The roles of Reg family proteins in Pancreatic Ductal Adenocarcinoma and *Diabetes Mellitus*

Abstract

Pancreatic ductal adenocarcinoma (PDAC), a malignancy derived from pancreatic exocrine ducts, and ***Diabetes mellitus*** (DM), a dysfunction involving the pancreatic endocrine islets, are two common pancreatic diseases that threatens the life of the human race. **Regenerating (Reg) proteins** are a group of C-type lectin-like proteins discovered in patients with pancreatitis and during pancreatic islet regeneration. Seven mouse and five human isoforms have been identified in the Reg proteins family. They are normally produced in the acinar and ductal cells of the pancreas in an age-dependent and isoform-specific manner. The overexpression of the Reg genes has been shown to promote cell proliferation and tumor growth, both *in vitro* and *in vivo.* On the basis of a decade-long study of Reg proteins in our laboratory, this thesis is focused on studying the roles of isoform-specific Reg proteins in the PDAC carcinogenesis, and the compensatory islet expansion in response to obesity and/or aging.

In PDAC, we found (1) elevated serum Reg1A and 1B levels, and increased Reg1A and 1B protein and mRNA levels in PDAC tissues, which were closely associated with cancer histological grades and patient survival; (2) increased expression of Reg1A, 1B and 3A in the two PDAC precursor lesions: acinar-ductal metaplasia (ADM) and pancreatic intraepithelial neoplasia (PanIN); (3) recombinant Reg3A protein induced ADM in 3-D culture of acinar cells

in vitro; and (4) tissue expressions of Reg1A, Reg1B and Reg4 could differentiate metastatic PDAC in the liver from intrahepatic cholangiocarcinoma (ICA) with 92% sensitivity and 95% specificity.

In DM, we found (1) that the normal expression of Reg2 gene was required for islet expansion in response to a high-fat diet (HFD) and aging; (2) Reg2 deficiency maintained glucose tolerance in HFD-fed young mice but caused its impairment in aged mice; (3) pancreatic β-cell specific overexpression of Reg3β did not protect mice from HFD-induced diabetes, but apparently exerted an opposite effect of worsening T2D through downregulation of GLUT2 and AMPK activity. Our observations suggest that both Reg2 and Reg3β are important in β-cell compensation in response to HFD-induced diabetes. However, excessive compensation may lead to the insulin resistance and deteriorated diabetes.

These novel insights into the roles of isoform-specific Reg proteins in PDAC and DM could help us in developing clinically applicable diagnostic and innovative therapeutic strategies to combat these two devastating diseases.

Résumé

L'adénocarcinome canalaire du pancréas (Pancreatic ductal adenocarcinoma, PDAC), une maladie des canaux pancréatiques exocrines et le Diabète sucré (Diabetes Mellitus, DM), une défaillance des îlots pancréatiques endocrines sont deux des principales maladies pancréatiques de l'être humain. Les protéines Reg (Régénération) sont un groupe de protéines de type C-lectine (*C-type lectin like proteins*) trouvées chez des patients atteints de pancréatite et chez ceux subissant un processus de régénération des îlots pancréatiques. Plusieurs isoformes des protéines Reg ont été décrites, cinq chez la souris et sept chez l'homme. Les protéines Reg sont normalement produites dans les cellules canalaires et acinaires du pancréas et suivant un processus dépendant de l'âge et spécifique à l'isoforme. Des études ont démontré que la surexpression des gènes Reg favorise la prolifération cellulaire et la croissance des tumeurs *in vivo* et *in vitro*. Notre laboratoire a étudié les protéines Reg pendant près de dix ans. Cette thèse est centrée sur l'étude des rôles des isoformes spécifiques des protéines Reg dans le processus de carcinogenèse du PDAC et dans le processus d'expansion des îlots pancréatiques, en réponse à l'obésité et/ou au vieillissement.

Dans les cas d'adénocarcinome canalaire du pancréas (PDAC), nous avons trouvé : (1) des niveaux élevés des protéines Reg1A et 1B dans le sérum et les tissus ainsi qu'une augmentation des niveaux de mARN dans les tissus. Les niveaux des protéines Reg1A et Reg1B ont été étroitement associés à la classification histologique du cancer et à la survie des patients; (2) une expression accrue des protéines Reg1A, 1B et 3A dans les deux lésions précurseurs du PDAC: les métaplasies acino-canalaires (acinar-ductal metaplasia, ADM) et les lésions PanIN (Pancreatic Intra epithelial Neoplasia); (3) que l'utilisation de la protéine recombinante Reg3A pouvait induire la formation d'ADM dans la culture 3-D de cellules acineuses *in vitro*; (4) que

les niveaux d'expressions tissulaires de Reg1A, Reg1B et Reg4 permettent de différencier les métastases du PDAC dans le foie, des cholangiocarcinomes intrahépatiques (ICA) avec 92% de sensibilité et 95% de spécificité.

Dans les cas de DM, nous avons trouvé que (1) l'expression normale du gène Reg2 est nécessaire pour l'expansion des îlots pancréatiques en réponse à une diète riche en gras (high-fat diet HFD) et au vieillissement; (2) la déficience en Reg2 chez de jeunes souris ayant une diète riche en gras a maintenu leur tolérance au glucose mais causé une intolérance au glucose chez des souris âgées; (3) la surexpression du Reg3β dans les cellules pancréatiques de type β n'a pas protégé la souris du diabète induit par une diète riche en gras et a plutôt produit un effet opposé de détérioration du diabète par une régulation négative de l'activité du GLUT2 et de l'AMPK. Nos observations suggèrent l'importance des protéines Reg2 et Re3β dans le mécanisme de compensation des cellules β suite au diabète induit par une diète riche en gras. Cependant, une compensation excessive peut provoquer une résistance à l'insuline et une détérioration du diabète.

Ces nouvelles découvertes sur les rôles des isoformes spécifiques des protéines Reg dans le PDAC et le DM pourraient aider au développement de nouvelles stratégies diagnostiques et thérapeutiques pour combattre ces deux maladies dévastatrices.

Preface

This thesis was written in accordance with the guidelines for a manuscript-based thesis issued by the Faculty of Graduate and Post-Graduated (doctoral) studies of McGill University. It consists of a general introduction and literature review, two published articles (Chapter 2 and 4), one manuscript under 1st revision of Endocrinology (Chapter 3), a general discussion and perspectives, a section describing my major contribution to original research and summary.

Appendix 1 contains biohazard, radioactivity and animal certificates. Appendix 2 contains permissions to reproduce figures and materials. Appendix 3 contains reprints derived from Chapter 2 and 4, a book chapter cited in Chapter 1, as well as another two articles I contributed towards.

Contributions of authors

Chapters 1 and 5 were written entirely by me with editorial comments and corrections by my supervisors Drs. Liu and Gao. Chapter 1 is partially composed of a book chapter I authored, entitled "The Contribution of Reg Family Proteins to Cell Growth and Survival in Pancreatic Islets" published on "Islets of Langerhans" Second Edition, 2014 (1).

Chapter 2: Li Q, Wang H, Zogopoulos G, Shao Q, Dong K, Lv F, Nwilati K, Gui XY, Cuggia A, Liu JL, Gao ZH. Reg proteins promote acinar-to-ductal metaplasia and act as novel diagnostic and prognostic markers in pancreatic ductal adenocarcinoma. Oncotarget. 2016 Oct 24. [Epub ahead of print]

I designed and performed most of the experiments and did data analysis and presentation except Fig 3A, which was generated by Dr. Hao Wang. I wrote the first draft of the manuscript, which was revised by Drs. Gao and Liu.

Chapter 3 is a manuscript currently under 1st revision of *Endocrinology*

The design of experiments, lab work and data analysis were all done by myself except for the data presented in Fig 1 and Fig 2A, B and E, which were initiated by Dr. Bing Li. I wrote the first draft of the manuscript, which was revised by Dr. Liu.

Chapter 4: Xiong X*, Li Q*, Cui W, Gao ZH, Liu JL. Deteriorated high-fat diet-induced

diabetes caused by pancreatic β-cell-specific overexpression of Reg3β gene in mice. Endocrine. 2016 Jun 3. [Epub ahead of print] *Equal contribution

I contributed equally to the work with Dr. Xiaoquan Xiong. I performed the experiments and generated the data in Table 1, figures 4 and 5 and revised the entire manuscript.

Other publications relevant to the thesis project:

1. Qing Li, Jun-Li Liu, Zu-Hua Gao, REG3β Plays a Key Role in IL17RA Protumoral Effect-Letter. Cancer Res. 2016; 76(7):2050.

2. Subrata Chowdhury, Xiao Wang, Coimbatore B. Srikant, Qing Li, Min Fu, Ying Jia Gong, Guang Ning, and Jun-Li Liu, IGF-I Stimulates CCN5/WISP2 Gene Expression in Pancreatic β-Cells, Which Promotes Cell Proliferation and Survival Against Streptozotocin Endocrinology 2014; 155:5, 1629-1642

Contributions to the original research

In chapter 2, I demonstrated 1) the upregulation of Reg1A and Reg1B in the serum and tissues of PDAC patients compared to healthy subjects. Their expression levels are positively correlated with precursor PanIN lesions. Additionally, I showed higher levels of Reg1A and Reg1B in well-differentiated tumors compared to poorly differentiated tumors, and it predicted better survival rate in PDAC patients. 2) Interestingly, there was upregulation of Reg1A and Reg3A in tumor-adjacent ADM areas, indicating their involvement in ADM and carcinogenesis of PDAC. I also demonstrated that recombinant human Reg3A directly promoted the ADM transition *in vitro*. 3) The combination of Reg1A, Reg1B and Reg4 proteins showed high specificity and sensitivity in differentiating PDAC from intrahepatic cholangiocarcinoma. Together, my study suggests a new panel of diagnostic and prognostic biomarkers for PDAC and putative therapeutic targets to inhibit PDAC development.

In chapter 3, I studied Reg genetic engineered mice, that is, Reg2 knockout mice. Both aged and HFD-induced Reg2KO mice showed less β-cell mass and decreased serum insulin levels compared to wild-type mice, suggesting Reg2 was required for β-cell compensation under these conditions. Aged Reg2KO mice also showed impaired glucose tolerance and increased fat mass, suggesting more severe insulin resistance compared to wild-type. However, we demonstrated that under HFD, Reg2KO mice showed normalized blood glucose and insulin levels, compared to hyperglycemia and hyperinsulinemia in wild-type mice. It may be explained by the limitation in insulin levels that could potentially improve the insulin sensitivity, therefore maintaining the normal glucose levels. Our study is the first *in vivo* study that systematically

explores the role of Reg2 in HFD-induced diabetes. We provide a new understanding of Reg2 function in β-cell compensation and HFD-induced diabetes.

In chapter 4, I investigated the role of pancreatic specific Reg3β overexpression in HFD-induced diabetes. In contrast to its protective effect in streptozotocin-induced diabetes, Reg3β overexpressing mice showed hyperglycemia, impaired glucose tolerance and decreased insulin sensitivity compared to wild-type in response to HFD. Moreover, aged mice showed increased fat weight hepatosteatosis. In general, Reg3β overexpression is detrimental to the HFD-induced diabetes.

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During my Ph.D. study, I have been very blessed by so many people, including professors, labmates, friends and family.

First, I would like to sincerely thank my supervisor Dr. Liu. He is a very kind and warm-hearted person. He helped me to adapt to the new environment smoothly, not only in my study, but also in life. He guided me to sort out how to manage my study and research efficiently and conduct experiments independently. At McGill, I have been fortunate to meet Dr. Gao, who became my co-supervisor. He brought out my project and provided very professional opinions on my pathology study. Through him, we were able to have full access to the tissue bank. I would also like to thank him for the generous contribution to my research funding. I am fortunate to have both of them as my supervisors.

Second, I would like to thank all professors who helped me in my projects, especially Dr. Srikant and my committee members, including Dr. Larose, Dr. Bateman and Dr. Rocheleau. Whenever I had questions about my research study, they were always there to discuss them with me and to give me more materials to study and suggestions. I would like also to thank our collaborators, Dr. Zogopoulos and Dr. Wang. I would like to thank SCRiBBR and my colleague Milada for helping me in my thesis editing. Thanks to Dr. Andrea.Gomez and Marie Lamarche for their help in the French abstract.

Third, I would like to thank all the current and previous members in Fraser Labs. I would like first to thank Dr. Junting Liu and Dr. Subrata Chowdhury, who taught me the general principle of the lab and basic techniques when I was brand new to the lab. I would also like to thank my colleagues and friends, Kate, Karam, Larson, Xiaoliang, Bo, and Dr. Zhang, with

whom I have been able to discuss my research plan, failures and success in my experiments, as well as my holiday plans. You guys are amazing! I know I am going to miss the time at McGill for the rest of my life.

Last but not the least, I would sincerely thank my entire family, including my parents, my parents-in-law and my husband. During my Ph.D., I had my beloved baby Alexander born. He has made my life shining. All family rotated to help me take care of him so that I can focus on my study. I am truly thankful for them.

I hope this is not the end of my study. I will keep going and pursue my further career in medicine.

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List of Abbreviations

ADM	Acinar-to-ductal metaplasia
AMPK	AMP activated protein kinase
BrdU	Bromodeoxyuridine
CA19-9	Carbohydrate antigen 19-9
CEA	Carcinoembryonic antigen
CK19	Cytokeratin 19
CRP	C-reactive protein
DAB	3,3'-diaminobenzidine
DM	*Diabetes Mellitus*
ECA	Extrahepatic cholangiocarcinoma
EGFR	Epidermal growth factor receptor
ER	Endoplasmic reticulum
EXTL3	Exostosin like glycosyltransferase 3
FN1	Fibronectin 1
GLUT2	Glucose transporter 2
GSIS	Glucose stimulated insulin secretion
HFD	High-fat diet

HIP	Hepatocellular carcinoma-intestine-pancreas protein
ICA	Intrahepatic cholangioarcinoma
IL-6	Interleukin-6
INGAP	Islet neogenesis-associated protein
IPMN	Intraductal papillary mucinous neoplasm
MAPK	Mitogen-activated protein kinase
MCN	Mucinous cytoplasmic neoplasm
MMP	Matrix metalloproteinase
NAFLD	Non-alcoholic fatty liver disease
Ngn3	Neurogenin 3
PanIN	Pancreatic intraepithelial neoplasia
PAP	Pancreatitis-associated protein
PDAC	Pancreatic ductal adenocarcinoma
Pdx-1	Pancreatic and duodenal homeobox-1
PI3K	Phosphoinositide 3-kinase
PSP	Pancreatic stone protein
PTP	Pancreatic thread protein
Reg	Regenerating proteins

RELP	Regenerating protein-like protein
SMO	Smoothened homolog
STZ	Streptozotocin
T1D	Type 1 diabetes
T2D	Type 2 diabetes
TGF-α	Transforming growth factor-α
TNF-α	Tumor necrosis factor-α

Chapter 1. General introduction and literature review

1.1 Introduction of Reg proteins

1.1.1 General information

Reg and Reg-related genes constitute a family within the C-type lectin superfamily (2-4). In the last two decades, over 29 Reg genes have been discovered in different species. These secretory proteins share structural and functional properties associated with tissue injury, inflammation and carcinogenesis in the pancreas, liver, neurons and gastrointestinal tracts (5-9). Ever since Reg1 was discovered, special attention has been paid to the therapeutic potential of Reg proteins in the regeneration of pancreatic islets and their roles in carcinogenesis (10).

1.1.2 History of studies on Reg proteins in our lab

In 2006, our lab identified that three Reg family proteins (Reg2, Reg3α and Reg3β) were upregulated in the pancreas of Pdx-1-Cre mediated whole pancreas inactivation of IGF-1 mice (11). These IGF-1 deficient mice exhibited increased islet mass and were protected from streptozotocin-induced diabetes. The Reg proteins were re-expressed in both the endocrine and exocrine portions of the pancreas, suggesting that they may have protective effects against β-cell disruption. In 2008, we reviewed the roles of Reg family proteins in several systems, including their cell regenerating roles in the pancreas. Since then the research scope has expanded significantly to include their pathophysiological functions in the pancreas, intestine, immunity and cancer. More importantly, in communicating our research findings, we feel the need for further classification of the seven independent genes among key species. A more uniform terminology should help us understand their isoform-specific functions and/or mode of activation.

1.2 Exocrine and endocrine pancreas

1.2.1 Basic composition of the pancreas and its functions

The mammalian pancreas is composed of three major cell types: exocrine acini, ducts and endocrine islets. The endocrine islets constitute about 5% of the volume and consist of α, β, δ, ε and pancreatic polypeptide (PP) cells that produce glucagon, insulin, somatostatin, ghrelin and pancreatic polypeptide, respectively (12, 13). Insulin and glucagon are two hormones with opposing roles. They work together to maintain the balance of glucose storage and utilization (14). Somatostatin and pancreatic polypeptide exert inhibitory effects on both pancreatic endocrine and exocrine secretions (15, 16). Ghrelin regulates insulin secretion and the expression of genes essential for β-cell biology, promotes β-cell proliferation and survival, and inhibits β-cell apoptosis (17). The exocrine cells that are organized into acini and ducts constitute about 85% of the pancreas. Acinar cells secrete digestive enzymes, such as amylase, elastase and trypsinogen, into the pancreatic ducts. These enzymes, along with bicarbonate and other electrolytes secreted by ductal cells, constitute pancreatic juice and are drained into the duodenum through the main duct (12). The terminal ductal epithelial cells that interfere with acinar cells are called centroacinar cells, which are composed of squamous-like epithelium and have been shown to be associated with transdifferentiation between the different cell types. Additionally, increasing interest has been paid to the interactions between the exocrine and endocrine portions of the pancreas with respect to their anatomy and functions. For example, insulin secreted from islet cells can potentiate the secretion of amylase from acinar cells, which is defined as the "islet-acinar axis" (18). Most studies so far have been focused on the effects of endocrine hormones on the exocrine pancreas through neurocrine, endocrine and paracrine

avenues; however, enzymes and proteins secreted from the exocrine pancreas also affect the endocrine pancreas, such as Reg proteins. I will further address their interactions in this thesis.

1.2.2 Embryonic development of the pancreas

The pancreas arises from the foregut endoderm and emerges to form primitive epithelial tubules at 7 weeks of gestation in humans. These tubules undergo branching, with their ends forming acini. Endocrine cells begin from the central ductal area and move to the periphery with development of the pancreas at around 10 weeks. By the end of the first trimester, both exocrine and endocrine portions of the pancreas develop their mature morphology and organization (19). In mice, there is a transition period defined as the "secondary transition" starting from E13.5 to E14.5, when there is a burst of pancreatic cell proliferation and differentiation occurring (19).

Both the endocrine and exocrine pancreas emerge from common progenitor cells. During development, there are increased expressions of cell markers and transcriptional factors that are essential for the formation and maturation of the pancreatic cells. Among them, Pdx-1 (pancreatic and duodenal homeobox 1) is a critical transcriptional factor that contributes to the development of all pancreatic cell lineages. It is especially required for endocrine cell specification and maintenance of β-cell maturation (20). Pdx-1 is upregulated in response to pancreatic injury such as pancreatic ductal ligation (PDL). By enhancing the activity of another endocrine transcriptional factor neurogenin 3 (Ngn3), Pdx-1 promotes the proliferation and differentiation of pancreatic β-cells (21). The ductal cells have been shown to retain their ability as progenitor cells due to the lack of endocrine transcriptional factor and can be reprogrammed to islet-like structures under the drive of specific endocrine transcriptional factors (22). Sox9, along with Hnf1β and Hnf6, are three transcriptional factors that are critical for maintaining the

progenitor pool of the ductal cells. A lineage tracing study has shown that Sox-9 positive cells give rise to all ductal, acinar and endocrine cells, and are required to maintain the exocrine compartment (23). Loss of Sox9 can result in the degeneration of all cells and hypoplasia of the pancreas. In addition to these transcriptional factors, cytokeratin 19 (CK19) is a key cell membrane marker for ductal cells and can be used to monitor the ductal cell differentiation status. The differentiation level of the acinar cells is in between that of endocrine cells and ductal cells. Its development is regulated by two bHLH transcriptional factors: Ptf1a (known as p48) and Mist1. In mice, Mist1 becomes important in the secondary transcription stage in the pancreatic embryonic development and is required for the maintenance of acinar organization. Otherwise, the acinar cells could undergo transdifferentiation into ductal cells with activation of Kras gene mutations and TGF-α/EGFR signaling pathways (24) (Fig 1.1). Balance between the endocrine and exocrine pancreas is regulated by various signaling pathways, but Hedgehog, Kras and Wnt/β-catenin signaling pathways are the three major ones. They will be further discussed in the reprogramming of acinar to ductal cells.

1.2.3 Association between diabetes, pancreatitis and pancreatic cancer

Diabetes and pancreatic cancer are two common diseases that occur in the endocrine and exocrine pancreas. They interact with each other in multiple ways due to their close anatomical location. Long-standing diabetes and obesity predispose patients to a higher risk for pancreatic cancer. Hyperinsulinemia and insulin resistance not only predict cancer risk but also are early symptoms of cancer in the exocrine pancreas (hazard ratio [HR] 2.13) (25). About 25% of pancreatic cancer patients develop DM at diagnosis and another 40% manifest impaired glucose tolerance (26, 27). This tendency may be due to the fact that pancreatic cancer cells destroy their surrounding islets, especially when the cancer involves the tail of the pancreas, therefore leading

to impaired glucose tolerance and hyperglycemia. Some diabetes can be cured by resection of PDAC (28-33). Pancreatitis is another well-known risk factor for PDAC in human beings. Chronic pancreatitis causes a 16-fold increase in relative risk for PDAC, and this risk is increased to 50-fold in patients with hereditary chronic pancreatitis (34). In genetically engineered mice predisposed to pancreatic cancer, inflammation largely accelerated the cancer development (35). Some studies also suggest that β-cell dysfunction and diabetes may contribute to the risk of developing PDAC through the activation of cytokines and inflammation in the pancreas (36). The World Health Organization has classified these pancreatogenic DM as Type 3 DM (T3DM), characterized as hyperglycemia caused by acute pancreatitis, chronic pancreatitis, cystic fibrosis, pancreatectomy and pancreatic cancer. Among them, the most common cause is chronic pancreatitis (about 78.5%) and the second is pancreatic carcinoma (8%) (37). Novel findings have shown that metformin, a well-known metabolic drug used to treat diabetes through activating AMP-activated protein kinase (AMPK) is effective in protecting patients from the development of pancreatic cancer (38). Other molecular studies also support the close interaction between diabetes and pancreatic cancer. Pdx-1 is important for insulin expression in adults. It has also been found to be overexpressed in both pancreatic cancer and insulinoma patients. Pdx-1 knockdown can reverse the development of the cancer by inhibiting cell proliferation (39). Diabetes also promotes pancreatic cancer growth through IGF-1/insulin signaling in a hyperglycemic state (40). However, the cause-and-effect associations between pancreatic cancer and diabetes remain unclear even after decades of studies.

1.3 Pancreatic cancer

1.3.1 Relevant biology and genetics

Pancreatic ductal adenocarcinoma (PDAC) is the most common cancer type in the exocrine pancreas. Although the morbidity is low, it is the fourth leading cause of cancer-related deaths and the 5-year survival rate is only 5% (41). There are multiple risk factors for this deadly disease, such as aging, smoking, familial chronic pancreatitis, *diabetes mellitus* and obesity. Patients with advanced PDAC usually presented clinical signs of severe abdominal or back pain, obstructive jaundice and weight loss. However, patients at early stages only present some non-specific symptoms such as indigestion, abdominal discomfort and/or malnutrition. Due to the cancer's deep anatomical location and non-specific early presentations, the patients are usually only diagnosed at late stages. Only 10–20% of tumors are resectable at diagnosis (42). Furthermore, PDAC patients have poor response to most chemotherapeutic drugs, among which the most commonly used is gemcitabine. Therefore, understanding the biological mechanisms and progression of the disease are critical for early diagnosis and finding therapeutic targets.

Genome-wide associations have revealed genetic predispositions in PDAC patients. The most common mutations are activation of the oncogene Kras (90%) and inactivation of the three tumor suppressor genes Cyclin-dependent kinase inhibitor 2A (CDKN2, 90%), TP53 (50–75%) and SMAD4 (over 50%) (43). Other low-frequency mutations (<20%) occur at BRCA2, liver kinase B1 (LKB1), MutL homolog 1 (MLH1) and so on (44, 45). The advanced genomic sequencing allows researchers to subtype the cancer based on their gene profiles. Collisson et al. identified three subtypes (classical, quasimesenchymal and exocrine-like), and they are associated with clinical outcomes and therapeutic responses of the PDAC patients (46). Mottiff et al.

subclassified the cancer into classical and basal subtypes with normal or activated stroma and correlated them with patients' survival rates (47). They further compared their subtyping with the previous study by Collisson and found their classical subtype was identical with previous classical subtypes, while quasimesenchymal may be more related to stromal gene expression because of the impurity of the cancer cell with stromal cells in the previous study. Therefore, the classification approach that excludes stroma from cancer cells is more accurate. A more recent subtyping study identified an aberrantly differentiated endocrine exocrine (ADEX) subtype (48), which correlates with the different histopathological characteristics. In this subtype, an upregulation of transcriptional factors was observed in the exocrine and endocrine pancreatic differentiation, including MIST1, INS, Neurogenic differentiation factor 2 (NeuroD2), NK2 homeobox 1 (NKX2-1) and MAF BZIP transcription factor A (MAFA), which further indicated the close correlation between pancreatic cell differentiation and PDAC development. The ADEX subtype is closely correlated with the previously identified exocrine-like subtype by Collisson et al., which shows high expression of Reg3A (pancreatitis-associated protein) and PRSS1. Based on these studies, PDAC can be generally subdivided into classical, quasimesenchymal (stroma-activated) and exocrine-like (endocrine-exocrine) subtypes. Subtyping PDAC can further promote the study of personalized medicine and the development of novel therapeutic targets in different molecular mechanisms (49).

1.3.2 Morphological characteristics of the invasive PDAC

1.3.2.1 General pathological features of the invasive PDAC

The histopathological study of PDAC is very critical for its diagnosis. The typical PDAC cancer is composed of glandular (ductal) epithelial cells surrounded by a dense desmoplastic

stroma, consisting of fibroblasts and inflammatory cells such as macrophages. The gland formation can vary from well-formed glands, small poorly formed glands to sheets of tumor cells without obvious gland formation. Intracellular mucin can be appreciated in some tumor glands. (50). Other histological subtypes of PDAC include colloid, sarcomatoid and adenosquamous cancer. There are eight features that can distinguish invasive cancer glandular cells from benign non-neoplastic glands summarized by Hruban R.H. (51): 1) haphazard growth pattern, 2) gland grows adjacent to muscular vessels, 3) peri-neural invasion, 4) intravascular invasion, 5) nuclear variation, 6) fat-touching glands, 7) gland incomplete, and 8) intraluminal necrosis (50). Even though PDAC has its distinct pathologic features, in some cases it can be difficult to distinguish a reactive gland of chronic pancreatitis from a well-differentiated pancreatic cancer gland. Therefore, some immunohistological biomarkers can be used to aid the diagnosis, such as carcinoembryonic antigen (CEA) and mesothelin (52).

1.3.2.2 Cancer differentiation grades

The cancer differentiation grade is another pathological feature of the tumor malignancy. Cellular differentiation refers to the process that one cell type develops into another, usually from a less specified cell type to a more specified one (53). Cancer differentiation grades are used to describe the morphological similarity with the normal cells. Usually, individual cancer glands have their own differentiation characteristics, varying from the well differentiated to the poorly differentiated. Well-differentiated carcinomas usually form relatively complete duct-like glands and cells are cuboidal to columnar with round or oval nuclei, while poorly differentiated ones show incomplete and irregularly organized glands with pleomorphic and enlarged nuclei (54). Different from the clinical stages (TNM staging) of the cancer, which refers to the progression of cancer in individuals, cancer differentiation grade refers to the initial features of

the tumor malignancy. Therefore, some tumors, although small in size and early in TNM staging, can have poor differentiation when diagnosed, and they manifest very aggressive behavior and metastasize quickly. In contrast, well-differentiated tumors can grow big locally before metastasis.

1.3.3 Precursors to PDAC: ADM and PanIN lesions and their pathological characteristics

As PDAC is a very malignant disease and is usually diagnosed at advanced stages, the detection of premalignant lesions and preventive intervention could be effective approaches before it fully develops. This could benefit subjects with genetic predispositions and aid the surveillance of at-risk individuals. Traditionally, it is believed that PDAC starts from a distinct precursor lesion named pancreatic intraepithelial neoplasia (PanIN) and progresses to invasive carcinoma through a series of genetic events (55). Recent studies suggest that PDAC can also derive from acinar-to-ductal metaplasia (ADM), with additional mutations such as K-ras and TP53. Initially, ADM is believed to be one of the protective mechanisms of acinar cells in response to inflammatory stimulants such as chronic pancreatitis (Fig 1.2). The genetic profile of ADM is consistent with what has been described in ADEX or exocrine-like subtype of the PDAC. Understanding the molecular mechanisms of these precursors could help us find early biomarkers and preventive therapeutic targets for PDAC patients.

1.3.3.1 Pancreatic intraepithelial neoplasia (PanIN)

PanIN lesions, as the most common precursor for PDAC, are neoplasia confined to pancreatic ducts. They are classified into four grades based on their neoplasia: PanIN-1A, -1B, -2 and -3, or carcinoma in situ. PanINs (<5mm in diameter) are microscopically visible but not in

the pancreatic gross appearance. As they proceed from the low to high grade, the appearance of PanIN duct epithelium transforms from a flat to a columnar structure, and the nucleus normally located in the basement membrane loses its polarity. PanIN-3, which is also called PDAC in situ, is characterized by "budding off" clusters into the ductal lumen. Once the neoplasia invades beyond the basement membrane, it transforms into invasive PDAC. Some common genes have been found to be expressed in both PanINs and invasive PDAC. The mice develop sporadic PanIN lesions after 3 weeks of activated Kras mutation (56).

1.3.3.2 Acinar-to-ductal metaplasia (ADM)

Metaplasia has been defined as a transdifferentiation process from one differentiated cell type to another in order to adapt to external insults such as inflammation. There are increased risks of certain tumor types due to metaplasia, such as cervical cancer and Barrett's esophagus. Acinar-to-ductal metaplasia (ADM) was first described as a morphological change from pancreatic acinar cells to ductal epithelial cells in response to chronic inflammation in 1985 (57, 58). It is frequently seen in chronic pancreatitis and the background of PDAC tissues. Only recently has its contribution to PDAC development with genetic predisposition drawn the attention of oncologists and cancer researchers. It is estimated that ADM causes a 16-fold increase in relative risk for PDAC, and this risk increases to 50-fold in patients with familial chronic pancreatitis. Kras mutation, in synergy with chronic pancreatitis, could lead to the development of ADM/PanIN lesions and further progression to PDAC. This progression is transient with inactivation of Kras (56). In addition, TGF-α, a ligand for the EGF receptor, has been well established as promoting ADM (59). Both endogenous overexpression or exogenous treatment with TGF-α in primary acinar cells can effectively induce ADM transformation. The acinar transcriptional factor Mist1 can effectively inhibit $Kras^{G12D}$ mutation-induced ADM and

maintain the phenotype of acinar cells (24). However, the transcriptional factors in ductal cells including Hnf6 and Sox9 have been shown to be upregulated in human ADM as well as in mouse models (60). Acinar cells are usually resistant to the oncogenic Kras mutation and repression of tumor suppressor gene Ink4a. However, when exposed to inflammatory injury, these acinar cells can undergo metaplasia and transform to PanIN lesions and PDAC (61). Treatment of the inflammation in chronic pancreatitis showed senescent PanIN lesions, which suggests that anti-inflammatory drugs may be useful in preventing PDAC in patients with familial pancreatitis (61).

1.3.4 Diagnosis of PDAC

Recognizing the pathologic features of PDAC and its precursors allows for its definitive diagnosis. However, early detection before any symptoms or biopsy is extremely critical because it will prolong the patients' curable window. A 5-year survival rate is up to 60% in early small carcinoma of the pancreas (diameter less than 2cm defined as s-PC), compared to 5% upon late detection (62). Although genetic studies help us to better understand the molecular mechanisms of PDAC development, population genetic screening is still very challenging due to the lack of specificity and low cost benefit. The best approaches for early diagnosis remain increasing imaging resolution and finding sensitive biomarkers.

1.3.4.1 Current early diagnosing approaches

High-quality pancreatic-protocol CT scans are commonly used for identifying advanced pancreatic cancer and for initially determining cancer stages. To identify the small lesions, endoscopic ultrasound (93%) is more widely used compared to other imaging approaches, such as MRI (81%) and CT (27%) (63). Fine-needle aspiration and pancreatic fluid collection guided

by endoscopy have been introduced to collect susceptible cancer cells and proteins secreted by cancer cells. Urine has also been shown to be useful in aiding the diagnosis because some biomarkers are elevated in the urine (64).

1.3.4.2 Diagnostic, prognostic and predictive biomarkers for PDAC

Based on their clinical use, biomarkers can be classified into: the diagnostic biomarkers that aid early diagnosis in non-invasive ways; the prognostic biomarkers that predict survival rate and metastasis, and monitor tumor relapse; and the predictive biomarkers that predict the treatment response and direct the personalized treatment (65). Some biomarkers in other tumors harbor satisfying sensitivity and specificity in clinical practice, and therefore have been widely used for screening in high-risk populations. For example, BRCA1 and BRCA2 mutations predict a high risk of developing breast cancer and ovarian cancer, and have been widely applied to the clinical practice (66, 67).

CA19-9, the only FDA-approved biomarker for PDAC, is limited for predicting treatment responses and monitoring tumor relapses (68). Due to its low sensitivity, it is not used for cancer screening among the general population (only 1.9% identified) (69). The sensitivity and specificity increased to 70% and 87%, respectively, in high-risk individuals with pancreatobiliary diseases (70). Furthermore, there have been thousands of biomarkers identified by various sources in the last two decades, including plasma, pancreatic fluid, tissues from laser capture microdissection and circulating cancer cells. Hu-antigen R (HuR or ELAV-like protein 1) activates deoxycytidine kinase, which is essential for the activation of gemcitabine in the treatment of PDAC patients. Therefore, higher HuR predicts a better response to gemcitabine and prolonged survival rate after treatment (71). Some studies use mass spectrometry-based proteomic analysis to identify the potential diagnostic biomarkers and combine them as a panel

(72, 73). A panel containing Reg1B, syncollin, anterior gradient homolog 2 protein (AGR2) and lysyl oxidase-like 2 (LOXL2) showed an improved performance compared to CA19-9 in distinguishing PDAC patients from normal subjects (AUC 0.92 vs. 0.82) (73).

New forms of biomarkers in addition to cell surface antigen or secreted proteins from cancer cells are emerging. MicroRNAs are important for cancer development and the modulation of drug responses. The levels of miRNA-21 was elevated in both tissues and plasma of PDAC patients. A higher level of miRNA-21 predicted a worse overall survival rate and a poorer response to gemcitabine (74, 75). Moreover, detection of circulating tumor cells has become a new approach to predicting the prognosis of PDAC; however, these cells are only detectable in advanced and/or metastatic stages, limiting their applications in the early diagnosis (76, 77).

1.3.4.3 Differential diagnosis of PDAC

Ideal biomarkers would be those that not only diagnose at early stages, but also distinguish PDAC from the precursor lesions, such as ADM and PanINs. In this way, the development of advanced cancer could be prevented since PDAC spreads very rapidly once developed. It also needs to differentiate them from other benign disorders, such as intraductal papillary mucinous neoplasm (IPMN), mucinous cytoplasmic neoplasm (MCN) and chronic pancreatitis caused by obstruction. These benign disorders, to some extent, could also contribute to the development of PDAC. Moreover, liver is the most common metastatic site for PDAC. If metastasized to liver, it also needs to be distinguished from intrahepatic cholangiocarcinoma (ICA), a primary bile duct carcinoma occurring in the liver. Their morphological and immunological features are essentially identical, but the management and clinical outcomes differ completely. ICA is less malignant compared to PDAC, with a 5-year survival rate that

varies from 10–20% (78-80). Therefore, seeking specific biomarkers to distinguish PDAC from other diseases is also clinically essential.

1.3.5 Therapeutic targets in major signaling pathways of PDAC development

The studies on cascades of ADM-PanIN-PDAC transition help the understanding of the cancer development and further provide potential therapeutic targets for PDAC. Among various pathways, some of the classical pathways have been well studied and used as therapeutic targets in clinical practice or trials, while others are still in the basic research phase. The application of basic research to translational medicine is a key approach for developing new therapeutic drugs for PDAC. Since identifying and treating the cancer at its early stage is the key for improving the survival rate of PDAC patients, the following review will focus on the major early mutations and signaling changes in PDAC development (Fig 1.2).

1.3.5.1 Ras-Raf-Mek-Erk signaling pathway

The activation of the mitogen-activated protein kinase (MAPK) pathway is very common in cancer, especially gastrointestinal cancers. Epidermal growth factor receptor (EGFR) can be activated by binding to its ligands, such as EGF and TGF-α. Upon stimulation, the tyrosine kinase domain of EGFR is phosphorylated and the downstream Ras-Raf signaling is activated. Subsequently, the downstreams Mek/Erk are activated. In addition, EGFR also activates the PI3K-Akt signaling pathway, and it has multiple cross-talks with the MAPK pathway. Both of them result in proliferation and anti-apoptosis of the cells. The roles of the MAPK and PI3K/Akt signaling pathways in the carcinogenesis of PDAC have been extensively studied.

EGFR and its ligands TGF-α and EGF have been shown to be elevated in PDAC (81). Overexpression of TGF-α induced ADM transition and promoted the progression to PDAC by

binding to EGFR and activating its downstream signaling (59). Erlotinib, an EGFR inhibitor, has been shown to be superior when combined with gemcitabine to gemcitabine alone in treating patients with advanced PDAC (82). This therapeutic protocol has been approved for the clinical management of advanced PDAC patients.

The Ras family contains three members: Kras, Nras and Hras. Kras is the most common mutation in PDAC (90%). With mutation, it is excessively activated due to the decrease of GTPase activity independent of growth factor stimulation. The Kras mutation is required both in the initiation and maintenance of PDAC as studied by engineered inducible Kras mutated mice (56). In synergy with pancreatitis stimulation, Kras mutated mice develop ADM/PanIN lesions in 3 weeks and PDAC in 10 weeks (61). Inactivation of the pancreas-specific Kras mutation reversed the ADM and regressed the established PanIN lesions. With other subsequent loss of tumor suppressor genes, such as p53 or Ink4a, Kras mutated mice can develop PDAC within weeks. Farnesyl transferase inhibitor (FTI) Tififarnib has been used to inhibit Ras activity by increasing the GTPase activity. However, its efficiency is still debated when compared with gemcitabine alone (83).

It is difficult to inhibit the Ras activity due to its lack of binding pockets. The downstream signaling provides alternative targets for inhibiting the cancer growth. MAPK signaling is activated in response to the pancreatitis with the Kras mutation (84). Inhibition of Mek can prevent or even reverse the carcinogenesis by inhibiting PanIN lesions and promoting the redifferentiation of ductal to acinar cells (84). Another downstream signaling of Kras-driven oncogenesis is PI3K/PDK1, which are key regulators of cell proliferation and carcinogenesis. Pancreatic-specific overexpression of PI3K mimicked the effects of Kras-induced ADM and PanIN lesions. Reversely, elimination of PI3K and/or PDK1 effectively blocked the Kras-driven

oncogenesis (85). However, clinical trials targeting MAPK and PI3K signaling pathways failed to inhibit the cancer growth, since it results in the de-repression of their upstream molecules (86-88). Although the EGFR-Ras-MAPK/PI3K signaling is very common in cancer, it is difficult to find effective targets to treat PDAC. Therefore, discovering alternative signaling pathways is critical to finding new therapeutic targets.

1.3.5.2 Hedgehog signaling

Hedgehog signaling is essential in the pancreatic embryonic development. The Hedgehog ligands bind to protein patched homolog1 (PTCH1), relieving its inhibitory effect on smoothened homolog (SMO) and leading to the activation of GLI transcription factors. Hedgehog signaling is excessively activated in the early development of PanIN lesions and its level is positively correlated with the progression of PDAC (89). However, its role in the pancreatic cell plasticity is controversial. It has been shown that the loss of intact Hedgehog signaling impaired the regeneration of pancreatic acinar cells and led to the persistent cell metaplasia in response to the caerulein-induced pancreatitis (90). However, another study has shown that pancreas-specific Hedgehog overexpression promoted the metaplasia from acinar to ductal cells and the formation of stroma around the cancer (91). This finding suggests that the Hedgehog pathway may be required for cell regeneration and re-differentiation to normal exocrine pancreas, but may also promote cell transdifferentiation if forced to express. A synthesized SMO inhibitor IPI-926 has been shown to reduce the desmoplasia of the stroma and increase the uptake of gemcitabine by tumors. This, along with another Hedgehog inhibitor IPI-26GDC-0449, is under phase II clinical trials in patients with metastatic PDAC in combination with gemcitabine and other nanoparticled drugs (NCT01088815 and NCT01130142).

1.3.5.3 Wnt/β-catenin signaling

Like Hh signaling, Wnt/β-catenin signaling is also important in the embryonic development of the pancreas. There are three types of Wnt signaling pathways: the canonical cascade, the non-canonical planar cell polarity pathway and the Wnt/Ca^{2+} pathway (92). The canonical pathway has been shown to be associated with PDAC development. In the canonical pathway, upon the binding of Wnt ligands, the Frizzled family receptor interacts with its co-receptor LRP5/6 and inactivates a complex of cytoplasmic proteins that promote the degradation of β-catenin. This leads to the accumulation of β-catenin in the cytoplasm and its translocation to the nucleus, which can further bind to TCF/LEF and activate its downstream targets. Accumulation of β-catenin is observed in PDAC and its amount is correlated with grades of PanIN lesions and progression to invasive cancer (93). Surprisingly, stabilized β-catenin does not give rise to PDAC but suppresses it through promoting acinar cell regeneration (94). In wild-type mice, β-catenin was accumulated and promoted acinar cell regeneration followed by caerulein-induced pancreatitis. However, this signaling is blocked in Kras-mutated mice, characterized by ADM transformation and progression to PanIN/PDAC (94). Therefore, β-catenin signaling is critical to inhibit Kras/pancreatitis-induced acinar to ductal reprogramming and PDAC development. However, once the reprogrammed ductal cells cross the threshold and develop cancer, β-catenin has its positive effects on PDAC progression.

1.4 Diabetes and β-cell regeneration

1.4.1 Diabetes biology and its molecular mechanisms

Excessive proliferation in the exocrine pancreas may be detrimental and increases risks of developing cancer. However, it may be beneficial for diabetic patients to have β-cell proliferation in order to regain their islets function in the endocrine pancreas. Diabetes is becoming a leading world health problem as a result of rapid changes in the modern lifestyle. In 2012, about 29.1 million Americans, or 9.3% of the population, are living with diabetes and 86 million with prediabetes, according to the statistics from the American Diabetes Association. Among them, about 1 million people have type 1 diabetes (T1D), usually youths, and the remaining 28 million have type 2 diabetes (T2D). There are also some less common types, such as the maturity onset diabetes of the youth (MODY), caused by the inherited mutations of Hnf1α, Hnf4α or glucokinase genes (95) and the latent autoimmune diabetes of adults (LADA). T1D is an autoimmune disease. In T1D, the β-cell is destroyed by the immune system, which results in insufficient insulin secretion to maintain the glucose homeostasis. In contrast to T1D, T2D patients usually present with normal or hyperinsulinemia, but their insulin sensitivity is impaired. Obesity and aging are closely associated with insulin resistance and the development of type 2 diabetes. In early onset of obesity, β-cells can compensate the increased need for insulin in the human body through the expansion of islets and increase of insulin secretion (β-cell compensation). The stimulants contribute to this process via increased nutrient supply such as a high-fat diet, growth factors that promote β-cell replication and increased levels of incretins such as glucagon-like peptide-1 (GLP-1) (96). At this stage, the glucose tolerance is normal or impaired, but the blood glucose level is still maintained within the normal range. As the presence

of stimulants becomes prolonged, the β-cell is no longer able to sustain its normal function (β-cell failure), and consequently the patients present with hyperglycemia and develop diabetes (96) (Fig 1.3). The β-cell failure is further caused by decreased β-cell regeneration and increased apoptosis. This decompensation of the β-cell is more prevalent in patients with a predisposing genetic background, particularly PPARγ coactivator 1-α (PGC1α) (97, 98).

B-cell dysfunction occurs early in the impaired glucose tolerance stage and increases in severity as T2D develops. The molecular mechanisms involved in the β-cell failure include endoplasmic reticulum (ER) stress, glucolipitoxicity, islet amyloid deposition, oxidative stress and other inflammatory stresses that could destroy β-cells and impair their normal function. Only major mechanisms that are relevant to the present study will be discussed. More detailed mechanism reviews can be found elsewhere (96, 99).

1.4.1.1 ER stress

The ER is the major site for protein synthesis and folding. In response to obesity, insulin secretion is increased to compensate the reduced insulin sensitivity. This compensatory response requires increased synthesis of pro-insulin from the ER. The excessive protein load in the ER results in an accumulation of misfolded and unfolded proteins, termed ER stress. This could activate an adaptive response called unfolded protein response (UPR) (100). UPR can help to attenuate the stress and restore ER homeostasis by increasing protein folding efficacy and protecting cell survival. However, when the ER stress is unresolvable, the UPR fails to restore ER homeostasis, therefore leading to cell apoptosis through activation of IRE1α and/or PERK (101). ER stress now is considered an important mechanism of β-cell failure in T2D patients. The three important initiators of the UPR include inositol requiring 1 (IRE1), PKR-like ER

kinase (PERK) and activating transcription factor 6 (ATF6), which are also considered as markers for ER stress (101).

1.4.1.2 Glucolipotoxicity

Another mechanism that directly destroys β-cell is glucolipotoxicity. Glucolipotoxicity refers to the fact that chronic hyperglycemia and hyperlipidemia cause detrimental effects on insulin secretion and β-cell survival (102) (Fig 1.4). In the early stage of hyperglycemia, high glucose causes permissive effects on lipid metabolism by increasing lipolysis from peripheral fat tissues and fatty acid oxidation. As the stress is prolonged, the combined excessive glucose and free fatty acids in the circulation can cause a decrease of insulin secretion and increase of β-cell apoptosis. *In vitro* studies have shown that chronic exposure to high saturated fatty acid such as palmitate in isolated islets can lead to the impairment of glucose stimulated insulin secretion (GSIS), accompanied by decreased β-cell specific transcriptional factors Pdx-1 and MafA (103-106). This chronic exposure also leads to the activation of oxidative stress and ER stress.

In addition, elevated free fatty acid in the blood results in its increased delivery to the liver. Consequently, there will be increased triglyceride synthesized and accumulated in the liver, causing non-alcoholic fatty liver disease (NAFLD), also called fatty liver (107). It becomes more severe when the condition of insulin resistance is prolonged because diminished insulin suppression on lipolysis can further lead to more fatty acid flow into the liver.

1.4.1.3 Adipose tissue inflammation

Besides targeting the β-cell itself, T2D is associated with multiple systemic imbalances. New understandings suggest that T2D can be considered as an inflammatory disease due to the activation of various cytokines and inflammatory signaling pathways (99). Levels of cytokines

including interleukin -1β (IL-1β), IL-6 and C-reactive protein (CRP) have been shown to be elevated in T2D and can be used as biomarkers to predict other microvascular diseases associated with T2D (108, 109). Adipose tissue is a major source of cytokines in obesity and T2D. It is now considered as an endocrine organ due to its function of secreting pro-inflammatory and anti-inflammatory adipokines (110). Previously, it was believed that cytokines associated with obesity are produced from adipocytes; but this is now thought to be due to the macrophages in the adipose tissues. There is a large number of macrophages infiltrating the adipose tissue and they contribute to the inflammatory response in obesity, T2D and other metabolic diseases (111). There are two types of macrophages: M1 phenotype is characterized by the expression of tumor necrosis factor-α (TNF-α), IL-6 and iNOS; M2 phenotype is characterized by the expression of YM1 (also known as CHI3L3), arginase 1 and anti-inflammatory cytokine IL-10 (112). In HFD-induced obesity, there is a switch from M2 phenotype to M1 phenotype and it contributes to the progression from obesity to insulin resistance and T2D (113). T2D is characterized by increased M1/M2 ratio in adipose tissue and is a predictor for the severity of T2D. Activation of M2 phenotypic macrophages can protect obese mice from developing T2D. In addition to macrophages, other immune cells also have shown increased infiltration and activation in adipose tissue in T2D, such as mast cells (114). Mast cell-deficient mice exhibited improved insulin sensitivity compared to obese controls. Mast cell-derived IL-6 and IFN-γ contribute to insulin resistance and T2D (115).

1.4.1.4 AMPK

Besides adipose tissue inflammation, T2D is also systematically regulated by metabolic factors. AMP-activated protein kinase, or AMPK, is a systemic energy sensor and plays a critical role in regulating metabolic homeostasis in the human body. It is activated when the ratio of AMP/ATP

increases during fasting, hypoxia, exercise and heat shock (116). Adiponectin and leptin, adipokines secreted from adipose tissue, can also activate AMPK (117, 118). Upon activation, AMPK leads to the increase of the energy production (catabolic process) such as glucose uptake, glycolysis, fatty acid oxidation and the decrease of energy consumption (anatabolic process) such as lipogenesis and gluconeogenesis. High glucose suppresses the activity of AMPK and its downstream p70 ribosomal S6 kinase (p70S6K) while low glucose stimulates its phosphorylation (119). Defects of AMPK signaling in the pancreas and liver contribute to the development of insulin resistance, T2D and other metabolic disorders (116). Pharmacological AMPK activator metformin is the first-line medication for the treatment of T2D. Further, AMPK has also been shown to inhibit tumorigenesis by modulating key factors in cell proliferation, cell cycle progression and metabolism (120, 121). The use of metformin is now under Phase II clinical trials, in combination with other chemotherapy drugs such as gemcitabine and erlotinib (NCT01210911, NCT02005419).

There are also other mechanisms leading to insulin resistance, β-cell failure and T2D. All these molecular signaling pathways are not independent; they interact with each other and lead to further β-cell damage with the progression of T2D. For instance, AMPK signaling is impaired in the process of glucolipotoxicity and can further lead to the excessive hyperglycemia and hyperlipidemia (96). Increase of free fatty acid leads to the β-cell apoptosis through activation of UPR and NFkB in inflammatory response (122).

1.4.2 β-cell regeneration

As T1D and T2D progress, the common characteristics they show is loss of β-cell mass and β-cell dysfunction. Therefore, promoting β-cell regeneration and improving their function become alternative therapeutic approaches for both T1D and T2D.

1.4.2.1 Physiological characteristics of β-cell regeneration in the development of the pancreas

In both normal and pathophysiological states, β-cell mass is determined by changes in the rate of replication, individual cell volume, and cell death rate (123). Even though neogenesis from progenitor cells can be an alternative source of beta cells, β-cell self-duplication still remains the major source of β-cells. A high rate of β-cell replication has been observed in the embryos of late gestation and in newborns. A similar increase in the activity of cellular apoptosis also occurs during the newborn and postpartum period in the mother. The dynamic changes in the replication and apoptosis may contribute to the remodeling of β-cell mass during these periods (123-126). They are both reduced significantly beyond 3 months of life, and remain with low activity unless under physiological/pathological situations. Low rate of β-cell replication lasts throughout the lifespan, which is closely correlated to the body weight increment. Early in life, increases of β-cell size and number contribute to the increase of β-cell mass. However, in aged animals, the β-cell hypertrophy is mainly responsible for the increased β-cell mass (126).

1.4.2.2 Different approaches to promote β-cell regeneration

There are three major efforts to promote β-cell regeneration:

1) *To promote the self-replication and reverse the destruction of existing β-cells.* A number of growth factors have been reported to promote β-cell expansion in animal models,

such as IGF-I (127, 128), gastrin (129, 130), transforming growth factor (TGF)-α (131-133), GLP-1 (134), exendin-4 (135-138), and Reg family proteins Reg1 (139, 140) and INGAP (Reg3δ) (141). Transgenic mice with β-cell specific overexpression of IGF-I displayed increased β-cell mass in parallel with a higher rate of neogenesis and β-cell replication. Hence, the mice showed better recovery from the hyperglycemia and hypoinsulinemia induced by streptozotocin compared to control animals (127). In the pancreatic regeneration model stimulated by duct ligation, gastrin expression was strongly induced in the ligated part of the pancreas shortly after surgery (142). Mice that overexpress both gastrin and TGF-α showed significant increase in islet cell mass, suggesting a synergistic effect of the two factors on stimulating islet cell growth (132). GLP-1 or exendin-4 treatment increased pancreatic insulin content and β-cell mass and decreased basal glucose level in streptozotocin-treated neonatal rats in both the short and long term (137). The contribution of Reg family proteins to cell growth and survival in pancreatic islets will be introduced in detail in the following sections.

2) *To directly differentiate stem cells or pancreatic progenitor cells into β-cells*. Stem cells have the ability to differentiate from one precursor cell to multiple specialized terminal types, whereas progenitor cells can only be differentiated into their (one-and-only) direct targets. In the adult pancreas, β-cells preserve a limited ability to replicate and generate new cells from progenitor cells under stress or injury. Therefore, identifying these progenitor cells and factors influencing their differentiating outcome represents a promising avenue towards rescuing diabetes. A differentiation process that converts human embryonic stem cells (ESCs) to endocrine cells has been developed by using a series of transcriptional factors at different stages (143). It is generally accepted that pancreatic progenitor cells can redifferentiate into β-cells, as evidenced by the observation of islet cell budding from the ductal structures during

embryogenesis or postnatal growth (144). Although replication is the major source of β-cell renewal, around 30% of new β-cells can arise from neogenesis from non-β-cell precursors in adult rats (145). Neogenesis of β-cells was also observed in response to various stresses, including 90% pancreatectomy (139) and partial obstruction of the pancreas (146). Recent studies revealed that progenitor cells located in the ductal epithelium can be induced to express Ngn3 and become new β-cells after partial duct ligation in the mouse pancreas (147). In addition, adult ductal and acinar cells could be de-differentiated into a progenitor state and then re-differentiated into β-like cells by a combination of pancreatic islet transcriptional factors, including Pdx1, Neurogenin (Ngn) 3 and Maf A(148).

3) *To transdifferentiate from other endogenous pancreatic cells.* Cells in the periphery of islets in the neonatal pancreas strongly express ductal markers CK19 and CK20, and may serve as islet progenitors (149). Shortly after birth, CK19 expression expands to the whole pancreas and is turned off in differentiated islet cells (150). This transient expression of CK19 suggests that new islets may arise from ductal cells. In response to appropriate stimuli, ductal epithelial and acinar cells can be transdifferentiated to regenerative islet cells (151). Gastrin alone, or in combination with epidermal growth factor (EGF), increased the expression of Pdx1 and insulin in isolated CK19-positive human ductal cells (129). Overexpression of TGF-α upregulated the Pdx1-expressing epithelium characterized by the expression of Pax6 and initiates islet neogenesis (131). Exendin-4, an agonist of GLP-1 with a longer half-life, also facilitates β-cell neogenesis in rat and human pancreatic ducts (138).

1.5 Isoform-specific functions of Reg proteins in cell growth and differentiation in PDAC and diabetes

After reviewing the development of PDAC and diabetes in the exocrine and endocrine pancreas, we are able to gain a general impression of how the two diseases are impacted by the pancreatic development and interact with each other. Reg proteins, mainly produced by acinar cells, modulate the functions of the endocrine and exocrine pancreas in various ways. Before reviewing the roles that Reg proteins play in these two diseases, their structure, expression and biological functions will be briefly introduced.

1.5.1 Classification of Reg proteins based on protein sequence

In mice, seven unique Reg genes have been discovered, all of which are located on chromosome 6C except Reg4 (Fig 1.5A). Subsequently, four Reg genes in rats and five in humans have been identified. The molecular relationships based on sequence comparison and alignments are summarized in Table 1.1 Based on sequence homology, and the phylogenetic analysis data retrieved from BLAST database in NCBI, Reg proteins can be divided into four groups: Reg1, Reg2, Reg3 and Reg4 (Fig 1.5B) (10). With the exception of Reg4, which has seven exons, all of the other Reg family genes are structured into six exons separated by five introns spanning about 3 kb. The first exon encodes the 5'-UTR. The second exon encodes the remainder of the 5'-UTR, the ATG start codon and the initial protein coding sequence. Exons 3 to 6 encode the body of the proteins with the 3'-UTR located in the sixth exon (152). In Reg3 subfamily proteins, there is a common 5-aa insertion in the C-terminal regions (152). Based on the high levels of domain/sequence identities, the gene family is probably derived from the same ancestor gene through gene duplication events accumulated during evolution. Currently, Reg2

and Reg3δ/INGAP are only found in mice and hamsters; more Reg3 isoforms should be discovered in rats and humans.

Table 1.1 Members of the Reg family proteins in the mouse, rat and human.

Several alternate names for Reg proteins occur in the literatures, such as HIP, gene expressed in hepatocellular carcinoma-intestine-pancreas; PAP, pancreatitis-associated protein; PSP, pancreatic stone protein; PTP, pancreatic thread protein; and RELP, regenerating protein-like protein. Reg stands for **reg**enerating islet-derived. The data is mostly based on NCBI collections. Based on the levels of sequence identity, mouse Reg1 seems to correspond to two human genes, Reg1A and Reg1B, and Reg3α and Reg3β correspond to two unique rat genes and one human gene, respectively. When identical proteins were repeatedly submitted to Genbank, NCBI or UniProtKB/Swiss-Prot, "=" is used to list identical protein IDs and orthologues in a single cell.

Mouse genes	**Orthologues**	**Transcript**	**Polypeptide**
Reg1 (93-95)	Reg, PTP, PSP, lithostathine	NM_009042	NP_033068 = P43137
	Rat Reg1/1α (153, 154)	NM_012641	NP_036773(139, 155) =P10758
	Human Reg1A, PSP, PTP, lithostathine	NM_002909	NP_002900(156, 157) =P05451
	Human Reg1B, RegL (158), PSP2	NM_006507	NP_006498.1

			=P48304.1
Reg2 (93; 97)	PTP2, PSP2, lithostathine 2	NM_009043	NP_033069.1(159, 160) =Q08731
Reg3α (92)	PAP2, PAP II, Reg IIIalpha	NM_011259	NP_035389(152, 161) =O09037
	Rat Reg3α (REG3A), Rat Reg III	NM_001145846 NM_172077.2	NP_001139318 =NP_742074.2 (155, 162)
	Rat PAP II	L10229	AAA02980.1 =P35231.1(162, 163)
	Human Reg3G , PAPIB, Reg III	NM_198448 =NM_001008387.2 AB161037 AY428734	NP_940850.1(164, 165) =Q6UW15.1 =NP_001008388.1 =BAD51394.1 =AAR88147.1
Reg3β (92; 100; 101)	PAP, PAP1, PAP I, HIP, Reg IIIbeta,	NM_011036	NP_035166(159, 166) =P35230

	Rat Reg3β, PAP	NM_053289	NP_445741 (155, 167) =P25031.1
		M98049	=AAA16341.1(168)
	Rat Reg-2, Reg2	S43715	AAB23103.1(169, 170)
	Human Reg3A, HIP, PAP, INGAP (171)	NM_002580.2	NP_002571.1(161, 172) =Q06141.1 =NP_620354.1 =NP_620355.1 =AAH36776.1
		NM_138937.2	
		NM_138938.2	
		BC036776	
		M84337.1	AAA36415.1(173)
Reg3γ (92)	PAP3, PAP III, Reg IIIgamma	NM_011260	NP_035390.1(152, 174) =O09049
	Rat Reg3γ, PAP III	NM_173097	NP_775120.1(175, 176) =P42854.1
Reg3δ (91; 105)	INGAP, Reg3d, RegIII delta	NM_013893	NP_038921(177, 178) =Q9QUS9
		NM_001161741.1	NP_001155213.1(178)
	INGAP-related protein	AB028625.1	BAA92141.1 (177)

Reg4 (106-108)	RELP, Reg IV	NM_026328	NP_080604.2(179, 180) =Q9D8G5
	Rat Reg4	NM_001004096.1	NP_001004096 (181) =Q68AX7.1
	Human Reg4	NM_032044 =NM_001159352.1	NP_114433.1(4, 182) =Q9BYZ8.1 =NP_001152824.1
		NM_001159353.1	NP_001152825.1

All members of the Reg family are synthesized on the rough endoplasmic reticulum and secreted to the pancreatic exocrine drainage system from zymogen granules, similar to other secreted proteins from the pancreas. They all contain the typical C-type lectin-like domain (CTLD) (Fig 1.5C) that is subject to trypsin cleavage at the Arg-Ile bond located at 11th residue of N-terminal. It results in the formation of insoluble fibrils (183). This sensitive cleavage site is conserved in 18 Reg/Reg-related proteins from 6 different species (human, bovine, mouse, hamster, pig and rat). Trypsin cleavage converts three of the soluble 16 kDa Reg proteins (Reg1, Reg3β, Reg3γ) into 14 kDa insoluble products that are completely resistant to trypsin and partially resistant to other proteases from the pancreatic juice. Reg3α is also processed into the 14-kDa form, but its product remained soluble and is only resistant to trypsin, but not other proteases. How this cleavage affects the function of Reg proteins remains to be understood. It was found that trypsin-activated insoluble isoforms of Reg1, Reg3β and Reg3γ polymerize into highly organized fibrillary structures with helical configurations (184). The C-terminal cleavage product of rat Reg1 spontaneously precipitates at a neutral pH (185). This insoluble form may

play a key role in forming protein plugs in chronic pancreatitis. In more than one half of patients with pancreaticobiliary malfunction, Reg1, together with trypsinogen and activated trypsin, were detected in both the bile-duct bile and the gallbladder bile, whereas none of the pancreatic enzymes or Reg1 were detected in the controls (186).

1.5.2 Expression pattern during the embryonic development in humans and rodents

The expression pattern of Reg genes during development may vary from gene to gene depending upon the types of tissue and the age of development. The expressions of Reg1 and Reg2 genes have been investigated in the whole mouse embryos from 8.5 to 12 days of the development, as well as that of Ins1 and Ins2 genes. *Reg1* mRNA becomes detectable at day E9, following the onset of Ins2 expression at day E8.5. *Reg2* mRNA is not detectable until E12, when Ins1 transcription takes place (187). This suggests that the two insulin genes and the two Reg genes are induced and expressed differentially during the early development of the mouse. In the human fetus, Reg1A expression is observed only in the pancreas, in contrast to its widespread expression in adults (188). The level of human pancreatic Reg1A transcript is low before 16 weeks of gestation, at which time it increases dramatically and reaches a similar level as in the adult by 20-week gestation (189). Despite its early expression, the Reg1A/Reg1 gene might not be involved in β-cell or acinar cell growth during human and rat fetal development due to a lack of coordination between Reg mRNA levels and insulin gene expression (190, 191). Human Reg1B transcript is present not only in the pancreas, but also in the colon and brain of the fetus (188). Interestingly, expression of Reg1A was higher than Reg1B in human fetal pancreata, but the reversed expression pattern is observed in the adult pancreas where Reg1B is higher than Reg1A (192). Reg3A/PAP mRNA expression displays a broad distribution in the

human fetus, which has been observed abundantly in the pancreas, stomach, jejunum, colon and, to a much lower level, pituitary gland. The expression of Reg3A/PAP transcript in these tissues lasts throughout the adult lifespan, being especially high in the jejunum (188). Reg3A/PAP protein is first detectable at 8 weeks for an embryo in the endocrine nests co-stained with chromogranin A. The expression of Reg3A/PAP reaches a level comparable with that of the adult pancreas at 10 weeks, being detected only in the glucagon-producing cells. No expression is detected in the exocrine pancreas of the fetus (193). Mouse INGAP/Reg3δ is present in cells that co-expressed insulin or somatostatin, but not glucagon, in the developing pancreatic bud of the embryo. Surprisingly, the colocalization of glucagon and INGAP occurred in the mouse islet cells to a significant level postnatally (194). This switched expression in the endocrine cells suggests that INGAP can be used as a marker in the embryonic development of the pancreas. The expression pattern of the other Reg isoforms including Reg3G and Reg4 has not been systematically studied yet in the embryos of humans or rodents.

Postnatal expression of the Reg proteins has only been analyzed in rodents. After birth, total *Reg* expression in the pancreas showed an age-dependent decline, which decreased by 55% at 30-month old versus 1-month old in mice. *Reg1* mRNA level in the pancreas decreases progressively with age, and the changes of its level was in parallel with that of insulin. However, *Reg2* mRNA level does not decline significantly, indicating that Reg1 and Reg2 expressions in the pancreas have differential age-dependent regulation (195). This is in contradiction with what has been observed in our lab. We have shown that both the mRNA and protein levels of Reg2, Reg3α and Reg3β were very low at Day 7 after birth, boosted at Day 30 and decreased significantly at Day 90. INGAP exhibited a different expression pattern, with its level increased constantly from Day 7 to Day 90. In addition, Reg1 was detectable in the duodenum and

pancreas of newborn rats, and was dramatically increased at 3 weeks of age. Reg3β mRNA was undetectable in neonatal rats, and displayed a sudden increase in the ileum around the time of weaning. A decline of Reg1 and Reg3β expressions in the ileum was observed in older rats (3).

1.5.3 Biological features of Reg proteins and their receptors

1.5.3.1 The regulation of Reg proteins expression

IL-6, in synergy with dexamethasone, has been shown to significantly induce the expression of Reg1, Reg2 and PAP/Reg3β in pancreatic acinar AR42J cells and/or islet MIN6 cells (159, 196, 197). These effects were mediated by the binding of transcriptional factors poly (ADP-ribose) synthetase/polymerase (PARP) and inhibited by PARP inhibitor nicotinamide (159). Another cytokine IL-22 has also been shown to induce Reg3β expression in acinar cells via activation of STAT3 (198). In addition, Reg3β/hReg3A can also induce its own mRNA and protein expression via a positive feedback mechanism. Several transcriptional regulatory elements have been identified after examining the 5'-flanking sequences of Reg family genes. IL-6 response elements, mediating putative acute-phase responses, are located in the 5'-flanking region of all mouse Reg genes. Pan-1 motif sequences (CACCTG) are located in the promoter regions of mouse Reg3α and Reg3β, rat Reg1 and hamster INGAP genes. Pit-1 element, which mediates pituitary-specific transcription, is located in the promoter regions of mouse Reg3α, Reg3δ, Reg3γ and rat Reg3β genes (152). In addition to the IL-6 and Pit-1 response elements shared with other Reg3 genes, Reg3δ also contains consensus motifs for Myo D and IRf 1/IRf 2 binding sites in the promoter region, which suggests an isoform-specific expression and response (199).

1.5.3.2 The potential receptors of Reg proteins

The putative interactions of Reg proteins and Reg receptor(s) have not been sufficiently elucidated to date. The Reg1α receptor cDNA was isolated from rat islets in an open reading frame of 2760 bp. The 919-amino acid protein was suggested to be a type II transmembrane protein with a long extracellular domain (868 aa), a single transmembrane domain (residues 29-51), and a short N-terminal intracellular region (200). The rat receptor is homologous to human EXTL3/EXTR1, a member of the EXT gene family. EXTL3 is a glycosyltransferase that is involved in the heparan sulfate biosynthesis and can modulate NF-κB signaling upon stimulation by TNF-α (201). The mRNA of Reg1α receptor was detected in normal pancreatic islets, regenerating islets and insulinoma RINm5F cells. The receptor transcript was also expressed in a wide range of other tissues, including liver, kidney, spleen, thymus, testis, adrenal gland, stomach, ileum, colon, pituitary gland and brain, but not in the heart or jejunum (200). Reg1α receptor-expressing RINm5F cells showed significant Reg1-dependent growth acceleration as indicated by BrdU incorporation. However, the expression of Reg1α receptor remained unchanged in regenerating islets as compared to normal ones, suggesting that both proliferation and apoptosis of pancreatic β-cells are primarily regulated by the expression of the *Reg genes,* but not the receptor (200).

Beside the isolation of Reg1α receptor, the mechanism of Reg3δ/INGAP action on RIN-m5F cell proliferation was also explored (202). Both the full-length recombinant protein and bioactive peptide of INGAP (INGAP-P, a pentadecapeptide corresponding to amino acids 104-118) stimulated cell regeneration via binding to Gi-protein coupled receptor and activating Ras/Raf/Erk signaling pathway. Activation of ERK1/2 can be blocked by pertussis toxin, a

reagent that can prevent the G proteins from interacting with corresponding receptors on the cell membrane. Further, the PI3K/Akt pathway was also activated after INGAP administration. However, to date, the sequence of this particular INGAP receptor has not been determined.

1.6 The pathophysiological functions of isoform-specific Reg proteins in diabetes and PDAC

1.6.1 Reg1 [hReg1A and hReg1B]

1.6.1.1 The identification of Reg1 and its expression in the pancreas

Pancreatic stone protein (PSP) and Reg1 were identified separately in the exocrine and endocrine pancreas and proved identical later. PSP/lithostathine was first found in the study of pancreatitis in 1979, which was proposed to promote the formation of calcium carbonate crystals in the pancreas (203). The term Reg1 was then used to describe its roles in the regenerating islets after 90% pancreatectomized rats (139). The Reg1 gene was highly expressed in rat regenerating islets and induced by the treatment with aurothioglucose, a drug that induced islet hyperplasia (139). As human homologues of mouse Reg1, both Reg1A and Reg1B proteins consist of 166 amino acids and differ only by 22 a.a. (204); their protein sequences share 87% identities (158).

Under physiological conditions, a very low concentration of Reg1 protein was detected in the islets or ductal cells. Most of the protein was located in acinar cells (205) and its level increased significantly under acute inflammatory stress or other forms of pancreatic injury. Its mRNA level was significantly elevated in response to interleukin (IL)-6, interferon (IFN) or tumor necrosis factor (TNF)-alpha, but decreased by dexamethasone (206). Expression of Reg1 mRNA in the pancreas was also increased by 12-fold after a 2-week 75% high-protein diet (154).

A consistent increase of Reg1 protein in the pancreatic juice was also detected within 2 weeks of an 82% high-protein feeding (153). In addition, Reg1 is also normally expressed in the gastrointestinal tract, including the duodenum and jejunum, and gastric mucosa, and becomes even upregulated in gastric cancer (139, 154, 160, 195, 207, 208). The highly conserved element at -81/-70 bp region of the Reg1 promoter has been proven essential for the activations by both IL-6/dexamethasone and IL-6/dexamethasone/nicotinamide treatments (209).

1.6.1.2 Reg1 in β-cell function and diabetes

Reg1 expression is closely associated with pancreatic β-cell function. It is proposed to promote proliferation and differentiation of cells in the digestive and endocrine systems through a paracrine or endocrine manner (210). In regenerating islets, Reg1 was found to co-localize with insulin in secretory granules, suggesting that Reg1 is synthesized in and secreted from regenerative β cells (140). Reg1 mRNA levels were increased 3-fold within 2 days in the rat pancreas that received surgical wrapping, which correlated with ductal proliferation and emerging insulin staining within ductal epithelia in the wrapped lobe. However, the induced Reg1 gene expression was localized in the exocrine tissue, suggesting that Reg1 may be involved in the maintenance of normal islet function through induction of new islet formation from precursors of ductal origins (211).

1.6.1.2.1 Reg1 in the replication of existing β-cells

Using isolated rat islets, Reg1 protein stimulated β-cell replication as evidenced by increased [^{3}H] thymidine incorporation in a dose-dependent manner (212). Reg1 proteins isolated from human and bovine pancreata were mitogenic to both ARIP ductal and RIN β-cell lines in a dose-dependent manner, but had no effect on AR42J acinar cells or isolated mature

islets (213). Isolated human and rat Reg1 proteins were also mitogenic to primary ductal cells and may modulate the expansion of the pancreatic ductal population during islet regeneration *in vitro* (214). It suggests that Reg1 can potentiate proliferation of ductal cells and islet β-cells, but not acinar cells. Administration of rat Reg1 protein to depancreatized rats ameliorated the surgical diabetes after 2 months, as evidenced by decreased blood glucose and preserved insulin-producing capacity (212). Diabetic NOD mice treated with recombinant human Reg1A protein showed increased β-cell mass and decreased mortality rate compared to untreated animals. This was interpreted as a result of Reg1A-induced maturation of β-cell precursors in NOD mice (215).

The roles of endogenous Reg1 in islets regeneration have also been studied by the gene knockin or knockout strategies. The rate of [^{3}H] thymidine incorporation was low in cultured pancreatic islets from Reg1-deficient mice (216), but high in those from β-cell specific Reg1-overexpressing (Ins-Reg) mice, indicating that Reg1 protein secreted from the islets stimulated DNA synthesis through an autocrine mechanism. The Reg1-deficient mice had a significant smaller β-cell mass than control animals following goldthioglucose treatment, a drug which induced hyperplasic islets. This finding suggests that endogenous Reg1 might be essential for the cell cycle progression in pancreatic β-cells. The NOD mice carrying the Reg1 transgene showed a delayed onset of diabetes, which coincided with a 3-fold increase in the islet cell volume (216). These data further support the notion that Reg1 promotes the regeneration of β-cells, which, as a consequence, compensates for the β-cell loss and delays the onset of autoimmune diabetes. It is thus conceivable that Reg1A level in the serum of both T1D and T2D patients was significantly elevated (217). However, controversies existed in an early study of transgenic mice overexpressing Reg1 protein in the islets. The transgenic mice became diabetic as a result of

increased β-cell apoptosis, as well as the development of various tumors. This result was explained by the high level of endogenous Reg1 leading to the cell apoptosis via binding to the MKP-1 and inactivating its downstream signaling (218). Nevertheless, most evidence supports that Reg1 protein can be used as a replacement therapy for diabetes.

On the other hand, autoimmunity to Reg1 may be associated with the development of diabetes. A significant increase in anti-Reg1 autoantibodies was found in both T1D and T2D patients compared with healthy subjects. Serum from diabetic patients with Reg1 autoantibodies demonstrated significantly attenuated BrdU incorporation induced by Reg1, while non-diabetic serum without the autoantibodies had small effect (219).

1.6.1.2.2 Reg1 in the neogenesis of β-cells from pancreatic progenitor cells and other exocrine cells

Reg1 expression was detected in mouse embryonic stem cells (ESCs) and can be activated by the Wnt/β-Catenin signaling pathway. β-catenin is important for the maintenance of ESC in an undifferentiated state (220). It indicates that Reg1 might play an important role during the embryonic development. Attempts have been made to assess the effect of Reg1 protein on ESC differentiation by adding recombinant protein and overexpressing the Reg1 gene. However, no significant effect was observed in the cell growth or differentiation compared with controls. Nevertheless, the potential effect of Reg1 on stem cells should be re-examined. If it has protective and/or proliferative effects on stem cells, it can be used along with other transcriptional factors to facilitate the differentiation of ESCs into β-cells.

In addition to the stem cells, the role of Reg1 in pancreatic progenitor cells was also examined. In the early stages of streptozotocin-induced diabetes, both Reg1 expression and BrdU

incorporation can be induced in residual β-cells, indicating a role in β-cell regeneration (221). In the meantime, co-expression of Reg1 and cytokeratin 19 in acini-ductal cells suggested that Reg1 may participate in the transdifferentiation of acinar and/or ductal cells to islet cells (222). In murine pancreatic tumors with acinar-specific overexpression of the gastrin receptor (CCK2R), expressions of Reg1 and Reg3α proteins were strongly upregulated in duct-like cells in pre-neoplastic lesions, or at the periphery of tumors and adjacent acini. The CCK2R transgenic mice showed improved glucose tolerance, increased insulin secretion and doubled insulin contents compared to control animals (223), which indirectly indicated that Reg1 may promote the pancreatic cell proliferation and/or transdifferentiation from exocrine cells. This CCK2R overexpressing mouse model also builds links between the pro-tumor function of Reg proteins in the exocrine pancreas and their β-cell proliferating effects in the endocrine pancreas.

1.6.1.2.3 The signaling associated with Reg1 function in β-cell regeneration

The identification of the putative receptor for Reg1, EXTL3, supports its direct effect on islet proliferation. Reg1 stimulates DNA synthesis in islet β-cells via activating the phosphoinositide 3-kinase (PI3K) and downstream targets including ATF-2 and cyclin D1 (224). In Reg1 knockout islets, the levels of phospho-ATF-2 and cyclin D1 were decreased, resulting in the decreased rate of DNA synthesis. Alternatively, the ERK1/2 pathway was activated during mitogenesis of ductal and β-cell lines triggered by Reg1 overexpression or recombinant protein treatment (225). Using cDNA microarray, significant elevations of mitogen-activated protein kinase phosphatases (MKP-1) and cyclins were detected in Reg1-treated cells (Fig 1.6).

In addition, the action of Reg1 exhibits a dose-dependent manner. Endogenously expressed Reg1 may form a complex with EXTL3, bind to MKP-1 and inactivate JNK, leading to cell

apoptosis or the differentiation into other cells (218). On the other hand, low-dose Reg1 protein promotes the cell proliferation of β-cells and ductal cells. In fact, it has been reported that high extracellular levels of Reg1 over 100 nM could inhibit cell growth (226). It was explained that under a low-dose of Reg1, the protein could bind to its receptor and activate MAPK-cyclin D1 pathway. When overexpressed within cells or cultured in high concentration, Reg1 can inhibit growth by binding to MKP-1, leading to differentiation into other types of cells (Fig 1.6).

1.6.1.3 Reg1 (hReg1A and Reg1B) in PDAC, as potential biomarkers

There are not many studies on the Reg1 subfamily in PDAC. It was reported that Reg1A was upregulated in PDAC tissues compared with normal tissues (227). However, the tumor adjacent neoplastic areas showed even higher levels when compared to tumors, suggesting a possible role of Reg1A in the precursor lesions of PDAC. Moreover, this study also demonstrated that patients with diabetes had higher Reg1A expression compared to those without (227), suggesting an interacting role of Reg1A plays between PDAC and diabetes. But the cause and effect are not clear yet. A recent proteomic analysis identified a 3-biomarker panel in urine to distinguish PDAC from healthy subjects, including LYVE-1, REG1A and TFF1. The biomarkers combination achieved a higher specificity and sensitivity than the widely used biomarker CA19-9 (64). This study also excluded the changes seen in chronic pancreatitis, which is a benign lesion that needs to be distinguished from PDAC in clinical practice. However, the results of Reg1A level in chronic pancreatitis have been controversial, as some of the results showed decreased levels compared to controls whereas others showed no change (228-230). These different results could be partially explained by different forms of Reg1/PSP, either soluble or insoluble, being secreted and detected in different methods. Nevertheless, exclusion of

chronic pancreatitis is critical to establish Reg1 proteins (Reg1A and Reg1B) as a biomarker for diagnosing PDAC. This is also part of our current study (Chapter 2).

Humans have another Reg1 orthologue defined as Reg1B, which shares an 87% identity with Reg1A. Surprisingly, higher expression of Reg1B is more observed in colorectal carcinoma without than with invasion or lymph node metastasis, which correlates with a better survival rate (231). Similar to the identification of Reg1A as a biomarker, Reg1B, along with syncollin (SYCN) and anterior gradient homolog 2 protein (AGR2), was identified in PDAC patients versus controls in a 2-D liquid chromatography–mass spectrometry (LC-MS/MS). Combining these biomarkers with CA19-9 showed better sensitivity and specificity in diagnosing early stages (I–II) PDAC than CA19-9 alone (73). These finding may suggest that Reg1B is most likely involved in the early development of PDAC.

1.6.1.4 Reg1 in other gastrointestinal diseases

Reg1 expression is also associated with other pancreatic pathologies. In patients with cystic fibrosis (CF), Reg1 immunostaining was induced in the duct-like cells of the tubular complexes co-stained with the ductal marker CK19 (232). *In vitro*, Reg1 inhibited proliferation and migration of pancreatic stellate cells and stimulated fibrolysis by increasing the ratio of matrix metalloproteinases (MMPs) to tissue inhibitors of matrix metalloproteinases (TIMPs). Hence, it might rescue pancreatitis by promoting the resolution of fibrosis (233).

Additionally, the action of Reg1 is not only restricted to the pancreas. Reg1A (also named RegIα) is a good candidate of prognostic biomarker for gastric cancer. Once induced by IL-6 treatment, Reg1 can mediate anti-apoptotic signaling *in vitro* through activation of the JAK2/STAT3 signaling pathway (234, 235). Reg1 knockout mice had a greater number of severe

lesions in the small intestines induced by indomethacin, a non-steroidal anti-inflammatory drug. These intestinal injuries were rescued by the administration of Reg1 protein, indicating a physiological role for Reg1 in maintaining the intercellular integrity in the small intestine (236). In addition, Reg1 regulates cell growth that is required for the maintenance of the villous structure of the small intestine (237). In the regenerating rat liver, Reg1 was significantly induced after 2-acetylaminofluorene administration and subjection to 70% partial hepatectomy (2-AAF/PH), with increasing formation of bile ductules (238). Hence, the level of Reg1 expression is closely associated with the extent of regeneration of the small intestines and liver.

1.6.2 Reg2

1.6.2.1 Expression characteristics of Reg2 gene

Reg2 was identified in mice but has not been found in rats or humans thus far. The previously identified rat Reg2 was identical with Reg3β and has been corrected by our lab (169, 239). Aside from being highly expressed in the pancreas, Reg2 protein is also found in the mouse liver, duodenum, small intestine and colon (160, 195). There have been different opinions on the precise cellular source of Reg2 from the pancreas. Sanchez et al. reported that the expression of Reg2 mRNA and protein was restricted to the exocrine tissue regardless of the age, the presence of insulitis and/or diabetes (240). In our previous study, we only detected Reg2 immunostaining in peri-islet acinar cells of normal mice (159, 241). However, Gurr et al. demonstrated expression of Reg2 in the endocrine cells of NOD mice (242).

1.6.2.2 Activation of Reg2 expression promotes β-cell survival

There have been numerous reports on the induction of the Reg2 gene as part of islet protection or regeneration mechanisms. After 5 days of 50% pancreatectomy, Reg2, 3β and 3γ

were the most abundantly induced (>10 fold) transcripts during islet regeneration (243). Exendin-4 and INGAP-p, which stimulate β-cell replication and/or neogenesis, also increased Reg2 gene expression. This evidence suggests that Reg2 may play a role in the islet survival and/or regeneration(244). In the NOD mice, Reg2 expression was more significantly increased compared to that of Reg1, irrespective of sex or the state of the diabetes (245). Following mycobacterial adjuvant treatment, Reg2 expression was significantly increased, followed by an increase in the number of newly formed small islets and improved glucose tolerance in NOD mice (246).

Reg2 was suggested to be a β-cell-derived autoantigen in NOD mice since vaccination with the C-terminal fragment of Reg2 delayed the onset of T1D (242). We have reported that Reg2 overexpression protected MIN6 insulinoma cells from streptozotocin-induced cell apoptosis by attenuating the activation of caspase-3 and the cleavage of PARP. Moreover, Reg2 reversed the persistent suppression of JNK phosphorylation caused by streptozotocin (247). Our recent data suggested that Reg2, as well as Reg3β, can be activated by glucocorticoids and IL-6 in pancreatic acinar and islet cells and may serve a protective role in response to pancreatitis (159). Moreover, Reg2 can also induce glucose-regulated peptide 78 (GRP78) via activating Akt-mTOR signaling pathway and preventing thapsigargin-induced unfolded protein response (UPR) in insulinoma cells *in vitro* (248). UPR is now considered one of the most important mechanisms of β-cell disorders in type 2 diabetes. However, acinar-specific overexpression of Reg2 failed to protect mice from streptozotocin-induced type 1 diabetes or pancreatitis (249). It may suggest that the protective effects of Reg2 on β-cells may be overridden by inflammatory stress or other compensation, which further incited our study on the function of Reg2 *in vivo* by using Reg2 knockout mice (Chapter 3).

No study has been conducted on the role of Reg2 in PDAC or any other tumors thus far.

1.6.3 Reg3α

1.6.3.1 The identification of Reg3α protein and its expression

Different from other Reg family members, the sequences of Reg3 subfamily (Reg3α, 3β, 3γ, and 3δ) proteins are characterized by the extra 5 amino acids close to the C-terminus in their primary structure. Reg3α belongs to a subfamily of pancreatitis-associated protein (PAP/PAP I/Reg3β/peptide 23/HIP), which was first discovered in the pancreatic juice and homogenate of the rat pancreatitis, but not the normal pancreas (250, 251). Rat Reg3α (PAP II), which shares 74% amino acid identity with PAP/Reg3β, was first discovered in 1993 (162). Its human orthologue is Reg3G/RegIII/PAPIB, which shares 66% identity with mouse Reg3α and 65% identity with another mouse homology Reg3γ in the protein sequence (164, 165, 172, 252). To avoid confusion on the names, the function of Reg3G will be discussed with Reg3γ (Reg3gamma) even though their protein sequence is closer to Reg3α.

Normally in rats, Reg3α is hardly detectable but can be drastically induced after pregnancy and pancreatitis, indicating its involvement in the functional adaption (162, 253, 254). In the endocrine pancreas, Reg3α was reported to be expressed by mouse islet α-cells (242). It was also detected in the small intestine, the proximal colon, and the pancreatic primordium (193).

1.6.3.2 Upregulation of Reg3α in response to islet generating factor or diabetes

Acinar-specific overexpression of gastrin/CCK2 receptor induced an upregulation of Reg3α/RegIII protein, which is proposed to be involved in the adaptive and regenerative

responses of the islets (223). C-Myc is a potent driver of β-cell proliferation (255). Activation of Myc transcription factor in mouse islets significantly promoted cell cycle progression, with steady induction of the mRNAs of Reg2, -3α, -3β, and -3γ up to 2-fold within 24 h. In NOD mice, pancreatic Reg3α mRNA level (but not other isoforms) was increased 1.5-fold after onset of insulitis leading to T1D (GEO profile ID 15736194, 15738507). Among the changes brought by diabetes, hyperglycemia seemed to have a specific effect on Reg3α. In primary rat islets, increasing glucose concentrations from 2 to 10 mM caused no change in the expression of Reg3α. However, further increase to 30 mM that is known to be detrimental or proliferative to islets significantly doubled its mRNA level (GEO profile ID 59898598). This response was isoform-specific and did not occur to other Reg proteins.

1.6.3.3 Roles of Reg3α in the islet proliferation

In order to explore whether Reg3α can directly stimulate islet β-cell replication, we overexpressed its cDNA in stably transfected MIN6 cells (256). Using real-time PCR and Western blots, Reg3α expression was barely detectable in vector-transfected cells; in contrast, the levels of its mRNA and protein in pcDNA-Reg3α-expressing clones were increased 10- and 6-fold respectively. Reg3α protein was being released into the culture medium, which is consistent with its secretion to the patients' sera (217). In MTT cell viability assay, Reg3α-overexpression caused ~2-fold higher rate of growth vs. vector-transfected cells. In order to investigate possible intracellular mechanisms, we detected an average 1.8-fold increase in Akt phosphorylation, 2.2- and 2.5-fold increased levels of cyclin D1 and cdk-4 in these cells vs. vector-transfected. These effects were not revealed when Reg2 or -3β gene was transfected, indicating isoform specificity. It is well established that β-cell replication is associated with increased cyclin D1 and cdk-4 levels(257); deficiency in cdk-4 or cyclin D2 results in β-cell loss

and diabetes (258, 259). Both Reg1 and Reg3δ cause PI3K-mediated increases in cyclin D1 and cdk-4 levels (224, 260-263). Our result thus established that Reg3α can stimulate β-cell replication by activating Akt kinase and increasing the levels of cyclin D1/cdk-4 (256). Identical effects of rat Reg3α on proliferation have since been confirmed in embryonic stem cells (ESCs), with increased expression of islet transcription factors NeuroD, Nkx6.1 and Pax6(264).

1.6.4 Reg3β (hReg3A, pancreatitis-associated protein, PAP, hepatocarcinoma-intestine-pancreas protein, HIP)

1.6.4.1 The identification of Reg3β as a pancreatitis-associated protein and its expression in the pancreas

In 1986, Reg3β protein was found in the rat and named pancreatitis-associated protein (PAP) due to its induction during experimental pancreatitis. It level was correlated with the severity of pancreatitis (265). In fact, Peptide-23 gene was primarily detected in primary cultures of rat pituitary and was then proved to be identical with Reg3β (3). Pancreatic expression of Reg3β was strong in glucagon-producing islet cells and acinar cells close to the islets, but quite low in other endocrine cells located in the center of islets or ductal cells (193, 266). This suggests Reg3β may act as an endocrine or paracrine modulator of β-cell function and PDAC development. Reg3β is also expressed in the ileum and to a lesser extent in the jejunum and duodenum (267). Its homologue in human is hReg3A/HIP, with 70% identities. In a panel of 36 adult human tissues, human Reg3A was only expressed in the pancreas and small intestines, with 100-fold higher levels than others, including that in the pituitary gland (profile ID 10124642, Gene Expression Omnibus (GEO), www.ncbi.nlm.nih.gov/geo). The drastic induction of Reg3β/PAP by pancreatic injuries supports its role as a stress protein in the pancreas.

1.6.4.2 Roles of Reg3β (hReg3A) in diabetes

IL-6 induced the release of Reg3β protein in primary islets taken from T1D patients, indicating that Reg3β is involved in a local inflammatory response in diabetic islets (268). The pancreatic mRNA level of Reg3β in NOD mice was significantly higher than that in control mice (266). The protein has been shown to be expressed in the islets and ductal epithelia in pancreata of prediabetic and diabetic NOD mice, in contrast to its restricted expression in acinar cells and peri-islet cells in non-diabetic controls. The lymphocytes from islets infiltrates and pancreatic lymph nodes of 7-week-old NOD mice showed a strong proliferative response to Reg3β, suggesting a possible role as an autoantigen (268).

In order to test whether Reg3β can promote islet cell growth or survival against experimental damage, we previously established a pancreatic islet-specific overexpression of the Reg3β mouse model using rat insulin I promoter in our lab. We evaluated the changes in normal islet function, gene expression profiles and the response to streptozotocin-induced diabetes. Significant and specific overexpression of Reg3β was achieved in the pancreatic islets of RIP/Reg3β mice, which exhibited normal islet histology, β-cell mass and insulin secretion in response to glucose stimulation. Upon streptozotocin treatment, in contrast to wild-type littermates that became hyperglycemic in 3 days and lost 15% weight, RIP/Reg3β mice were significantly protected from hyperglycemia and weight loss. To identify specific targets affected by Reg3β overexpression, cDNA microarray on islet RNA isolated from the transgenic mice revealed more than 45 genes were either up- or down-regulated significantly. Among them, islet-protective osteopontin/SPP1 and acute responsive nuclear protein p8/NUPR1 were significantly induced. These results were further confirmed by real-time PCR, Western blots and

immunohistochemistry (269). This suggests that, compared to the regenerative effects of other Reg proteins on islets, Reg3β is more likely an islet protective factor released in response to stress and inflammation. The role of Reg3β overexpression in HFD-induced diabetes is further studied in my thesis project and will be introduced in Chapter 4.

In regard to the effects of human Reg3A in pancreatic regeneration, the debate still remains whether the regeneration of pancreatic β-cells is achieved by self-replication, neogenesis or both (270-273). Based on rodent experimental evidences, the adult pancreas may harbor a small progenitor population, which is perhaps located among centroacinar or ductal cells; and it can be activated by injury and inflammation and give rise to new islet cells (274). Transcription factors and extracellular regulators control this islet neogenesis process. Direct evidence was demonstrated by a 5-day injection of a 15-aa peptide based on human Reg3A (Human proIslet Peptide: IGLHDPTQGTEPNGE). It increased the volume of small extra-islets, insulin-positive clusters by 1.5-fold in the mouse pancreas and showed a tendency of increased Ngn3 and Nkx6.1 expression in IHC (275). This was the first report indicating that human Reg3A peptide has the bioactivity *in vivo* to promote new islet formation by elevations of transcription factors. Earlier, a similar 16-aa peptide based on Reg3A (WIGLHDPTQGTEPNGE) had been shown to prevent streptozotocin-induced diabetes by increasing the islet cell mass in mice (276, 277).

1.6.4.3 Reg3β in pancreatitis-PDAC transition

Reg3β was initially shown to be associated with pancreatic acinar cells adaption to inflammation and other injures. Reg3β knockout mice displayed increased apoptosis in acinar cells in response to pancreatitis, as evidenced by elevated levels of caspase-3 and cleaved PARP; pre-treatment with Reg3β protein reversed these effects and protected the pancreas(278). The

pancreas in this mouse also showed more neutrophil infiltration and higher levels of inflammatory cytokines, including TNF-α, IL-6 and IL-1β. All parameters were significantly reduced when the mice were pretreated with Reg3β, further supporting its anti-inflammatory role (278). Consistently, antisense knockdown and antibody neutralization of Reg3β worsened the symptoms of pancreatitis induced by sodium taurocholate in rats(279). Thus, Reg3β is anti-apoptotic by inhibiting caspase-3 and PARP activation and anti-inflammatory by controlling cytokine output. Transcription factors NF-κB family are crucial in controlling the inflammatory responses and cell survival. Pancreatic-specific deletion of RelA/p65 thus NF-κB signaling abolished Reg3β induction and resulted in more severe pancreatitis, suggesting Reg3β protects the acinar cells disruption from pancreatitis (280). The mechanism of Reg3β action in pancreatic acinar cells involves modulation of MAPK(281) and/or cytokine receptor-mediated activation of JAK and the transcription factor STAT3. In acinar AR42J cell line, Reg3β activated JAK and caused phosphorylation and nuclear translocation of STAT3. Meanwhile, it was shown to induce JAK-dependent NF-κB inhibition, pointing to a cross-talk between the JAK/STAT and NF-κB signaling pathways (282).

Induction of Reg3β can protect acinar cells from the inflammatory stress, but excessive activation could contribute to the transition from pancreatitis to PDAC. In humans, Reg3A (homologue of mouse Reg3β) has been shown to be upregulated in pancreatic juice from PDAC patients compared to healthy subjects. However, it is not directly secreted from pancreatic cancer duct epithelial cells, but from the tumor adjacent acini (283). A recent *in vitro* study showed that Reg3A promoted pancreatic cancer cell growth through activation of the JAK2/STAT3 signaling pathway and inhibition of the cell apoptosis (284). It can be either stimulated by IL-6 or itself through a positive feedback loop (Fig 1.7). Deficiency of Reg3β in mice impaired the tumor

growth by modulating macrophage polarization in the tumor stroma and inhibiting IL-17 induced ADM/PanIN transition to PDAC (285, 286). This is also mediated by the JAK2/STAT3 signaling pathway (286). As introduced before, ADM is initially a protective cell transition in response to pancreatitis, when the cells reduce the digestive enzyme secreted from acini by differentiating to epithelial ductal cells. Therefore, Reg3β can protect cells from pancreatitis by either inhibiting acinar cell apoptosis or promoting ADM transition. But excessive activation of Reg3β may lead to ADM/PanIN-PDAC transition with genetic predisposition. Loncle C. et al. demonstrated that upregulation Reg3β in $Kras^{G12D}$ mice with pancreatitis. Moreover, Reg3β-deficient mice were protected from PDAC development (286). However, we have found some defects in this study regarding gp130 as the potential receptor for Reg3β. A commenting letter was published afterwards (287).

1.6.4.4 Reg3β in other regenerative diseases

Aside from being in the pancreas, Reg3β is also a crucial mitogenic and anti-apoptotic protein in the liver. Deficiency of Reg3β caused cellular apoptosis and impaired liver regeneration (170) (mislabeled as Reg2, see our editorial correspondence(239)). In the hepatocellular carcinoma, a strong immunoreactivity was detected for Reg3A and a less pronounced signal for Reg1A. Using the Huh7 hepatoma cell line, the Reg3A gene was upregulated upon activation of Wnt/β-catenin signaling by lithium chloride (LiCl). This induction was abolished by the inhibition of the β-catenin signaling with siRNA (288). The overexpression of Reg3A and the activation of the Wnt/β-catenin pathway were also detected in colon adenoma from familial adenomatous polyposis, but not in the pediatric liver tumor hepatoblastoma (289). Implantation of Reg3β-expressing hepatocytes into SCID mice enhanced

liver regeneration following hepatectomy, probably through modulating the effects of TNF-α, IL-6 and STAT3 and shortening the cell cycles (290). In Reg3β-deficient mice, there was a delayed ERK and AKT signaling activation but a persistent activation of TNF-α/IL-6/STAT3 pathway during liver regeneration following hepatectomy. This delay may result in delayed liver regeneration and persistent inflammatory condition (170).

1.6.5 Reg3δ (INGAP)

1.6.5.1 The expression of islet neogenesis associated protein (INGAP), also named Reg3δ

Reg3δ was first identified and purified in hamsters after partial obstruction of the pancreatic duct in 1997 (171). It is well known as islet neogenesis associated protein (INGAP) since it was identified as a local pancreatic protein that reversed streptozotocin-induced diabetes presumably by the induction of islet neogenesis. Normally, it is expressed in cells in the stomach, duodenum and glucagon-producing α-cells and ductal cells (199, 291, 292). The expression level of INGAP increased significantly in acinar cells of cellophane-wrapped pancreata, but not in endocrine cells (171). Pancreatic transcription factors (such as Pdx1, Ngn3, NeuroD and Isl-1) can activate the INGAP promoter individually or in combination (194). There has been no orthologue of INGAP identified in humans so far.

1.6.5.2 INGAP directly promotes β-cell replication

To demonstrate a direct effect, a 15-amino acid INGAP peptide was administrated to non-diabetic and diabetic rodents. It caused increases in the islet cell number and mass, as well as new islets formation (small foci of islet-like cells budding from intralobular and terminal ductules). INGAP peptide was also capable of reversing the hyperglycemic state in diabetic

animals (141, 293). Petropavlovskaia M et al. then explored the mechanisms of INGAP on the proliferation of insulin producing RIN-m5F cells (202). Both the recombinant protein and bioactive peptide stimulated cell regeneration via binding to the Gi-protein coupled receptor and activating Ras/Raf/Erk (202) and PI3K/Akt signaling pathways. *Ex vivo* studies showed that INGAP peptide could enhance glucose- and amino acid-stimulated insulin secretion from both adult and neonatal rat islets without affecting the islets' survival rate. A significant increase of β-cell size was observed in the cultured islets in the presence of INGAP peptide compared to controls (291). A microarray analysis of INGAP peptide-treated neonatal islets revealed upregulation of multiple genes, especially those related to islet metabolism, insulin secretion, β-cell mass and islet neogenesis. The most predominant gene changes included hepatocyte nuclear factor 3β (Hnf3β), upstream stimulatory factor 1 (Usf1), K^+-channel proteins (Sur1 and Kir6.2), Ins1, glucagon, MAPK1 and Pdx1 (294).

Meanwhile, targeted overexpression of INGAP in the pancreatic β-cells (IP-INGAP) improved glucose tolerance and significantly delayed the development of hyperglycemia in streptozotocin-induced mice. Isolated islets from these INGAP-overexpressing mice displayed increased insulin release in response to glucose stimulation in the presence of streptozotocin. This is partially due to a decreased induction of apoptosis and oxidative stress in the islets of the transgenic mice, indicated by lower levels of caspase-3, NADPH oxidase and NOX1 (295).

1.6.5.3 The roles of INGAP in the neogenesis of the β-cells

In the β-cell neogenesis, INGAP seems to play a more active role than other Reg family proteins. Similar to Reg1, INGAP-positive cells were also present in the embryonic pancreatic buds in mice (194). Based on the fact that INGAP and some growth factors are essential for the

islet development, a combination of INGAP, EGF and GLP-1 was able to induce the expansion and differentiation of the pancreatic progenitor cells into the islets (202). Moreover, intra-peritoneal or muscular administration of INGAP stimulated the growth of new endocrine cells with mature islet appearance in streptozotocin-treated hamsters or healthy dogs (296, 297). The INGAP peptide can also normalize blood glucose and insulin levels; the mechanism of this protective effect seems to include increased expression of Pdx1 in ductal and islet cells(141).

Also, INGAP can promote the transdifferentiation of other pancreatic cells into β-cells. Overexpression of INGAP in mouse pancreatic acinar cells caused a significant increase in both the β-cell mass and pancreatic insulin content, which was mainly attributed to the increased number of small islets. These mice were resistant to the β-cell destruction caused by streptozotocin treatment and had a markedly preserved islet structure (298). In addition, several transcriptional factors that were crucial for the β-cell differentiation were introduced in order to promote the transdifferentiation of ductal cells into islet-like clusters (ILCs) (299). The mass of ILCs was much larger in the group treated with INGAP in combination with nicotinamide, exendin-4 and TGF-β than that with a scrambled peptide. There were increased expression of Pdx1, insulin and glucagon in these ILCs. Furthermore, high levels of insulin and C-peptide secretion were detected during the differentiation process, illustrating a gain in the secreting capacity. In the meantime, INGAP or INGAP peptide can stimulate the proliferation of ductal cells, thereby maintaining a pool of possible precursors of islet cells. In human islets, short-term incubation of INGAP peptide induced a re-differentiation from primitive duct-like structures to ILCs. Those newly generated islets resembled freshly isolated islets with respect to the number and topological arrangement of cells within an islet, and the capacity of glucose-stimulated insulin secretion. Furthermore, these ILCs also express islet-specific transcription factors Pdx1,

ISL-1 and Nkx-2.2. The levels of GLUT2 expression and C-peptide were comparable to those in freshly isolated islets (171). In total, these *in vitro* evidences support the role of INGAP in new β-cell formation through a process of re-differentiation and transdifferentiation.

1.6.5.4 Clinical trials of INGAP in the treatment of diabetes

Among different isoforms of Reg proteins, INGAP is the most well-studied protein in its application of the diabetes treatment. Ratner RE et al. reported a double blind, placebo controlled clinical trial on INGAP peptide therapy, which induced the islet neogenesis and improved the insulin secretion both in T1D and T1D patients. In more details, INGAP peptide (600 mg/d) increased C-peptide secretion in T1D and reduced HbA1c levels in T2D patients (300). The Phase 2 clinical trial is currently running, with its safety and efficacy still undetermined (NCT02204397, NCT00995540).

1.6.6 Reg3γ (hReg3G)

1.6.6.1 Expression of Reg3γ in normal tissues and diabetes

Mouse Reg3γ was identified along with Reg3α and Reg3β (152). Consistent with other members of the Reg3 subfamily, it also expresses in the pancreas and small intestine. Its expression also becomes elevated in response to pancreatitis and other injuries in the skeletal muscles and nerves (301). Using laser-captured microdissection, the elevated mRNA levels of Reg1A, Reg1B, Reg3A and Reg3G were detected in the β-cells of T2D patients versus non-diabetic subjects, indicating their involvement in the pathogenesis of diabetes (302). However, there has been no direct evidence suggesting the roles of Reg3γ in islet cell proliferation thus far.

1.6.6.2 Recent progress on human Reg3G effects on pancreatitis and PDAC

Until recently, attentions have been paid on Reg3G in the cancer field. Reg3G, as another pancreatitis-associated protein (PAP II), is also upregulated in response to long-term inflammation exposure. Injection of lentivirus-mediated Reg3G to mice exacerbated the transition of caerulein-induced pancreatitis to PDAC (303). The signaling pathways include activation of the STAT3 and NFκB signaling pathways, which is consistent with other Reg isoforms. This function shares some similarity with that of Reg3A. More studies should be done to further determine the role of Reg3G in the development of PDAC.

1.6.6.3 Reg3γ as an antimicrobial protein in the intestine

Most studies about Reg3γ focus on its antimicrobial function in the intestine, where it is produced along with lysozyme and cryptdin by Paneth cells (304). These cells constitute innate intestinal immunity and protect intestinal epithelial cells from the inflammatory stress. After 2-day food deprivation, Reg3γ mRNA and proteins levels were both decreased significantly in the ileal tissues. These decreases were associated with increased bacterial translocation into mesenteric lymph nodes (305). Similar to Reg3β, Reg3γ limited the epithelial contact with bacteria in the small intestine by directly interacting with peptidoglycan carbohydrate (306). It is important to maintain the homeostasis of the intestinal microbiota. In Reg3γ-deficient mice, more bacteria reached the small intestinal epithelium (307, 308).

1.6.7 Reg4

1.6.7.1 The distinction of Reg4 from other isoforms of Reg proteins

Reg4 is a distinct isoform in the family, starting from its structure and chromosomal

location to its high expression in gastrointestinal cancers. Human Reg4, also named RELP (REG-like protein), is the latest Reg protein discovered from a high-throughput screening of the inflammatory large bowel disease library. The *Reg4* cDNA consists of 7 exons rather than the 6 found in other Reg family genes (180). The protein differs from the 5-amino acid insertion (P-N/D-G-E/D-G) present in Reg3 proteins and the 6 residues (S/A-Q-T-E-L-P) near the N-terminus found in all other Reg family proteins. Its chromosome location also differs from the rest. It is located at chromosome 1 instead of 2p12 in humans, and chromosome 3 but not 6C in mice. Reg4 is normally expressed in the prostate, testes, stomach, duodenum, jejunum, ileum and colon (4, 309). Cells positive for Reg4 are mostly enteroendocrine and mucin-producing goblet cells in the GI tracts. Immunofluorescent dual labeling demonstrated its colocalization with chromogranin A in the neuroendocrine cells of the duodenal epithelium (310).

1.6.7.2 Reg4 is a promising diagnostic and predictive biomarker for PDAC

Reg4, as the most studied Reg isoform as a biomarker, was first tested in pancreatic cancer in 2006 after its discovery (311). The elevation of Reg4 was detected in 6 out of 11 PDAC patients. Another study further demonstrated that Reg4 had better specificity and sensitivity when compared to CA19-9 (312). However, due to the small sample size, it is difficult to estimate the correlation with the progression and prognosis of the cancer. Additionally, Reg4 has also been recognized as a potential predictive biomarker for advanced PDAC. Cells overexpressing Reg4 were resistant to γ-ray and gemcitabine (313), and the xenografts with Reg4 overexpression showed a worse survival rate and metastasis compared with vector controls (314). The use of Reg4 as a diagnostic biomarker was also demonstrated in other gastrointestinal tumors, including gastric and colon cancer. As a part of the GI tracts, pancreatic cancer may be a

good site for Reg4 to be secreted and act as a pro-tumor factor. In our study, Reg4 is used as a positive control due to its well-established elevation in PDAC patients.

1.6.7.3 Targeting Reg4 may block the cancer growth in PDAC

Reg4 has been shown to be upregulated in both human PDAC tissues as well as cancer cell lines BxPc-3 (315). The treatment of recombinant Reg4 or conditioned medium stimulated the growth and migration of pancreatic cancer cell lines by upregulation of MMP-7 and -9 (315). On the other hand, antibody against Reg4 or its knockdown by siRNA neutralized the cancer cell growth (311). Further molecular study revealed that GLI, a key transcription factor in Hedgehog signaling in PDAC development, directly induced the expression of Reg4 by binding to its promoter (225). This may suggest that with the activation of various signaling pathways in PDAC, cancer cells can secrete high levels of Reg proteins and stimulate its growth and metastasis in an autocrine manner. If this positive loop can be targeted, the cancer growth will be blocked efficiently. However, as the studies on the Reg4 protein targeting therapy are very limited, it is still too early to conclude whether Reg4 could be a potential therapeutic target for PDAC.

1.6.7.4 Reg4 is a pro-tumor factor in other gastrointestinal cancers, including gastric and colorectal cancers

In the Protein Atlas, expression of Reg4 is clearly demonstrated in the glandular cells of the small intestines, colon and rectum. The protein was strongly expressed in the cryptal epithelium in the ulcerative colitis (316). It was also upregulated in the goblet cells of the glands representing intestinal metaplasia in the esophagus and the gastric antrum (180). Recombinant Reg4 protein caused increased weight and size of the tumors, and worsened the survival in nude

mice injected with gastric cancer cells, whereas Reg4 knockdown reversed these changes (317). Compared to the low or no-expression of other Reg genes (Reg1A, Reg1B, Reg3), Reg4 has been shown to be expressed in 71% colorectal tumors (310, 318). Moreover, Reg4 was highly expressed in the drug-resistant colon cancers compared to the drug-sensitive cancers. Reg4 positive colorectal cancer patients showed a significantly worse prognosis than those of negative patients (319). Reg4 also plays important roles in tumor progression and deterioration in prostate cancer (320). Applications of specific Reg4 antibodies or small interfering RNAs against Reg4 resulted in increased cell apoptosis and decreased proliferation, leading to decreased tumor size and increased host animal survival (321). All these data indicate that Reg4 may play an important role in tumor formation and growth, and it might be a potential therapeutic target for gastrointestinal cancers.

1.6.7.5 Roles of Reg4 in the pancreatic islets

Reg4 expression in normal islets has not been determined. In a single report, positive Reg4 staining was shown in mouse insulin-producing islet cells (309), but we could not confirm it in the normal or malignant pancreas (data not shown). Also, there was no expression found in the islet-derived neuroendocrine tumor (322). So far, there is no evidence showing that Reg4 is related to cell proliferation of the pancreatic islets.

1.7 Rationale and objectives of the study

Although isoform-specific roles of Reg proteins have been studied in diabetes and PDAC fields over the last several decades, there are still many unanswered questions. In the field of PDAC research, first, no study has systematically examined the isoform-specific expression of

all Reg proteins in the acinar, ductal and islet cells in PDAC patients versus healthy subjects. We performed a single study addressing the expression of Reg proteins in PDAC precursor lesions (PanIN and ADM) and their roles in the formation of ADM directly. Second, there is no study correlating the expression of Reg proteins with the progression of PDAC, including its precursors ADM and PanIN lesions, and the patients' survival. Third, there is no study using Reg proteins to differentiate the metastatic PDAC in the liver from intrahepatic cholangiocarcinoma. Lastly, the potential of using Reg proteins as therapeutic targets has not been well studied. Our aims were to identify the roles of Reg proteins in the ADM and PDAC development, and to identify whether they can be used as diagnostic biomarkers and therapeutic targets to block the initiation and progression of PDAC.

In the field of diabetes research, the roles of Reg proteins in β-cell proliferation have been widely studied in different isoforms, especially Reg1 and the INGAP subfamily. We have previously established that overexpression of Reg2 and Reg3β can protect insulinoma cells or genetic engineered mice from streptozotocin-induced diabetes (T1D). However, the role of Reg proteins in HFD-induced T2D have not been studied before. Therefore, our aim was to identify functions of the two isoforms in the β-cell compensation in the development of obesity and T2D.

1.8 Figures for Chapter 1

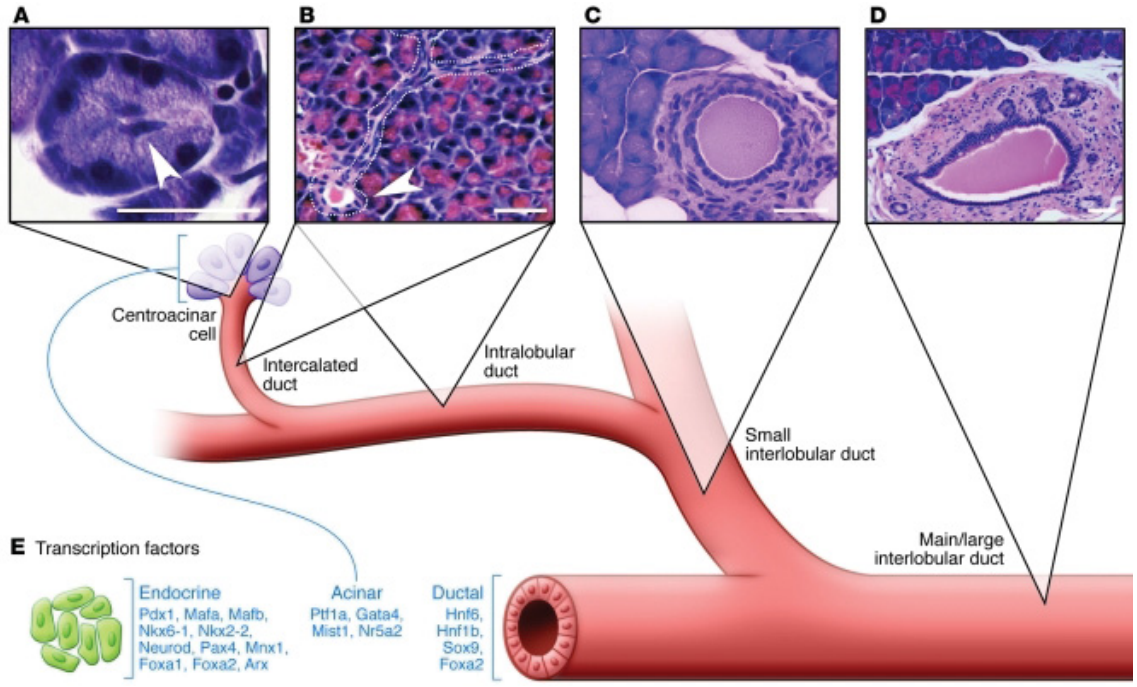

Figure 1.1 Morphology of pancreas and critical transcriptional factors involved in the embryonic development of pancreas.

Pancreas is composed of exocrine tissue, including acinar, centroacinar (A) and ductal cells, and endocrine cells. Braches of ductal cells include intercalated duct, intralobular duct (B), small interlobular duct (C) and main/large interlobular duct (D). There are various key transcriptional factors involved in the development of different cell types (E). Adapted from (Reichert, M. 2011).

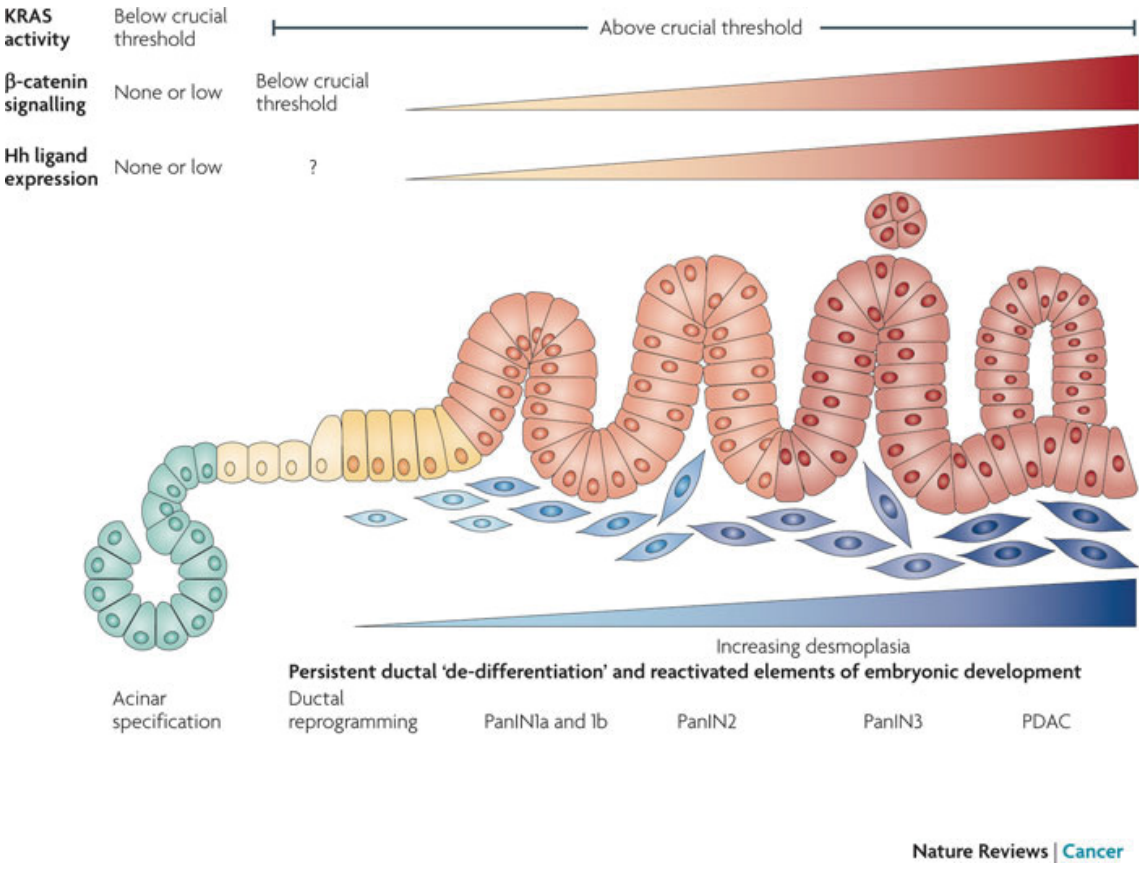

Figure 1.2 The scheme of PDAC initiation and progression and associated signaling pathways.

Traditionally, it is believed PDAC originated from neoplastic ductal epithelial cells, defined as pancreatic intraepithelial neoplasia (PanIN), and it contains three grades, PanIN1a and b, PanIN2 and PanIN3. With the increased desmoplasia, the Kras, Hedgehog signaling and Wnt/β-catenin signaling pathways get activated subsequently. Recent studies have shown acinar cells also contribute to the initiation of PDAC through reprograming, defined as acinar-to-ductal metaplasia (ADM). This transition is in response to chronic pancreatitis and it is reversible unless with genetic predisposition, such as Kras. Adapted from (Morris J.P 2010)

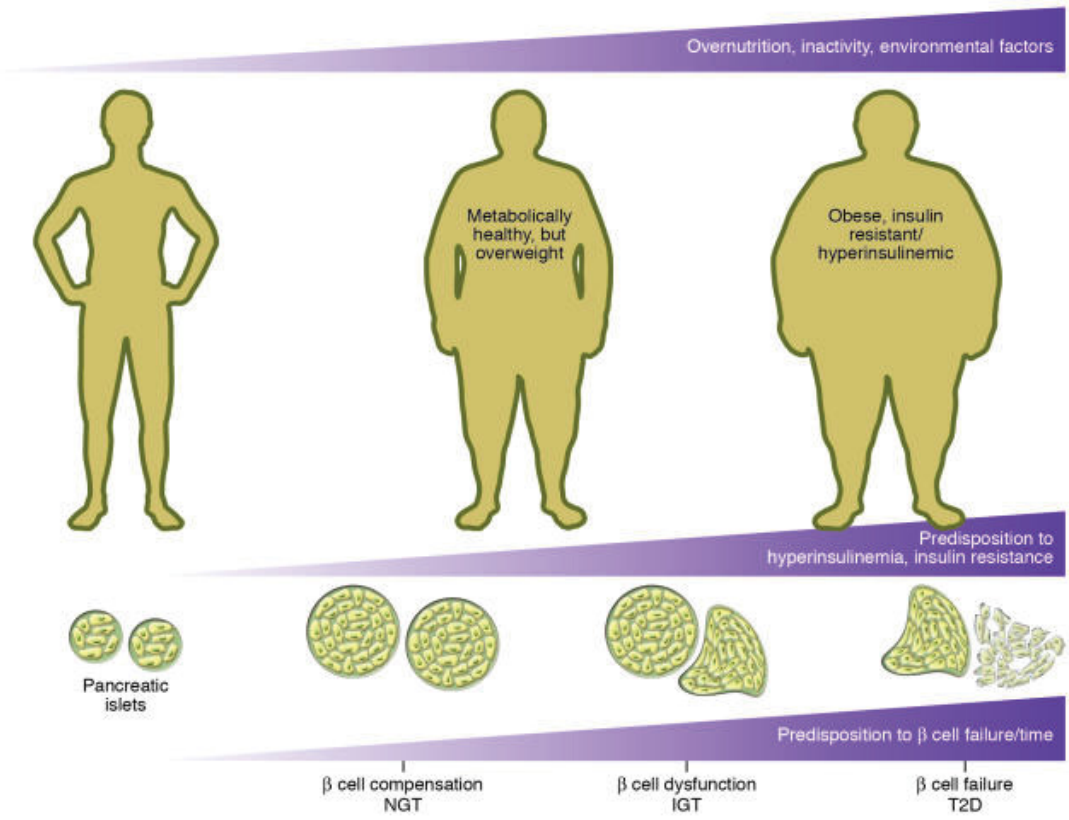

Figure 1.3 Development of obesity-associated T2D and β-cell failure.

β-cell compensation is stimulated by overnutrition, inactivity and other environmental factors, resulting in hyperinsulinemia but the glucose tolerance normal. The subjects can progress to impaired glucose tolerance (IGT) and T2D with predisposition to insulin resistance. β-cell regeneration and insulin secretion function were progressively impaired in this progression. Adapted from (Prentki, M. 2006)

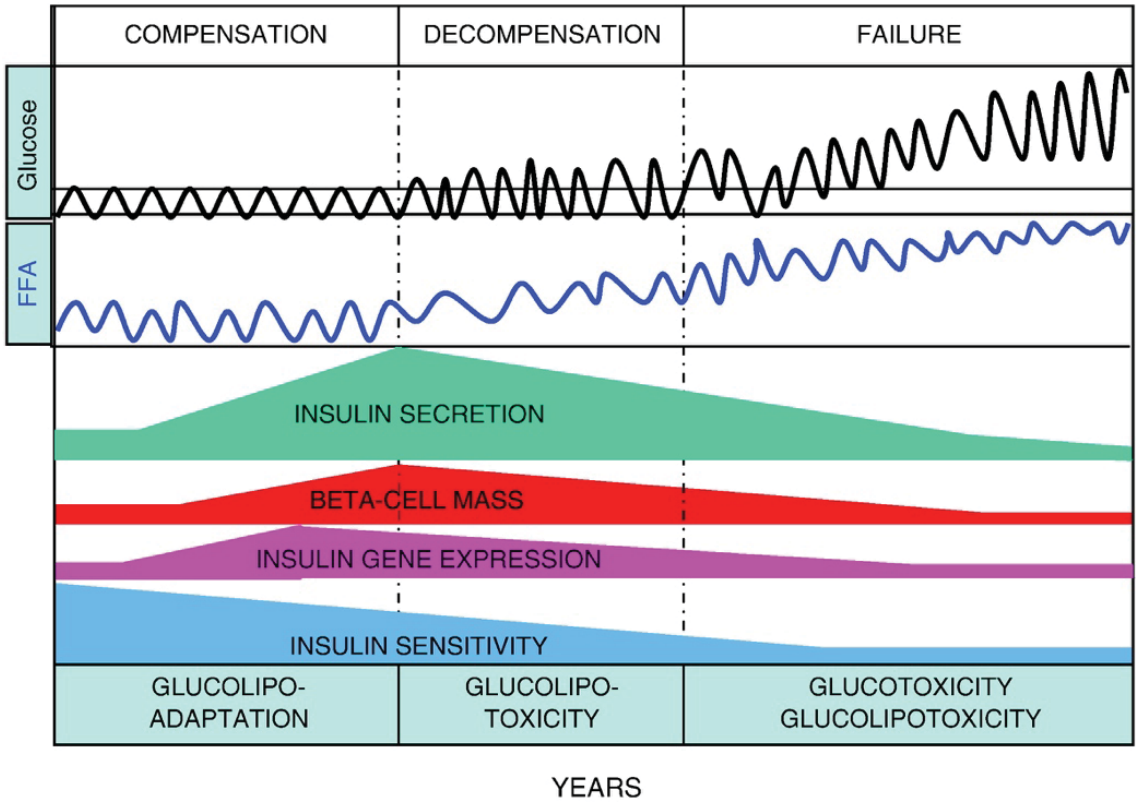

Figure 1.4 Mechanism of glucolipotoxicity leading to β-cell compensation to failure in insulin resistance and T2D.

In response to high glucose and free fatty acid (FFA), β-cell can compensate by increasing insulin secretion, β-cell expansion, and insulin gene expression, while the insulin sensitivity decreases gradually. With prolonged exposure of glucose and FFA and genetic predisposition, β-cell presents decompensation and failure, with decreased insulin secretion and insulin sensitivity. Adapted from (Poitout V, 2010).

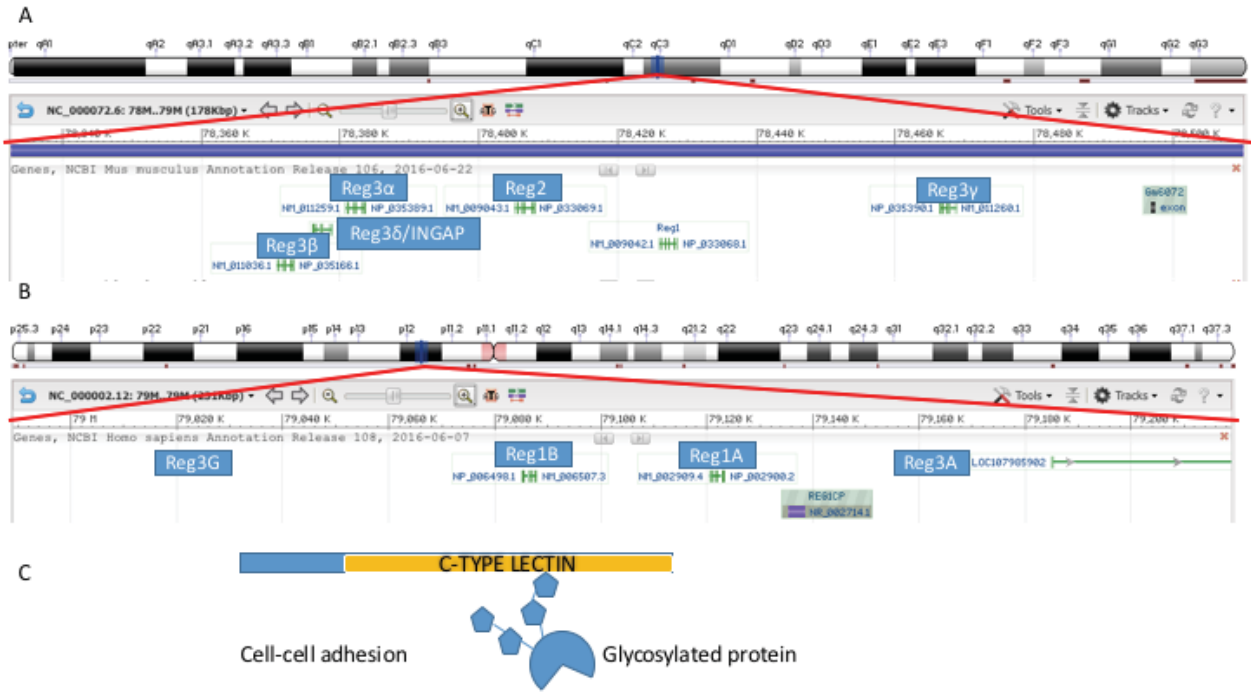

Figure 1.5 Chromosome location of Reg proteins in the mouse and human (except Reg4) and the main structural elements of Reg proteins.

A. Chromosome 6 in the mouse showing the location of Reg proteins except Reg4, located in chromosome 3. **B**. Chromosome 2 in the human showing the location of Reg proteins except Reg4, located in chromosome 1. **C**. The main structural elements of Reg proteins. It contains a N-terminal signal peptide (in blue) and a C-terminal C-typle lectin domain (CTLD, in yellow). The CTLD binds to the glycosylated proteins and promotes the cell-cell adhesion. (partially adapted from NCBI Gene).

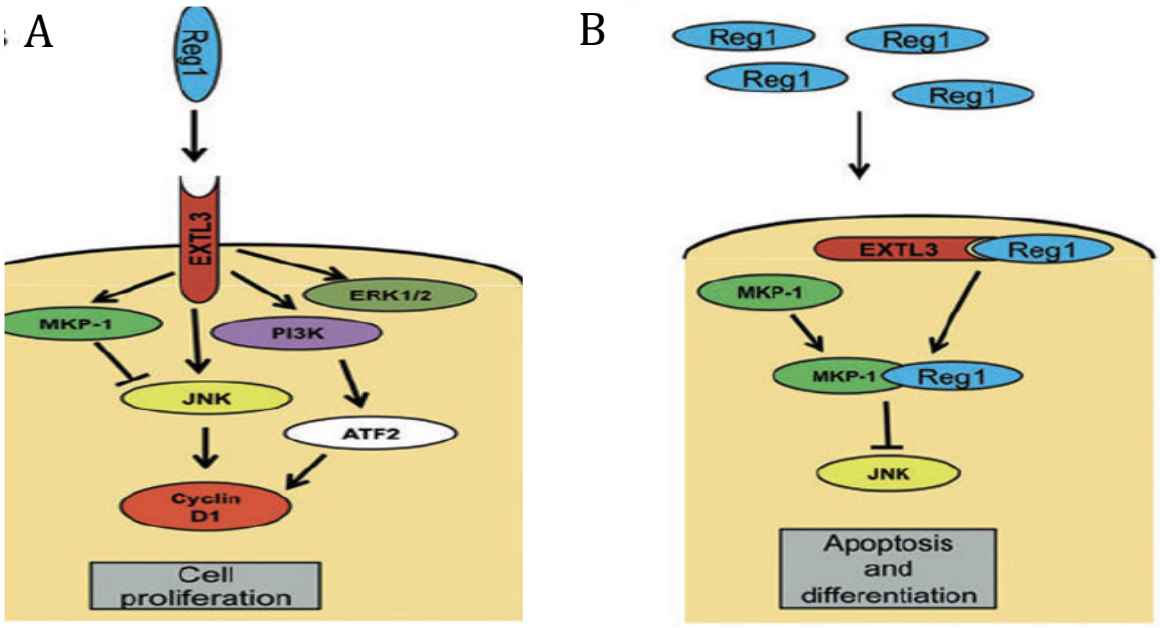

Figure 1.6 Potential mechanisms of Reg1 protein signaling.

A) Secreted extracellualr Reg1 binds to its receptor EXTL3 and activates multiple signaling pathways, PI3K/ATF2, Erk1/2 and JNK. They can further activate the downstream cyclin D1 and promotes cell proliferation. Simultaneously, JNK activity can be inhibited by EXTL3-indued MKP-1 activation. At high concentrations of exogenouse Reg1, it can form a complex with EXTL3 and intereact with MKP-1. The MKP-1/Reg1 complex leads to cell apoptosis and differentiation through inhibiting JNK. Adapted from (Parikh A. 2012)

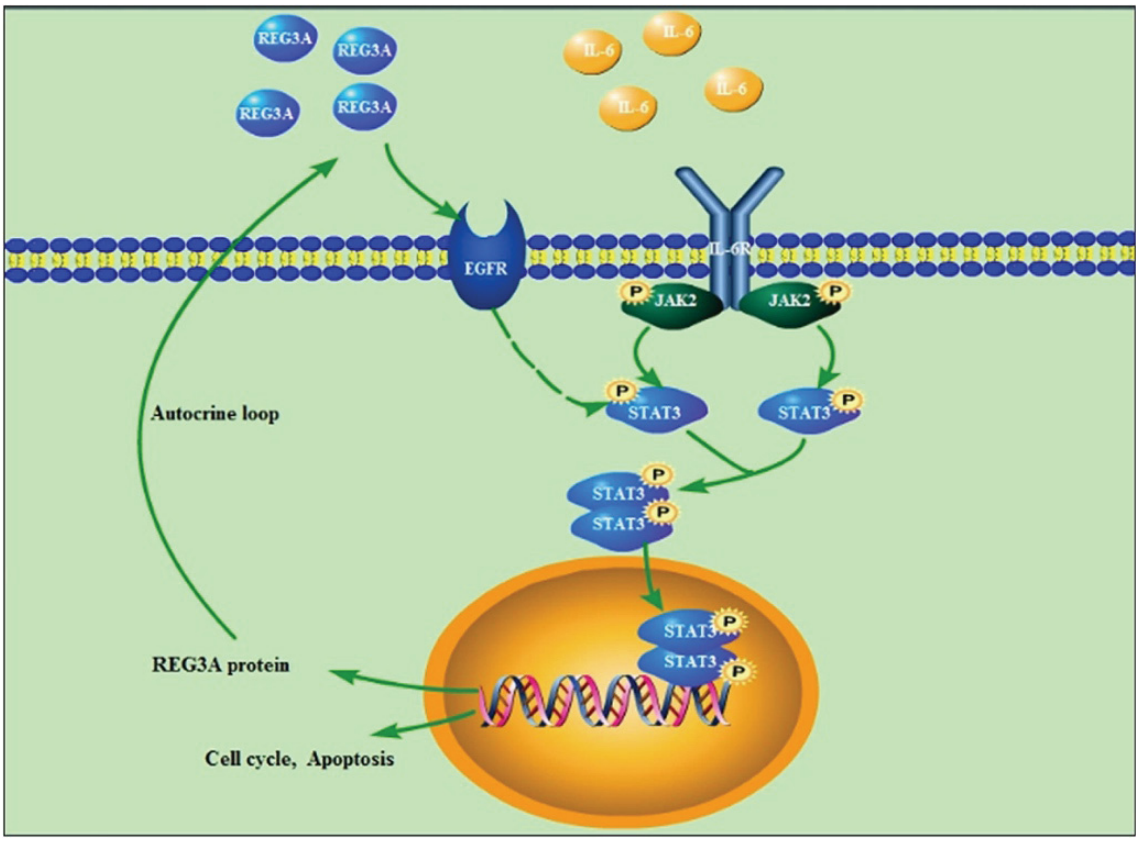

Figure 1.7 Reg3A, in synergy with IL-6, promotes pancreatic cancer cell growth through triggering the Reg3A-JAK2/STAT3 positive-feedback loop.

In response to inflammation, IL-6 binds to its receptor and activates the downstream JAK2/STAT3 singling pathway, resulting in STAT3 translocation to the nucleus and promote cell cycle and anti-apoptosis. In the meantime, it promotes the synthesis and secretion of Reg3A proteins. Reg3A can subsequently stimulate JAK2/STAT3 signaling through binding to its potential receptor, leading to the positive feedback of the IL-6/JAK2/STAT3 loop. In general, this loop promotes cancer growth in PDAC cell liens. Adapted from (Liu X. 2015)

Chapter 2. Reg proteins promote acinar-to-ductal metaplasia and act as novel diagnostic and prognostic markers in pancreatic ductal adenocarcinoma

Qing Li[1]* MD, Hao Wang[2]*MD, George Zogopoulos[3, 4] MD, PhD, Qin Shao[5], Kun Dong[6] MD, Fudong Lv[5] MD, Karam Nwilati[1] B.Sc, Xian-yong Gui[7] MD, Adeline Cuggia[4] Msc, Jun-Li Liu[1] PhD, Zu-hua Gao[4] MD, PhD

[1]*Fraser Laboratories for Diabetes Research, Department of Medicine, McGill University Health Centre, Montreal, Canada*

[2]*Department of Oncology, Qingdao Municipal Hospital, School of Medicine, Qingdao University, Qingdao, China*

[3]*Department of Surgery, McGill University Health Centre, Montreal, QC, Canada*

[4]*Quebec Pancreas Cancer Study, McGill University Health Centre, Montreal, QC, Canada.*

[5]*Department of Pathology, McGill University Health Centre, Montreal, QC, Canada.*

[6]*Department of Pathology, You An Hospital, Capital Medical University, Beijing, China*

[7]*Department of Pathology, University of Calgary, Calgary, AB, Canada*

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2.1 Preface

Reg proteins have been well known in their proliferative and anti-apoptotic functions in pancreatic islets. Previous studies in our lab demonstrated that the overexpression of Reg3α in insulinoma cells stimulated cell replication by activating PI3K/Akt signaling pathway and increasing cell cycles (256). Reg2 protected cells from UPR- or streptozotocin-induced apoptosis (247, 248). However, excessive proliferation may contribute to the cancer development. Reg4 has been shown to be upregulated in various cancer types, including gastric, colorectal and pancreatic cancers (314, 323, 324). Therefore, in my study, I firstly determined the levels of all Reg protein isoforms in tissues and sera of PDAC patients versus healthy subjects. I showed higher levels of Reg1A and Reg1B in PDAC patients compared with controls. Their levels were correlated with patients' survival rate. Besides, Reg1A and Reg3A showed upregulation in two precursors of PDAC, including ADM and PanIN lesions. I have demonstrated a direct stimulation of Reg3A on ADM formation by activating MAPK signaling pathway in 3-D cultured primary acinar cells. Lastly, the combination of Reg1A, Reg1B and Reg4 clearly differentiated PDAC from another malignant disease intrahepatic cholangiocarcinoma. This differential diagnosis is critical in the clinical practise due to their different management and outcomes. This is the first study that systematically explores the expression of Reg proteins in PDAC patients and their correlations with the cancer precursor lesions and patients' prognosis. I have also shown that Reg3A directly promotes ADM formation and may contribute to the initiation of PDAC.

2.2 Abstract

Pancreatic ductal adenocarcinoma (PDAC) is a highly aggressive malignant tumor. Acinar-to-ductal metaplasia (ADM) and pancreatic intraepithelial neoplasia (PanIN) are both precursor lesions that lead to the development of PDAC. Reg family proteins (Reg1A, 1B, 3A/G, 4) are a group of calcium-dependent lectins that promote islet growth in response to inflammation and/or injuries. The aim of this study was to identify the roles of Reg proteins in the development of PDAC and their clinical value as biomarkers. We found that Reg1A and Reg3A/G were highly expressed in the ADM tissues by immunohistochemistry. In the 3-dimensional culture of acinar cells, Reg3A promoted ADM formation with concurrent activation of mitogen-acitvated protein kinase pathway. Upregulation of Reg1A and Reg1B was observed as benign ductal epithelium progresses from PanIN to invasive PDAC. Patients with PDAC showed significantly higher serum levels of Reg1A and Reg1B than matched healthy controls. These results were further validated by the Reg 1A and 1B mRNA levels in the microdissected tissues (22- and 6-fold increases). Interestingly, patients with high levels of Reg1A and 1B exhibited improved survival rate, than those with low levels. Furthermore, tissue expressions of Reg1A, Reg1B, and Reg4 could differentiate metastatic PDAC in the liver from intrahepatic cholangiocarcinoma with 92% sensitivity and 95% specificity. Overall, our results demonstrated the upregulation of Reg proteins during PDAC development. If validated in large scale multicenter studies, Reg1A and Reg1B could become clinical markers for detecting early stages of PDAC, monitoring therapeutic response, and predicting patient's prognosis.

2.3 Introduction

Pancreatic ductal adenocarcinoma (PDAC) is the fourth leading cause of cancer-related death (325). Traditionally, it is believed that PDAC starts from a distinct precursor lesion named pancreatic intraepithelial neoplasia (PanIN) and progresses to invasive carcinoma through a series of genetic events. The activation of the K-ras oncogene and inactivation of tumor suppressor genes including CDKN2A and TP53, and transcriptional factor SMAD4/DPC4 have all been implicated (55). Recent studies suggest that PDAC can also derive from acinar-to-ductal metaplasia (ADM), with additional mutations in K-ras and TP53 (24, 326). ADM is also a protective mechanism of acinar cells in response to inflammatory stimuli, such as chronic pancreatitis or interleukin-17 (327, 328).

Due to the deep anatomical location of the human pancreas, tumor-specific symptoms of PDAC, such as abdominal mass, jaundice, and weight loss, typically emerge only after the tumor has reached advanced stages. It is either unresectable or has already metastasized to the liver or other organs (329). In order to implement an effective therapy and improve patients' prognosis, sensitive and specific biomarkers to aid in early diagnosis are urgently needed. Moreover, when PDAC metastasizes to the liver, it needs to be differentiated from primary intrahepatic cholangiocarcinoma (ICA). The therapeutic approaches and prognoses for ICA and metastatic PDAC in the liver are completely different. Surgery is the primary therapeutic option for ICA with a 5-year survival rate up to 40% for patients with resectable ICA (330). Metastatic PDAC, however, is usually unresectable and the treatment option is limited to palliative chemoradiotherapy. This clinical demand poses a huge challenge to surgical pathologists because the histomorphological and immunohistochemical profiles of ICA and PDAC are essentially identical. Therefore, clinically applicable biomarkers that can clearly differentiate

these two malignant tumors are needed to guide appropriate therapy and provide more accurate staging information for predicting patient prognosis.

The family of Regenerating (Reg) proteins is a group of C-type lectin-like proteins discovered in patients with pancreatitis and during pancreatic islet regeneration (4). Five Reg family members including Reg1A, Reg1B, Reg3A, Reg3G and Reg4 have been identified in humans. The overexpression of the Reg1A gene in pancreatic cancer cells has been shown to result in accelerated cell proliferation and tumor growth, both *in vitro* and *in vivo* (227). Reg3 subfamily, including Reg3A and Reg3G, are known as pancreatitis-associated proteins due to their activation in response to inflammatory stimulants (161). Recently, Reg3A has been reported to accelerate pancreatic cancer cell growth in response to interlukin-6 via the JAK2/STAT3 signaling pathway (284, 285). Reg4, the most recently discovered member of the family, was reported to be activated in PDAC and proposed to be a diagnostic marker (312, 315). Moreover, a proteomic analysis of pancreatic juice demonstrated increased levels of Reg1A, Reg1B and Reg3A proteins in PDAC, in comparison to normal subjects and patients with pancreatitis (331). However, the involvement of Reg proteins in the onset and progression of PDAC has yet to be elucidated. In the present study, we first demonstrated the presence of Reg proteins in precursors to PDAC, including ADM and PanIN. We then evaluated the diagnostic and prognostic value of Reg proteins in PDAC by measuring the serum levels and tissue expression of Reg proteins in association with the malignant progression of PDAC and patients' prognoses. Lastly, we assessed the role of Reg proteins in differentiating metastatic PDAC from ICA by comparing their expression between these two groups of cancer tissues.

2.4 Materials and Methods

2.4.1 Patients and tissue samples

Tissues samples of PDAC (n=60), intrahepatic cholangiocarcinoma (ICA) (n=27), and extrahepatic cholangiocarcinoma (ECA) (n=13) were obtained from patients who underwent resections at the Department of Pathology at the University of Calgary (Canada), the Beijing You An Hospital (China), and McGill University (Canada) between 2009 to 2014. The tumor diagnoses, histological grades and stages were reassessed by two pathologists (ZHG and KD). Ampullary adenocarcinoma, duodenum adenocarcinoma, mucinous adenocarcinoma, hepatocellular carcinoma, and bile duct adenoma were excluded from the study. Clinical information was obtained from the original pathology requisitions and physician's notes in the charts. The study was approved by the institutional Research Ethics Board (REB) (332).

2.4.2 Serum samples

Peripheral blood samples were obtained from 61 healthy donors, 9 chronic pancreatitis patients and 41 PDAC patients at the Affiliated Hospital of Qingdao University and McGill University Health Center between September 2012 and March 2015. Samples were centrifuged for 10 min at 1000×g in a swing bucket rotor at 4°C, and the serum was stored in cryovials at −80°C until examination. All the PDAC patients involved in this study were newly diagnosed before any medical treatment or surgery.

2.4.3 Immunohistochemistry and immunofluorescence

For the detection of Reg proteins in human PDAC tissue samples, 4 μm tissue sections were deparaffined in xylene and rehydrated in graded ethanol, 0.1% Triton X-100, and 3%

hydrogen peroxide were used prior to 10% blocking serum. Sections were incubated overnight with primary antibodies (hReg1A, hReg1B and hReg3A/G from Santa Cruz, TX, USA; hReg4 from R&D, MN, USA). Corresponding secondary antibodies were used, followed by the addition of DAB (SK-4105, Vector, CA, USA). The slides were then counterstained with hematoxylin (Thermo Fisher, MA, USA) and mounted with hydrophobic medium. For immunofluorescence, Cytokeratin 19 (TROMA-III, DSHB) and Reg1A antibodies were used to detect colocalization of these two markers on the epithelium that had undergone acinar-ductal metaplasia in PDAC patients. Corresponding Alexa Fluor dyes were used for fluorescent detection. DAPI was used for nuclear counter staining.

2.4.4 Evaluation of Reg proteins immunostaining

The immunohistochemical staining on whole slides was independently evaluated under 100x magnifications by two pathologists. The immunoreactive scoring (IRS) system that evaluated the proportion of positivity and the intensity of staining was adapted from previous work on Reg4 and other proteins (333, 334). The percentage of positive tumor cell staining was scored as 0 (negative), 1 (<25%), 2 (26-50%), 3 (51-75%), and 4 (>75%). The intensity of tumor cell staining was graded 0 (negative), 1 (light yellow color), 2 (brownish-yellow), and 3 (brown). Grades 0-1 were defined as low expression, and 2-3 were defined as high expression. The two scores were multiplied, and the final IRS value was determined. A final score equivalent to <2 was considered negative. Compound positivity was recorded when Reg1A, Reg1B and Reg4 were all positive.

2.4.5 Three-dimensional culture and Western Blotting

Primary acinar cells were isolated from C57BL/6 mice by Collagenase P (Sigma). Cells were then suspended in 5% matrigel/medium (v/v) and seeded on 8-chamber slides pre-coated with matrigel (Cat No. 354248, Corning). Reg3A (100nM, mouse isoform, Biomart) was added to the RPMI 1640 culture medium containing 10% fetal bovine serum (FBS), soybean trypsin inhibitor (0.1mg/ml, Sigma) and dexamethasone (1μg/ml, Sigma), and the medium was changed every 2 days. Cells were cultured up to 14 days. DAPI was used to stain the nuclei. Pictures were taken under inverted microscope.

To determine the activity of Erk, primary acinar cells were cultured in the medium containing 2% FBS. Cells were collected after 30min of treatments. Western Blot was performed to determine the levels of phosphorylated and total Erk1/2 (sc-16982R and sc-154, Santa Cruz, TX, USA). Pictures were taken by using ChemiDoc Touch Imaging System (Bio-Rad).

2.4.6 Enzyme-linked immunosorbent assay (ELISA)

Reg protein (Reg1A, 1B, 3A, 3G and 4) concentrations were determined by means of ELISA (Uscn Life Science Inc. China). An Avidin-Biotin system was used to develop colors and changes were measured at a wavelength of 450 nm (Perkin-Elmer, Enspire 2300, MA, USA). Results were expressed as ng/ml.

2.4.7 Microdissection and quantitative RT-PCR in PDAC tissue vs. paired adjacent non-neoplastic tissues

Representative tissue blocks were selected to perform microdissection based on H&E staining in pancreatic cancer FFPE tissue sections (15μm-thick, 10 consecutive slides). Total RNA was prepared by using RecoverAll Total Nucleic Acid Isolation Kit for FFPE (AM1975, Ambion). Quantitative real-time PCR was performed by using PowerUp SYBR Green Master Mix (A25742, Applied Biosystems). Primers for Reg1A, Reg1B and GAPDH were designed and synthesized from Life Technologies (sequences listed in Table S2.1). The relative expression levels were normalized by GAPDH, and fold changes were calculated by comparing cancer vs. its paired tumor-adjacent non-neoplastic tissues.

2.4.8 Statistical analysis

All data were expressed as Mean ± SEM. One-Way ANOVA and student's two-tailed t-test were used for comparison of ELISA, qPCR and immunoreactive scores results. The correlation analyses were done by using Spearman's test. Chi-square test and Fisher's exact test were used for comparisons of Reg protein expression in different differentiation grades and in PDAC and ICA. The sensitivity, specificity, and predictive values of combining Reg1A, Reg1B, and Reg4 immunohistochemical staining for differentiating ICA from PDAC were calculated using GraphPad Prism 6.0 program. $P<0.05$ was considered as statistical significant. Data management was performed by using the GraphPad Prism 6.0 and SPSS statistics software (version 21).

2.5 Results

2.5.1 The clinical and pathological features

The clinicopathological information gathered from PDAC, chronic pancreatitis and cholangiocarcinoma patients and their matched healthy controls in the ELISA and IHC studies are summarized in Tables 2.1 and 2.2. There were no statistical differences among the groups in terms of gender and age distribution, lymphatic invasions, and metastasis. However, PDAC cases showed significantly more advanced tumor stages than ICA and ECA cases, corresponding directly with their more aggressive behavior.

2.5.2 Reg proteins were involved in PDAC precursors including ADM and PanIN lesions

2.5.2.1 Reg1A and Reg3A/G were involved in acinar-to-ductal metaplasia

ADM is defined as a transdifferentiation of acinar cell to ductal cell phenotypes. It is characterized by the formation of duct-like structures, decreased expression of acinar biomarkers such as amylase, and increased expression of ductal biomarkers such as cytokeratin 19 (CK19). Mounting evidence supports the involvement of ADM in the initiation of PDACs (335). We screened the expression of all Reg protein isoforms in human PDAC tissues vs. normal tissues. No expression of Reg1A or Reg3A/G was observed in normal acini and ducts (Fig 2.1A). However, Reg1A and Reg3A/G positive duct-like structures were observed in tumor-adjacent acinar areas (Fig. 2.1A). To validate the ductal phenotypes of these structures, CK19 was co-stained with Reg1A using immunofluorescence, confirming that the Reg protein positive structures were ADM (Fig. 2.1B).

To directly study the role of Reg proteins in promoting ADM, we established a 3-D culture model for primary acinar cells *in vitro* by using matrigel. Acinar cells treated with Reg3A exhibited increased duct-like cysts formation (19.3±2.9% cysts per area), comparable with the positive control TGF-α treated cells (26.5±1.5%). However, cells treated with vehicles remained only acinar clusters, with only 5% sporadic cysts formation. (Fig. 2.1C, D). This suggests that Reg3A could promote the transition of acinar cells to ductal cell phenotypes.

To understand the underlying mechanism, primary acinar cells were treated with Reg3A or TGF-α for 30min and the status of Erk phosphorylation was assessed using Western Blotting. Compared with vehicle treated cells, Reg3A treated cells showed a significantly higher level of Erk phosphorylation (Fig 2.1E). The level of Erk phosphorylation was comparable with the positive control TGF-α treated acinar cells. This data suggests that the promoting effect of Reg3A on ADM may involve an activation of mitogen-activated protein kinases (MAPK) pathway, which is known to mediate the effect of TGF-α.

2.5.2.2 Reg1A and Reg1B were highly expressed in PanIN lesions and their progression to PDAC

Pancreatic intraepithelial neoplasia (PanIN) is the most common precursor to PDAC with four histological grades (IA, IB, II and III), based on the degree of cytological and architectural atypia (Fig. 2.2a-d) (50). Immunohistochemically, no expression of Reg proteins was found in normal duct epithelium. As PanIN progresses from low to high histological grades, stepwise increases of Reg1A staining intensity were observed (Fig. 2.2e-h), whereas Reg1B expression remained elevated during the whole progression from PanIN to PDAC (i-l). Different

grades of PanIN lesions were observed within the same duct, and positively correlated with the staining intensity of Reg1A (Fig. 2.2g, 2.2k).

2.5.3 Reg proteins act as diagnostic biomarkers for PDAC

2.5.3.1 Elevation of the serum Reg protein levels in PDAC patients

Reg proteins are known to be secreted into the circulation under certain conditions (1, 336). We performed a whole panel of Reg proteins ELISA on the sera of PDAC patients and matched healthy subjects, as well as chronic pancreatitis patients. In comparison to matched healthy subjects, PDAC patients showed significantly higher serum levels of Reg1A, Reg1B and Reg4 (Fig. 2.3A). The differences in serum Reg4 levels appeared less dramatic than those of Reg1A and Reg1B. There were only mild but statistically insignificant elevations of Reg1A and Reg1B in the sera of chronic pancreatitis compared to the normal controls (Fig. 2.3A, mid column). There were no statistically significant differences in the serum levels of Reg3A or 3G between PDAC, chronic pancreatitis patients, and matched healthy individuals.

2.5.3.2 Increased expression of Reg proteins in PDAC tissues

Immunohistochemically, the infiltrative PDAC cancer glands stained strongly positive for Reg1A and Reg1B, but negative for Reg3A/G (Fig. 2.3B). The intensity of staining for Reg1A and Reg1B in these cancer cells was much stronger than that of Reg4 (Fig. 2.3B), despite the fact that Reg4 had previously been reported as a biomarker of PDAC (311-314). Unlike the strong and evenly distributed staining of Reg1A and Reg1B in malignant glands, the staining of Reg4 was uneven and varied from mild to intermediate and strong in some areas of the malignant glands. Distinct from other isoforms, Reg3A/G showed strong staining exclusively in the stroma, which is composed of extracellular matrix proteins, stellate cells, fibroblasts, and lymphocytes. Increased stromal Reg3A/G expression may contribute to the reduced penetration of chemotherapy drugs in the cancer tissue, and associated with drug resistance (337).

2.5.3.3 Increased Reg1A and Reg1B mRNA levels in the PDAC tissue

To further validate the serological and immunohistochemical findings, Reg1A and Reg1B mRNA levels were measured in microdissected cancer tissues and adjacent non-neoplastic acinar tissues (Fig. 2.3C). PDAC cancer tissues showed 22-fold and 6-fold increases of Reg1A and Reg1B mRNA levels than the adjacent non-neoplastic acinar tissues (P=0.025 and 0.016; Fig. 2.3D). None of the other isoforms showed significant changes (data not shown).

2.5.4 Reg proteins act as prognostic biomarkers for PDAC

2.5.4.1 Serum level of Reg proteins negatively correlated with the histological grade of PDAC

To study whether the serum levels of Reg proteins could predict the malignant progression of PDAC, we performed a correlation analysis between Reg protein serum levels and PDAC clinical stages, grades and metastatic profiles. Interestingly, negative correlation

between the histological grades of PDAC and serum Reg1A and Reg1B levels were observed (p=0.003 and 0.028, Fig. 2.4A, Table S2.2). The correlation data generated for other isoforms of Reg protein and other clinical parameters did not exhibit any statistical significance (Table S2.2).

2.5.4.2 Tissue expressions of Reg proteins negatively correlated with the histological grades of PDAC

The negative correlation between the histological grades of PDAC and the serological levels of Reg1A and Reg1B were further validated by the IHC data in tissue samples. There was a gradual decrease pattern of Reg1A and Reg1B expression as PDAC progressed from well and moderate differentiation to poor differentiation (Fig. 2.4B). In low differentiation grades (G1-G2), 83% of the cases showed high Reg1A expression levels and 86% showed high Reg1B expression levels, while in high differentiation grades (G3), 86% and 100% of the cases showed low Reg1A and Reg1B levels ($P<0.01$ and $P<0.0001$, Fig. 2.4C), respectively.

2.5.4.3 Higher expression of Reg proteins was correlated with better patients' survival rates

The clinical relevance of the negative correlation between tissue expressions of Reg proteins and histological grades of PDAC was analyzed by incorporating patients' survival data. High grade PDAC with low levels of Reg1A and Reg1B showed a statistically significant lower survival rate after tumor resection, when compared to those with low grade PDAC and high levels of Reg1A and Reg1B ($P<0.0001$, $P<0.001$ and $P<0.001$, Fig. 2.4C). It suggess that the prediction value of Reg proteins in PDAC patient survival rate is dependent on their histological grades.

2.5.5 Reg proteins can clearly differentiate ICA from PDAC

Unlike in PDAC, the expression of Reg proteins has not been reported in hepatic cholangiocarcinoma. To investigate whether the tissue expression of Reg proteins could be used to differentiate ICA from metastatic PDAC in the liver, we compared the immunohistochemical expression of Reg proteins in 60 PDAC, 27 ICA and 13 ECA patients. Reg proteins, especially Reg1A and Reg1B, were clearly overexpressed in the PDAC cases, but absent in the ICA cases (Fig. 2.5A, a-c and d-f). The mean scores of Reg1A, Reg1B, and Reg4 in PDAC were 3.2, 3.7, and 2.1 fold of those in ICA, respectively (Fig 2.5A, g-i). Additionally, the mean scores of Reg1B and Reg4 in ECA did not show any statistical difference with those in PDAC, indicating the closer relations of ECA with PDAC, as compared to ICA.

Based on previous studies, a cut-off value of 2 was determined for categorizing positive from negative IHC results (338). Among 60 cases of PDAC, 95.6% were Reg1A positive, 100% were Reg1B positive, and 93.3% were Reg4 positive. However, only 33.3% of the ICA cases were Reg1A and Reg1B positive; and 44.4% were Reg4 positive (Fig. 2.5B). Used independently in the differential diagnoses of PDAC and ICA, Reg1A, Reg1B or Reg4 demonstrated high sensitivity (95.56%, 100% and 93.33%, respectively) but low specificity (66.67%, 66.67% and 55.56%, respectively, Fig. 2.5B). However, by combining the scores of all three proteins together, the test specificity increased to 95.24% and the sensitivity remained at 91.67%. The positive and negative predictive values of combining Reg1A, Reg1B and Reg4 in differentiating PDAC from ICA were 0.9524 and 0.8333, respectively.

2.6 Discussion

An increasing number of reports have revealed the contributions of pancreatitis-associated ADM in PDAC initiation (24, 327, 328, 339). As the pancreatitis-associated Reg3 subfamily of proteins are known to contribute to the cell regeneration in several cell types, it is conceivable that Reg3 may contribute to the transition from pancreatitis to PDAC. Recently, both mouse Reg3β and Reg3g have been reported to promote the transition from chronic pancreatitis to PDAC by using a caerulein-induced pancreatitis mouse model (303, 327). In this study, we were able to validate at multiple levels, on the involvement of Reg proteins in the ADM process. Firstly, increased Reg1A and Reg3A/G expressions in ADM tissues were demonstrated by immunohistochemistry. Secondly, co-localized expression of CK19 and Reg1A in ADM glands was observed by immunofluorescence. Thirdly, Reg3A treatment can directly induce the formation of ADM in a 3-D culture model *in vitro*. We were able to demonstrate the upregulation of phosphorylated Erk upon Reg3A stimulation, indicating the activation of MAPK signaling pathway. MAPK has been shown to be required in the formation of ADM and PanIN lesions. Inhibition of MAPK signaling promotes a re-differentiation of the duct-like cells back to normal acinar cells and prevents the development of PanIN lesions in Kras mutated mice (84). Therefore, we believe that the induction of ADM by Reg3A is at least partially through MAPK signaling pathway. Interestingly, we have also found that as PanIN advanced from PanIN-1 to PanIN-3, and finally to invasive PDAC, there were stepwise increases in the tissue expressions of Reg1A and Reg1B proteins. Since both ADM and Pan IN are established precursors for PDAC, our data supports the notion that there is a close correlation between Reg protein levels and the disease progression from ADM/PanIN to PDAC.

One of the important reasons for the high mortality rate of PDAC is that most patients are at the advanced stages and with very limited therapeutic options at the time of the initial diagnosis. This phenomenon was also reflected in the clinical characteristics of patients in our study group (Table 2.1 and 2.2). Sensitive and specific biomarkers could aid in early diagnosis, facilitate effective therapy and improve a patient's prognosis (340). In this study, we found significant elevations of Reg1A and Reg1B in the sera of pancreatic cancer patients in comparison to normal healthy subjects. We further validated the increases of these two proteins in both mRNA and proteins levels in cancer cells. Our data suggests that serum levels of Reg1A and Reg1B are superior than previously reported Reg 4, and could be used as clinically applicable biomarkers for the early diagnosis of PDAC (312, 315, 341, 342). We also observed statistically insignificant elevations of Reg1A and Reg1B in the sera of chronic pancreatitis compared to the normal controls. These elevations could be associated with ADM, which is frequently observed in pancreatitis tissues. These data further support the clinical values of Reg1A and Reg1B as early diagnostic biomarkers. If validated in large-scale multi-center studies, Reg1A and Reg1B serological testing should be made available as a routine screening test for detecting PDAC in the at-risk patient population, and for monitoring PDAC and metastasis after therapy.

To our surprise, both the serum levels and tumor tissue expression of Reg1A and Reg1B showed negative correlations with the histological grades of invasive PDAC. This observation suggests that a certain level of pancreatic duct differentiation may be required for the sufficient expression and secretion of Reg1A and Reg1B proteins. Loss of ductal differentiation will likely cause a decrease in Reg protein levels. Clinically, low grade tumors tend to have larger tumor volumes when they are diagnosed, and consequently may have the

capability to secrete more Reg proteins into the circulation. In our study population, patients with poorly differentiated PDAC and low levels of Reg1A and Reg1B demonstrated higher mortality than those with well to moderately differentiated PDAC and high levels of Reg1A and Reg1B. The association between poor survival and high histological grade PDAC was established in previous studies (343, 344). Therefore, the negative correlation between serum Reg1A and Reg1B levels and patients' survival is believed to be dependent on the histological grades. Our observation suggests that Reg proteins could be used as prognostic biomarkers for PDAC patients. Furthermore, this observation also indicates that well to moderately-differentiated tumors may be more sensitive to Reg protein-targeted therapy than poorly differentiated tumors.

As PDAC progresses, it can metastasize to the regional lymph nodes, liver and other less common sites of the body. When PDAC metastasizes to the liver, it needs to be differentiated from ICA. This clinical demand typically presents itself in the following two scenarios: through the execution of a small liver mass biopsy, and when a small lesion is found in the liver during surgery for a pancreatic tumor. Both scenarios are nightmarish for pathologists because the histological and immunohistochemical profiles of ICA and PDAC are essentially identical. Recognizing the clinical importance of differentiating ICA from PDAC and their associated pathological challenges, dozens of studies have attempted to resolve this issue from different angles. Collins et al. attempted to use microRNA profiling to differentiate these two cancers and found that 15 microRNAs were dysregulated in both tumor types, with seven of them demonstrating opposite expression patterns between ICA and PDAC (345). Hooper et al. showed that HPC2 expression was observed in 80% of PDACs, and 32% of ICAs, while N-cadherin antibody stained 27% of the PDAC resections versus 58% of the ICA resections (346). Recently, a novel technology using branched DNA-enhanced albumin RNA

in situ hybridization was found to be able to distinguish hepatocellular carcinoma and ICA from metastatic PDAC (347, 348). Although promising, none of these previous studies have found their way into the current routine clinical practice due to their low specificity or sensitivity, or technical complexity. By contrast, our study demonstrated the highest sensitivity and specificity (92% and 95%, respectively) to date, and the combination of Reg1A, Reg1B, and Reg4 IHC staining is easy to conduct compared with previous studies (345, 346, 349, 350). The only limitation of this study is its relatively limited case numbers. However, the differences observed in this study strongly suggest the potential applicability of Reg proteins in routine clinical practice and warrants further validation in larger multi-institutional studies.

In summary (Fig. 2.6), this is the first systematic assessement of the diagnostic and prognostic values of all Reg protein isoforms using serological, immunohistochemical and molecular technologies in close correlation with clinical follow-up data.We have shown, for the first time, that the expression of Reg proteins in the precursor lesions of PDAC (ADM and PanIN lesions). In the 3-dimensional culture of acinar cells, Reg3A promoted ADM formation through the activation of MAPK signaling pathway. The combination of Reg 1A, Reg1B, and Reg4 tissue expressions could clearly differentiate metastatic PDAC from ICA with very high sensitivity and specificity.

2.7 Acknowledgements

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2.8 Tables for Chapter 2

Table 2.1 Clinical information of healthy subjects (N=61), chornic pancreatitis (n=9) and PDAC patients (N=41) for the study of Reg proteins by ELISA

	Healthy (n=61)	Chronic Pancreatitis (n=9)	PDAC (n=41)	P value
Age (years, median, mean± S.E.M.)	56 ± 1.4	63 ± 6.0	59 ± 2.0	0.99
Sex (M: F)	41:20	6:3	27: 14	
Differentiation Grades				
G1	-	-	0	
G2	-	-	19	
G3	-	-	22	
Lymphatic invasion				
Absent	-	-	26	
Present	-	-	15	
Metastasis				
Absent	-	-	20	
Present	-	-	21	
TNM staging				

IA/IB	-	-	4
IIA	-	-	10
IIB	-	-	5
III	-	-	3
IV	-	-	19

Table 2.2 Clinical information of PDAC, ECA and ICA patients whose tissues were used for the immunohistochemistry study.

	PDAC (n=60)	ECA (n=13)	ICA (n=27)	P value
Age (years, mean± S.E.M.)	69±1.3	60±1.8	62±2.3	#0.0004 ***
Sex				
M	48	10	18	0.37
F	12	3	8	
Differentiation Grades (%)				
G1	3	0	3	0.47
G2	45	10	21	
G3	12	3	3	
Lymphatic invasion (%)				
Absent	14	6	12	0.12
Present	46	7	15	
Metastasis (%)				
Absent	56	12	27	0.40
Present	4	1	0	
TNM staging				
IA/B	5	5	6	0.0017**
IIA	9	2	8	
IIB	41	4	6	
III	4	1	3	
IV	1	0	4	

Data analysis was done by using a one-way ANOVA. All the other analyses were done by using a Chi-square test. * $P<0.05$, **$P<0.01$, ***$P<0.001$.

Supplementary Table S2.1 Nucleotide sequences of primers used for quantitative PCR

Gene name	Forward Primer	Reverse Primer
Reg1A	GAGAAGCCAACTCAGACTCAG	TGAGACAGAAACATCAGGCAG
Reg1B	GGTCCTGCAATTACTATGAAGTCAAA	AAGATCAGCGATGCAAACTCATT
GAPDH	TGACAACTTTGGTATYCGTGGAAGG	AGGCAGGGATGATGTTCTGGAGAG

Supplementary Table S2.2 Correlation of serum Reg proteins levels with TNM staging and histology grading of PDAC. N=41.

		Reg1A	**Reg1B**	**Reg3A**	**Reg3G**	**Reg4**
TNM stages	Correlation Coefficient	0.101	0.313	-0.158	-0.191	0.141
	P value	0.530	0.047*	0.324	0.232	0.378
Differentiation Grades	Correlation Coefficient	-0.446	-0.343	-0.273	0.004	0.095
	P value	0.003**	0.028*	0.084	0.980	0.554

The statistical analysis was performed by using a Spearman's test. *P<0.05.

2.9 Figures for Chapter 2

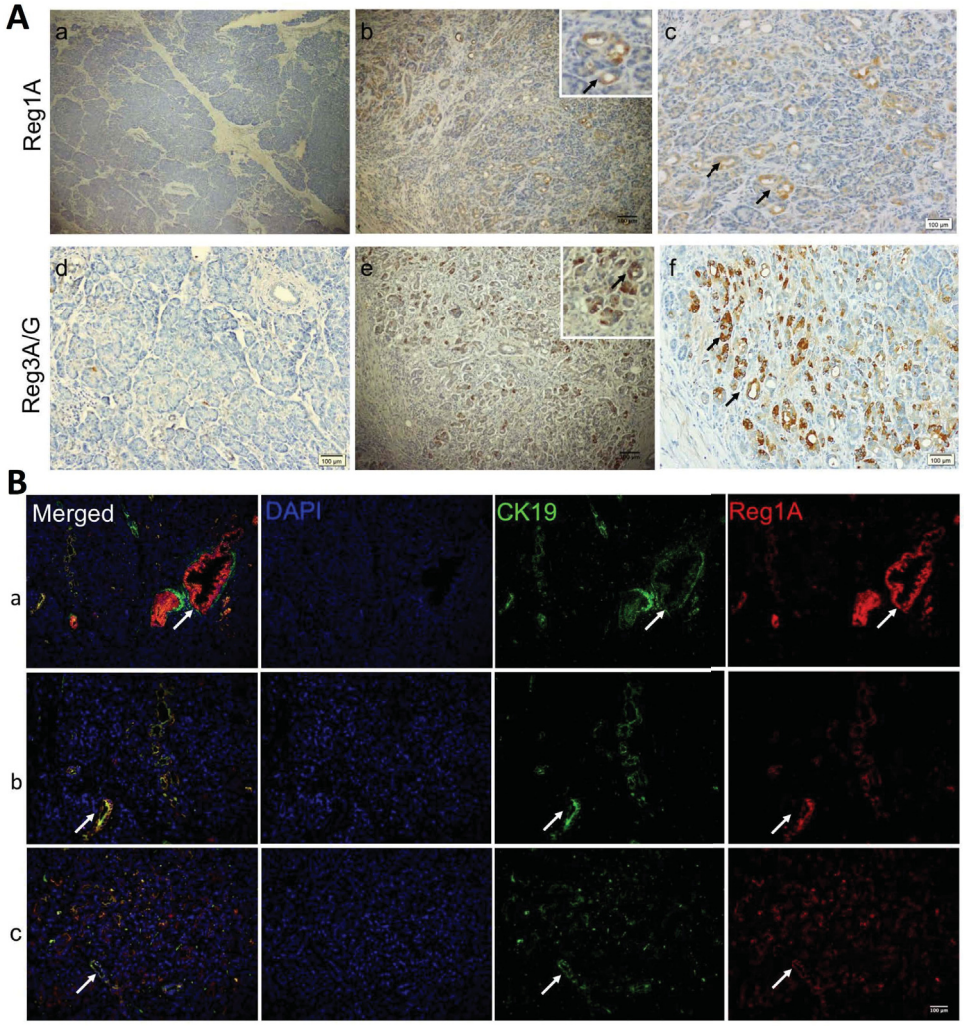
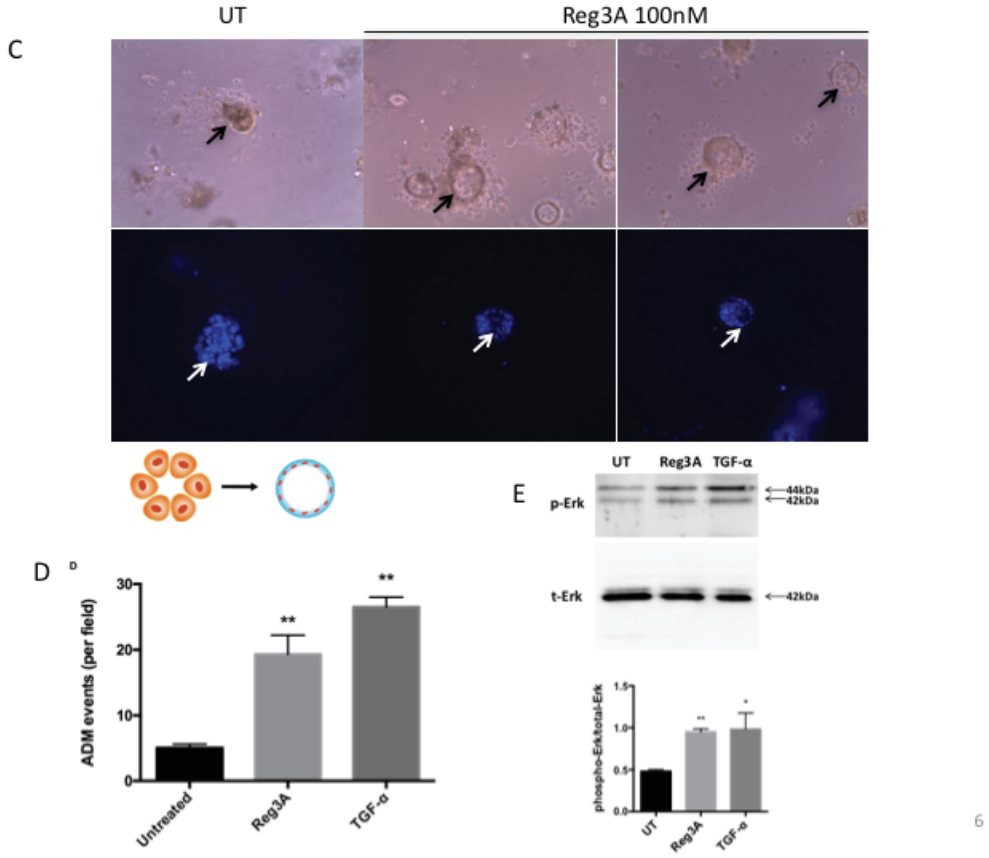


Figure 2.1 Reg1A and Reg3A/G (pancreatitis-associated proteins) were associated with ADM

(A) Increased Reg1A and Reg3A/G protein expressions in acinar cells undergoing ADM, compared to normal tissues. A, d) normal tissues, b, e) areas undergoing ADM, c, f) magnified pictures showing ADM clusters, positive for Reg1A and Reg3A/G. Arrows: duct-like structure in tumor adjacent acini (B) Co-localization of Reg1A and CK19 in cancer epithelium and duct-like

structures. a) arrows: cancer cells, as positive controls, b, c) arrows: duct-like structures. Blue: DAPI, Green: CK19, Red: Reg1A.



C) Reg3A (100 nM) promoted the formation of duct-like structure in primary acinar cells in 3-D culture. Arrows: duct-like structures. Bottom: blue DAPI showing cell nuclei. The graphic illustrates the model of ADM formation. D) Quantification of the cysts formation from primary acinar cells. Data was presented as cysts formation per area. The images were taken under 100x magnification, and the analysis was done by Image J. E) Western Blotting of the phosphorylated and total Erk1/2 in primary acinar cells after 30min treatment with Reg3A or TGF-α. The upper panel showed the representative membrane selected from 3 repeats of the experiment. The lower panel was the quantification of phosphorylated Erk corrected by total Erk levels (Image Lab). *p<0.05, **p<0.01.

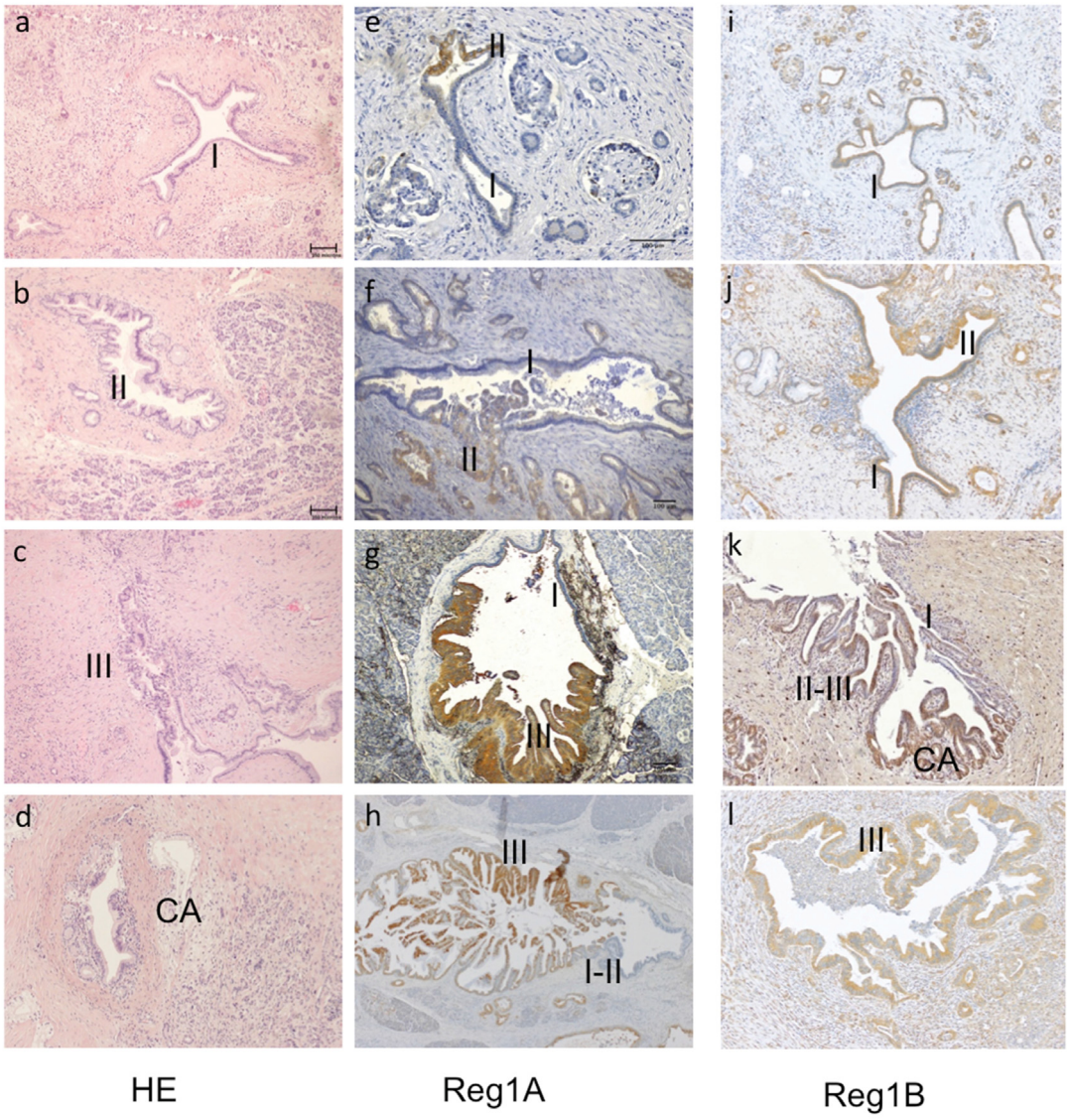


Figure 2.2 Association of Reg1A and 1B tissue expressions with the histological grades of PanIN lesions and invasive PDACs.

a-c) Representative illustrations of the different grades of PanIN lesions, from PanIN-I, II to III. d) Invasive cancer adjacent to a PanIN-II lesion. I-III: PanIN I, II and III; CA: cancer (100x). e-h) The staining intensity of Reg1A was positively associated with different grades of PanIN, marked as I, II, III. i-l) Reg1B staining was strongly positive with different grades of PanIN seen in ducts. Representative images were selected from at least 10 fields, each at 100x magnification.

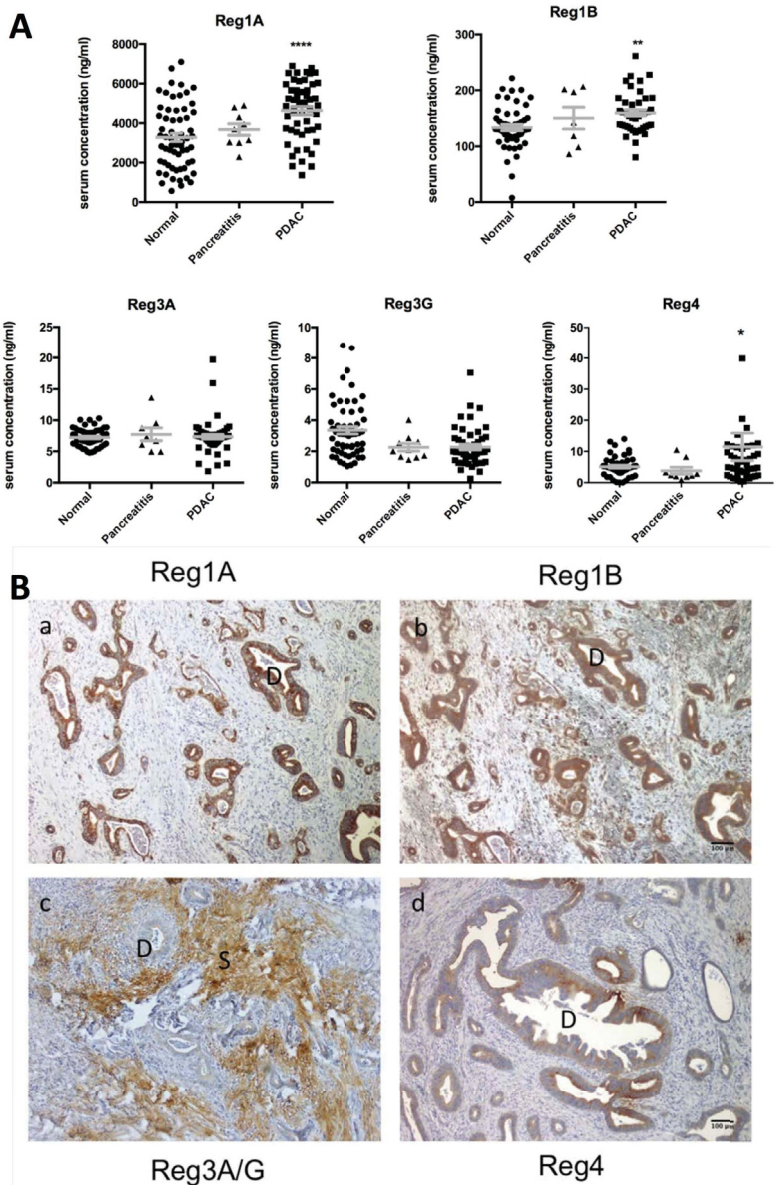
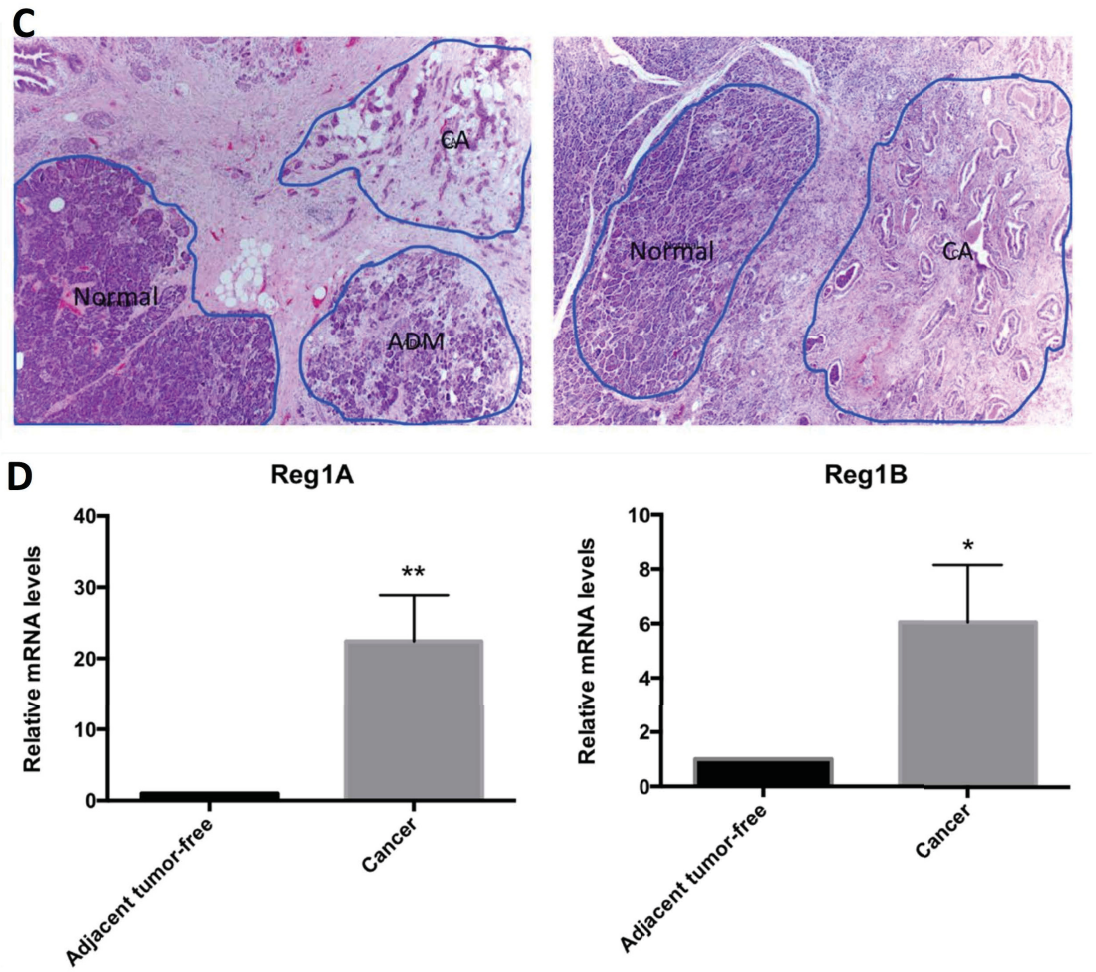


Figure 2.3 Upregulation of Reg1A and Reg1B in the sera and tissues of PDAC patients.

(A) Serum Reg1A and 1B levels in PDAC patients (n=41), healthy controls (n=61) and chronic pancreatitis pateints (n=9). The concentrations of Reg protein isoforms were determined using ELISA. The comparisons were performed using One-Way ANOVA. *P<0.05, **P<0.01, ****p<0.0001. (B) Immunohistochemistry profiles of Reg proteins in pancreatic ducts and stroma

in PDAC patients. The color brown represents positive staining of various Reg protein isoforms. D: ducts, S: stroma. Representative images were selected from at least 10 fields each.



(C) Microdissection to collect tissues from cancer and adjacent normal areas. Representative images showing how the microdissections were done. ADM areas were excluded for this study. (D) Relative mRNA levels of Reg1A and 1B in microdissected cancer tissues vs. adjacent tumor-free tissues. N=5; 7. Levels of mRNA in cancer tissues were calculated as fold changes, compared to those in their paired adjacent tissues. *P<0.05, **p<0.01.

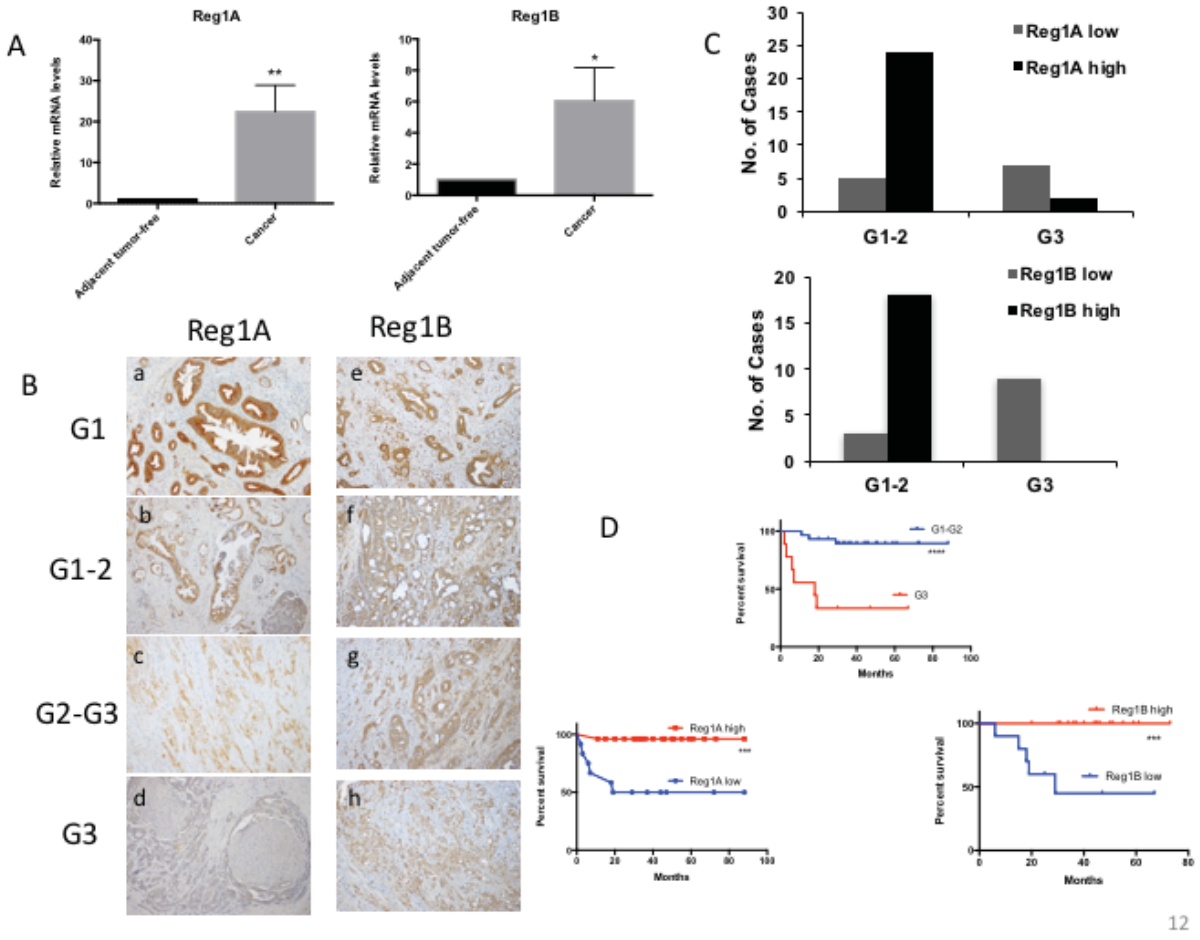


Figure 2.4 High levels of Reg1A and Reg1B were associated with low differentiation grades of cancer cells and predicted better prognosis.

(A) Serum levels of Reg1A and Reg1B were negatively correlated with cell differentiation grades in PDAC patients. Correlation analysis was done by Spearman's test *P<0.05, **P<0.01. (B) Immunohistochemistry showing Reg1A and Reg1B expression in different differentiation grades. G1: well-differentiated cancer, G2: medium-differentiated cancer, G3: poorly-differentiated cancer. (C) Positivity of Reg1A and 1B in low and high grades of PDAC. Cases were divided into high expression and low expression groups, based on their immunohistochemical staining intensity. IRS≥9 was considered as high expression. G1-2 was

defined as a low differentiation grade; G3 was defined as a high differentiation grade. (D) Survival rate of patients with low and high Reg expression levels and differentiation grades. Cases were divided into high expression and low expression groups, based on their immunohistochemical staining intensity. G1-2 was defined as a low differentiation grade; G3 was defined as a high differentiation grade. ***P<0.001, ****P<0.0001.

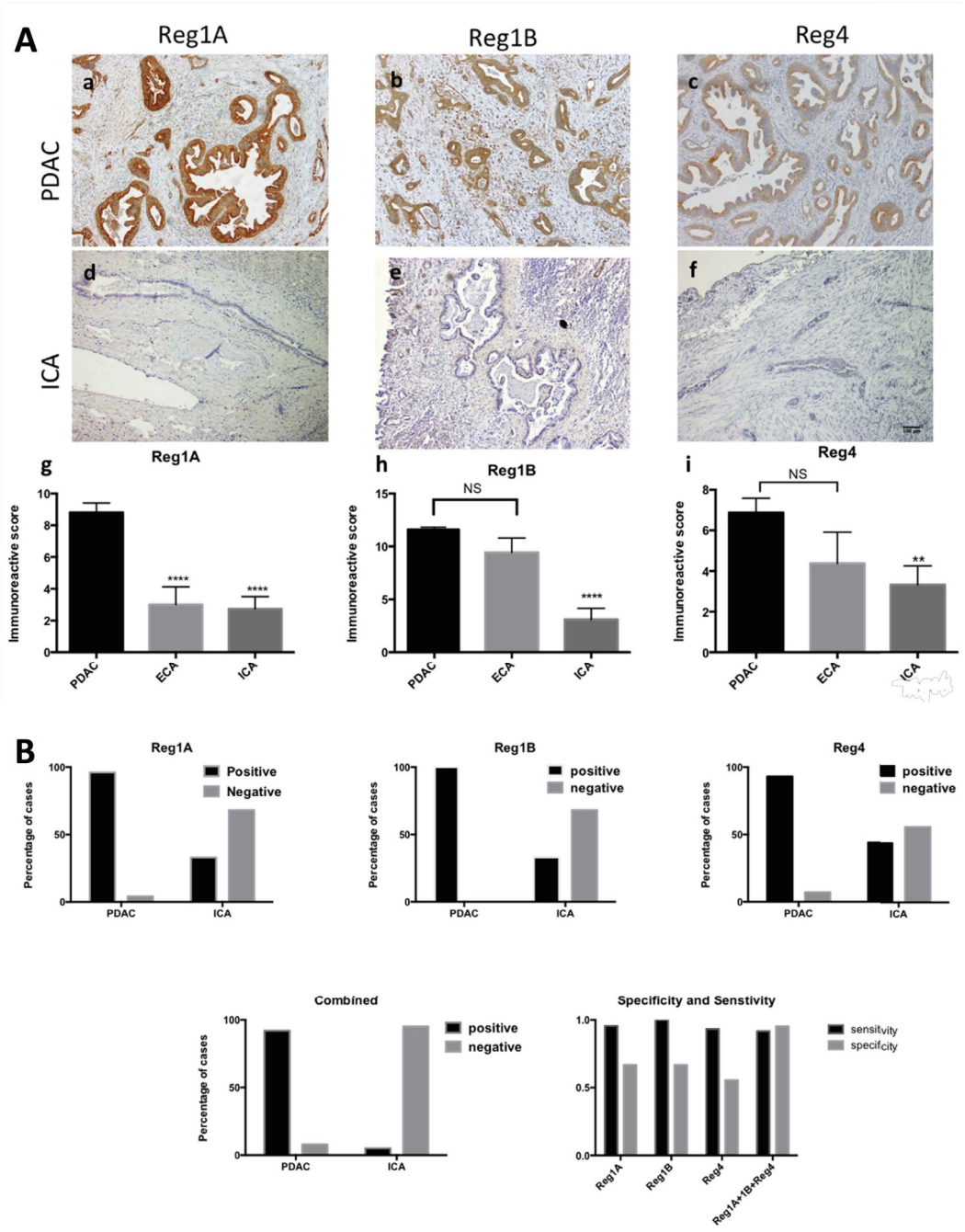


Figure 2.5 Tissue expression of Reg1A, Reg1B, and Reg4 could differentiate PDAC from ICA with high specificity and sensitivity.

(A) Immunoreactive scores (IRS) of Reg1A, Reg1B and Reg4 in PDAC were significantly higher than ICA. a-c) Reg1A, Reg1B and Reg4 expression in PDAC; d-f) Reg1A, Reg1B, and

Reg4 expression in ICA; g-i) comparisons of Reg1A, Reg1B, and Reg4 IRS among PDAC, ECA, and ICA. **P<0.01, ****P<0.0001, NS: no significance. (B) The sensitivity and specificity of combining Reg1A, Reg1B, and Reg4 immunohistochemical staining in distinguishing PDAC from ICA. Positive and negative cases of PDAC and ICA for Reg1A, Reg1B, and Reg4 were plotted on column graphs. Combined positivity was recorded when Reg1A, Reg1B, and Reg4 were all positive. Sensitivity, specificity and predictive values were calculated by using GraphPad Prism.

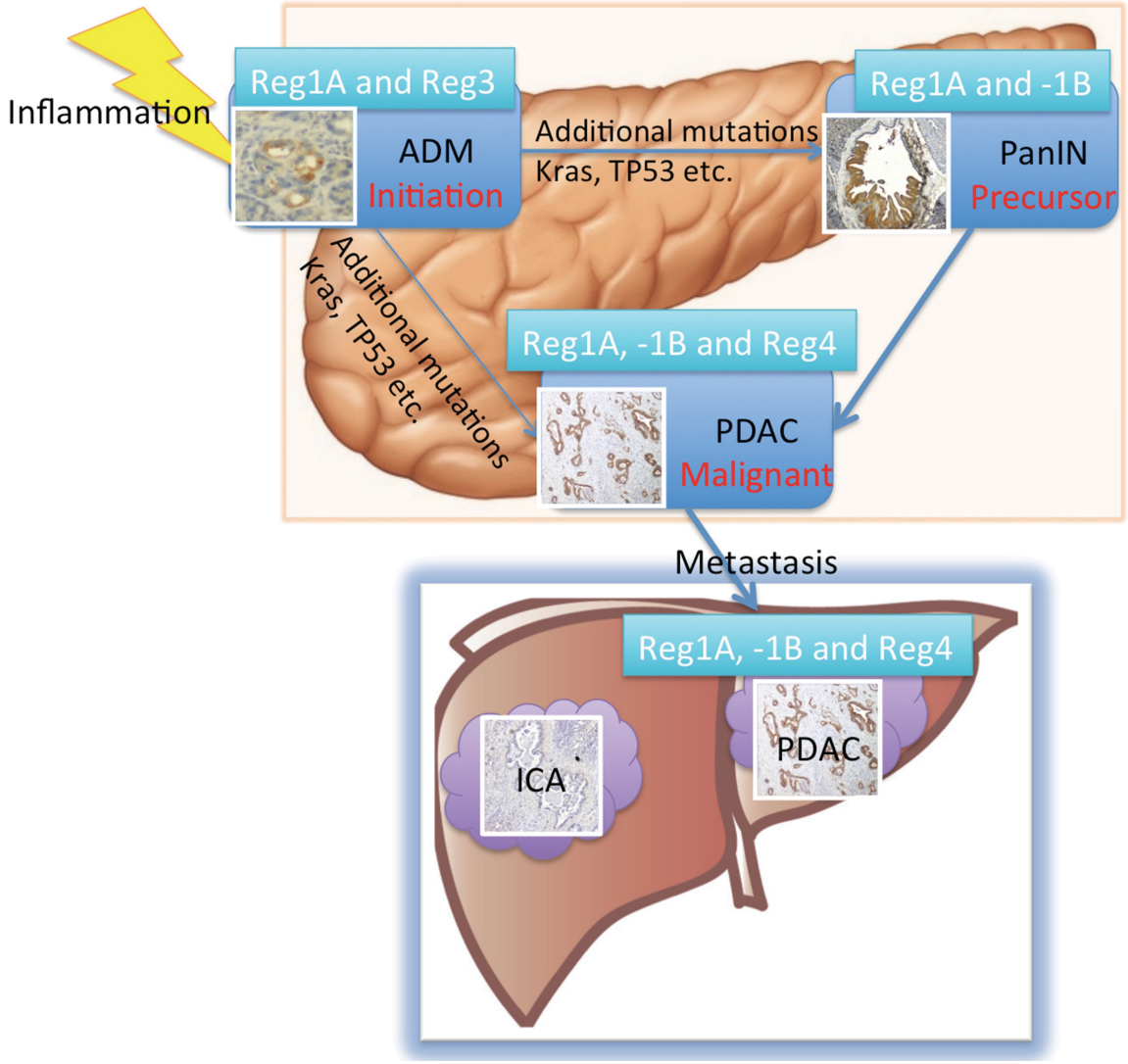


Figure 2.6 Summary of the expression of Reg proteins in the progression of ADM/PanIN to invasive cancer and metastasis in liver.

Expression of Reg1A and Reg3 is upregulated in ADM and Reg3 can directly promote ADM formation in vitro. With additionally mutations such as Kras and TP53, ADM can transform to PanIN lesions and PDAC (56). Reg1A and Reg1B are both upregulated in the sera and tissues of PDAC patients. Upregulation of Reg proteins can be used to differentiate PDAC from ICA.

Chapter 3. Reg2 expression is required for pancreatic islet compensation in response to aging and high fat diet-induced obesity

[1]Qing Li, [1]Bing Li, [2]Xiaoliang Miao, [1]Christopher Ramgattie, [3]Zu-hua Gao*, [1,4]Jun-Li Liu*

[1]Fraser Laboratories for Diabetes Research, Department of Medicine, McGill University Health Centre, Montreal, Canada;

[2]School of Life Science and Technology, China Pharmaceutical University, Nanjing, Jiangsu, China;

[3]Department of Pathology, McGill University Health Centre, Montreal, QC, Canada;

[4]Montreal Diabetes Research Centre, Montreal, Canada.

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3.1 Preface

In the previous chapter, I showed the upregulation of Reg proteins in PDAC and its precursors. Excessive activation of Reg proteins in the exocrine pancreas may be involved in the initiation and progression of the cancer. However, this activation may be beneficial for patients with type 1 or type 2 diabetes due to their ability to stimulate proliferation in β-cell via the paracrine manner. Additionally, the upregulation of Reg proteins in the endocrine pancreas also provides an alternative approach for β-cell proliferation and anti-apoptosis in response to islet stress. Previous studies in our lab have shown that Reg2 overexpression protects insulinoma cells from apoptosis induced by streptozotocin or UPR (247, 248). Therefore, I further investigated whether Reg2 was required for the β-cell compensation in response to HFD-induced diabetes. We established a general Reg2 knockout mouse model. There was no obvious phenotype change in young Reg2 deficient mice. However, I observed a decrease in the islet volume and β-cell size, and impaired glucose tolerance in aged Reg2 knockout mice compared to their wild-type littermates. We further challenged the mice with HFD, which is known to induce islet compensation. Consistently, we observed smaller islets and lower insulin levels in Reg2 deficient mice, suggesting Reg2 is required for the islet compensation under HFD. Surprisingly, we detected lower blood glucose in these Reg2 deficient mice. It may be explained by that the limit on β-cell growth decreased insulin levels in the circulation, therefore improving insulin sensitivity in HFD-induced mice. This may be beneficial for the HFD-induced obesity and diabetes. In general, Reg2 expression is required for islet compensation both in aged mice and mice under HFD.

3.2 Abstract

Maintaining pancreatic β-cell mass and function is essential for normal insulin production and glucose homeostasis. Regenerating islet-derived 2 (Reg2, Reg II, human orthologue Reg1B) gene is normally expressed in pancreatic acinar cells and significantly induced in responses to diabetes, pancreatitis, high-fat diet and during pancreatic regeneration. In order to evaluate the role of endogenous Reg2 production in normal β-cell function, we characterized Reg2 gene deficient mice normally and when subjected to several pathological challenges. At a young age, Reg2 gene deficiency caused no obvious change in normal islet morphology or glucose tolerance. There was no change in the severity of streptozotocin-induced diabetes or caerulein-induced acute pancreatitis in the Reg2 deficient mice, indicating that the increased Reg2 expression under those conditions was not essential to protect the islet or acinar cells. However, 13-14 months old, aged Reg2 gene deficient mice developed impaired glucose tolerance associated with significantly decreased islet β-cell ratio and serum insulin level. Similarly, after young mice were fed high-fat diet for 19 weeks, diminished islet mass expansion and serum insulin level were observed in Reg2 deficient vs. wild-type mice. This was associated with a decline in the rate of individual β-cell proliferation measured by Ki67 index. In both conditions, the β-cells were smaller in size in gene deficient vs. wild-type mice. Our results indicate that normal expression of Reg2 gene is required for appropriate compensations in pancreatic islet proliferation and expansion in response to obesity and aging.

3.3 Introduction

The increasing prevalence of obesity and its associated metabolic disorders, such as type 2 diabetes (T2D), has posted a great challenge for public health. T2D is characterized by defects in both insulin production and responsiveness and its prevalence increases substantially with age (351). Pancreatic β-cell is the only source of insulin production in humans and most mammalian animals, and its growth potential determines the scope of insulin secretion. In obesity, the β-cells exhibit excessive expansion to compensate for the increased demand of insulin in order to maintain euglycemia (352, 353). However, insulin resistance and eventually T2D occur when islet compensation cannot be sustained. On the other hand, increased islet cell mass but decreased β-cell regeneration upon aging have been reported in rodents (354). Obesity, aging and β-cell malfunction are thus dynamically interconnected with the mechanism of insulin resistance and diabetes (355). Maintaining a normal mass/function of β-cells and the sensitivity of peripheral organs to insulin are vital for controlling the onset of insulin resistance and diabetes.

The expression of Regenerating (Reg) proteins in pancreatic tissue is induced by islet hyperplasia, diabetes or inflammatory stimuli. Among the seven isoforms, Regenerating islet-derived 2 (Reg2, Reg II, human orthologue Reg1B) is normally expressed in the pancreatic acinar cells in mouse but significantly induced in response to pancreatitis, high-fat diet (HFD), the treatment of exendin-4 and during regeneration after partial pancreatectomy (195, 244, 356-360). We have demonstrated the activation of Reg2, Reg3α and Reg3β genes in the pancreas of IGF-I gene deficiency and after streptozotocin-induced diabetes (11). However, acinar overexpression of Reg2 gene did not offer protection to the islet cells in response to experimental diabetes induced by streptozotocin (361). In order to further explore the normal physiological roles of Reg2 gene in regulating β-cell function and glucose homeostasis, we hereby characterized a systemic gene

deficiency in mice. This is a new model and completely different from previously reported Reg3β gene deficiency which was mislabeled as Reg2 (170, 239). Although normal islet cell ratio and function were unaffected, Reg2 gene deficient mice displayed impaired islet compensations in response to both aging and HFD-induced obesity.

3.4 Materials and Methods

3.4.1 Reg2 gene deficient (Reg2-/-) mice

The Reg2-/- mice were created by replacing the 2502-bp Reg2 gene (MGI: 97896) on chromosome 6 with a 6-kb ZEN-Ub1 cassette using homologous recombination, by Knockout Mouse Project (UC Davis; www.komp.org). To achieve homozygosity, Reg2+/- heterozygous male and female pairs were intercrossed and the offspring were weaned at 4-week old. The animals were ear-tagged and 0.5-cm tails were collected for DNA isolation and genotyping. Three PCR primers were used, Reg2GenoF (5'-TAT AGA ACC GTC GTT GGC AC), NeoF2 (5'-TGT GGT TTC CAA ATG TGT CAGT), and SD (5'-GAG AGA TAA AGA ATG CAT GGAG). The Reg2F/SD pair was used to amplify the endogenous Reg2 (628 bp) and NeoF/SD the deleted allele (349 bp) (Fig 3.1A). Deletion of Reg2 gene was further confirmed using Western blot and immunohistochemistry of pancreas in 1-month-old mice. Mice had free access to water and food unless otherwise indicated for special treatments. All animal procedures were approved by McGill University Animal Care Committee.

3.4.2 Streptozotocin-induced diabetes and caerulein-induced pancreatitis

Streptozotocin (75 mg/kg) was prepared freshly in 0.1 M sodium citrate (pH 4.5) and injected into 8-10 weeks old Reg2-/- and wild-type littermates for 5 consecutive days. To

monitor onset of diabetes, body weight and blood glucose were recorded every 3 d until 12 d. For induction of pancreatitis, 6-8 weeks old mice were fasted for 12 h before given seven hourly injections of caerulein (50 μg/kg i.p.) or vehicle. Mice were euthanized after 11 h of the injection and pancreas and serum were collected for further analysis.

3.4.3 High-fat diet (HFD) feeding and aged mice

Homozygous Reg2-/- and wild-type littermates were fed with HFD containing 60% fat (Cat #D12492, Research Diets Inc.) starting from 2-3 months old and lasted for 19 weeks before being sacrificed. For aging study, Reg2-/- mice and age-matched wild-type mice were fed with chow diet until reaching 13-14 months old. For glucose tolerance, mice were injected with 1 or 2 g/kg glucose intraperitoneally. Blood samples were taken from the tail vein and measured by the OneTouch Ultra glucose meter at 0, 15, 30, 60, and 120 min after the injection. For insulin tolerance test, mice were injected with 0.75 U/kg insulin and blood glucose was measured at 0, 20, 40, 60 min after the injection. Changes in fat and lean masses were determined using an EcoMRI System. To reflect the extent of inflammation in adipose tissues, tholudine blue staining for mast cell was performed using standard protocol. Insulin sensitivity index was calculated as 1/(log[Serum insulin]+log[Blood glucose]) according to the report (362).

3.4.4 Tissue collections and serum insulin and amylase tests

At the end of each experiment, mice were euthanized with a cocktail injection of ketamine, xylazine and acepromazine followed by cervical dislocation. Blood were collected and supernatant serum was stored at -20°C before further fanalysis. The pancreas and liver were removed and half was used to extract protein and another half was fixed in 10% formalin to prepare paraffin sections. Serum insulin level was measured by ELISA following a standard protocol (ALPCO). Serum α-

amylase level was tested using an enzymatic assay kit (Sigma).

3.4.5 Immunohistochemistry and immunofluorescence

Pancreatic sections of 5 μm were de-waxed and hydrated with graded ethanol. They were permeabilized with 0.1% Triton X-100 and endogenous peroxidase activity was blocked by 3% hydrogen peroxide. The sections were further blocked by 10% serum before being incubated with primary antibodies overnight at 4°C. Corresponding horseradish peroxidase (HRP)-conjugated secondary antibodies were applied, followed by the incubation with DAB substrate (Vector Labs, Burlingame, CA). The slides were counterstained with hematoxylin for the nucleus and mounted with hydrophobic mounting medium. Ki67 and caspase-3 IHC were processed using BenchMark Ultra Automated IHC/ISH slide staining system (Ventana, Roche). For immunofluorescence, corresponding fluorescent antibodies were conjugated with either Alexa 488 or 596. Primary antibodies used in this study include: Reg2 (R&D Systems), insulin (Cell Signaling Technology), glucagon and Ki67 (Ventana, cat #790-4286), and cleaved caspase-3 (Cell Signaling Technology, cat # 9661S). Histological images were captured using a Zeiss light and fluorescent microscope under 100x and 400x magnifications. The pancreatic islet ratio was quantified by the areas of insulin-positive cells divided by total tissue area using Image J software. Average β-cell size was calculated by the islet area divided by cell numbers of an individual islet. The quantification of Ki67 positive cells was performed by dividing the number of positive cells by total cell numbers in each islet. Each data was created by selecting 5-8 representative images in each mouse.

3.4.6 Western blotting analysis

Pancreatic proteins were extracted by a lysis buffer (10 mM Tris, pH 7.5, 1 mM EDTA, 150 mM NaCl, 1% Triton X-100) containing protease inhibitor cocktail (Bioshop) and separated

by SDS-PAGE. The proteins were then transferred to a nitrocellulose membrane. Nonspecific bindings were blocked by 10% skim milk followed by the incubation with primary antibodies at 4°C for overnight. HRP-conjugated secondary antibodies were incubated for 1 h followed by ECL substrates. The signals were captured using ChemiDoc Touch Imaging System (Bio-Rad). Antibodies used include phospho-Akt at Ser-473 and total Akt (Cat#4058 and 9272), phospho- and total Erk1/2 (Cat#9101 and 9102, Cell Signaling Technology), Reg3α, Reg3β (R&D systems), and β-actin (MM-0164-P, Medimabs). Data was quantified using Image J and Image Lab (Bio-Rad).

3.4.7 Statistical analysis

All data were presented as Mean ± S.E. and analyzed by using Students' t-test, One-Way and Two-Way ANOVA using GraphPad Prism version 6.0. Area under curve was calculated by using Prism. $P<0.05$ was considered as statistically significant.

3.5 Results

3.5.1 Reg2 gene deficiency did not alter glucose tolerance in young mice

To confirm the deletion of Reg2 gene, three primers were designed to amplify different fragments by PCR from Reg2 gene or ZEN-Ub1 cassette (Fig 3.1A). The homozygous Reg2-/- mice showed a 349-bp band, while wild-type showed a 628-bp band and heterozygous Reg2+/- mice had both (Fig 3.1B). To verify the deletion of Reg2 gene at the protein level, Western blotting was performed by using the pancreas of 1-month old mice. Reg2 was expressed in the wild-type while absent in Reg2-/- mice (Fig 3.1C). Using immunofluorescence, we observed Reg2 expression in exocrine pancreas of wild-type mice as previously reported (159), but not in Reg2-

/- mice (Fig 3.1D), further confirming the ablation of Reg2 gene. Both wild-type and gene deficient mice showed similar ratio of glucagon immunostaining and islet cell ratio. Unlike previous reports, Reg2 expression did not co-localize with the β-cells (242). The glucose tolerance in Reg2-/- mice was normal in 3-4 months old males and females (Fig 3.2A, B). In the pancreas by Western blots, we also tested the expression of other Reg protein isoforms that may compensate for Reg2 gene deficiency and proliferative signals of Akt and Erk. None of them showed a significant change in Reg2-/- vs. wild-type mice under normal conditions (Fig 3.2C).

3.5.2 Reg2 Deficiency did not affect streptozotocin-induced diabetes and caerulein-induced acute pancreatitis

As the deficiency of Reg2 gene did not alter normal islet phenotype in young mice, we challenged the animals with streptozotocin or caerulein in order to test whether endogenous Reg2 production protects the islets from T1D or the acini against pancreatitis. Multiple low-dose streptozotocin has been shown to mimic autoimmune insulitis and produce insulin dependent diabetes (T1D) (363). Wild-type and Reg2-/- mice of 8-9 weeks old receiving vehicles did not exhibit any change in blood glucose and body weight (data not shown). After streptozotocin injections, blood glucose level reached above 400 mg/dL after 6 d and remained high until being sacrificed at 12 d in all animals (Fig 3.2D). No significant difference was observed in either male or female Reg2-/- vs. wild-type mice regarding to their body weight and blood glucose level.

As a cholecystokinin (CCK) analogue, hourly injections of a super maximal doses of caerulein induce acute pancreatitis, characterized by hyperamylasemia, pancreatic edema and acinar vacuolization (364). In caerulein-injected wild-type mice, we observed a ~4-fold increase in serum amylase level (Fig 3.2E), significant neutrophil-infiltration and acinar cell death revealed

using IHC (data not shown) (361). However, there was no difference between Reg2-/- and wild-type mice, indicating Reg2 deficiency did not affect either streptozotocin-induced diabetes or caerulein-induced pancreatitis; the increased Reg2 expression under those conditions is not essential to protect the islet or acinar cells.

3.5.3 Reg2 gene deficiency impaired insulin production and glucose tolerance in aged mice

Young mice may compensate for the lack of Reg2 expression better than the old; aging is closely associated with increasing incidences of obesity and diabetes (365). To explore the effect of aging, some Reg2-/- and wild-type mice were kept on chow diet for 13-14 months. At that age, Reg2-/- mice displayed a significantly impaired glucose tolerance vs. wild-type littermates. Higher glucose levels were observed at 30, 60 and 120 min after glucose injection (Fig 3.3A). This was caused at least in part by decreased serum insulin level and islet cell ratio, indicating an essential role of endogenous Reg2 production in maintaining a normal islet mass/function in old mice. We observed obviously smaller islet area using both H&E staining and immunohistochemistry for insulin in Reg2-/- vs. wild-type mice (Fig 3.3B); the quantification of % islet area to total tissue showed a significant 60% decrease in Reg2-/- mice (Fig 3.3C). As the rate of β-cell proliferation in aged mice can be neglected (no Ki67 labeling), at least part of the decrease can be attributed to a diminished β-cell size (Fig 3.3D). Consistent with the islet hypotrophy, serum insulin level was also decreased by more than 50% (Fig 3.3E). On the other hand, Reg2-/- mice exhibited a slightly improved insulin tolerance (Fig 3.3F), further supporting that islet hypotrophy and diminished insulin production contribute to glucose intolerance in aged Reg2-/- mice. As glucose intolerance may lead to or be caused by lipid disorders in liver and fat tissues, we observed increased lipid deposition (steatosis) in the liver of aged Reg2-/- vs. wild-type mice (Fig 3.3G). As well, aged

Reg2-/- mice showed a 30% increase in % fat mass and increased mast cell infiltration (Fig 3.3H, I), a sign of increased inflammation, all of which may further contribute to impaired glucose tolerance. There was no change in the overall body weight or that of the pancreas (data not shown).

3.5.4 Reg2 gene deficiency protected mice from high-fat diet-induced diabetes

Another challenge to Reg2-/- mice would be obesity-induced islet compensation. As Reg2 gene expression is stimulated by diet-induced obesity and highly expressed in the regenerating pancreas (358, 359), its deficiency was expected to be detrimental to obesity-induced islet expansion and the onset of diabetes. Reg2-/- and wild-type littermates were fed for 19 weeks on HFD, both demonstrated the same scale of weight gain, 2-fold (Fig 3.4A), and similar extents of hepatic steatosis and fat mass increase (data not shown). Wild-type mice became significantly hyperglycemic starting from 12 weeks and reached a peak of 254 mg/dL towards the end of the 19 weeks on HFD (Fig 3.4B). Reg2-/- mice, although they gained the same weight, exhibited a much slower and smaller increase in blood glucose level, reaching a transient peak of 200 mg/dL only. The difference was statistically significant at most time points from 11 to 19 weeks. The attenuated hyperglycemia in the face of Reg2 gene deficiency, instead of a more severe T2D, was surprising.

The level of Reg2 gene expression in the pancreas has been known to be dependent to age which peaks at 30 d but vanishes after 90 d (356). Although our mice were studied at 7-8 months of age by the end, HFD and obesity are known to induce Reg2 re-expression (358). Indeed, using caerulein-induced pancreatitis as a positive control (Fig 3.4C, upper panels), we detected patchy expression of Reg2 in the exocrine pancreas of wild-type mice after 19 weeks of HFD (middle panels); which was totally ablated by Reg2 gene deficient (lower panels).

3.5.5 Diminished islet compensation to high-fat diet caused by Reg2 gene deficiency

After the mice have been sacrificed, we studied islet histology and serum insulin. As shown in Fig 3.5A, middle panels, the compensation in islet area (indicating volume) to HFD in wild-type mice vs. chow diet (left panels) was very obvious, especially on the size of the islets. However, Reg2-/- littermates showed very little islet expansion vs. chow diet (Fig 3.5A, right panels); the % islet area was only 25% of that of wild-type mice after HFD (Fig 3.5B), suggesting that normal Reg2 expression is required for obesity-induced islet expansion. The diminished islet expansion was at least in part caused by a significant, 20% reduction in average β-cell size (Fig 3.5C). Moreover, serum insulin levels were increased 6-fold by 19 weeks of HFD in wild-type mice; Reg2 gene deficient mice only displayed a mere 1.3-fold increase (Fig 3.5D).

The compensation in islet cell number could be caused by either increased cell proliferation or decreased cell death. Indeed, HFD caused a significant increase in islet cell proliferation as indicated by Ki67 labeling in wild-type islets in the middle panels and column (from 0.95% to 6%), which was largely blunted by Reg2 gene deficiency in the right panels and column (1.7%; Fig 3.5E, F). There was no significant change in the rate of cell apoptosis due to HFD, measured by the IHC staining of caspase-3 cleavage (data not shown). Although we have not measured insulin sensitivity directly during the course of HFD experiment, Reg2-/- mice demonstrated significantly increased insulin sensitivity index (Fig 3.5G). The result is in favor of a possibility that in response to HFD and obesity, Reg2 gene deficiency caused islet hypotrophy and hypoplasia, supporting its normal stimulatory role by the endogenous gene expression.

3.6 Discussion

In this initial characterization of Reg2 gene deletion, we found no change in body weight, blood glucose level and glucose tolerance in young adults. Reg2 gene deficiency did not affect the severity of streptozototin-induced diabetes nor caerulein-induced acute pancreatitis, despite that Reg2 is normally expressed in acinar cells, and its overexpression protected insulinoma cells in vitro (247, 356). However, in aged mice Reg2 deficiency caused diminished islet mass turnover, decreased insulin level and impaired glucose tolerance which may be associated to certain extent of hepatosteatosis, obesity and adipose tissue inflammation. Similarly, in HFD-induced obesity, we demonstrated diminished compensations in the rate of islet cell proliferation, cell size and % islet area and decreased serum insulin level, together with a seemingly increased insulin sensitivity may collectively contribute to the surprise *protection* of Reg2-/- mice from obesity-induced T2D. These are definite proofs that under specific conditions Reg2 expression becomes important and essential for islet maintenance and compensation.

In rodents, Reg2 belongs to a large C-type lection superfamily. This family contains seven isoforms that are clustered in their chromosome localization. They share the overlapping tissue/cell distribution and high degree of sequence identities (160, 366). Gene redundancy within this family may at least in part explain the obvious lack of phenotype in normal islet growth and glucose homeostasis after the deletion of Reg2 gene alone. Moreover, it has been known that gene deficient mice may not exhibit significant abnormality unless being challenged with additional stresses (367). Similarly, in streptozotocin-induced diabetes, not only Reg2 but also Reg3α, -3β and -3γ are significantly induced which may compensate for the loss of putative protection normally provided by Reg2 (11). Subfamily Reg3 members are also known as pancreatitis-associated proteins, several of which as well as Reg1 are highly induced by acute

pancreatitis (167, 254, 368-373). Based on previous report and Fig 3.4C top panels, Reg2 expression was also highly induced by acute pancreatitis (360). Demonstrated using gene deficiency, endogenous Reg3β is anti-apoptotic and anti-inflammatory (278); administration of recombinant Reg3α and Reg4 proteins significantly prevented L-arginine- or caerulein-induced acute pancreatitis (179, 374); and Reg3α antibody exacerbated the pancreatitic damage (279), indicating a general protective role by various Reg proteins. It is thus reasonable to assume gene redundancy in explaining why Reg2 gene deficiency alone did not affect the severity of pancreatitis in this study. However, the same cannot be said in the changes caused by obesity and aging.

In wild-type mice, normal aging is known to result in diminished β-cell proliferation but an overall hypertrophic expansion as a way to compensate for decreased insulin sensitivity (354). While in Reg2-/- mice, decreased β-cell hypertrophy and overall β-cell ratio were observed, indicating that the normal production of Reg2 promotes β-cell growth (at least in size) in aged mice and the effect cannot be compensated by other Reg isoforms. Indeed, although both Reg1 and Reg2 are presumed to play important roles in aging, only Reg2 level was maintained but Reg1 expression was significantly decreased in 30- vs. 3-month-old mice (195). Further to decreased β-cell hypertrophy, a decreased cell proliferation could have occurred between 3 to 13 months which was not measured; the lack of Ki67 labeling in the aged mice was inconclusive. In future studies, it would be interesting to treat aged Reg2-/- mice with recombinant Reg2 protein to directly rescue β-cell hypotrophy and/or hypoplasia if there is. The decrease in β-cell mass may at least in part lead to decreased insulin production, together with increased adiposity, adipose inflammation and hepatic steatosis, causes glucose intolerance. On the other hand, there was a slight increase or at least no sign of decrease in insulin sensitivity. Glucose intolerance was

not due to impaired insulin sensitivity. Decreased insulin level would relieve the inhibition on lipolysis and cause increased release of fatty acids to further feed hepatic steatosis. Whether Reg2 gene deficiency cause the changes in adipose tissue and the liver directly or secondary to the islet change is unclear at the moment, although Reg2 is not known to be expressed in those two tissues (356).

In HFD-induced obesity, the wild-type mice were able to compensate significantly in β-cell proliferation and hypertrophy and serum insulin level. These are the normal responses to the obesity-induced insulin resistance that can delay the onset of glucose intolerance. In Reg2-/- mice after HFD, we detected diminished β-cell hypertrophy and proliferation. These changes may contribute to an overall decrease of the β-cell ratio to 1/3 of that of wild-type level and even more decrease in serum insulin level (Fig 3.5). Since only the level of Reg2, but not other Reg isoforms, was significantly elevated after HFD (also confirmed in Fig 3.4C, middle panels), a total deletion *is expected* to have a significant effect (358). Although more direct evidence is required to be developed using in vitro cultured primary islets and in vivo rescue of Reg2 gene deficiency, our results seem to indicate that normal production of Reg2 is essential for promoting β-cell expansion in response to HFD and obesity. An alternative explanation would be that a possible increase in insulin sensitivity caused by Reg2 deficiency in the face of HFD-induced obesity results in reduced hyperglycemia and drive for β-cell expansion. We have yet to carefully analyze insulin sensitivity using hyperinsulinemic, euglycemic clamp in future studies; and Reg2 is not known to be expressed in major insulin target tissues such as skeletal muscle, adipose tissue and liver. Indirectly, however, we found increased insulin sensitivity index in these mice.

The only controversy in our view is the partial prevention of hyperglycemia in HFD fed Reg2-/- mice. Because diminished or lack of compensations in β-cell mass and insulin

production in gene deficient mice are expected to worsen T2D vs. wild-type littermates; the opposite was observed (Fig 3.4B). Nevertheless, this result seems consistent to Johnson's model developed through combinational deletions of the two insulin genes. They propose that HFD causes hyperinsulinemia first, which promotes obesity, followed by insulin resistance (375). Based on that model, our Reg2-/- mice with demonstrated decreases in β-cell ratio and insulin level would have (1) prevented obesity, but we detected the same degree of weight gain and obesity from wild-type (Fig 3.4A); and (2) resulted in improved insulin sensitivity and glucose tolerance. We indeed found higher level of insulin sensitivity index but no change in glucose tolerance.

Interestingly, although we found decreased islet ratio and insulin level in both aged and HFD-fed Reg2-/- mice, their glucose tolerance showed a tendency of opposite changes, i.e. aged Reg2-/- mice were impaired while young ones after HFD had unchanged glucose tolerance vs. WT-HFD. Glucose tolerance is determined by a dynamic interplay of the levels of insulin release and insulin sensitivity. As recently reported, hyperinsulinemia itself may cause obesity and insulin resistance (375, 376). In our study, wild-type mice after HFD showed hyperinsulinemia and hyperglycemia. However, the insulin level in Reg2-/- mice fed HFD was not significantly raised from that of WT-Chow; their blood glucose level was significantly diminished from WT-HFD at most time points. This would be consistent with the notion that limited compensation in insulin secretion improves insulin sensitivity and limits the development of insulin resistance and hyperglycemia. We suspect that in young mice fed HFD, improved insulin sensitivity compensates for the lack of insulin production, resulting in an unchanged glucose tolerance. However, after 13-14 months' exposure to Reg2 deficiency, the decreased β-cell growth may dominate over the improved insulin sensitivity, therefore impairs their ability to dispose glucose

effectively (Fig 3.6). If that was true, T2D pathophysiology may be managed better, e.g. in prediabetic stages, we should prevent over compensating islet expansion and insulin production in order to delay the onset of glucose intolerance and T2D.

In summary, based on the evidence presented here, normal expression of Reg2 gene seems to be required for islet expansion in response to HFD and aging. Reg2 gene deficiency maintained glucose tolerance in HFD-fed young mice but caused its impairment in aged mice. This may be explained by an interplay between decreased insulin level and increased insulin sensitivity. Although Reg2 expression does not seem to be required normally for maintaining islet function and glucose homeostasis, pancreatic islet compensation in responses to aging and HFD-induced obesity seems to depend on it.

3.7 Acknowledgement

This work was support by Canadian Diabetes Association (OG-3-11-3469-JL) and by the Research Institute of McGill University Health Centre to Drs. Liu and Gao. QL and XLM were both supported by China Scholarship Council. Reg2 knockout mice were provided by Knockout Mouse Project, UC Davis. Dr. Xiaohong Liu of RI-MUHC helped in setting up EcoMRI.

3.8 Figures for Chapter 3

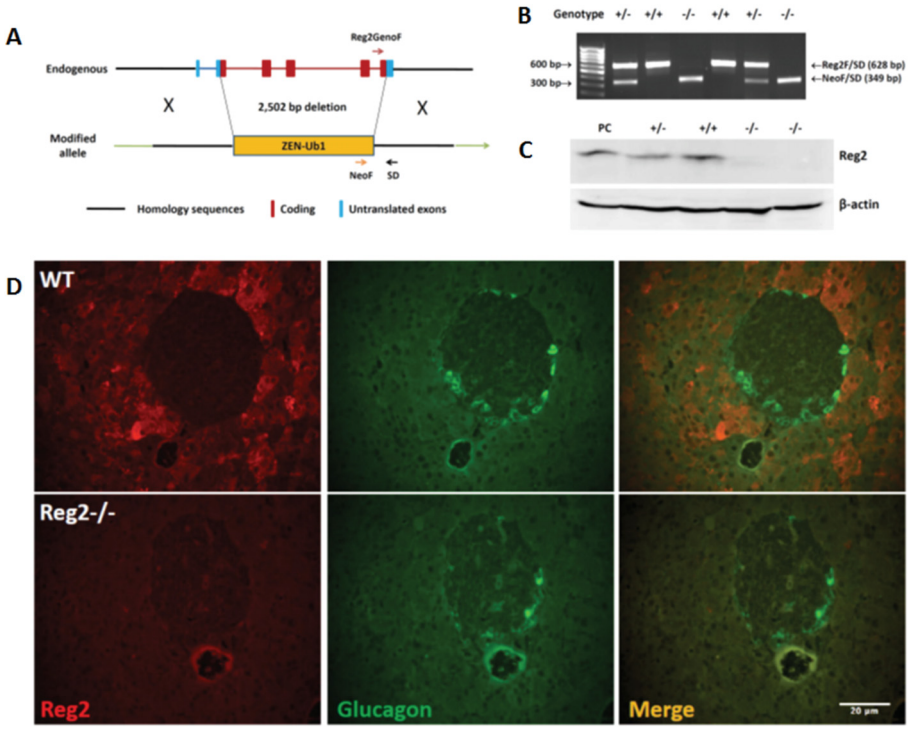

Figure 3.1 Genomic deletion of Reg2 gene.

A. Gene targeting strategy. A 2,502 bp genomic region of Reg2 gene on chromosome 6 covering all the protein coding axons (red) was replaced by a ZEN-Ub1 cassette via homologous recombination (more details on web site: www.komp.org/alleles.php). **B.** Result of genotyping PCR reactions: primers Reg2F/SD amplify a 628 bp endogenous allele, and NeoF/SD a 349 bp Reg2 deleted allele. **C.** Western blots of pancreatic protein extracted from wild-type and Reg2-/- mice of 1 month old, showing Reg2 and β-actin. PC: positive control of Reg2-rich pancreatic extract from acute pancreatitis. **D.** Immunofluorescent dual labeling of Reg2 (red) and glucagon (green) in pancreatic sections of wild-type and Reg2-/- male mice of 1 month old. Reg2 was stained in peri-islet acinar cells, as reported (356). Glucagon was used to mark the islet. Images were taken at 400x oil. Scale bar 20 microns.

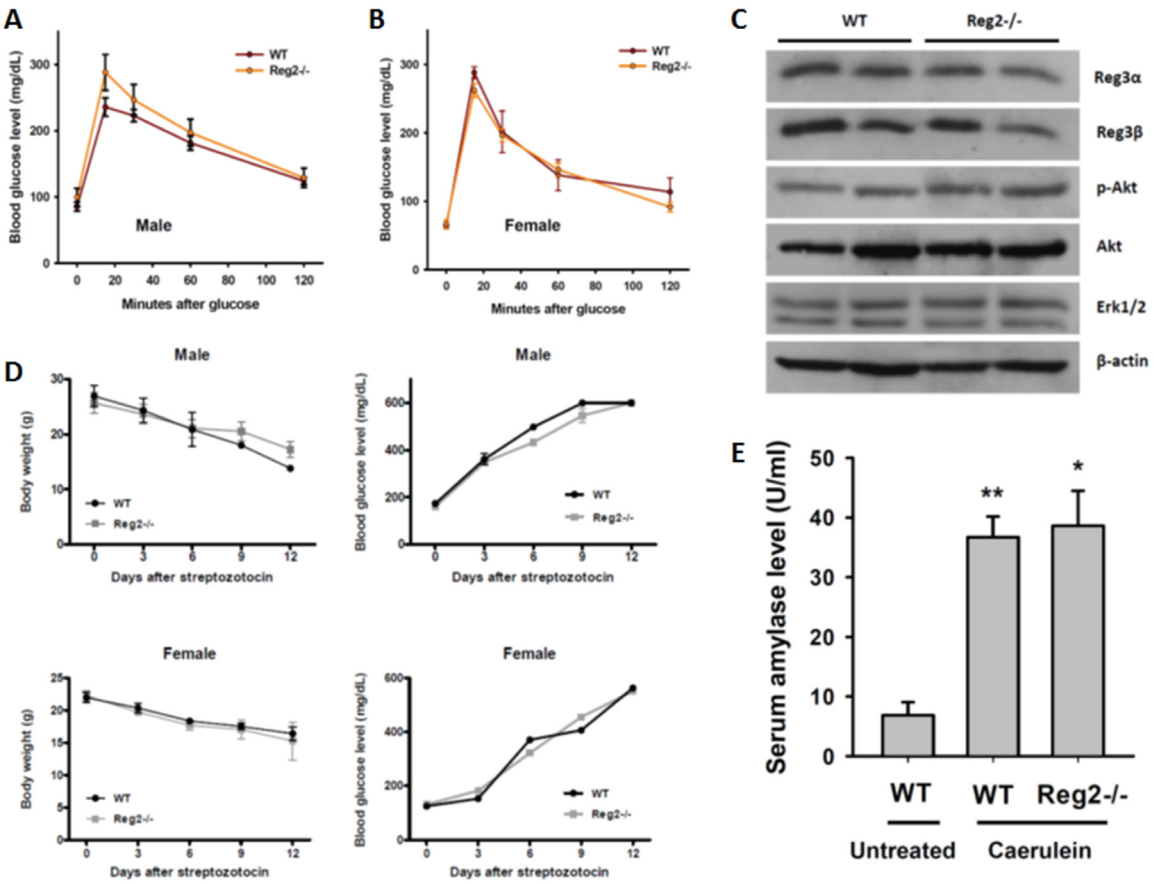

Figure 3.2 Lack of Reg2 expression did not affect glucose tolerance and the severity of streptozotocin-induced diabetes and caerulein-induced acute pancreatitis.

A and **B.** Normal glucose tolerance in both male and female knockout mice of 8-10 weeks old. Glucose was injected i.p. at 1 g/kg, N=4-7. **C.** Western blot of Reg3α, Reg3β, phospho- and total Akt, Erk1/2, and β-actin in the pancreas of 1-month old male mice, N=2. **D.** Streptozotocin-induced diabetes. Mice of 8-9 weeks' old were injected with multiple low doses of streptozotocin (75 mg/kg) on 5-consecutive days. Body weight and blood glucose were monitored at 0, 3, 6, 9, 12 d. N=8-10. **E.** Caerulein-induced acute pancreatitis. Mice at 6-8 weeks' old were injected 7 hourly doses of caerulein (50 μg/kg) to induced pancreatitis, serum amylase level was measured at 11 h. N=8-10, *P<0.05, **P<0.01 vs. wild-type untreated.

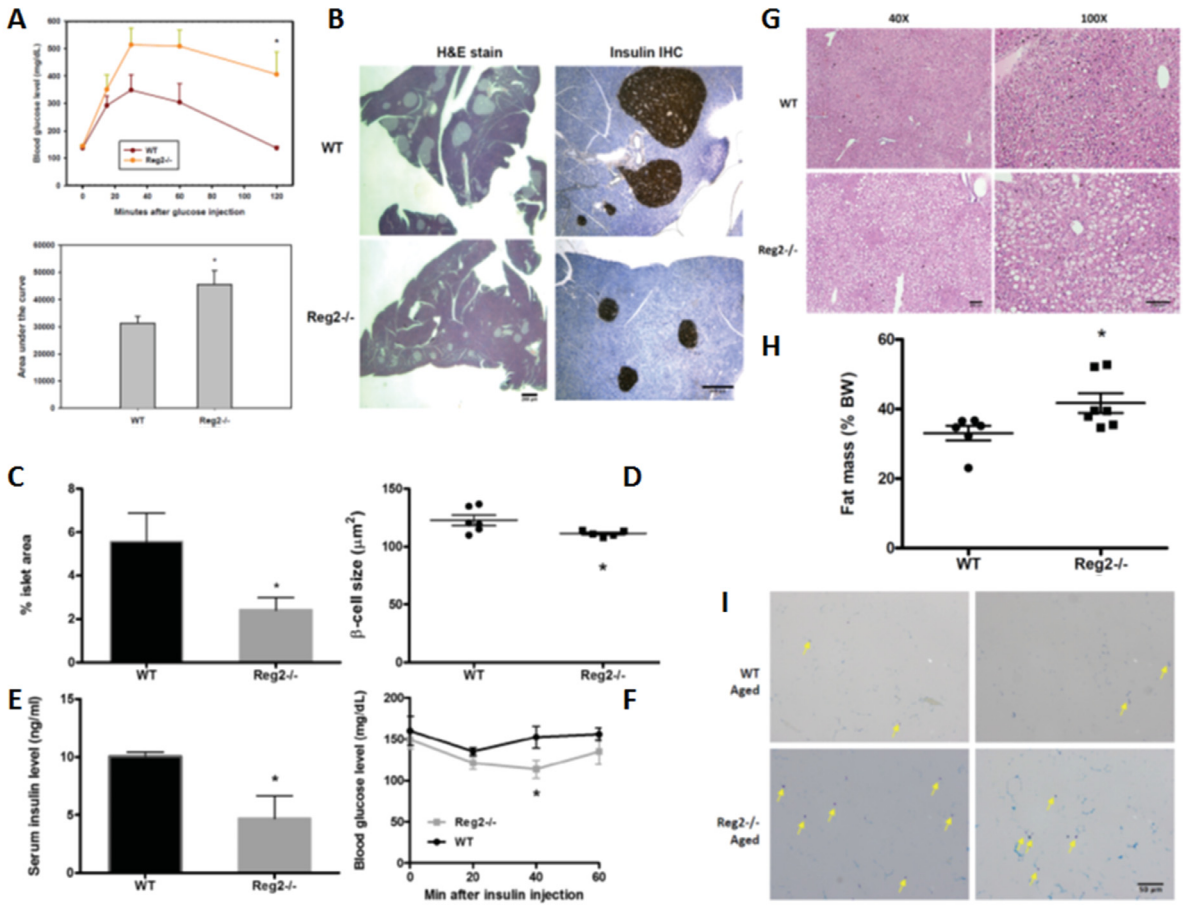

Figure 3.3 Reg2-/- mice exhibited impaired islet mass expansion and glucose tolerance at an old age.

A. Glucose tolerance test in 13-14 months old wild-type and Reg2-/- mice. Glucose (2 g/kg, i.p.) was injected to mice at random fed. AUC was calculated for each curve by using GraphPad Prism. N=6-8, *P<0.05 vs. wild-type mice using one-way ANOVA (graphs) and t-test (AUC) respectively. **B.** H&E staining of pancreatic sections (left, 25x) and insulin immunohistochemistry (right, 100x) of wild-type and Reg2-/- mice. Representative images from N=6-8. **C.** Percentage of islet area was quantified using Northern Eclipse software, as reported (377, 378). N=5, *P<0.05 vs. wild-type littermates. Scale bar was 200 microns. **D.** Average β-cell size was calculated by dividing the β-cell area by the numbers in each islet. **E**. Serum insulin level was determined using

ELISA. **F.** Result of insulin tolerance test. The experiment was repeated twice. N=5-7, *P<0.05. **G.** Reg2-/- mice showed hepatic steatosis vs. wild-type control. Images were taken under 40x and 100x magnifications from 5 mice each and representatives were selected for presentation. Scale bar was 200 microns. **H.** Reg2-/- mice have increased fat mass per body weight than wild-type control measured by EcoMRI. N=4-5, **P<0.01. **I.** Increased mast cell infiltration in adipose tissue of Reg2-/- mice as stained by tholudine blue (yellow arrows). Images were taken at 100x magnification. The scale bar was 50 microns.

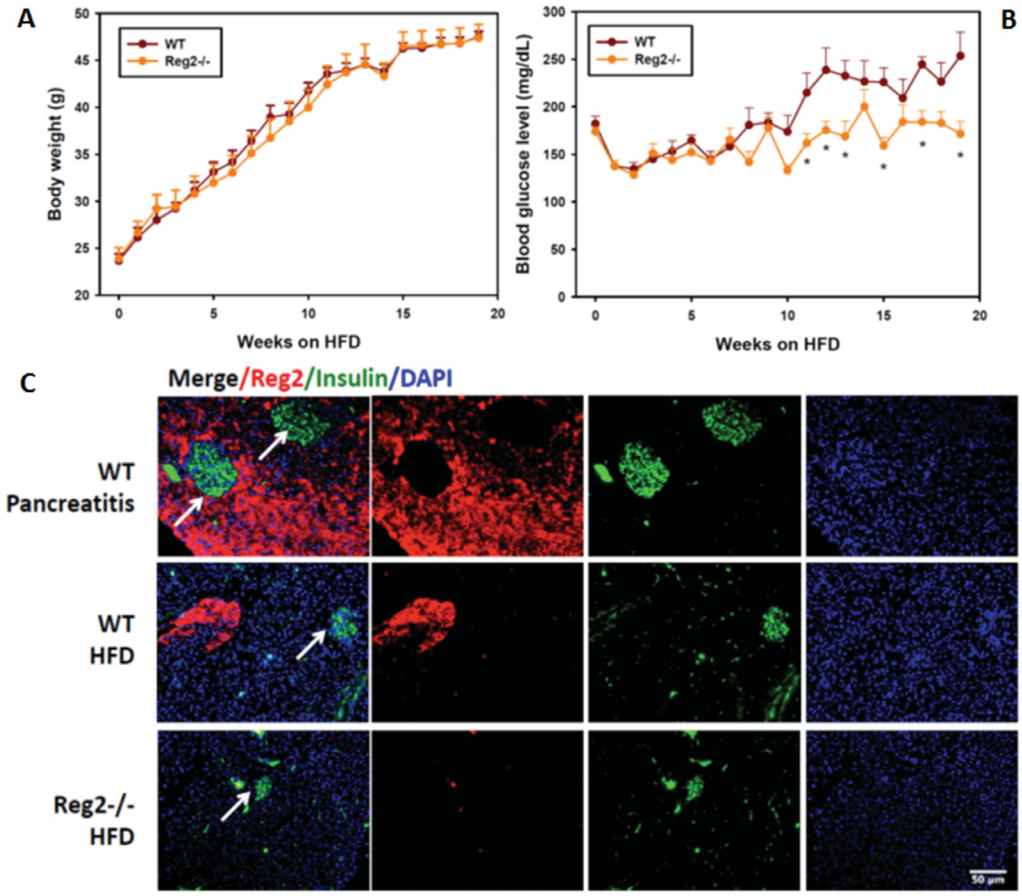

Figure 3.4 Reg2 gene deficiency protected mice from high-fat diet-induced diabetes.

A and B. Changes in body weight and blood glucose level (at random fed) of Reg2-/- and wild-type littermates during a 19-week HFD. The experiments were repeated twice. Mice used were male, 2-3 months old. N=8-10, *P<0.05 vs. wild-type. **C.** Induction of Reg2 expression by diet-induced obesity. Upper panels: as a positive control, Reg2 expression was induced by caerulein in the exocrine pancreas in acute pancreatitis; middle panels: pancreas from wild-type mice after 19-week HFD, the Reg2 expression was induced in exocrine patches; bottom panels: pancreas from Reg2-/- mice after the HFD, confirming a lack of Reg2 expression. White arrows point to the islets stained green for insulin. Representative images were taken under 100x magnification. Scale bar 50 microns.

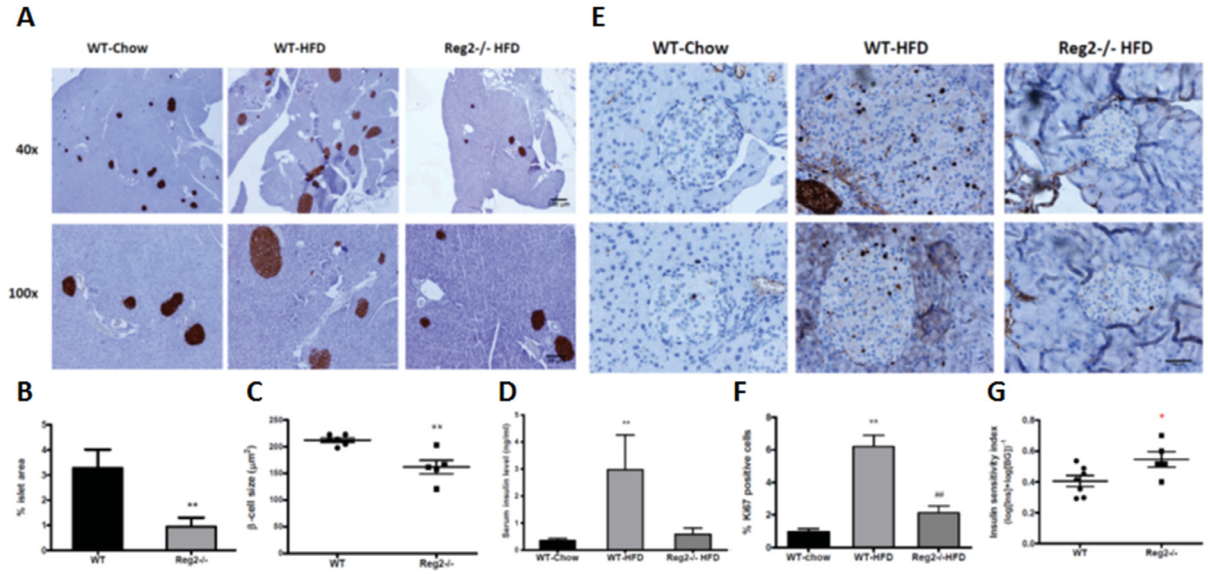

Figure 3.5 Diminished islet compensation to HFD in the absence of Reg2 gene expression.

A. Immunohistochemistry for insulin (brown color) in WT-Chow, WT-HFD and Reg2-/- HFD mice. Images were taken under 40x and 100x magnifications respectively. Scale bars are 50 or 100 microns. **B.** Quantification of % islet area, each based on 5 representative sections. **P<0.01 vs. WT-HFD. **C.** Average β-cell size was calculated by dividing the β-cell area by the numbers in each islet; N=5, **P<0.01 vs. WT-HFD. **D.** Serum insulin levels after 19-week HFD. N=8-10, **P<0.01 vs. WT-Chow. **E.** Immunohistochemistry of Ki67 in the islets of Reg2-/- and wild-type mice after 19-week HFD or Chow. Magnification: 400x. Scale bar 50 microns **F.** The number of Ki67 positive nuclei of each islet was quantified by using Image J software. N=5, **P<0.01 vs. WT-Chow, $^{\#\#}$P<0.01 vs. WT-HFD. G. Insulin sensitivity index calculated as 1/(log[Insulin]+log[Glucose]). N=5-7, P<0.05.

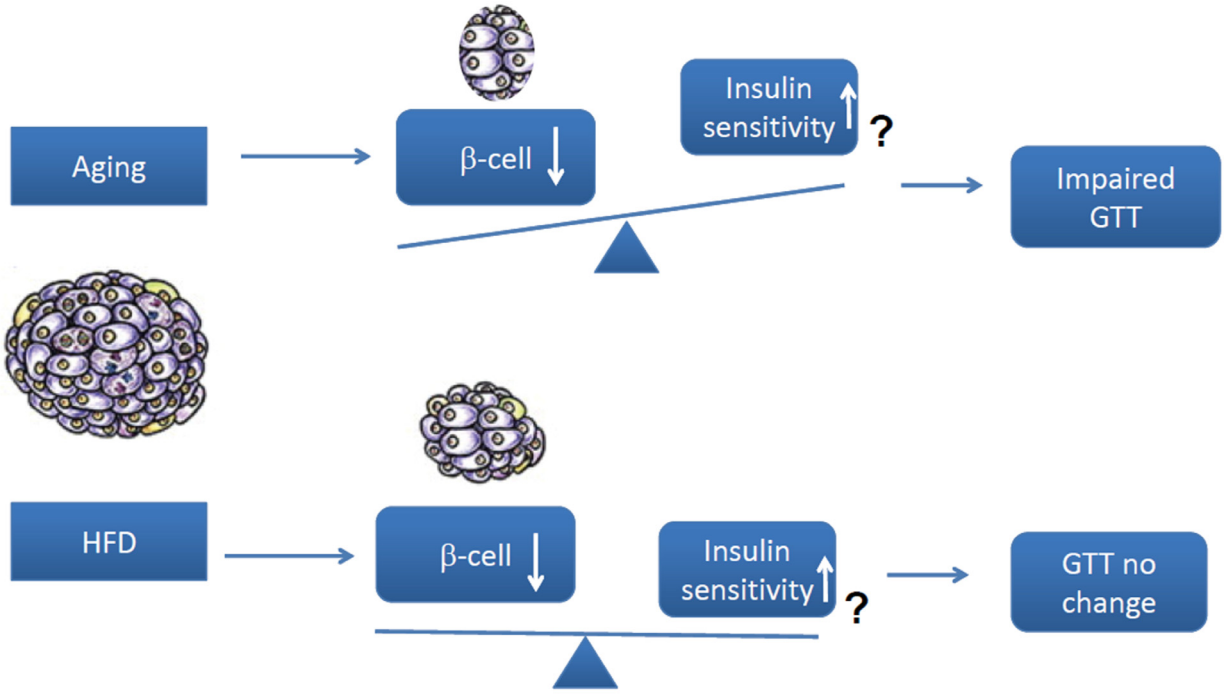

Figure 3.6 A cartoon illustrating the dynamic interplay between the changes in β-cell mass (insulin production) and insulin sensitivity caused by Reg2 gene deficiency in aged mice and HFD-induced obesity.

In aging, decreased β-cell mass seems to dominate over improved or normal insulin sensitivity which results in an impaired glucose tolerance. In HFD challenged Reg2-/- mice, insulin sensitivity might be elevated despite a decrease in β-cell mass and insulin production which results in unchanged glucose tolerance. Question marks indicate the need for further and more detailed demonstrations.

Chapter 4. Deteriorated high-fat diet-induced diabetes caused by pancreatic β-cell-specific overexpression of Reg3β gene in mice

[1]Xiaoquan Xiong*, [1]Qing Li*, [2]Wei Cui, [3]Zu-Hua Gao, [1,4]Jun-Li Liu

[1]Fraser Laboratories for Diabetes Research, Department of Medicine, McGill University Health Centre, Montreal, Canada;

[2]Department of Endocrinology, The First Affiliated Hospital of Xi'an Jiaotong University, Xi'an, China;

[3]Department of Pathology, McGill University Health Centre; 4Montreal Diabetes Research Centre, Montreal, Canada.

**These authors contributed equally*

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4.1 Preface

In 2011, our lab published a paper demonstrating islet-specific overexpression of Reg3β can significantly protect islets from disruption caused by streptozotocin-induced diabetes. The mechanisms seem to involve the upregulation of several islet proliferating factors, such as p8 and osteopontin, and downregulation of GLUT2 to reduce the uptake of streptozotocin. However, whether Reg3β overexpression played a role in T2D had not been explored. Therefore, it prompted my project studying the roles of Reg3β in HFD-induced diabetes. In contrast to the protective effects of Reg3β in streptozotocin-induced diabetes, Reg3β was detrimental to the islets under HFD. We observed higher blood glucose, more impaired glucose and insulin tolerance, and more fat accumulation in the transgenic mice compared to their wild-type littermates after 10 weeks of HFD. This may be due to the decreased expression of GLUT2 in the cell membrane and impairment of AMPK levels to maintain the glucose homeostasis. Furthermore, I observed the similar phenomenon in aged transgenic mice. The islets showed less insulin expression and there was more steatosis observed in the liver, suggesting a more severe insulin resistance state. This gives us a new understanding on the roles of Reg3β in different diabetic conditions.

4.2 Abstract

Purpose: Reg family proteins have long been implicated in islet β-cell proliferation, survival, and regeneration. In our previous study, we reported that Reg3β overexpression did not increase islet growth but prevented streptozotocin-induced islet damage by inducing specific genes. In order to explore its role in type 2 diabetes (T2D), we established high-fat diet (HFD)-induced obesity and diabetes in RIP-I/Reg3β mice.

Methods: Glucose and insulin tolerance tests, immunofluorescence for insulin, eIF2α, and GLUT2 in islets, Western blots on phosphorylated AMPKα and hepatic histology were performed.

Results: Both RIP-I/Reg3β and wild-type mice gained weight rapidly and became hyperglycemic after 10 weeks on the HFD. However, the transgenic mice exhibited more significant acceleration in blood glucose levels, further deterioration of glucose intolerance and insulin resistance, and a lower intensity of insulin staining. Immunofluorescence revealed similar magnitude of islet compensation to a HFD. The normal GLUT2 distribution in the transgenic β-cells was disrupted and the staining was obviously diminished on the cell membrane. HFD feeding also caused a further decrease in the level of AMPKα phosphorylation in the transgenic islets.

Conclusion: Our results suggest that unlike its protective effect against T1D, overexpressed Reg3β was unable to protect the β-cells against HFD-induced damage.

4.3 Introduction

Regenerating (Reg) gene family of proteins were first discovered in 1988, following an extensive search for factors that promote pancreatic islet regeneration (139, 152, 207, 357, 379). In mouse, these C-type lectins can be classified into four subclasses, Reg1, Reg2, Reg3 (α, β, γ, δ) and Reg4, based on the similarities in their primary structures (10, 380). Over the last three decades, Reg protein isoforms have been discovered in several mammalian species and implicated in cell proliferation, survival and regeneration in the pancreas, liver, neuron and gastrointestinal tract (366, 380). While collectively they are involved in tissue injury, inflammation, diabetes and carcinogenesis, each individual isoform also retains its unique expression pattern and functional characters depending on the tissue and pathological conditions (290, 381). Reg1 and islet neogenesis-associated protein (INGAP, Reg3δ) produced from pancreatic acinar/ductal cells stimulate β-cell proliferation and/or regeneration (141, 224). Both acinar and islet β-cell-specific overexpression of INGAP increase β-cell mass, improve insulin secretion and protect the animals from streptozotocin-induced diabetes (298, 382). In recent years, we have demonstrated the cytoprotective role of Reg2 against various cellular stresses to β-cells and a proliferative effect of Reg3α overexpression (247, 256, 383).

Together with other Reg proteins, Reg3β, pancreatitis-associated protein (PAP), or the gene expressed in hepatocellular carcinoma-intestine-pancreas (HIP) shares a short signal peptide at the N-terminal and a conserved carbohydrate recognition domain toward the C-terminal (2, 4, 11, 360, 369). Its expression is normally undetectable in the pancreas or only identified at the edge of pancreatic islets in certain strains of mice (356). Reg3β, however, can be highly induced in experimental diabetes and acute pancreatitis (11, 360, 369). Results from a knockout study clearly

indicates this protein to be mitogenic, anti-apoptotic and anti-inflammatory in the liver and pancreatic acini (278). In non-obese diabetic mice, significant upregulation of Reg3β gene expression was detected in acinar cells; suggesting an interaction between endocrine and exocrine cells (266). In order to test whether Reg3β can promote islet cell growth or survival against experimental damage, we developed β-cell-specific overexpression of Reg3β using the rat insulin I promoter. In our previous report, overexpressed Reg3β gene induced the expression of several pro-islet genes, and protected mice against streptozotocin-induced diabetes; it did not, however, affect normal β-cell mass and insulin secretion (378). To further characterize its effect in type 2 diabetes (T2D), we established high-fat diet (HFD)-induced obesity and diabetes.

4.4 Materials and Methods

4.4.1 High-fat diet-induced obesity

Male RIP-I/Reg3β mice (378) and wild-type littermates of 3-4 month old were fed a HFD (rodent diet with 60% kcal fat, Research Diets Inc.) or chow diet. Blood glucose level was measured from the tail vein once a week (between 2-3 pm at a random fed status) using the OneTouch Ultra glucose meter. Change in body weight was assessed. Serum insulin levels were measured at 0, 8, and 10 weeks following the HFD using ELISA (Alpco). At the 5^{th} and 7^{th} week, half of the mice were randomly picked and subjected to glucose or insulin tolerance testing on separated days. After ten weeks on the HFD, mice were sacrificed; half of the pancreas was dissected, weighed and fixed in 10% formalin; and the other half was stored at -80°C for protein extraction. Serum was collected for insulin measurement. Other mice were sacrificed, and the whole pancreas was used to isolate the islets, which were hand-picked for protein extraction,

followed by Western blot analysis, as previously reported (378). Pancreas were stained with insulin and pancreatic β-cell percentage was quantified by using Northern Eclipse software, as previously reported (377, 378). For glucose tolerance, mice were fasted overnight and given an I.P. injection of 10% glucose at 1 g/kg body weight. To measure glucose, blood was taken from the tail vein before, and at 15, 30, 60 and 120 min after the injection. For insulin tolerance, mice were given an I.P. injection of insulin (1 U/kg), and blood glucose levels were measured at 0, 20, 40 and 60 min respectively. The McGill University Animal Care Committee approved all animal handling procedures.

4.4.2 Western blot analysis

Freshly isolated islets from 3-4 month old male mice subjected to a 10-week chow diet or HFD were handpicked and sonicated in 150-200 μl lysis buffer supplemented with protease inhibitor tablet (Roche Diagnostics). Western blotting was performed to quantify the levels of GLUT2 (1:4000, Millipore, MA), p-AMPKα (1:2000, #2535, Cell Signaling), total AMPKα (1:5000, #2532, Cell Signaling, Danvers, MA), p-eIF2α (1:1000, Invitrogen, MA) and β-actin (1:2000, Santa Cruz, CA). Protein levels in the individual lanes were quantified using the FluorChem 8900 imaging system (Alpha Innotech) and normalized to the level of β-actin.

4.4.3 Pancreatic immunohistochemistry

Paraffin sections were dewaxed, rehydrated, and blocked with 10% host sera of the secondary antibodies followed by overnight incubation with a primary antibody at 4°C. The following antibodies were used: guinea pig anti-insulin (1:50, Biomeda), rabbit anti-GLUT2 (1:100, Millipore), goat anti-glucagon (1:50, Santa Cruz), and rabbit anti-p-eIF2α (1:100, Invitrogen). For fluorescent microscopy, the samples were incubated for 3 h with suitable

secondary antibodies: Alexa-Fluor 488 goat anti-guinea pig (1:100, Invitrogen), Rhodamine donkey anti-rabbit (1:100, Jackson ImmunoResearch Laboratories), or dylight 488 donkey anti-goat (1:100, Jackson Immuno Res Labs). The nuclei were stained using fluorogen DAPI contained in mounting medium (Vector Laboratories). For confocal microscopy, images were collected on an LSM-510 Meta laser scanning microscope with a 63· oil immersion lens (Carl Zeiss). Other images were analyzed using an Axioshop 2 Plus microscope (Carl Zeiss), Retiga 1300 digital camera, and Northern Eclipse software (Empix Imaging).

4.4.4 Statistical analysis

Data were expressed as means ± S.E. and plotted using SigmaPlot version 11 (Systat Software). An unpaired Student's t-test was performed using InStat version 3 software (GraphPad Software, San Diego, CA). To test whether the means among different ages within a given diet-treatment group were statistically different, a one-way ANOVA was performed using SigmaPlot followed by a post hoc Turkey analysis. A two-way ANOVA test was applied for the comparison of the three groups after diet treatment.

4.5 Results

4.5.1 RIP-I/Reg3β mice developed more severe diabetes in response to HFD-induced obesity

We previously reported a transgenic mouse strain with pancreatic β-cell-specific overexpression of Reg3β, and demonstrated its resistance against streptozotocin-induced diabetes (378). The animals showed normal body weight and serum insulin levels, but slightly higher glucose levels on the chow diet. To further characterize the effect of Reg3β overexpression, we

hereby established HFD-induced obesity and T2D. As shown in Figure 4.1A, both RIP-I/Reg3β and wild-type littermates demonstrate rapid weight gain upon feeding with the HFD. At 10 weeks, the average weight gains vs. chow-fed mice were 51-53%. This gain was largely attributed to 2-2.5-fold increases in visceral fat mass in both wild-type and transgenic mice (fat/BW in WT-Chow 0.019±0.004%, **WT-HFD 0.048±0.005%, **RIP-I/Reg3β-HFD 0.043±0.004%, N=6-7, **P<0.01). Consequently, blood glucose level started to elevate in wild-type mice after 4 weeks, reaching a maximum mean of 238 mg/dL by 10 weeks (Fig 4.1B). RIP-I/Reg3β mice exhibited a similar pattern of hyperglycemia, except the level elevated faster and higher, and peaked at 330 mg/dL. The increased glucose level in RIP-I/Reg3β mice was significantly higher than in wild-type mice from 6 to 10 weeks, indicating a worsening of T2D. Halfway through the experiment (at 5 weeks), we recorded impaired glucose tolerance in wild-type mice on HFD and a significantly worsening in RIP-I/Reg3β mice, as indicated by the area under the curves (Fig 4.1C). After 7 weeks on the HFD, transgenic mice exhibited more severe insulin resistance too (Fig 4.1D). After 10 weeks on the HFD, serum insulin concentration in wild-type mice was elevated 8.6-fold, and was similarly increased in RIP-I/Reg3β mice (Fig 4.1E). In contrast to our previously reported protection against T1D (378), these results indicate that Reg3β overexpression in pancreatic β-cells did not prevent HFD-induced glucose intolerance and T2D, but rather accelerated the disease progression.

4.5.2 Reg3β overexpression did not seem to affect the levels of ER stress, islet proliferation or apoptosis in response to HFD

To investigate what causes the deterioration of T2D in RIP-I/Reg3β mice, overexpression of the Reg3β gene in β-cells might result in the accumulation of unfolded or misfolded proteins

within the endoplasmic reticulum (ER). ER stress is an important cause of β-cell death in T2D (384). To detect whether the β-cells of RIP-I/Reg3β mice were under increased ER stress, we assessed the phosphorylation of eukaryotic translational initiation factor (eIF2α), at Ser-51 by the ER transmembrane sensor PKR-like ER kinase (PERK). The phosphorylation of eIF2α in turn leads to an unfolded protein response (UPR) and a global repression of protein synthesis (385). When fed a chow diet, the level of eIF2α phosphorylation was low in both wild-type and RIP-I/Reg3β mice, and was mainly detected in the α-cells (Fig 4.2A left panels). When the effect of Reg3β overexpression was quantified in freshly isolated islets from both wild-type and transgenic mice on a chow diet, the levels of phosphorylated eIF2α were undistinguishable (Fig 4.2B). The HFD induced a significant increase in eIF2α phosphorylation mostly in α-cells, and to a similar extent in both wild-type and transgenic mice (Fig 4.2A, middle and right panels). This observation seems to indicate that the islets from the HFD-fed RIP-I/Reg3β mice were not under *increased* ER stress. The restricted expression of p-eIF2α in α-cells was consistent with highly phosphorylated eIF2α in pancreatic islets, as previously reported (386).

Increased β-cell apoptosis or decreased proliferation may affect islet compensation against a HFD. To explore the impact of Reg3β overexpression and a HFD, we performed Ki67 and TUNEL staining respectively. Similar ratios of Ki67-positive cells were detected in wild-type and RIP-I/Reg3β mice fed either the chow diet or HFD (data not shown). Although HFD treatment led to a slight increase in TUNEL-labeled cells, there was no significant difference between wild-type and RIP-I/Reg3β animals. Therefore, the worsening of T2D in Reg3β-overexpressing mice is unlikely to be caused by increased ER stress, or the change in β-cell survival or proliferation.

4.5.3 Deterioration in insulin and GLUT2 staining in islet β-cells of RIP-I/Reg3β mice

We next studied pancreatic immunohistochemistry to determine whether Reg3β overexpression impaired islet compensation for obesity-induced insulin resistance (Fig 4.3). Previously, we reported decreased insulin and GLUT2 staining in the islets of RIP-I/Reg3β vs. wild-type mice fed a chow diet (378). These results are confirmed in the present study, as seen in Figure 4.3B vs. 4.3A, and Figure 4.3F vs. 4.3E, respectively (378). After 10-weeks on the HFD, the same tendency of decreased insulin staining was maintained (Fig 4.3D vs. 4.3C), along with a clear compensatory increase in the islet size (percentage), and noticeable interlobular fat deposits within the pancreatic tissues of both wild-type and transgenic mice (Suppl Figure 4.1, brown arrows).

Similar to our previous report, both increased intracellular retention of GLUT2 and reduced localization on the plasma membrane were observed in most islets of RIP-I/Reg3β mice (Fig 4.3F white arrows vs. 3E) (378). After HFD feeding, decreased staining of GLUT2 was observed in the β-cells of wild-type mice, while GLUT2 distribution was further impaired in RIP-I/Reg3β mice. Although GLUT2 staining was still detectable in the cell membrane of wild-type β-cells, it was largely abolished in the RIP-I/Reg3β islets (Fig 4.3G vs. 4.3H). Interestingly, the HFD induced the formation of "vessel- or duct-like" structures in the islets of RIP-I/Reg3β mice (Fig 4.3H, yellow arrows). They were recognized by GLUT2 and DAPI, but not by insulin antibodies, and islet amyloid or fibrosis was also ruled out. As GLUT2 is essential for glucose uptake into β-cells and the regulation of insulin secretion, decreased GLUT2 and insulin staining in islet β-cells might contribute in part to the deterioration of T2D.

4.5.4 Decreased AMPKα phosphorylation in the islets in response to a HFD and Reg3β overexpression

Disruption in GLUT2 expression and/or localization in the islets of RIP-I/Reg3β mice would negatively affect glucose uptake and ATP production in the β-cells. As a systemic energy sensor, AMPK activity is sensitive to changes in ATP level. To provide further evidence, we measured the change in Thr-172 phosphorylation of AMPKα in freshly isolated islets using Western blot analysis. As shown in Figure 4.4, there was a 33% reduction in the amount of phosphorylated AMPKα (p-AMPKα) in RIP-I/Reg3β vs. wild-type mice on a chow diet (first two lanes). HFD feeding for 10 weeks caused a further reduction of 47% in p-AMPKα level in wild-type mice, with a proportionate decrease in RIP-I/Reg3β mice (last two lanes). Taken together, the impaired AMPKα response to energy deficiency and defects in GLUT2 and insulin, may all contribute to deteriorated diabetes. The suppression of AMPK activity is consistent with a previously reported, wide-spread decrease of AMPK activity in HFD-induced obesity (387).

4.5.5 Aged RIP-I/Reg3β mice also exhibited impaired insulin staining and hepatic steatosis

Aging is another challenge testing the effect of Reg3β overexpression, in some aspects resembling HFD-induced obesity. Preliminary observations indicated that 13-14-month old RIP-I/Reg3β mice were significantly overweight and hyperglycemic, when compared to their wild-type littermates (Table 4.1). Upon histological examination, these transgenic mice showed decreased insulin staining and evidence of hepatic steatosis, i.e. accumulated lipid droplets within the hepatocytes (Fig 4.5), in clear contrast to their wild-type littermates. These phenomenon are consistent with the possibility that Reg3β overexpression causes functional

defects in the β-cells and/or insulin resistance, indirectly supporting the deteriorated diabetes seen after HFD feeding.

4.6 Discussion

To test the role of Reg3β in islet growth or protection, we previously reported β-cell-specific overexpression. These transgenic mice are slightly hyperglycemic, low in islet GLUT2 expression and significantly protected from streptozotocin-induced diabetes (378). In the present study, however, Reg3β overexpression was unable to prevent mice from developing HFD-induced diabetes. In fact, RIP-I/Reg3β mice exhibited accelerated hyperglycemia and deteriorated T2D. The glucose intolerance and insulin resistance, found in wild-type mice after 10 weeks of HFD feeding, were further deteriorated. Although similar extents of compensation in islet size were observed in all animals after HFD feeding or in aging, the intensity of insulin staining was lower, implying exhausted secretion and/or depleted storage in RIP-I/Reg3β mice. This detrimental effect was unlikely to be caused by an increase in ER stress in the β-cells, as the level of eIF2α phosphorylation was not elevated vs. wild-type mice. Instead, the decreased level of GLUT2 and AMPKα phosphorylation in RIP-I/Reg3β mice on a HFD suggests impaired glucose uptake and metabolism. These results clearly argue against the notion that Reg3β is a general growth/survival factor that promotes β-cell compensation against diabetes.

The decreased GLUT2 level and membrane localization caused by Reg3β overexpression, which contributes to the protection against streptozotocin-induced T1D (378), may also partially cause a worsened T2D. Decreased GLUT2 levels would affect glucose uptake and metabolism, gene expression, and membrane transport (388). In the long run, and even worse in aged animals, it could lead to defects in β-cell function, as evidenced by the elevation of blood glucose level

(378). This tendency of hyperglycemia would facilitate a further loss of GLUT2 (389), and lead to impaired glucose sensing and insulin secretion, and early onset and deteriorated diabetes. As observed in the present study, RIP-I/Reg3β mice on a HFD lost most of their β-cell surface expression of GLUT2, with only residual GLUT2 vesicles trapped in the cytoplasm. This defect occurred on top of an already compromised translocation of GLUT2 to the plasma membrane after HFD, with some intracellular accumulation in wild-type mice.

The decreased level of GLUT2 could be caused by an interruption of post-translational modification necessary for its trafficking and docking to the cell membrane, or accelerated degradation. On the one hand, HFD feeding is known to reduce glycosylation of GLUT2 in the ER and Golgi apparatus in β-cells; with diminished cell-surface localization and elevated intracellular retention (390). On the other hand, galectin 9, a member of the lectin family, has been identified to efficiently crosslink glycosylated GLUT2, and thus decrease its endocytosis (391). Reg3 proteins bind peptidoglycans in both calcium-dependent and independent manners (392). It is possible that overexpressed Reg3β, which shares a conserved carbohydrate recognition domain with galectin 9, may also interact with glycosylated GLUT2. Although further study is necessary to decipher a direct interaction of Reg proteins with GLUT2, it might be worthwhile to *reconsider* some of the protective effects of the Reg family proteins. For instance, a possible change in GLUT2 level should be considered for Reg2-mediated protection against streptozotocin-induced apoptosis in insulinoma cells (247). However, no change in GLUT2 has been observed in the protective effects of either acinar- or β-cell specific overexpression of INGAP against streptozotocin diabetes (298, 382).

In addition to decreased GLUT2 expression, we also observed decreased phosphorylation of AMPK, a systemic energy sensor, in the islets of RIP-I/Reg3β mice. On the one hand, the lower

GLUT2 expression in RIP-I/Reg3β mice would affect glucose uptake and ATP production, therefore, promoting AMPK activation. On the other hand, the activity and phosphorylation of AMPK are inversely correlated to glucose level in pancreatic β-cells (393, 394). In two β-cell lines HIT-T15 and INS-1, glucose removal caused AMPK activation (393). In the present study, HFD feeding caused a significant elevation in blood glucose level and a decline in AMPK phosphorylation at the catalytic subunit. Indeed, the p-AMPK level was decreased by 33% in the islets of RIP-I/Reg3β vs. wild-type littermates on a chow diet, and decreased further with HFD feeding. It is likely that the inhibition of high glucose on AMPK activity dominated the effect of energy deprivation-induce AMPK activation. If that was the case, AMPK activation would not compensate for impaired glucose homeostasis in RIP-I/Reg3β mice.

In pancreatic β-cells, low glucose activates AMPK, which increases membrane localization of K_{ATP} channels, and contributes to hyperpolarization and decreased insulin release (395). Activation of AMPK by metformin and AICAR or adenovirus-mediated overexpression of AMPKα-1CA in β-cells blocks high glucose-stimulated insulin secretion, which may provide a relief to the β-cells (396). Dysregulation of AMPK is observed in metabolic syndromes including T2D, cardiovascular disease and hypertension. In HFD-induced diabetic animals, there is an increase of insulin secretion in response to high glucose, accompanied by a decrease in AMPK activity (397). Conversely, the reduction of AMPK activation may improve insulin secretion in the islets in order to meet the whole-body demand for insulin upon HFD feeding. However, this unregulated insulin release may subsequently deplete insulin storage, as evidenced by the poor immunological staining of insulin in the RIP-I/Reg3β islets, and further cause β-cell failure and insulin resistance. Moreover, insulin resistance contributes to liver steatosis by directly promoting *de novo* lipogenesis and indirectly increasing fatty acid transportation to the liver from peripheral

lipolysis (398). This is also consistent with fatty liver observed in aged RIP-I/Reg3β mice. Nevertheless, how exactly AMPK phosphorylation was suppressed in RIP-I/Reg3β mice remains to be clarified.

In summary, in contrast to the protection it provides against streptozotocin-induced diabetes, pancreatic β-cell specific overexpression of Reg3β did not protect mice from HFD-induced diabetes, but apparently exerted an opposite effect of worsening T2D. The mechanism for this effect may include decreased levels of insulin and GLUT2 in the islets, thus deteriorated glucose and insulin tolerances, and the suppression of AMPK activity which contribute to further impairment of β-cell function. These results provide new evidence that the effect of Reg proteins on islet function is isoform- and indeed model-specific.

4.7 Acknowledgements

We would like to thank Dr. Louise Larose for her instructions on ER stress tests and Carolynna Olha for the English revision and editing of this manuscript. This work was supported by the Canadian Institutes of Health Research (grant MOP-84389), Canadian Diabetes Association (OG-3-11-3469-JL) and a bridge fund from the Research Institute of the McGill University Health Centre (RI-MUHC) to JLL. QL received support from the China Scholarship Council (201208370055). ZHG was supported by the RI-MUHC.

4.8 Tables for Chapter 4

Table 4.1 Increased body weight and blood glucose level of aged RIP-I/Reg3β vs. wild-type mice.

Male mice 13-14 months old were littermates and pooled. N=5

	WT	RIP-I/Reg3β	P value
Body weight (g)	39.8 ± 0.5	42.5 ± 0.8	0.04
Blood glucose (mg/dL)	152 ± 16	222 ± 16	0.033

4.9 Figures for Chapter 4

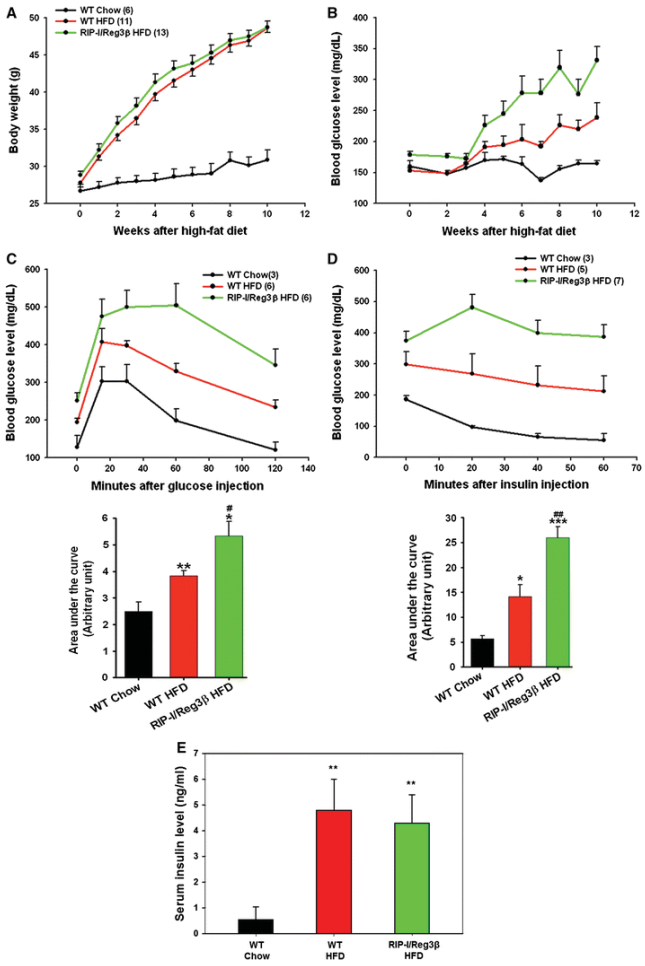


Figure 4.1 RIP-I/Reg3β mice exhibited accelerated diabetes and impaired glucose tolerance in response to HFD-induced obesity.

Male mice of 3-4 months old were fed a HFD or chow diet for 10 weeks, as marked. **A**. Changes in body weight are expressed as Mean ± S.E. The number of animals is indicated in parenthesis. The weight changes within the same groups are tested using a one-way ANOVA: WT Chow, NS; WT-HFD, $P<0.001$; RIP-I/Reg3β-HFD, $P<0.001$. **B.** Changes in blood glucose level. The result of two-way ANOVA comparing two curves: RIP-Reg3β-HFD vs. WT-HFD, $P<0.001$. **C.** Changes in glucose tolerance after five weeks on a HFD. Mice were fasted overnight before being injected with glucose at 1 g/kg, i.p. The column graph depicts the change in the area under the curves. **D.** Changes in insulin tolerance after seven weeks on a HFD. Insulin was injected at 1 U/kg (i.p.) and blood glucose level was measured at the indicated time. The column graph depicted the differences in the area under the curves. **E.** Changes in serum insulin level after HFD-induced diabetes. Blood was taken at the end of the 10-week HFD or chow diet. *$P<0.05$, **$P<0.01$, ***$P<0.001$ vs. wild-type littermates on the chow diet. $^{\#}P<0.05$, $^{\#\#}P<0.01$ vs. wild-type on HFD, all using unpaired t-test.

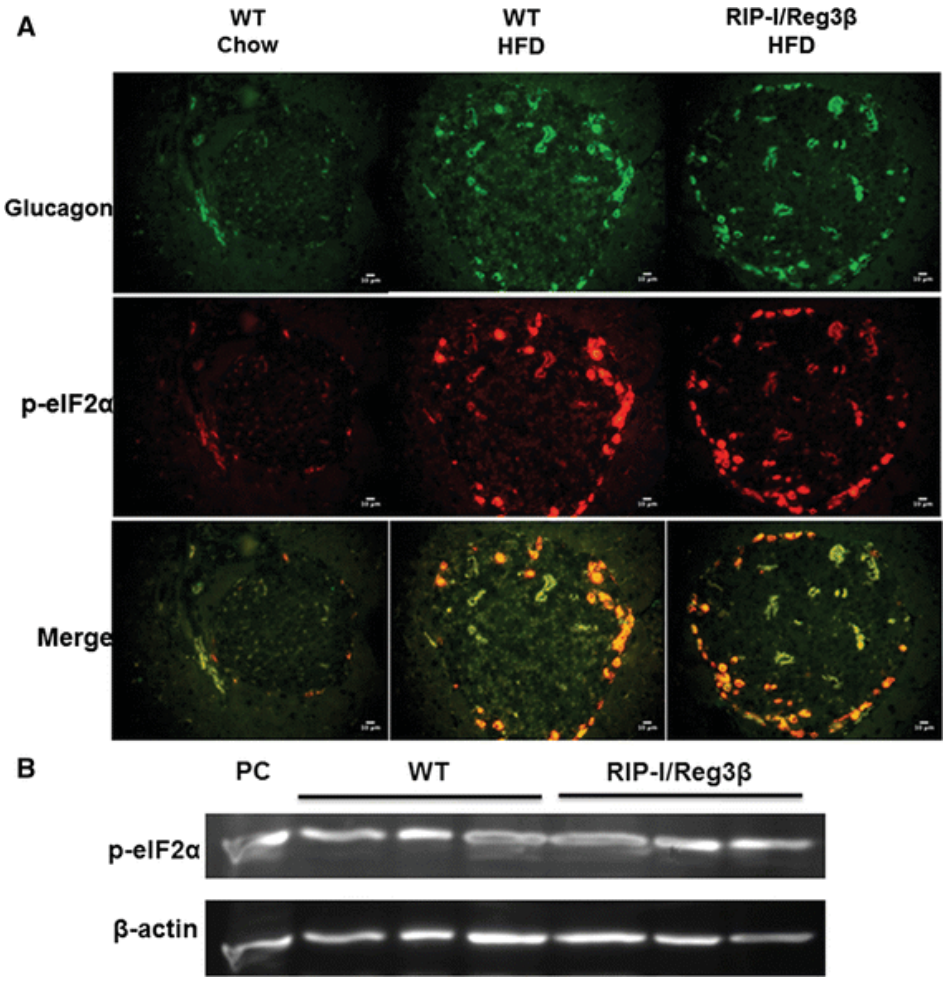


Figure 4.2 HFD caused similarly elevated ER stress in islet cells of both wild-type and RIP-I/Reg3β mice.

A. The pancreas was taken from RIP-I/Reg3β and wild-type mice after 10 weeks on the HFD or chow diet. Top and middle panels: paraffin sections were stained for glucagon (Dylight 488, green) and p-eIF2α (Rhodamine, red) consecutively using immunofluorescence. The two images are merged at the bottom panels. Representative islets from >10 images and 5 mice in each group are illustrated. Original magnification, ·400 oil; the scale bar is 10 μm. **B**. Western blotting of p-eIF2α and β-actin using proteins from freshly isolated islets extracted from animals fed on chow diet. Representative blots from N >5. PC: positive control.

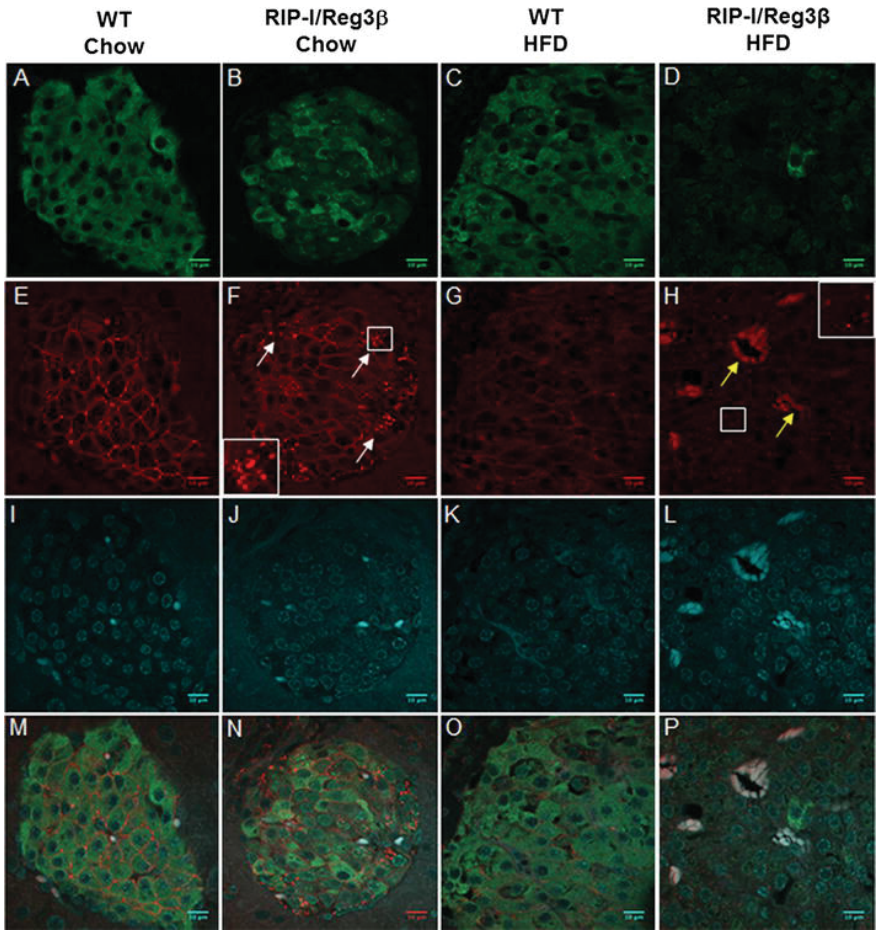


Figure 4.3 HFD caused further deteriorated insulin and GLUT2 staining in the islets of RIP-I/Reg3β mice.

Pancreas were taken from RIP-I/Reg3β and wild-type mice after 10 weeks on the HFD or chow diet. Paraffin sections were stained (from top to bottom) for insulin (green), GLUT2 (red) and DAPI (blue) consecutively using immunofluorescence. The three images are merged into the bottom panels. Representative islets from >10 images of 5 mice in each group are illustrated. Original magnification, ·630 oil; the scale bar was 10 μm. The boxes in panels F and H showed 2x magnification of original pictures. To be noted, some "vessel/duct-like" structures emerged in panels H and L, stained positively with GLUT2 and DAPI (yellow arrows), but did not react to the insulin antibody.

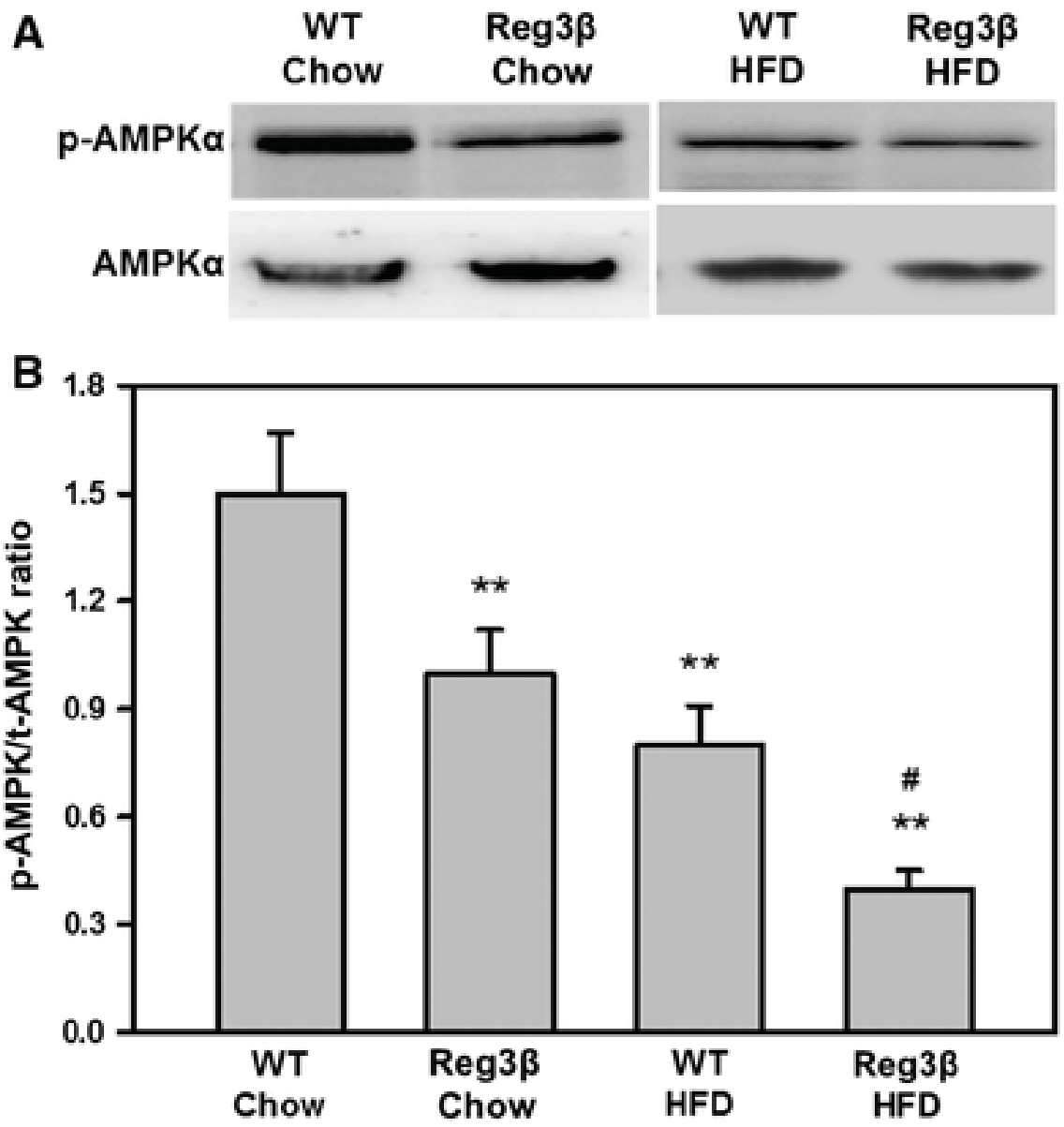


Figure 4.4 Decreased AMPKα phosphorylation caused by HFD and Reg3β overexpression.

A. Western blot analysis of p-AMPKα (Thr-172) and total AMPKα using proteins extracted from freshly isolated islets. A representative blot from 4-6 mice in each group is shown. **B.** Quantification of the changes in p-AMPKα level normalized to total AMPKα. **P<0.01 vs. WT chow; $^{\#}$P<0.01 vs. Reg3β chow.

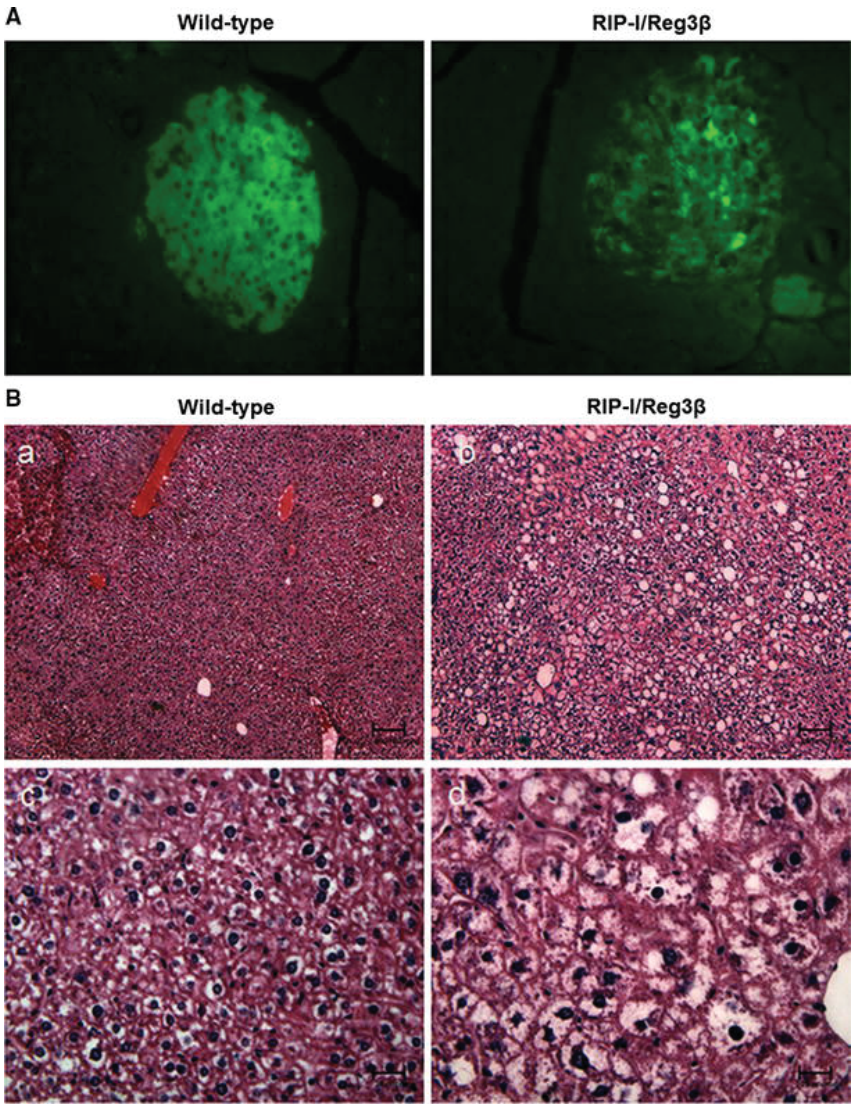
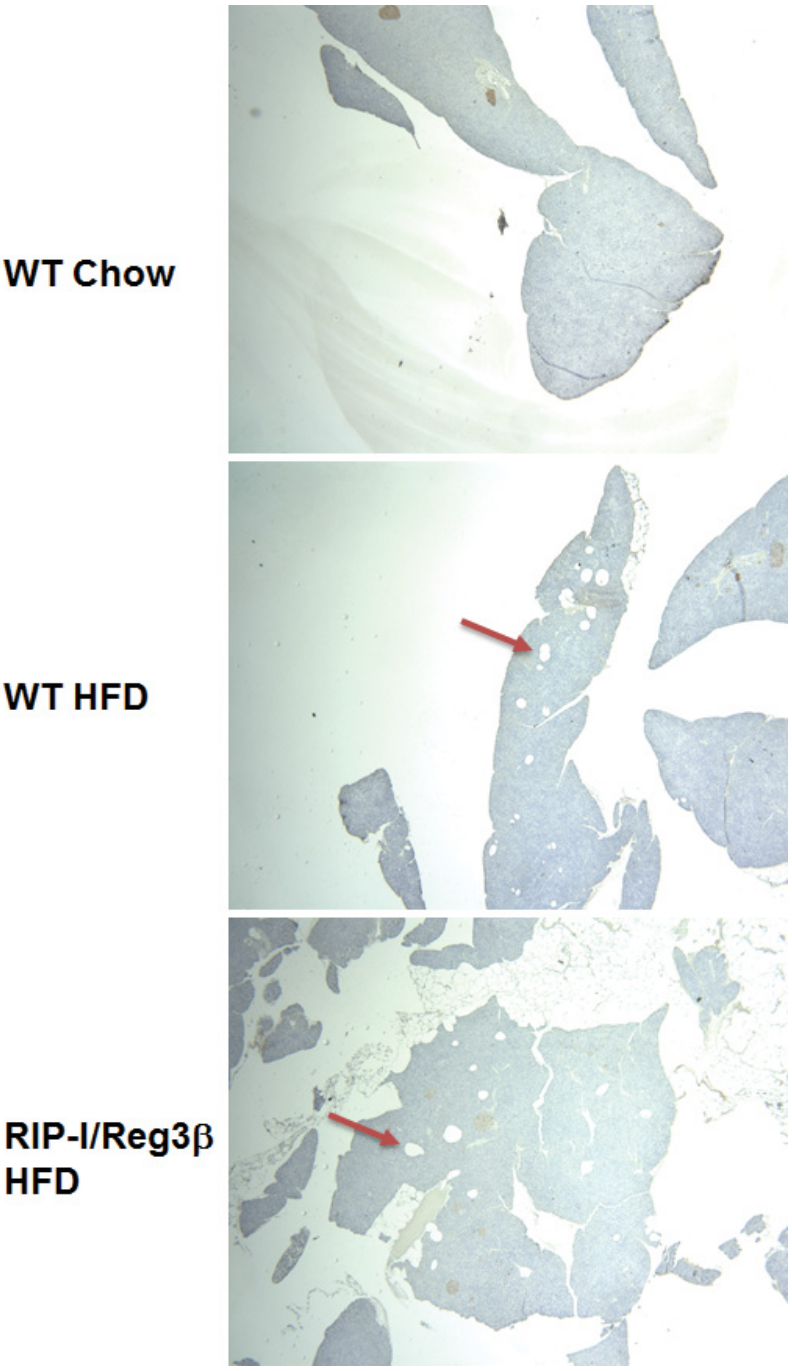


Figure 4.5 Evidence of impaired insulin staining and hepatic steatosis in aged RIP-I/Reg3β mice.

Wild-type and transgenic mice of 13-14 months old were studied after the measurement of body weight and blood glucose. **A**. Insulin immunofluorescence in pancreatic islets. **B**. Sign of hepatic steatosis in aged RIP-I/Reg3β mice. Hepatic sections were stained using hematoxylin and eosin. Significantly enlarged lipid droplets can be seen in the hepatocytes of RIP-I/Reg3β mice compared with age-matched wild-type. The scale bars at upper and lower panels are 200 and 25 μm respectively.



Supplemental Figure S4.1 Interlobular fat deposition within the pancreatic tissues of HFD- but not Chow-fed mice.

At the end of the 10-week HFD feeding, pancreatic sections were stained with HE, the brown arrows indicate fat deposition in the form of bubbles. Representative images of N=5.

Chapter 5. General discussions and future perspectives

5.1 General discussion of the findings

5.1.1 The classification of Reg proteins in different species

Reg proteins have different isoforms and orthologues that vary among species. It has been quite confusing for both the researchers and readers to identify their isoform-specific functions. Some isoforms have an identical name but different identities, while others have received different names for the same proteins. It is necessary to clarify the nomenclature for the study of Reg proteins. Therefore, we generated a table comparing different orthologues in between species. All the identities are based on the gene bank BLAST database. We use capital letters to name human Reg proteins, while Greek letters to name mouse isoforms to distinguish them. The isoforms can also be characterized based on their expression patterns in the pancreas and other tissues. For example, it is quite confusing that human Reg3A is identical to mouse Reg3β, but not mouse Reg3α. Using immunohistochemistry, we showed that the expression of hReg3A in the islets is colocalized with glucagon expressing α-cell and peri-islets acini. This is very similar with the mouse Reg3β expression pattern, but not Reg3α. Moreover, both hReg3A and mReg3β are named hepatocarcinoma-intestine-pancreas (HIP)/pancreatitis-associated protein (PAP) based on their upregulation in pancreatitis and hepatocellular carcinoma. It further supports the fact that hReg3A and mReg3β are two orthologues in different species.

Despite the fact that no Reg2 or Reg3δ (INGAP) has been identified in humans, an INGAP peptide containing amino acid 104-118 of the full length INGAP in hamsters has been shown to effectively increase the β-cell mass and control the glucose level in diabetic mice (141). This INGAP peptide has been involved in Phase II clinical trials and shown to be effective in glucose control in diabetic patients. In a preliminary study, we used INGAP antibody

generated from hamster antigen to react with human PDAC samples and found a similar expression pattern of INGAP in the glucagon-producing α cells as in mice. It is likely that human INGAP protein exists but has yet to be further identified. In my study, Reg2 seems a unique isoform in the β-cell compensation in HFD-induced diabetes. Even though it has no orthologue in humans, Reg2 or its bioactive fragments may be proved to be druggable targets for the treatment of diabetes with further pharmacological studies.

5.1.2 The contributions of Reg1 and Reg3 subfamilies in the development of PDAC

Although Reg proteins share some identities in their structure and chromosome location, their functions may vary in different isoforms. The roles of Reg1 and Reg3 subfamilies in pancreatitis and PDAC differ significantly. Reg3A was elevated in acute and chronic pancreatitis and play direct roles in promoting ADM in response to inflammation, whereas Reg1A was only mildly elevated when there was existing ADM/PanIN formation and became highly expressed in invasive cancer (Chapter 2). We should further identify the isoform-specific function of Reg proteins in more detail by using genetically engineered mice ($Kras^{G12D}$ mutated mice) in combination with drug-induced pancreatitis. The time points of the Reg1A and Reg3A expression are critical for detecting PDAC and its precursors. Furthermore, understanding their expression pattern can provide new therapeutic targets in the progression of PDAC. In general, Reg3A should be detected in transformed acinar-ductal cells in inflammatory stages, while its expression becomes limited to stroma but not the malignant ductal epithelial cells in invasive cancer. Reg1A can be detected in cancer in situ or invasive cancer stages and its levels are positively correlated with increasing grades of PanIN lesions. Moreover, higher expression levels of Reg1A and Reg1B predict better survival rate in PDAC patients, supporting its value in predicting the cancer prognosis. Therefore, in regard to the use of diagnostic and prognostic

biomarkers, Reg1A has advantages over Reg3A. However, as PDAC is a very malignant cancer type, it is usually in advanced stages and may metastasize very quickly at diagnosis. In the preliminary test, we tried to target invasive cancer cells with siReg1B and achieved 50% decrease of cancer proliferation. However, these effects may not be efficient to inhibit cancer growth in patients with advanced tumors. Finding early therapeutic targets or even preventive targets will be a good option for high-risk populations. Under this condition, targeting on Reg3A may have advantages because they are overexpressed in chronic pancreatitis and ADM.

The preventive treatment has become a valuable option for various malignancies. For instance, women with cervical squamous intraepithelial neoplasia 3 (CIN3) and HPV infection usually undergo cervical resection to prevent the development of cervical cancer (399). Carriers of BRCA mutation are susceptible to breast cancer and ovarian cancer, and therefore may be advised to perform preventive resection or personalized chemotherapy before the cancer development (400). We have established that Reg3A is expressed in ADM transformation and may contribute to the early carcinogenesis of PDAC. Moreover, Reg3A can directly promote ADM, mimicking the effects of TGF-α. As is known, Reg3A can protect acinar cells from inflammatory stress, such as acute and chronic pancreatitis (401). This protection can be achieved either by inhibiting acinar cell apoptosis or ADM transition. The result of both changes is to reduce enzyme outputs from acinar cells; therefore, the self-digestion in pancreatitis is inhibited. These processes are reversible when the inflammatory stress is attenuated. However, excessive Reg3A activation with predisposed genetic mutation of PDAC may lead to permanent lesions and contribute to the initiation of PDAC. Inactivation of mouse Reg3β (human Reg3A) has been shown to prevent Kras-induced PanIN lesions. Therefore, Reg3A could be a good chemopreventive candidate to target in early carcinogenesis of PDAC and high-risk populations

with pancreatitis. The roles of Reg3A in the transition of ADM to PanIN/PDAC provide a new path in treating and preventing PDAC in early stages.

5.1.3 The association of Reg protein levels with the histological grades of PanIN and differentiation grades of PDAC

Besides detecting the upregulation of Reg proteins in PDAC and exploring their potential function as therapeutic targets, we also analyze the association of their levels with histological grades.

As presented in the introduction, Reg proteins express in normal prenatal pancreas tissues, but the expression declined with the aging of mammalians and humans. In a normal pancreas, we were unable to detect Reg protein expression in ductal epithelial cells. The expression of Reg1A and Reg1B was increased stepwise with PanIN 1–3 grades to invasive cancer, suggesting a close correlation with the progression of PDAC. Regaining Reg proteins in cancer tissues indicates a reversed differentiation towards the premature status. This is consistent with other malignancies. For example, AFP is secreted in the prenatal liver while diminished in postnatal liver tissues. Hepatocellualr carcinoma (HCC) regains the expression of AFP, which is a biomarker for HCC. Also, the proliferating and/or anti-apoptotic effects of Reg1A in the gastric, pancreatic and colorectal cancers have been well established by gene knockin and knockouts (208, 227, 402). Therefore, upregulation of Reg1A and Reg1B may accelerate the progression of PDAC.

To our surprise, higher levels of Reg1A and Reg1B predict better survival rates in malignant PDACs. Theoretically, if a protein can promote cancer growth, its expression would remain being elevated with the advanced cell differentiation grades and malignancies, while our

observation is the opposite. Nevertheless, our finding is consistent with several previous reports. For example, the positivity of Reg4 is more frequently seen in well- and mediate-differentiated gallbladder cancer compared to poorly differentiated ones (403). Patients with high expression of Reg4 showed more prolonged survival rate compared to those with low expression. Other protein biomarkers also displayed similar expression patterns, such as the well-established biomarker CEA in the colorectal cancer. It also shows higher expression in well-differentiated cancer glands compared to poorly differentiated ones. More importantly, its level increased with the increased clinical stages and metastasis, which is another indicator of tumor development (404, 405). It is apparent that a certain level of tumor gland differentiation is required for the synthesis and secretion of Reg proteins. Higher levels of Reg proteins would be expected to positively correlated with tumor volume and clinical stages. Therefore, poorly differentiated tumors may have less Reg proteins secretion due to the smaller tumor volume. Due to the limited sample size, we were unable to show this significant correlation, but still the trend was observed. Elevated serum level of Reg1A tends to be correlated with increased clinical stages and chances of lymph node involvement and metastasis. Further study and collaborations with multiple institutions need to be conducted before we can draw any conclusions.

5.1.4. Reg2 and Reg3β functions in β-cell compensation and HFD-induced diabetes

In mice, the proliferating effects of Reg proteins on pancreatic β-cells provide a putative revenue of treating type 1 and type 2 diabetes. Both the aged Reg2KO mice and mice under HFD showed smaller islets compared to the wild-type counterparts, indicating its proliferating role in islet compensation. In our previous study, overexpression of Reg3β protected islets from the streptozotocin-induced diabetes partially by decreasing GLUT2 expression and upregulating pro-

islets growth factors, such as p8 and osteopotin (406). *In vitro* overexpression of Reg2 in insulinoma MIN6 cells also protected cells from streptozotocin- or UPR- induced cell apoptosis by upregulating GRP78 (247, 248). Surprisingly, pancreatic-specific overexpression of Reg2 or Reg3β does not show any proliferating effects in normal mice compared to wild-type (249), suggesting that they may not be able to efficiently promote β-cell proliferation *in vivo*. They may only show their protective effects under certain stresses, such as streptozotocin-induced β-cell disruption.

Under HFD, Reg2KO mice showed lower blood glucose compared to their wild-type littermates, while Reg3β overexpressing mice showed impaired glucose tolerance and higher blood glucose compared to their littermates. These results seem controversial with their aforementioned β-cell proliferating effects. However, this is in consistent with what has been observed in INS1 knockout mice, which showed improved insulin sensitivity along with decreased insulin level in the serum (407). The limitation in β-cell growth may be reversely beneficial to patients with insulin resistance and T2D because hyperinsulinemia leads to insulin resistance in multiple tissues independently (407). As Reg proteins are secreted proteins, they may also systematically affect other metabolic organs, such as liver and adipose tissue. We have observed increased lipid deposit in the liver and higher volume of adipose tissue in Reg3β overexpressing mice, indicating additional broad effects in metabolic homeostasis and insulin resistance. In general, Reg proteins may act in different manners in direct β-cell disruption (T1D) and insulin resistance (T2D).

To be noted, pro-tumor effects of these Reg proteins should be considered when treating diabetes. It has been shown Reg3β can promote the carcinogenesis in hepatocellular carcinoma.

In the Reg3β-overexpressing mice, we also noticed that some mice had tumors formed in the liver and the head of the pancreas. Further statistical analysis need to be done before any conclusions are drawn.

5.2 Future perspectives

5.2.1 How to further establish Reg1A as a diagnostic and prognostic biomarkers

In our study, we have detected higher levels of Reg1A and Reg1B in the sera of PDAC patients and upregulation in both protein and mRNA levels in PDAC tissues compared to adjacent normal tissues. Their expression levels correlate with PanIN lesions and tumor differentiation grades. To further establish them as diagnostic and prognostic biomarkers, we need to collect a larger scale of tissues and sera through multi-institutional collaborations. The results will be further correlated with patients' clinical stages (TNM stages), cell differentiation and clinical outcomes, including time of relapse, metastasis and survival rate. We also need to compare the sensitivity and specificity with established biomarkers such as CA19-9. I believe through a more in-depth and large-scale study, we will be able to establish a new panel of biomarkers containing Reg1A and Reg1B proteins in diagnosing PDAC.

To determine the dynamic changes of isoform-specific Reg proteins in PDAC development, genetically engineered mice harboring Kras mutation shall be utilized. Dynamic changes of Reg proteins will be quantified and correlated with parameters such as tumor size, tumor stages and occurrence of precursors, including ADM and PanIN. It will help us to better understand different roles of Reg protein isoforms in the initiation and/or development of PDAC.

5.2.2 Targeting Reg proteins in ADM-PDAC and the underlying mechanisms

As Reg proteins are upregulated in ADM and PDAC, we propose they can be potential therapeutic targets in inhibiting cancer development.

First, since the trophic and anti-apoptotic effects by overexpressing Reg proteins or conditioned medium in cancer growth are well established, we will use gene knockdown to inhibit their expression. In our preliminary study, we have obtained a 50% decrease of cell proliferation of PDAC cell lines when inhibiting Reg1B expression by siRNA. Further exploration of the cell behavior in invasion, migration and cell apoptosis needs to be performed. Xenografted nude mice carrying PDAC cells can be done to further study the putative inhibition of Reg proteins in the cancer growth and metastasis.

Second, as ADM is a newly identified mechanism of PDAC initiation, we would like to identify the role of Reg proteins in ADM and its transition to PDAC. We have observed upregulation of Reg1A and Reg3A in tumor-adjacent ADM areas. Treatment of recombinant Reg3A protein seems to directly promote ADM in primary acinar cells in a 3-D matrigel culture. Further knockdown of Reg gene expression should be performed in order to demonstrate whether endogenous Reg proteins are required in the development of ADM and cancer.

Third, the mechanism of Reg proteins in ADM and PDAC development has not been fully studied, especially in ADM. A recent study has demonstrated a direct interaction of Reg3A with fibronectin 1 (FN1) and integrin receptor α5β1, resulting in PI3K/Akt activation. Reg3A has been shown to affect the phosphorylation of PI3K/Akt and MAPK signaling in pancreatic β-cells and other cell types (161, 256). Additionally, FN1 also activates MAPK signaling. Therefore, FN1-PI3K/Akt and/or MAPK signaling may be involved in the Reg3A action. As matrix

metalloproteinase-7 and -9 (MMP-7 and MMP-9) are critical in ADM and PDAC development, they are also regulated by the Akt and MAPK signaling pathways; we thus propose that ADM formation can be promoted through MMP-7 and -9. In our preliminary study, we demonstrated the upregulation of Reg3A and FN1 mRNA levels in ADM tissues as compared to normal controls by using microdissection. The level of MMP-7 was prompted upon Reg3A treatment in ADM *in vitro*. Co-immunoprecipitation and pull-down experiments need to be performed to further establish the direct interaction of Reg3A and FN1. The downstream mechanism should also be determined in order to fully characterize this signaling pathway (Fig 5.1).

5.2.3 Insulin secretion and sensitivity differentially regulated by Reg2 and Reg3β

Upon HFD, the two mice studies showed striking changes in glucose tolerance. Reg3β overexpressing mice showed hyperglycemia and impaired glucose tolerance while Reg2KO mice showed normalized glucose levels. This finding seems contradictory with their roles in promoting β-cell proliferation. We traditionally believe that β-cell proliferation could increase β-cell mass, and therefore increase insulin secretion and rescue the β-cell failure in T1D and T2D. However, new understandings in the diabetes mechanism indicates that hyperinsulinemia may independently lead to insulin resistance. Excessive β-cell compensation may not always be beneficial for insulin sensitivity and β-cell function. Therefore, we need to further assess insulin sensitivity in these two mouse models under HFD. Moreover, the direct GSIS should also be performed on isolated islets *in vitro*. With these efforts, we will be able to shed light on the mechanisms underlying the changes in glucose tolerance in these two mice.

In all, even though Reg proteins have been long established as proliferating factors, their isoform-specific functions vary significantly. In recent years, their roles in inflammation-

associated cancer and insulin resistance have drawn increased attention due to their exocrine and endocrine expression in the pancreas. This thesis focuses on their roles as biomarkers for PDAC and therapeutic targets for both PDAC and diabetes. I provided reasonable evidence that: 1) Reg1A and Reg1B are correlated with the initiation and development of PDAC; 2) Reg3A can directly promote the ADM in cancer initiation; and 3) mouse Reg2 and Reg3β contribute to the β-cell compensation and insulin resistance differently in response to the HFD-induced T2D. Collectively, data reported in this thesis provides a new understanding of Reg proteins in the two pancreatic diseases PDAC and diabetes.

5.3 Figure(s) for Chapter 5

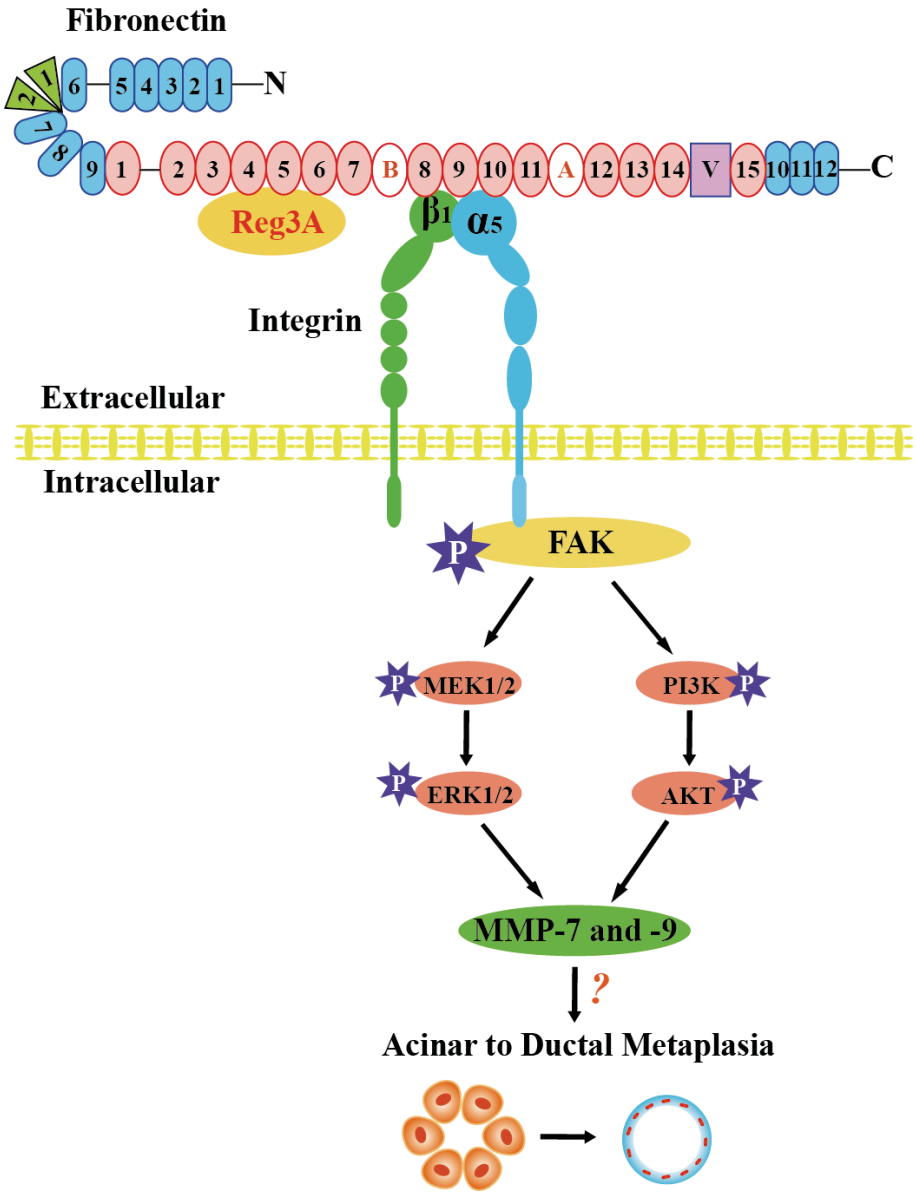


Figure 5.1 Proposed interaction of Reg3A and fibronectin 1 in ADM formation.

The binding of Reg3A and fibronectin activate integrin and cause the phosphorylation of FAK. It leads to further activation of PI3K/Akt and MAPK signaling pathways and promotes actin reorganisation. This may further lead to the ADM. Partially adopted from (Jennifer Veevers-Lowe 2011)

Summary and conclusions

In this thesis, I demonstrate the roles of isoform-specific Reg proteins in two major pancreatic diseases happening in exocrine (PDAC) and endocrine (diabetes) pancreas.

In PDAC, I discovered high levels of Reg1A and Reg1B in cancer ductal epithelial cells and in the sera. Their levels were closely correlated with the grades of precursor PanIN lesions. High Reg1A and 1B levels also predict better survival rates. In addition, Reg3A was overexpressed in ADM and directly promoted ADM *in vitro*. I thus establish that Reg1A and Reg1B can be potential diagnostic and prognostic biomarkers for PDAC. The combination of Reg1A, 1B and Reg4 can clearly differentiate PDAC from ICA. Moreover, upregulation of Reg proteins indicate their involvement in the initiation and progression of ADM and PanIN to PDAC transitions.

In HFD-induced diabetes, pancreatic-specific overexpression of Reg3β caused higher levels of glucose and impaired glucose tolerance. Also, hepatosteatosis and more obesity were observed in aged Reg3β overexpressing mice. Generally, Reg3β overexpression deteriorates HFD-induced diabetes. The inverse effects were observed in Reg2 knockout mice. The knockout limits the β-cell compensation, as evidenced by smaller β-cell mass and lower insulin levels. This result is partially due to the decreased β-cell proliferation under Reg2 deficiency. However, this may become beneficial for HFD-induced insulin resistance and T2D, as evidenced by decreased blood glucose level.

In conclusion, the work presented in this thesis strongly supports that Reg proteins have their proliferating effects on both an exocrine and endocrine pancreas. They may act through both endocrine and paracrine approaches. In PDAC, upregulation of Reg proteins can be used as

biomarkers for diagnosis and disease monitoring, as well as therapeutic targets to control cancer growth. In diabetes, Reg proteins can be considered as an alternative for β-cell replacement for T1D and β-cell failure stages of T2D. On the other hand, some isoforms of Reg proteins may be detrimental as it contributes to hyperinsulinemia and insulin resistance in T2D.

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