Studies on Some Factors Critical for the Development of Pancreatic Progenitor Cells Derived from Human Fetal Pancreas

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Abstract

Diabetes Mellitus, a life threatening disease, results in more than 300 million deaths every year. Restoration of beta cell mass by transplantation is encouraging as this not only repair Type I Diabetes Mellitus but also compensate deficiency in Type II Diabetes Mellitus. Clinical studies showed that 10% pro-transplantation patients could be insulin free for 5 years and achieve a better living standard. However, shortage of islets donation has hampered transplantation.

Over the last decades, advances in stem cell and regeneration therapy, producing insulin-producing cells that can be transplantable, benefit Replacement Therapy. Yet, available islet engineering methods are still immature and considerable efforts are required for perfection of the Therapy. Most successful protocols used for islet engineering mimic *in vivo* pancreatic organogenesis by stepwise resembling different stages of development. As a result, additional developmental clues would help in maturation of protocols.

Vitamin A modulates most tissues development and vitamin D affects beta-cell insulin secretion, while both vitamin ligands act through heterodimerization with the retinoid X receptor (RXR). However, their mono- and combinatory-effects in modulating pancreatic development in human have not been well characterized. Besides, considering the storage and regulation of vitamin A and vitamin D by liver, it also raises interests in whether fetal liver stroma can induce pancreatic differentiation.

Considering inherent difference between human and animals, our group has developed isolation, characterization and culture of a population of pancreatic progenitor cells (PPCs) from human fetal pancreas for investigations of islet differentiation regulated by different morphogens and later, different microenvironments. Aims of my project are (1) to identify the proliferative and differentiative effects of vitamin A and vitamin D on PPCs; (2) to evaluate the Islet-like Cell Clusters (ICCs) induction potential by human fetal liver stroma microenvironment; (3) to further elucidate factors produced by liver stromal cells responsible for ICC differentiation.

Isolation of pancreatic progenitor cells (PPCs) and liver stromal cells (LSCs) from discarded fetal tissues were regulated under Clinical Research Ethnics Committee (CREC-2005.461). Reverse Transcription Polymerase Chain Reactions (RT-PCR), Western blotting and immunocytochemistry were used to examine the expression and localization of vitamin D receptor (VDR), retinoic acid receptor (RAR), and Retinoid X Receptor (RXR) in PPC. Effects of exogenous addition of all-trans retinoic acid (atRA, a form of vitamin A) or/and calcitriol (activated form of vitamin D) on PPC were determined by quantitative estimation of cell viability, proliferation, cell death and neurogenin-3 (NGN3) genes transcription. On the other hands, PPC and LSC were co-cultured under modified medium. Resulted PPC growth and PPC derived Islet-like Cell Cluster (ICC) differentiation and functions were determined using quantitative estimation of cell viability, multiple genes transcription and insulin production. Furthermore, by making use of the differential characteristics between 1st and 2nd trimester fetal liver derived LSC, candidates responsible were under investigations.

Results showed that RAR and RXR were localized in nuclei while VDR were expressed in nuclei, cytoplasm and plasma membrane of PPCs. atRA and calcitriol each increased PPC viability and proliferation; atRA additionally decreased PPC apoptosis. Co-addition of atRA and calcitriol had no additive effects on cell viability but increased NGN3 mRNA expression. In addition, 2nd trimester liver derived LSC co-culture enhanced PPC growth, ICC differentiation and functions without exogenous addition of growth factors. Plenty of growth factors' genes were expressed in LSC and insulin-like Growth Factor 1 (IGF1), a controversial morphogen involved in regeneration but not development, were found contributing for the improvement.

In conclusion, RAR, RXR and VDR were expressed in PPCs and PPC proliferation was promoted by calcitriol or atRA and co-treatment enhances NGN3 expression in PPC. On the other hands, LSC microenvironment induced PPC growth and ICC differentiation and possible factors contributing to improvement was Insulin-like growth factor-I (IGF1). Data were valuable for elucidating mechanisms underlying islet development and further modifications of stroma microenvironment would be an alternative more effective, economic and efficient way for islets differentiation, where these data could together contribute to development of clinical islet transplantation.

論文摘要

糖尿病是一種致命的疾病，每年導致 300 多萬死亡個案。使用移植來恢復胰腺中的β細胞不僅能修復第一型糖尿病，而且能彌補第二型糖尿病體內的胰島素不足。臨床研究表明，10%的胰島移植病人可免外源性的胰島素注射達 5 年，以得到更好的生活水平。然而，胰島的捐贈短缺卻阻礙了胰島移植的可行性。

在過去的十年中，因幹細胞研究和再生治療的發達而製造出可以被移植的胰島細胞能彌補胰島的捐贈短缺。然而，現有的胰島製造工程方法仍然不成熟，需要相當大的努力去完善製造方法。而現時最成功的製造方法是建基於模擬體內不同階段的胰島器官的發展。因此，胰島器官發展的線索將有助於完善製造方法。

維生素 A 調節大多數組織的發展和維生素 D 影響β細胞胰島素的分泌，而這兩個維生素通過和維甲酸 X 受體 (Retinoid X Receptor)互相结合形成异双元体(heterodimer)以發揮其功效。然而，它們對調節胰腺發育的影響還沒有得到完善的研究。此外，考慮到肝臟負責儲存和管理體內的維生素 A 和維生素 D 的濃度，提出了胎兒的肝臟是否能誘導胰腺分化。

考慮到內在的人與動物之間的差異，我們已開發了從人類胎兒胰島分離，鑑定和培殖胰腺祖細胞 (Pancreatic progenitor cell) 的方法去調查受不同的因素和不同的微環境對祖細胞的分化的影響。我的項目目標是：（1）去識別維生素 A 和維生素 D 對胰腺祖細胞的增殖和分化效果，（2）去評估人胎肝基質微環境誘導類小島

細胞球 (Islet-like cell cluster) 的分化的潛力，（3）進一步去闡明人胎肝基質細胞 (Liver stromal cell)產生的哪一些因素負責對類小島細胞球的分化。

胚胎組織分離的胰腺祖細胞和肝基質細胞的使用是由临床研究伦理委员会規管的 (CREC-2005.461)。我們先使用逆轉錄聚合酶鏈反應（RT- PCR）技術，免疫印跡 (Western blotting) 和免疫細胞化學 (Immunocytochemistry) 的方法去檢測胰腺祖細胞的維生素 D 受體 (Vitamin D receptor)、維甲酸受體 (Retinoic acid receptor)和維甲酸 X 受體的表達和定位，之後再定量評估全反式維甲酸（All Trans Retinoic Acid），維生素 A 的一種形式或/和骨化三醇 (Calcitriol) ，活化形式的維生素 D 對胰腺祖細胞的活力，增殖，細胞死亡和 NGN3 的基因的表達。另一方面，我們共培養胰腺祖細胞和肝基質細胞在修改培養液下，之後再採用細胞活力，多基因的表達，胰島素的生產定量估算共培養對胰腺祖細胞的增長和類小島細胞球的分化和功能的影響。此外，再通過利用第一和第二孕期肝基質細胞的不同因素之間的表達去調查哪一些因素負責影響。

結果表明，維甲酸受體和維甲酸 X 受體在胰腺祖細胞定位於細胞核，而維生素 D 受體表達在細胞核，細胞質和細胞膜中。全反式維甲酸和骨化三醇增加了胰腺祖細胞的活力和增殖，此外全反式維甲酸還降低胰腺祖細胞的死亡率。聯合全反式維甲酸和骨化三醇沒有增大對細胞的生存能力和增殖的影響，但是增加了 NGN3 的表達。此外，在無外源性生長因子下，第二孕期肝基質細胞共培養增強對胰腺祖細胞的增長，類小島細胞球的分化和功能。肝基質細胞表達了大量的生長因

子的基因，發現其中一個具爭議性的生長因子胰島素樣生長因子 1（Insulin-like growth factor）改善分化。它原被認爲影響再生但不影響分化。

總結，在胰腺祖細胞表達了細胞的維生素 D 受體、維甲酸受體和維甲酸 X 受體。全反式維甲酸和骨化三醇促進了胰腺祖細胞的增殖，其合作也增強了 NGN3 的表達。在其他方面，肝基質細胞微環境誘導胰腺祖細胞的增長，類小島細胞球的分化和功能，其可能造成分化的因素是胰島素樣生長因子- I（IGF1）。這結果將有助於完善開發胰島胰腺器官的發展進一步的修改，此外，進一步修改基質細胞的微環境將是一個更有效和更經濟的方式替代以外源性生長因子直接誘導方式去促進臨床胰島移植的發展。

List of Publications

Original Articles:

Ng KY, Ma MT, Leung KK, Leung PS. Vitamin D and vitamin A receptor expression and the proliferative effects of ligand activation of these receptors on the development of pancreatic progenitor cells derived from human fetal pancreas. *Stem Cell Rev.* 2011;7:53-63.

Ng KY, Liang J, Ronnald, Xia Y, Leung PS. Human fetal liver stromal niche enhances pancreatic progenitor cells growth and differentiation into islet-like cell-clusters.
(In submission to *Gastroenterology*)

Review Articles

Leung PS, Ng KY. The current progress on stem cell research and its potential for islet cell transplantation. (In submission to *Current Molecular Medicines*)

Poster Presentations:

Ma MT, Ng KY, Leung KK and Leung PS. The Proliferative Effects of Vitamin A and Vitamin D on Human Pancreatic Progenitor Cells.
(Physiology symposium, 2009)

Ng KY and Leung PS. Inductive Molecules Derived from Human Fetal Liver Stromal Cells Enhance the Growth and Differentiation of Human Pancreatic Progenitor Cells. (International Society for Stem Cell Research 9th Annual Meeting, 2011)

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(Stem Cell Rev. 2011;7:53-63)

Chapter 4

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ADP	Adenosine diphosphate
ADHs	Alcol dehydrogenase
AFP	Alphafetoprotein
atRA	All-trans retinoic acid
ATP	Adenosine-5'-triphosphate
BMP	Bone morphogenetic protein
BMSC	Bone marrow stromal cells
BSA	Bovine serum albumin
BrdU	5-bromo-2'-deoxyuridine
CK	Cytokeratin
CD	Cluster of Differentiation
CPT1	Carnitine palmitoyl trnasferase 1
CYP24	25(OH)D_3-24-hydroxylase
CYP26	Cytochrome P450, family 26
CYP27	Cytochrome P450, family 27
DAPI	4',6'-Diamidino-2-phenylindole
EGF	Epidermal growth factor
ELISA	Enzyme-linked immunosorbent assay
Ep-CAM	Epithelial cell adhesion molecule
ESC	Embryonic stem cells
FBS	Fetal bovine serum
FGF	Fibroblast growth factor
GH	Growth Hormone
HBSS	Hanks Balanced salt solutions
HES-1	Hairy and enhancer of split 1
HGF	Hepatocyte growth factor
HNF	Hepatocyte nuclear factor 6
HSCs	Hepatic stellate cells
IBMIR	Instant blood-mediated inflammatory reaction
ICC	Islet like cell cluster
IGF	Insulin-like growth factor
IGFR	Insulin-like growth factor receptor
IGF-BP	Insulin-like growth factor binding protein
iPSCs	Induced pluripotent stem cells
IL	Interleukin
IR	Insulin receptor

IRS	Insulin receptor substrate proteins
ISL-1	Insulin gene enhancer protein
K_{ATP} channel	ATP-sensitive potassium channel
LADA	Latent autoimmune diabetes of the young
LID	Liver IGF1 deficiency
LSC	Liver stromal cell
MAF	V-maf musculoaponeurotic fibrosarcoma oncogene
Malony-CoA	malonyl coenzyme A
M-6-PR	Mannose-6-phosphate receptor
MHC	Major histocompatibility complex
miRNA	Micro RNA
MODY	Maturity-onset diabetes of the young
MSC	Mesenchymal stem cell
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
N-Cad	Neural-Cadherin
NEUORD	Neurogenic differentiation
NGF	Nerve growth factor
NGN3	Neurogenin3
NIDDM	Non-insulin-dependent diabetes mellitus
NKX2.2	NK2 homeobox 2
NKX6.1	NK6 homeobox 1
PAK	Pancreas-after-kidney transplant
PAX	Paired box gene
PBS	Phosphate buffered saline
PCR	Polymerase Chain Reaction
PDX1	Pancreatic and duodenal homeobox 1
PFA	Paraformaldehyde
PID	Pancretic IGF1 deficiency
PPAR	Peroxisome proliferators-activated receptors
PPC	Pancreatic progenitor cell
P/S	Penicillin/streptomycin
RA	Retinoic acid
RALDH	Retinaldehyde dehydrogenase
RAR	Retinoic acid receptor
RARE	RAR responese elements
RDHs	Retinol dehydrogenase
RXR	Retinoid X receptor
RT-PCR	Reverse transcriptase-polymerase chain reaction;

SCF	Stem Cell Factor
SHH	Sonic hedgehog
SPK	Simultaneous pancreas-kidney transplant
STOP	Surgical termination of pregnancy
STZ	Streptozotocin
T1DM	Type 1 diabetes mellitus
T2DM	Type 2 diabetes mellitus
TAE	Tris-Acetate-EDTA
TGF	Transforming growth factor
VDR	Vitamin D receptor
VDRE	Calcitriol response element
VEGF	Vascular endothelial growth factor

Chapter 1

General Introduction

1.1 The Pancreas

Pancreas is an elongated compound gland and composed of exocrine and endocrine portions, which are responsible for digestion and absorption of nutrients as well as maintaining glucose homeostasis in our body.

1.1.1 Anatomy of Pancreas

Pancreas lies retroperitoneally and transversely across posterior abdominal wall while being posterior to the stomach between the duodenum on the right and the spleen on the left. Structurally, it consists of four parts, i.e. head, neck, body and tail.

The head of pancreas, which is an expanded part of pancreas, lies within the concavity of the duodenum. The ucinate process emerges from the inferior part of the head, and lies deep to superior mesenteric vessels. Exchange of oxygen, nutrients and wastes were conducted by the superior pancreaticoduodenal artery from gastroduodenal artery and the inferior pancreaticoduodenal artery from superior mesenteric artery.

The neck is short and constricted part which connects between the head and the body, overlying superior mesenteric vessels. Blood supply is from pancreatic branches of splenic artery. The body lies behind the stomach and left of the superior mesenteric artery, where blood is supplied from pancreatic branches of splenic artery.

On the other hand, the tail is closely related to spleen and passes between layers of the aplenorienal ligament with the splenic vessels. The pancreatic branches of splenic artery also supply blood to the tail of the pancreas (Keith LM and Anne MRA, 2007). Figure 1.1 schematically summarizes the anatomy and structure of the pancreas.

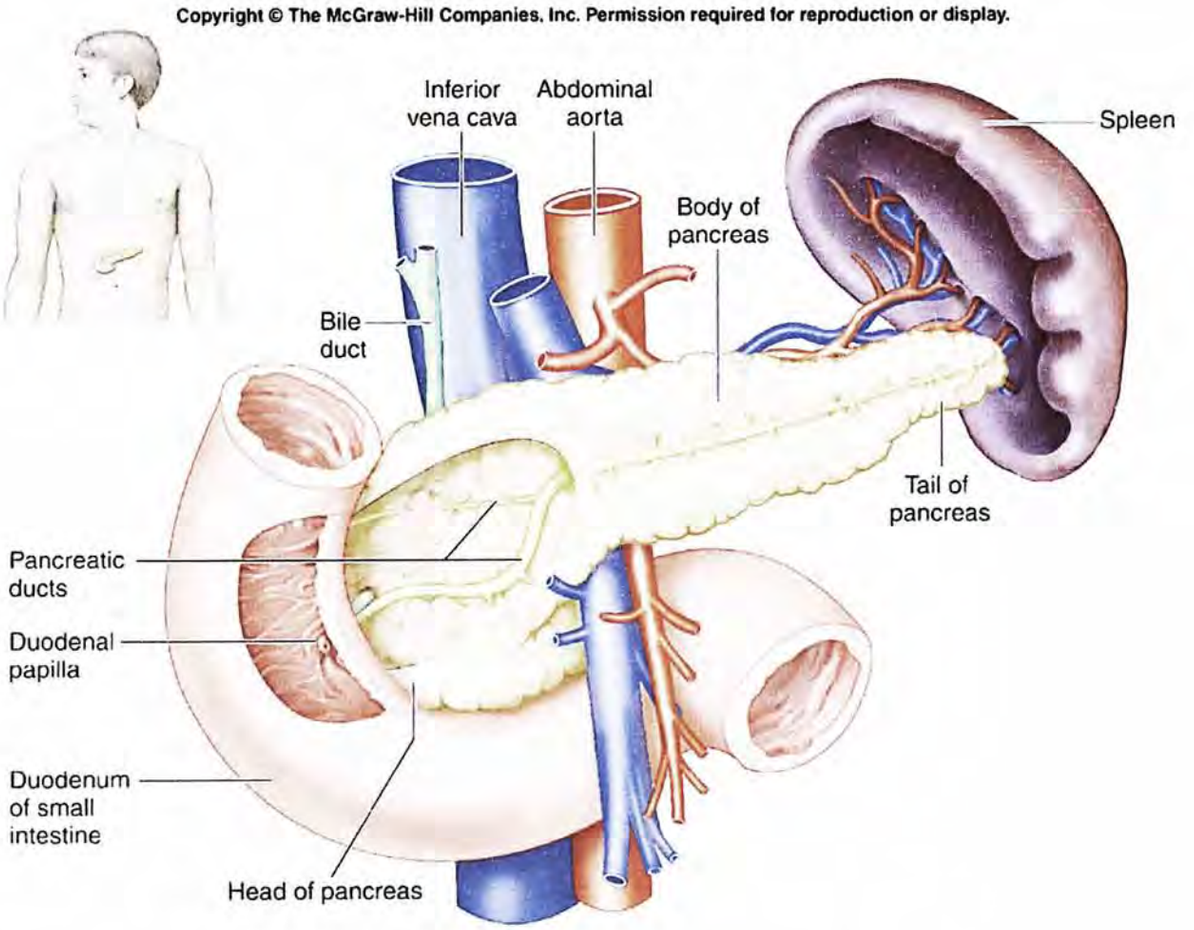

Figure 1.1 The anatomy and structure of the pancreas.

1.1.2 The Exocrine Pancreas

The exocrine pancreas has ducts that are arranged in clusters called acinus (Figure 1.2). Pancreatic juice, including alkaline-rich sodium bicarbonate and digestive enzymes, is secreted into the lumen of the acinus, and released into the intralobular ducts; its secretions are drained to the main pancreatic duct, starting from the tail of the pancreas and running into the head and finally merged with the bile duct to form hepatopancreatic ampulla, opens at summit of the major duodenal papilla into the descending part of the duodenum (Ushiki et al., 1988).

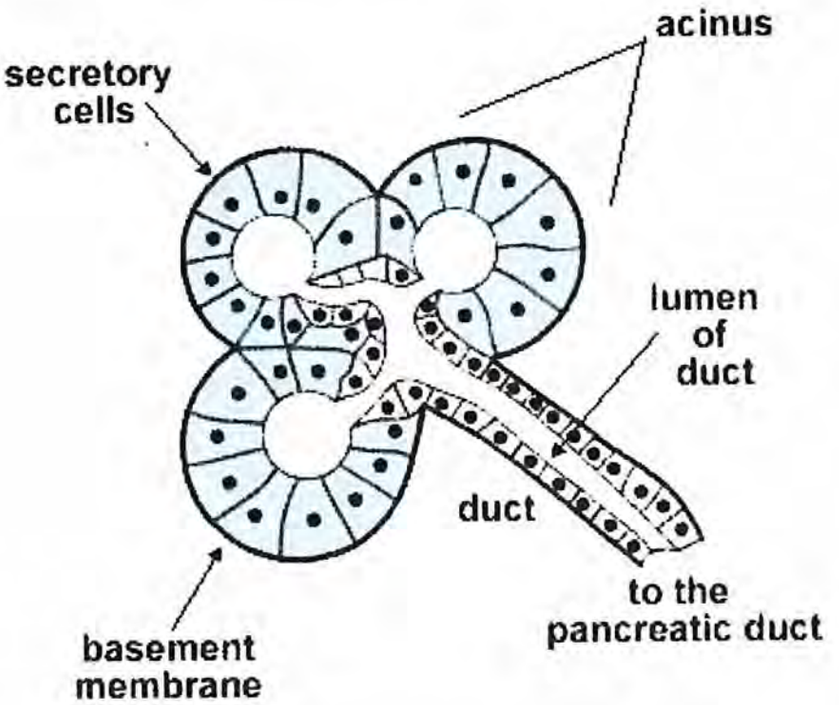

Figure 1.2 Structure of exocrine portions of pancreas.

1.1.3 The Endocrine Pancreas

The endocrine pancreas is made up by 1 million of individual pancreatic islets which are called Islet of Langerhans, distributed throughout the whole pancreas. They are generally oval or clover leaf shape. They consist of 1-2% mass of the pancreas with each size being around ~50 to 500 mm in diameter. The major function of pancreatic islets is responsible for the regulation of glucose homoeostasis by means of releasing a number of peptide hormones in response to absorption of nutrients, via neural and hormones as well as autocrine and paracrine mechanisms (Kulkarni et al., 2004).

1.1.3.1 Structure of Islets

In rodents, adult islets are composed of a central core of β-cells (insulin-secreting), surrounded by a mantle of α-cells (glucagons-secreting), δ-cells (somatostatin-secreting), and PP-cells (pancreatic polypeptide-secreting), while ε-cells disappear after birth (Figure 1.3). The islets are innervated by nerves and in intimate contact with blood vessels, thus controlling insulin secretion by nerve signals and close monitoring of serum glucose levels, respectively. It is estimated that there are 2000 – 4000 β-cells, making up of 70 – 80% islets; while there are about 5% δ-cells and 15 – 20% α-cells or PP-cells. In human, there is, however, no such

architecture when compared with rodents; instead, more complex and with several mantle core subunits are observed and precise data on patterning are still under determination. The architecture between humans and rodents as described above might provide evidence for the inherent difference during their development of the pancreas (Kulkarni et al., 2004).

Replication rate and turnover rate of β-cells in adult are formed either by slow replication of existing β-cells or by differentiation of stem cells located in ductal regions. Half-life of islets is approximately 47 days in rodents while it remains to be unknown in human (Magami et al., 2002).

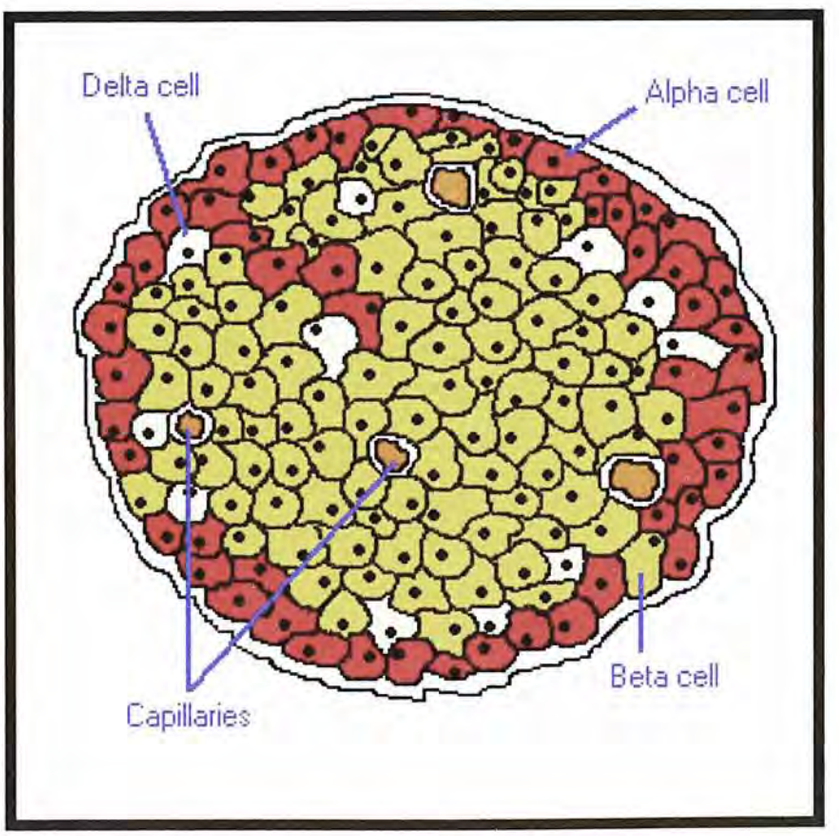

Figure 1.3 Structure of islets of Langerhans of mouse, showing α-, β-and γ-cells.

1.1.3.2 Functions of α-, β-, γ-, δ-, ε- and PP-cells in Islets

In adult human, α-, β-, δ-, ε-and PP-cells are identified in Islet of Langerhans. They produce glucagons, insulin, somatostatin, ghrelin and pancreatic polypeptide, respectively, via mediation of hormonal, autocrine and/or paracrine regulation. Within these 4 cell types, β-cells are the major type of cells which is responsible for insulin secretion so as to maintain glucose homeostasis.

β-cell synthesizes and secretes insulin primarily in response to glucose stimulus but also other stimuli, such as nutrients, hormones and nervous signals. Glucose stimulates biphasic insulin secretion which is involved in several pathways, say K_{ATP} channel dependent and K_{ATP} channel independent pathway. The response begins after glucose-sensing 1 – 2 mins and peaks at 3 – 4 mins followed by a decline rapidly at 8 mins. Initial spikes of insulin release refer to the 1st wave and second wave increases gradually and plateau at 25 – 30 mins.

(a) K_{ATP} channel dependent pathway (first wave)

Glucose can induce the closure of K_{ATP} channel and depolarize the β-cell to result in an increase in Ca^{2+} entry to the cell via voltage-dependent Ca^{2+} channel. This, in turn, increases cellular concentration of Ca^{2+} and triggered a small pool of insulin granules release (Figure 1.4).

(b) K_{ATP} channel independent pathway (second wave)

i) The first hypothesis is that glucose induces an increased production of glutamine in mitochondria and is exported to cytosol. Glutamine permeabilizes β-cells and increases Ca^{2+} secretion, thus leading to a larger pool release of insulin granules.

ii) The second hypothesis is that glucose induces an increased ratio of ATP/ADP and GDP/GTP ratio, thus leading to activation of K_{ATP} channel and triggering a larger pool release of insulin granules.

iii) The third hypothesis is that glucose stimulates an increase in mitochondria citrate level which later release to cytosol. This in turn increases malony-CoA which inhibits carnitine palmitoyl trnasferase 1 (CPT1). This results in decrease of fatty acid production and permeabilizes β-cells and increases Ca^{2+} secretion which leads to a larger pool release of insulin granules. (Straub et al., 2002; Bratanova-Tochkova et al., 2002)

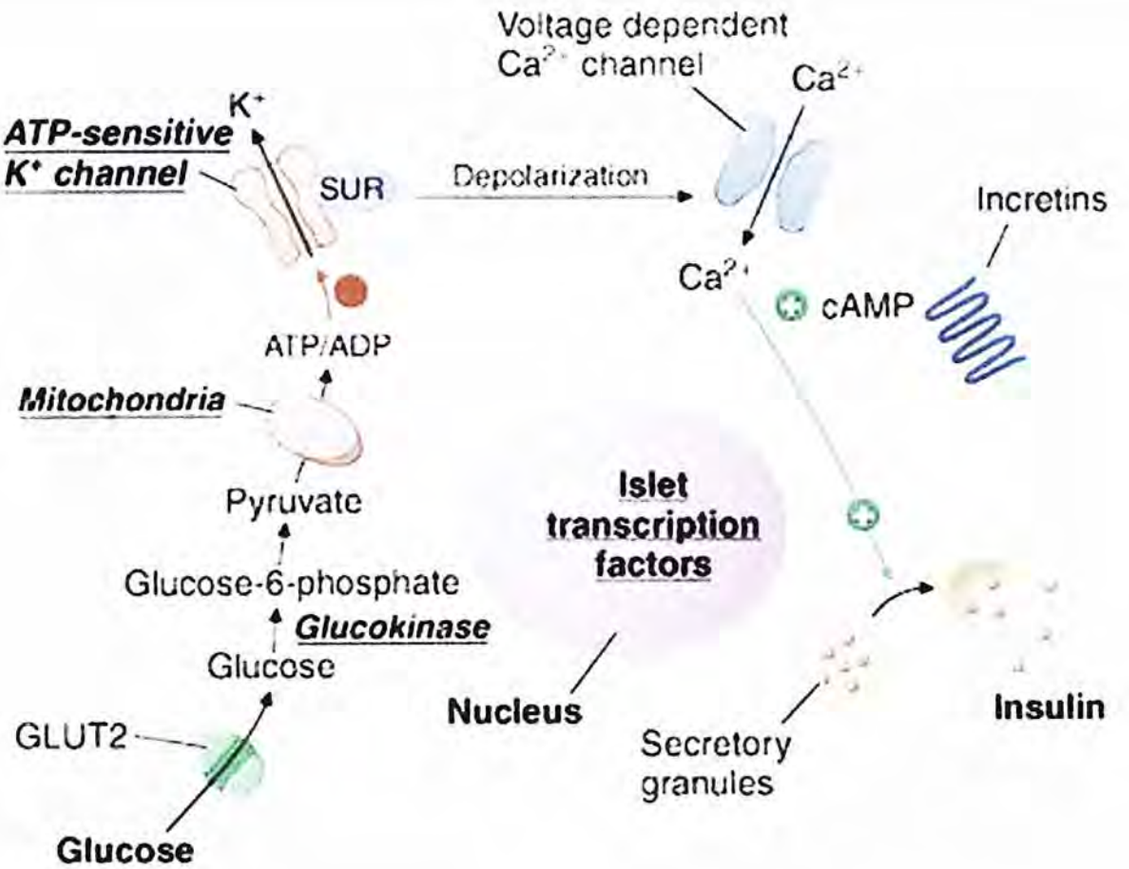

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Figure 1.4 Glucose stimulates insulin secretion (K_{ATP} channel dependent pathway).

1.1.4 Overview of Pancreas Development

Our understandings in human pancreatic development are derived from animal studies (Tiso et al., 2009; Pearl et al., 2009), human embryonic cells differentiation (D'Amour et al., 2006; Segev et al., 2004), clinical cases on heterogeneous loss-of-function, and histological analysis of discarded tissues from aborted fetus (Du et al., 2011; Ozcan et al., 2009). It is believed that most of the transcriptional factors

in human pancreatic development are similar to those in mouse (Smith et al., 2010; Du et al., 2011).

Pancreas growth and development occur in a stepwise process based on observation of organ morphology (Jørgensen et al., 2007), cyto-differentiation (Jensen et al., 2004) and change in expression of transcription factors (Sander et al., 1997). The development is shown below in Table 1.1 and 1.2.

1.1.4.1 Organ Morphology

Endocrine islets, exocrine acini and pancreatic ducts are all derived from the endodermal epithelium through a series of inductive interactions with surrounding tissues.

The mouse pancreas is formed by the fusion of a dorsal and ventral epithelial buds generated by evagination of foregut endoderm (Spagnoli et al., 2007). Dorsal part forms splenic portion (tail and body) and ventral part forms the duodenal portion (head) of pancreas. Control of evagination is by the factors released in 6-10 somites (Edlund et al., 2002). An evagination of the foregut endoderm starts at approximately embryonic day (E)8.5 which marks the start of pancreas development. At this stage, it is initially under the influence of notochord (Herrera et al., 1991). The ventral and dorsal buds appears in (E)9.5 and after fusion of dorsal aorta, factors release from

epithelium of dorsal aorta affects its specification and differentiation. At (E)12.5, ventral pancreas and dorsal pancreas fuse and form a intact pancreas. Similar to mouse pancreas development (Kaestner et al., 2003) (Figure 1.6), figure 1.5 summarizes human pancreas development.

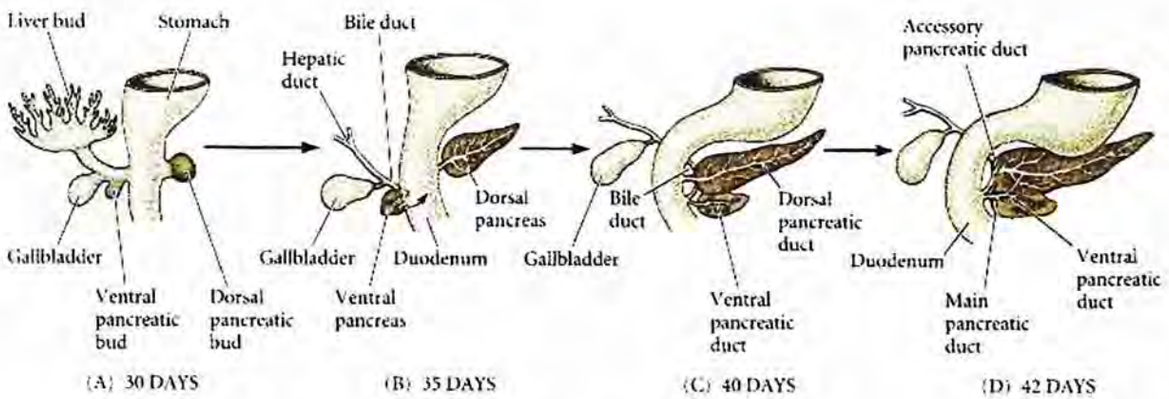

Figure 1.5 Organ morphology in Pancreas Development in Human.

(A) At 30 days, the ventral and dorsal pancreatic buds appear. (B) By 35 days, ventral pancreas begins migrating posteriorly and (C) fuses with the dorsal pancreatic bud during the sixth week of development. (D) In most individuals, the dorsal pancreatic bud loses its duct into the duodenum; however, in about 10% of the population, the dual duct system persists.

1.1.4.2 Cyto-differentiation

Table 1.1 Important observations in mouse pancreas development.

(E)8.5	Pancreatic specification
(E)9.5	Ventral and dorsal pancreas bud appeared
(E)8.5 – (E)12.5	1st transition
(E)9.5	Glucagon and insulin co-positive cells
(E)12.5	Dorsal and ventral bud fusion
(E)12.5 – (E)16.5	2nd transition
(E)12.5	Neural crest cells
(E)13	Single hormone expression cells
(E)16.5 – birth	3rd transition
(E)18	Emergence of all 4 major endocrine cells in vascularized and innervated islets

(Murtaugh LC, 2003)

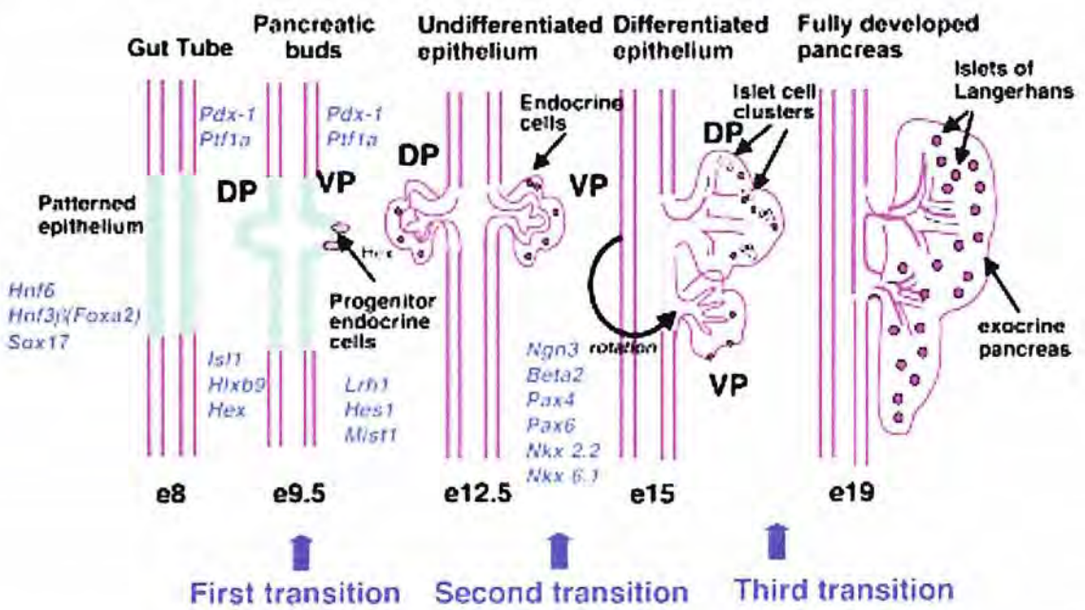

Figure 1.6 Schematic diagram of pancreatic development in the mouse.

Table 1.2 Important observations in human pancreas development.
(Desdicioglu et al., 2010)

4-5 weeks	Evagination of human foregut to form ventral and dorsal pancreas
6-7 weeks	Fusions of ventral and dorsal pancreas
9-11 weeks	Mesenchymal tissues infiltrated by ductal epithelial structures Small clusters of insulin or glucagons positive cells scattered along or residing inside the ductal epithelium
9-19 weeks	PDX1 positive cells
8 weeks	Glucagon positive cells
9 weeks	Insulin positive cells (1 – 2% cells co-expressed both insulin and glucagons but negative for Ki67)
9 weeks	CK19 positive cells
11 weeks	Ghrelin and pancreatic polypeptide positive cells (ε and pp markers)
11 weeks	CEL positive cells (Pro-acinar markers)
>19 weeks	PDX1 positive only in insulin cells
9-23 weeks	Ki67 positive cells
23 weeks	Vascularized and Glu-2 positive islets - Irresponsive to glucose *in vitro*
23 weeks	Amylase positive cells

1.1.4.3 Control by Transcriptional Factors

Table 1.3 Temporal expression of transcription markers for mice.
(Kaestner et al., 2003)

(E)5.2-6.5	HNF3β
(E)7.5	HNF3α
(E)8	HB9
(E)8.5	PDX1
(E)9	ISL-1, PAX6
(E)9.5	NGN3, HES-1, HNF6, BETA2/NEUROD, PAX4, NKX2.2, NKX6.1, MAFA, MAFB

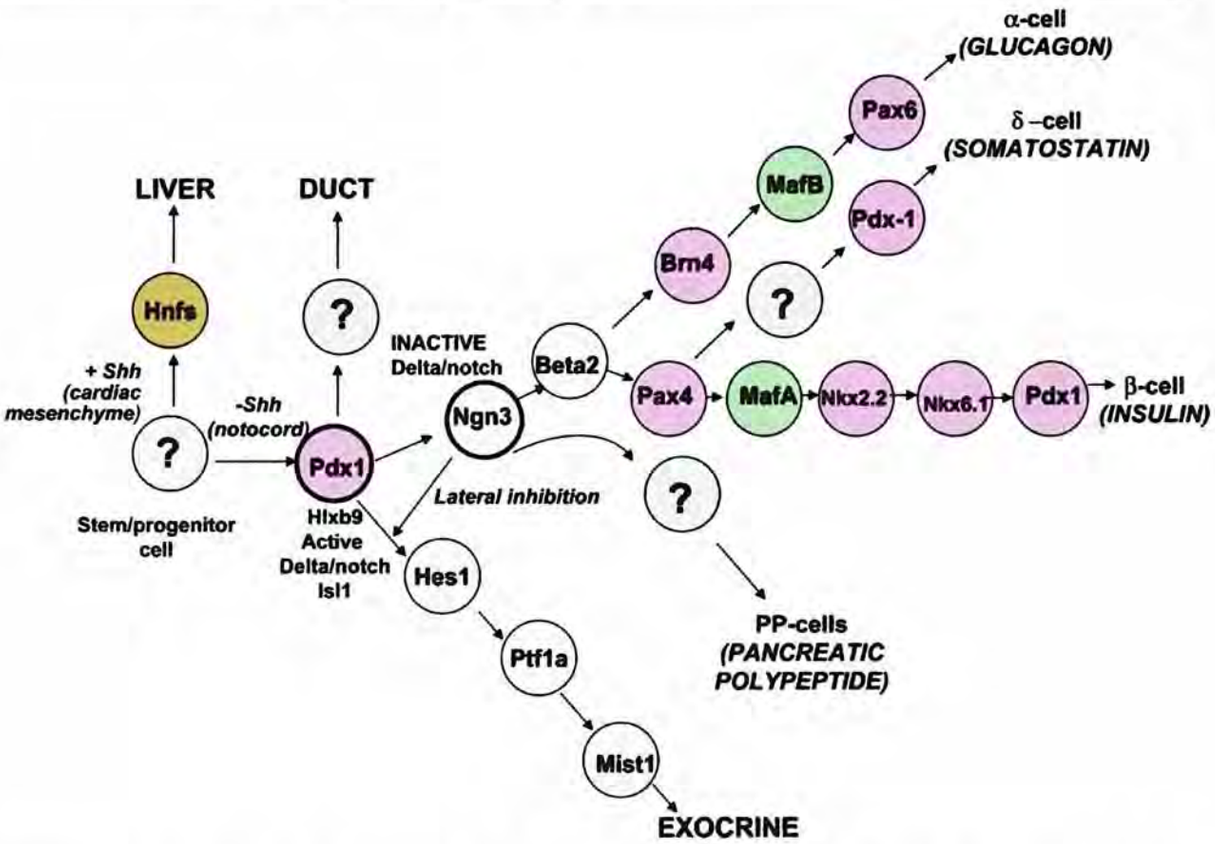

Figure 1.7 A simplified hierarchy of transcription factor expression in the developing pancreas.

(a) LIM homeobox transcription factor (ISL-1)

ISL-1 plays a promoting role in pancreatic development (Elshatory et al., 2007). It is both an extrinsic factor and intrinsic factor. It can either produced by dorsal mesenchyme or expressed in pancreatic progenitor cells. It is expressed at (E)9.0 in the dorsal pancreatic epithelial cells to regulate PDX1 expression. The expression in dorsal mesenchyme is required for maintenance and indirect differentiation for exocrine pancreas while intrinsic expression is necessary for endocrine pancreas differentiation.

(b) Pancreatic and duodenal growth factor 1 (PDX1)

PDX1 is a master transcription factor that appears in the earliest stage and marks specifically for pancreatic lineage. The expression of PDX1 starts in progenitor cells (ventral pancreas at (E)8.5 and (E)9 at dorsal pancreas) at the definite endoderm, which was triggered by Hnf6, Hb9 and Isl1 in the dorsal bud and Hex1 in the ventral bud. Maintenance of the expression is done by the constitutive signals, FGFs, Notch and Wnt/b-catenin. It continues its expression until maturation in β-cells but not exocrine lineages and other endocrine cells. Null PDX1 embryo shows an absence of pancreas. In human, heterozygous mutations in PDX1 is highly susceptible to the onset of type 2 diabetes mellitus and complete loss-of-function leads to pancreatic aplasia.

Initial expression helps to determine the final pancreas size as it controls the proper proliferation and commitment of pancreatic progenitor cells. It also involves in the early commitment of exocrine lineage as it triggers expression of Ptf1a.

PDX1 expression elevates in the late gestation during islets formation and a threshold increase is necessary for the activation of differentiation. PDX1 control expression of NGN3, Arx and other transcription factors to determine the specification and ratio of α-, β-, δ-, ε-and PP-cells.

The PDX1, accompanied with NEUROD and MafA, is one of the activators of insulin expression by binding to the insulin promoter. It is also a necessary transcriptional regulation for glucokinase and glut-2 (Fujimoto et al., 2009).

(c) Pancreas specific transcription factor 1a (Ptf1a, p48)

Accompanied with PDX1, it can convert pancreatic endoderm to pancreatic precursors in Xenopus and Zebrafish embryo. It is later, only specifically expressed in pancreatic exocrine portions (Jiang et al., 2008).

(d) Neurogenin3 (NGN3)

Neurogenin3 is the master regulator of endocrine specification in pancreatic islet differentiation and regeneration. NGN3 belongs to a family of basic helix-loop-helix transcription factors. NGN3 expression is first found expressing at (E)9.5 and peak at (E) 15.5 and disappeared in adult pancreas, except during regeneration. The NGN3

expression is correlated with the 1st and 2nd wave where endocrine cells started being detected.

In NGN3 knockout mice, no endocrine cells are found and over-expression of NGN3 results in early maturation of endocrine cells. Although the mechanisms by which NGN3 is involved in endocrine specification remain unknown, its downstream signal molecules have been identified; they include, but name a few, PAX4, ARX, NEUROD, NKX2.2, NKX6.1 and NGN3 itself (Rukstalis et al., 2009).

(e) NKX transcription factors 2.2

NKX2.2 starts expression in pancreatic bud and contributes to NGN3 expression. Yet, it is responsible for the regulation of β-cell development which is in parallel with the expression of Pax4 because Null NKX2.2 embryo shows absent of β-cells only but not others. NKX2.2 binds to MafA promoter to promote expression of MafA, a regulator for insulin expression. NKX2.2 also exerts functions via its downstream transcription factor NKX6.1 in regulation of insulin expression (Sander et al., 2000).

(f) BETA2/neuronal determination factor

NEUROD, accompanied with PDX1 and MafA, is a potent transcription activator of insulin by binding to insulin promoter. It is believed to be downstream target of NGN3 (Naya et al., 1997).

(g) Others (Pax4, Arx, Pax6, MafA, MafB)

Pax4 is responsible for the suppression of glucagons, ghrelin and Arx and is expressed in pluripotent endocrine progenitors. Arx expression promotes α-cells development and Null Arx embryo show no expression of α-cells. Pax6 is a paired homeodomain transcription factor, which is expressed at (E)9.5 to (E)10.5 and in endocrine progenitor cells. It is essential for the expansion of endocrine lineage and development of α-cells. MafA is a marker of mature β-cells and accompanied with NEUROD and PDX1 to promote insulin expression. MafB is essential for the differentiation of α-and β-cells in the second waves. Mutant embryo shows a decrease of 50% α and β-cells. MafB exerts its effect by binding to insulin, glucagons and MafA promoters. (Bonal et al., 2008)

1.1.5 Postnatal Pancreas Development and Regeneration

In order to maintain glucose homeostasis, it is critical to maintain sufficient insulin production. A constant turnover of β-cells is required to compensate for the loss of β-cells and increased insulin demand during growth. Postnatal capacity for β-cells renewal appears to diminish rapidly in the mouse (Bonner-Weir S, 2000). At neonatal period, β-cells are compensatory at a rate of roughly 18% of the total number of β-cells per day and followed by a severe reduction in growth rate by 2-3% per day (Kemp et al., 2003). This is observed in the neonatal regeneration model

starting with different Streptozotocin (STZ) injection day. Butler and co-workers concluded that there are residual β-cells after 1 year of the onset of autoimmunity in T1DM patients (Butler et al., 2007). This suggested that even in human, there may be regeneration exists.

Tissues regeneration can be attained by proliferation of pre-existing cells (Dor et al., 2004) and neogenesis (Bouwens, 1998; Wang et al., 1995) (Figure 1.8). Although pancreatic β-cells were thought to be unable to significantly increase in mass in the past, clinical evidence on the pancreatic hyperplasia in obesity and pregnancy has recently overthrown this idea (Bernard-Kargar and Ktorza, 2001). β-cells can be compensated with an increase in the demand of insulin.

1.1.5.1 Proliferation of Pre-existing β-cells

Proliferation of β-cells has been demonstrated in the rodent pancreas using BrdU labeling (Teta et al., 2005). Several studies have shown that proliferative ability is decreased with increasing age (Teta et al., 2005; Chen et al., 2009; Perl et al., 2010). Under special conditions, say hyperglycemia, pregnancy and obesity, replication rate of β-cells increases significantly (Bernard-Kargar and Ktorza A, 2001).

However, studies of adult human pancreas can only be done by using immunocytochemistry in cadaveric or diseased tissues. In human fetal pancreas, Ki67

staining can be detected significantly within 9-23 weeks (Sarkar et al, 2008). In human adult pancreas, most of the islets showed negative for Ki67 staining; in tissues from patients with T2DM, Ki67 staining has not shown a significant increase in amount (Hao et al, 2006), suggesting that replication does not seem to be the major source of regeneration in adult human.

1.1.5.2 Neogenesis from Precursor Cells

Bonner-Weir and colleagues have shown that, in mouse pancreas, new β-cells can be regenerated by ductal precursors using lineage tracing approach (Laybutt et al., 2002) despite that some studies exhibit a negative role in neogenesis (Dor et al., 2004). This discrepancy may be due to the difference in the use of model. In human pancreas, nesidioblastosis/islet neogenesis was observed in clinical diagnosis of chronic pancreatitis (Phillips et al., 2007) or asymptomatic pancreatic fibrosis (Woo et al., 1976). In patients with recurrence of autoimmunity, insulin-positive cells were detected in the ductal region of the pancreas (Martin-Pagola et al., 2008). In chronic pancreatitis, α-cells increase significantly by the neogenesis of ductal precursors (Butler et al., 2003).

1.1.5.3 Transdifferentiation of other cells

Trandifferentiation was found important in producing insulin positive cells in adult mouse. In human, presence of insulin-glucagon positive cells during development and in pancreatitis is reported (Desdicioglu et al., 2011; Gao et al., 2005). This suggested a possibility of trandifferentiation of α-cells into β-cells. And in chronic pancreatitis, ductal origin differentiation of α-cells provides large pool of cells capable in differentiating to β-cells (Gao et al., 2005).

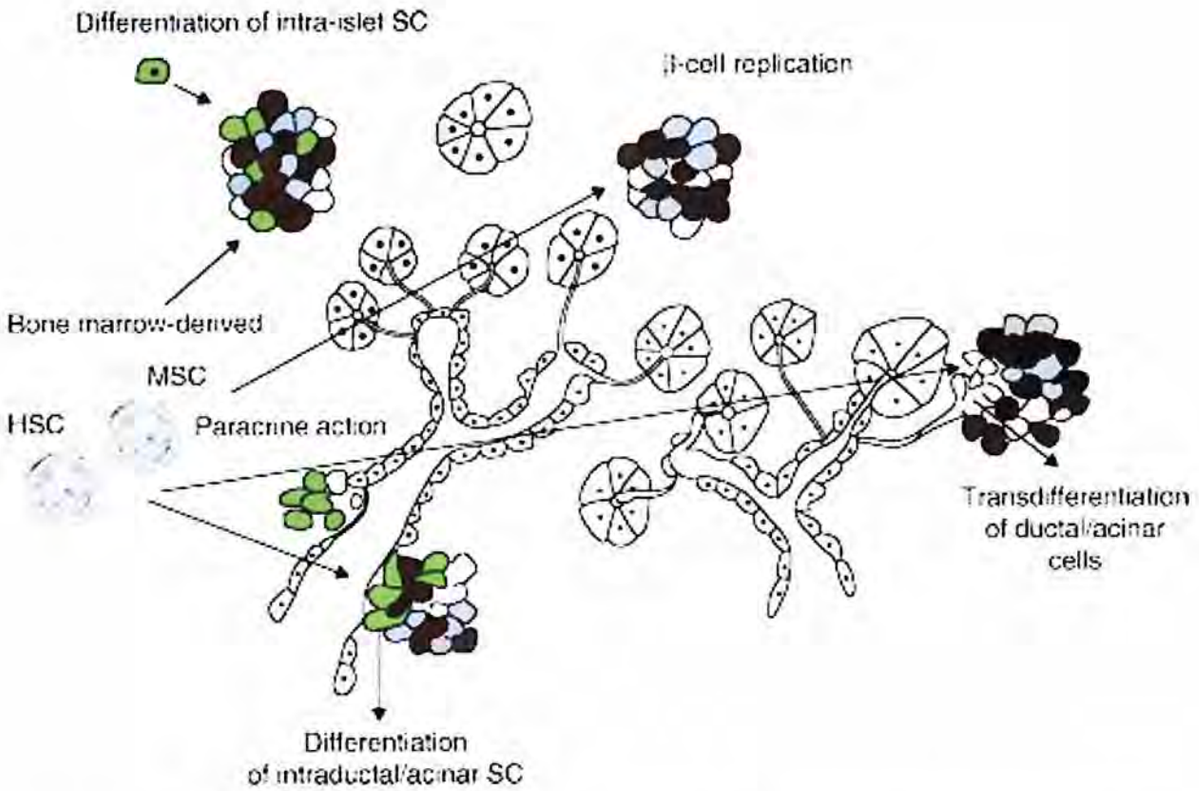

Figure 1.8 Possible mechanisms of β-cell regeneration.

1.2 Diabetes Mellitus

Endocrine portion of pancreas is involved only a small part of the pancreas; however, its function is indispensable for our human body. It is responsible for glycemic control in our bodies by producing several regulatory hormones, such as insulin. Hypofunction or hyperfunction of the endocrine pancreas has an adverse impact on our glucose homeostasis, which, in turn, affects such glucose sensitive organs as the brain and subsequently on all parts of the body (Henderson et al., 1981; Schulingkamp et al., 2000). There are several types of pancreatic disorders, including insulinoma (Athanasopoulos et al., 2011), somatostatinoma (Deschamps et al., 2010) and pancreatitis (Braganza et al., 2011). Among them, Diabetes Mellitus is the most common in term of mortality and morbidity (Sesti, 2002).

According to WHO, "Diabetes is a chronic disease that occurs when the pancreas is not able to produce sufficient insulin, or when the body does not effectively utilize the insulin that is produced by the pancreas. Hyperglycemia, or high concentration of blood glucose, is a common complication in patients with uncontrolled diabetes and over time it leads to serious damage to many of the body's organ systems, especially the nerves and blood vessels." Every year, it causes about 5% of deaths globally and are likely to increase by more than 50% in the next 10 years if there is no urgent actions in preventing the widespread. Besides, Diabetes and its complications cause a

significant economic burden on individuals, families, health systems and countries worldwide. WHO estimates that China will lose $558 billion in foregone national income in the period 2006-2015 (Data from WHO, 2009).

Under normal circumstances, human plasma glucose level would be around 3.8-6.7mM, however, in the diabetes condition, it rises to 11.1mM. (Data from WHO, 2011) Prolong high glucose concentration, named hyperglycemia, would cause various complications, say retinopathy (Oluleye et al., 2010), cardiovascular disease (Lefrandt et al., 2010) and diabetic feet (Powlson et al., 2010). Diabetes Mellitus can be divided into Type I Diabetes Mellitus (T1DM), Type II Diabetes mellitus (T2DM), gestational diabetes and secondary diabetes. (Hober et al., 2010; Athyros et al., 2010; Syed et al., 2011)

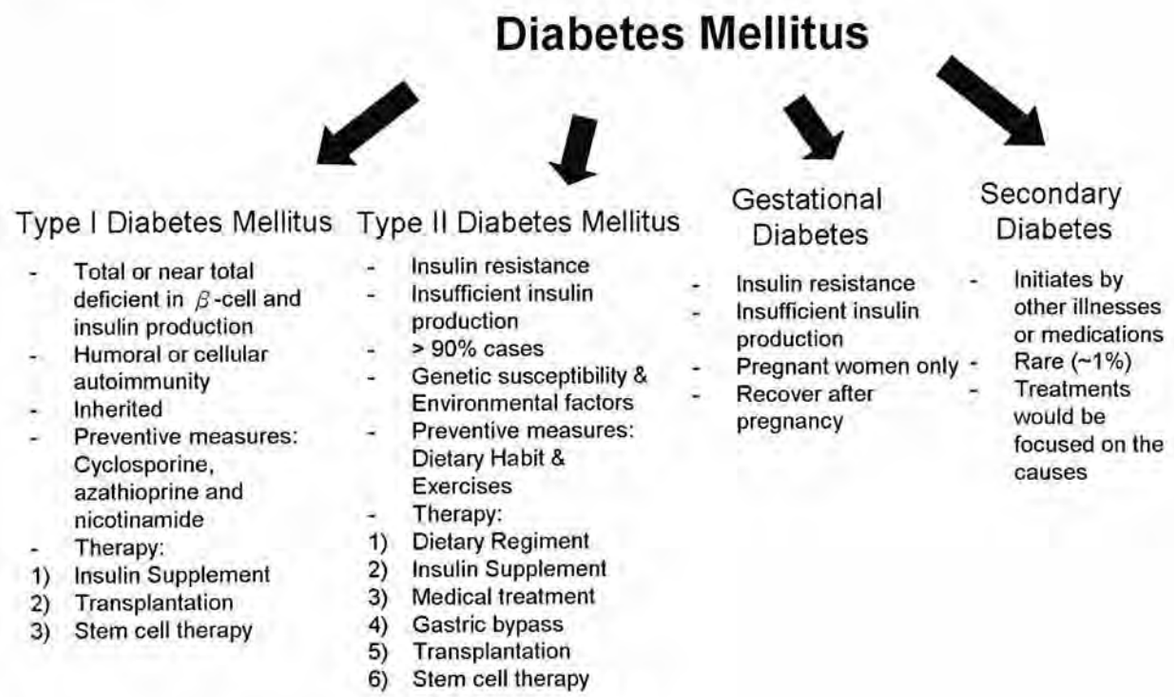

Figure 1.9 Classification of Diabetes Mellitus.

1.2.1 Pathophysiology of Diabetes Mellitus and Current Treatments

1.2.1.1 Type I Diabetes Mellitus

T1DM, characterized by total or near total deficient in β-cell and insulin production, is not the most common type of diabetes in adults but it's a frequent affecting youth due to its early onset of diabetes. Clinical studies found that it may be an inherent disease from the male family member and susceptible genes may locate at the HLA class II locus on chromosome 6 (Cheung et al., 2011; Friday et al., 1999). Yet, causes for the onset of autoimmune are still under investigations. The destruction of β-cells may due to humoral or cellular autoimmunity (Ludvigsson J, 2009). In the pass, lack of major immunological markers in β-cell has provided evidence on T-cell mediated autoimmune attack. Yet, this may not be true now as auto-antibodies against β-cell specific antigens are found in more than 90% patients and this supported the hypothesis on humoral autoimmune (William et al., 2002; Schranz et al., 1998).

Theoretically, T1DM cannot be prevented. However, more therapies and dietary habits have proposed to slow down or delay the onset of T1DM. Cyclosporine, azathioprine and nicotinamide could be used alone or in combination to protect against the immune attack (Palmer, 1996). Insulin therapy is most traditional and effective ways in treating T1DM. Yet, due to its non-physiological nature, precise control is needed to prevent occurrence of complications (Kansagara et al., 2011).

Replacement therapy, such as pancreas transplantation, islets transplantation (de Kort et al., 2011) and kidney-pancreas transplantation (Becker et al., 2002) accompany with long term introduction of immunosuppressive drugs are useful therapies for improving glucose control without insulin supplement. There are patients with transplantation, lives a normal live for more than 5 years (de Kort et al., 2011). Yet, lack of organs donations has hampered treatment.

Recent advance in stem cell therapy provide islets engineering (D'Amour, 2006) and bone marrow derived mesenchymal stem cell immunomodulatory (Delis et al., 2006), which can provide more physiological and permanent cure to diabetes. Yet, before clinical applications, considerable efforts are still required.

1.2.1.2 Type II Diabetes Mellitus

Type II Diabetes Mellitus, previously termed non-insulin-dependent diabetes mellitus (NIDDM) or adult-onset diabetes, is the most common form of diabetes, accounting for more than 90% cases. It is characterized by progressive loss of insulin secretion, reduced β-cell mass and insulin resistance in peripheral tissues, say livers, muscles and adipocytes. Classic symptoms, such as polyuria, glucoseuria, polydipsia, polyphagia, fatigue, and weight loss are resulted. Similar to T1DM, complications, say retinopathy, neuropathy and diabetes feet, etc. could be resulted. (Data from

WHO) Onset of T2DM mostly occurs in middle age or later stage of life and usually initiates by combination of genetic and environmental factors. Obesity, lack of exercise and over-consumption of alcohol could be the environmental factors. Clinical research on genes relating includes TCF7L2, PPARγ, FTO, KCNJ11, NOTCH2, WFS1, CDKAL1, IGF2BP2, SLC30A8, JAZF1, and HHEX, etc. (Eriksson et al., 2001; Krupanidhi et al., 2009; Ahlqvist et al., 2011)

The initial response of β-cell to T2DM is to increase its mass in order to compensate insufficient of insulin (Okamoto et al., 2006). However, higher insulin secretion results in further insensitize peripheral responsive tissues which result in higher demand of insulin. Elevated glucose level resulted from irresponsive of peripheral tissues impaired β-cells functions by glucotoxicity, lipotoxicity and β-cell exhaustion, etc. (Takeshi et al., 2010) The temporal increase in β-cell mass starts to decrease and insulin resistance also increases (Okamoto et al., 2006; Takeshi et al., 2010). Apart from β-cell, dysfunction of α-cell is also involved in T2DM. Due to inability to regulate blood glucose, elevated level affects glucagons secretion, which results in malfunction of α-cells (Ehses et al., 2009).

Although it is a more complex situation comparing to T1DM, early stages can be reversed by dietary regiments and plenty of exercises (Gulve, 2008). In later stage, insulin replacement is required for β-cell exhaustion and insulin resistance. First line

medical treatment for T2DM is Metformin while in later stage sulfonylureas, nonsulfonylurea secretagogues, α-glucosidase inhibitors and thiazolidinediones are introduced (Cheng et al., 2005). In serious case, surgical method, gastric bypass are currently considered as an elective procedure. However, due to relative high failure rate of surgery and side effects, it is not recommended (Schauer et al., 2003).

1.2.1.3 Gestational Diabetes

Gestational Diabetes occurs in pregnant women who have never been diagnosed for any type of diabetes ahead. Pregnant women experience in high blood glucose due to insufficient insulin production and insulin resistance (Reece et al., 1998). In U.S., approximately 4% of pregnant women are diagnosed for gestational diabetes. (Data from WHO) Only few symptoms are observed, thus, it is diagnosed only during pregnancy screening. Women with family history of T2DM, high maternal age and obesity need to take good care about it (Reece et al., 1998; Ben-Haroush et al., 2004).

Usually, gestational diabetes only diagnosed during pregnancy and after pregnancy, blood glucose will return to normal level. However, to mother, rate of recurrence of diabetes during life time increases and to babies, "big head" babies, hypoglycemia and jaundice may result. Various risk factors are found related to gestational diabetes, say weight gain, obesity and higher blood pressure, etc

(García-Patterson et al., 1998). Administration of exogenous insulin, plenty of exercises and dietary regimens focus on lowering blood glucose are mostly applied therapies to prevent mother and fetus suffering from complications.

1.2.1.4 Secondary Diabetes

Secondary Diabetes, a rare case of diabetes, occupies only 1% of diabetes and initiates by other illnesses, such as pancreatitis, Cushing's syndrome and autoimmune diseases, or medications, say glucocorticoids, antiretrovirals and opiates (Choudhuri et al., 2009; Catargi et al., 2003; Resmini et al., 2009). It is a temporarily increase in plasma blood glucose. Besides, secondary diabetes also includes cases, for example, maturity-onset diabetes of the young (MODY) (Fajans et al., 1994), latent autoimmune diabetes of the young (LADA) (Zachariah et al., 2008) and Wolfram syndrome (Barrett et al., 1995), etc., that cannot be classified as T1DM, T2DM or gestational diabetes. Treatments would be focused on the causes, say stop medication if it is triggered by certain drugs.

1.3 Stem Cell therapy

Recent stem cell therapy for Diabetes focused in several aspects, including islet engineering, immune modulation and regeneration in vivo. Islet engineering, using embryonic stem cells (ESCs), induced pluripotent stem cells (iPSCs) or mesenchymal stem cells (MSCs), provided unlimited sources of islets ready for transplantation in order to compensate insufficient islets demand. Secondly, post-transplantation immuno-rejections of grafts could be solved by transplanting low-immunogenicity iPSC/ESC induced islets, immuno-modulating MSCs and cells fusions by bone marrow derived MSCs. Finally, localization and repairment after transplantation could be attained by development of vascular and nervous system by undifferentiated stem cells, in vivo transdifferentiation of adult cells into insulin producing cells and investigation in transplantation sites.

1.3.1 Stem Cell

Stem cell, or unspecialized cell, is characterized by its renewal power and ability to differentiate into all different tissues or organs under certain physiologic or experimental conditions while progenitor cell (precursor cell), or specialized cell, is characterized by its limited renewable capacity and limited potential to differentiate to

certain lineages. It can be classified based on its degree of potency, i.e. totiopotency, pluripotency, multipotency, oligopotency, and unipotency (Nancy, 2005).

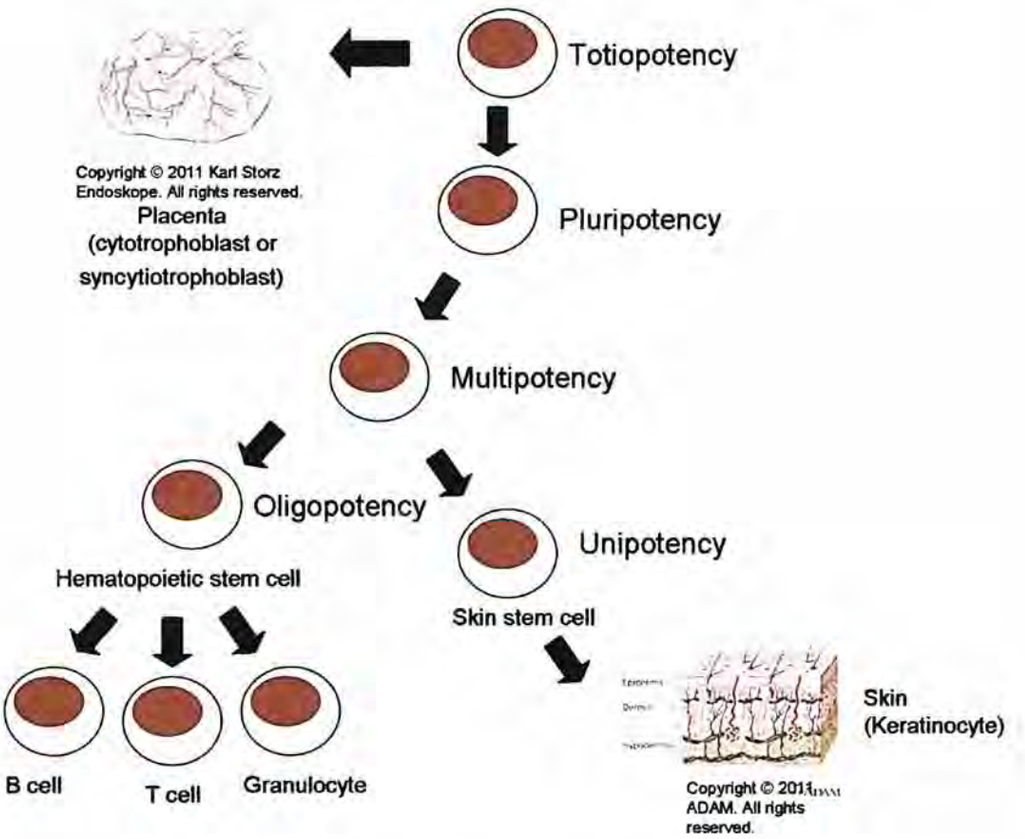

Figure 1.10 Stem cells classified based on the degree of potency.

Totipotent and pluripotent stem cells are the cells that have not begun/barely started differentiating and be capable of developing into any other type of body cell while their difference lies in the ability in differentiating cells in placenta say cytotrophoblast layer or syncytiotrophoblast. Multipotency defined by its limited power in differentiating to multiple but not all cell types. Oligopotency is the ability of progenitor cells to differentiate into a few cell types in a given family. Examples of oligopotent stem cells are the hematopoietic stem cells. They can become any of the

many types of blood cell, say red blood cells, white blood cells and platelets, etc., but not islets nor neurons. Unipotent stem cells can only differentiating to single lineage but are considered as stem cells because of their unlimited proliferation ability. For example, skin cells can renew themselves indefinitely but cannot become any other type of cells (Nancy, 2005).

1.3.1.1 Mesenchymal Stem Cell

MSCs are multipotent stem cells that can be isolated from adult or fetal tissues and organs, such as liver, adipocyte and pancreas. It is characterized by the expression of a raft of primitive markers, say CD90, CD105 and CD166, etc. They are capable in differentiating into osteoblasts, chondrocytes and adipocytes but not into hematopoietic stream (Bernardo et al., 2009).

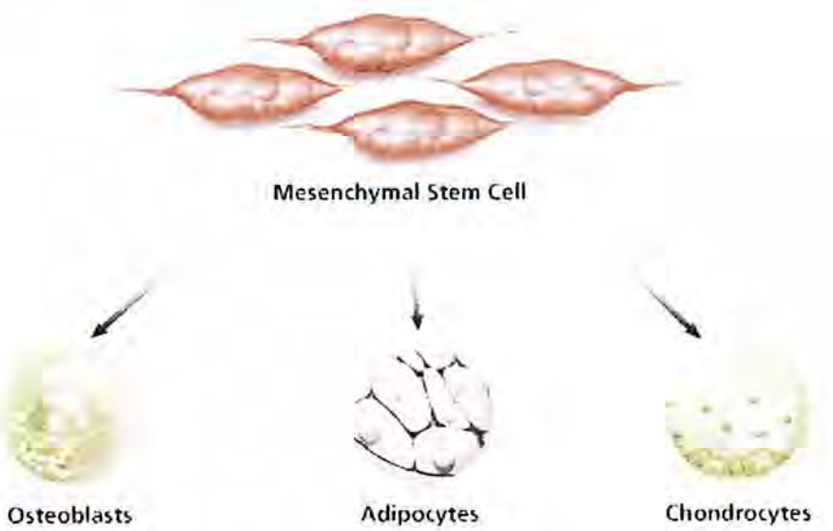

Figure 1.11 Mesenchymal Stem Cell Pathway.

(a) Umbilical cord blood derived stem cells

The youngest, most primitive MSC can be obtained from the umbilical cord tissue, namely Wharton's jelly and the umbilical cord blood (Bernardo et al., 2009). Comparing to embryonic tissues and bone marrow, they are much easier to obtain and there is no ethical issues concerning as they are discarded tissues. They can be induced into islet-like cell clusters *in vivo* through stepwise culturing in neuron-condition medium (Chao et al., 2008). After xenotransplantation, they are capable in reversing glucose-tolerance stages in the absence of immuno-suppressants.

(b) Bone marrow derived mesenchymal stem cells

It is readily available and easily collected (Bernardo et al., 2009). Apart from islet engineering, it also capable in regenerating damaged tissues by direct injection to recipients (Barbash et al., 2003). Research suggested that after injections, they are automatically home to the regenerating pancreas and improve insulin secretion and reduce blood glucose level in hyperglycemia mice (Li et al., 2008). Besides, cytokines produced by bone marrow stem cell microenvironment has enhanced isolated β-cells survival and improved their functions (Jung et al., 2011). In addition, it also improved T2DM in metabolic control and reduced insulin requirement (Estrada et al., 2008).

(c) Pancreas derived mesenchymal stem/progenitor cells

The advantages of using pancreas derived stem cells or progenitor cells rest on their commitment towards the pancreas. Less manipulation is required to obtain fully functional β-cells. As they are less pluripotent and more easily directed, potential of triggering tumouriogenesis reduced. Progenitor/stem cells are reported to be present in both fetal and adult pancreas that originate from islets, ducts or mesenchymal cells.

i) Duct epithelial cells

During development and regeneration, many groups have found that progenitor cells reside in ductal origin (Wang et al., 1995). Genetic lineage tracing experiment shows that cells expressing carbonic anhydrase II in the ductal region will develop to new β-cells after regeneration (Gu et al., 1994). Moreover, other groups showed that NGN3 positive cells are found in the ductal region during regeneration (Liu et al., 2007). This suggested that in the ductal region, there are a population of stem cells which can differentiate to new β-cells. Besides, isolated rodent duct cells have been shown to differentiate into insulin-producing cells *in vitro* (Bonner-Weir S et al., 2000).

ii) Acinar cells

Acinar cells are the most common cell type making up the pancreas and share a common population of precursors with islets (Slack et al., 1995). With the process of dedifferentiation by Notch signalling, acinar cells were shown capable in

differentiating into insulin-producing cells *in vitro* (Esni et al., 2004). Besides, with genetic manipulation in PDX1/NGN3/MAFA, acinar cells were shown improve fasting glucose levels in hyperglycemic mice *in vivo* (Zhou et al., 2008). This process involved dedifferentiation and activation of cell cycles, which believe to be less risk of tumoriogenesis by pluripotent stem cells. However, utilization of virus raises concerns in insertional mutagenesis triggered tumoriogenesis.

iii) Islets

Using cells reside in islets may provide a lower barrier for reprogramming into insulin producing cells. Lineage tracing has demonstrated that newly generated β-cells derived from pre-existing β-cells in adult (Dor, 2006). However, extremely slow rate of regeneration has hampered further investigation on underlying mechanisms. Some groups suggested that there are precursors reside in islet while some suggested that it is the proliferation of pre-existing cells (Dor, 2006; Segev et al., 2004). Under extreme condition of β-cells lost, pancreatic α-cells can transform into β-cells *in vivo* (Lu et al., 2010; Thorel et al., 2010). NGN3 positive cells found suggested that dedifferentiation occurs. Collombat et al., group has shown that reprogram by using transcription factor PAX4 expression can convert α-cells into insulin producing cells (Collombat et al., 2009). Although transgene cannot be used in

therapy, the idea of conversion of α-cells into β-cells can be apply in T2DM with relative reduction in β-cells but increased α-cells.

(d) Liver derived mesenchymal stem cells/progenitor cells

Successful cases are reported by transdifferentiation of fetal/adult liver progenitor cells into insulin producing cells raises interest in investigating potential of liver cells as a source of islet engineering (Zalzman et al., 2003; Yang et al., 2002).

Problems encountered are difficulty in isolating stem cell from adult tissues as they are very scarce and invasive (Garg et al., 2008). Comparing to other stem cells, they have limited proliferation and differentiation potential (Watt et al., 2000; Pittenger et al., 1999). Yet, they are more easily directed than other stem cell type as they may, to certain extent, have committed lineage (Pittenger et al., 1999). Besides, this is also less tumorigenic.

MSCs having immunomodulatory properties and their power to differentiate into insulin-secreting cells make it a promising therapeutic target for diabetes (Le Blanc, 2003; Fiorina et al., 2009; Karnieli et al., 2007). However, ethical debates have limited the use of MSC.

1.3.1.2 Embryonic Stem Cell

ESCs are pluripotent stem cells derived from the inner cell mass of the blastocyst, an early-stage embryo. They are capable in differentiate into all derivatives of the three primary germ layers: ectoderm, endoderm, and mesoderm. Cell lines, without any contaminations, derived from human embryos under completely cell- and serum-free conditions are available commercially. Problems encountered are their high tumoriogenicity, difficult to control its fate and immunogencity. Moreover, ethical debate has also limited the use of human ESC (Baharvand et al., 2004; van der Bogt et al., 2006; Blum et al., 2008).

Shi Y et al., have introduced a novel three-step approach in inducing ESCs to differentiate into pancreatic beta cells (Shi et al., 2005) and Chen S et al., also showed that a small molecule can direct differentiation of human ESCs into the pancreatic lineage (Chen et al., 2009). These provide excellent potential for ESC to be the unlimited sources of islets.

1.3.1.3 Induced Pluripotent Stem Cells

iPSCs are a pluripotent stem cell artificially derived from a non-pluripotent origin by genetic modifications (Takahashi and Yamanaka, 2006; Takahashi et al., 2007), miRNA (Anokye-Danso et al., 2011) or proteins (Zhou et al., 2009). Yamanaka and Takahashi et al., group has first created iPSCs from mouse skin cells, later, from

human skin cells (Takahashi and Yamanaka, 2006; Takahashi et al., 2007). Later, Thatava T, et al., has shown that Indolactam V/GLP-1 is capable in mediating differentiation of human iPSCs into glucose-responsive insulin-secreting progeny (Thatava T et al., 2011), which provide promising potential for the use of iPSCs in islet engineering.

As iPSCs obtained from same host, immunogenicity encountered is resolved (Fairchild, 2010). Problem encounter while using iPSCs are its low efficiency in induction to iPSCs from mature somatic cells, virus utilized during induction and genetic mutations leaving after differentiation (Utikal et al., 2009).

1.3.2 Islets Engineering

1.3.2.1 Genetic Modification

MSCs and mature cell types can be induced to differentiate into functional insulin producing cells when PDX1 is introduced via recombinant adenoviral vector (Li et al, 2007; Noguchi et al., 2006). By using adenovirus to mediate PDX1/NGN3/NEUROD/PAX4 expression in duct cells, Noguchi et al., demonstrate that NEUROD was the most effective inducer (Noguchi et al., 2006) while Collombat

et al., group also has shown that overexpression of PAX4 only can α-cells direct into insulin producing cells (Collombat et al., 2009).

1.3.2.2 Directed Differentiation

Directed differentiation is the hottest and well-studied methods in islet engineering. Most successful protocol recently available by D'Amour et al. group is also utilizing this method (D'Amour et al., 2006). By stepwise addition of different morphogens in culture medium to direct stem cells fate has successfully induced islets formation. Directed differentiation of ESC or iPSC involves differentiation into definite endoderm, establishment of the primitive foregut endoderm, patterning of the posterior foregut and specification and maturation pancreatic endoderm and thus endocrine precursors. Yet, in vitro differentiation resulted insulin producing cells still encountered major problems, such as non-functional, irresponsive to glucose, incomparable c-peptide expression to adult islets.

1.3.2.3 Microenvironment

Cells in culture can "condition" the medium where they grow, changing its capacity to promote further cell growth or transformation of other cell type and its created microenvironment can restore isolated cells' functions (Timmers et al., 2011),

maturation of stem cells (Vicente-Salar et al., 2011) and regenerating tissues architectures (Schonfeld et al., 1981). Mechanical force, extracellular matrix, condition medium and co-culture techniques are commonly employed to mimic microenvironment (Nathaniel et al., 2008). Among them, co-culture was predicted as the most powerful tools for maintaining the physiological functions of isolated cells because of the presence of cell-cell communications, contact and signalling.

Recently, Ledran et al., group showed that stromal niche derived from organs involved in hematopoiesis can efficiently differentiate ESC into hematopoietic lineage (Ledran et al., 2008). Besides, Chen et al., group has promoted β-cell differentiation by using adult islet cells (Chen et al., 2009). These data have provided us insight in microenvironment direct stem cell fate can provide a supportive environment for maturation.

1.3.2.4 *In vivo* Regeneration

Final succeed only comes with successful transplantation of functional organs in patients. As a result, many groups have tried to transplant their semi-functional/ immature organs, even undifferentiated stem cells into different sites of the mouse for investigations. Many cases have shown that after transplantation, immature organs have reached certain extend of maturation and stem cells can partial recover and

regenerate the damaged/ malfunctions organs. From above data, people believe that direct transplantation of stem cells or differentiated tissues can recover the disease state (Chapel et al., 2003; Orlic et al., 2001).

1.3.2.5 Cell Fusions

Cell fusion is another possible way to produce viable cells and to induce cells from one tissue into another after transplantation (Nygren et al., 2004). After transplantation of pluripotent stem cells or other cells with committed lineage, instead of transdifferentiation of original tissues or differentiation of new comers, they fused with cells in the site of transplantation to perform a new functions and no immuno-rejection occurs. This has been shown by bone marrow-derived cells fused with hepatocytes in liver, Purkinje neurons in the brain and cardiomyocytes in the heart (Alvarez-Dolado et al., 2003). Furthermore, it has been demonstrated that liver transplanted with ICC will lead to the discovery of insulin positive and nuclei positive liver cells (Sapir et al., 2005). They contain single nuclei, mitochondrion-rich cytoplasm, less glycogen storage granules and multiple insulin secretory granules.

1.3.2.6 Combinatory Treatment

Single direction in differentiation may not be succeeded as organs development is a complicate process. As a result, some groups are trying to utilize a mixed method to achieve more mature and better glucose responsive insulin-producing cells. For example, *in vitro* hematopoietic differentiation of human ESC is promoted by co-culture of human bone marrow stromal cells and with exogenous addition of low dose of cytokines (Wang et al., 2005).

1.4 The Vitamin A and vitamin D system

1.4.1 The Vitamin A

Vitamin A has been long been discovered as a signalling molecule essential for several metabolic pathways in adult. Pioneer studies regarding the effects of vitamin A in rodents by feeding vitamin A deficient diet have shown its paramount importance in several organ developments (Burn et al., 1941). Subsequent studies in embryogenesis have shown that vitamin A cooperates with fibroblast growth factors (FGFs) and sonic hedgehog (SHH) in embryonic patterning (Chiang et al., 2001). Recent advances have shown that exceeding 500 genes are thought to be regulated by retinoic acid and functions including normal growth and development, normal vision, a healthy immune system, normal reproduction, and healthy skin and barrier functions (Balmer et al., 2002; Gerster et al., 1997).

As vitamin A cannot be synthesized in sufficient quantities by human, it must be obtained from the diet. In adult, excess intake of vitamin A is converted by small intestine in the form of retinol during absorption and stored in liver hepatocytes or hepatic stellate cells (HSCs) which are liver-specific mesenchymal/ stromal cells (Blomhoff et al., 1990). In embryo, vitamin A is stored as two forms, namely retinoaldehyde and beta-carotene in eggs (Niederreither et al., 2008). In early fetus,

vitamin A is stored in hepatoblasts or bipotent hepatic parenchymal progenitors (Kutoba et al., 2007).

Vitamin A includes the compounds retinoic acid (RA), retinal, retinol, and four known carotenoids, i.e. alpha-carotene, beta-carotene, gamma-carotene; and the xanthophyll beta-cryptoxanthin, where only retinoic acid, retinal and beta-cryptoxanthin are the active functional forms. Among them, RA is found responsible in many developmental processes, including pancreatic axial patterning and development.

Vitamin A is a lipophilic morphogen, which can readily across cell membrane and bind to nuclear receptor to exert effects. Retinoic acid receptor (RARs), consists of RAR alpha, beta and gamma, belong to the superfamily of nuclear receptor.

atRA exerts its effect by binding to RAR alpha, beta or gamma depending on its functions which triggers receptors conformational change and release co-repressors. Then, it starts to recruit co-activators and RAR will form heterodimer with RXR, including RXR alpha, beta and gamma. The heterodimers will then bind to the RAR response elements (RARE) and induced signaling cascades. In the absence of RA, RAR also recruit RXR and co-repressor to bind to RARE and make the DNA become inaccessible for transcription. This may be interpreted as a negative transcriptional effect (Niederreither et al., 2008).

Although hundreds of genes have been reported to activate by RA, only approximately 20 genes were unambiguously to be contained in RARE. Recent researches have suggested that diverse repertoire of RA-regulated genes can directly or indirectly induce cascades of signals that are responsible for the effects (Balmer et al., 2002). Recent research also suggested that RA act on other steroid orphan receptors, say Peroxisome proliferator-activated receptors (PPARs) to exert effects (Desvergne et al., 1999).

1.4.2 Vitamin A Metabolism

Dietary Retinoid, in the form of retinol, retinyl ester or carotenoids, absorpted through intestine are converted into retinol (Blomhoff et al., 1990).

Developmental effects of vitamin A rely tightly on the distribution and amount of RA within the tissues through synthesis, metabolism and diffusion gradients. Retinol is converted into RA through 2 consecutive reactions. Retinol is firstly converted into retinoaldehyde by 2 enzyme families, which is the cytosolic alcohol dehydrogenase (ADHs) and microsomal retinol dehydrogenase (RDHs). The retinoaldehyde are then oxidized via retinoaldehyde dehydrogenase (RALDH 1, 2, 3) which is located in different position during embryogenesis. The conversion of retinoaldehyde to RA is the rate limiting step. An alternative pathway cleaving

beta-carotene by beta-carotene15,15'- monooxygenase to retinoaldehyde has been suggested during embryogenesis.

Excess RA can lead to developmental abnormalities and should be converted into more polar form (4-OH and 4-oxo RA) by cytochrome P450 26 A1, B1 and C1 (CYP26A1, B1 and C1). Although more polar form was suggested to be inactive, recent advance in search found that they may exert certain actions (Niederreither et al., 2008).

Tissue-specific anabolism and catabolism of RA have been studies for the regulation of RA, a morphogen, for patterning in development. RALDH displays significant patterning in different part of the embryo. For example, RALDH is firstly expressed in primitive streak and mesodermal cells but absence in posterior embryonic regions, which implies that RA is essential only in primitive streak and mesodermal cells but not in posterior embryonic regions (Swindell et al., 1999). Mutant of RALDH will cause malformations of embryo which is lethal (Mark et al., 2006). Figure 1.9 summarizes vitamin A metabolisms.

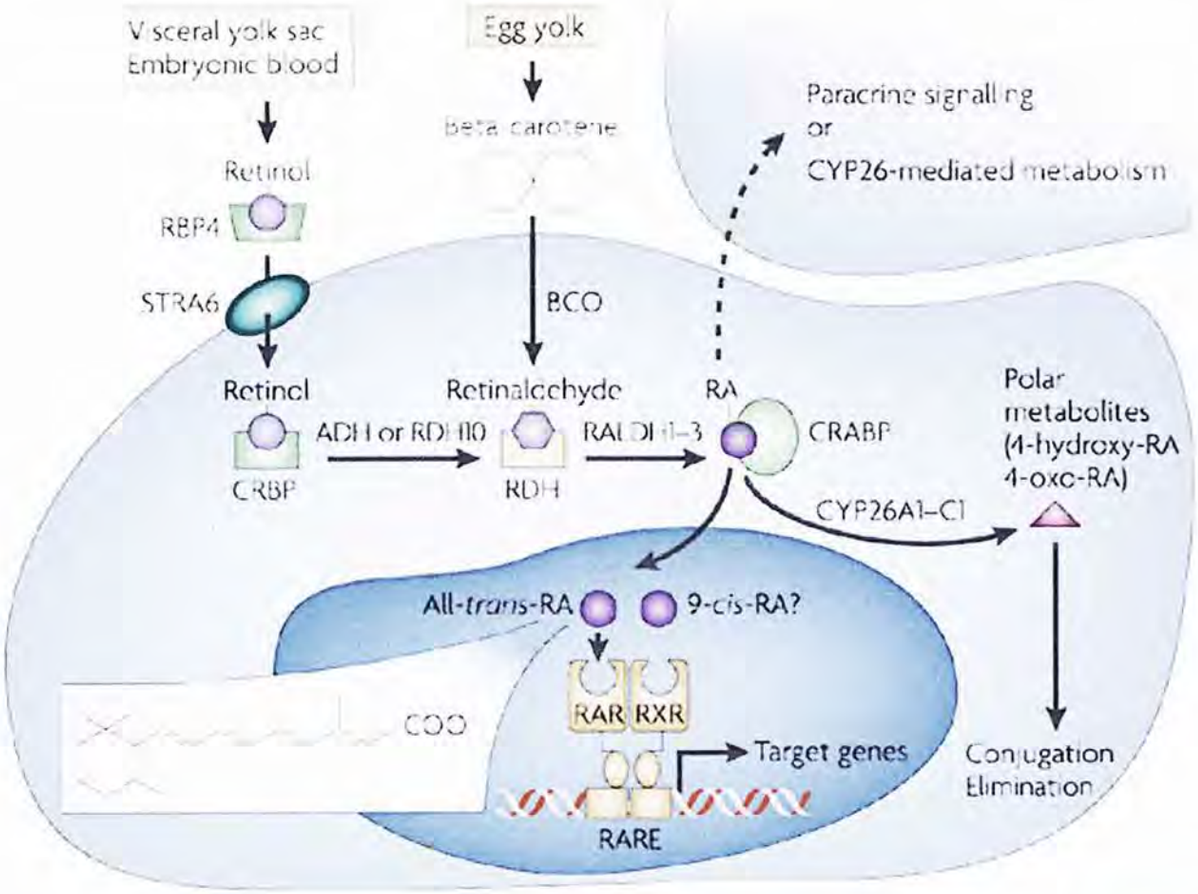

Figure 1.12 Vitamin A metabolism.

1.4.3 Roles of Vitamin A in Pancreatic Development

Gain-of-function and loss-of-function has been introduced to study the effect of RA in embryos and fetus. In both RALDH or RAR deficient zebra-fish and mice, they lack pancreatic cell types (Mark et al., 2006; Alexa et al., 2009). Besides, RA is an instructive signal to anterior endoderm and trigger development of pancreatic cells (Stafford et al., 2006). Studies across other species, say amphibian and avian also

possess similar results show that RA system is conserved during evolution (Stafford et al., 2004).

RA is essential for several steps of development, to begin with, formation of definite endoderm, anterior-posterior axis patterning of the pancreas, induction of specification to pancreatic fate and later generation of β-cells (Stafford et al., 2006).

The position and extent of pancreatic buds not only depend on inductive signals from surrounding tissues but also competence of the endoderm. After gastrulation, endoderm formed in the receipt of adjacent mesectoderm signals, mediated by FGF4. Later the anterior-posterior axis patterning by generation of alternative levels of RA by diffusion or site of generation, and bone morphogenetic proteins (BMP) proteins. In this case, RA acts as a posteriorising agent of endoderm (Kumar et al., 2003).

Pancreatic specification depends on the anterior-posterior axis patterning of the endoderm and RA derived from adjacent anterior paraxial mesoderm directly act on definite endoderm to induce a population of pancreatic precursors. RALDH2 was positively expressed in anterior paraxial mesoderm which indicate it is the source of RA and sufficient for pancreatic induction (Stafford et al., 2006).

Besides, the generation of NGN3+ endocrine progenitor cells and further differentiation into β-cells also depends on the presence of RA, proven by the generation of Ipf1/RAR403 transgene mice. The RA induced expression of

transcription factors NGN3 which specify the endocrine lineage and preferentially generating β-cells (Oström et al., 2008).

1.4.4 The Vitamin D

Vitamin D, is a pro-hormone that can not only regulate calcium homeostasis and bone mineralization but also module a wide range of fundamental biological functions in different part of the body, including keratinocytes differentiation and immuno-modulation. In term of human history, it was firstly discovered during industrial revolution. It was discovered in children who worked in sunless factories. They were found with severe growth retardation, widening of the ends of the long bones and bending legs. By the late 1600s, rickets was recognized as major health problem in children. Although nowadays working conditions have improved, many cases of vitamin D deficient are still found in people lacking sunlight exposure in wintertime, a particular mode of dress and malnutrition in developing countries (Soram Khalsa, 2009).

As vitamin D cannot be synthesized in sufficient quantities by human, it must be obtained from the diet. However, little is known about its first made. Bills et al., suggested that vitamin D was found primary in oily fish and fish liver oils and it was obtained by eating green plankton. A single fish consumed about 1.2% of its body

weight every 24hrs and through concentrating power of the food of the food chain, they obtained a large amount of vitamin D in fat and livers (Holick MF, 2003).

VDR belongs to the nuclear receptor superfamily. It contains transactivation domain, termed AF-1, a central DNA-binding domain with 2 zinc-finger motifs and carboxyl-terminal ligand-binding domain. AF-1 is recognized by coactivator proteins and other transcriptional factors and carboxyl terminal ligand-binding domain is conserved among different species but diverse enough to recognize specific ligands (Carlberg et al., 2007)

Vitamin D is a lipophilic morphogen, which can readily across cell membrane and bind to nuclear receptor to exert effects. VDR functions with heterodimerization with Retinoid X receptors (RXRα, β, γ) and binds to calcitriol response element (VDRE). Some reports showed that VDR sometimes binds to other nuclear receptors, say thyroid hormone receptor and RARs but with strongest binding affinity towards RXRs. Besides, there is also secondary responsive gene apart from VDRE but the inductions showed a delay response, say a few hours or even a day. They are most probably mediated by primary VDRE (Carlberg et al., 2007).

1.4.5 Vitamin D Metabolism

Vitamin D is a prohormone, required conversion in kidney and liver, to become active form calcitriol and bind to its target receptor vitamin D receptor (VDR) to engender biological functions. It is mainly produced in the skin in response to sunlight and obtained from diets. Major sources of vitamin D are oily fish, eggs and livers (Figure 1.10).

Exposure of skin to sunlight with wavelength approximately 295nm can causes photolytic conversion of 7-dehydrocholeterol to previtamin D_3 followed by thermal-isomerization to Vitamin D_3. Vitamin D_3, also called Cholecalciferol, is converted by the hydroxylation steps. Firstly, it is hydroxylation at carbon-25 to 25-hydroxylvitamin D_3 by CYP2R1 and sometimes other hepatic cytochrome P450s, which occurs primarily in livers. This is the rate-determining steps and level of 25-hydroxylvitamin D_3 is commonly used as an indicator of vitamin D status.

25-hydroxylvitamin D_3 is afterwards subjected to another hydroxylation at carbon-1 to produce 1α, 25-hydroxylvitamin D_3 by CYP27B1, which occurs mostly in kidney. Yet, recent researches showed that it also presented in other tissues, say keratinocytes to act as autocrine or paracrine functions. Feedback mechanisms by 1α, 25-hydroxylvitamin D_3 can inhibit over production and minimize vitamin D toxicity.

Elevating 1α, 25-hydroxylvitamin D_3 can cause an increase in serum calcium and phosphate level. 25(OH)D_3-24-hydroxylase (CYP24) oxidize at Carbon-23 and

Carbon-24 which lead to cleavage of side chain and inactivation. Cooperation of CYP24 and CYP27B1 is of vital important in regulating calcitriol level in growth and differentiation [Holick, 2003; Carlberg et al., 2007; Kochupillai, 2008; Feldman et al., 2005].

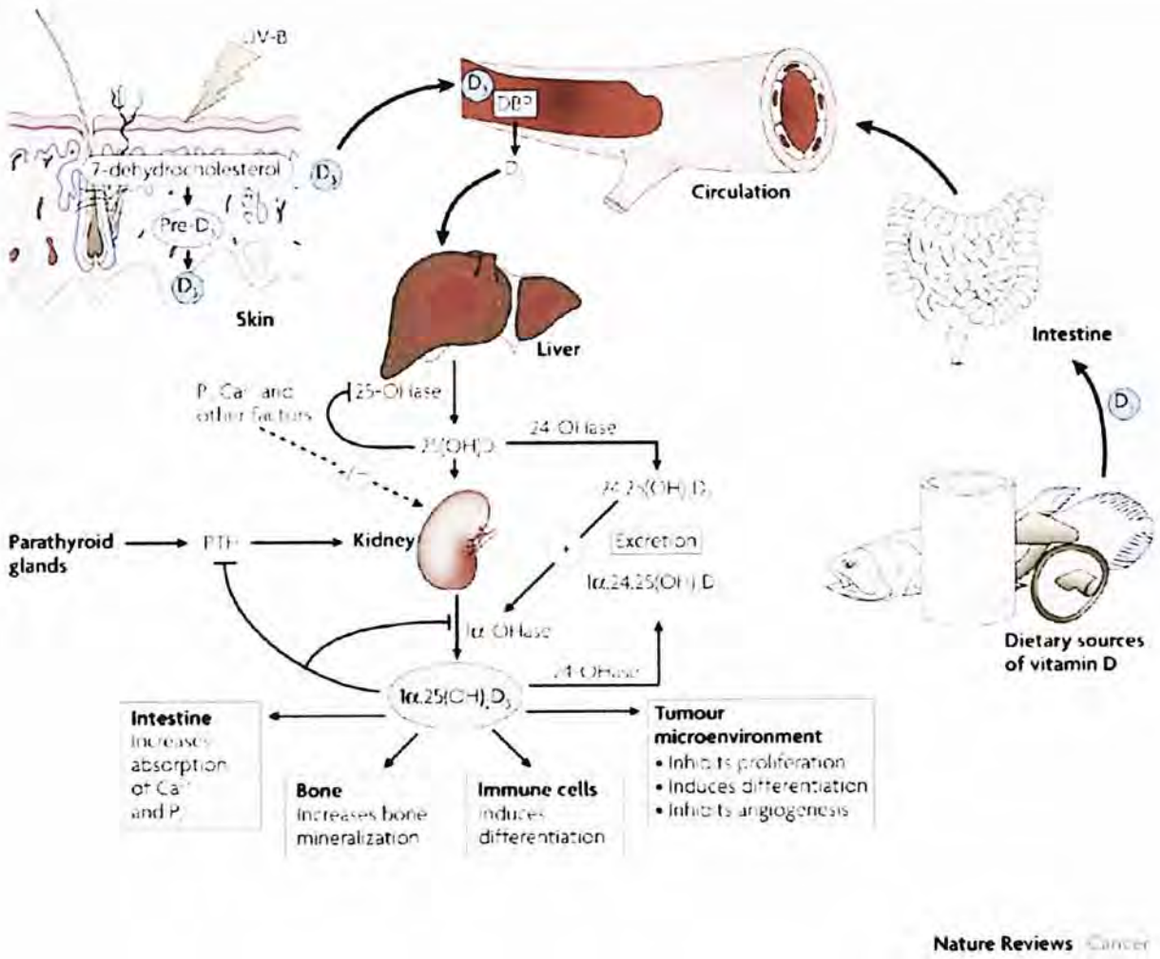

Figure 1.13. Vitamin D metabolism.

1.4.6 Metabolic Functions of Vitamin D in Islets

(A) Type 1 Diabetes Mellitus

Several clinical studies have correlated the vitamin D deficient with change in serum glucose or insulin level (Scragg et al., 1995) and sometimes, insulin sensitivity (Borissova et al., 2003). In animal studies, administration of vitamin D or its analogues can inhibit the development of STZ induced Diabetes and inflammation induced diabetes (Del Pino-Montes et al., 2004). Also, studies in infants with consumption of cod liver oil supplement, rich in vitamin D can reduce risk of T1DM development. In T1DM, caused by deficient of islets, this implied vitamin D play a certain roles in improving insulin secretion of islets. Beneficial effects of vitamin D mainly lie on glucose-induced insulin secretion after 3 hrs administration whereas not affecting fasting glucose and insulin concentration (Hyppönen et al., 2001; Harris, 2005).

(B) Type II Diabetes Mellitus

Non-obese and young mice fed on a vitamin D-depleted diet has higher incident in developing T2DM. On the contrary, vitamin D benefits animal models of diabetes, say obese Wistar rats with T2DM. Regular consumption of fish, rich in vitamin D by adult have been associated with 60% reduction in developing glucose intolerance. T2DM, characterized by insufficient insulin secretion and insulin insensitivity, insulin secretion can be improved while insulin insensitivity improvement is still controversial (Tai et al., 2008).

1.4.7 Cod Liver Oil

Cod liver oil, a natural nutritional supplement derived from liver of cod fish, was once commonly given to pregnant women for its high value of omega-3, EPA and DHA for fetal development and children to prevent rickets or other vitamin D deficient symptoms. However, with the high dose of vitamin A presented, it made things controversial, especially to pregnant women. Because vitamin A is a morphogen, with precise dose would enhance fetal development, however, overdose will affect normal fetal development and also cause gestational hypertension to mother. As a result, cod liver oil is now only recommended to ease the pain and joint stiffness associated with arthritis even with its proven positive effect on wound healing, heart, bone, hair and nails growth (Masterjohn, 2009).

1.4.8 Interactions between Vitamin A and Vitamin D

Studies have shown that vitamin A and vitamin D may have modulating effects in different type of cells. (1) For example, they can synergistically inhibit the clonogenicity of MCF-7 and T-47D cells [Koga et al., 1991; Wang et al., 2000]. (2) However, excess RA levels antagonise calcitriol's roles in Ca^{2+} homeostasis (Ørnsrud et al., 2009). Although mechanisms are still unclear, (3) Retinoid X receptors are suspected candidates as they are the common target. Retinoid X receptors (RXRα, β,

γ), receptors for 9-cis-retinoid acid (9-cis-RA), are able to form heterodimerization partners for several nuclear receptor, say RAR, VDR and Peroxisome proliferators-activated receptors (PPARs), etc (Feldman et al., 2005). As specific ligands 9-cis-RA are not detected in any adult or embryo tissues and with exogenous addition of 9-cis-RA or any synthetic ligands, embryo defects can be rescued (Niederreither et al., 2008), this excludes its effect as a stereoisomer but forming a inter-relation molecules with different receptors.

In additional to sharing common target, (9) research showed that RA cross-talk with calcitriol by inhibiting the expression of CYP24 in human prostate cancer (Lou et al., 2005). There is also study suggested that (10) RARgamma2 can interact with VDREs and affect vitamin D activity (Koszewski et al., 2010). Besides, (11) RA was found to stimulate VDR expression in both tumour and non-tumour bone cells of rats (Grigoriadis et al., 1986).

1.5 The Relations of Liver and Pancreas Development

During embryogenesis, development of neighbouring tissues is under similar combinations of transcription factors, differing by just one or a few factors (Chia-Ning et al., 2003). In developmental studies, emergence of ventral pancreas is related to the emergence of liver as they are specified at the same time and in the same general domain of cells (Deutsch et al., 2001). FGF signalling from the cardiac mesoderm diverts this endoderm to express genes for liver instead of those for pancreas, which implies that devoid of FGF signalling from cardiac mesoderm would lead to the development of pancreas (Deutsch et al., 2001, Zaret et al., 2008). In addition, lower organisms such as Nile Tilapia, pancreas and liver are not separated as hepatopancreas, which shows that their development may be associated (Morrison et al., 2004). In addition, vitamin A and D, whose serum level was regulated by liver, enhanced islets specification (Ng et al., 2011), which showed that liver and pancreas are closely related during development. Further studies in adult pancreatic regeneration during obesity, neuronal relay from the liver control regulation of β-cells mass (Imai et al., 2009).

1.5.1 Endoderm Induction for Hepatic and Pancreatic Differentiation of ESCs

Both hepatic and pancreatic developments are originated from the budding from embryonic endoderm. Thus, development of endoderm represents an important step and has an integral common role in initiating the early stages differentiation of ESCs into hepatocytes and insulin producing cells.

Common signalling events can induce SOX17 and FOXA genes in definite endoderm, i.e. signalling from Activin/Nodal family members and inhibitory signalling from PI3K through insulin/IGF1 family members. Patterning of the definite endoderm to foregut, midgut and hindgut is also of vital importance. Both liver and pancreas are budding from the midgut instead of foregut and hindgut (Soto-Gutierrez et al., 2008).

1.5.2 Bipotential Precursor Population within Embryonic Endoderm

Fate mapping has shown that the liver bud is generated in part from the paired lateral domains of the endoderm, immediately anterior to and, partially, intermingled with the ventral pancreatic progenitor. Vertebrate endoderm gives rise to epithelial lining of the gut and to organs, such as the thymus, liver and pancreas. Specifically, the liver and ventral pancreas are out bud at the embryonic endoderm that is next to each other and derived from homogenous bipotential precursor populations. The developmental fate of liver and pancreas is determined by cardiac mesoderm. Cardiac

mesoderm and FGF signalling induce local expression of sonic hedgehog and this will in turn inhibit pancreatic specification but not liver development (Deutsch et al., 2001).

1.5.3 Pancreatic Islets Promote Mature Liver Hepatocyte Proliferation

Existence of the liver-trophic substances in the portal venous blood was found and the origin of these factors was from the pancreas. In light of this background, Kaufmann et al., have investigated the ability of pancreatic islets to enhance growth of mature hepatocytes. Islets and hepatocytes were harvested from Lewis rats. It was found that pancreatic islets could provide a stimulatory effect on the number of hepatocytes *in vitro*. Legrelle et al., have also shown that a higher proliferation rate of hepatocyte is detected in the presence of pancreatic islets. Besides, co-culture of pancreatic islets also enhances hepatocyte's albumin secretion rate in higher islets to hepatocytes ratio. By selective blockage of insulin and glucagons, proliferative effect on hepatocytes by islets was inhibited (Kaufmann et al., 1999).

1.5.4 Transdifferentiation

Transdifferentiation takes place when the conversion of cells from one differentiated cell type to another. Transdifferentiation involves dedifferentiation of a

committed cell back to stem cells and differentiation into new target lineage. It was believed that this cannot occur in nature. However, Tsonis group has demonstrated new lens regenerates from the dorsal iris after removal of the previous lens in newts and salamanders. (Del Rio-Tsonis et al., 1995) Some researchers also regard regeneration of β-cells from ductal that is originated in pancreas as transdifferentiation; however, this has yet to be investigated (Bouwens, 1998).

(A) Retinoic acid signalling

Retinoic signalling is one of the vital mechanisms required for both pancreas and liver development. Retinoic acid (RA) binds to retinoic receptor and heterodimerize with retinoic X receptor to activate many genes required for development. Inhibition of Retinoic signalling inhibits early development of endoderm for pancreatic and hepatic progenitors. RA signalling suppresses endodermal sonic hedgehog expression, which suppresses dorsal pancreatic development and excess RA signal can ablate liver development. However, absent of RA signal has no effects on ventral pancreas and liver development. This shows that excess RA signal has a similar negative effect on ventral pancreas and livers and more researches are required to further elucidate reasons behind (Deutsch et al., 2001).

(B) Wnt signalling family

Wnt signalling promoted liver development (Lemaigre et al., 2004) while inhibiting in pancreas development (Hebrok, 2003). Metheus mutant embryo defects in initial budding of liver but later a liver rudiment was found in the ductular region of the pancreas. Metheus mutants include a mutation in the Wnt-family and wnt2bb (Zaret, 2008).

(C) BMP & FGF family

Cardiac mesoderm BMP and FGF signalling induces local expression of sonic hedgehog and this, in turn, inhibits pancreatic specification but not liver development. Consistence with developmental studies, combined functions of endogenous BMP and supplemented FGF can induce differentiation of hepatocytes from human ESCs and shift developmental pathways from hepatic to pancreatic cell differentiation (Mfopou et al., 2010).

In light of these backgrounds, many groups have tried to differentiate pancreatic stem cells to hepatocyte and vice versa. It has reported that hepatic oval cells can be induced in vitro to form islet like cell clusters, expressing pancreatic markers and transplantation could further improve glycemic control of diabetic mice (Yang et al., 2002). Both in a pancreatic cell line, AR42J-B13, and in organ cultures of pancreatic buds from mouse embryos can be converted into hepatocytes by treatment with a synthetic glucocorticoid, dexamethasone (Shen et al., 2000).

1.5.5 Transplantation in Liver Niche Promotes Maturation of Insulin-producing Cells

Recent advances in stem cell therapy have shown that current *in vitro* induction protocol cannot promote successful islet engineering. However, it is observed that immature and unpurified islets after transplantation have attained more mature phenotype and functional. Pancreatic progenitor cells transplantation can be divided into orthotopic and heterotopic sites. The former includes pancreas, port vein region of liver, spleen and greater omentum and the latter includes subcutaneous, renal capsule and the brain.

Transplanting back into pancreas should be the best place where progenitors can be easily localized and thus, repaired injury sites. Yet, due to recurrence of autoimmune or pancreatitis, in some cases, it is not recommended to transplant in pancreas. Transplantation in other sites, say spleen and greater omentum, localization of transplanted precursors is difficult and absent of nerve fibers and less dense vascular network in islets observed. For subcutaneous, it is easily operated but immune attack on cells are severe. Transplantation in brain is not an easy task comparing to other sites.

Kidney capsule, due to its immune privileged, and portal vein of liver are hottest site for transplantation. Recent researched found that liver has an important role for

pancreatic regeneration. When comparing to kidney capsule, glycemic control after transplantation in liver has greater improvement and better response to intraperitoneal glucose challenge. Besides, there are marked differences with regard to the pattern of reinnervation of islets as nerve control is of vital importance of insulin secretion. The islets implanted into the liver contained a denser network of sympathetic nerve fibers than kidney capsule. This indicate transplant in livers promote maturation of pancreatic precursors [Chen et al., 2009; Merani et al., 2008].

1.5.6 Neuronal Relay from the Liver to Pancreatic β-cells in Regulation of β-cell Mass

β-cells proliferate significantly in obesity-related insulin resistance. However, underling mechanisms are still unknown. A group has identified a neuronal relay originate from liver enhanced insulin secretion and increase in β-cells mass. Blockage of the relay showed an inhibition in compensatory response during obesity. Obesity insulin-resistance induced hepatic selective extracellular signal-regulated kinase phosphorylation signalling cascade which activate the afferent splanchnic nerve. It transmits signals to the central nervous system and after process, the efferent vagus sends signals to control the hyperplasia in pancreas (Figure 1.14). This

suggested that an inter-organ communication between liver and pancreas existed (Imai et al., 2009; Imai et al., 2008).

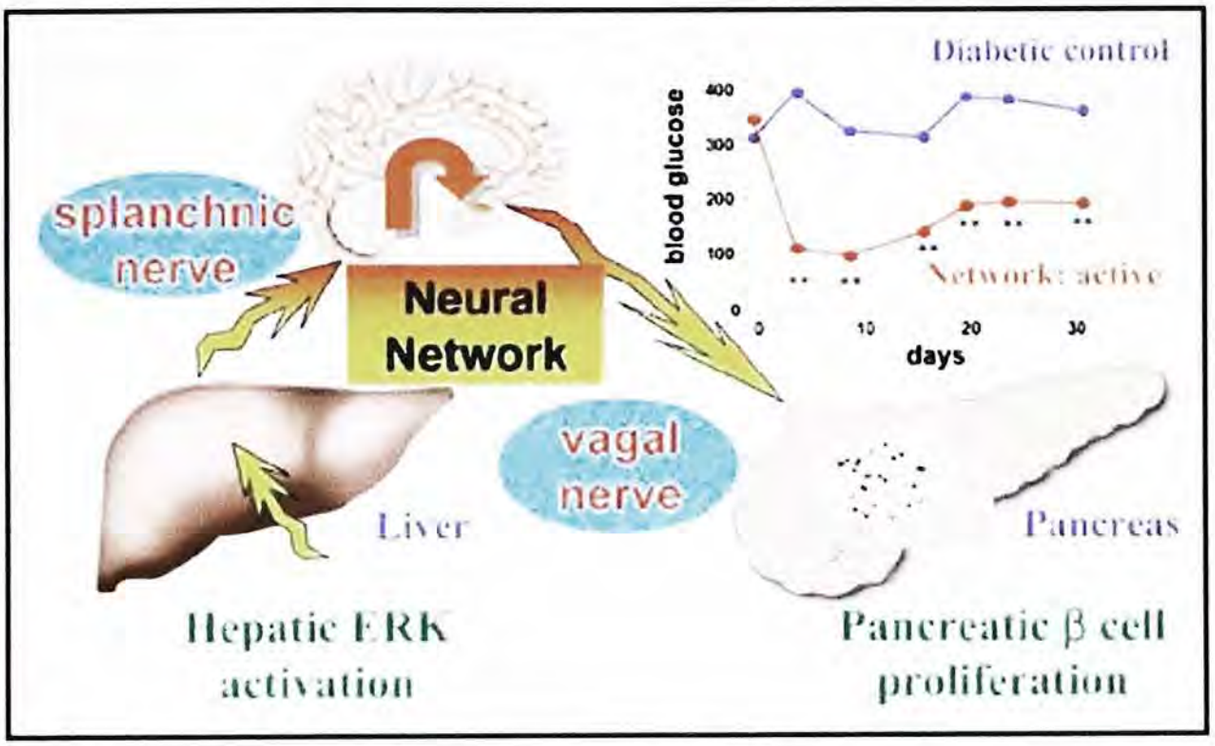

Figure 1.14 Obesity induced hepatic ERK activation promotes pancreatic β-cell proliferation via the neuronal system consisting of afferent and efferent nerves and the central nervous system.

1.5.7 Development of Islets in the *Nile Tilapia*

In Nile tilapia, *Oreochromis niloticus*, was born with a compact organ, consisting endocrine and exocrine as mammals. Yet, after birth, structures modification starts. The exocrine pancreas later becomes disseminated, extending along blood vessels through the mesenteries and into liver. This migration of the exocrine pancreas appears continue throughout the lifespan.

A single principle islet with scare scattering secondary islets around it, arise from a group of endocrine cells that differentiate in parallel with the surrounding exocrine cells in the embryo and early larval stage. A close relationship between small secondary islets and pancreatic ducts in young tilapia is reported by Agulleiro et al. group. Isolated insulin-positive cells in young tilapia are found at pancreatic duct epithelium. It is important to highlight islet development occurs in liver after exocrine migration, although interaction of developing islets with mature liver during larval stages was not described (Morrison et al., 2004).

1.6 The Insulin-like Growth Factor-I (IGF1)

1.6.1 IGF1 System

Insulin-like growth factors I (IGF1) (70 amino acid) consists of 4 domains, namely A, B, C and D with a molecular weight of approximately 7.5 kDa. Due to its structural homology with insulin, it is termed as insulin-like growth factors. There is also another family member, named Insulin-like growth factors II (IGF2), which has 70% similarity with IGF1.

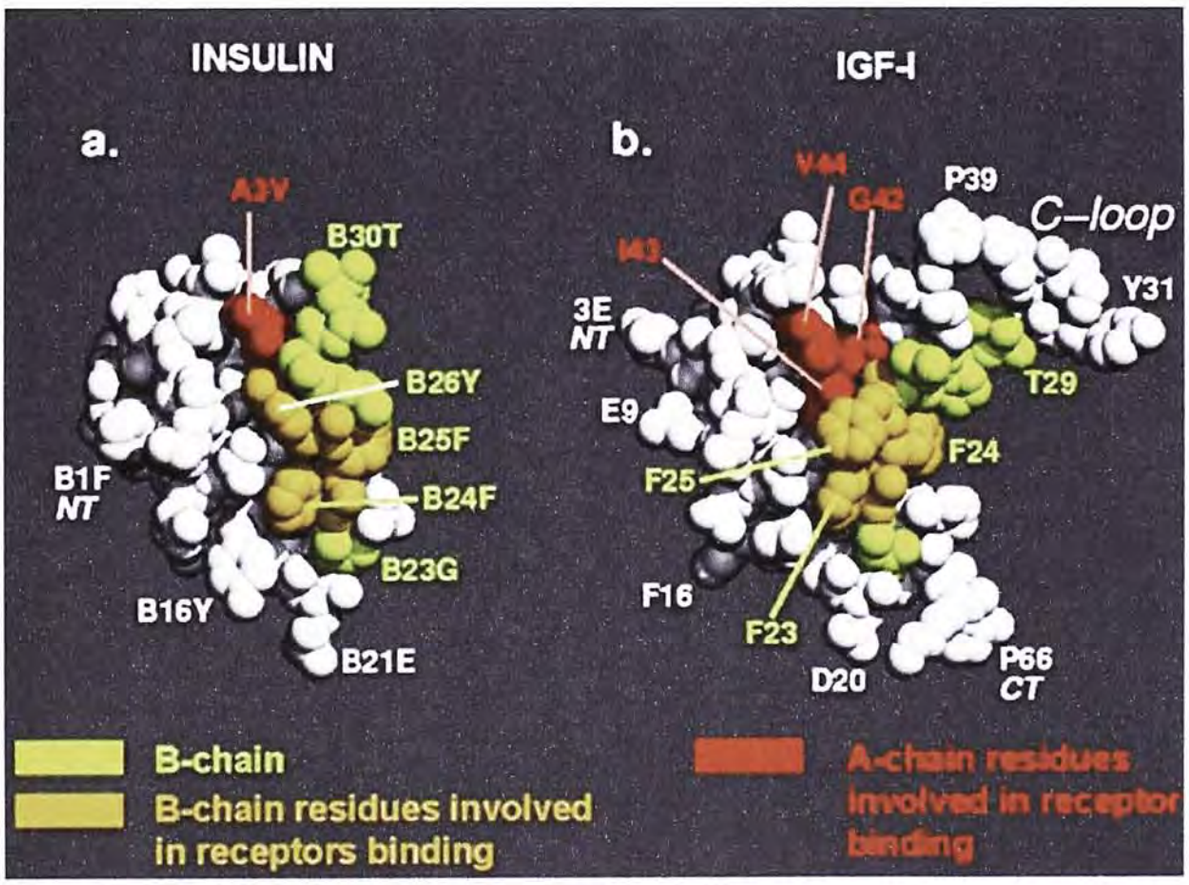

Figure 1.15 Structural hot-spots targets in human insulin and IGF-1.

Both IGF1 and IGF2 exert their biological effects via binding to the receptor insulin-like growth factor I receptor (IGF1R), a cell surface tyrosine kinase receptor. The IGF1R is a heterotetrameric glycoprotein consisting of two extracellular α-subunits and two transmembrane β-subunits linked y disulphide bond. It is 70% structurally similar to insulin receptor (IR). Binding of IGF1 to IGF1R causes autophosphorylation of the receptor and activation tyrosine kinase. It then subsequently phosphorylates other cellular component to exert its effect. Its downstream components are insulin receptor substrate proteins (IRS-1, IRS-2, IRS-3, IRS-4, IRS-5 and IRS-6), Src-homology collagen protein and growth receptor bound-2 associated binder-I, etc. In each case, they activate different cascades, namely, Akt, Ras-Erk and PIP3 etc. Sometimes, it also forms dimerization with IR and increases its affinity for IGF1 while decreasing its insulin affinity.

In contrast to IGF1R, binding to insulin-like growth factor II receptor/ mannose-6-phosphate receptor (IGF2R/M-6-PR) causes a considerable debate. In terms of structure, it is significantly different from IGF1R. It is a monomeric receptor, with no intrinsic tyrosine kinase activity. It is composed of 15 cysteine-based repeat in extracellular domain (Kulkarni, 2005; Dupont et al., 2003).

1.6.2 IGF1 Regulation

IGF1 is regulated by a system of receptors, binding proteins and related protease. Although the liver is so far identified as the major organ for IGF1 production accounting for 80% in the circular levels, recent researches have shown that many peripheral tissues, such as the pancreas, muscle and bone, also produce IGF1 in an autocrine/ paracrine manner. The secretion of IGF1 is modulated by production of Growth Hormone (GH) from anterior pituitary gland and can be retarded by under-nutrition, growth hormone insensitivity, lack of growth hormone receptors, or failures of the downstream signalling pathway post GH receptor including SHP2 and STAT5B. Besides, IGF1 in the bloodstream exerts negative feedback regulation on hypothalamus and pituitary gland, reduce GH secretion in order to reduce IGF1 production.

Approximately 98% of IGF-1 is always bound to one of 6 binding proteins (IGF-BP). IGF-1 binds to IGF to transport IGF1 in the bloodstream, cerebrospinal fluid and body fluid. They determine the bioavailability of IGF1, increase its half-life and prevent over-stimulation of cells. IGFBP3 is the predominant IGFBP and accompany with acid-labile subunit, a liver derived GH-regulated glycoprotein binds to IGF1 in a 1:1:1 molar ratio forming ternary complex. In tissues, IGFBPs interact with cellular matrix constituents or on cell membrane to regulate interaction between IGF1 and IGF1R. IGFBP proteases are mainly responsible for the catabolism of

IGFBP, which reduce affinity of IGFBP to IGF1, and thus reduce the serum level of IGF1.

Serum IGF1 level increased gradually after birth, increase slowly at puberty and eventually decrease due to aging. In embryo and fetus, the IGF1 level is approximately around 30- 50 % to adult, increase with gestational weeks. Figure 1.16 summarizes IGFs regulations insides human body. (Kulkarni, 2005; Dupont et al., 2003)

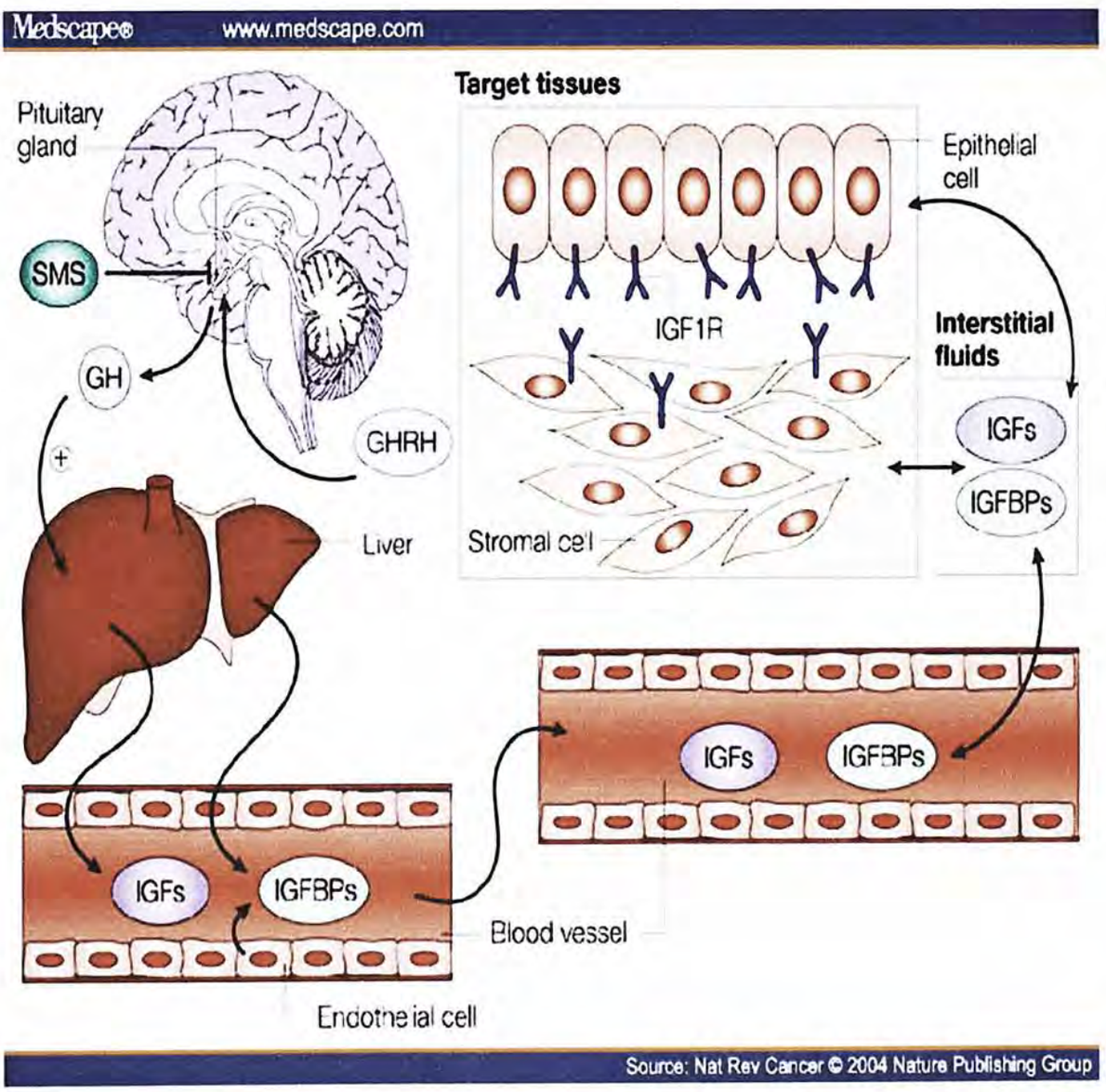

Figure 1.16 Regulation of circulating and tissue levels of insulin-like growth factors.

1.6.3 Roles of IGF1 in Pancreatic Development and Regeneration

IGF1 signal provides essential conserved signals for the control of embryonic and postnatal development in many species. Studies through transgenic and gene knock out mice have demonstrated considerable variety of tissue-specific and developmental stage-specific effects.

For IGF1 knockout mice, neonates have reduced birth weight by 40% and die shortly after birth, suggesting that IGF1 is essential for development. In IGF2 knockout mice, it also shows a reduction in weight but normal growth afterwards. It, thus, proposes that IGF2 is responsible for intrauterine growth. In double mutant of IGF1 and IGF2 mice, it affects growth more severely, indicating that they act independently to stimulate growth. In IGF1R knockout mice, severe growth retardation has been observed while IGF2R has an increase in placental mass more than 25%, which suggested that IGF1R is essential for embryo development but not IGF2R. In natural deletion of exons 4 and 5 IGF1 gene, human suffers from severe pre- and postnatal growth, developmental defects and mental retardation (Dupont et al., 2003).

The roles of IGF1 and IGF2 in development have been proposed by their expression in human and rat fetal and neonatal islets (Swenne et al., 1987; Rabinovitch et al., 1982). Exogenous addition of IGF1 promotes islet cells DNA

synthesis and overproduction of IGF1 in β-cells has resulted in islet hyperplasia. However, IGF1 and IGF1R deficient mice also resulted in pancreas hyperplasia, which may be due to direct inhibition of development and indirect effects. IGF1R deficiency in β-cells showed a decrease in expression of glucokinase and Glut2, which result in failure of glucose-induced insulin secretion and impaired glucose tolerance (Yu et al., 2003; Lu et al., 2004). It is hard to determine whether IGF1 would be important for islet development as the result may be due to direct or indirect effects of IGF1 knockout. In this regard, extra investigations are required.

On the contrary, IGF1 is consistently important for pancreas regeneration. During regeneration, IGF1 expression in pancreas is increase significantly (Smith et al., 1991). Exogenous injection of IGF1 improved regeneration and IGF1 over expression mice showed improved regeneration. Research suggested that IGF1 mediates regeneration of endocrine pancreas by increase cell replication, recruitment of stem cells for differentiation and protect islets against inflammation (Agudo et al., 2008; Warzecha et al., 2003; Hess et al., 2003). However, exact reasons are not fully understood.

1.7 Aims and Objectives of Study

In light of the literature review in the aforementioned introduction, convergent evidence has supported for potential roles of vitamin A, vitamin D and liver stroma in the development of pancreatic progenitor cells (PPCs) into islet like cell clusters (ICCs). In order to test this hypothesis, a series of novel objectives which will be adequately addressed by our established methodology in this project as described below:

1) To study the effects of vitamin A and vitamin D alone or in combination on PPC proliferation and specification.
2) To establish the novel co-culture system for liver stromal cells (LSCs) and PPC
3) To study the effects of LSC on PPC viability and PPC-derived ICC differentiation.
4) To further study some morphogenic factors identified from LSC that are responsible for the above effects.

Chapter 2

General Materials and Methods

2.1 Isolation and culture of PPCs and LSCs

2.1.1 Tissue procurement

Human fetal pancreata and livers employed in this study were approved by our Institutional Committees on Human Ethics. Maternal consent was obtained by the outpatient clinic of the Prince of Wales Hospital before Surgical Termination of Pregnancy. Ethical approval for the use of fetal tissue from the Clinical Research Ethnics Committee was obtained (CREC-2005.461). 9^{th} – 16^{th} weeks of early gestational ages from human fetal pancreata and livers were collected from surgical termination of pregnancy (STOP) by dilation and extraction. Isolation of pancreata and livers were under high power dissecting microscope and they were then rinsed under sterilized phosphate buffered saline (PBS) (Invitrogen, Carlsbad, CA) as previously described (Suen et al., 2008; Leung et al., 2009).

2.1.2 PPC and LSC culture

PPCs were isolated as previously described (Suen et al., 2008; Leung et al., 2009). In brief, pancreas was rinsed in distilled PBS and subjected to digestion by 3 mg/ml collagenase P (Roche, Mannheim, Germany) in Hanks Balanced salt solutions (HBSS) (Gibco Life Technologies, Inc., Grand Island, NY) for 5-8 mins in a vigorous shaking 37°C water bath. After digestion, HBSS was added to dilute the Collagenase

P solution. The tissues were subjected to centrifugation for 5 mins 1600 rpm. Collagenase P solution was removed and HBSS was added to rinse the digested tissues. It was then subjected to another centrifugation for 5 mins at 1600 rpm. The centrifuged pellet was resuspended in modified RPMI1640 medium (Gibco Life Technologies, Inc., Grand Island, NY) supplemented with 10% fetal bovine serum (FBS) (Gibco Life Technologies, Inc., Grand Island, NY), 20 mM HEPES (Sigma, St Louis, MO), 1 mM sodium pyruvate (Sigma, St Louis, MO), 1% penicillin/streptomycin (P/S) (Gibco Life Technologies, Inc., Grand Island, NY) and 71.5 μM β-mercaptoethanol (Sigma, St Louis, MO) in non-adherent Petri-dish (Corning Incorporated, Corning, NY).

Rounded and non-adherent cell clusters were formed after 24 hrs incubation and they were transferred to a new Petri-dish to remove attached fibroblasts and dead cells in the pertri-dish. The rounded and non-adherent cells were cultured for another 24 hrs and then transferred to adherent culture dish (Corning Incorporated, Corning, NY) with exogenous addition of 20 ng/ml basic fibroblast growth factor (bFGF) (Sigma, St Louis, MO) and epidermal growth factor (EGF) (Invitrogen, Carlsbad, CA). PPC outgrowth was induced and migrated out to form a monolayer of cells within 1 week. Upon confluence, monolayer of cells was trypsinized using 2.5% TRYPLE (Gibco

Life Technologies, Inc., Grand Island, NY) for 6 mins under 37°C and passed until 8 times at maximum (Suen et al., 2008; Leung et al., 2009).

Human fetal liver was rinsed under distilled PBS and subjected to digestion by 5 mg/ml collagenase A (Roche, Mannheim, Germany) in HBSS for 30 mins in a vigorous shaking 37°C water bath. After digestion, HBSS was added to dilute the Collagense A solution. The tissues were subjected to centrifuge for 5 mins 1600 rpm. Collagenase A solution was removed and HBSS was added to rinse the digested tissues. It was then subjected to another centrifuged for 5 mins at 1600 rpm. The remaining centrifuged pellet was resuspended in modified 1:1 DMEM/F12 medium (Gibco Life Technologies, Inc., Grand Island, NY) supplemented with 10% FBS and 1% P/S in T25 filter-capped flask (Corning Incorporated, Corning, NY). The cells attached on the flask and formed a monolayer of cells within 1 week. Upon confluence, monolayer of cells was trypsinized using 2.5% TRYPLE for 6 mins under 37°C and passed until 8 times at maximum (Lee et al., 2011).

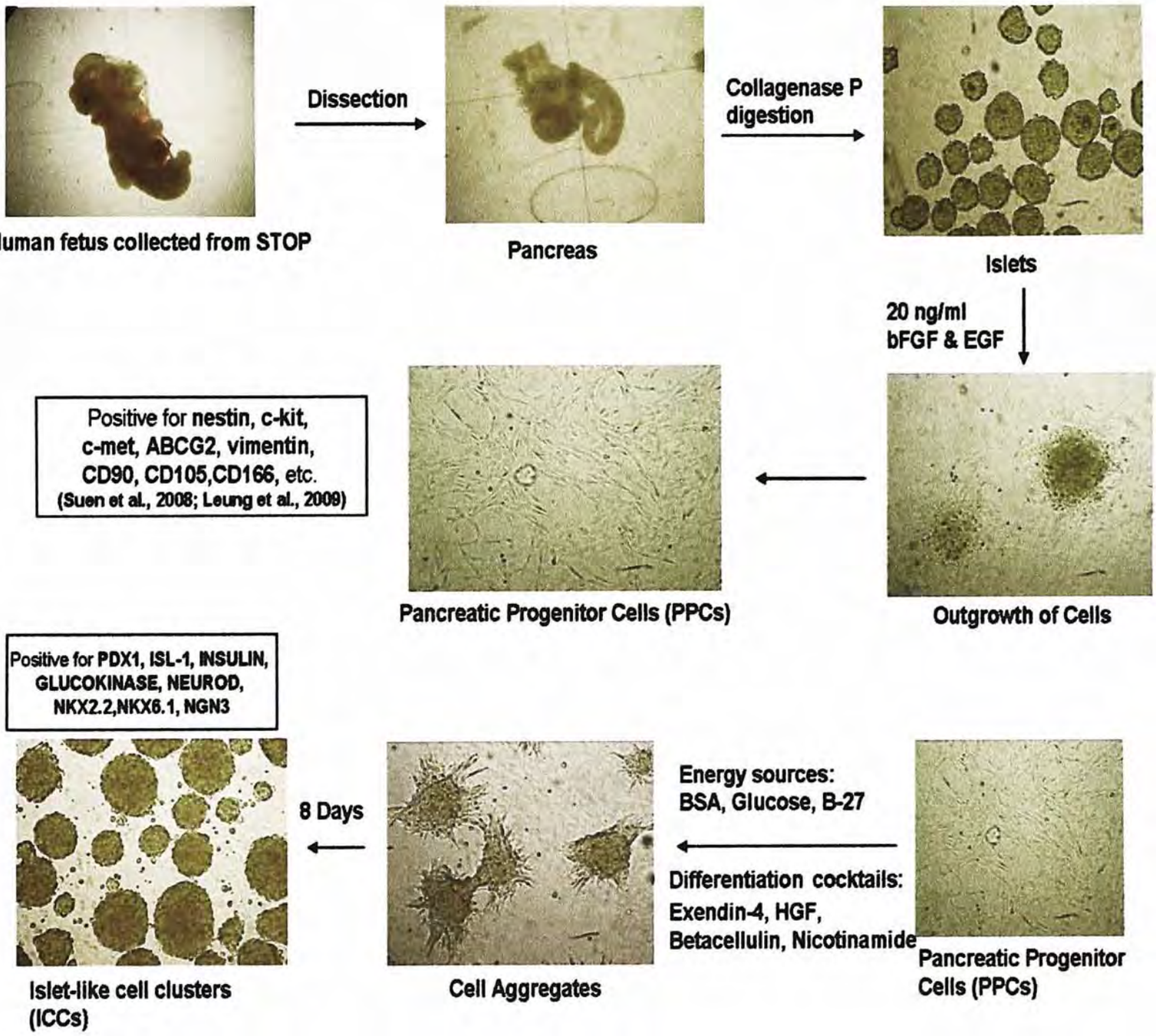

Figure 2.1 Procedures for PPC isolation and PPC-derived ICC formation.

2.1.3 Treatments of vitamin A, vitamin D and IGF1

PPCs were seeded at a concentration of 3 $x10^4$ cells in a 96-well plate (Corning Incorporated, Corning, NY) and 1.5 $x10^5$ cells in a 6-well plate (Corning Incorporated, Corning, NY). They were allowed for attachment for at least 4 hours. After attachment, they were incubated in serum free medium overnight to arrest cell cycles. Vitamin A (10^{-12} – 10^{-5} M) (Sigma, St Louis, MO), vitamin D (10^{-14} – 10^{-4} M) (Sigma, St Louis, MO) and IGF1 (1, 10, 50, 100 ng/ml) (Sigma, St Louis, MO) were added exogenously to PPC and incubated for 2 days or 3 days.

2.1.4 Culture of Caco-2, HepG2 and DU-145 cell lines

Positive controls used for verifying primers newly designed, Caco-2, HepG2 and Du-145 were cultured as followed. DU-145 cells (a gift from Dr. H. Wise, The Chinese University of Hong Kong) were grown in DMEM (Gibco Life Technologies, Inc., Grand Island, NY) supplemented with 10% FBS, 100 U/ml of penicillin G (Sigma, St Louis, MO) and 100 μg/ml streptomycin sulphate (Sigma, St Louis, MO). Caco-2 cells were grown in MEME medium (Gibco Life Technologies, Inc., Grand Island, NY) supplemented with 2 mM Glutamine (Sigma, St Louis, MO), 1% NEAA (Sigma, St Louis, MO) and 10% FBS. HepG2 were grown in DMEM medium (Gibco Life Technologies, Inc., Grand Island, NY) supplemented with 1% P/S and 10% FBS.

2.2 Induction of PPC-derived ICC Differentiation

2.2.1 *In vitro* Directed Differentiation

PPC-derived ICC differentiation was performed as reported previously (Suen et al., 2008; Leung et al., 2009). Briefly, PPCs (9^{th} to 16^{th} week gestation and 3-8 passages) were trypsinized and suspended in ultra-low attachment plate (Corning Incorporated, NY, USA) under 8 days differentiation in high glucose (11 mM) DMEM/F12 medium supplemented with 1X B27 (Gibco Life Technologies, Inc., Grand Island, NY), 0.05% bovine serum albumin (BSA), 10 ng/mL Hepatic Growth Factor (HGF) (Invitrogen, Carlsbad, CA), 10 nM Exendin-4 (Sigma, St Louis, MO), 500 pM Betacellulin (Sigma, St Louis, MO) and 10 mM Nicotinamide (Sigma, St Louis, MO). Medium was replaced every 2 days. Resultant ICCs were handpicked by micro-pipette under an inverted light microscope (Suen et al., 2008; Leung et al., 2009).

2.2.2 *In vitro* LSC Microenvironment

In the co-culture model, LSC were seeded on Transwell (Millipore Filter Corporation, Bedford, USA) and co-culture with ICC in ultra-low attachment plate, i.e. high glucose (11 mM) DMEM/F12 1:1 medium with basal energy source (1X B27, 0.05% BSA) supplemented with or without growth factors (differentiation cocktails),

i.e. 10 ng/mL HGF, 10 nM Exendin-4, 500 pM Betacellulin and 10 mM Nicotinamide. Medium was replaced every 2 days. Resultant ICCs were handpicked by micro-pipette under an inverted light microscope.

2.3 RNA Expression Detection

2.3.1 RNA isolation

Total RNA was isolated from PPCs, ICCs, LSCs, Caco-2, DU-145, HepG2 and homogenized human fetal livers, kidneys and other tissues used for positive control. 1 ml TRIzol (Invitrogen, Carlsbad, CA) was used according to manufacturer's protocols. After homogenization, samples were incubated at room temperature for 4 mins to allow complete dissociation of nucleotideprotein complexes. Chloroform was used for extraction and isopropanol was used to precipitate RNA. 75% Ethanol was used to remove organic solvent leaving behind. Finally, the resulted pellet containing RNA was dissolved in RNAase free water.

The absorbance of RNA was determined by UV-spectrophotometer (Hitachi, Japan). The value of absorbance at wavelength 260 nm obtained x 40 x dilution factor would equal to the concentration. The purity of RNA was determined by comparing ratio of O.D.280 and O.D. 260.

2.3.2 Reverse Transcription

First strand cDNA was generated from 2 μg mRNA in a 20 μl volume using SuperScript III reverse transcriptase (Invitrogen, Carlsbad, CA) with 500 μg oligo(dT)188 (Invitrogen, Carlsbad, CA), 5X RT-buffer (Invitrogen, Carlsbad, CA),

40 units/μl RNAaseOUT (Invitrogen, Carlsbad, CA), 0.1 M DTT (Invitrogen, Carlsbad, CA) and 10 mM dNTP (Invitrogen, Carlsbad, CA) according to manufacturer's protocol.

2.3.3 Polymerase Chain Reaction (PCR)

PCR and real-time PCR primers were designed using online primer 3 version 0.4.0 according to manufacturer's protocol. Each primer was tested by conventional PCR using positive control. Primers used for subsequent experiment were stated in Table 2.3

PCR was performed in 25 μl reaction volume containing 1 μl cDNA template, 1.5 mM $MgCl_2$ (Invitrogen, Carlsbad, CA), 0.2 mM dNTP, 0.3 μM forward primer (Invitrogen, Carlsbad, CA), 0.3 μM backward primer (Invitrogen, Carlsbad, CA), PCR buffer (Invitrogen, Carlsbad, CA) and *Taq* DNA polymerase (Invitrogen, Carlsbad, CA) according to manufacturer's protocol. Annealing temperature for each primer was summarized in Table 2.1.

PCR products were then electrophoresized in a 2% agarose gel in 1X Tris-Acetate-EDTA (TAE) buffer pre-stained with ethidium bromide for 20 mins at 100V. Then resulted band were visualized using UV illuminator (FluroChem8000 Advanced Fluorescence, Chemiluminescence and Visible Light Imaging, Alpha

Innotech Corporation, CA, USA). Target band sized for each primer was stated in Table 2.3.

2.3.4 Real-time PCR

Real-time PCR was performed according to manufacturer's protocol using i-Cycler Thermal Cycler (Version 3.1, Bio-Rad Laboratories Incorporation, Munich, Germany). Real-time PCR was done in triplicate, in 25 μl reaction volume containing SYBER Green PCR Mastermix (Bio-Rad, Munich, Germany), 0.3 μM forward primer and 0.3 μM backward primer (Figure 2.3). Melting curve was performed to confirm specification of the PCR product. Amplification data was collected using i-Cycler Detector and Sequence Detection System Software.

Fold changes of target genes were calculated using $2^{-\Delta\Delta CT}$ method. The ΔC_T value was calculated by subtracting C_T derived from house keeping gene (in our case, β-actin). $\Delta\Delta C_T$ was obtained by difference between treatment and control ΔC_T. It was then substituted back to $2^{-\Delta\Delta CT}$ equation to calculate the folds change.

house keeping gene	*Forward Primer*	*Backward Primer*	*Temp*	*Size*	*Reference*
h β -actin	TGGCACCACACCTTCTACAATGAGC	GCACAGCTTCTCCTTAATGTCACGC	/	/	2
h β -actin	TGTCCACCTTCCAGCAGATGT	CGGACTCGTCATACTCCTGCTT	/	/	2
r β -actin	AGCTATGAGCTGCCTGACG	GGATGCCACAGGATTCCA	/	/	5
Expression of rat gene					
rIGF-I	AAGCCTACAAAGTCAGCTCG	GGTCTTGTTTCCTGCACTTC	/	/	6
rIGF-1R	AAA ACC ATC GAT TCT GTG ACG	GGT TCT TCA GGA AGG ACA AGG	/	/	6
rNgn3	TGGCGCCTCATCCCTTGGATG	CAGTCACCTGCTTCTGCTTCG	/	/	7
Characterization of LSC					
hEp-CAM	GCTCTGAGCGAGTGAGAACC	ACGCGTTGTCATCTCCTTCT	60	116	/
hN-cadherin	CCTCCATGTGCCGGATAG	AATGCGATTTCACCAGAAGC	60	97	/
hVimentin	AAGCAGGAGTCCACTGAGTACC	GAAGGTGACGAGCCATTTCC	/	/	8
hCD45	AGCACCTACCCTGCTCAGAA	TTCAGCCTGTTCCTTTGCTT	/	/	8
hCD90	ATGAACCTGGCCATCAGCATCG	CACGAGGTGTTCTGAGCCAGCA	/	/	8
hCD105	GAAACAGTCCATTGTGACCTTCAG	GATGGCAGCTCTGTGGTGTTGACC	/	/	8
hCD166	TGATCTCCGCCACCGTCTTCAG	CTCTTTTCATCACTGATCCTTGCA	/	/	8
hAFF	GCAAAGCTGAAAATGCAGTTGA	GGAAAGTTCGGGTCCCAAAA	/	/	9
hAlbumin	CCAGGAAGACATCCTTTGC	CCTGAGCCAGAGATTTCC	/	/	9
hCK19	AGGTGGATTCCGCTCCCGGCA	ATCTTCCTGTCCCTCGAGCA	/	/	10
hCK18	TGGTCACCACACAGTCTGCT	CCAAGGCATCACCAAGATTA	/	/	10
Expression of RAR, VDR and RXR system					
hVDR	*CTCAAACGCTGTGTGGACAT*	*ACTGTCCTTCAAGGCCTCCT*	*60*	*117*	/
hRAR α	*GACCAGATCACCCTCCTCAA*	*GTCCGAGAAGGTCATGGTGT*	*60*	*99*	/
hRXR α	*CTGCTCATGGCCTCCTTCT*	*ACACAAGCTCCGTCAGCAC*	*60*	*142*	/
hCYP24	*GAAACCAGGGGAAGTGATGA*	*AAGGACCATTTGTTCAGTTCG*	*60*	*135*	/
hCYP26	*CCAGAAAGTGCGAGAAGAGC*	*GGGATTCAGTCGAAGGGTCT*	*60*	*136*	/
Expression of soluble factors					
hHGF	CGAGGCCATGGTGCTATACT	TTCTCCTTGACCTTGGATGC	60	134	/
hVEGFA	CCTGCAAAAACACAGACTCG	GAGAGATCTCGTTCCCGAAA	60	137	/
hGF1	GGAGGCTGGAGATGTATTGC	GCCAACCTTTCCTTCTCTGA	60	150	/
hGF2	CGTTGAGGAGTGCTGTTTCC	GGGGTATCTGGGGAAGTTGT	60	130	/
hSCF	ACACCACTGTTTGTGCTGGA	GATTCCTGCAGATCCCTTCA	60	134	/
hGLP-1	CGTTCCCTTCAAGACACAGAG	GTGAATGTGCCCTGTGAATG	62	125	/
hPro-BTC	TGGGAATTCCACCAGAAGTC	CCTTTCCGCTTTGATTGTGT	59	96	/
hReg 4	AGGAGGAAGATGGCTTCCAG	GGTAAAACCATCCAGGAGCA	62	118	/
hFGF1	GGGCTTTATACGGCTCACA	CTTGAGGCCAACAAACCAAT	60	126	/
hFGF2	TGGCTATGAAGGAAGATGGAA	ACTGCCCAGTTCGTTTCAGT	60	150	/
hFGF7	CAGGCAGACAACAGACATGG	CAGTTGCTGTGACGCTGTTT	60	87	/
hNGF	AGTCGTCGTGCAGTCCAAG	GTGGCCAGGATAGAAAGCTG	60	108	/
hGastrin	CCAGCCTCTCATCATCGAA	TCCATCCATCCATAGGCTTC	62	119	/
hPTHrP	GCTCAAGACACCTGGGAAGA	GGAGGTGTCAGACAGGTGGT	62	145	/
hFNg	TTCAGCTCTGCATCGTTTTG	TCCGCTACATCTGAATGACCT	60	112	/
hIL6	GGCTGAAAAAGATGGATGCT	GCTCTGGCTTGTTCCTCACT	60	141	/
hFGF21	ACCTGGAGATCAGGGAGGAT	GCACAGGAACCTGGATGTCT	59	131	/
hTGFa	CCCTGGCTCTCCTTATCATC	CTGTTTCTGAGTGGCAGCAA	59	147	/
hTGFb1	GCAGGGATAACACACTGCAA	GGCCATGAGAAGCAGGAA	60	107	/
hTGFb2	ACAACACCCTCTGGCTCAGT	CTCCATTGCTGAGACGTCAA	60	135	/
hTGFb3	AGGAGATGCATGGGGAGAG	TTTAGGGCAGACAGCCAGTT	60	134	/
hTNFa	TCAGCCTCTTCTCCTCCTG	GCCAGAGGGCTGATTAGAGA	60	124	/
hTNFb	AACCTGCTGCTCACCTCATT	TGCTCAAGGAGAAACCATCC	60	102	/
h3MP2	CGCAGCTTCCACCATGAAGAA	CCTGAAGCTCTGCTCAGGTGATA	/	/	11
h3MP4	AGGAGCTTCCACCACGAAGAAC	TGGAAGCCCCTTTCCCAATCAG	/	/	12
h3MP6	GTGAACCTGGTGGAGTACGACAA	AGGTCAGAGTCTCTGTGCTGATG	/	/	12
h3MP5	TTCTTCAAGGCGAGTGAGGT	AGGCTTTGGTACGTCGTCAG	/	/	13
h3MP7	CAGAGCATCAACCCCAAGTT	AGGATGACGTTGGAGCTGTC	/	/	13
h3MP9	TGTACAACAGGTACACGTCCG	TGAATGTCCTGGGACACCAG	/	/	
Characterization of PPC					
hGFR1	AGGATGCACCATCTTCAAGG	AATGCCGGATCTTCACGTAG	60	125	/
hGFR2	GGCCCTGTTGCTCTACAAGA	CCATCAGCCACTCTCTTTCA	60	149	/
Developmental markers of ICC					
hPDX1	ACTCCACCTTGGGACCTGTTT	TTAAGGTACTCGGCCCAGCTT	/	/	2
hNGN3	TGTGGGTGCTAAGGGTAAGG	GGGAGAAGCAGAAGGAACAA	/	/	2
hNEUROD	TCCAAAATCGAGACTCTGCGC	GCAAAGCGTCTGAACGAAGGA	/	/	2
hISL-1	GATCAAATGCGCCAAGTGCAG	CAGCGGAAACACTCGATGTGA	/	/	2
hNKX2.2	TCTCCTTGGAGTGGCAGATTC	AAACACGGCGTAGAGTTCAGC	/	/	2
hNKX6.1	GACGGGAAGAGAAAACACACG	ACTCTCTGTCATCCCCAACGA	/	/	2
hGLUCOKINASE	CTCCATGGGGAAGTGCTC	CACCGAAAAACTGAGGGAAG	/	/	2
hINSULIN	CAGCCTTTGTGAACCAACAC	GGTCTTGGGTGTGTAGAAGAAGC	/	/	2

Table 2.1 Sequence of the specific PCR primers and their expected product size of different target genes.

2.4 Immunocytochemistry

LSC and PPC monolayers were cultured on coverslips coated with 3% gelatine (Sigma, St Louis, MO) in PBS and fixed with 4% paraformaldehyde (Sigma, St Louis, MO) in PBS for 60 mins at room temperature. Samples were permeabilized with 0.01% Triton-X (Sigma, St Louis, MO) in 1% BSA/PBS for 5 mins at room temperature, then blocked with 6% normal donkey serum (Invitrogen, Carlsbad, CA) in 1% BSA/PBS at room temperature for 1 hour and then incubated with primary antibodies (Table 2.2) at 4°C overnight. Samples were then washed with PBS several times and incubated with Alexa Fluor 568 donkey anti-rabbit antibodies (1:500) (Invitrogen, Carlsbad, CA), FITC-conjugated anti-mouse (1:200) or anti-goat (1:300) secondary antibody (Jackson ImmunoResearch, PA, USA) at room temperature for 1 hr and 4',6'-Diamidino-2-phenylindole (DAPI) (1:1000) (Invitrogen, Carlsbad, CA) for 15 mins. Omission of primary antibody was used as negative control. The slips were washed thoroughly with PBS several times, mounted in Vectashield and imaged using a fluorescence microscope equipped with a DC 200 Digital camera (Leica Microsystems, Buffalo Grove, IL). Different images were captured separately with a 580 nm long-pass emission filter (Alexa 568), FITC band pass 520 – 560 nm barrier filter and excitation filter 360–370 nm (DAPI). Afterwards, images were overlapped using image software, Adobe Photoshop CS2 (Adobe Systems, San Jose, CA).

Primary antibody	Dilution	Host Species	Brand
RAR	1:100	Rabbit	Santa Cruz Biotechnology
VDR	1:100	Rabbit	Santa Cruz Biotechnology
RXR	1:100	Rabbit	Santa Cruz Biotechnology
IGF1R	1:25	Rabbit	Santa Cruz Biotechnology
AFP	1:100	Mouse	Santa Cruz Biotechnology
Albumin	1:100	Goat	Santa Cruz Biotechnology
Dlk	1:500	Rabbit	Abcam
CD29	1:100	Rabbit	Abcam
CD90	1:100	Mouse	eBioscience

Table 2.2 Antibodies used in the immunocytochemistry.

2.5 Western Blotting

2.5.1 Protein extraction and quantification

Cell lysates from PPCs were obtained using Cytobuster Protein Extraction Reagent (Novagen, Darmstady, Germany) according to manufacturer's protocol. Cells were incubated with Cytobuster in room temperature for 15 mins and subjected to centrifuge at 16,000 g for 15 mins at 4°C. Protein concentrations were determined using Bio-Rad Protein Bradford Kit (Bio-Rad, Munich, Germany) according to manufacturer's protocol. 2 μl (5X dilution or 10X dilution) samples were added to 198 μl Bradford solution. Resulted absorbance was read at 595 nm with a spectrometer. Relative concentration was determined comparing to 0.1 – 0.5 mg/ml BSA standard curve.

2.5.2 Western Blotting

Western blotting was performed according to manufacturer's protocol with minor modifications. Laemmli Buffer (Bio-Rad, Munich, Germany) containing 5% β-mercaptoethanol was added to protein samples and denatured at 100°C for 5 mins. 15 mg protein mixture was added to each lane and electrophoresized in a 12% SDS-polyacrylamide gel at 100V for 2 hrs. It was then electrotransferred to a nitrocellulose membrane (Bio-Rad, Munich, Germany) by a Semi-Dry Transfer

System at 17V for 1 hr. The membrane was blotted by using 5% skim milk in 0.1% Tween20/PBS (PBST) at room temperature for 1.5 hrs. It was then incubated with primary antibody (Table 2.3) in 1% BSA/PBST overnight at 4°C, followed by peroxidase-labelled sheep anti-rabbit antibody or rabbit anti-mouse antibody (Abcam, Cambridge, UK) in 1% BSA/PBST. After throughout washing, bands were visualized on autography film using Enhanced Chemiluminescent (ECL) and Western Blot Detection Reagents mixtures (Amersham Pharmacia Biotechnology, Buckinghamshire, UK). Intensity of the bands was quantified using an image analyzer.

Primary antibody	**Dilution**	**Host Species**	**Brand**
RAR	1:300	Rabbit	Santa Cruz Biotechnology
VDR	1:1200	Rabbit	Santa Cruz Biotechnology
RXR	1:500	Rabbit	Santa Cruz Biotechnology
IGF1R	1:100	Rabbit	Santa Cruz Biotechnology
β-actin	1:8000	Mouse	Abcam
Total Smad	1:1000	Mouse	Cell Signalling
Phospho-smad1,5,8	1:1000	Mouse	Cell Signalling

Table 2.3 Antibodies used for Western blotting.

2.6 Enzyme-linked Immunosorbent Assay (ELISA)

2.6.1 Detection of cell viability

PPC viability was measured using 3-(4,5-Dimethylthiazol-2-yl) -2,5-diphenyltetrazolium bromide (MTT) ELISA (Sigma, St Louis, MO). Reductions of MTT in mitochondria took place only when reductase enzymes were active, and therefore conversion was often used as a measurement of viable cells.

PPCs were seeded in 96-well plate with a concentration of 3 $x10^4$ cells. Cell cycle were arrested by serum-free overnight. Exogenous atRA, calcitriol or IGF1 were added and incubated for 3 days. The measurement was done according to manufacturer's protocol. In brief, addition of MTT solution and incubated for 2-4 hrs at 37°C under 5% CO_2. MTT solution was removed and DMSO (Sigma, St Louis, MO) was added to dissolve the purple formazan formed. The absorbance of each well was measured at 540 nm using a MicroKinetics plate reader (PerkinElmer, CA, USA).

2.6.2 Detection of cell proliferation

PPC proliferation was measured by detecting the DNA replication using 5-bromo-2'-deoxyuridine (BrdU) ELISA (Amersham Biosciences, UK). BrdU was incorporated into newly syntheisized DNA of cells.

PPCs were seeded in 96-well plate with a concentration of 3 $x10^4$ cells. Cell cycle was arrested by serum-free overnight. Exogenous atRA and/or calcitriol was added and incubated for 3 days. BrdU incorporation assay was performed according to the manufacturer's instructions. Briefly, cells were incubated with BrdU solution for 4 h at 37°C under 5% CO_2. Cells were fixed by for 30 min and subsequently treated with blocking reagent for 30 min. After removal of the blocking solution, the cells were incubated with peroxidase-labeled monoclonal mouse anti-BrdU for 120 mins. TMB substrate was added to each well and incubated until color change. It was then stopped by the addition of 25 µl 1 M sulphuric acid. The absorbance was measured at 450 nm (ref. 490 nm) using a MicroKinetics plate reader.

2.6.3 Measurement of cell death

Cell death was characterized by membrane blebbing and cytoplasmic condensation and resultant Mono- and oligonucleosomes in cytoplasmic fractions were detected.

PPCs were seeded in 96-well plate with a concentration of 3 $x10^4$ cells. Cell cycle was arrested by serum-free overnight. Exogenous atRA and/or calcitriol were added and incubated for 3 days in medium not supplemented with FBS, which can induce cell apoptosis by nutrient deprivation. Enzyme-linked immunosorbent cell

death assays (Roche, Mannheim, Germany) were performed to assess cell death according to the manufacturer's instructions. Briefly, cells were lysed and lysates were centrifuged at 200 × g for 10 min. The supernatants were collected, added to the strip, immuno-reagent was added and shaken at 300 rpm for 2 h. before the solutions were removed by suction and rinsed with incubation buffer ×3. ABTS solution was added and upon color development, ABTS stop solution was added. The absorbance was then measured at 450 nm (ref. 490 nm) using a MicroKinetics plate reader.

2.6.4 Measurement of IGF1 levels in condition medium

LSC was cultured under DMEM/F12 1:1 medium supplemented with 1X B27, 0.05% BSA in high glucose (11 mM) and IGF1 assay (Ray Biotech, Inc., Norcross, GA) was performed according to the manufacture's protocol. The medium collected was immediately chilled in ice and added to wells overnight at 4°C. The following procedures were performed under room temperature. Solution was discarded and washed with Wash Solution. Biotin antibody provided were added to each well and incubated for 1 hr. Solution was discarded and washed with Wash Solution. Streptavidin solution was added to each well and incubated for 45 mins. TMB One-Step Substrate Reagent was added and incubated for 30 mins. Stop solution was

added and absorbance was then measured at 540 nm using a MicroKinetics plate reader.

2.6.5 Measurement of glucose-induced insulin secretion

To measure glucose-stimulated insulin secretion (GSIS) and potassium-stimulated insulin secretion (KSIS), handpicked ICCs with similar size were rinsed by PBS and incubated in Krebs-Ringer bicarbonate buffer supplemented with 10 mM HEPES and 0.1% BSA for 90 mins. It was then subjected to low concentrations of glucose (2 mM) or potassium (2 mM) before resultant solution was collected. Afterwards, it was subjected to high concentrations of glucose (25 mM) or potassium (25 mM) challenge before resultant solution was collected.

The collected solution was measured using human ultrasensitive Insulin ELISA (Mercodia, Uppsala, Sweden). It was performed according to the manufacture's protocol under room temperature. The collected solution and Enzyme-conjugate Solution were added to each well and incubated for 1 hr. The solution was discarded and washed with Wash Solution. Substrate TMB was added into each well and incubated for 30 mins. Stop Solution was added and absorbance was measured at 450 nm using a MicroKinetics plate reader.

To normalize the insulin produced, ICCs were then lysed by Cytobuster Protein Extraction Reagent and total protein was quantified by Bradford Assay.

2.7 Regeneration model

2.7.1 Regeneration model of neonatal-STZ rat

Work on animal tissues was approved by Institutional Committees on Animal Ethics. Pregnant Sprague-Dawley rats (time pregnancy 17 days) were obtained and caged individually with free access to standard diet and water. They were checked regularly from 0900 to 1800 daily for delivery of pups. One-day-old neonates were injected intraperitoneal with 100 μg/g body wt of streptozotocin (STZ) freshly dissolved in 0.05 mmol/l citrate buffer (pH 4.5) (Li et al., 2004). Blood glucose of all neonates after STZ treatment was measured by glucometer (Bayer Corp., Tarrytown, NY). Only those with blood glucose ranged 11.1 mM – 19.44 mM after the day of STZ treatment were chosen for subsequent experiment. To assess whether differentiation was involved in pancreas regeneration, NGN3 mRNA expression in whole pancreas was investigated.

2.7.2 Change in IGF1 expression in pancreas and liver

To investigate whether liver-production and local-production IGF1 were involved in pancreatic regeneration, whole pancreata and livers were homogenized in TRIzol. Extracted RNAs were subjected to reverse-transcription and real-time PCR in order to analyze the change in expression levels.

2.8 Statistical Data Analysis

Data were expressed as means ± SEM for all experimental groups. Comparison probabilities (P values for chance differences between experimental groups) were made using Student's independent two-tailed t-test. Multiple comparisons between groups were performed using analyses of variance (ANOVAs) followed by Tukey's post hoc tests. For all comparisons, $P < 0.05$ was considered statistically significant. For quantitative real-time RT-PCR, relative expression was normalized to β-actin and calculated using the comparative CT method; the fold change was defined as $2^{-\Delta\Delta Ct}$. Statistical analyses and graphics design were performed using GraphPad Prism 5 (GraphPad Software, San Diego, CA, USA).

Chapter 3

Vitamin D and vitamin A receptor expression and the proliferative effects of ligand activation of these receptors on the development of pancreatic progenitor cells derived from human fetal pancreas.

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3.1 Abstract

The growth and development of pancreatic islet cells are regulated by various morphogens. Vitamin A modulates in vitro differentiation of islet cells and vitamin D affects beta-cell insulin secretion, while both vitamin ligands act through heterodimerization with the retinoid X receptor (RXR). However, their effects in modulating pancreatic development have not been determined. In this study, cultured human pancreatic progenitor cells (PPCs) isolated from human fetal pancreas were stimulated to differentiate into islet-like cell clusters (ICCs). RT-PCR, Western blotting and immunocytochemistry were used to examine the expression and localization of vitamin D receptor (VDR), retinoic acid receptor (RAR), and RXR in PPCs. The effects of added all-trans retinoic acid (atRA, a form of vitamin A), calcitriol (activated vitamin D) and of these ligands together on PPC cell viability, proliferation and apoptosis were assessed by MTT, BrdU and ELISA assays, respectively. Post-treatment neurogenin-3 (NGN3) expression, necessary for islet-cell lineage development, was examined by real-time RT-PCR. Results showed that RAR, RXR and VDR were expressed in PPCs. RAR and RXR were localized in nuclei, and the VDR in nuclei, cytoplasm and plasma membrane. atRA and calcitriol each increased PPC viability and proliferation; atRA additionally decreased PPC apoptosis. Co-addition of atRA and calcitriol had no additive effects on cell viability but did

increase ngn3 responses. In conclusion, RAR, RXR and VDR are expressed in human fetal PPCs and PPC proliferation can be promoted by calcitriol, atRA or both together, data valuable for elucidating mechanisms underlying islet development and for developing clinical islet transplantation.

3.2 Introduction

Type 1 diabetes mellitus (T1DM) is a severe disease with major acute complications such as hyperglycemia and ketoacidosis and chronic complications, leading to blindness, renal failure and cardiovascular disease (Kitabchi et al., 2009; Ovalle et al., 2008; Simó et al., 2009; Marshall et al., 2006) that increasingly affects human populations world-wide. It is characterized by absolute deficiency of insulin secretion following autoimmune destruction of pancreatic islet beta-cells leading to loss of glucose homeostasis (Kitabchi et al., 2009; Marshall et al., 2006). Current treatments such as administration of exogenous insulin and dietary regimens focus on lowering blood glucose (Simó et al., 2009; Renner et al., 1990; Girish et al., 2006). These approaches do not, however, restore glucose homeostasis physiologically nor alter the disease state. Transplantation of a complete vascularized pancreas or of pancreatic islets provides more promising approaches to long-lasting and effective treatment. Despite active organ donation programs, an insufficient supply of compatible donor pancreata remains a problem (Lerner et al., 2008).

Biological research on stem and islet cells has led to the development of experimental islet cell replacement using harvested islets and efforts to solve this problem using islets grown from stem cells therapy. The signals necessary for effective promotion of islet beta-cell development must be identified for stem cells

therapy to become an effective treatment, or even a cure, for T1DM and a treatment option for type 2 diabetes mellitus (T2DM) (Kodama et al., 2004; Evans-Molina et al., 2009). Our laboratory has successfully isolated and characterized a population of fetal pancreatic progenitor cell (PPC) model from first trimester human fetal pancreas using a basic protocol developed for differentiating PPCs into insulin-secreting islet-like cell clusters (ICCs) (Suen et al., 2008; Leung et al., 2009). Further identification of all the factors necessary for islet beta cell differentiation is required before translation of this work into clinical practice can be achieved.

All-trans retinoic acid (atRA), a vitamin A derivative, is well known for its importance in mammalian development (Niederreither et al., 2008; Cheung et al., 2003; Wohl et al., 1998). It supports pancreas axial patterning and formation during early embryonic development (Niederreither et al., 2008; Oström et al., 2008; Stafford et al., 2003; Alexa et al., 2009; Shi et al., 2005). The RA synthesizing enzyme Radlh1 is present in the human fetal pancreas during the stage at which the main generation of beta-cells takes place (Oström et al., 2008). Given that Radlh1 is involved in the tissue-specific patterning of RA (Niederreither et al., 2008), this finding suggests that RA, at appropriate concentrations, may play a role in the development of mature pancreatic beta-cells (Oström et al., 2008). We therefore set out to examine the biological function of different concentrations of RA in our PPC system and whether

the retinoic acid receptor (RAR), necessary for RA effectiveness [12], was present during PPC differentiation.

In addition, the active hormonal form of vitamin D, 1,25-dihydroxycholecalciferol (1,25-(OH)2D3; calcitriol) (Feldman, 2005; Carlberg et al., 2007; Kochupillai et al., 2008) promotes insulin secretion and release from healthy mature beta-cells (Lee et al., 1994; Norman et al., 1980; Tanaka et al., 1984; Nyomba et al., 1984; Bourlon et al., 1999). Calcitriol exerts this effect on glucose induced secretion of insulin but has little effect on serum insulin concentrations during fasting (Cade et al., 1987). Calcitriol is also involved in the development of certain stem cells (Alon et al., 2002), such as keratinocytes (Cianferotti et al., 2007) and the vitamin D receptor (VDR), provide effector pathways for genomic and the more rapid non-genomic effects of calcitriol through the nuclear and plasma cell membrane caveolar VDRs respectively since VDRs are located in both these sites in islet beta cells. (Feldman, 2005; Carlberg et al., 2007; Kochupillai et al., 2008)

Vitamin A and vitamin D are fat soluble vitamins whose active metabolites, atRA and calcitriol, can pass through the cell membrane on carrier proteins potentiated by factors such as megalin and cubulin to bind to their receptors. The nuclear VDR and the RAR are members of the nuclear receptor superfamily which modulate transcriptional regulation of many body functions, including reproduction

and development as well as many aspects of metabolism (Niederreither et al., 2008; Feldman D et al., 2005). atRA and calcitriol act synergistically through RXR:VDR heterodimers to effect many of vitamin D's known effects; they also interact to inhibit the proliferation of breast (MCF-7) and ovarian cancer (NIH:OVCAR3) (Saunders et al., 1995) and promote the differentiation of human promyeloid leukemia cells (HL-60) into monocytes (Brown et al., 1994). The RAR and VDR can each heterodimerize with the retinoid X receptor (RXR), accounting for further interactions of the atRA and calcitriol signaling pathways (Niederreither et al., 2008; Feldman et al., 2005; Popadic et al., 2008; Wang et al., 2000; De Vos et al., 1997) and also of the cytochrome P450 superfamily enzymes CYP26 and CYP24 Niederreither et al., 2008; Feldman et al., 2005; Lou et al., 2005). AtRA also down-regulates cellular levels of CYP24 while enhancing the actions of calcitriol (Lou et al., 2005).

Given these potential interacting pathways, experimental co-treatment with both atRA and calcitriol or of whole animals with vitamins A and D, can lead to additive, synergistic or even antagonistic effects. In order to develop clinically applicable stem cell therapy we aimed to characterize the expression and location of the RAR, RXR and VDR, the effects of atRA and calcitriol, individually and in combination on PPC proliferation and maturation.

3.3 Materials and Methods

3.3.1 Fetal Tissue Procurement

The human fetal pancreata used in these experiments were obtained by the Department of Obstetrics and Gynecology in the Prince of Wales Hospital at The Chinese University of Hong Kong (Suen et al., 2008; Leung et al., 2009) following surgical termination of pregnancy (STOP) by dilation and extraction at 9–15 weeks gestation, with informed consent. Ethical approval for the use of fetal tissue was obtained from the Clinical Research Ethnics Committee (CREC-2005.461).

3.3.2 Culture of Pancreatic Progenitor Cells

Each experiment was performed using preparations from a single isolated fetal pancreas for pancreatic progenitor cell preparation, as previously described (Suen et al., 2008; Leung et al., 2009). In brief, the pancreas was immersed in chilled RPMI 1640 medium and then minced and digested in 3 mg/ml collagenase P (Roche, Mannheim, Germany) at 37°C for 5 min. before being resuspended in modified RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS), 10 mM HEPES buffer, 1 mM sodium pyruvate (Invitrogen, Carlsbad, CA, USA), 100 U/ml of penicillin G, 100 μg/ml of streptomycin sulphate (Gibco Life Technologies, CA, USA), and 71.5 μM betamercaptoethanol (Sigma Aldrich, St. Louis, MO, USA) in 60

mm culture dishes (Corning Incorporated, NY, USA) at 37°C for 48 h. Rounded, non-adhesive cell clusters were obtained, transferred to a new culture dish, for incubation for 48 h to remove fibroblasts. Themedia were changed with 10 ml modified RPMI 1640 medium containing 8 ng/ml basic fibroblast growth factor (bFGF) (Sigma) and 10 ng/ml epidermal growth factor (EGF) (Invitrogen) in a T75 flask to induce cell outgrowth. After 24 h, monolayers of cells migrated out from the cell clusters, taking about 3 days to reach 90% confluence, the media being renewed daily to support the cell growth. Upon reaching 90% confluence, the cell monolayer was harvested with TRYPLE (Gibco Life Technologies) and the cells replated for up to 20 passages; passages 3–10 being used for these experiments. DU-145 cells (a gift from Dr. H.

Wise, The Chinese University of Hong Kong) were grown in DMEM supplemented with 10% FBS, 100 U/ml of penicillin G and 100 μg/ml streptomycin sulfate. Caco-2 cells were grown in MEME medium (Sigma) supplemented with 2 mM Glutamine, 1% NEAA and 10% FBS.

3.3.3 RNA Expression Analysis by Reverse Transcription-Polymerase Chain Reaction (RT-PCR)

Total RNA was extracted from PPCs using Trizol (Invitrogen) according to the manufacturer's protocol. As previously described in our laboratory (Suen et al., 2008; Leung et al., 2009), first strand cDNA was reverse transcribed using Superscript III (Invitrogen) from5 μg RNA in a reaction volume of 15 μl. Each PCR was performed in a 25-μl reaction volume with 1 μl cDNA. Quantitative real-time PCR analyses of different genes were performed using i-Cycler Thermal Cycle (version 3.1) (Bio-Rad Laboratory Inc., Munich, Germany) (see Table 1 for specific annealing temperatures). The reactions were performed in triplicate in 25 μl volumes with 1 μl cDNA, SYBR Green PCR Master Mix (Bio-Rad Laboratory Inc.), and 0.3 μM of each primer (Invitrogen). Melt-curve data through temperature gradients (+0.5°C every 10 s) were analyzed at the end of each RT-PCR experiment to guarantee the specificity of each amplified target PCR product. Amplification data were collected using an i-Cycler Detector and then analyzed using Sequence Detection System Software (Biorad Inc., Hercules, CA). Transcript levels relative to those of β-actin were calculated using the 2-ΔΔCt statistical method (see "Statistical data analysis"). The primer sequences employed in this study are shown in Table 3.1.

3.3.4 Western Blot Analysis

Total proteins were isolated from the cultured PPCs using CytoBuster™ Protein Extraction Reagent (Novagen, Darmstadt, Germany) according to the manufacturer's protocol and our laboratory (Suen et al., 2008; Leung et al., 2009). In brief, the CytoBuster™Protein Extraction Reagent was added to the supernatant fromthe PPC lysate and incubated for 10–15min at roomtemperature before centrifugation at 16000g for 15 min at 4°C to remove the cellular debris. Supernatant lysate protein content was then quantified using Bio-Rad protein assay reagents (Bio-Rad Laboratory Inc.) using aliquots containing 15–30 μg/lane for electrophoresis on 12% (wt/vol) polyacrylamide gel and

electrotransferred to polyvinylidene fluoride transfer membrane. (GE Osmonics Labstore,Minnetonka,MN, USA). Nonspecific proteins were blocked by incubation in 5% (wt/vol) skim milk in phosphate-buffered saline (PBS; Invitrogen) with 0.1% Tween-20 in PBS (PBS-T) for 1 h at room temperature. After rinsing twice with PBS-T the membranes were incubated overnight at 4°C in PBS-T containing primary antibodies (Santa Cruz Biotech., Santa Cruz, CA, USA.) to the proteins of interest: rabbit anti-RAR antibody (1:300), anti-RXR antibody (1:500), anti-VDR antibody (1:1200) and mouse anti-actin antibody (1:8000). After the membranes had been re-washed twice, they were incubated in PBST with secondary anti-rabbit (1:3700) or anti-mouse (1:5000) antibodies at room temperature for 1 h. Positive bands were

detected using enhanced chemoluminescense (ECL) and western blotting detection reagents (Amersham, Buckinghamshire, UK) followed by membrane exposure to X-ray film (Amersham, Buckinghamshire, UK). Protein band intensities were then quantified using image analyzer (Molecular Dynamics Image Quant, Sunnyvale, CA, USA).

3.3.5 Immunocytochemistry

PPC monolayers were cultured on coverslips coated with 3% gelatin in PBS and fixed with 4% paraformaldehyde in PBS for 30 min at room temperature. The samples were then permeabilized with 0.01% Triton-X in 1% bovine serum albumin (BSA)/PBS for 5 min at room temperature, before being blocked with 6% normal donkey serum in 1% BSA/PBS at room temperature for 1 h before being incubated with primary rabbit anti-RAR (1:100), anti-RXR (1:100) or anti-VDR (1:100) antibodies (Santa Cruz Biotech.), at 4°C overnight, as described previously (10, 11). The samples were then washed with PBS several times and incubated with Alexa Fluor 568 donkey anti-rabbit antibodies (1:500) at room temperature for 1 h and 4´,6´-diamidino-2-phenylindole (DAPI) (1:1000) nuclear counterstain for 15 min. Omission of primary antibodies was used as a negative control. The slips were then washed thoroughly with PBS several times, mounted in Vectashield, and imaged

using a fluorescence microscope equipped with a DC 200 digital camera. (Leica Microsystems, German) Multiple images were captured separately at different laser wavelengths before being merged.

3.3.6 PPC Proliferation Assays

PPCs were plated at 1.5×10^4 cells per well in 96-well plates with modified RPMI medium. The cells were then incubated in serum-free conditions overnight to arrest the cell cycle. atRA and calcitriol were then added at concentrations, ranging from 10^{-12} to 10^{-5} M and 10^{-14} to 10^{-4} M, respectively, to cells in modified RPMI medium supplemented with 0.5% FBS. Two assays were used to measure cell proliferation rates: 5-bromo-2-deoxyuridine (BrdU) uptake and 3-(4,5-dimethylthiazol-2-yl) -2-5-diphenyltetrazolium bromide (MTT) assay for mitochondrial activity, which have been described previously in our laboratory (Suen et al., 2008). The MTT assay (Sigma) was performed according to the manufacturer's protocol. Briefly, after removal of the modified RPMI medium, the cells were incubated with MTT solution (0.05 mg/ml) for 3 h at 37°C under 5% CO2. The formazan was immediately dissolved by addition of 100 μl DMSO. The absorbance for each treatment (n=6 per treatment) was then measured at 540 nm. The BrdU incorporation assay (Amersham Biosciences, UK) was performed according to the manufacturer's instructions. Briefly,

after removal of the modified RPMI medium, the cells were incubated with BrdU in 0.5% FBS modified RPMI medium for 4 h at 37°C under 5% CO2. After removal of the labeling medium by suction, the cells were fixed by fixative solution provided for 30 min at room temperature and the fixative was then removed. The cells were treated with blocking reagent and incubated for 30 min at room temperature. After removal of the blocking solution the cells were incubated with peroxidase-labeled monoclonal mouse anti-BrdU for 120 min at room temperature before being washed three times with the washing solution. 100 µl of room temperature equilibrated TMB substrate was added to each well and the plate was then covered with aluminum foil to keep it dark and incubated at room temperature for 10 min. The reaction was then stopped by the addition of 25 µl 1 M sulphuric acid. The absorbance of each well was measured at 450 nm (ref. 490 nm) using a MicorKinetics plate reader (PerkinElmer, CA, USA). Values were averaged for each treatment group (n=3).

3.3.7 PPC Cell Death Assays

PPCs were treated with atRA and calcitriol, as described above for the cell proliferation assays, except that the medium was not supplemented with FBS. Enzyme-linked immunosorbent cell death assays (ELISAs; Roche Diagnostics Indianapolis, IN, USA) were performed to assess cell death according to the

manufacturer's instructions. Briefly, after PPCs were treated with atRA for 72 h, the cells were treated with lysis buffer at room temperature for 30 min. The lysates were collected and centrifuged at 200 × g for 10 min. The supernatants were collected, added to the strip, immuno-reagent was added and shaken at 300 rpm for 2 h. before the solutions were removed by suction and rinsed with incubation buffer ×3. ABTS solution was added and upon color development, ABTS stop solution was added. The absorbance of the well contents was then measured at 450 nm (ref. 490 nm) using a MicorKinetics plate reader (PerkinElmer). Values were averaged for each treatment group (n=3).

3.3.8 Statistical Data Analysis

Data are presented as means ± SEM for all groups. Comparison probabilities (P values for chance differences between experimental groups) were made using Student's independent two-tailed t-test. Multiple comparisons between groups were performed using analyses of variance (ANOVAs) followed by Tukey's post hoc tests. For all comparisons, $P < .05$ was considered statistically significant. For quantitative real-time RT-PCR, relative expression was normalized to β-actin and calculated using the comparative CT method; the fold change being defined as 2 $\Delta\Delta$Ct. Statistical

analyses and graphics were carried out using GraphPad Prism 5 (GraphPad Software, San Diego, CA, USA).

3.4 Results

3.4.1 Expression and Localization of RAR, VDR and RXR, CYP26 and CYP24 in PPCs

RAR, VDR and RXR mRNA expression was present in 12 & 13 weeks gestation PPCs (Figure 3.1A). Detectable amounts of RAR, RXR and VDR protein were also documented in first trimester fetal PPCs and neither receptor protein nor expression profiles of these receptors varied with gestational age (between 9 and 15 weeks) of the source material (Figure 3.1B, C, D). Subsequent experiments did not, therefore, distinguish data from experiments on PPCs by gestational age of the donor fetus. In order to further localize the RAR, VDR and RXR in PPCs, immunofluorescence labeling was employed (Figure 3.2); results showed that the RAR (Figure 3. 2A & B) and the VDR (Figure 3.2C & D) were both present in the plasma membrane and in the nucleus as well as being scattered throughout the cytoplasm. However, the RXR was located exclusively in the nucleus (Figure 3.2E & F). Specificity of immunoreactivity has been validated by controls incubated without primary antibodies in each case (Figure 3.2G & H). On the other hand, neither the catabolic enzyme for atRA or for calcitriol, i.e. CYP26 and CYP24, respectively, was expressed in our experimental PPC system (Figure 3.3).

3.4.2 Incubation of PPC with atRA Enhances PPC Viability due to Increased Proliferation and Anti-apoptosis

MTT assays indicated that PPC viability was affected by 72-h treatment with atRA in the 10^{-12} to 10^{-5} M range in a dose-dependent manner. At 10^{-12} M, atRA treatment nearly tripled cell viability compared to control incubated cells (Figure 3.4A). BrdU proliferation assays indicated that treatment with atRA at concentrations in the range of 10^{-12} to 10^{-5} M for 72 h resulted in significantly enhanced cell proliferation (by ~200% at 10^{-12} M atRA vs. Vehicle controls) (Figure 3.4B). Cell death rates for PPCs in serum-free media were almost 6-fold those seen in PPCs incubated in full serum media. Treatment with 10^{-12} M atRA for 72 h decreased cell death in serum-free incubated PPCs almost approximately ~50% (Figure 3.4C).

3.4.3 Incubation of PPCs with Calcitriol Enhances Viability due to Increased Proliferation

MTT assays revealed that calcitriol, at 10^{-13} to 10^{-14} M for 72 h, enhanced cell growth which was maximal at 10^{-14} M, by ~50% (Figure 3.5A) and significant effects were not observed with higher concentrations. BrdU proliferation assays also showed that calcitriol treatment, at 10^{-13} to 10^{-14} M for 72 h, significantly enhanced

cell proliferation (by almost 50%) but that other concentrations had no effect on cell proliferation (Figure 3.5B).

3.4.4 Both atRA and Calcitriol Induce Up-regulation of both the RAR and the VDR but not the RXR

VDR and RAR (Figure 3.6A & B), but not RXR (Figure 3.6C) mRNA levels were elevated 13-fold and 3-fold respectively following 48-h exposure to calcitriol (10^{-14} M); in addition, RAR and VDR (but not RXR) mRNA levels were elevated 3-fold following 48-h exposure to atRA (10^{-12} M) (Figure 3.6).

3.4.5 Combination Treatment with atRA and Calcitriol on Cell Viability and NGN3 Expression

Combined treatment of PPCs with maximally effective concentrations of atRA (10^{-12} M) and of calcitriol (10^{-14} M) in serum-free media for 72 h did not increase cell viability relative to untreated controls and less than either alone (Figure 3.7A). Real-time PCR analysis of cDNA reverse transcribed from RNA derived from isolated PPCs after combined treatment with both atRA and calcitriol indicated that co-treatment greatly increased NGN3 expression by about 6 folds, compared to vehicle incubated control cells (Figure 3.7B) though atRA treatment alone decreased

NGN3 expression and calcitriol treatment alone did not alter NGN3 expression, relative to ngn3 expression in untreated control cells.

3.5 Discussion

The present study is the first characterization of the effects of retinoic acid and hormonal vitamin D in our human primary culture derived-pancreatic progenitor cell (PPC) system (Suen et al., 2008; Leung et al., 2009). We have demonstrated that PPCs express the RAR and VDR in the nucleus, cytoplasm and plasma membrane but the RXR only in the nucleus, suggesting that effector systems for atRA and calcitriol match those seen in adult tissues, including interaction with the RXR in the nuclei (Niederreither et al., 2008; Feldman et al., 2005; De Vos et al., 1997). There was no variation in expression of these receptors with gestational age of PPC donors, indicating that vitamins A and D are likely to have consistent effects, such as growth and differentiation, in PPC preparations from 9 to 15 weeks gestational age donors.

Neither of the enzymes catabolic for hormonal vitamin D and atRA, i.e. CYP26 and CYP24 (Feldman et al., 2005; Popadic et al., 2008) were detectable in our PPC system; thus the usual down-regulatory systems for these vitamins appear to be absent in our PPC preparations. Whilst atRA and calcitriol can have antagonistic effects on each other (Feldman et al., 2005; Popadic et al., 2008) this seems unlikely to be the case in our PPC system since co-treatment with atRA and calcitriol had no such effects. Thus, the mechanisms for regulation of the effects of vitamin A and D in our PPCs require further investigation. The well known morphogen atRA has dose-effects

(Niederreither et al., 2008). Similarly, we found dose dependent variation in its effects on PPC cell viability though the effects on proliferation were not dose-dependent, suggesting that there were anti-apoptotic effects on PPCs, as confirmed by direct measurements. Consistent with the known dose-dependent effects of calcitriol on cell proliferation (Alon et al., 2002), PPC viability was increased by calcitriol but only at concentrations of 10^{-13}–10^{-14} M, i.e. about the physiological level of fetal serum, which is lower than maternal serum level (Salle et al., 2000) and this effect appeared to be due to increased cell proliferation.

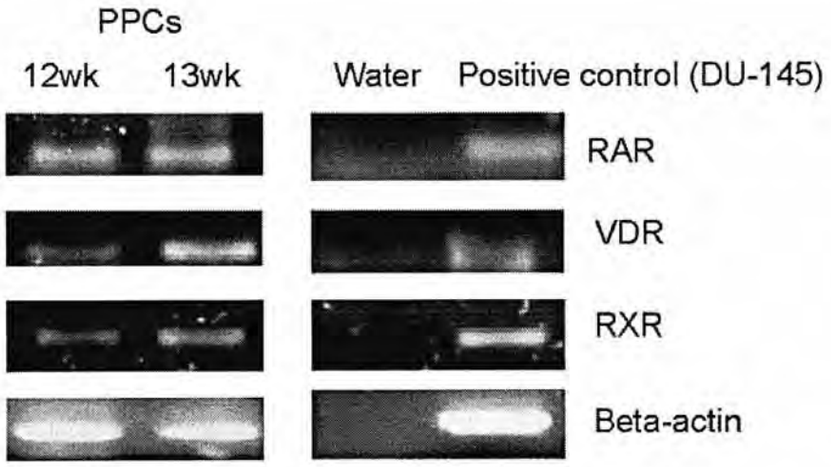
Upregulation of both RAR (Chomienne et al., 1991; Wuarin et al., 1994; Friedman et al., 1993) and VDR (Albrechtsson et al., 2003; Jensen et al., 2002; Wiese et al., 1992; Li et al., 1999) expression with exposure to either atRA or calcitriol, individually, suggests binding of each of these ligands to response elements on the genes of both receptors. The fact that there was greater RAR and VDR mRNA expression following longer incubations with either ligand is compatible with this view; atRA can be a signaling partner with calcitriol but this has been thought to reflect VDR:RXR heterodimerization (Feldman et al., 2005; Carlberg et al., 2007) rather than cross stimulation of the genes for these receptors. The finding of the RXR in our PPC system as well as the VDR and RAR is consistent with the possibility of cross-talk between the actions of atRA and calcitriol being mediated through the

interactions of each of these receptors with the RXR (Niederreither et al., 2008; Feldman et al., 2005; De Vos et al., 1997). However, we found, surprisingly, that combined treatment with atRA and calcitriol did not enhance PPC viability, a negative finding that may reflect potentiation of differentiation since expression of NGN3, a marker for endocrine cell specialization (Gradwohl et al., 2000) was increased over 6-fold by combined treatment as compared to the effect of either ligand alone. If confirmed, this would imply that the policy of giving vitamin D supplement along in pregnancy instead of cod liver oil would need adjustment. Cod-liver oil, act as natural supplement of vitamin A and vitamin D, is well known for its beneficial effects on growth in infants and children. (Masterjohn, 2009; Morgan et al., 1937; Metz et al., 1985; Clerk et al., 1962 ; Clerk et al., 1963) Yet, due to vitamin A's teratogenicity, vitamin D supplement along is used instead. From other research, specification of endocrine cells occurred within 20–23 weeks gestation. (Sarkar et al., 2008) As a result, intake of cod liver oil might prove to have specific benefits for endocrine islet cell formation within 20–23 week gestations.

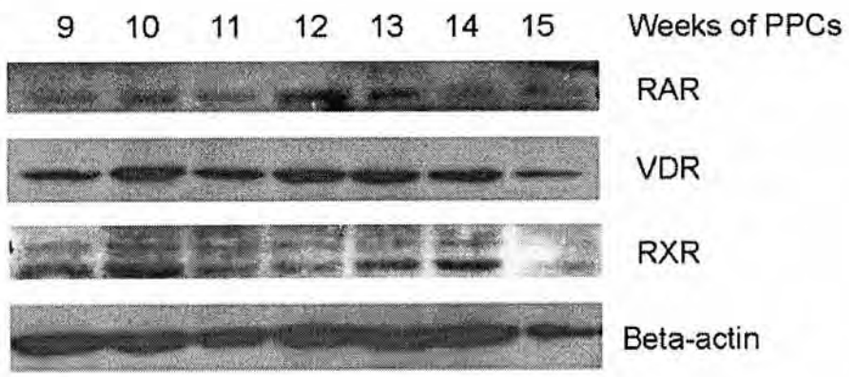
In conclusion, RAR, VDR and RXR were confirmed to be present in first trimester human fetal pancreatic progenitor cells and atRA and calcitriol were each shown, individually, to be capable of increasing PPC viability. While further investigations are needed to delineate the differentiation properties of PPCs and

clarify the roles of vitamin A and vitamin D in islet development, especially of the beta cell, the present data suggests that both vitamin A and vitamin D are involved in PPC development. These findings may contribute to the development of insulin sensitive ICCs which is suitable for subsequent transplantation for the treatment of T1DM, and possibly also of T2DM.

A.


PPCs
12wk
13wk
Water
Positive control (DU-145)
RAR
VDR
RXR
Beta-actin

B.


9
10
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Weeks of PPCs
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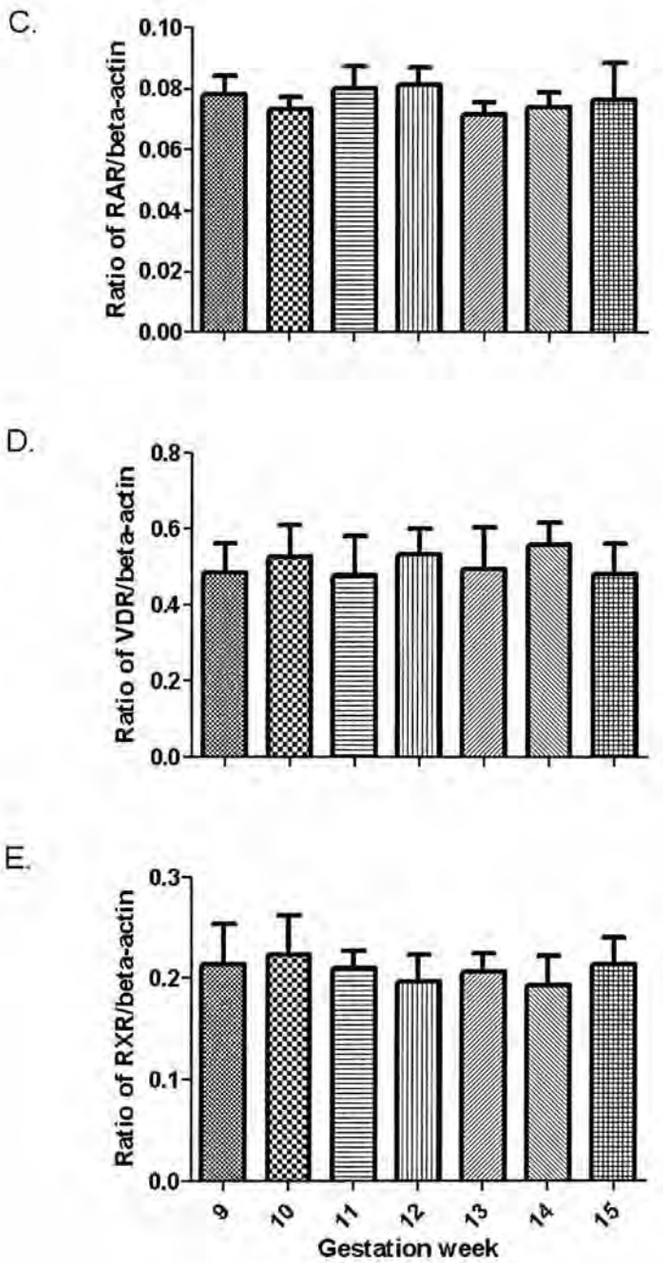


Figure 3.1 Characterization of RAR, VDR and RXR expression and protein content in PPCs. A. RT-PCR analysis of RAR, VDR and RXR gene expression from 12 to 13 weeks pancreas PPC preparations; controls (-RT) lacked cDNA; human prostate cancer cells (DU-145) provided positive controls (De Vos et al., 1997). B. Western blot analysis of RAR, VDR and RXR protein in PPCs derived from 9 to 15 week human fetal pancreas. C-E. Relative expression of RAR, VDR and RXR to beta-actin of PPCs derived from 9 to 15 weeks of pancreas. Each gene or protein was amplified in triplicate from three to five separate PPC samples prepared from different fetal pancreata. The protein/actin ratio did not vary with gestational sample age.

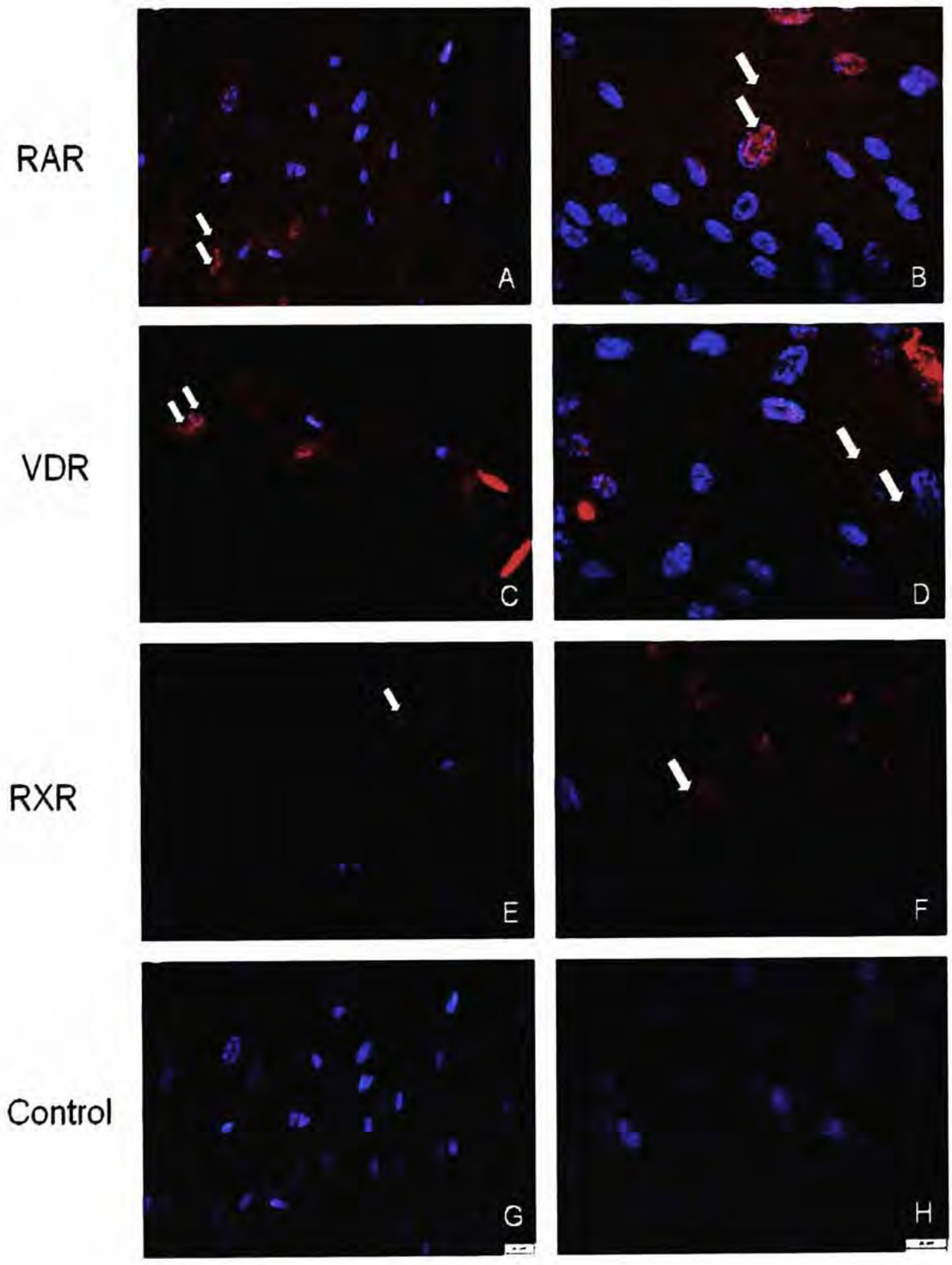


Figure 3.2 Localization of RAR, VDR and RXR proteins by immunoreactivity in PPCs. PPCs derived from pancreata across a range of gestational ages were immunolabeled with antibodies against RAR, A. ×400, B. ×630; against VDR, C. ×400, D. ×630; against RXR, E. ×400, F. ×630; negative controls of RAR, VDR and RXR immunoreactivity, incubated without any primary antibodies, H. ×400, G. ×630

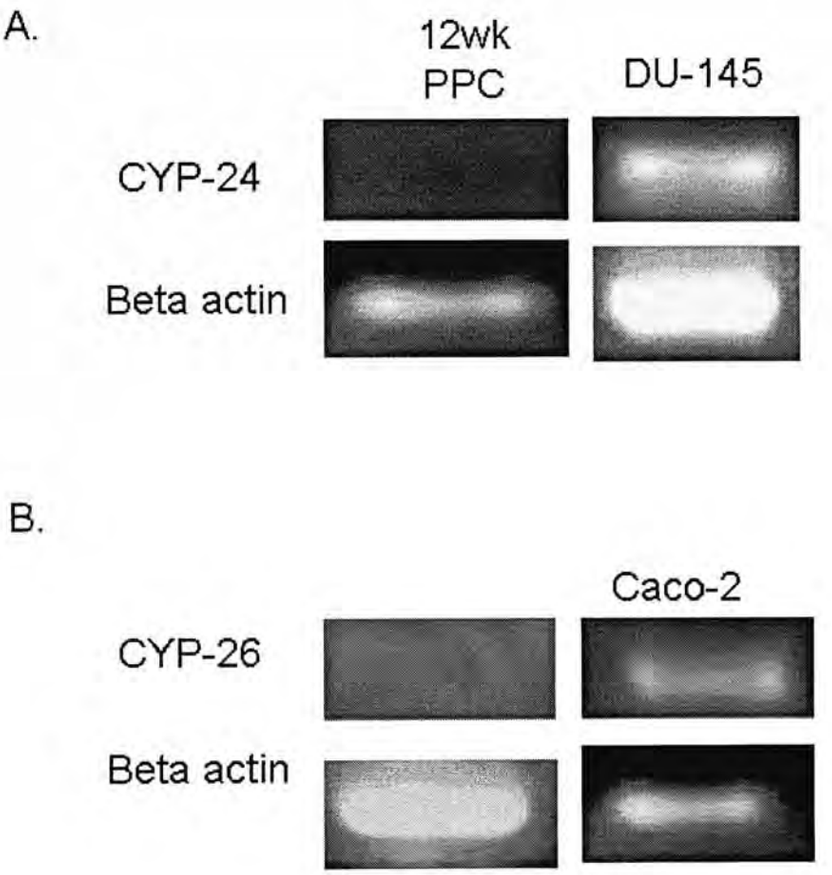


Figure 3.3 Characterization of enzymes catabolic for atRA and calcitriol (CYP26 and CYP24). Expression of these genes was not detectable in our 12-wk pancreas PPC preparations. Human prostate cancer cells (DU-145) (Muindi et al., 2007) and human colon cancer cells (Caco-2) (Lampen et al., 2001) served as positive controls.

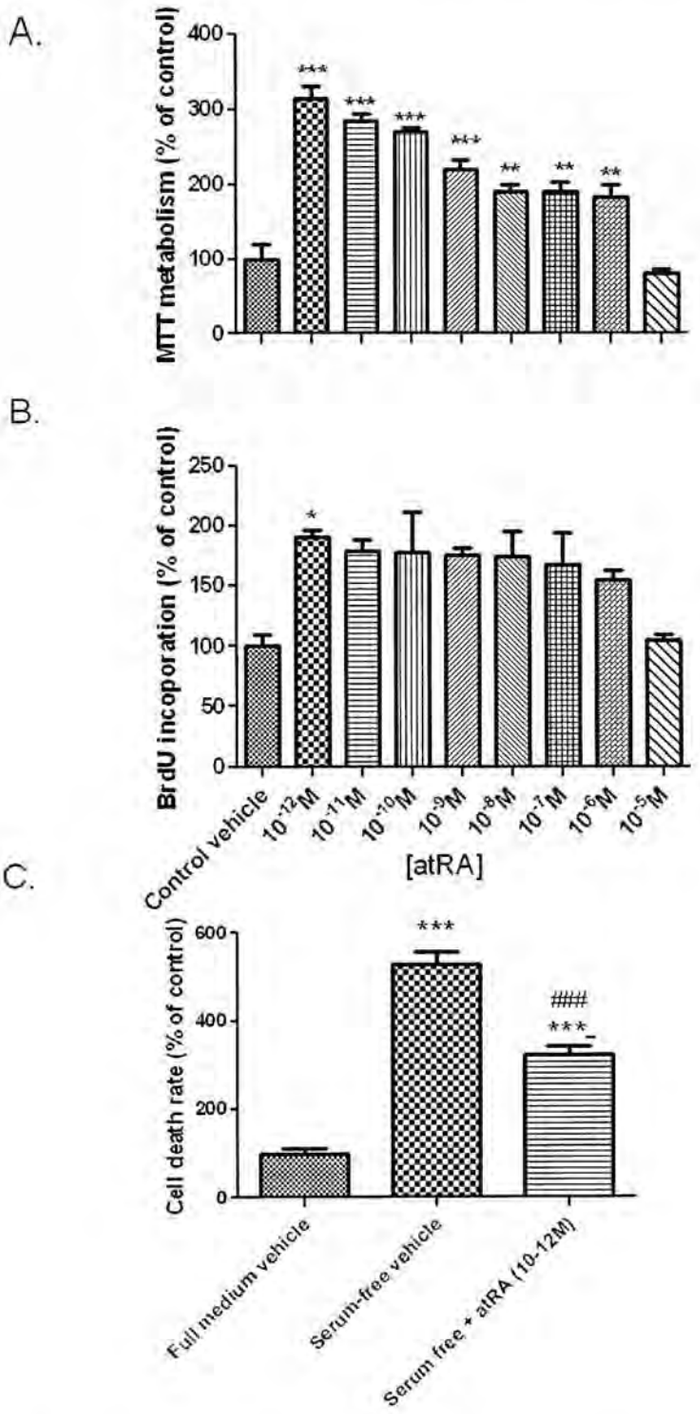


Figure 3.4 The effects of atRA on PPC proliferation. A. MTT proliferation assays and B. BrdU proliferation assays of PPCs exposed to atRA (10−12 M to 10−5 M for 72 h using DMSO as the vehicle control; showed dose-dependent effects, maximal at 10−12 M atRA. C. Antiapoptotic effect of atRA at 10−12 M (in serum-free conditions for induction of cell death). All data were expressed as means ± SEM of three experiments with three independent fetal samples (n=3 for each group; *p<0.05, **p<0.01, ***p<0.001 vs. vehicle control and #p<0.05, ##p<0.01, ###p<0.001 vs. serum-free vehicle)

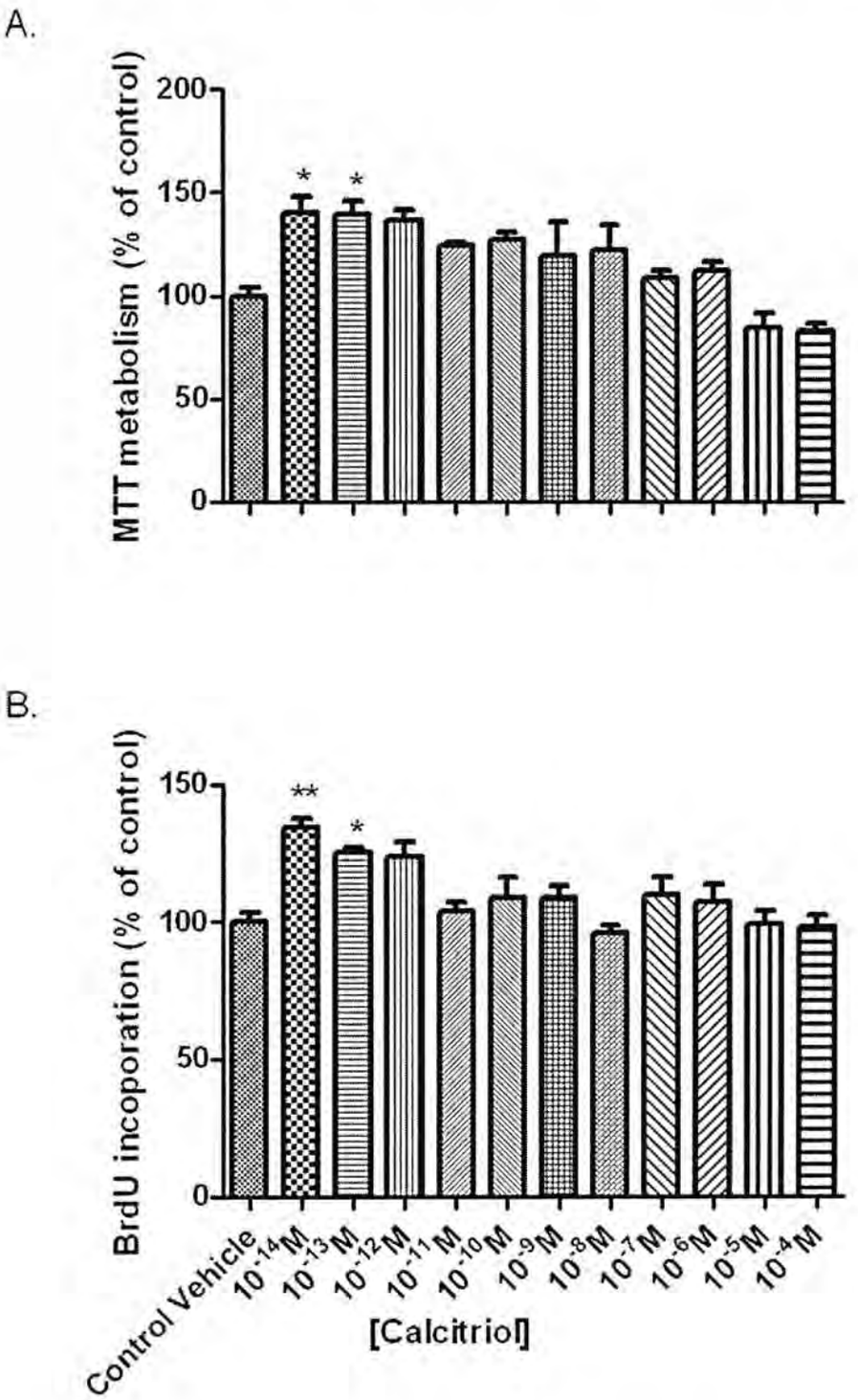


Figure 3.5 The effects of calcitriol on PPC proliferation. A. MTT and B. BrdU proliferation assays of PPCs treated with calcitriol (10−14 M to 10−4 M for 72 h) using absolute ethanol as the vehicle control showed increased cell viability at concentrations of 10−14–10−13 M. All data are expressed as means ± SEM of three experiments with three independent fetal samples (n=3 for each group. *$p<0.05$, **$p<0.01$, ***$p<0.001$ vs. vehicle control)

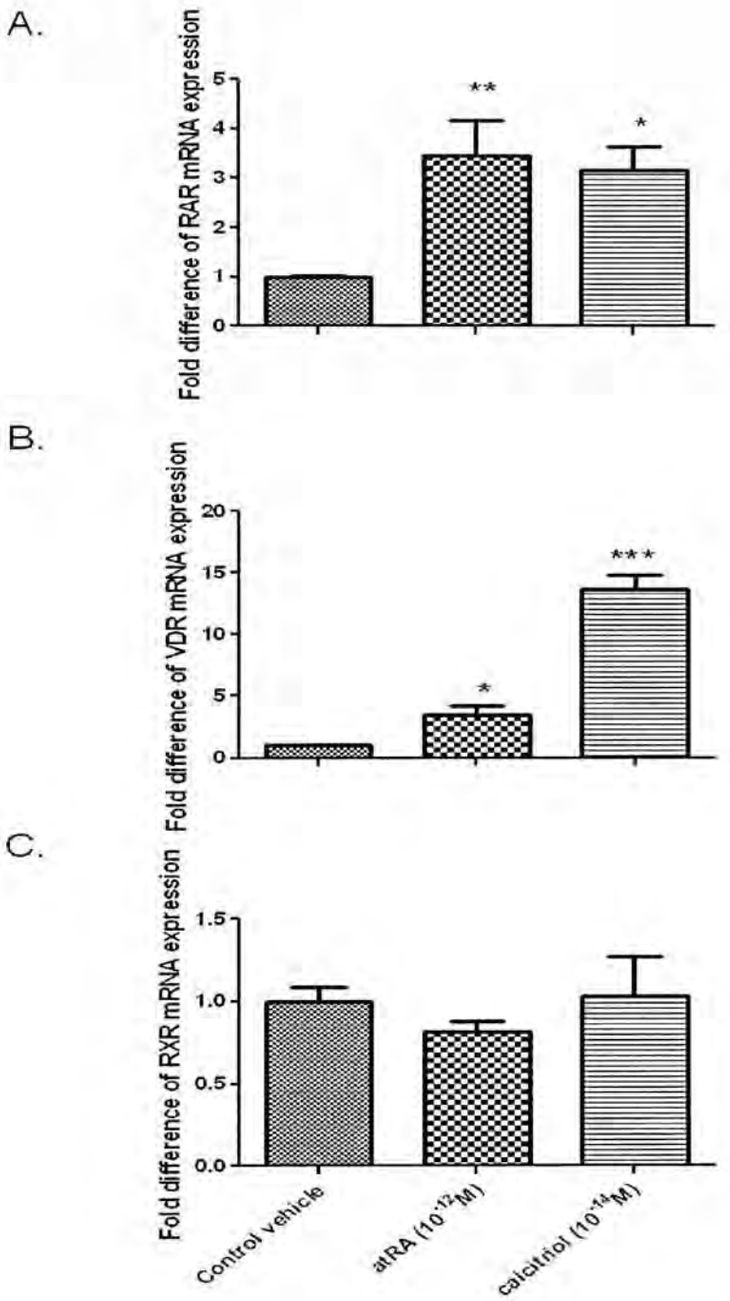


Figure 3.6 Effects of the RAR agonist atRA and the VDR agonist calcitriol on the mRNA expression of the RAR, VDR and RXR. A. RAR expression was increased in PPC preparations by exposure to AtRA (10−12 M, 48 h) and by exposure to calcitriol (10−14 M, 48 h); B. VDR expression was increased in PPC preparations by exposure to AtRA (10−12 M, 48 h) and by exposure to calcitriol (10−14 M, 48 h); C. There was no effect on RXR expression in PPC preparations of exposure to either AtRA (10−12 M, 48 h) or to calcitriol (10−14 M, 48 h). All expression concentrations were normalized to beta-actin and relative levels defined as 2 ΔΔCT. All data were expressed as mean ± SEM (n=6 per group. *$p<0.05$, **$p<0.01$, ***$p<0.001$ vs. vehicle controls)

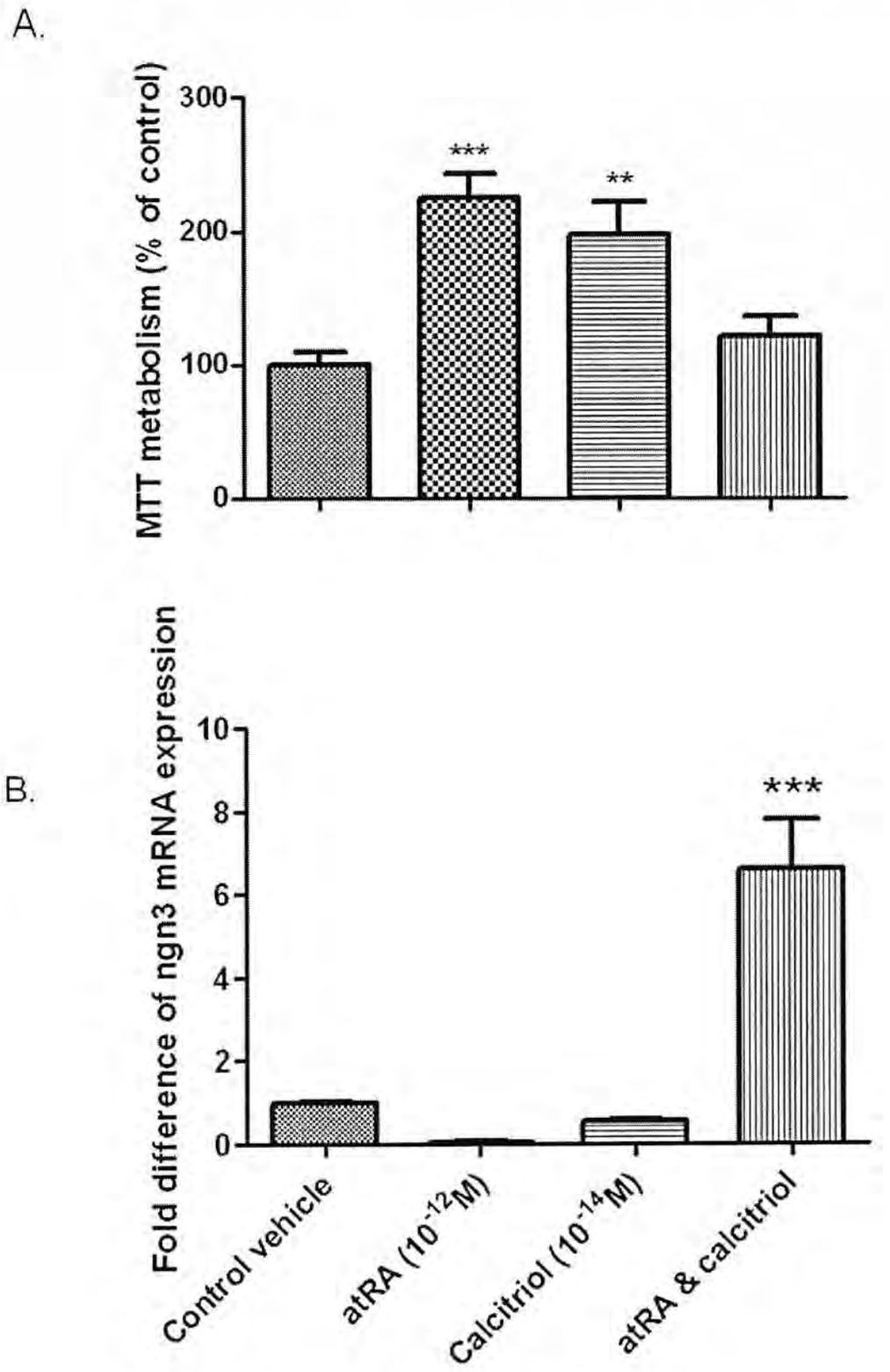


Figure 3.7 The effects of atRA combined with calcitriol on cell proliferation and 'maturation' in PPC preparations. A. Exposure to atRA (10−14 M) / calcitriol (10−12 M) in combination for 72 h did not enhance cell viability in comoparson with that produced by either ligand alone. B. Expression of the endocrine specification marker ngn3, measured by real-time PCR, was markedly increased by exposure to these two ligands in combination as compared to exposure to either alone.

Table 1 Sequence of the specific PCR Primers and their expected product size of different target genes

Gene	Sense primer	Antisense primer	Annealing temperature (°C)	Product size (b.p.)
For RT-RCR				
β-actin	TGGCACCACACCTTCTACAATGAGC	GCACAGCTTCTCCTTAATGTCACGC	60	396
RARα	GACCAGATCACCCTCCTCAA	GTCCGAGAAGGTCATGGTGT	60	99
RXRα	CTGCTCATCGCCTCCTTCT	ACACAAGCTCCGTCAGCAC	60	142
VDR	CTCAAACGCTGTGTGGACAT	ACTGTCCTTCAAGGCCTCCT	60	117
CYP24	GAAACCAGGGGAAGTGATGA	AACGACCATTTGTTCAGTTCG	60	135
CYP26	CCAGAAAGTGCGAGAAGAGC	GGGATTCAGTCGAAGGGTCT	60	136
For real-time PCR				
β-actin	TGTCCACCTTCCAGCAGATGT	CGGACTCGTCATACTCCTGCTT	62	51
RARα	GACCAGATCACCCTCCTCAA	GTCCGAGAAGGTCATGGTGT	60	99
RXRα	CTGCTCATCGCCTCCTTCT	ACACAAGCTCCGTCAGCAC	60	142
VDR	CTCAAACGCTGTGTGGACAT	ACTGTCCTTCAAGGCCTCCT	60	117
NGN3	TGTGGGTGCTAAGGGTAAGG	GGGAGAAGCAGAAGGAACAA	60	99

Table 3.1 Sequence of the specific PCR Primers and their expected product size of different target genes

Chapter 4

Human fetal liver stromal cell co-culture enhances the growth and differentiation of pancreatic progenitor cells into islet-like cell clusters
(The content of this chapter has been submitted to ***Gastroenterology***)

4.1 Abstract

BACKGROUND & AIMS: Recent advances in directed differentiation of pancreatic stem cells offer potential for the development of replacement therapy for diabetes patients. Existing differentiation protocols, however, are complex, time-consuming, and costly; thus there is a critical need for alternative protocols. Given the common developmental origins of the liver and pancreas, we sought to develop a novel protocol devoid of growth factors by using a microenvironment established by liver stromal cells (LSCs) derived from human fetal liver. We examined the effects of this LSC microenvironment on the growth and differentiation of established pancreatic progenitor cells (PPCs) into islet-like cell clusters (ICCs). **METHODS:** PPCs and LSCs were isolated from 1st and 2nd trimester human fetal tissues and underwent co-cultures. The growth, differentiation, and functionality of PPCs and ICCs were determined by examining cell viability, expression levels of critical markers and insulin secretion ability for pancreatic beta-cells. We also compared the differential expression profile of growth factors from the LSCs derived from 1st versus 2nd trimester fetal livers. **RESULTS:** Co-cultures with 2nd trimester LSCs enhanced PPC growth, ICC differentiation and ICC functionality without the addition of exogenous growth factors. A host of morphogenic factors were expressed in LSCs, and insulin-like growth factor 1 (IGF1) was identified as being responsible for the above effects. **CONCLUSIONS: This is the first report showing that LSC microenvironment can induce PPCs derived from human fetal pancreas to grow and differentiate into ICCs while also enhancing the functionality of ICCs. Further modifications of the stroma microenvironment may offer an alternative, efficient and cost-effective approach providing islet transplantation.**

4.2 Introduction

Transplantation of human pancreatic islets is the ideal curative option for replacement of beta-cell mass in diabetic patients (de Kort el al., 2011; Shapiro et al., 2003; Ryan et al., 2005). Despite the active promotion of organ donation, lack of islet donors has adversely affected the availability of this treatment modality. The need for alternative sources of islets has prompted a rapid rise in stem cell therapy and islet regeneration research (Yechoor et al., 2010; Kordowich et al., 2010; Juhl et al., 2010). Stem cells, characterized by their capacity for pluripotency and potential to differentiate into islet cells, thus, could be used to help to replace absent, damaged, or malfunctioning islets. Hence, stem cells offer the potential of being an unlimited source of human islets for diabetes research and transplantation; in view of this, considerable efforts have been invested in investigating islet engineering (de Kort el al., 2011; Shapiro et al., 2003; Ryan et al., 2005; Yechoor et al., 2010; Kordowich et al., 2010; Juhl et al., 2010; Guo et al., 2009).

Combined treatment with activin A and retinoic acid has been demonstrated to efficiently induce the generation of insulin-producing cells from mouse embryonic stem cells (Shi et al., 2005). However, this simple protocol was not effective when applied to human embryonic stem cells (Jiang et al., 2007), raising concerns about the ability to translate research conducted with mouse stem cells into human stem cells given the inherent inter-species differences (Shi et al., 2005; Jiang et al., 2007; Joglekar et al., 2009; Suen et al., 2006; Suen et al., 2008). Our group has established a system for the isolation and culture of pancreatic progenitor cells (PPCs) derived from human fetal pancreas tissue. These PPCs have been shown to have a high capacity for proliferation and differentiation when cultured with appropriate differentiation cocktail, and the effects of several morphogens, e.g. sPDZD2, vitamin

A and vitamin D related to human pancreatic development have been studied in this system (Suen et al., 2008; Ma et al., 2011; Ng et al., 2011; Leung et al., 2009).

Recently, different approaches have been utilized for islet engineering, such as genetic modifications, directed differentiation and *in vivo* stem cell injection (Yechoor et al., 2010; Kordowich et al., 2010; Juhl et al., 2010; Guo et al., 2009). Within these approaches, directed differentiation is the most popular means of islet engineering (Yechoor et al., 2010). D' Amour and colleagues have reported a complicated step-wise protocol (D'Amour et al., 2006) for differentiating human embryonic stem cells into islets that mimics *in vivo* development and produces an insulin content similar to that of human adult islets. Though this result is exciting, the protocol is variable among different stem cells, notably different cell lines of embryonic stem cells. Furthermore, substantial difficulties, such as the high costs of the large doses of growth factors needed, remain for directed differentiation. Therefore, with the idea of mimicking the developmental environment, we aim to develop more economical and convenient methods to achieve differentiation using a microenvironment, or stem cell 'niche', for islet cell development.

Mesenchymal/stromal cells consist of a heterogenous population of cells that provide structural and physiological support for somatic cells (Alphonso et al., 2009; Krebsbach et al., 1999). Recent research has shown that they support differentiation (Lee et al., 2011; Soto-Gutierrez et al., 2008) survival, and functional improvement (Sordi et al., 2010; Luo et al., 2007). It would be of interest, therefore, to assess the differentiation potential induced by a human fetal stroma generated microenvironment. Since to date there are no well established protocols available for isolating pancreatic stroma and organ development is a complicated and interactive process involving many different organs (Ranjan et al., 2009; Guney et al., 2009), in this study, human

fetal liver stroma was introduced into cellular microenvironment because methods for its isolation and culture (Lee et al., 2011) are well-established and liver stroma cells (LSCs) are developmentally closely related to pancreatic cells (Ji et al., 2009; Imai et al., 2009; Meivar-Levy et al., 2003; Deutsch et al., 2001; Odom et al., 2004; Si-Tayeb et al., 2010; Zaret et al., 2008; Morrison et al., 2004; Imai et al., 2008).

During embryogenesis, neighboring tissues are under the influence of similar combinations of transcription factors, differing by just one or a few factors (Odom et al., 2004; Si-Tayeb et al., 2010). Developmentally, emergence of the ventral pancreas has been shown to be related to the emergence of the liver; their specifications are at the same time and in the same general domain of cells (Deutsch et al., 2001; Odom et al., 2004; Zaret et al., 2008). Fibroblast growth factor (FGF) signaling from the cardiac mesoderm diverts endodermal cells to express genes for liver instead of those for pancreas, implying that a lack of FGF signaling from the cardiac mesoderm would lead to the development of pancreatic tissues (Deutsch et al., 2001; Zaret et al., 2008). Such findings further encourage us in pursuing the possibility that stromal cells which support liver development could also support pancreatic development. Moreover, in lower organisms (e.g. *Nile Tilapia*), there is a single hepatopancreas rather than a separate pancreas and liver, further indicating that the development of these two organs is likely to be associated (Morrison et al., 2004). Interestingly, adult liver also shows a regulation of pancreatic cell mass by neuronal pathway (Imai et al., 2008). Additionally, we have shown that vitamin A and D, whose serum levels are regulated by the liver, enhance islet specification (Ng et al., 2011).

In light of the aforementioned findings, we hypothesize that the liver plays a positive role in islet development via regulation of growth factors and cytokines secretion and we now report experiments designed to provide better insight into LSC

actions on PPC growth and differentiation. These data are essential for understanding liver-pancreas interactions in development as well as for revealing the potential of stem cell microenvironment for the promotion of stem cell proliferation and differentiation.

4.3 Materials and Methods

4.3.1 Use of human and animal tissues

The work on human and rat tissues was approved by our Institutional Committees on Human Ethics and Animal Ethics, respectively, The Chinese University of Hong Kong. Maternal consent for use of fetal tissues was obtained by the outpatient clinic of the Prince of Wales Hospital before surgical termination of pregnancy. Ethical approval for the use of human fetal tissue from the Clinical Research Ethnics Committee (CREC-2005.461) was obtained.

4.3.2 Cell preparation, characterization, and differentiation

Human PPCs and LSCs were obtained as described in the Supplementary Materials and Methods section. LSC characterization was performed using reverse transcriptase-polymerase chain reaction (RT-PCR) and immunocytochemistry. The procedure of human islet-like cell cluster (ICC) differentiation is described in the Supplementary Materials and Methods.

4.3.3 Examination of PPC growth and ICC differentiation and functions with LSC co-culture

To establish the co-culture model, human LSCs were first separated into 1st (from 9 up to 12 weeks) and 2nd (from 13 up to 16 weeks) trimester groups according to ultrasound measurements of fetal growth parameters, as previously reported (Suen et al., 2008; Ma et al., 2011; Ng et al., 2011; Leung et al., 2009). To determine the optimal irradiation for LSCs to stop proliferation, LSC aliquots were seeded on transwells under different irradiation levels (2000, 4000, 6000, 8000, and 10000 rad). After 8 days, cell counts were carried out to determine any changes in cell numbers and 8000 rad were selected for further study. To optimize the cell number effect of

LSCs on PPC cell viability and number, we co-cultured 9-16weeks LSCs (2, 4, 6, 8, or 10×10^4 cells) on transwells with PPCs (1.5×10^5 cells) in 6-well plates. After 3 days, PPCs were subjected to MTT metabolism assays and cell counts. To determine the effect of 1st and 2nd trimester LSCs on PPC cell viability and number, LSCs (8×10^4 cells) were co-cultured in transwells with PPCs (1.5×10^5 cells) in 6-well plates. After 3 days in co-culture, PPC number and viability were determined by cell counts and MTT metabolism assays. MTT and cell count procedures are described in detail in the Supplementary Materials and Methods.

To assess the effects of LSCs on ICC differentiation, LSCs were co-cultured in transwells with PPC-derived ICCs, with or without a differentiation 'cocktail'. Total RNA of the resultant ICCs were collected and subjected to real time PCR for the following genes: PDX1, NGN3, ISL-1, NKX2.2, NKX6.1, NEUROD, GLUCOKINASE, INSULIN, MHC-1, and MHC-II. In addition, the resultant ICCs were subjected to an enzyme-linked immunosorbent assay (ELISA) to determine their insulin secretory capacities. The detailed procedures used for real-time PCR and insulin ELISA are provided in the Supplementary Materials and Methods, and the primers sequences are listed in Table 4.1.

4.3.4 Identification of growth factors and investigation of their effects

Total RNA samples from 1st and 2nd trimester LSCs or homogenized human fetal livers were collected and subjected to RT-PCR and real-time PCR. LSC conditioned media samples were collected for insulin-like growth factor I (IGF1) ELISA. The detailed procedures employed for IGF1 ELISA were reported in the Supplementary Materials and Methods and the primer sequences are listed in Table 1.

IGF1 receptor (IGF1R) expression and localization in PPCs were determined

by RT-PCR, Western blotting, and immunocytochemistry. To determine the effects of IGF1 and IGF2 on PPC cell viability, PPCs (3×10^4 cells) were seeded in 96-well plates and exposed to a range of IGF1 or IGF2 concentrations (1, 10, 50, and 100 ng/ml). After 3 days, PPC viability was measured by MTT metabolism assay. Likewise, IGF1 or IGF2 (1, 10, 50, or 100 ng/ml) was added to the differentiation cocktail for the culture. Total RNA samples of the resultant ICCs were collected and subjected to real-time PCR for the PDX1, NGN3, NKX2.2, NKX6.1, ISL-1, NEUROD, GLUCOKINASE and INSULIN genes. The detailed procedures employed for real-time PCRs are described in the Supplementary Materials and Methods section and the primers sequences were listed in Table 4.1.

To determine the contribution of the IGF1R, MAB391 neutralizing antibodies were supplemented in PPC growth and ICC differentiation experiments. PPC viability experiments were performed to determine the dose response profile for MAB391 (0.001 - 10 μg/ml) (described in detail in the Supplementary Materials and Methods).

4.3.5 Statistical analysis

Data are presented as means ± SEM for all groups. For all comparisons, *p*-values less than 0.05 were considered statistically significant. Statistical analyses and development of graphics were performed using GraphPad Prism 5 (GraphPad Software, San Diego, CA).

4.4 Results

4.4.1 Isolation, culture, and characterization of LSCs

9 to 16-gestational week human fetal liver tissues were digested with collagenase A into single cells and cultured in modified DMEM/F12 1:1 medium with 10% FBS and 1% penicillin/streptomycin (P/S). Populations of LSCs with different morphologies, such as stellate-shaped cells and cuboidal-shaped cells, etc. appeared to adhere on the plastic culture flask after overnight incubation and free floating cells were discarded. Cells grew slowly during the first week of incubation, culminating in the development of a monolayer of cells. After a single passage, a monolayer of single stellate-shaped cells was obtained (Figure 4.1A). These cells were expanded to more than 20 passages over a period of 4 to 5 months (data not shown).

As shown in Figure 4.1B, RT-PCR analysis of stromal cells at passages 3-8 showed that the cells expressed detectable amounts of mRNA for hepatic precursor markers (CK18, CK19, EpCAM, N-CAD and CD45) and stromal cell markers (CD90, CD105, CD166 and VIMENTIN). The presence of liver stromal cells' markers, say Dlk, CD29, and CD90 proteins were localized by immunocytochemistry, which was found almost in every single cell (Figure 4.1C). In contrast, alpha-fetoprotein (AFP) and albumin, markers for hepatocytes and hepatoblasts, were detected neither at the mRNA nor at the protein levels (Figure 4.1B & 4.1C). Only cells that fulfilled the above conditions were used for subsequent experiments while other cells were discarded.

4.4.2 Establishment of the LSC co-culture system

To prevent blockage of transwells by overwhelming growth of LSCs, optimal irradiation (8000 rad) for LSC was determined; there was no significant change in cell

number after irradiation for 8 days (data not shown). PPC viability and cell count data indicated that the optimal LSC number per culture for preventing depletion of medium nutrients was ~8×10^4 cells (Figure 4.2A, 4.2B). Moreover, it was found that, upon co-culture with LSCs (8×10^4 cells), PPC viability increased 1.5-fold ($P < .001$ vs. no co-culture control) as measured by MTT metabolism (Figure 4.2A) and PPC counts increased 4.5-fold ($P < .001$ vs. no co-culture control) (Figure 4.2B). On the other hand, LSCs were separated into 1st trimester (9 - 12 weeks) and 2nd trimester (13 - 16 weeks) groups, according to the gestational age of the source fetus and co-culture of PPCs enriched with LSCs derived from 1st trimester versus 2nd trimester fetal livers did not differ from each other in terms of MTT metabolism or cell number (Figure 4.2C, 4.2D).

4.4.3 LSC co-culture enhances PPC-derived ICC differentiation

ICC co-culture with LSCs plus the addition of a differentiation cocktail[12-15] showed enhanced mRNA expression of PDX1 (8-fold, $P < .05$), NGN3 (7-fold, $P < .001$), NKX2.2 (6-fold, $P < .001$), NKX6.1 (4-fold, $P < .05$) (Figure 4.2E), GLUCOKINASE (5-fold, $P < .001$) and INSULIN (5-fold, $P < .001$) (Figure 4.2F). The induction effects of 1st and 2nd trimester derived LSCs were similar (data not shown). Subsequent experiments were designed to distinguish from the effects of a differentiation cocktail versus the effects of 1st and 2nd trimester LSC co-culture alone. Compared to non-LSC control (with a differentiation cocktail only), ICC co-culture enriched with 1st trimester LSCs, there is no significant change in transcription markers and functional genes, except for PDX1 (3-fold, $P < .05$) (Figure 4.3A, 4.3B). While ICC co-culture enriched with 2nd trimester LSCs showed a greater upregulation of PDX1 (4-fold, $P < .001$), NGN3 (11-fold, $P < .001$), ISL-1 (6.5-fold, $P < .01$),

NKX2.2 (14.5-fold, $P < .05$), NKX6.1 (9-fold, $P < .001$), NEUROD (4-fold, $P < .01$) (Figure 3A), GLUCOKINASE (7.5-fold, $P < .001$), and INSULIN (15-fold, $P < .05$) (Figure 3B) comparing to control.

ICCs co-cultured with 2nd trimester LSCs showed increased basal secretion of insulin (2-fold, $P < .001$ vs. no LSC co-culture), but no significant glucose-stimulated insulin secretion (GSIS) (Figure 4.3C). However, Potassium stimulated-insulin secretion (KSIS) experiments designed to evaluate the functionality of differentiated ICCs showed that ICCs incubated in LSC co-culture had increased KSIS (1.5-fold, $P < .001$ vs. no LSC co-culture control) (Figure 4.3D). Although the LSC for co-culture was derived from a different fetus, no significant alterations in the expression of MHC-I nor MHC-II were observed in the resultant PPCs (Figure 4.3E). On the other hand, a 2-fold ($P < .05$) increase in MHC-I and MHC-II expression was observed when ICCs were co-cultured with 1st trimester LSCs and there is no significant alterations in the expression of MHC-I or MHC-II were observed in the resultant ICCs that co-culture with 2nd trimester LSCs (Figure 4.3F).

4.4.4 Differential expression of mRNA for cytokines and growth factors between 1st and 2nd trimester LSCs

PCR showed that LSCs from both 1st and 2nd trimester fetal tissues expressed SCF, NGF, VEGFA, FGF1, FGF2, FGF7, IGF1, IGF2, TGFb, HGF, IL-6, BMP2, BMP4, and BMP6 mRNAs (Figure 4.4A), but not Reg4, GASTRIN, PTHrP, BETACELLULIN, GLP-1, TNFα, TNFβ, IFNγ, FGF21, BMP5, BMP7, or BMP9 mRNAs (data not shown). Several molecules (IGF1, IGF2, HGF, NGF, SCF, BMP2, BMP4 and BMP6) appeared to show differential expression between 1st and 2nd trimester LSCs by RT-PCR (data not shown). Therefore, real-time PCR was

performed, showing that IGF1 (35-fold, $P < .001$), IGF2 (10-fold, $P < .05$), BMP4 (5-fold, $P < .05$) and BMP6 (5-fold, $P < .05$) mRNA expression was much greater in 2nd trimester LSCs than in 1st trimester LSCs (Figure 4.4B). Within them, IGF1 showed the most significant difference in expression so human fetal liver tissues were used to verify the difference. Human fetal liver tissues from 2nd trimester also had higher IGF1 mRNA expression than 1st trimester liver (10-fold, $P < .001$) (Figure 4.4C). In addition, IGF1 ELISA demonstrated the presence of IGF1 protein in both 1st and 2nd trimester LSC-conditioned medium and conditioned medium from 2nd trimester LSCs had greater amounts of IGF1 protein than conditioned medium from 1st trimester LSCs (1.5-fold, $P < .01$) (Figure 4.4D).

4.4.5 Characterization of IGF1 receptors in PPCs and the effects of exogenous IGF1 on PPC growth and ICC differentiation

Expression of IGF1R, IGF2R, IGF1 and IGF2 mRNAs was confirmed in fetal PPCs (9 - 16 gestational weeks). Consistently, expression of the IGF1R protein, target functional receptors for IGF1 and IGF2, was confirmed and located in the cell cytoplasm and at the cell membrane of PPCs (Figure 4.5A). As shown in Figure 4.5B, 2nd trimester LSCs were found to have 100-fold ($P < .001$) higher levels of mRNA expression of IGF1 than 1st and 2nd trimester PPCs.

Exogenous 50 ng/ml IGF1 produced an augmentation of growth effect (1.5-fold, $P < .001$) on 1st trimester PPC but not 2nd trimester PPC (Figure 4.5C), thus, we have chosen 1st trimester PPC for subsequent IGF1 growth analysis. In the case of IGF2, both trimesters PPC also shows no significant change in cell viability (Figure 4.5C). In addition, we have evaluated whether dose effect existed in both IGF1 and IGF2 on 1st trimester PPC cell viability. For IGF1, 50 ng/ml exerts the maximal effect

(1.5-fold, $P < .001$) (Figure 4.5D) while there is no dose dependent effect for IGF2 (Figure 4.5E).

Exogenous 1 ng/ml IGF1 produced an augmentation effect on PDX1 expression on 2nd trimester ICC (>7-fold, $P < .05$ vs. no IGF1 control) but not 1st trimester ICC (Figure 4.6A), thus, we have chosen 2nd trimester for subsequent IGF1 differentiation analysis. In the case of IGF2, both trimesters ICC also shows no significant change in PDX1 expression (Figure 4.6A). Following 1 ng/ml IGF1 treatment, ICCs showed a significant upregulation in mRNA expression of such developmental genes as PDX1 (8-fold, $P < .01$), NGN3 (16-fold, $P < .001$), ISL-1 (6.5-fold, $P < .05$), NKX2.2 (4-fold, $P < .01$), NKX6.1 (11-fold, $P < .01$), NEUROD (15-fold, $P < .05$) (Figure 4.6B) as well as functional genes GLUCOKINASE (>40-fold, $P < .01$) and INSULIN (10-fold, $P < .01$) (Figure 4.6C). In addition, we have evaluated whether dose effect existed in both IGF1 and IGF2 on PDX1 expression of 2nd trimester ICC. For IGF1, 50 ng/ml exerts the maximal effect (20-fold, $P < .001$) while there is no dose dependent effect for IGF2 (Figure 4.6D and 4.6E).

4.4.6 Neutralizing antibodies against IGF1R inhibit ICC differentiation

Given that only IGF1 but not IGF2 appears to have measurable effects on PPC growth and ICC differentiation, blockade of IGF1R using MAB391 neutralizing antibodies should selectively reveal the effects of IGF1 on PPC growth and ICC differentiation. We observed that the growth effect triggered by 50 ng/ml IGF1 on PPCs reported above was inhibited in the presence of .01 μg/ml MAB391 (Figure 4.7A). The growth effects on PPCs triggered by LSC co-culture, however, were not inhibited by MAB391 (data not shown). Meanwhile, the enhanced expression of PDX1 mRNA in ICCs induced by IGF1 was reduced by 50% ($P < .001$) in the

presence of exogenously addition of 1 μg/ml MAB391 (Figure 4.7B). Exogenous addition of 1 μg/ml MAB391 reduced PDX1 mRNA expression to 33% of control levels in ICCs co-cultured with 2nd trimester LSCs ($P < .001$ vs. no MAB391) (Figure 4.7D), but not in ICCs co-cultured with 1st trimester LSCs (Figure 4.7C).

4.5 Discussion

This work is the first study to demonstrate that a co-culture system with primary LSCs isolated from human fetal liver can enhance PPC growth and ICC differentiation. There were several important aspects of our results that should be noteworthy. Firstly, we observed better differentiation using co-cultures with 2nd trimester fetal liver derived LSCs than with co-cultures containing 1st trimester fetal liver derived LSCs. This LSC gestational-age effect should not be surprising given that glucagon and insulin are expressed in human endocrine cells during the 2nd trimester, but not during the 1st trimester (Sarkar et al., 2008). The extreme rarity of abortions beyond 16 gestational weeks has so far precluded the possibility of conducting investigations employing cells from more mature fetal tissues. In addition, methodologically, it is important to observe that these enhancing effects were obtained in the absence of exogenous addition of growth factors. Moreover, the use of human cells, rather than mouse cells, avoids translational challenges related to inherent differences between human and mouse tissues (Joglekar et al., 2009).

The human fetal liver stroma derived LSCs supporting PPC growth and ICC differentiation in the present study consisted of a cell population defined by cells adherent to culture plates and positive for the stromal cell marker vimentin (Krebsbach et al., 1999), the fetal liver cell markers Dlk, EpCAM, N-CAD, CK18, CK19 and CD45 (Zhang et al., 2008; Tanimizu et al., 2004; Jensen et al., 2004) and the primitive cell markers CD29, CD90, CD105, and CD199 (Krebsbach et al., 1999), while being negative for AFP and albumin (hepatoblast and hepatocyte markers, respectively) (Zhang et al., 2008; Tanimizu et al., 2004; Jensen et al., 2004). The cell isolation and culture methods employed in these experiments were investigated extensively by other groups and the resultant LSC populations were found to be

capable of hematopoiesis (Lee et al., 2011; Ledran et al., 2008). Similar to hematopoiesis, we found that our LSC co-culture consistently improved ICC differentiation in the presence of differentiation cocktail, as evidenced by increased mRNA expression of developmental genes (PDX1, NGN3, NKX2.2, and NKX6.1) and functional genes (GLUCOKINASE and INSULIN).

The present observations show that human fetal liver derived LSC co-culture enhances PPC cell number and viability as well as enhancing the expression of PPC-derived ICC developmental and functional genes. These indicate that LSC co-culture increases PPC proliferation and induces ICC differentiation. In particular, 2nd trimester LSC, devoid of growth factors, were demonstrated to be capable of inducing greater upregulation of developmental and functional genes in ICC than differentiation cocktail previously described (Suen et al., 2008; Ma et al., 2011; Ng et al., 2011; Leung et al., 2009). Moreover, differential expression of growth factors was found between 1st and 2nd trimester LSC in our system.

The discrepancy between 1st and 2nd trimester LSC may be due to the difference in cell population as they were isolated from different gestational week livers with variable degree of maturity (Campagnoli et al., 2000; Terrace et al., 2009). As a result, further clarification of their identity would be useful for further investigations.

Confirmation of ICCs with insulin-secreting ability is critical for translating scientific research into a clinical replacement therapy protocol. Although our co-culture ICCs were able to secrete insulin in response to potassium, they had a limited or negligible glucose responsive capability, similar to cells differentiated under other directed differentiation protocols (Segev et al., 2004; Brolén et al., 2005). These results suggest that fetal LSCs alone, at least with the current protocol, cannot induce the formation of fully mature and functional ICCs. This may be due to the absence of

critical factors derived from a fully functional liver (e.g. vitamin A, D (Ng et al., 2011) in stellate cells and neuronal signals (Imai et al., 2008)), other supporters (e.g. epithelial cells of blood vessel (Ranjan et al., 2009; Lammert et al., 2001)), and others unknown reasons. With our preliminary efforts in seeking for additional supporter cells, it is plausible to propose that a mixed source of cells, i.e. LSC and bone marrow stromal cells (BMSC) co-culture, could enhance greater up-regulation of PDX1, NGN3, and GLUCOKINASE in ICCs than a single cell type (Suppl. Figure 1A).

Immuno-property changes are an important consideration in the development of stem cell therapy. The slightly elevated MHC-I and MHC-II mRNA expression observed in ICCs after LSC co-culture may be due to further ICC maturation. As PPCs showed no drastic change in MHC-I and MHC-II mRNA expression, cells co-culture obtained from different fetus appeared not to affect the immunogenicity. In combination with our previous study on reduced immunogenicity in 1st trimester PPC (Ma et al., 2011), an investigation into using 1st and 2nd trimester PPC with LSC co-culture, coupled with extra immuno-marker expression, would assist in reducing immuno-rejection for future transplantation.

RT-PCR experiments were used to screen for potential expression of developmentally critical candidate molecules. Of the many factors previously described as related to islet development or functions, (i.e., SCF, NGF, VEGFA, FGF1, FGF2, FGF7, IGF1, IGF2, TGFb, HGF, IL-6, BMP2, BMP4, BMP6) (Ranjan et al., 2009; Lammert et al., 2001; Li et al., 2006; Kanaka-Gantenbein et al., 1995; Movassat et al., 2003; Ameri et al., 2010), we found that the expression of four molecules, i.e. IGF1, IGF2, BMP4, and BMP6, differs significantly between 1st and 2nd trimester LSCs. Thus, the altered expression of these four factors could explain, at least in part, the LSC gestational age-related difference in enhancement of ICC differentiation. In

this regards, our subsequent experiments identified a positive role for IGF1 and negative roles of IGF2 and BMP4 in ICC differentiation (Suppl. Figure 4.1B & 4.1C).

The present results provide additional interesting data on the effects of IGF1, which has previously been reported to be necessary for pancreatic regeneration but to inhibit pancreatic development (Yu et al., 2003; Lu et al., 2004; Swenne et al., 1987; Rabinovitch et al., 1982; Wang et al., 1995; Smith et al., 1991; Warzecha et al., 2003; Kulkarni et al., 2005). Contrary to reports of developmental inhibition in studies of liver IGF1-deficient (LID) (Yu et al., 2003) and pancreas IGF1-deficient (PID) mice (Lu et al., 2004), our data together with earlier findings from *in vitro* culture studies on rat fetal islets (Swenne et al., 1987; Rabinovitch et al., 1982), suggest that IGF1 enhances PPC growth and ICC differentiation. Notably, we showed here that IGF1 enhanced growth of 1st trimester PPCs and enhanced differentiation of 2nd trimester PPC-derived ICCs through IGF1R, but not vice versa. Hence, it appears that IGF1 exerts different effects on stem cells from different stages of gestation; such stage-specific effects may explain those apparently contradictory observations in regeneration and development of IGF1 (Yu et al., 2003; Lu et al., 2004; Swenne et al., 1987; Rabinovitch et al., 1982; Wang et al., 1995; Smith et al., 1991; Warzecha et al., 2003; Kulkarni et al., 2005). Different stem cells were identified in mature pancreas for regeneration (e.g. ductal stem cell (Wang et al., 1995)) and during fetal development (e.g. mesenchymal stem cell in pancreas (Sarkar et al., 2008)).

Since results obtained under culture conditions may not necessarily reflect the *in vivo* state, a rat neonatal streptozotocin regeneration model (Li et al., 2004) was then designed to address this issue (Suppl. Figure 4.2A and 4.2B). Our preliminary data showed that IGF1 expression in neonatal rat pancreas appears to temporarily increase (Suppl. Figure 4.2C), which was consistent with those in regenerating pancreas as

reported previously (Smith et al., 1991); these observations might be, at least partly, attributed to, stimulatory production of interleukin-10 that could be protective against inflammation (Warzecha et al., 2003). Interestingly, a temporary decrease in IGF1 expression was detected in neonatal rat livers (Suppl. Figure 4.2D) which may be related to regulation of homeostasis. However, IGF1 expression in liver did not exhibit an appreciable change in the adult regeneration model (Smith et al., 1991). Therefore, the decrease we observed in neonatal rats could reflect the 'mimicking' action of fetal IGF1 levels. Indeed, we found that ICCs exposed to higher levels of IGF1 (100 ng/ml) have lower expression of PDX1 than ICCs exposed to 50 ng/ml IGF1. Likewise, clinical studies have shown that human fetuses have lower circulating IGF1 levels (30-50%) than adults (Mark et al., 2008; Bennett et al., 1983). Nevertheless, extensive investigations are required for further confirmation of our speculation.

In conclusion, the present study has shown that human fetal LSC co-culture enhances PPC growth and ICC differentiation apparently through, at least in part, secretion of IGF1. Further investigations of the ability of stromal microenvironments in the induction of ICC differentiation are warranted, particularly with respect to determining how combining stromal cells from multiple sources may optimize *in vitro* induction of ICC differentiation. It is because optimal stromal-microenvironment has the potential for development into an efficient and cost-effective alternative, for obtaining transplantable ICCs, to the direct differentiation islet engineering and other stem cell therapies.

4.6 Supplementary Materials and Methods

4.6.1 Cell preparation and culture

Human fetal pancreata and livers used in these experiments were provided by the Department of Obstetrics and Gynecology at the Prince of Wales Hospital of the Chinese University of Hong Kong. The fetal organs were collected from fetuses obtained from surgical termination of pregnancy (STOP) by dilation and extraction at 9 - 16 weeks of gestation (CREC-2005.461).

PPC isolation and culture were performed as previously described (Suen et al., 2008; Ma et al., 2011; Ng et al., 2011; Leung et al., 2009). Briefly, the pancreata were minced and digested in 3 mg/ml collagenase P (Roche, Mannheim, Germany) at 37 °C for 6 min. The digested cell mixtures were re-suspended in modified RPMI medium supplemented with 10% fetal bovine serum (Gibco Life Technologies, Inc., Grand Island, NY), 10 mM HEPES buffer, 1 mM sodium pyruvate, 71.5 μM Beta-mercaptoethanol (Sigma, St Louis, MO) solution with 100 U/ml of penicillin G and 100 μg/ml of streptomycin (Gibco Life Technologies, Inc., Grand Island, NY) in non-adherent 60-mm dishes (Corning Incorporated, Corning, NY) at 37 °C. Round and non-adherent cell clusters formed within 48 h of incubation and 20 ng/ml basic fibroblast growth factor (Sigma, St Louis, MO) and epidermal growth factor (Invitrogen, Carlsbad, CA) were used to induce PPC outgrowth. Cells from passages 3 - 10 were used for the experiments.

All experiments were performed using LSCs prepared from one isolated human fetal liver. Briefly, the isolated liver was immersed in chilled DMEM/F12 1:1 medium, and then minced and digested in 5 mg/ml collagenase A (Roche, Mannheim, Germany) at 37 °C for 60 min. The digested cell mixture was re-suspended in modified DMEM/F12 1:1 medium supplemented with 10% fetal bovine serum, 100 U

of penicillin G, and 100 μg/ml of streptomycin sulfate (Gibco Life Technologies, Inc., Grand Island, NY) in T75 culture flasks (Corning Incorporated, Corning, NY) at 37 °C for 24 h. Non-adhesive cells were discarded and a monolayer of cells adhered to the culture flask. Upon reaching 80% confluence (~3 day), the monolayer of cells was harvested with TRYPLE (Gibco Life Technologies, Inc., Grand Island, NY) and replated for up to 20 passages. Cells from passages 3 - 10 were used for the experiments.

Caco-2 cells were grown in EMEM medium (Sigma, St Louis, MO) supplemented with 2 mM Glutamine, 1% NEAA, 1% P/S, and 10% FBS, while hepG2 cells were grown in DMEM medium (Sigma, St Louis, MO) supplemented with 1% P/S and 10% FBS. The Caco-2 and hepG2 cells served as positive controls in the RT-PCR experiments.

4.6.2 In vitro ICC differentiation

PPC derived ICC differentiation was performed as previously reported (Suen et al., 2008; Ma et al., 2011; Ng et al., 2011; Leung et al., 2009). Briefly, PPCs (9 to 15-week gestation) were trypsinized and suspended in ultra-low attachment plates (Corning Incorporated, Corning, NY) for 8-day differentiation in high glucose (11 mM) DMEM/F12 medium supplemented with 1× B27, 0.05% bovine serum albumin (BSA), 10 ng/mL hepatic growth factor (HGF), 10 nM exendin-4, 500 pM betacellulin, and 10 mM nicrotinamide. LSCs were seeded on Transwells (Millipore Filter Corporation, Bedford, Mass.,) and co-cultured with ICC in ultra-low attachment plates (Corning Incorporated, Corning, NY) in high glucose (11 mM) DMEM/F12 1:1 medium with a basal energy source (1× B27, 0.05% BSA), with or without growth factors (differentiation cocktail), namely 10 ng/mL HGF, 10 nM exendin-4, 500 pM

betacellulin, and 10 mM nicrotinamide (Sigma, St Louis, MO). The formed ICCs were handpicked under an inverted microscope.

4.6.3 RNA expression analysis

Total RNA was extracted from PPCs, ICCs, and LSCs using Trizol (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol. First strand cDNAs were reverse transcribed using Superscript III (Invitrogen, Carlsbad, CA) from 3 μg of RNA in 20-μl reaction volumes. PCR was performed in 25-μl reactions with 1 μl of cDNA per reaction. Quantitative real-time PCR analyses of different genes were performed using an i-Cycler Thermal Cycle, version 3.1 (Bio-Rad Laboratory Incorporated, Munich, Germany). The reactions were performed in triplicate in 25-μl volumes with 1 μl cDNA, SYBR Green PCR Master Mix (Bio-Rad Laboratory Incorporated, Munich, Germany), and 0.3 μM of each primer (Invitrogen, Carlsbad, CA). Melt-curve data across temperature gradients (+0.5 ^{o}C every 10 s) were analyzed at the end of each RT-PCR experiment to guarantee the specificity of each amplified target PCR product. Amplification data were collected using an i-Cycler Detector and then analyzed using Sequence Detection System software. Transcript levels relative to those of β-actin were calculated using the comparative C_T method, with fold change defined as $2^{-\Delta\Delta Ct}$. Sequences of the primers employed in this study are listed in Table 1.

4.6.4 Immunocytochemistry

LSC and PPC monolayers were cultured on coverslips coated with 3% gelatin in PBS, and fixed with 4% paraformaldehyde in phosphate buffered saline (PBS) for 60 min at room temperature. Samples were permeabilized with 0.01% Triton-X in 1% BSA/PBS for 5 min at room temperature, blocked with 6% normal donkey serum in

1% BSA/PBS at room temperature for 1 h, and then incubated with rabbit anti-IGF1R (1:25 dilution) (Santa Cruz Biotechnology, Santa Cruz, CA), rabbit anti-DLK (1:500 dilution) (Abcam, Cambridge, UK), and rabbit anti-CD29 (1:100 dilution) (Abcam, Cambridge, UK) or mouse anti-CD90 (eBioscience, San Diego, CA), and mouse anti-AFP (Santa Cruz Biotechnology, Santa Cruz, CA) or goat anti-albumin (Santa Cruz Biotechnology, CA) primary antibodies at 4°C overnight. After several PBS washes, the samples were incubated with Alexa Fluor 568 donkey anti-rabbit antibodies (1:500) (Invitrogen, Carlsbad, CA), FITC-conjugated anti-mouse (1:200) or anti-goat (1:300) secondary antibody (Jackson ImmunoResearch, PA, USA) at room temperature for 1 h, and then incubated with 4',6'-diamidino-2-phenylindole (DAPI) (1:1000) (Invitrogen, Carlsbad, CA) for 15 min. Primary antibodies were omitted in negative controls. The slips were washed thoroughly with PBS several times, mounted in Vectashield, and imaged using a fluorescence microscope equipped with a DC 200 digital camera (Leica Microsystems, Buffalo Grove, IL). Different images were captured separately at different laser wavelengths and superimposed fro examination.

4.6.5 PPC viability and cell count assays

PPCs were plated at 1.5×10^5 cells per well in 6-well plates or 3×10^4 cells per well in 96-well plates with modified RPMI medium. Before LSC co-culture or treatment with human recombinant IGF1/IGF2 (Sigma, St. Louis, MO), PPCs were incubated in a serum free condition overnight to arrest the cell cycle. They were then co-cultured with LSCs or subjected to IGF1/IGF2 treatments (1, 10, 50, or 100 ng/ml) or MAB391 (0.001–10 μg/ml) (R&D Systems, Inc., Minneapolis, MN) plus IGF1 (50 ng/ml) for 3 d in modified RPMI medium with heat-inactivated FBS.

The MTT assay (Sigma, St. Louis, MO) was performed in accordance with the

manufacturer's protocol. Briefly, after removal of the modified RPMI medium, the cells were incubated with MTT solution (0.05 mg/ml) for 2.5 - 4 h at 37°C under 5% CO_2. The formazan that formed was immediately dissolved by addition of DMSO which was quantitated by measuring absorbance at 540 nm. For the cell counting assay, PPCs were visualized by Trypan Blue (Sigma, St. Louis, MO) and counted under a light microscope.

4.6.6 IGF1 and insulin ELISA

LSCs were cultured under DMEM/F12 1:1 medium supplemented with 1X B27 and 0.05% BSA, in the presence of high (11 mM) glucose. IGF1 assays (Ray Biotech, Inc., Norcross, GA) were performed according to the manufacturer's protocol using medium that had been collected immediately, chilled in ice and added to wells that were kept 4 °C overnight. The remaining procedures were performed at room temperature. Excess solution was discarded and the cells were washed with assay kit's Wash Solution. Biotin antibodies were added to each well and allowed to incubate for 1 h. Again, the solution was discarded and the cells were washed with the assay kit's Wash Solution. Streptavidin solution was added to each well and allowed to incubate for 45 min. TMB One-Step Substrate Reagent was added and allowed to react for 30 min. Stop solution was added and absorbance was measured immediately at 540 nm.

ICCs that formed were rinsed in PBS and incubated in Krebs-Ringer buffer supplemented with 10 mM HEPES and 0.1% BSA for 90 min. The ICCs were then serially subjected to low and glucose/potassium conditions and the solutions were collected after each exposure. The collected solutions were analyzed using an ultrasensitive human insulin ELISA (Mercodia, Uppsala, Sweden) according to the

manufacturer's protocol at room temperature. The collected solution and enzyme-conjugate solution were added to wells and incubated for 1 h. After discarding the excess solutions, and washing the ICCs with Wash Solution, substrate TMB was added to each well and allowed to develop for 30 min. Stop Solution was added and absorbance was measured immediately at 450 nm. ICCs were then lysed by Cytobuster Protein Extraction Reagent (Novagen, Darmstady, Germany) and total protein was quantified using a Bradford Assay (Bio-Rad Laboratory Incorporated, Munich, Germany).

4.6.7 Western blotting analysis

Total protein was extracted from PPC lysates using Cytobuster Protein Extraction Reagent (Novagen, Darmstady, Germany). Protein content was quantified using a Bradford Assay (Bio-Rad Laboratory Incorporated, Munich, Germany). Western blotting was carried out as previously described[11-15]. Briefly, total protein (10 μg/lane) was subjected to electrophoresis on a 12% (wt/vol) SDS-polyacrylamide gels and electrotransferred to nitrocellulose membranes (Bio-Rad Laboratory Incorporated, Munich, Germany). The membranes were blotted using 10% skim milk in 0.1% Tween-20 in PBS (Invitrogen, Carlsbad, CA) for 1 h at room temperature. The membranes were then incubated with rabbit anti-IGF1Rb (c-20) (1:100 dilution) (Santa Cruz Biotechnology, Santa Cruz, CA) or mouse anti-beta-actin (1:8000 dilution) (Abcam, Cambridge, UK), overnight at 4 °C followed by a peroxidase-labeled anti-rabbit IgG antibody (1:2600 dilution) or anti-mouse IgG antibody (1:2500 dilution) (Santa Cruz Biotechnology, Santa Cruz, CA) for 1 h at room temperature. Positive bands were revealed using Enhanced Chemiluminescense (ECL) detection reagents and autoradiography film (Amersham Pharmacia Biotechnology,

Buckinghamshire, UK).

4.6.8 Neonatal streptozotocin regeneration model

Pregnant Sprague-Dawley rats (time in pregnancy, TP-17) were obtained and caged individually with free access to standard diet and water. They were checked regularly from 09:00 to 18:00 daily for delivery of pups. One-day-old neonates were injected intraperitoneally with 100μg/kg body wt of streptozotocin (STZ) (Sigma, St. Louis, MO) freshly dissolved in 0.05mmol/l citrate buffer (pH 4.5). Blood glucose of all neonates after STZ treatment was measured by glucometer (Bayer Corp., Tarrytown, NY). Only those with blood glucose ranged 11.1mM - 19.44mM after the day of STZ treatment were used for subsequent experiments. RNA of pancreata and livers of neonates were collected and stored at for realtime PCR analysis. Every experiment was repeated with 3 neonates from different batches.

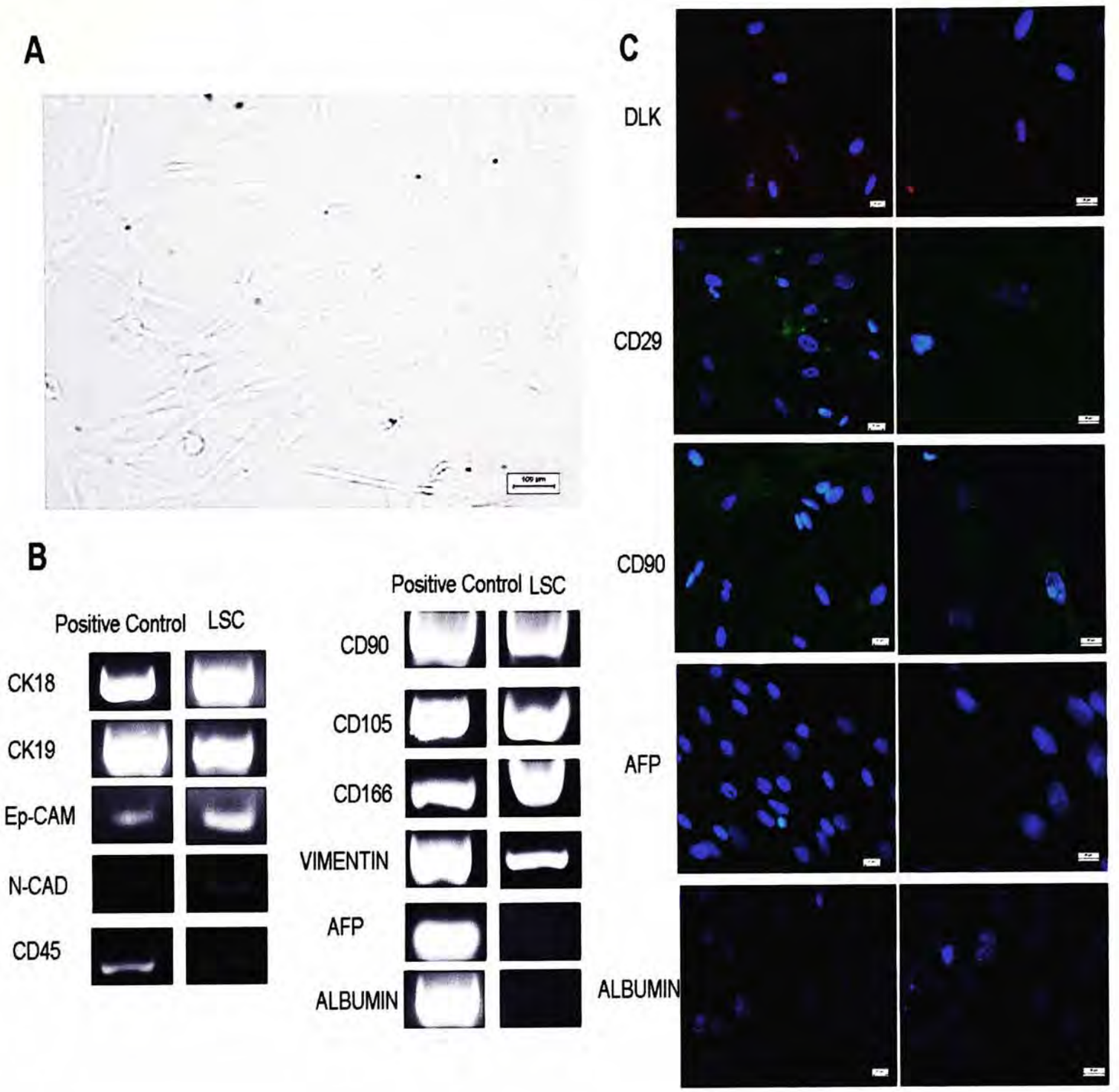


Figure 4.1 Characterization of LSCs isolated from human fetal liver..

All PPC (N = 3) and LSC (N = 3) ranged from 9 – 15 weeks were used for all the experiment. (A) Fibroblast-like shaped LSCs on plastic culture dishes. (B) Expression of selected liver precursors (CK18, CK19, Ep-CAM, N-CAD and CD45) and stromal cell markers (CD90, CD105, CD166 and VIMENTIN) was detected by RT-PCR while hepatoblast and hepatocyte markers (AFP and ALBUMIN) were not expressed. (C) Expression of DLK, CD29, CD90, AFP and albumin was examined by immunocytochemistry, where DLK, CD29 and CD90 were positively expressed and located in the cell cytoplasm and cell membrane while AFP and albumin were negatively expressed. Magnification of objective lens at 10×, 40×, and 63×.

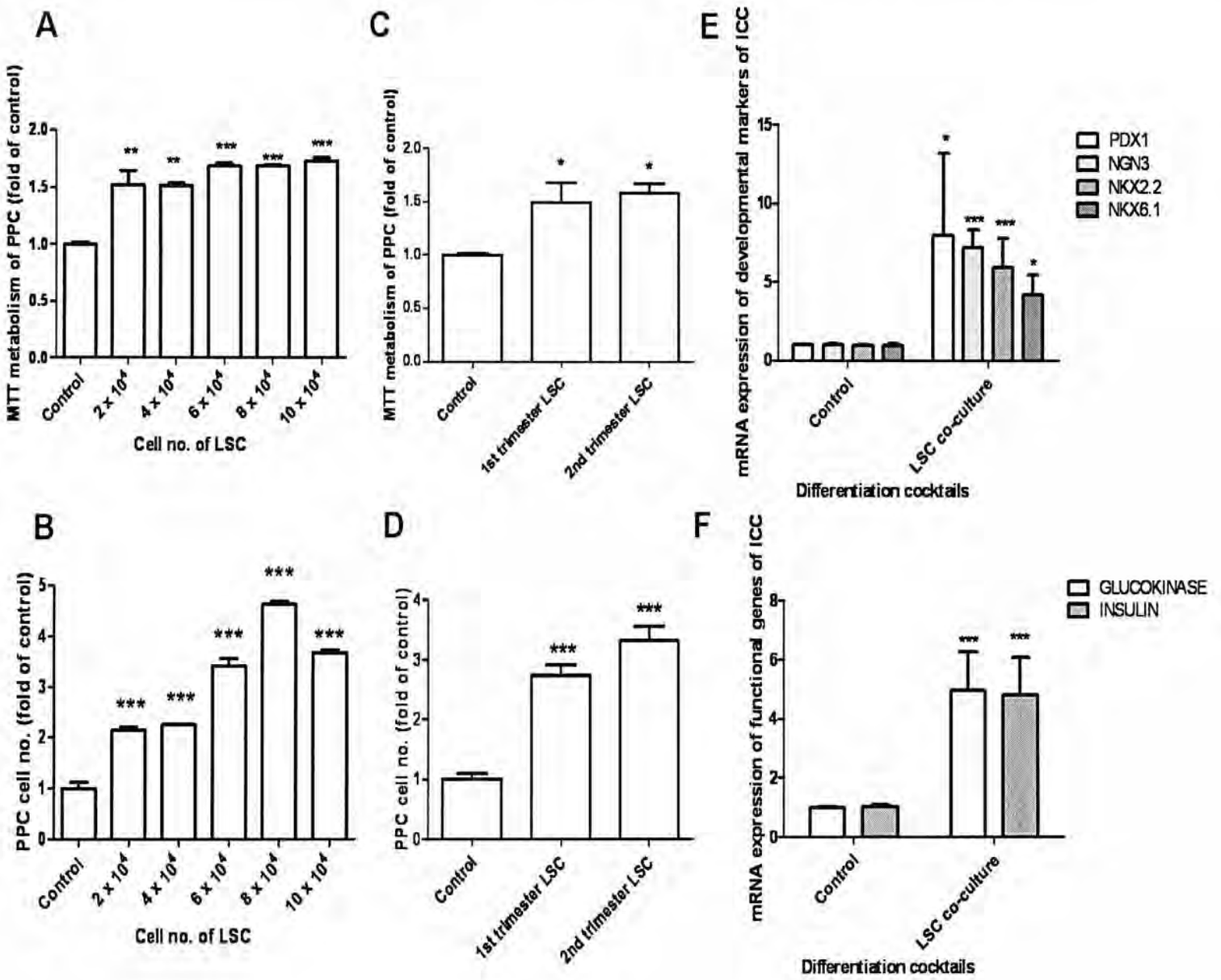


Figure 4.2 LSC co-culture enhances PPC growth and promotes ICC differentiation in the presence of differentiation cocktail.

All PPC (N = 3) and LSC (N = 3) ranged from 9 – 16 weeks were used for the experiment unless specified as 1st and 2nd trimester LSC (N = 3 from each group). In general, (A) MTT metabolism and (B) cell number increase while co-culture with LSC and MTT metabolism (1.5-fold) and cell number (4.5-fold) peaked at LSC co-culture with 8 × 104 cells. (C) MTT metabolism and (D) cell number were not significantly changed in PPCs when co-cultures with 1st and 2nd trimester LSCs. ICCs incubated in LSC co-cultures with a differentiation cocktail were found to have higher mRNA expression levels of (E) several developmental markers (PDX1, NGN3, NKX2.2 and NKX6.1) and (F) functional genes (GLUCOKINASE and INSULIN) than ICCs induced by differentiation cocktail alone. All data are expressed as means ± SEM for three experiments in each group. *$p < .05$, **$p < .01$ and ***$p < .001$ vs. Control.

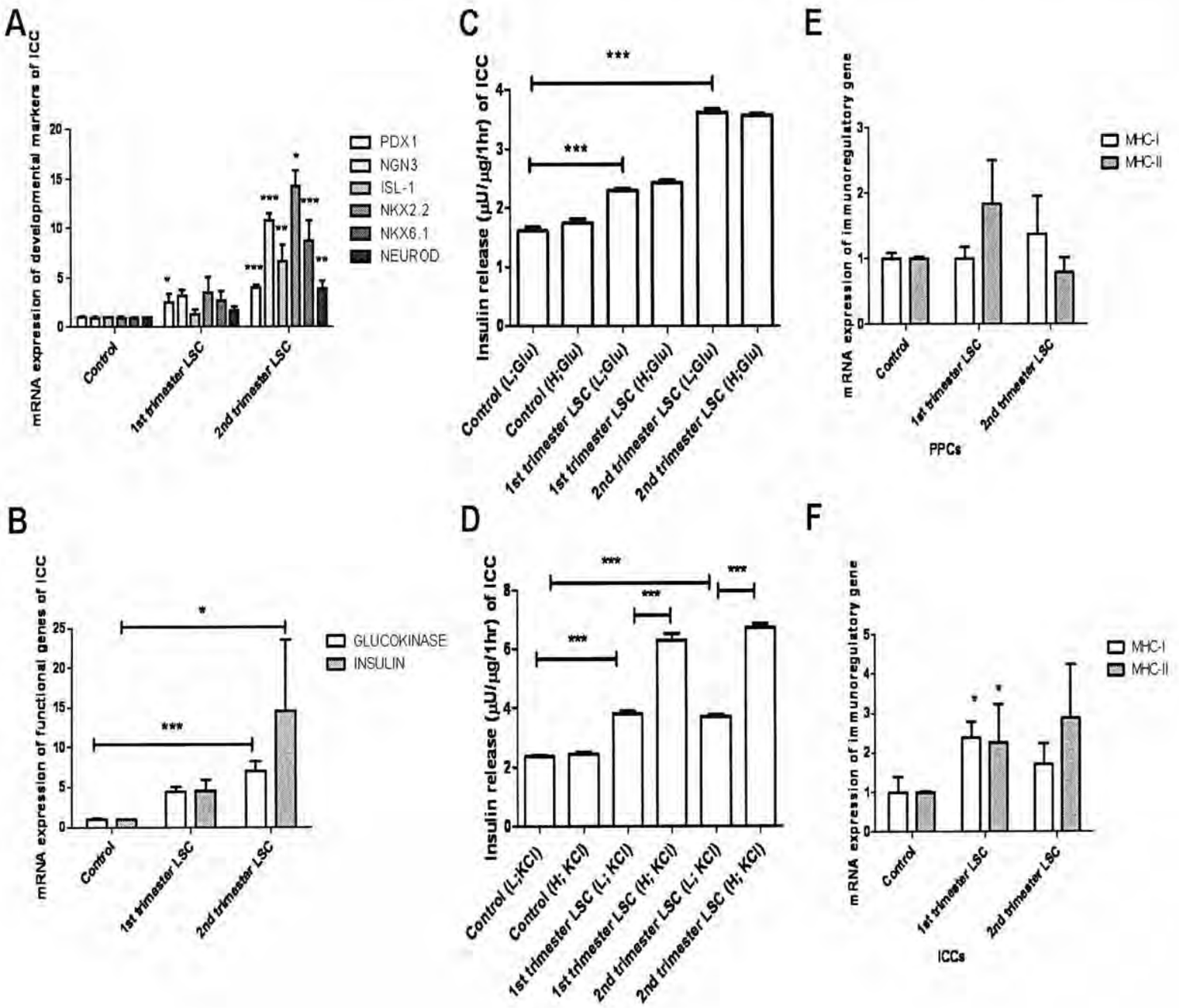


Figure 4.3 Characterization of ICCs grown in LSC co-culture.

All PPC (N=3) ranged from 9 – 16 weeks were used for the experiment while LSC were divided into 1st and 2nd trimester (N = 3 from each group). (A) Expression of developmental genes (PDX1, NGN3, ISL-1, NKX2.2, NKX6.1 and NEUROD) and (B) functional genes (GLUCOKINASE and INSULIN) in ICCs co-cultured with 1st trimester LSCs in the absence of differentiation cocktails only a slight up-regulation from that of ICCs induced by differentiation cocktails (Control) while co-cultured with 2nd trimester LSCs showed a significant up-regulation. Compared to Control, ICC with LSC co-cultures were found to have (C) higher basal insulin secretion level and (D) better response to KSIS. The mRNA expression of MHC-I and MHC-II in both (E) PPCs and (F) ICCs incubated in LSC co-cultures were not significantly altered relative to non-co-culture Control. All data are expressed as means ± SEM for three experiments in each group. *$p < .05$, **$p < .01$ and ***$p < .001$ vs. Control.

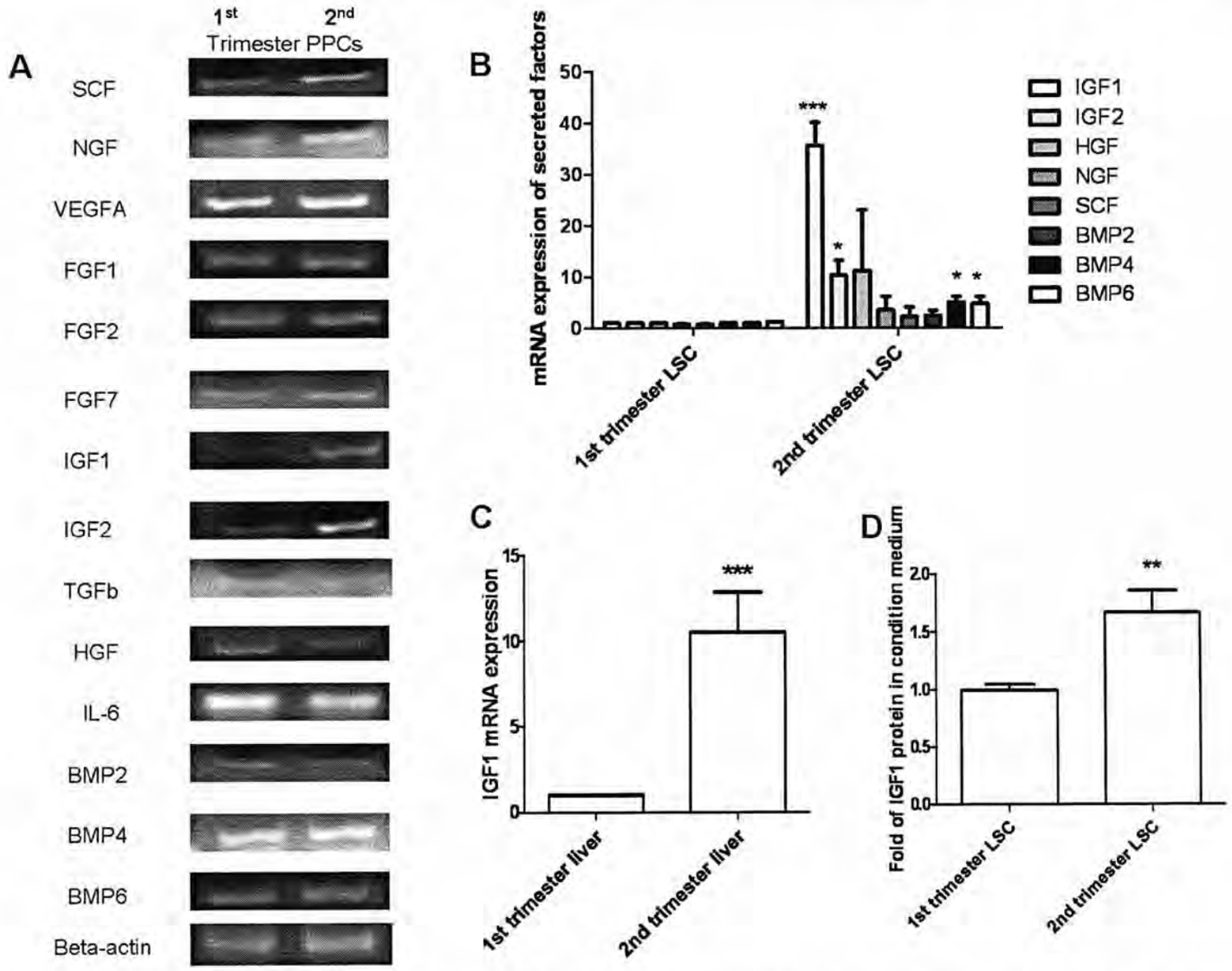


Figure 4.4 Expression of selected cytokines and growth factors in LSCs derived from 1st and 2nd trimester livers.

LSC used in this part were divided into 1st and 2nd trimester (N = 3 from each group). (A) mRNA expression profile of selected cytokines and growth factors (SCF, NGF, VEGFA, FGF1, FGF2, FGF7, IGF1, IGF2, TGFb, HGF, IL-6, BMP2, BMP4 and BMP6) in LSCs by RT-PCR. (B) Differential expression of selected cytokines and growth factors (IGF1, IGF2, HGF, NGF, SCF, BMP2, BMP4 and BMP6) between 1st and 2nd trimester LSCs. (C) Greater IGF1 mRNA expression in 2nd trimester versus 1st trimester human fetal livers. (D) The IGF1 protein content in 2nd trimester LSC conditioned medium was greater than that in 1st trimester LSC conditioned medium. All data were expressed as means ± SEM; N = 3 per group. *p < .05, **p < .01 and ***p < .001 vs. 1st trimester LSCs.

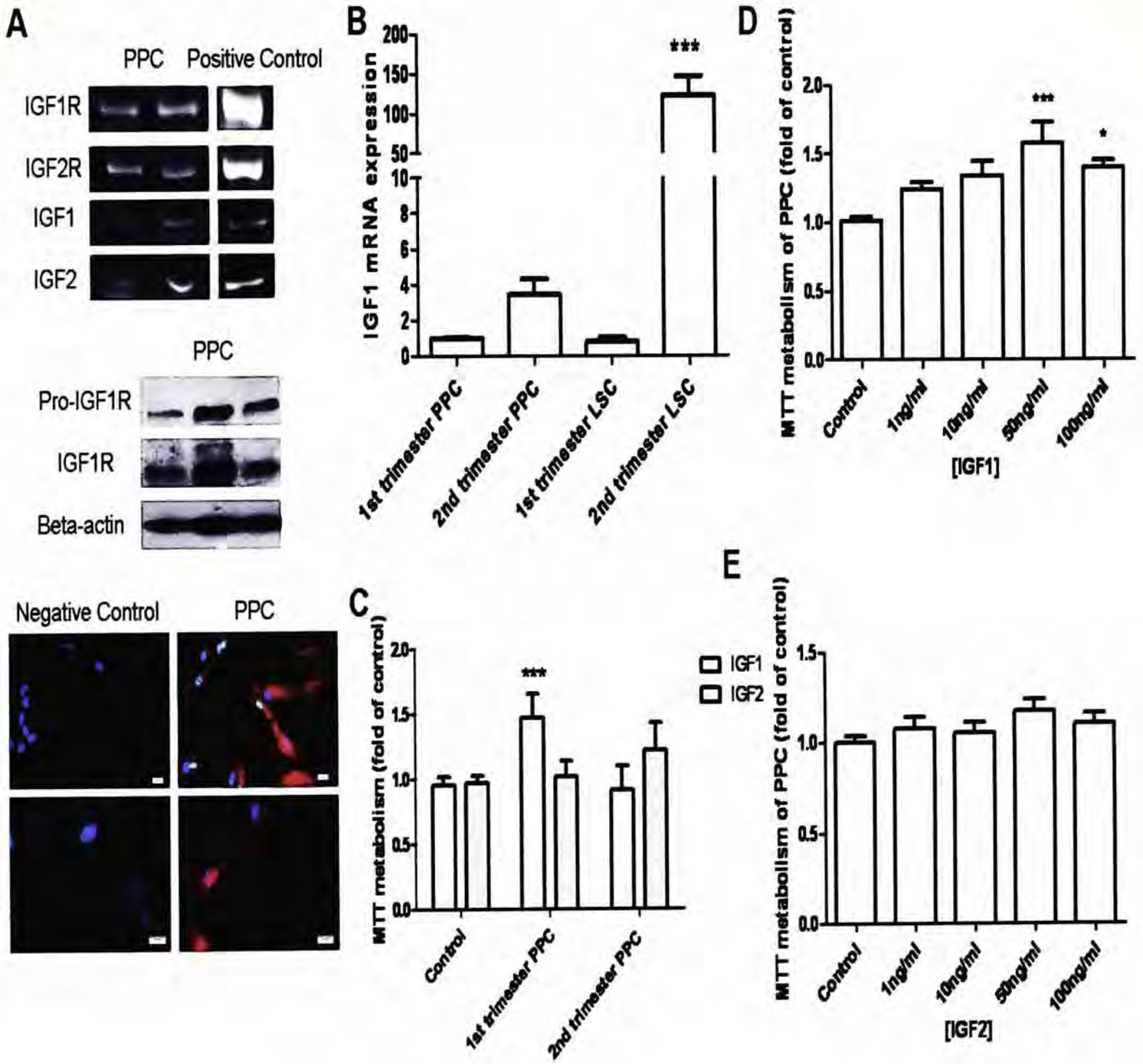


Figure 4.5 IGF1R characterization in PPC and IGF1 but not IGF2 enhances PPC growth.

PPC and LSC used in this part were divided into 1st and 2nd trimester (N = 3 from each group). (A) Expression of IGF1R, IGF2R, IGF1, and IGF2 mRNA, IGF1R proteins, and their localization in PPCs. (B) Differential expression of IGF1 in PPC and LSC derived from 1st and 2nd trimester fetal tissues. (C) Growth effect of PPC was only observed in 1st trimester PPC but not 2nd trimester PPC. (D) Dose dependent effect of IGF1 and (E) no effect of IGF2 on PPC viability as indexed by MTT metabolism. All data are expressed as means ± SEM; N = 3 per group. *p < .05, **p < .01 and ***p < .001 vs. Control.

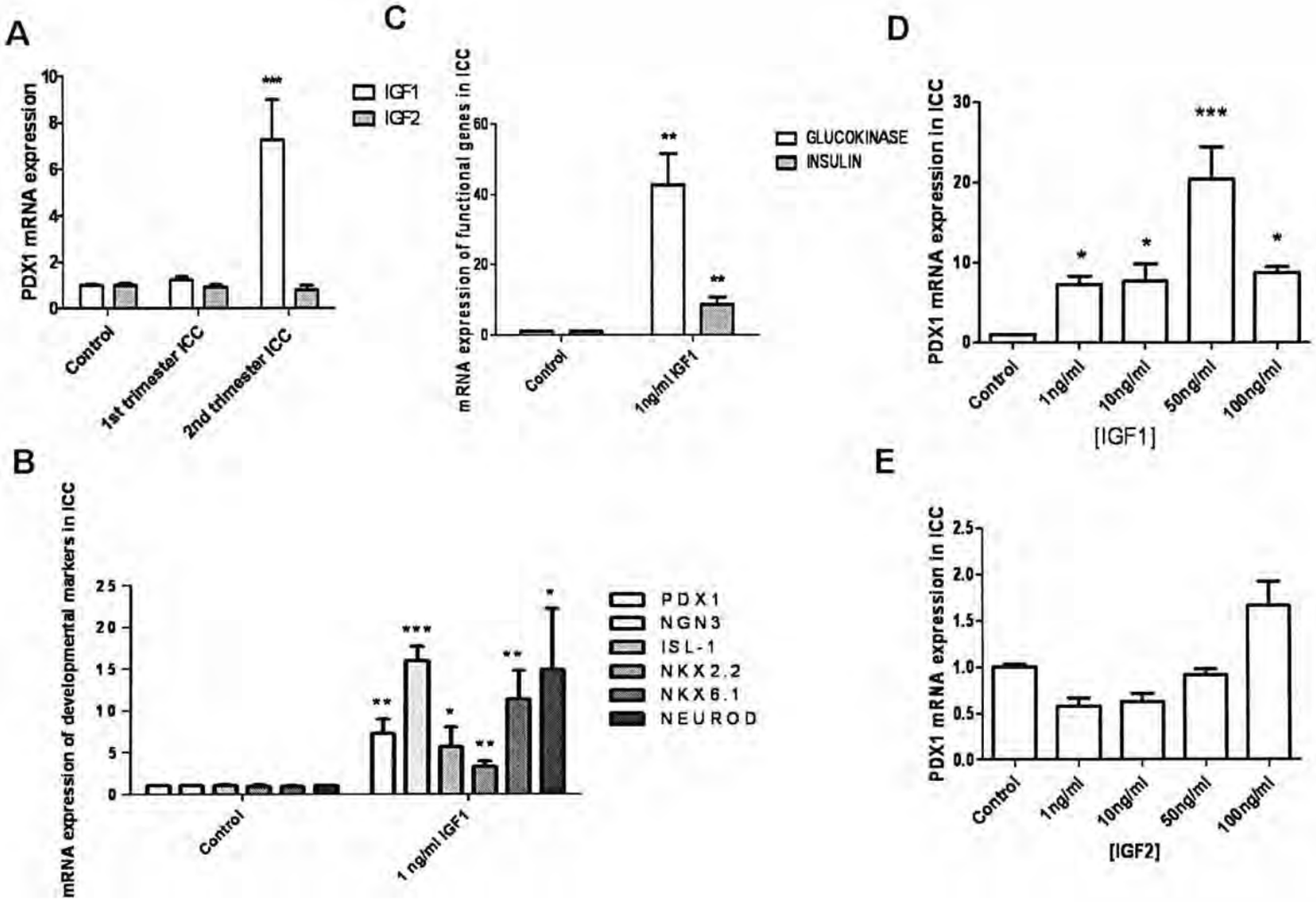


Figure 4.6 IGF1 but not IGF2 enhances PPC derived ICC differentiation.

PPC used in this part were divided into 1st and 2nd trimester (N = 3 from each group). (A) Enhanced PDX1 expression was only observed in 2nd trimester ICC but not 1st trimester ICC. (B) Dose dependent effects of IGF1 and (C) no effect of IGF2 on PDX1 expression in ICC was observed. ICCs that were differentiated under the influence of exogenous IGF1 (1 ng/ml) had higher expression levels of several (D) developmental markers (PDX1, NGN3, ISL-1, NKX2.2, NKX6.1 and NEUROD) and (E) functional genes (GLUCOKINASE and INSULIN) than ICCs that were differentiated in the absence of exogenous IGF1 (Control). All data are expressed as means ± SEM; N = 3 per group. *$p < .05$, **$p < .01$ and ***$p < .001$ vs. Control.

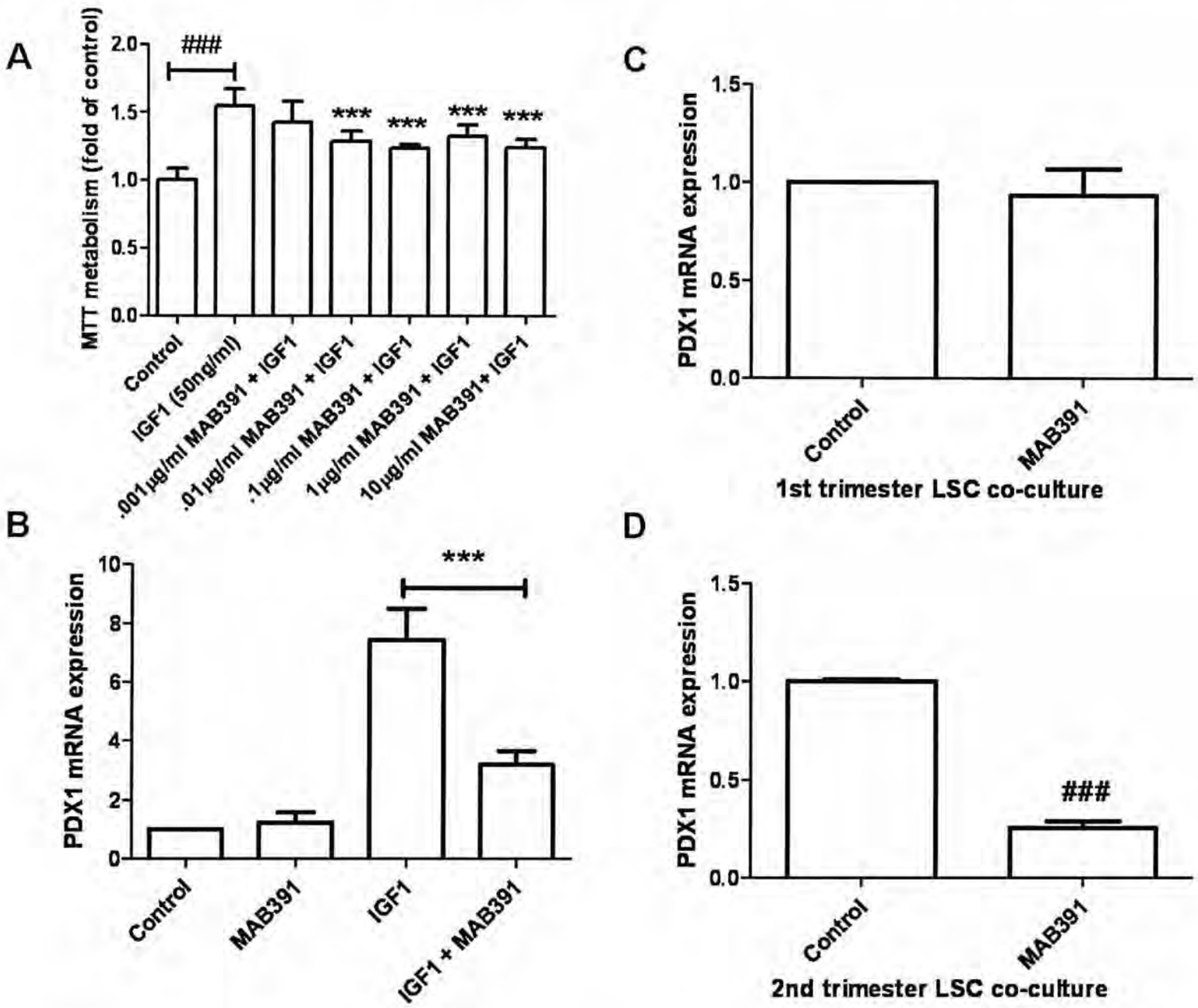
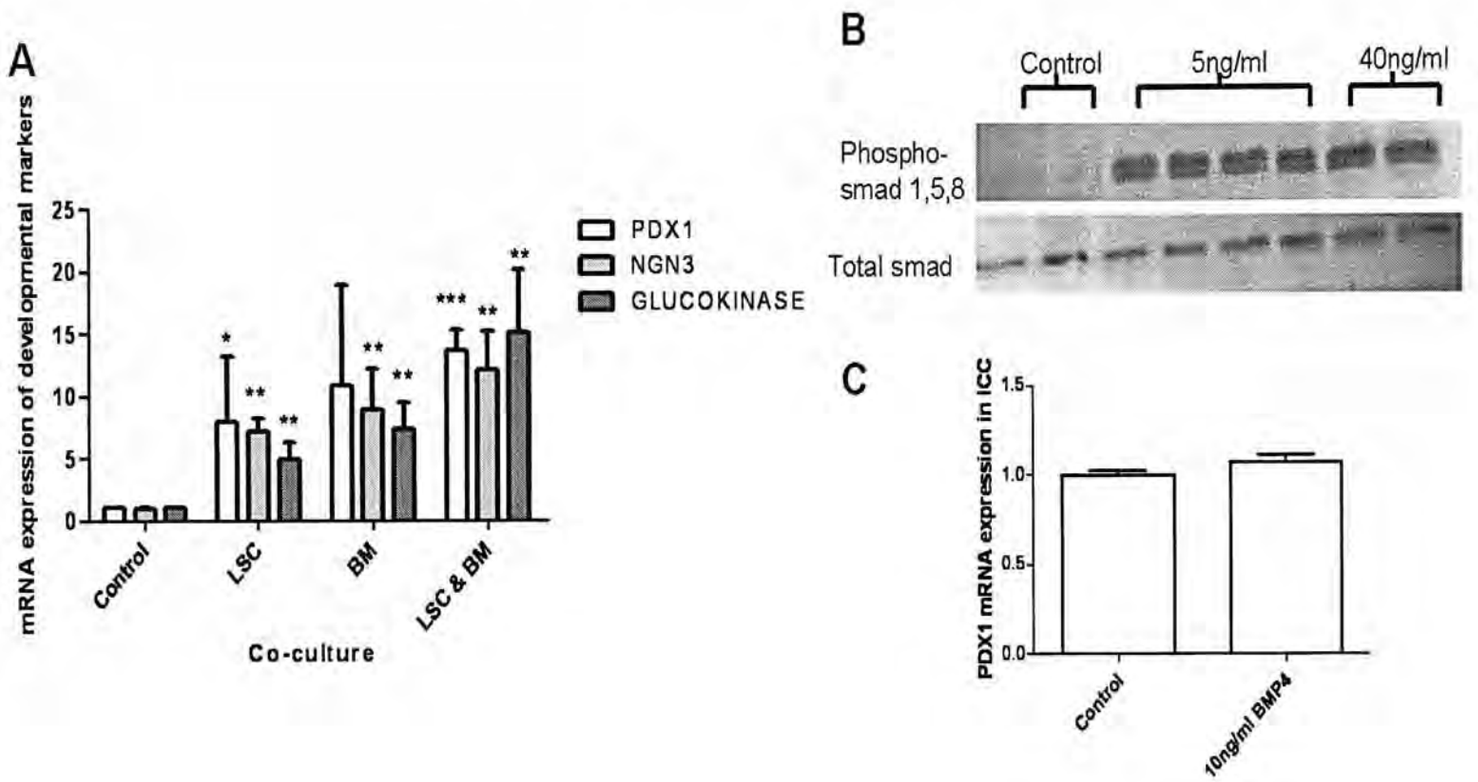
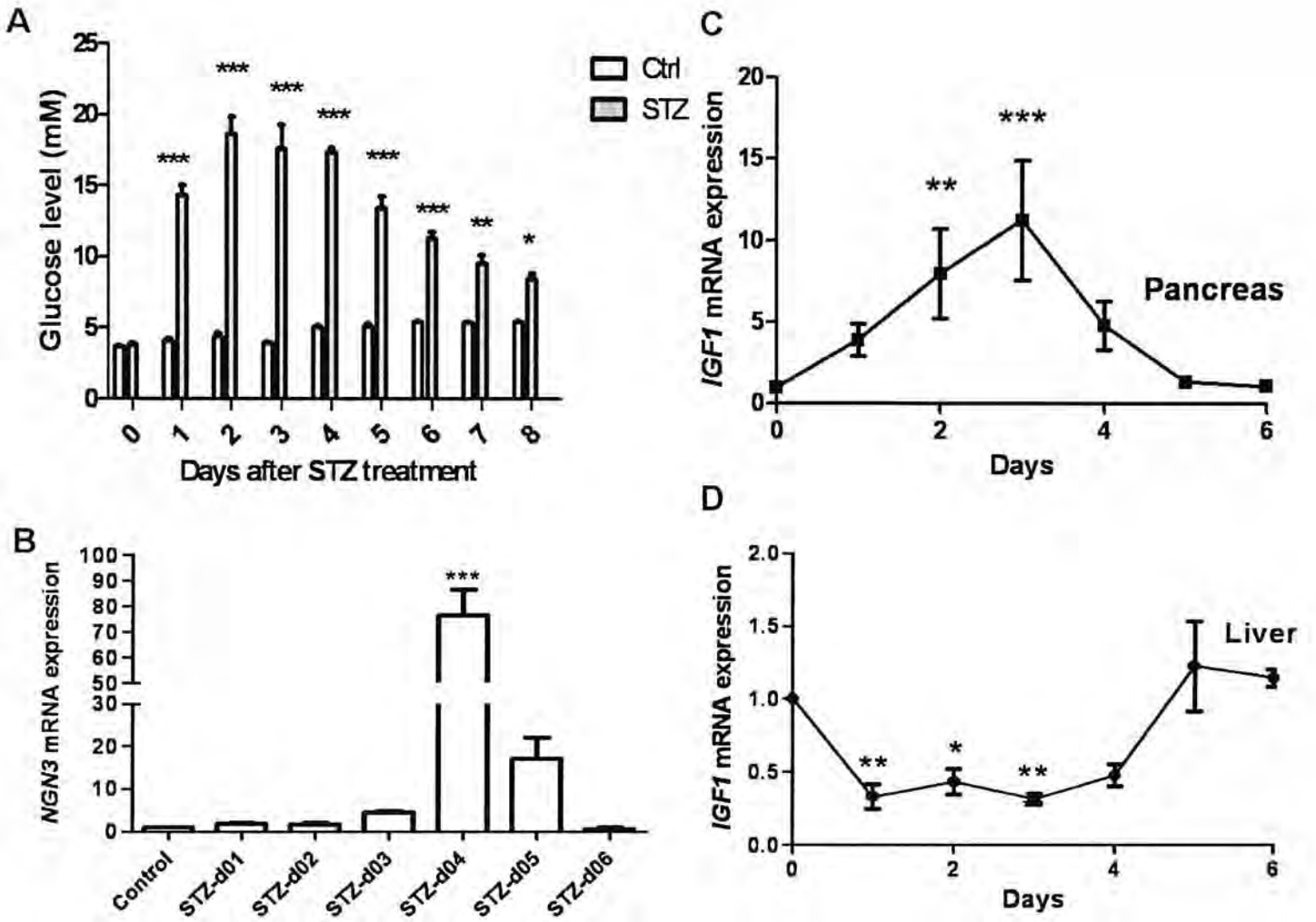


Figure 4.7 The IGF1R neutralizing antibody MAB391 inhibits PPC growth and ICC differentiation.
PPC used in (A) was derived from 1st trimester while in (B) was derived from 2nd trimester. In (C) and (D), PPC used were ranged from 9 -15 weeks. (A) The growth effect of IGF1 (50 ng/ml) on PPCs was inhibited in the presence of MAB391 (.01 – 10 µg/ml). (B) The enhanced PDX1 expression in ICCs produced by IGF1 (1 ng/ml) was inhibited in the presence of MAB391 (1 µg/ml). Addition of MAB391 (1 µg/ml) (C) had no effect on PDX1 mRNA expression in ICCs with 1st trimester LSC co-cultures but (D) reduced PDX1 mRNA expression in ICCs with 2nd trimester LSC co-cultures. All data are expressed as means ± SEM for three experiments in each group. *p < .05, **p < .01 and ***p < .001 vs. IGF1 treatment group while #p < .05, ##p < .01 and ###p < .001 vs. Control.



Supplementary Figure 4.1 Effects of BM co-culture microenvironment and BMP4 on ICC differentiation. PPC, LSC and BM (N=3) were derived from 9 – 20 gestational weeks. (A) A combined LSC plus bone marrow stromal cell (BMSC) co-culture upregulated developmental markers and functional genes (PDX1, NGN3 and GLUCOKINASE) more than co-culture with either alone. (B) Exogenous addition of BMP4 (5 ng/ml and 40 ng/ml) triggers phosphorylation of Smad1,5,8 in PPCs. (C) Exogenous addition of BMP4 (10 ng/ml) had no significant effects on PDX1 expression in ICCs. Each value represents the mean ± SEM of three separate experiments. *p < .05, **p < .01 and ***p < .001, vs. Control group.



Supplementary Figure 4.2 Expression profile of IGF1 in pancreas and liver across 6 days of neonatal regeneration

(A) One-day-old rat pups received injections of STZ (100 μg/kg) and their change of plasma glucose concentration was measured and peaked at day 2 after STZ treatment. (B) Ngn3 mRNA expression was assessed daily across 6 day of regeneration and peaked at d04 after STZ injection. IGF1 mRNA expression profile in (C) liver and (D) pancreas across the 6-day regeneration period. Each value represents the mean ± SEM of three separate experiments. *p < .05, **p < .01 and ***p < .001, vs. day0 or Control group.

House keeping gene	*Forward Primer*	*Backward Primer*	*Temp*	*Size*	*Reference*
h β -actin	TGGCACCACACCTTCTACAATGAGC	GCACAGCTTCTCCTTAATGTCACGC	/	/	15
h β -actin	TGTCCACCTTCCAGCAGATGT	CGGACTCGTCATACTCCTGCTT	/	/	15
r β -actin	AGCTATGAGCTGCCTGACG	GGATGCCACAGGATTCCA	/	/	61
Expression of rat gene					
rIGF-1	AAGCCTACAAAGTCAGCTCG	GGTCTTGTTTCCTGCACTTC	/	/	62
rIGF-1R	AAA ACC ATC GAT TCT GTG ACG	GGT TCT TCA GGA AGG ACA AGG	/	/	62
rNgn3	TGGCGCCTCATCCCTTGGATG	CAGTCACCTGCTTCTGCTTCG	/	/	63
Characterization of LSC					
hEp-CAM	GCTCTGAGCGAGTGAGAACC	ACGCGTTGTGATCTCCTTCT	60	116	/
hN-cadherin	CCTCCATGTGCCGGATAG	AATGCGATTTCACCAGAAGC	60	97	/
hVimentin	AAGCAGGAGTCCACTGAGTACC	GAAGGTGACGAGCCATTTCC	/	/	64
hCD45	AGCACCTACCCTGCTCAGAA	TTCAGCCTGTTCCTTTGCTT	/	/	65
hCD90	ATGAACCTGGCCATCAGCATCG	CACGAGGTGTTCTGAGCCAGCA	/	/	65
hCD105	GAAACAGTCCATTGTGACCTTCAG	GATGGCAGCTCTGTGGTGTTGACC	/	/	65
hCD166	TGATCTCCGCCACCGTCTTCAG	CTCTTTTCATCACTGATCCTTGCA	/	/	65
hAFP	GCAAAGCTGAAAATGCAGTTGA	GGAAAGTTCGGGTCCCAAAA	/	/	66
hAlbumin	CCAGGAAGACATCCTTTGC	CCTGAGCCAGAGATTTCC	/	/	67
hCK19	AGGTGGATTCCGCTCCGGGCA	ATCTTCCTGTCCCTCGAGCA	/	/	67
hCK18	TGGTCACCACACAGTCTGCT	CCAAGGCATCACCAAGATTA	/	/	67
Expression of soluble factors					
hHGF	CGAGGCCATGGTGCTATACT	TTCTCCTTGACCTTGGATGC	60	134	/
hVEGFA	CCTGCAAAAACACAGACTCG	GAGAGATCTGGTTCCCGAAA	60	137	/
hIGF1	GGAGGCTGGAGATGTATTGC	GCCAACCTTTCCTTCTCTGA	60	150	/
hIGF2	CGTTGAGGAGTGCTGTTTCC	GGGGTATCTGGGGAAGTTGT	60	130	/
hSCF	ACACCACTGTTTGTGCTGGA	GATTCCTGCAGATCCCTTCA	60	134	/
hGLP-1	CGTTCCCTTCAAGACACAGAG	GTGAATGTGCCCTGTGAATG	62	125	/
hPro-BTC	TGGGAATTCCACCAGAAGTC	CCTTTCCGCTTTGATTGTGT	59	96	/
hReg 4	AGGAGGAAGATGGCTTCCAG	GGTAAAACCATCCAGGAGCA	62	118	/
hFGF1	GGGCTTTTATACGGCTCACA	CTTGAGGCCAACAAACCAAT	60	126	/
hFGF2	TGGCTATGAAGGAAGATGGAA	ACTGCCCAGTTCGTTTCAGT	60	150	/
hFGF7	CAGGCAGACAACAGACATGG	CAGTTGCTGTGACGCTGTTT	60	87	/
hNGF	AGTGGTCGTGCAGTCCAAG	GTGGCCAGGATAGAAAGCTG	60	108	/
hGastrin	CCAGCCTCTCATCATCGAA	TCCATCCATCCATAGGCTTC	62	119	/
hPTHrP	GCTCAAGACACCTGGGAAGA	GGAGGTGTCAGACAGGTGGT	62	145	/
hIFNg	TTCAGCTCTGCATCGTTTTG	TCCGCTACATCTGAATGACCT	60	112	/
hIL6	GGCTGAAAAAGATGGATGCT	GCTCTGGCTTGTTCCTCACT	60	141	/
hFGF21	ACCTGGAGATCAGGGAGGAT	GCACAGGAACCTGGATGTCT	59	131	/
hTGFa	CCCTGGCTGTCCTTATCATC	CTGTTTCTGAGTGGCAGCAA	59	147	/
hTGFb1	GCAGGGATAACACACTGCAA	GGCCATGAGAAGCAGGAA	60	107	/
hTGFb2	ACAACACCCTCTGGCTCAGT	CTCCATTGCTGAGACGTCAA	60	135	/
hTGFb3	AGGAGATGCATGGGGAGAG	TTTAGGGCAGACAGCCAGTT	60	134	/
hTNFa	TCAGCCTCTTCTCCTTCCTG	GCCAGAGGGCTGATTAGAGA	60	124	/
hTNFb	AACCTGCTGCTCACCTCATT	TGCTCAAGGAGAAACCATCC	60	102	/
hBMP2	CGCAGCTTCCACCATGAAGAA	CCTGAAGCTCTGCTGAGGTGATA	/	/	68
hBMP4	AGGAGCTTCCACCACGAAGAAC	TGGAAGCCCCTTTCCCAATCAG	/	/	69
hBMP6	GTGAACCTGGTGGAGTACGACAA	AGGTCAGAGTCTCTGTGCTGATG	/	/	69
hBMP5	TTCTTCAAGGCGAGTGAGGT	AGGCTTTGGTACGTGGTCAG	/	/	70
hBMP7	CAGAGCATCAACCCCAAGTT	AGGATGACGTTGGAGCTGTC	/	/	70
hBMP9	TGTACAACAGGTACACGTCCG	TGAATGTCCTGGGACACCAG	/	/	69
Characterization of PPC					
hIGFR1	AGGATGCACCATCTTCAAGG	AATGGCGGATCTTCACGTAG	60	125	/
hIGFR2	GGCCCTGTTGCTCTACAAGA	CCATCAGCCACTCTGTTTCA	60	149	/
Developmental markers of ICC					
hPDX1	ACTCCACCTTGGGACCTGTTT	TTAAGGTACTCGGCCCAGCTT	/	/	15
hNGN3	TGTGGGTGCTAAGGGTAAGG	GGGAGAAGCAGAAGGAACAA	/	/	15
hNEUROD	TCCAAAATCGAGACTCTGCGC	GCAAAGCGTCTGAACGAAGGA	/	/	15
hISL-1	GATCAAATGCGCCAAGTGCAG	CAGCGGAAACACTCGATGTGA	/	/	15
hNKX2 2	TCTCCTTGGAGTGGCAGATTC	AAACACGGCGTAGAGTTCAGC	/	/	15
hNKX6.1	GACGGGAAGAGAAAACACACG	ACTCTCTGTCATCCCCAACGA	/	/	15
hGLUCOKINASE	CTCCATGGGGAAGTGCTC	CACCGAAAAACTGAGGGAAG	/	/	15
hINSULIN	CAGCCTTTGTGAACCAACACC	GGTCTTGGGTGTGTAGAAGAAGC	/	/	15

Table 4.1 Sequence of the specific PCR primers and their expected product size of different target genes.

Chapter 5

General Discussion and Future Studies

5.1 General Discussion

Diabetes, characterized by insufficient insulin for glycemic control due to the absence or malfunctioning β-cells (Alsaleh et al., 2010; Vantyghem et al., 2011; Richter et al., 2011) can be treated by replacement therapy such as insulin injection, pancreas transplantation and islet transplantation (Vantyghem et al., 2011). However, due to unphysiological nature and difficulty in glycemic control, insulin injection is not a very suitable replacement therapy. Comparatively, transplantation of pancreas or islets provides a physiological and reliable glycemic control as well as minimizing chronic complications arsing from diabetes (Vantyghem et al., 2011; Richter et al., 2011). Yet, owing to the lack of pancreas or islets donations, transplantation is hampered and alternative substitution is required. Intensive research has been focused on newly developed stem cell therapy (Hori et al., 2009) and many laboratories have paid considerable time and effort in islet engineering; yet, there are still a lot of difficulties encountered (Leung et al., 2009; D'Amour et al., 2006; Segev et al., 2004; Brolén et al., 2005); they include, for example, unresponsiveness to glucose, inefficient and complicate stepwise induction process, as well as involvement of costly growth factors (Leung et al., 2009; D'Amour et al., 2006; Segev et al., 2004). These issues have adversely affected the clinical applications of islet engineering for transplantation.

Existence of the differences in islet architecture and differential flexibility for differentiation of ESC derived from different species make research difficult to directly translate to human. Avoidance of translational challenge (Joglekar et al., 2009; Cabrera et al., 2006), it is plausible to use stem cells that derived from human. In view of the less flexibility of adult stem cells and tumoriogenecity of ESCs, we have used human fetal pancreas derived PPC and fetal liver derived LSC, which strike a balance between flexibility and tumoriogenecity, to address our hypothesis in this study.

In view of the lacking of pancreatic developmental clues in relations to vitamin A, vitamin D and liver stroma, we have firstly investigated the potential roles of vitamin A and vitamin D and their interactions in regulating islet cell development. Secondly, we have investigated effects of the presence of LSC on ICC differentiation. To our surprised, LSC microenvironment can be made used as a novel induction technique devoid of exogenous addition of growth factors for islet engineering with higher efficiency, convenience and economy. The data we obtained may contribute to future direction and formulation of islet engineering.

5.1.1 Proliferative effects and enhanced expression of NGN3 by vitamin A and vitamin D

The expression of RAR, RXR and VDR in the nucleus, cytoplasm and plasma membrane has indicated that their respective ligands, atRA and calcitriol, may exert certain effects in PPC. With no variations in expression of these receptors between different gestational age of PPC donors, vitamins A and D are likely to have consistent actions in PPC preparations from 9 to 15 weeks gestational age donors. Interestingly, down regulatory system, CYP26 and CYP24, catabolic enzymes of vitamin A and vitamin D, respectively, were not detected in PPCs. It has been reported that vitamin A and vitamin D act in an antagonistic manner (Rohde et al., 2005), however, this was not true in our case. This may be explained by the existence of another regulatory system or their metabolic enzymes are only expressed in the presence of their respective ligands (Feldman et al., 2005; Ozpolat et al., 2005) that required further investigations.

In consistent with the known dose-dependent effects of morphogen, atRA (Tocci et al., 1996), PPCs also exerted differential growth effects across different concentration of atRA (10^{-12}–10^{-6} M), peaking at 10^{-12}M. The increase in viability was partially contributed to cell proliferative and anti-apoptotic effect. Different from atRA, calcitriol only exerted growth effect on PPC at concentrations of 10^{-13}–10^{-14} M, i.e. about the physiological level of fetal serum (Tsang et al., 1981) and its effect was via cell proliferation.

As there are no effective commercial blockers or antagonists available for RAR and VDR, it is difficult to determine their functional roles with their ligands. However, up-regulation of RAR and VDR expression in response to atRA and calcitriol, respectively, suggests that atRA and calcitriol exert their effects probably via binding to response elements by respective receptors (Feldman et al., 2005; Tsang et al., 1981). Alternatively, atRA and calcitriol could up-regulate both RAR and VDR and, possibly, this might provide an insight into the signaling cooperation of atRA and calcitriol.

Unexpectedly, no enhance viability was observed in PPC after co-addition of atRA and calcitriol; this may be due to the antagonistic effects of atRA and calcitriol (Rohde et al., 2005). Yet, as they could cross-activated the expression of RAR and VDR, antagonistic effect should not be applied in our case. On the other hand, it is well believed that proliferation and differentiation cannot exist together and they are antagonistic. In view of this fact, we have analyzed the expression of some developmental markers and, in this case, NGN3, a critical marker for specification of endocrine lineage during islet development (Rukstalis et al., 2009) was selected. After co-treatment of atRA and calcitriol, NGN3 expression in PPCs increased to more than 6-fold. This implied that they are not antagonistic, instead, in the presence of complementary molecules, they can switch on mechanism of the specification of endocrine lineage.

Cod-liver oil, a natural supplement of vitamin A and vitamin D, is recommended in infants and children for a long time for its beneficial effects on growth (Masterjohn et al., 2009). However, due to recent findings on vitamin A's teratogenicity (Werler et al., 1996), vitamin D supplement along should be used instead. However, based on our result, policy of giving vitamin D supplementation along during pregnancy would need an adjustment. As specification of endocrine cells occurs within 20 – 23 weeks of gestation (Sarkar et al., 2008), intake of cod liver oil at this stage might be supportive for endocrine islets cell formation.

5.1.2 Induction of PPC derived ICC by LSCs

It is the first time that we have established a human fetal liver derived LSC microenvironment which is able to direct human fetal pancreas derived PPCs into insulin-secreting ICC within an 8-day protocol. This newly developed method is based on our previous study in directing specification of endocrine lineage of PPCs, via enhanced expression of NGN3, by combination treatment of vitamin A and vitamin D (Ng et al., 2011) and in additional to they are regulated by adult and fetal livers by transport and storage function (Senoo et al., 2010; Lehmann et al., 2010; Kubota et al., 2007; Gascon-Barré et al., 2001). In light of this background, it prompts

us to our investigation into the effects of LSC microenvironment on the PPC proliferation and differentiation into ICCs.

In this study, we have employed a human fetal liver stroma, or stem cell niche, which is a supportive unit for growth and development. Although there is no concrete ways for characterization at the moment, isolation and culture methods are well studied and the resultant cell population is consistently found supportive for hematopoiesis (Chagraoui et al., 2003). In the presence of differentiation cocktails, LSC consistently improved ICC differentiation evidenced by the increase expression of developmental (PDX1, NGN3, NKX2.2 and NKX6.1) and functional marker genes (GLUCOKINASE and INSULIN).

First of all, we have to optimize various conditions so as to establish the co-culture model. In order to prevent blockage of transwell and depletion of resources in medium by cell overgrowth, optimal cell number (8×10^4 cells) of LSC were exposed to 8000 rad to stop its proliferation. To avoid negative effect of co-culture, PPC cell viability under co-culture was also determined. Surprisingly, co-culture of LSC could enhance their viability. Afterwards, LSCs were separated into 1^{st} (9 – 12 wk) and 2^{nd} trimester (13 – 16 wk) as derived from different gestational weeks of human fetus as previously reported (Suen et al., 2008). There was no differential growth in PPCs between 1^{st} and 2^{nd} trimester LSC co-culture.

With the success for the establishment of our co-culture model, LSCs were used to induce PPC-derived ICC differentiation. In the presence of growth factors, ICCs formed expressed developmental genes (PDX1, NGN3, ISL-1, NEUROD, NKX2.2, NKX6.1) and functional genes (GLUCOKINASE and INSULIN). In the absence of growth factors, LSC co-culture could still induce ICC differentiation. When compared to differentiation cocktail induction, 2nd trimester LSC could enhance significantly the expression of developmental and functional genes expression. To confirm the resultant ICC's functions, glucose-stimulated insulin secretion (GSIS) and potassium-stimulated insulin secretion (KSIS) were examined. In consistent with the gene expression profile, 2nd trimester co-culture LSC had higher basal insulin secretion levels. Despite the unresponsiveness to glucose, there was a significant change in KSIS. This means that 2nd trimester LSC could improve functions of ICC.

In order to determine identities of growth factors or cytokines involved in enhancing differentiation, the expression of some growth factors was further analyzed in LSCs. In this regard, SCF, HGF, NGF, VEGFA, FGF1, FGF2, FGF7, IGF1, IGF2, TGFb, IL-6, BMP2, BMP4 and BMP6 genes were expressed and some of them were reported necessary for islet development (Ranjan et al., 2009; Lammert et al., 2001; Li et al., 2006; Kanaka-Gantenbein et al., 1995; Movassat et al., 2003; Ameri et al., 2010).

5.1.3 Potential effects of liver stroma derived IGF1 on PPC derived ICC differentiation

Making use of the differential effects of 1st and 2nd trimester LSC in ICC induction, differential mRNA expression of IGF1, IGF2, BMP4 and BMP6 between 1st and 2nd trimester LSC was found. They were then exogenous added into the differentiation cocktails to investigate their effects alone. We have identified positive roles of IGF1 and negative roles of IGF2 and BMP4 by examining the change in expression of PDX1. IGF1 enhanced PDX1 expression in ICC while others show no observable change.

9 – 15 week derived PPCs showed a positive mRNA expression of IGF1, IGF2, IGF1R and IGF2R. The IGF1R protein that binds to both IGF1 and IGF2 was localized at the cell cytoplasm and cell membrane of the PPCs. Application of exogenous IGF1 (1 – 100 ng/ml) to the PPCs showed an increase in cell viability at a concentration of 50 ng/ml while PPC-derived ICCs had an increased expression of PDX1, peaked at 50 ng/ml. When PPCs were separated into 1st and 2nd trimester, the growth response of IGF1 was observed only in 1st trimester PPC while differentiation response was observe only in 2nd trimester PPC derived ICC but not vice versa.

As IGF1, but not IGF2, enhances PPC growth and ICC differentiation, blockage of IGF1R would reveal IGF1 effects only (Hiden et al., 2009). To address this issue,

we used MAB391 neutralizing antibodies for IGF1R to block the effect via IGF1R. Growth effect triggered by 50ng/ml IGF1 on PPCs was inhibited at .01ug/ml MAB391. However, growth effect triggered by LSC co-culture was not inhibited. These observations may be due to presence of other growth factors which can also trigger proliferation effects.

Besides, the enhanced expression of PDX1 by IGF1 was reduced in ICC by MAB391. With exogenous addition of MAB391, there was no significant change in PDX1 expression in ICCs under 1st trimester LSC co-culture while PDX1 mRNA expression was reduced to 33% ($P < .001$) in ICCs under 2nd trimester LSC co-culture. These data indicates that the better induction by 2nd trimester LSC was, at least, partly attributable to IGF1.

From previous studies of Liver IGF1 Deficient (LID) (Yu et al., 2003) and Pancreas IGF1 Deficient (PID) (Lu et al., 2004) mice, IGF1 was necessary for pancreatic regeneration while inhibiting pancreatic development. In our *in vitro* culture results, IGF1 enhanced growth in 1st trimester PPC and enhanced differentiation in 2nd trimester PPC-derived ICCs through IGF1R but not vice versa. From this, we hypothesize that IGF1 exerts different effects on different stages of stem cells which explain the contractive results for regeneration and differentiation as the stem cells during regeneration and development are at different origin and stages.

Since results obtained under culture condition may not necessarily reflect *in vivo* condition, a rat neonatal STZ regeneration model has been used. Similarly to other pancreas regeneration model, IGF1 mRNA expression in pancreas has increased significantly for anti-inflammatory response (Smith et al., 1991). However, IGF1 mRNA expression in the liver has been decreased 2 to 3-fold after STZ injection. Change in IGF1 expression in liver and pancreas during pancreatic regeneration are in parallel with each other. This may be due to homeostasis regulation by liver in response to rise in IGF1 level; however, since in adult pancreatic regeneration model, IGF1 expression in the liver does not change, homeostasis regulation fails to explain the situation (Smith et al., 1991). Alternatively, the decrease of IGF1 in liver in neonates regeneration model may due to mimicking of IGF1 level in fetal development. From our result, we found that ICC exposed to higher level of IGF1 (100 ng/ml) may decrease PDX1 expression comparing to 50 ng/ml IGF1 and clinical studies showed that fetus has lower IGF1 circular level than adult (Mark et al., 2008; Bennett et al., 1983). Further investigation is required for confirmation of the hypothesis.

5.1.4 Significance of islet engineering in the management of diabetes

Replacement of deficient, damaged or malfunctioning islets by transplantation is undoubtedly the most effective and promising approach in treating and curing diabetes. Considerable clinical cases have reported that patients with successful transplantation under immunosuppression can lead to an insulin-independent life for a long period of time (Merani et al., 2006). In this respect, successful islet transplantation depends on several critical factors; they include, but are not limited to, sufficient number of islets, islet durability and islet functionality after islet transplant (Ryan et al., 2002). Therefore, it is in desperate need to search for renewable and unlimited source as well functional islets in response to glucose challenge for clinical islet transplantation in patients with diabetes.

In this study, we have attempted to examine three such critical factors as vitamin A, vitamin D and liver microenvironment that are essential to get involved in the involution of PPC growth, ICC differentiation and ICC functionality. Taken all data together from this study, we propose that vitamin A, vitamin D, IGF1 and several other inductive molecules derived from LSCs should be considered to introduce into a differentiation cocktail for direct differentiation of PPCs into ICCs as well as inducing functionally insulin-producing islet cell for clinical transplantation. On the other hand, further modifications of the microenvironment induction approach could be achieved by the application of other supporter cells, notably the bone marrow stromal cells.

Such a stem cell niche for islet cell development may offer an alternative, efficient and cost-effective approach in providing islet transplantation for the management of diabetic patients.

5.1.5 Conclusions

In the present study, RAR, VDR and RXR are found to exist in PPCs and their respective ligands, vitamin A and vitamin D are involved in PPC proliferation while combination treatment can up-regulate NGN3 expression in PPC. On the other hand, LSCs derived from human fetal liver can induce ICC differentiation with or without the presence of differentiation cocktails. These observations are due, at least partly, to the growth factors produced by LSCs and IGF1 is identified as being one of the inductive molecules and responsible for these effects.

In summary, vitamin A, vitamin D and LSC play a critical role for the development of human fetal derived PPCs into ICCs, thus providing a potential for clinical islet transplantation in patients with type 1 diabetes and some severe forms of type 2 diabetes.

5.2 Future Studies

As mentioned, vitamin A and vitamin D can enhance PPC proliferation and NGN3 expression. However, the underlying mechanism(s) whereby they are involved with particular reference to their interactions during islet cell development remains to be fully elucidated. In addition, it is worthwhile further investigating their effects in PPC-derived ICC differentiation so as to confirm their roles in islet development.

Based on the idea of LSC enhanced ICC differentiation through secretion of secret factors, it is advisable to screen these factors in 3 levels, i.e. proteins, small molecules and miRNA. Total protein content could be screen by the use of 2D gel electrophoresis and Mass Spectrometry while small molecules can be screened by Mass Spectrometry after filtering. Besides, miRNA and change in gene expression should be screened using microarray. Through studying of these 3 levels, all required components and their in-depth knowledge of interactions could be found.

Apart from the studies of LSC, it is also interesting to study if other potential cell sources from different organs, which are closely related to the pancreatic development, involves in the maturation of ICC. Based on other researches on the isolation techniques of different cells, we can co-culture them with PPC to examine whether they are involved. Afterwards, isolating specific cell types using Flow Cytometry and

converting them into cell line could make the experiment easily repeatable and thus, clinical applications would be viable.

Chapter 6

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6.1 Reference

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