Sci U S A *104*:175-180.

Sussel, L., Kalamaras, J., Hartigan-O'Connor, D.J., Meneses, J.J., Pedersen, R.A., Rubenstein, J.L. and German, M.S. (1998). Mice lacking the homeodomain transcription factor Nkx2.2 have diabetes due to arrested differentiation of pancreatic beta cells. Development *125*:2213-2221.

Takahashi, K., Tanabe, K., Ohnuki, M., Narita, M., Ichisaka, T., Tomoda, K. and Yamanaka, S. (2007). Induction of pluripotent stem cells from adult human fibroblasts by defined factors. Cell *131*:861-872.

Takahashi, K. and Yamanaka, S. (2006). Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors. Cell *126*: 663-676.

Tam, C.W., Cheng, A.S., Ma, R.Y., Yao, K.M. and Shiu, S.Y. (2006). Inhibition of prostate cancer cell growth by human secreted PDZ domain-containing protein 2, a potential autocrine prostate tumor suppressor. Endocrinology *147*:5023-5033.

Tam, C.W., Liu, V.W., Leung, W.Y., Yao, K.M. and Shiu, S.Y. (2008). The autocrine human secreted PDZ domain-containing protein 2 (sPDZD2) induces senescence or quiescence of prostate, breast and liver cancer cells via transcriptional activation of p53. Cancer Lett *271*:64-80.

Taquini, A.C. Jr. and Taquini, A.C. (1961). The renin-angiotensin system in hypertension. Am Heart J *62*:558-564.

Tateishi, K., He, J., Taranova, O., Liang, G., D'Alessio, A.C. and Zhang, Y. (2008). Generation of insulin-secreting islet-like clusters from human skin fibroblasts. J Biol Chem *283*:31601-31607.

Tatemoto, K. (1982). Isolation and characterization of peptide YY (PYY), a candidate gut hormone that inhibits pancreatic exocrine secretion. Proc Natl Acad Sci U S A *79*:2514-2518.

Teramura, Y., Kaneda, Y. and Iwata, H. (2007). Islet-encapsulation in ultra-thin layer-by-layer membranes of poly(vinyl alcohol) anchored to poly(ethylene glycol)-lipids in the cell membrane. Biomaterials *28*:4818-4825.

Teta, M., Long, S.Y., Wartschow, L.M., Rankin, M.M. and Kushner, J.A. (2005). Very slow turnover of beta-cells in aged adult mice. Diabetes *54*:2557-2567.

Theys, N., Bouckenooghe, T., Ahn, M.T., Remacle, C. and Reusens, B. (2009). Maternal low-protein diet alters pancreatic islet mitochondrial function in a sex-specific manner in the adult rat. Am J Physiol Regul Integr Comp Physiol. *297*:R1516-R1525.

Thomas, M.K., Tsang, S.W., Yeung, M.L., Leung, P.S. and Yao, K.M. (2009). The roles of the PDZ-containing proteins bridge-1 and PDZD2 in the regulation of insulin production and pancreatic beta-cell mass. Curr Protein Pept Sci *10*:30-36.

Thomas, M.K., Yao, K.M., Tenser, M.S., Wong, G.G. and Habener, J.F. (1999). Bridge-1, a novel PDZ-domain coactivator of E2A-mediated regulation of insulin gene transcription. Mol Cell Biol *19*:8492-8504.

Thyssen, S., Arany, E. and Hill, D.J. (2006). Ontogeny of regeneration of beta-cells in the neonatal rat after treatment with streptozotocin. Endocrinology *147*:2346-2356.

Trus, M., Corkey, R.F., Nesher, R., Richard, A.M., Deeney, J.T., Corkey, B.E. and Atlas, D. (2007). The L-type voltage-gated Ca^{2+} channel is the Ca^{2+} sensor protein of stimulus-secretion coupling in pancreatic beta cells. Biochemistry *46*:14461-14467.

Tu, J., Tuch, B.E. and Si, Z. (1999). Expression and regulation of glucokinase in rat islet beta- and alpha-cells during development. Endocrinology *140*:3762-3766.

Ueno, H., Yamada, Y., Watanabe, R., Mukai, E., Hosokawa, M., Takahashi, A., Hamasaki, A., Fujiwara, H., Toyokuni, S., Yamaguchi, M., Takeda, J. and Seino, Y. (2005). Nestin-positive cells in adult pancreas express amylase and endocrine precursor cells. Pancreas *31*:126-131.

Ushio-Fukai, M. and Urao, N. (2009). Novel role of NADPH oxidase in angiogenesis and stem/progenitor cell function. Antioxid Redox Signal *11*:2517-2533.

van der Windt, D.J., Echeverri, G.J., Ijzermans, J.N. and Coopers, D.K. (2008). The choice of anatomical site for islet transplantation. Cell Transplant *17*:1005-1014.

Verfaillie, C.M., Pera, M.F. and Lansdorp P,M. (2002). Stem cells: hype and reality. Hematology *Am Soc Hematol Educ Program* 369-391.

Verge, C.F., Gianani, R., Kawasaki, E., Yu, L., Pietropaolo, M., Jackson, R.A., Chase, H.P. and Eisenbarth, G.S. (1996). Prediction of type I diabetes in first-degree relatives using a combination of insulin, GAD, and ICA512bdc/IA-2 autoantibodies. Diabetes *45*:926-933.

Volinic, J.L., Lee, J.H., Eto, K., Kaur, V. and Thomas, M.K. (2006). Overexpression of the coactivator bridge-1 results in insulin deficiency and diabetes. Mol Endocrinol *20*:167-182.

Wang, J., Elghazi, L., Parker, S.E., Kizilocak, H., Asano, M., Sussel, L. and Sosa-Pineda, B. (2004). The concerted activities of Pax4 and Nkx2.2 are essential to initiate pancreatic beta-cell differentiation. Dev Biol *266*:178-189.

Wang, S., Jensen, J.N., Seymour, P.A., Hsu, W., Dor, Y., Sander, M., Magnuson, M.A., Serup, P. and Gu, G. (2009). Sustained Neurog3 expression in hormone-expressing islet cells is required for endocrine maturation and function. Proc Natl Acad Sci U S A *106*:9715-9720.

Watanabe, C., Kato, Y., Sugiura, T., Kubo, Y., Wakayama, T., Iseki, S. and Tsuji, A. (2006). PDZ adaptor protein PDZK2 stimulates transport activity of organic cation/carnitine

transporter OCTN2 by modulating cell surface expression. Drug Metab Dispos *34*:1927-1934.

Weinhaus, A.J., Tabiin, M.T., Poronnik, P., Palma, C.A., Cook, D.I. and Tuch, B.E. (2003). Insulin secretagogues, but not glucose, stimulate an increase in [Ca2+]i in the fetal human and porcine beta-cell. J Clin Endocrinol Metab *88*:2753-2759.

White, P., May, C.L., Lamounier, R.N., Brestelli, J.E. and Kaestner, K.H. (2008). Defining pancreatic endocrine precursors and their descendants. Diabetes *57*:654-668.

Wiebe, P.O., Kormish, J.D., Roper, V.T., Fujitani, Y., Alston, N.I., Zaret, K.S., Wright, C.V., Stein, R.W. and Gannon, M. (2007). Ptf1a binds to and activates area III, a highly conserved region of the Pdx1 promoter that mediates early pancreas-wide Pdx1 expression. Mol Cell Biol *27*:4093-4104.

Williams, R.L., Hilton, D.J., Pease, S., Willson, T.A., Stewart, C.L., Gearing, D.P., Wagner, E.F., Metcalf, D., Nicola, N.A. and Gough, N.M. (1988). Myeloid leukaemia inhibitory factor maintains the developmental potential of embryonic stem cells. Nature *336*:684-687.

Wong, K.C. and Wang, Z. (2006). Prevalence of type 2 diabetes mellitus of Chinese populations in Mainland China, Hong Kong, and Taiwan. Diabetes Res Clin Pract *73*:126-134.

Xu, C., Inokuma, M.S., Denham, J., Golds, K., Kundu, P., Gold, J.D. and Carpenter, M.K. (2001). Feeder-free growth of undifferentiated human embryonic stem cells. Nat Biotechnol *19*:971-974.

Xu, X., D'Hoker, J., Stangé, G., Bonné, S., De Leu, N., Xiao, X., Van de Casteele, M., Mellitzer, G., Ling, Z., Pipeleers, D., Bouwens, L., Scharfmann, R., Gradwohl, G. and Heimberg, H. (2008). Beta cells can be generated from endogenous progenitors in injured adult mouse pancreas. Cell *132*:197-207.

Yamane, T., Dylla, S.J., Muijtjens, M. and Weissman, I.L. (2005). Enforced Bcl-2 expression overrides serum and feeder cell requirements for mouse embryonic stem cell self-renewal. Proc Natl Acad Sci U S A *102*:3312-3317.

Yan, D., Wu, Y., Feng, Y., Lin, S.C. and Lin, X. (2009). The core protein of glypican Dally-like determines its biphasic activity in wingless morphogen signaling. Dev Cell *17*:470-481.

Yang, S.N. and Berggren, P.O. (2005). Beta-cell Ca_V channel regulation in physiology and pathophysiology. Am J Physiol Endocrinol Metab *288*:E16-E28.

Yao, S., Chen, S., Clark, J., Hao, E., Beattie, G.M., Hayek, A. and Ding, S. (2006). Long-term self-renewal and directed differentiation of human embryonic stem cells in chemically defined conditions. Proc Natl Acad Sci U S A *103*:6907-6912.

Yechoor, V. and Chan, L. (in press) Minireview: {beta}-Cell Replacement Therapy for Diabetes in the 21st Century: Manipulation of Cell Fate by Directed Differentiation. Mol Endocrinol *10 Mar 2010*. Epub ahead of print.

Yeung, M.L., Tam, T.S., Tsang, A.C. and Yao, K.M. (2003). Proteolytic cleavage of PDZD2 generates a secreted peptide containing two PDZ domains. EMBO Rep *4*:412-418.

Ying, Z., Giachini, F.R., Tostes, R.C. and Webb, R.C. (2009). PYK2/PDZ-RhoGEF links Ca2+ signaling to RhoA. Arterioscler Thromb Vasc Biol *29*:1657-1663.

Zalzman, M., Anker-Kitai, L. and Efrat, S. (2005). Differentiation of human liver-derived, insulin-producing cells toward the beta-cell phenotype. Diabetes *54*:2568-2575.

Zaret, K.S. and Grompe, M. (2008). Generation and regeneration of cells of the liver and pancreas. Science *322*:1490-1494.

Zecchin, E., Mavropoulos, A., Devos, N., Filippi, A., Tiso, N., Meyer, D., Peers, B., Bortolussi, M. and Argenton, F. (2004). Evolutionary conserved role of ptf1a in the specification of exocrine pancreatic fates. Dev Biol *268*:174-184.

Zhang, H., Maximov, A., Fu, Y., Xu, F., Tang, T.S., Tkatch, T., Surmeier, D.J. and Bezprozvanny, I. (2005a). Association of $Ca_V1.3$ L-type calcium channels with Shank. J Neurosci *25*:1037-1049.

Zhang, L., Hu, J., Hong, T.P., Liu, Y.N., Wu, Y.H. and Li, L.S. (2005b). Monoclonal side

population progenitors isolated from human fetal pancreas. Biochem Biophys Res Commun *333*:603-608.

Zhang, S.L., Moini, B. and Ingelfinger, J.R. (2004). Angiotensin II increases Pax-2 expression in fetal kidney cells via the AT2 receptor. J Am Soc Nephrol *15*:1452-1465.

Zhou, Q., Brown, J., Kanarek, A., Rajagopal, J. and Melton, D.A. (2008). In vivo reprogramming of adult pancreatic exocrine cells to beta-cells. Nature *455*:627-632.

Zhou, Q., Chipperfield, H., Melton, D.A. and Wong, W.H. (2007). A gene regulatory network in mouse embryonic stem cells. Proc Natl Acad Sci U S A *104*:16438-16443.

Zhou, Q. and Melton, D.A. (2008). Extreme makeover: converting one cell into another. Cell Stem Cell *3*:382-388.

Zuk, P.A., Zhu, M., Mizuno, H., Huang, J., Futrell, J.W., Katz, A.J., Benhaim, P., Lorenz, H.P. and Hedrick, M.H. (2001). Multilineage cells from human adipose tissue: implications for cell-based therapies. Tissue Eng *7*:211-228.