NOVEL GENES EXPRESSED IN THE PANCREATIC ISLETS AND REGULATED BY IGF-I

by

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Dedicated to

MY BELOVED PARENTS

MY DEAREST WIFE TANUSRI CHOWDHURY

MY SWEETEST DAUGHTER ANEETA CHOWDHURY ISHI

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Title: Novel genes expressed in the pancreatic islets and regulated by IGF-I

ABSTRACT

Insulin-like growth factor (IGF)-I is mainly produced by hepatocytes and other tissues including pancreas at lower level. Acting through its receptor, IGF-IR, it promotes embryonic development, postnatal growth and maturation of major organ systems. To evaluate the effects of increased IGF-I level on islet growth and glucose homeostasis, our lab previously characterized MT-IGF mice which over express IGF-I gene under the metallothionine I promoter and found that its expression was highly concentrated to β-cells. IGF-I overexpression led to a significant hypoglycemia in fasted animals, hypoinsulinemia and improved glucose tolerance. Moreover, MT-IGF-I mice were significantly resistance to streptozotocin-induced diabetes, with diminished hyperglycemia and abolished weight loss and reduce mortality.

Although IGF-I is known to stimulate protein synthesis, cell survival and proliferation, the specific target within the islets have not been screened in a systematic manner. In order to explore novel targets of IGF-I action, we performed a whole genome DNA microarray analysis on isolated islets from overexpressing IGF-I mice, and found 82 genes specifically up- or down-regulated. Prominent targets among those are HSD11B1 encoding 11β-hydroxysteroid dehydrogenase-1 (11β-HSD1) and CCN5/Wisp-2, previously not shown to be expressed in the pancreatic islets and regulated by IGF-I. In this study, we further checked 1) the localization of these targets in pancreatic islets both at the mRNA and protein level; 2) the effect of CCN5/Wisp2 in pancreatic islet proliferation and survival; 3) the effect of 11β-HSD1 under IGF-I regulation and assessed its functional relevance to type 2 diabetes.

The studies described in chapter II demonstrates that CCN5/Wisp2 is normally expressed in mouse β-cells and IGF-I directly stimulated its expression. CCN5 over expression increases the proliferation of mouse insulinoma cells, activates Akt and ERK kinases, and inhibits streptozotocin-induced cell death. Furthermore, use of recombinant CCN5 protein seems to reproduce the proliferative effect and the stimulation on Akt phosphorylation. All these findings suggest that CCN5 can regulate islet cell proliferation and survival and its increased expression may contribute to IGF-I stimulated islet cell growth and/or survival.

The study outlined in chapter III confirmed the presence of 11β-HSD1 exclusively on α-cells of the islet. We demonstrated an inhibitory effect of IGF-I on 11β-HSD1 expression and activity which may partially explain the observed increase in basal insulin levels in MT-IGF mice. The Inhibitory effect of IGF-I on 11β-HSD1 may render protection to islet cells by delaying apoptosis and thus can be proved beneficial for type 2 diabetes patients.

Titre : Gènes novateurs exprimés dans les îlots pancréatiques et leur régulation par IGF-I

RÉSUMÉ

IGF-I, le facteur de croissance analogue à l'insuline, est principalement produit par les hépatocytes, ainsi que par d'autres tissus incluant le pancréas à des niveaux plus bas. En interagissant avec son récepteur, IGF-IR, IGF-I promeut le développement embryonnaire, la croissance postnatale, et la maturation d'organes vitaux. Afin d'évaluer les effets d'une hausse des niveaux d'IGF-I sur la croissance des îlots pancréatiques et sur la régulation de l'homéostasie du glucose, le laboratoire du Dr Liu a précédemment caractérisé les souris transgéniques MT-IGF qui sur-expriment le gène du IGF-I sous le contrôle du promoteur métallothionéine I et a ainsi identifié l'expression d'IGF-I comme étant la plus concentrée aux cellules bêta. La surexpression d'IGF-I a mené à une hypoglycémie à jeûn significative, à l'hypoinsulinémie et à une amélioration de la tolérance au glucose. De plus, ces souris transgéniques MT-IGF-I étaient significativement résistantes au diabète induit par la streptozotocin, ce qui a par conséquence diminué l'hyperglycémie et aboli la perte de poids et la mortalité.

Bien qu'IGF-I soit connu pour sa capacité de stimuler la synthèse protéique et de promouvoir la survie et la prolifération des cellules, la véritable nature de sa cible dans les îlots pancréatiques n'a pas encore été détectée d'une manière systématique. Afin de découvrir des cibles novatrices de l'action d'IGF-I, nous avons conduit une analyse micropuces ADN du génome entier d'îlots isolés des souris sur-exprimant IGF-I ce qui nous a mené à l'identification de 82 gènes dont l'expression est stimulée ou supprimée par l'activité de la voie IGF-I. Parmi les cibles retrouvées, les plus importantes incluent HSD11B1 qui encode 11β-hydroxystéroïde déhydrogénase1 (11β-HSD1 in vitro) et

CCN5/Wisp-2 qui n'ont pas auparavant été démontrées d'être exprimées dans les îlots pancréatiques ni d'être régulées par IGF-I. Le but de cette étude est d'analyser davantage 1) la localisation de ces cibles dans les îlots pancréatiques en tant que mRNA et protéines; 2) les effets de CCN5/Wisp-2 sur la prolifération et la survie des îlots pancréatiques; 3) les effets de 11β-HSD1 sous la régulation d'IGF-I et l'évaluation de sa pertinence fonctionnelle dans le diabète type 2.

Les études décrites dans le chapitre II démontrent que CCN5 /Wisp2 est habituellement exprimé dans les cellules bêta murines et IGF-I stimule directement son expression. La surexpression de CCN5 stimule la prolifération des cellules murines pancréatiques de l'insulinome, active les kinases Akt et ERK, et inhibe la mortalité cellulaire induite par la streptozotocine. D'ailleurs, l'utilisation de la protéine recombinante CCN5 semble reproduire l'effet prolifératif et stimuler la phosphorylation d'Akt. Ces résultats suggèrent que CCN5 peut réguler la prolifération et la survie des îlots pancréatiques, tandis que l'augmentation de son expression peut contribuer à l'action stimulatrice d'IGF-I sur la croissance cellulaire et/ou la survie des îlots.

L'étude soulignée au chapitre III confirme la présence de 11β-HSD1 exclusivement dans les cellules alpha des îlots. Nous avons démontré un effet inhibiteur d'IGF-I sur l'expression et l'activité de 11β-HSD1, ce qui peut partiellement expliquer la hausse des niveaux d'insuline de base observés dans les souris transgéniques MT-IGF. Nous avons aussi constaté une baisse de sécrétion d'insuline par les cellules murines pancréatiques de l'insulinome qui sur-expriment 11β-HSD1. Il est possible que l'effet inhibitoire d'IGF-I sur 11β-HSD1 renforce la protection des cellules d'îlots pancréatiques en retardant

l’apoptose démontrant ainsi des bénéfices potentiels aux patients souffrant du diabète de type 2.

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Preface

In accordance with the guidelines published by McGill University, this thesis is organized as manuscript-based. It contains four chapters- one general introduction regarding literature review, two data chapters and finally a general discussion chapter. Chapter II and chapter III are composed of one published manuscript and one to be submitted, respectively.

Chapter II: Subrata Chowdhury, Xiao Wang, Coimbatore B Srikant, Qing Li, Min Fu, Ying Jia Gong, Guang Ning, Jun-Li Liu: IGF-I stimulates CCN5/WISP2 gene expression in pancreatic β-cells, which promotes cell proliferation and survival against streptozotocin. *Endocrinology* 2014 May; 155(5):1629-42.

Chapter III: Subrata Chowdhury, Larson Grimm, Kate Gong, Bing Li, Coimbatore B. Srikant, Zu-Hua Gao, Jun-Li Liu. Decreased 11β-hydroxysteroid dehydrogenase 1 level and activity in pancreatic islets caused by insulin-like growth factor I overexpression. Endocrinology 2014 (Submitted).

Copy of the paper in its published format has been included in the Appendices.

List of Abbreviations

11DHC	11-Dehydrocorticosterone
11β-HSD 1	11 beta-hydroxysteroid dehydrogenase Type 1
11β-HSD2	11 beta-hydroxysteroid dehydrogenase Type 2
17β-E2	17β-estradiol
ACT	Adrenocorticoid Tumor
ACTH	Adrenocorticotropic Hormone
ADA	American Diabetes Association
ADP	Adenosine diphosphate
AIF	Apoptosis inducing factor
PKB	Protein kinase B
AMPK	AMP-activated protein kinase
ANOVA	Analysis of variance
AP-1	Activator protein-1
Apaf1	Apoptosis protease-activating factor 1
APCs	Antigen Presenting Cells
ATP	Adenosine triphosphate
AVP	Arginine Vasopressin
Bad	Bcl-2-associated death promoter
Bak	Bcl-2 homologous antagonist killer
BAT	Brown adipose tissue
Bax	Bcl-2-associated X protein
Bcl-2	B cell lymphoma-2

BrdU	Bromodeoxyuridine
BrdU	5-bromo-2-deoxyuridine
BSA	Bovine serum albumin
C/EBPβ	CCAAT/enhancer binding protein, β
CAD	Caspase-activated DNase
cAMP	Cyclic adenosine monophosphate
CaP	Prostate Carcinoma
CBG	Corticosterone binding globulin
CCN	Cysteine-rich 61/connective tissue growth factor/nephroblastoma
CD	Crohn's disease
CDK4	Cyclin-dependent kinase 4
CGN	*cis*-Golgi network
CLIM	Cofactor of LIM Homeodomain Proteins
CNS	Central nervous system
CRH	Corticotropin-releasing Hormone
CSF	Cerebrospinal Fluid
CTGF-L	CTGF-like protein
DAB	Diaminobenzidine
DAPI	4,6-diamino-2-phenylindole
DBD	DNA Binding Domain
DEPC	Diethylpyrocarbonate
DMEM	Dulbecco's modified eagle medium

DNA	Deoxyribo-nucleic acid
DTT	Dithiothreitol
ECM	Extracellular Matrix
EDTA	Ethylene diamine tetraacetic acid
EGF	Epidermal growth factor
EMT	Epithelial- to mesenchymal transition
ER	Endoplasmic reticulum
Erk	Extracellular-signal-regulated kinase
ERK1/2	Extracellular signal-regulated kinase 1/2
ER-α	Estrogen Receptor α
Ex-4	Exendin-4
FADD	Fas-associated death domain containing protein
FFA	Free fatty acids
FOXO1	Forkhead box O1
G6P	Glucose-6-phosphate
G6Pase	Glucose-6-phosphatase
G6PDH	Glucose-6-phosphate dehydrogenase
G6PT	Glucose-6-phosphate transporter
Gab-1	Grb2-associated binding protein 1
GC	Glucocorticoid
GH	Growth hormone
GHR	Growth hormone receptor
GHRH	GH releasing hormone

GILZ	Glucocorticoid induced leucine Zipper
GK	Glucokinase
GLP-1	Glycogen-like Peptide-1
GLUT2	Glucose transporter 2
GLUT4	Glucose transporter 4
GR	Glucocorticoid Receptor
Grb2	Growth factor receptor bound protein 2
GREs	Glucocorticoid-response elements
GSH	Glutathione
GSIS	Glucose stimulated Insulin Secretion
GSK	Glycogen synthase kinase
GSK3β	Glycogen synthase kinase 3
GSSG	Glutathione disulfide
H6PDH	Hexose-6-phosphate dehydrogenase
HDL	High-density lipoprotein
hER-α	Human Estrogen Receptor-α
HPA	Hypothalamopituitary-Adrenal
HPA-axis	Hypothalamic-pituitary-adrenal -axis
HRP	Horse radish peroxidase
HSP	Heat shock protein
IAPP	Islet amyloid poly-peptide
IDF	International Diabetes Federation
IF	Immunofluorenscence

IFN-γ	Interferon γ
IGF	Insulin-like growth factor
IGFBP	Insulin-like growth factor binding protein
IGF-I	Insulin-Like Growth Factor-I
IHC	Immunohistochemistry
IL-10	Interleukin-10
IL-6	Interleukin-6
IL-8	Interleukin-8
IRE1α	Inositol requiring enzyme 1α
IRS	Insulin Receptor Substrate
IRS-2	Insulin receptor substrate
KATP	ATP-dependent K+ channel
KRB buffer	Krebs-Ringer bicarbonate buffer
LBD	Ligand Binding Domain
LC-CoA	Long Chain acyl-CoA
LDL	Low Density Lipoprotein
LPL	Lipoprotein lipase
MafA	v-maf musculoaponeurotic fibrosarcoma oncogene A
MafB	v-maf musculoaponeurotic fibrosarcoma oncogene B
MAP kinase	Mitogen-activated protein kinases
MAPK	Mitogen-activated protein kinase
MKP-1	Mitogen-activated Protein Kinase Phosphatase-1
MMPs	Matrix mettalloproteinases

MR	Mineralocorticoid Receptor
MT-IGF	Transgenic mice that overexpress IGF-I with metallothionein 1 promoter
mTOR	Mammalian Target of Rapamycin
mTORC2	Mammalian Target of Rapamycin Complex 2
MTT	3-(4,5- dimethylthiazol-2-yl)-2,5-diphenyltetrazolium
NAD	Nicotinamide Adenine dinucleotide
NADP	Nicotinamide adenine dinucleotide phosphate
NF-κB	Nuclear Factor Kappa B
NIDDM	Non-insulin dependent Diabetes
NO	Nitric Oxide
NOD	Non-obese Diabetic
NTD	N-Terminal Transactivation Domain
OVX	Ovariectomized Rat
p44/42	(MAPK3) Mitogen-activated protein kinase 3
p70S6K	p70S6 Kinase
PAP	Pancreatitis-associated Protein
PARP	Poly ADP-ribose Polymerase
PBS	Phosphate Buffered Saline
P-CREB	Phospho-cAMP response element-binding protein
PDGF	Platelet-derived Growth Factor
PDX1	Pancreatic and Duodenal Homeobox Gene 1
PEPCK	Phosphoenol Pyruvate Carboxykinase

PERK	Protein kinase RNA-like ER kinase
PI	Propidium Iodide
PI3K	Phosphotidylinositol 3'-kinase
PID	Pancreatic-specific IGF-I deficiency
PIP2	Phsphatidylinositol-4,5-bisphosphate
PIP3	Phosphatidylinositol-3,4,5-triphosphate
PKA	Protein Kinase A
PKC	Protein Kinase C
PPARPγ	Peroxisome Proliferator-activated Receptor Gamma
Reg	Regeneration associated gene
RER	Rough Endoplasmic reticulum
RLIM	Ring Finger LIM Domain Binding Protein
RNA	Ribo-nucleic acid
RNAi	RNA interference
ROIs	Oxidative stress inducers
ROS	Reactive oxygen species
RT	Reverse transcription
S6K	Ribosomal S6 protein kinase
SGK1	Serum-glucocorticoid Regulated Kinase 1
SGT	Salivary Gland Tumor
SH-2	Src-homology-2
siRNA	Small interfering RNA
STZ	Streptozotocin

T1D	Type 1 diabetes
T2D	Type 2 diabetes
TF	Transcription factor
TGF-α	Transforming growth factor α
TGF-β	Transforming growth factor beta
TGN	*trans*-Golgi Network
TIMPs	Inhibitors of Matrix Metalloproteinases
TNFα	Tumor necrosis factor α
TSP-1	Thrombospondin Type-1
TTP	Tristetraproline
USF-2	Upstream stimulatory factor 2
UTR	Untranslated region
VEGF	Vascular Endothelial Growth Factor
VSMC	Vascular Smooth Muscle Cell
VWFC	Von Willebrand Factor Type C
WAT	White adipose tissue
WHO	World Health Organization
WISP-2	WNT1Inducible Signaling Pathway Protein-2
WT	Wild type
βHLH	Basic helix-loop-helix

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Chapter 1

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Claim of Originality

This doctoral thesis provides important information regarding the role of novel IGF-I targets in pancreatic islets. These targets can be important for islet proliferation and survival

1) To identify the novel target of IGF-I we performed whole genome DNA microarray analysis on RNA extracted from IGF-I over expressing mice and found 82 genes which can be potential candidate for β-cell proliferation and survival. None of these targets were shown to be regulated by IGF-I before in pancreatic islet cells.

2) We identified CCN5 for the first time in islet β-cells in higher level compare to WT mice. CCN5 expression in increased level contributes to islet cell growth and/or survival.

3) We also found the decreased expression of 11β-HSD1 in α-cell due to overexpression of IGF-1 in pancreatic islet. Decreased expression of 11β-HSD1 may cause increased basal insulin secretion in MT-IGF mice. This may also contribute to islet survival.

Author Contributions

Chapter II: IGF-I stimulates CCN5/WISP2 gene expression in pancreatic β-cells, which promotes cell proliferation and survival against streptozotocin. In this manuscript, S. Chowdhury performed all experiments, presented the data and revised the manuscript, Xiao Wang performed microarray and Table 1, Coimbatore B Srikant and Guang Ning contributed to discussion and revision, Min Fu performed confocal microscopy and data analysis, Qing Li, Ying Jia Gong participated part of the experiments and their analysis, Jun-Li Liu designed the study, wrote the manuscript and approved the final revision.

Chapter III: Decreased 11β-hydroxysteroid dehydrogenase 1 level and activity in pancreatic islets caused by insulin-like growth factor I overexpression. In this manuscript, S. Chowdhury performed all experiments, presented the data. Kate Gong confirmed 11β-HSD1 overexpression in mouse insulinoma cell-line, Bing Li helped with experimental design analysis, C Srikant and Zu-Hua Gao contributed in discussion. Jun-Li Liu designed the study, wrote the manuscript.

Chapter I

GENERAL INTRODUCTION

1.1 Glucose homeostasis in the body

Glucose is quantitatively the most important fuel source for humans as well as other animals. It is the major building block for cellular structures. Glucose constitutes about 80% of absorbed dietary sugars. Glucose is also the primary fuel for the brain but if glucose is low it can use ketone bodies to replace about 20% of its glucose requirement. Gluconeogenesis provides the brain with a steady supply of glucose even during prolonged starvation. In healthy individuals, adipose tissue, muscle and liver rapidly absorb glucose to ensure postprandial blood glucose levels. While a surplus of caloric intake is not immediately risky, an increase in the anabolic processes can lead to the development of obesity and diabetes.

1.2 Regulation of glucose metabolism

The two main regulators of glucose metabolism are insulin and glucagon, which are both secreted by the islets of Langerhans in the pancreas. The metabolic effects are primarily exerted on the liver, muscle, and adipose tissue. The liver is the main site for several of the metabolic processes, such as gluconeogenesis or glycolysis. Skeletal muscle is capable of glucose uptake, where it can be oxidized or stored as glycogen, and it is also the major site of amino acid uptake and protein synthesis. Finally, adipose tissue is a major site for energy storage in the form of triglycerides, providing long term storage for energy dense lipids.

The levels of these two hormones are regulated in a reciprocal manner, such that as the plasma level of one increases, the other decreases. Insulin is the primary mediator of anabolism such as glycogenesis, lipogenesis and protein synthesis. Intake and digestion of food increases the secretion of insulin which then promotes the uptake and utilization of nutrients into muscles, adipose tissue, and liver. Glucagon, on the other hand, is a catabolic hormone and increases during

starvation and acts in an opposite fashion to insulin by promoting glycogenolysis in the liver and muscles, lipogenolysis in adipose tissue and proteolysis in muscle. Thus, insulin and glucagon coordinate to keep the blood glucose levels in a normal range. The principal modulator of these hormones is plasma glucose levels, although many other nutrients, as well as neuronal and hormonal inputs can also regulate secretion of these hormones. The blood glucose concentration is tightly regulated. According to ADA guideline (2014), blood glucose level under fasting condition ranges from 5.6 to 6.9 mM (100–125 mg/dL) and increases to 7.8-11 mM (140-199 mg/dL) postprandially (1). Maintenance of glucose levels in blood within these ranges is crucial in ensuring its maximal uptake and utilization in the tissues. Any alternative of that may lead to hypoglycemia and hyperglycemia with severe consequences for the body such as muscle weakness, impaired cardiac function, cerebral energy failure leading to brain damage and diabetes (2). Hypoglycemia is commonly caused by intensive therapy in type 1 and type 2 diabetes (3) and prolonged starvation.

1.3 Diabetes Mellitus

Diabetes mellitus is considered as a group of metabolic diseases characterized by, and diagnosed with, chronic hyperglycemia (WHO definition). The classical symptoms caused by chronic hyperglycemia are polydipsia (increased thirst), polyuria (frequent urination), and polyphagia (increased hunger).

1.3.1 Current world status of Diabetes

The International Diabetes Federation (IDF) reported that 382 million people worldwide or 7.5% of the population is suffering from diabetes in 2013, which is nearly 15 times more than reported in 1985 (IDF Diabetes Atlas, 6th Edition, 2013). Alarmingly, 46% of this population

remains undiagnosed. The condition is affecting more people of working age than previously predicted and no ethnic group has escaped from its increasing prevalence. The mortality rate has significantly increased because of diabetes complications and the financial burden on the health sector is rising exponentially. It is predicted that if the current rate of growth continues, the total number of diabetic patients will exceed 592 million in 2035 (IDF Diabetes Atlas, 6th Edition, 2013).

Diabetes is associated with long-term damage due to the chronic hyperglycemia. This causes many complications such as deregulation and failure of various organs including the eyes, kidneys, blood vessels, heart and nerves (4). In both forms of diabetes, the ultimate result is β-cell failure (4). Type 1 diabetes (T1D) results from a failure to produce insulin and requires life-long injections of insulin (although recent islet transplant therapy has opened the possibility to finally supersede this) (5,6). Type 2 diabetes (T2D) results from peripheral insulin resistance wherein cells fail to sense and respond to insulin properly. At least initially, T2D can be controlled with medications that improve insulin sensitivity before the need to administer insulin.

1.3.2 Type 1 Diabetes

T1D or insulin dependent diabetes is most often found among young adults or children, and is characterized by high blood and urine glucose levels. T1D is caused by an autoimmune reaction that reduces 80% reduction in β-cell mass in the islets of Langerhans of the pancreas and resulting in permanent insulin loss (7).

The possible factors behind the cause of T1D might be associated with genetic predisposition because of immunological origin (8). Also environmental factors such as stress (9-12), viral mediators (13), bacterial infection (14), food constituents (15-18) may also trigger the conditions required for disease onset. T1D is rapidly life threatening and there is a need for either

multiple daily insulin injections or continuous subcutaneous insulin infusion. Pancreatic or islet transplants have been used to treat T1D clinically, though this approach is still at the experimental trial stage and requires a lifetime of immunosuppression (5,6,19,20). A drive to create artificial glucose responsive pancreatic islet tissues is currently at the forefront of T1D research (21).

1.3.3 Type 2 diabetes

T2D used to be called non-insulin dependent diabetes (NIDDM) or adult-onset diabetes, and accounts for at least 90% of all cases of diabetes. T2D is characterized by peripheral insulin resistance, increased hepatic glucose production and relative insulin deficiency (22, 23). The diagnosis of T2D can occur at any age with any or all of these pathological alterations. Although the etiology of T2D is far from clear, researchers found its close association with inheritable genetic and environmental factors, such as stress (11), high fat diet (27) and a less active life style (24). Recent genome wide association studies in the human population identified several candidate genes of T2D, the majority of which are associated with β-cell function (25).

In T2D, there is a loss of up to 50% of the β-cell mass along with a 3 fold increased rate of apoptosis of the β-cells (29, 30). β-cell mass is negatively affected by dyslipidemia and hyperglycemia, which increase apoptosis (31, 32).

1.4 The Pancreas

The pancreas is a glandular organ in the digestive and endocrine system. It functions both as an endocrine gland (producing insulin, glucagon, and somatostatin) as well as an exocrine gland (secreting pancreatic juice containing digestive enzymes). These enzymes help in the further breakdown of the carbohydrates, protein, and fat in the chyme.

1.4.1 The Anatomy and histology of the Pancreas

The pancreas has three main sections: 1) Head: area of pancreas to the right of the left border of the superior mesenteric vein. 2) Body: The area of pancreas between the left border of the superior mesenteric vein and the left border of the aorta. 3) Tail: The area of pancreas between the left border of aorta and the hilum of the spleen. The pancreas is in direct contact with the gastro-intestinal tract (stomach, duodenum, spleen, and major vessels of the abdomen).

1.4.2 Functions of Pancreas

The pancreas is a dual-function gland, having features of both endocrine and exocrine glands.

Endocrine: The endocrine pancreas is made up of approximately a million cell clusters called islets of Langerhans. There are four main cell types in the islets: α cells secrete glucagon, β cells secrete insulin, δ cells secrete somatostatin, and PP cells secrete pancreatic polypeptide. The islets are a compact collection of endocrine cells arranged in clusters and cords and are interlinked by a dense network of capillaries. Endocrine cells in direct contact with vessels line the capillaries of islets, and most endocrine cells are in direct contact with blood vessels.

Exocrine: The exocrine pancreas produces digestive enzymes and an alkaline pancreatic juice, and secretes them into the small intestine through the exocrine ducts in response to intestinal hormones secretin and cholecystokinin. Digestive enzymes include trypsin, chymotrypsin, pancreatic lipase, and pancreatic amylase, are produced and secreted by acinar cells of the exocrine pancreas.

1.4.3 Anatomical and morphological origin of the pancreas and progenitor cell development

New insights from recent advancement studies in pancreas developmental biology with the focus on β-cell ontogeny have expanded our knowledge on the cause and treatment of diabetes. As the work involved in this thesis relates to the mouse, this section will highlight murine

pancreatic development. Both the pancreatic endocrine and exocrine cells derive from the embryonic endoderm. Prior to outgrowth of the pancreatic buds, a ventral and dorsal prepancreatic region is specified in the gut endoderm around e8.5. Pancreatic development starts from embryonic day e8.5-9.0 with the formation of dorsal and ventral buds. The dorsal bud lies in close proximity to the notochord and lateral to the dorsal aorta. The ventral bud comes from the hepatic diverticulum and becomes the pancreatic head. In mice, at e9.5, the dorsal and ventral buds begin a differential rotation. The duodenum rotates to the right along with the ventral bud. By e10.5, the partially differentiated epithelium of the two buds undergoes branching morphogenesis into a ductal tree that by e12.5 results in the formation of two primordial pancreas organs consisting predominantly of an undifferentiated ductal epithelium. Between e13 to e14, the ventral bud fuses with the much larger dorsal bud to form a single definitive pancreas (37). (Figure 1.2). Mouse pancreatic endocrine progenitor cells are present from the very beginning of development (e9.5). The pancreas development involves two major transitions: the first one between E9.5 and E12.5 related to organ determination resulting the conversion of pre-differentiated cells to a proto-differentiated state, characterized by low levels of pancreas-specific proteins. During the secondary transition from e12.5 until birth, the pancreatic epithelium continues to expand and branch into a complex yet highly ordered tubular network. At e15.5, the luminal plexus progressively remodels into a single-layered epithelium consisting of highly branched primitive ducts (also known as progenitor cords) and newly differentiated acinar cells. Ngn3-expressing endocrine precursors delaminate and migrate away from the progenitor cords to form endocrine clusters. Blood vessels are intercalated between nascent branches of the pancreatic ductal tree. In the mature pancreas, acinar cells cap the endings of small terminal ducts and form functional exocrine secretory units. Endocrine cells are clustered in so-called islets of Langerhans, which are

penetrated by a dense network of blood vessels (Figure 1.2)

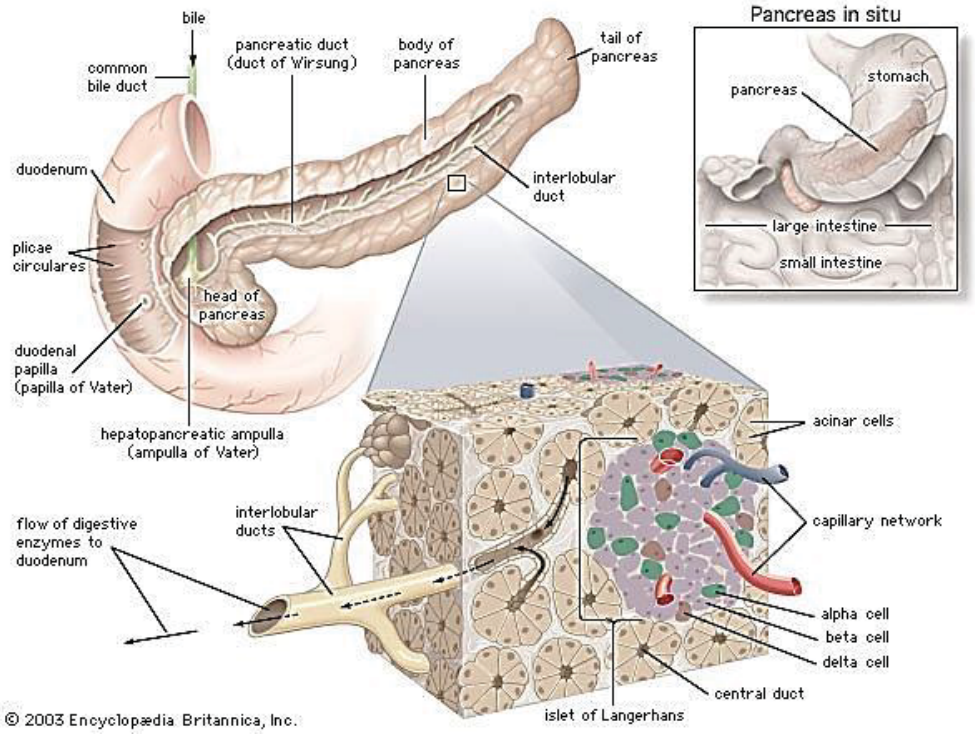

Figure 1.1 The structure of the Pancreas and islets of Langerhans: Islets are responsible for the endocrine function of the pancreas. Each islet contains beta, alpha, and delta cells that are responsible for the secretion of pancreatic hormones. Beta cells secrete insulin, a well-characterized hormone that plays an important role in regulating glucose metabolism (Adapted from Encyclopedia Britannica, Inc. 2003)

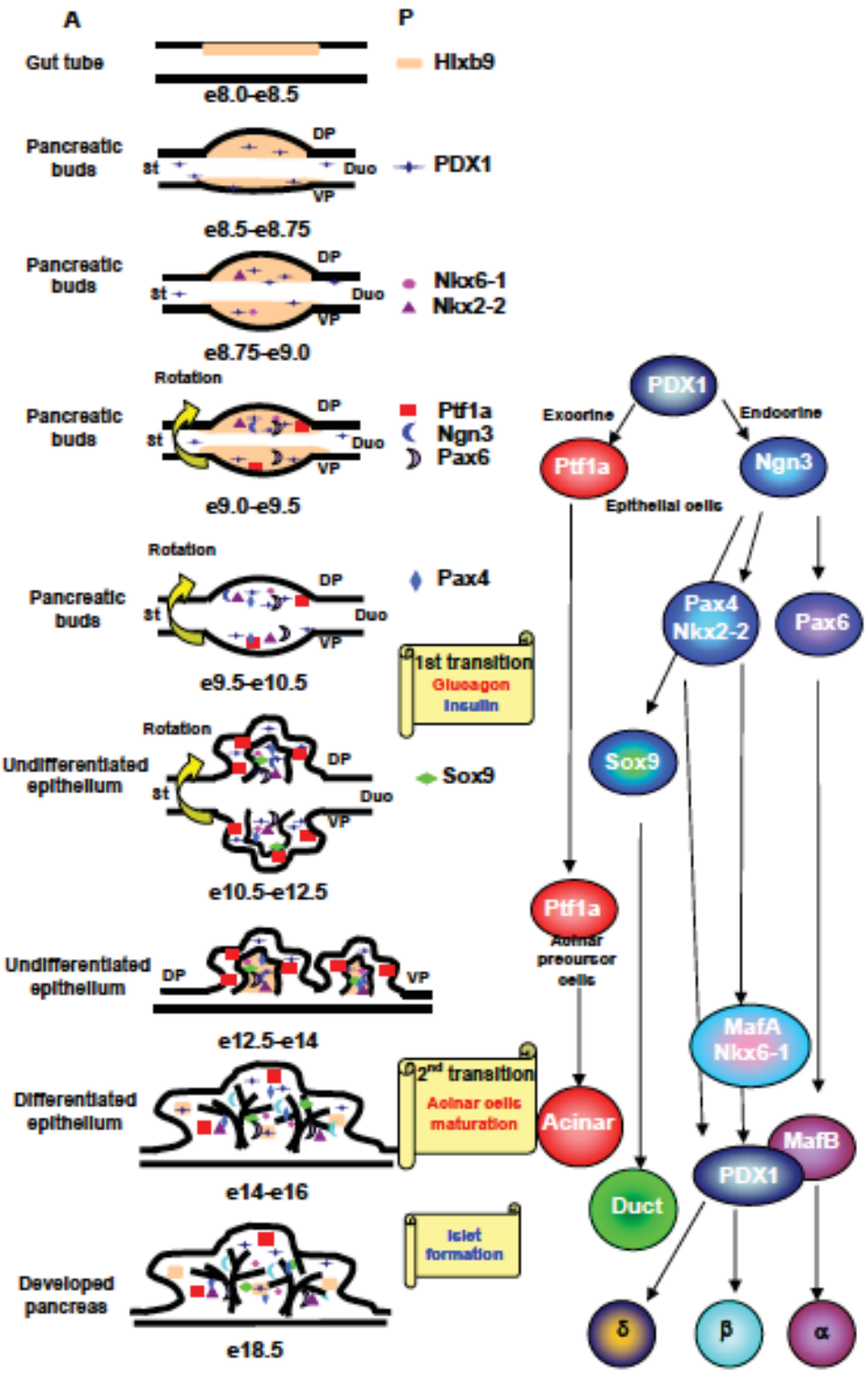
A
P
Gut tube
Hlxb9
e8.0-e8.5
Pancreatic buds
DP
st
Duo
VP
PDX1
e8.5-e8.75
Nkx6-1
Nkx2-2
e8.75-e9.0
Rotation
Ptf1a
Ngn3
Pax6
e9.0-e9.5
Pax4
e9.5-e10.5
1st transition
Glucagon
Insulin
Undifferentiated epithelium
Sox9
e10.5-e12.5
e12.5-e14
Differentiated epithelium
2nd transition
Acinar cells maturation
e14-e16
Islet formation
Developed pancreas
e18.5
PDX1
Exocrine
Endocrine
Ptf1a
Ngn3
Epithelial cells
Pax4
Nkx2-2
Pax6
Sox9
Ptf1a
Acinar precursor cells
MafA
Nkx6-1
MafB
PDX1
Acinar
Duct
δ
β
α

Figure 1.2 Schematic representation of morphogenetic events and expression of transcription factors during embryonic pancreas development. Hlxb9 is expressed in the dorsal endoderm before initiation of DP organogenesis; PDX-1 appears between e8.5 and e8.75 at the foregut domain, VP begins to form; Nkx2-2 co-expresses with PDX-1 at e8.75, Nkx6-1 expresses at VP; by e9.0 some cells turn on Ngn3 expression and go through the endocrine pathway, some cells expressing Ptf1a and co-expressing PDX-1 enter the exocrine pathway; at e9.5, VP starts to rotate to the right, several transcription factors co-express at DP and Pax4 appears. At this stage, glucagon and insulin can be detected (first transition); from e10.5 to e12.5, the uniform epithelial structure of the entire pancreatic epithelium becomes highly branched, and by e12.5, the undifferentiated ductal epithelium forms. Nkx2-2, Nkx6-1, Hlxb9-positive cells segregate to the central epithelium, whereas Ptf1a-positive cells are specifically found in the periphery and migrate to regulate acinar cell maturation; from e12.5 to e14, the VP fuses with DP to form a single pancreas organ; the buds begin to differentiate into endocrine and exocrine cellular lineages by e14 and proliferate and expand extensively, acinar cells complete maturation; from e13.5 to e14.5 (second transition) the endocrine cells organize into isolated clusters that condense into the islets of Langerhans by e18.5. Abbreviations: A, anterior; P, posterior; st, stomach; duo, duodenum; DP, dorsal pancreas; VP, ventral pancreas. **[Adapted with permission from (40)]**

1.4.4 Pancreatic β-cell growth and regeneration

1.4.4.1 β-Cell expansion in normal growth

The regulation of β-cell mass is a dynamic process. Any kind of change (increase or decrease) in terms of function and mass of the β-cell is associated with the maintenance of the glycemic level within a very narrow physiological range (41). The mechanisms responsible for changes at the level of replication and neogenesis in both normal and pathophysiological states can lead to changes in individual cell volume, and changes in cell loss or death rates. In rodents, cell replication is significantly higher during late gestation and the neonatal period than after weaning and changes little beyond 30–40 days of age except in response to physiological/ pathological changes (42-44). The frequency of apoptosis is low and unchanged before weaning (45, 46). In the initial months of life, the β-cell mass increases with body weight through increases in both cell number and size. Increase in this β-cell mass lasts throughout the life span but in the later stage of life (15–20 months), the increment in mass in rodents is largely accounted for by increased cell size (46).

1.4.4.2 Neogenesis of Pancreatic β-cell *in vivo*

Although replication is the major pathway of renewal of β-cells (58) some of the new β-cells arise from a non- β-cell precursor or stem cell in adult rats (59). During the fetal stage, differentiation is the major mechanism for forming new cells, but replication or self-duplication is enhanced during the perinatal period. During the neonatal period, cell replication continues, and significant neogenesis occurs under the normal physiological conditions. Exendin-4, an analog of GLP-1, has been reported to enhance both proliferation and neogenesis of pancreatic β-cells in rats with 90% pancreatectomy (60). Administration of exendin-4 caused a direct trophic effect on islet neogenesis in insulin-resistant obese *fa/fa* Zucker rats that was independent of glycemia and body

weight, and in addition improvement insulin sensitivity (61). GLP-1 or exendin-4 treatment increased pancreatic insulin content and β-cell mass and decreased blood glucose in STZ-treated neonatal rats in both short- and long-term (62).

1.4.4.3 *In vitro* expansion of β-cells:

In vitro expansion of pancreatic β-cells is an attractive strategy for β-cell transplantation. In the presence of growth factors and hormones, human β-cells exhibit the capability to proliferate in culture plates **(68-70)**. But these expansions of β-cells have very limited capacity to show β-cells phenotype in terms of insulin expression and secretion. Studies found that the phenotypic changes of pancreatic β-cells in culture sometimes resemble epithelial-to mesenchymal transition (EMT) in mouse and human pancreatic β-cells (**71-73**). According to these studies pancreatic β-cells of adult mice undergo dedifferentiation into mesenchyme- like fibroblastoid cells (i.e., EMT), and that the process is associated with the progression of the cell cycle. The dedifferentiated cells can redifferentiate into insulin-positive cells (**74**). However, some other studies (48–50) found that most proliferative mesenchymal cells migrating out from pancreatic islets *in vitro* were not derived from β-cells, and suggested that these cells do not represent a useful source for the generation of physiologically competent β-cells for treatment of diabetes (**75,76**).

There are several hormones and growth factors which can regulate the pancreatic β-cell mass. Regulators like growth hormone (GH), insulin, insulin-like growth factor-I (IGF-I), incretins, Glucagon-like peptide-1 (GLP-1) have been shown to promote islet cell function and/or growth (78-79) in different animal models of diabetes. In phases of discussion (section 1.5, 1.6) we will discuss about insulin and IGF-I.

1.5 Insulin

β-cells store a large amount of insulin in mature granules and release a small proportion of these granules immediately following glucose stimulation. To renew these stores, insulin biosynthesis is immediately stimulated and occurs mainly at the translational level. Under physiological conditions, the quantity of insulin released by β-cells is directly related to the prevailing glucose concentration, which may depend on the nature, quantity and route of administration or exposure to nutrients.

1.5.1 Insulin transcription, synthesis and release

Insulin is an important hormone in regulating metabolic homeostasis. Insulin is a peptide of ~6000 daltons and composed of 2 chains (alpha 21 amino acids and beta 30 amino acids) linked together by disulphide bonds A7-B7 and A20-B19. Most of the animals have one copy of the gene except the rat and mouse, which carry two copies of each (341). In rats, both insulin gene I and II are localized on the same chromosome 1 (80). In mice, the insulin gene I is located on chromosome 19 (64), and the insulin gene II is localized on chromosome 7 (65). Physiological insulin expression is restricted to pancreatic β-cells, although the insulin gene I is moderately expressed in the rat brain predominantly in the hippocampus (66). Insulin transcription is controlled by the insulin promoter that is a highly conserved ~340 bp region located upstream from the transcription start site (67). The insulin promoter binds β-cell specific as well as ubiquitous transcription factors (63, 67). The most critical transcription activation elements of the insulin promoters are the E, A and C sites (63) as well as additional sequences that may have more subtle regulatory effects. The insulin mRNA is translated as a single chain precursor (preproinsulin) and processed to proinsulin through the removal of the "pre" portion by a signal peptidase. Newly synthesized proinsulin in the RER is transferred to the CGN, through the Golgi apparatus, to the TGN, where immature β-

granules form. The TGN is the sorting point of β-granule, lysosomal-, and constitutively secreted proteins. There is a post-TGN editing mechanism (569, 570, 571) that is indicated as budding off of the clathrin-coated region of an immature β-granule. Proinsulin is targeted to the β-granule compartment, where it undergoes proteolytic conversion to insulin and C peptide. Granule maturation can be divided into three steps: (i) acidification of the granule lumen, (ii) conversion of proinsulin to insulin and C-peptide through proteolysis by endoproteases PC1/3 and PC2, followed by trimming of the carboxyl termini carboxypeptidase E, and (iii) loss of the coat protein clatherin (583, 595, 606). The granule luminal ATP-dependent proton pump serves to produce the acidification of the granule milieu that facilitates the conversion of proinsulin to insulin since both convert enzymes (endoproteases PC1/3 and PC2) display an acidic pH optimum (167). Mature β-granules are then held in an intracellular storage compartment in close association with Zinc and Calcium, and finally stored as a hexamer which is inactive (344). Insulin is released by exocytosis after glucose stimulation. The granules carrying insulin hexamers are released into the intercellular space where they dissolve to expose insulin monomers which are the active form of insulin (344).

1.5.2 The role of glucose in insulin secretion

Glucose is the main physiologic regulator of insulin transcription, translation and secretion. Basal insulin gene transcription occurs at low plasma glucose concentrations (< 5.6mM). The transcription of insulin increases at higher glucose concentrations, and is sensed through increased glucose metabolism by the high Km GLUT2 and GK activity within the β-cell (81). Glucose-induced insulin transcription is modulated by many factors which influence insulin transcription as mentioned above, and the insulin promoter boxes. β-cells release insulin in response to glucose metabolism (82). After entering the β-cell through the GLUT2 transporter, glucose is phosphorylated to G6P by glucokinase and metabolized via glycolysis to generate pyruvate which

is then utilized by the mitochondria (81, 83, 84). In the glucose oxidation pathway, approximately 50% of pyruvate is metabolized by pyruvate dehydrogenase producing acetyl-CoA which enters the tricarboxylic acid cycle (TCA) to generate ATP. This causes an increased intracellular ATP/ADP ratio (81, 83, 85) which results in closure of the plasma membrane K_{ATP}-channels that induces membrane depolarization, and triggers increased calcium concentrations in the cytoplasm through opening of voltage-gated calcium channels (86). The increased intracellular calcium level induces the insulin secretory granules to fuse with the membrane and releases insulin by exocytosis (87). This pathway, often termed the K_{ATP} channel-dependent pathway is considered to be the major triggering event for GSIS. Pyruvate can also be metabolized by pyruvate carboxylase to oxaloacetate, which ensures the provision of a carbon skeleton to the TCA cycle in the mitochondria, a process known as anaplerosis (85, 88, 89). (Figure 1.3)

1.5.3 Insulin signaling Network

Activation of specific insulin receptor (member of tyrosine kinase subfamily) is responsible for normal insulin signaling (90). The insulin molecule binds to the α-subunit of the receptor, releasing the inhibition of tyrosine auto-phosphorylation by the β subunit (91, 92). The receptor is auto-phosphorylated at distinct tyrosine residues. In contrast to most tyrosine kinase receptors, the activated insulin receptor directly phosphorylates IRS-1 to -4 on multiple tyrosine residues. There are currently four members of the IRS family known to be involved in insulin signaling, with IRS-1/2 being the most important for glucose transport (92, 93). The subcellular distribution of these proteins between the cytoplasm and low-density membrane compartments of the cell has been shown to play a vital role in transmitting the proper insulin response (93, 94). Tyrosine-phosphorylated IRS proteins then act as a binding site for signaling molecules containing SH-2

domains such as PI3K, GRB-2/mSOS, and SHP-2. These molecules bind the phosphorylated tyrosine residues of IRS proteins, forming a signaling complex to mediate downstream signaling. PI3K is the main signal mediator of the metabolic and mitogenic actions of insulin. It is composed of a p85 regulatory subunit, which binds to IRS proteins, and a p110 catalytic subunit. After the association of p85 with IRS-1/2, the p110 subunit has increased catalytic activity. This allows phosphorylation of its substrate, PtdIns (4,5)P2, on the 3′ position of the inositol ring to generate PtdIns(3,4,5)P3 (91). The second messenger, PtdIns (3,4,5)P3, recruits the serine kinases PDK-1, PKB/Akt, and PKC to the plasma membrane via their PH domains. The activation of these kinases results in several of insulin's responses, such as GLUT4 translocation to the membrane, glycogen synthesis by phosphorylation of GSK-3, and lipogenesis by up-regulating synthesis of the fatty acid synthase gene. In addition to insulin signaling via PI3K, insulin can activate the mitogen-activated protein kinase (MAPK) ERK, which leads to the gene expression of various cellular proliferation or differentiation components. After phosphorylation of IRS-1/2, the adaptor proteins GRB-2 and SOS are recruited and work together with a stimulated tyrosine phosphatase, SHP-2, to activate membrane-bound Ras. Activated Ras leads to a kinase cascade, allowing ERK to translocate to the nucleus for gene expression (92).

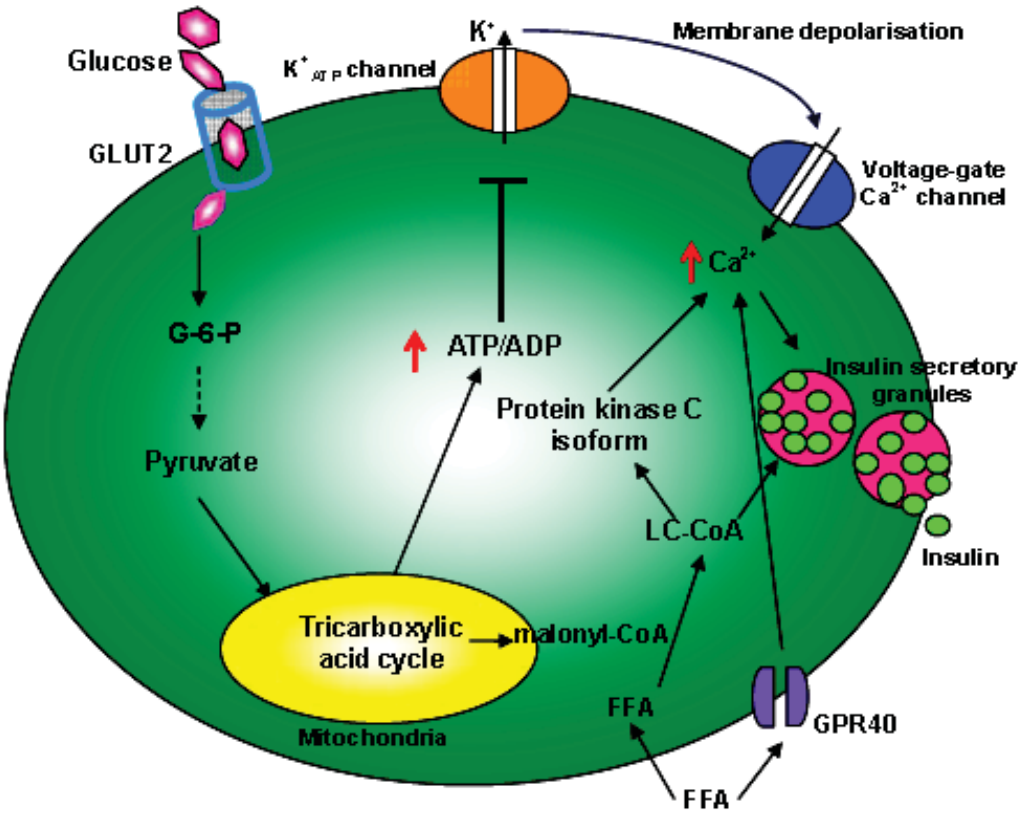

Figure 1.3 Glucose regulates insulin secretion. Glucose enters in the β-cell through the GLUT2 transporter. Glucose is metabolized through glycolysis and the tricarboxylic acid cycle to generate ATP, resulting in an increased intracellular ATP/ADP ratio, which causes closure of KATP-channels. This depolarizes the plasma membrane and increases calcium concentrations in the cytoplasm through voltage-gated calcium channels. The increased calcium causes insulin secretory granules to be released by exocytosis**.** Fatty acids influence insulin release by both directly activating GPR40 and through formation of malonyl-CoA to generate long chain acyl-CoAs (LC-CoA) which stimulate insulin granule exocytosis, either directly or through PKC-dependent mechanisms.

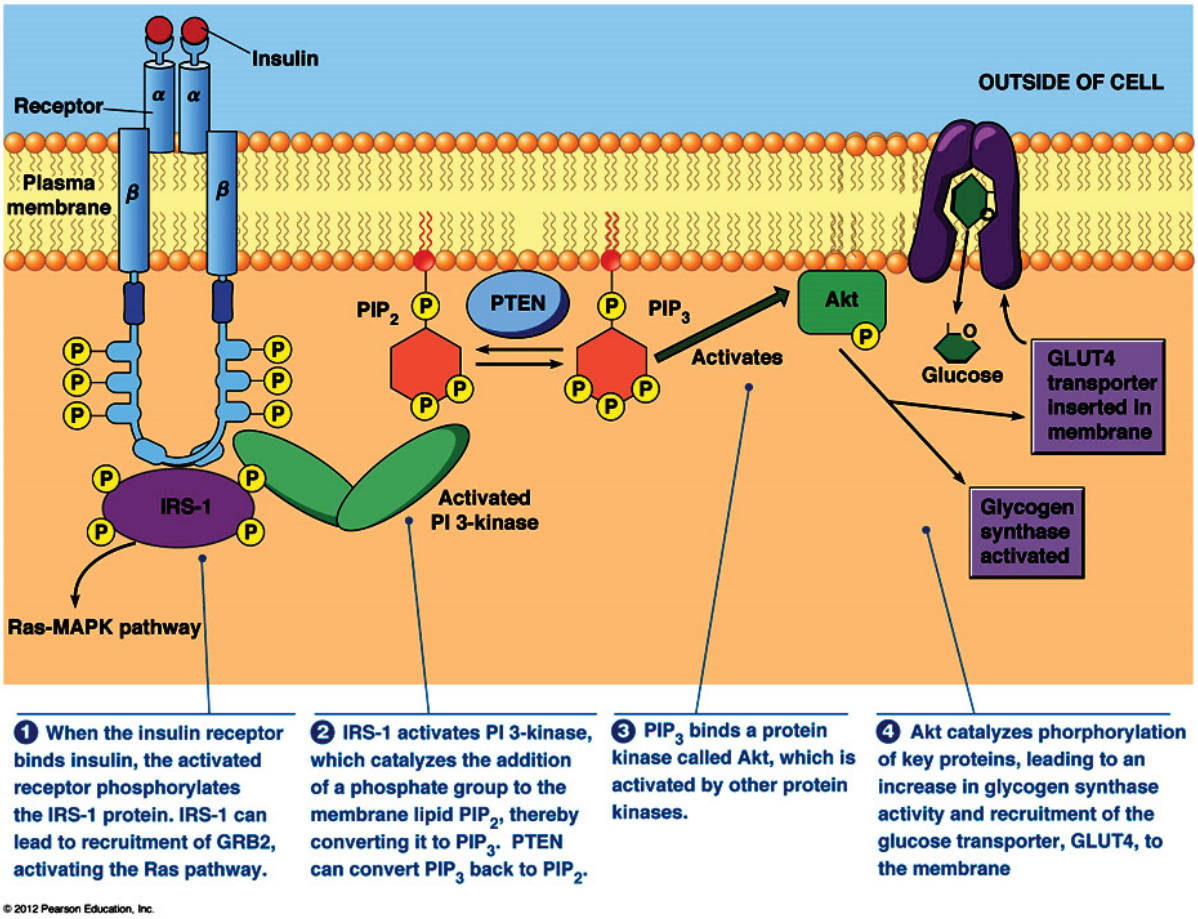

Figure 1.4 Insulin Signaling Cascade. The binding of insulin to its receptor activates the IRS proteins, this stimulates downstream pathways, resulting a diverse series of cellular processes. **[Adapted with permission from (613)]**

1.6 Insulin-like Growth Factor-I (IGF-I)

In recent years, new technologies have enabled many advances in the cascade of growth hormone axis. GH secretion from the anterior pituitary, is regulated by GH releasing hormone (GHRH) (96), somatostatin (GH secretion inhibiting hormone) and also by Ghrelin (97). They appear to act synergistically with GHRH by inhibiting somatostatin (98). The interplay between GHRH and somatostatin induces a pulsatile secretion of GH. GH induces the generation of insulin-like growth factor 1 (IGF-I, also called somatomedin 1) in the liver and regulates in many other tissues (99). In 1957 Salmon and Daughaday identified IGF-I and IGF-2 and designated "sulphation factor" by their ability to stimulate ^{35}-sulphate incorporation into rat cartilage. In 1972, this term was replaced by the term "somatomedin", denoting a substance under control and mediating the effects of GH (100). In 1976, Rinderknecht and Humbel isolated two active substances from human serum with structural similarity to proinsulin and were renamed as "insulin-like growth factor 1 and 2" (IGF- I and 2). IGF-I is the mediator of the anabolic and mitogenic activity of GH (101).

1.6.1 Chemical Structure:

The 7649 Da IGF-I molecule is 70-amino acids in length and contains three intra-chain disulfide bridges (102). Similar to insulin, IGF-I has an A (21 amino acids) and B chain (29 amino acids) connected by disulphide bonds. The C peptide region had 12 amino acids. The insulin-like proteins belong to a family of peptide hormones that include at least 9 different genes- two non-allelic insulin genes in rodents, *igf-I, igf-2*; relaxin; and four insulin-like peptides, *IGF-II to -6* (113). The human gene for IGF-I is 95kb and contains six exons on the long arm of chromosome 12 (12q23-23). The structural similarity to insulin explains the ability of IGF-I to bind to the insulin receptor with lower affinity. Multiple forms of IGF-I mRNA have been detected because of

alternative transcription initiation sites (103), alternative splicing (104) and alternative polyadenylation (105). On the other hand, IGF-II gene is located on chromosome 11 (Human) with 9 exons and 35kb in length (106). IGF-II is composed of a single polypeptide chain of 67 amino acids, with high degree of homology to IGF-I and proinsulin. IGF-II contains an N-terminal B domain (28 amino acids), C domain (12 amino acids), A domain (21 amino acids), and a D domain (6 amino acids) present at the carboxyl terminal. There are multiple variant IGF-II mRNAs present which arise through alternative splicing and the protein products account for 25% of circulating IGF-II levels; the significance of these variants is not clear (107) . IGF-II, similar to IGF-I, is synthesized as a precursor, with an E domain jutting off from the C- terminal; however, the E region is less conserved, and its function is unknown. Four promoters are located within the IGF-II gene in rodents (108). The presence of IGF-II differs between rodents and humans. In rodents, IGF-II expression diminishes after the first two weeks of life but in humans IGF-II is present throughout life at relatively high concentrations due to continuous activity of a specific promoter within the liver (109, 110).

1.6.2 IGF-binding proteins (IGF-BPs)

In the plasma, 99% of IGFs will form a complex with a family of binding proteins, which modulate the availability of free IGF-I to the tissues. There are six binding proteins (IGFBPs) in humans (111). The IGFs are bound by these well-characterized proteins with high affinity (Kd~10^{10}). However, upon proteolysis, the IGF binding is reduced with a concomitant increase in insulin binding as the insulin binding sites become revealed. These circulating IGFBPs act as 1) carrier proteins, 2) transport IGFs out of the circulation to target tissue, 3) prolong half-life of IGFs by protecting them from proteolytic degradation, 4) control the interactions between IGFs and their receptors and prevent hypoglycemia. The IGFBPs are cysteine-rich proteins (16-20 Cysteines).

The primary structure of IGFBPs have three domains- N-terminal domain with 80-93 amino acids, 58% structural similarity, contains 10-12 cysteine residues, highly structured to form 5-6 disulfide bonds and a local conserved motif GCCGCCxxC. The mid-region is 55-95 amino acids long, with only 15% similarity, works as a hinge and undergoes post-translational modifications (glycosylation and phosphorylation). The C-terminal is rather conserved with 34% similarity and form intradomain disulphide bonds through cysteine (6) residues (112).

The genes encoding the six IGFBP share a similar structure consisting of four exons-except *IGFBP3* which possess an additional 3' non-coding exon (113). Despite the general similarities of their structures, the different IGF binding proteins have distinct properties that might provide clues to their specificities. For example, IGFBP-1 and IGFBP-2 have Arg-Gly-Asp (RGD) sequences near their carboxyl termini that allow them to bind to cellular receptors (integrins) and extra-cellular matrix proteins (ECM). All the IGF binding proteins bind IGF-I and IGF-II with similar affinity, except for IGFBP-6 which has a marked preferential affinity for IGF-II.

IGFBP-1(25 kDal) is predominantly expressed in the liver and amniotic fluid and binds to IGF-I and IGF-II with equal affinity (114). Insulin is a positive regulator for IGF-I actions whereas IGFBP-1 usually does the opposite. **IGFBP-2** (32 kDal) is found in the postnatal stage at very high levels but decreases significantly in adults (115). Usually IGFBP-2 inhibits IGF-II actions, in some cells it may also stimulate IGF-I actions. **IGFBP-3** (46-53kDa) is N-glycosylated and is the most abundant binding protein in serum with commonly found in forming complexes. **IGFBP-4** (26kDa) is produced by bone cells, and inhibits IGF-I actions whereas **IGFBP-5** (29-31kDa) is expressed in almost all peripheral tissues and is the major IGFBP found in bone; it binds to fibroblast extracellular matrix (ECM) and increases DNA synthesis in presence of IGF-I. Recent data shows that IGFBP-5 is also capable of forming a complex with IGF and ALS (Somatomedin

Hypo 2001). **IGFBP-6** (28-34kDa) is present in serum and CSF and may have an anti-gonadotropic effect in the ovary (116).

In order to cross the circulation and reach its target tissues, IGFs will be dissociated from these large complexes to form smaller, 50kDa complexes (20-25% of total IGFs) with other IGFBPs (117). In addition to their major roles in the circulation, most target tissues also express IGFBPs, where they further regulate the local action of IGFs (118).

The roles of the six IGFBPs in modulating various IGF actions, such as cell proliferation, differentiation, survival, and migration, have been studied in a wide variety of cell types, including fibroblasts, osteoblasts, myoblasts, smooth muscle cells, breast, and prostate cancer cells (119,120). While IGFBP-4 and IGFBP-6 have been found to inhibit IGF actions, IGFBP-1, -2, -3, and -5 can both inhibit and potentiate IGF actions, depending on cell type, method of administration (adding exogenous protein, overexpression by transient or stable transfection, or depleting the endogenous proteins) and experimental conditions (121). For example, IGFBP-3 inhibits glucose uptake in fat cells, inhibits fibroblast proliferation, works in nuclear localization and binds to the RXRα receptor. IGFBP-3 is also found to significantly inhibit IGF-I-stimulated DNA synthesis in human skin fibroblasts when added together with IGF-I, but potentiated IGF-I actions when it was pre-incubated with those cells (122, 123). A recent study found increased IGFBP-3 involvement in insulin resistance and type 2 diabetes. IGFBP-5 has also been demonstrated to inhibit and/or potentiate IGF actions in cultured cells. In human fibroblast cells, IGFBP-5 bound to ECM and potentiated IGF-I- induced cell proliferation (124). Similarly, IGFBP-5 potentiated IGF-I-induced proliferation and migration in vascular smooth muscle cells (125). In kidney mesangial cells, however, intact IGFBP-5 inhibited IGF-I-induced cell chemotaxis, although an IGFBP-5 fragment had a stimulatory effect (126). Overexpression of

IGFBP-5 inhibits the growth of human breast cancer cells and murine myoblast differentiation (127). Recently, it was shown that the activities of IGFBP-5 can interact with a number of other proteins and these protein–protein interactions may determine its action in defined tissues. For instance, binding of IGFBP-5 with fibronectin abolishes the effect of IGFBP-5 in potentiating IGF-I-induced cell migration (128).

1.6.3 The IGF-I receptor

The human IGF-I receptor (type 1 receptor) is the product of a single gene spanning over 100 kb of genomic DNA at the end of the long arm of chromosome 15q25–26 (129). The gene contains 21 exons and its organization resembles that of the structurally related insulin receptor (Figure 1.6) (130). The type-1 IGF receptor gene is expressed in almost all tissues and cell types during embryogenesis (129). In the liver, the organ with the highest IGF-I ligand expression, IGF-I receptor mRNA is almost undetectable, possibly because of the down-regulation of the receptor by the local production of IGF-I.

The structure of the IGF-2 receptor is a type I transmembrane protein (domain with its C-terminus on the cytoplasmic side of lipid membranes) with a large extracellular/lumenal domain and a short cytoplasmic tail (129). IGF2 receptor functions to clear IGF2 from the cell surface to attenuate signaling, and to transport lysosomal acid hydrolase precursors from the Golgi apparatus to the lysosome. IGF2 receptors are accumulated in forming clathrin-coated vesicles and internalized after binding IGF2 at the cell surface (129).

1.6.4 Comparison between IGF-I and Insulin

Similarities: IGF-I can mimic all the actions of insulin. The A and B domain of IGF-I has almost 45% homology with insulin A and B chains. The folding pattern in the IGF-I 3D structure is

identical with that of proinsulin. There is also 50-60% overall structural homology between IGF-I and insulin receptor.

Differences: IGF-I has one chain with C and D domains whereas insulin has 2 chains without C and D domains. IGF-I is produced by the liver and many other tissues; in the case of insulin, it is produced from pancreatic islet β-cells. The physiological stimulus for IGF-I is growth hormone and the secretion pattern is constant and slow but glucose is the stimulus of insulin whereas the insulin secretion pattern is emiocytosis and pulsatile. IGF-I has 6 binding proteins (IGFBP 1-6) but insulin has none. Regarding the mode of action, IGF-I can be endo-, auto-, and paracrine whereas insulin is only endocrine.

1.6.5 Comparison between IGF-IR and IR

Not only is there considerable structural similarity between the IGF ligands (IGF-I and IGF-II) and insulin but there also is significant similarity between the IGF-1R and the IR, resulting in cross-talk between the two systems (134). The IGF-1R and IR are transmembrane tyrosine kinase receptors that are both synthesized as a precursor that is glycosylated on the extracellular regions, dimerized and proteolytically processed to yield separate α and β chains (138). Two extracellular α subunits (~135 kDa each) and two membrane spanning β subunits (95 kDa each) form a β-α-α-β arrangement (32–34). The domain organization of the receptors is reviewed in (31, 35, 36) and is shown in (Figure 1.5). The major ligand binding determinants are located within the α-subunits (139-41) and the intrinsic tyrosine kinase domain is located in the cytoplasmic portions of the β subunits (39). Ligand binding to the extracellular region causes a conformational change resulting in tyrosine phosphorylation of the intracellular β subunits, which then causes an increase in the intrinsic kinase activity of the receptor (142).

IGF-I, IGF-II and insulin bind to the IGF-1R and activate its intrinsic tyrosine kinase activity with different potencies. A variety of responses such as cell proliferation, differentiation, migration and

protection from apoptosis can result from activation of the IGF-1R (132,133). The insulin receptor exon 11+ (IR-B) isoform is the best known form for the classic metabolic responses induced upon insulin binding and this isoform has low affinity for the IGFs. However, it has recently been shown that IGF-II binds with high affinity to the insulin receptor exon 11-(IR-A) isoform of the IR. This isoform arises from alternative splicing of exon 11 encoded by the IR gene (135, 136). Activation of IR-A leads to mitogenic responses similar to those described for the IGF-1R. Up regulation of IR-A and IGF-II expression in fetal and cancer tissues has led to the hypothesis that the IR-A isoform plays a role in development and cancer (135–37). In addition, functional hybrid receptors can form between the IGF-1R and either the IR-A or IR-B, but their roles in cellular responses remain unclear and present a challenge to IGF/insulin researchers.

1.6.6 IGF on metabolic regulation:

IGF-I is structurally similar to insulin, and so it is not surprising that insulin and IGF-I have similar effects in regards to metabolism. In general, IGF-I has insulin-like effects, such as increasing glucose uptake and glycogen synthesis (144), and inhibiting hepatic glucose production (145). Although IGF-I has approximately one-thirteenth the potency of insulin (146), administering IGF-I to normal rats (147) or humans (148) resulted in increased peripheral glucose uptake, increased glycolysis and glycogen synthesis, and caused a slight decrease on hepatic glucose production; all these effects resulted in hypoglycaemia. The insulin receptor is expressed in all the major tissues involved in carbohydrate metabolism, while the IGF-IR is only abundant in skeletal muscle, with lowered expression in the other two tissues. It is therefore very likely that the hypoglycaemic effects of IGF-I are mediated through activation of the insulin receptor. In fact, *in vitro* studies on human and animal tissues have shown that direct action of IGF-I on the IGF-IR stimulates glucose uptake in skeletal muscle via the PI-3K pathway (144). Nevertheless, neither a

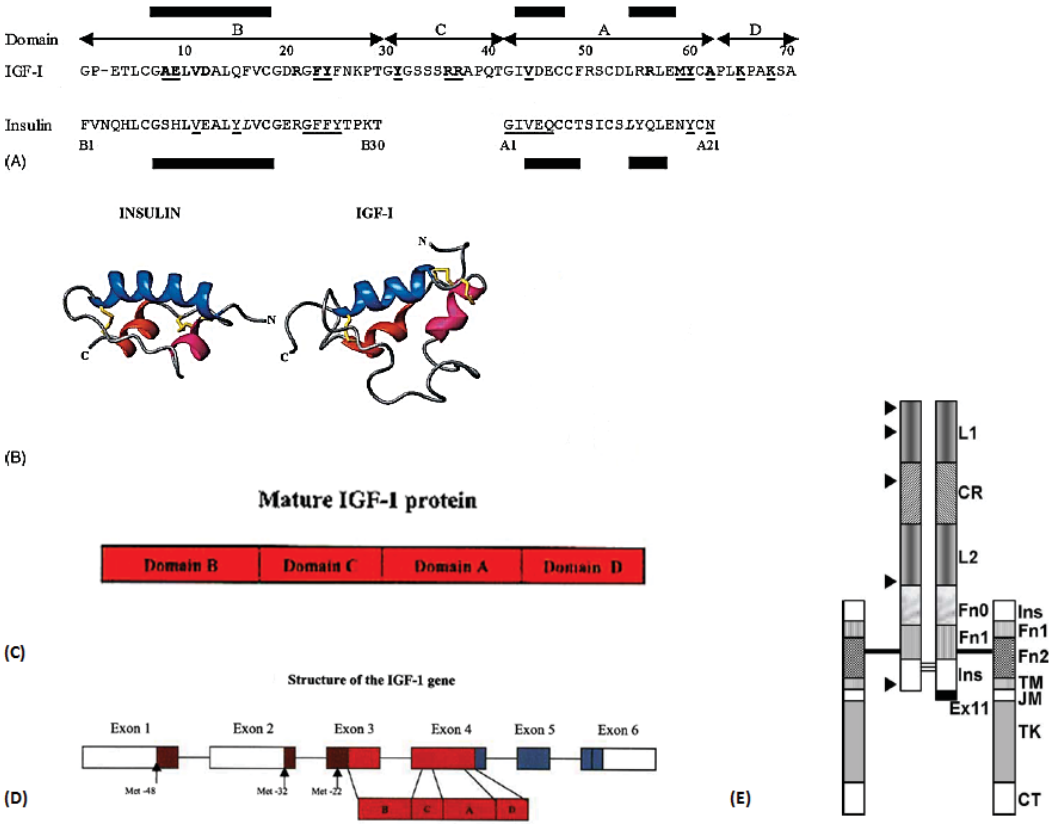

Figure 1.5 IGF-I, insulin and receptors (A) **Sequence alignment** highlighting domains and helices (boxes). IGF-I residue which has ≥5-fold (bold and underlined) or ≥2 (bold) lower IGF-1R binding affinity are shown. Insulin residues in the classic site 1 (underlined) or site 2 binding sites (italics) are also indicated. (B) **Ribbon structures of IGF-I and insulin** showing the B domain helix 1(blue), A domain helix 2 (pink) and A domain helix 3 (red). (C) **Domains of the mature IGF-1 protein**; (D) **Structure of the IGF-1 gene.** Regions that code for the mature IGF-1 protein are shown in red. Contributions of exons 3 and 4 to the mature peptide are indicated by lines connecting the mature peptide shown again below the gene. Parts of exons 1 and 2, which are present in the different signal peptides, are shown in dark red. Positions of the translation initiation codons are indicated by arrows. Parts of exons 4, 5, and 6, which contribute to the distinct E-peptides, are blue. (E) **Domain structure of the IGF-1R and IR.** The heterotetrameric IGF- 1R and IR share the same domain structure. L1 and L2, large domains 1 and 2 (leucine-rich repeats); CR, cys-rich domain; Fn0, Fn1, Fn2, fibronectin type III domains; Ins, insert domain; Ex11, residues encoded by exon11 of the IR; TM, transmembrane; JM, juxtamembrane; TK, tyrosine kinase and CT, C-terminal domains. Arrows indicate ligand binding regions. **[Adopted with permission from (616)]**

global deletion of the *igf-1* gene or a liver-specific *igf-1* gene deficiency in mice resulted in extreme glucose intolerance, indicating that IGF-I is not necessary for normal glucose regulation. The importance of IGF-I in energy homeostasis may be due to increase insulin sensitivity. For example, the liver-specific IGF-I deficient mice were glucose tolerant and found to have normal blood glucose levels during fasting, but became hyperinsulinemic and insulin-resistant (149, 150). Administering IGF-I normalized insulin levels and restored a normal insulin response in these mice, suggesting that IGF-I may be important for maintaining insulin sensitivity. However, a general overexpression of IGF-I in mice led to no effect on insulin secretion or insulin sensitivity, despite significant hypoglycaemia, hypoinsulinemia, and an improved glucose tolerance (505).

1.6.7 IGF-I and Pancreatic islets:

IGF-I and IGF-IR are expressed in all the major cell types (α-, β-, and δ) in islets of the pancreas in local autocrine/paracrine mode of action (152). IGF-I induces DNA synthesis in a glucose dependent manner in islet cells (153-156). Inactivation of the IGF-IR gene in islet β-cells decreases GLUT-2 and glucokinase expression leading to decreased glucose stimulated insulin secretion (162-163).

IGF-I also plays a direct role in increasing β-cell mass (152). IRS-2 plays an important role in the maintenance of a normal β-cell population (157), via activation of PI3K and AKT (158). Increased IRS-2 *in vitro* promotes β-cell replication, neogenesis and survival and decreased IRS-2 induces β-cell apoptosis (159). *In vivo* overexpression of IRS-2 promotes β-cell survival (160) while IRS-2 knockout mice exhibit a decrease in β-cell mass. Islet-specific IGF-I overexpression promotes islet cell regeneration in diabetic mice (162). IGF-I also shows anti apoptotic activity. Administration of IGF-I prevents Fas-mediated autoimmune β-cell destruction and delays the onset of diabetes in non-obese diabetic (NOD) mice by minimizing the insulitis (154,155,164,

165). The IGF-I-treated animals show a greater percentage of intact islets (49% vs. 2%), a decrease of lymphocytic infiltrated islets and an increased β-cell mass. The anti-apoptotic effect involves PI3K activation and phosphorylation of AKT (166). Overexpression of IGF-I specifically in pancreatic β-cells again led to animals with normal islets and β-cell mass under normal conditions, but displayed an increase in β-cell mass in response to streptozotocin treatment, as a result of increased β-cell neogenesis and decreased β-cell apoptosis (168). These results indicate that IGF-I may have a protective role in the β-cell, by activating neogenesis in response to β-cell damage (502).

1.7 Transgenic mouse model for functional analysis of IGF-1

The classical method for generating transgenic mice is the pronuclear injection. The transgene is injected into a fertilized mouse egg and integrated at random positions in the genome. The success rate of the method improved significantly as different companies developed innovative ES cell-based technology that significantly reduced the risks associated with the development of transgenic mouse lines by DNA pronuclear random integration. This approach allows the generation of a high number of founders with validated characteristics (no truncated transgene and low, medium or high transgene copy number). In MT-IGF mice, IGF-I is overexpressed under metallothionein promoter. For whole body IGF-I expression, a fusion gene pMIG65 containing a mouse metallothionein-I promoter, rat somatostatin sequence (to ensure secretion), hIGF-I-A cDNA and the 3' end of hGH ribonucleic acid (RNA) processing elements is constructed. This construct is stably expressed *in vitro* baby in hamster kidney cells and verified by 1) Northern blot to check transcription and correct RNA, 2) by Western blot to identify the secreted human IGF-1 peptide with correct cleavage. *KpnI-EcoRI* containing restriction fragment was obtained from the vector and injected into the pronuclei of fertilized eggs and implanted into

foster mothers and selection of the pups containing the foreign DNA is determined by Laser dot hybridization (169).

MT-IGF mice were first constructed 2 decades ago with IGF-I level increased by 2-3 fold at about 3 weeks after birth (505). More recent studies showed that IGF-I overexpressed mice demonstrated a small but significant (~1.4-fold) increase in the body weight at 2 months of age (505). Moreover, overexpressed IGF-I resulted in selective organomegaly in the spleen and pancreas (2.0- and 1.8-fold weight increases, respectively). There were no apparent differences in the weight of the major fat pads. Most of the organs of MT-IGF mice didn't exhibit any gross anatomical lesions except the kidneys with larger glomeruli with mild red cell hematopoiesis and the skin with microscopic abnormalities such as a thickened adipose layer and disrupted collagen bundles. The serum was analyzed and protein, albumin, and cholesterol were normal but triglycerides were elevated and insulin is reduced (170). However, the resulting level of IGF-I mRNA was 31-fold higher in the pancreas than in the liver; IGF-I content was increased 5,200-fold in the pancreas, with the absolute content 344-fold higher than in the liver (34). IGF-I overexpression resulted in significant hypoglycemia, hypoinsulinemia, and improved glucose tolerance but normal insulin secretion and sensitivity, and IGF-I overexpression significantly suppressed hepatic gluconeogenesis. Finally, MT-IGF mice were significantly resistant to streptozotocin-induced diabetes, with diminished hyperglycemia and with the prevention of weight loss and death- clearly showing an antidiabetic effect by improving islet cell survival (170). When MT-IGF mice were crossed with GH-deficient mice, the offspring exhibited increased organ and bone growth (171) as excess IGF-I fully compensated for the absence of GH at the level of the target tissue.

Another approach is to use the conditional knockouts using the *Cre-loxp* system to provide

new insights into the function of circulating IGF-I. This approach is highly recommended for studying growth and development specific gene of mouse. Furthermore, due to the compensatory mechanism, up-regulation of any gene with similar function may obscure the loss of function of the gene of interest. Thus, the conditional knockout at a target specific tissue provides tremendous scope to overcome the problem.

1.8 Pancreatic β-cell, Apoptosis and Diabetes

Apoptosis (programmed cell death) is an evolutionarily conserved energy-dependent, genetically regulated cell suicide process. Upon receiving an apoptotic signal, the cell uses its machinery to undergo a highly orchestrated and characteristic process with the commitment to die. In multicellular organisms, this process plays a critical role in all stages of life, from development, where apoptosis is important for tissue sculpting and remodeling, to maintenance of homeostasis during physiological turnover and the elimination of unwanted or harmful cells throughout the life of the organism. Evidence from both human disease and mouse models has shown that beta cell death in both type1 diabetes and type 2 diabetes occurs by apoptosis. Beta cell apoptosis is difficult to detect *in vivo*, due to the slow kinetics of the inflammatory process, rapid turnover and clearance of apoptotic cell debris by host macrophages and neighboring cells. Nevertheless, apoptotic beta cells have been detected *in situ* morphologically and by TUNEL and Ki67 staining in several studies (172-176).

The pancreatic β-cell mass is dynamic and tightly balanced to meet the body's demand for insulin (176). During the neonatal period, the β-cell population increases rapidly, and this burst in expansion of β-cell mass is followed by a transient increase in β-cell death (177, 178). With increasing age, the rates of β-cell apoptosis and proliferation and/or neogenesis equilibrate under steady-state conditions (179). In diabetes, a combination of genetic and environmental influences causes the balance to fail. Despite major advances in recent years, the nature of the gene–

environment interactions that promote β-cell apoptosis in diabetes remain unclear, as do many aspects of the apoptotic pathways involved. So far, researchers have found three pathways for islet β-cell apoptosis: The extrinsic pathway, the intrinsic pathway, and the ER stress pathway.

Apoptosis in Type 1 Diabetes: Type 1 diabetes is a chronic autoimmune disease that affects 0.5% of the population in the developed world and continues to increase in incidence (180). This disease is hallmarked by immune-mediated destruction of the pancreatic β-cells. Type 1 diabetics have an estimated 60–80% reduction in β-cell mass at diagnosis (181). The trigger for this destruction and the exact mechanisms of β-cell death remain enigmatic. It has been suggested that a perinatal wave of β-cell apoptosis may promote the presentation of β-cell auto-antigens and thus provoke an autoimmune response against β-cell. The current working model is that in genetically predisposed individuals, T lymphocytes are aberrantly activated by the antigen-presenting cells (APCs) in the pancreatic draining lymph nodes (Figure 1.6). The activated T lymphocytes then circulate, target, and invade the islets. These activated T cells can then proceed to destroy the islets (180). Recent genome-wide association studies have identified several single nucleotide polymorphisms that contribute modest risk of type 1 diabetes. Most of these genes target immune cells and were demonstrated to modulate pancreatic β-cell apoptosis. Thus, genes responsible for the type 1 diabetes may also affect β-cell death directly. The mechanisms by which β-cell are selectively killed by the immune system involves multiple pathways such as activation of "death receptors"-FAS and tumor necrosis factor receptor, perforin and granzyme B induced granule-mediated cytotoxicity, CD8+ cytotoxic T cells activation, cytokine-induced cell death and oxidative stress.

Apoptosis in Type 2 Diabetes: Both functional defects and decreased β-cell mass contribute to β-cell failure in type 2 diabetes (T2D). Pancreas sections from human organ donors and autopsy material have shown apoptosis, detected by TUNEL staining which is the sign of β-cell death in

T2D (173). Apoptosis is evident in pancreata from both lean and obese T2D patients (173). Although the primary cause of this apoptosis is not yet clear, islet amyloid polypeptide, glucose and palmitate have been proposed to induce cellular stress through activation of endoplasmic reticulum (ER) stress, oxidative stress or NLRP3 inflammasome activation and IL-1β production (Fig 1.6). These stresses induce the intrinsic apoptosis pathway through activation of Bcl-2 family molecules. IAPP has also been shown to directly activate the extrinsic apoptosis pathway.

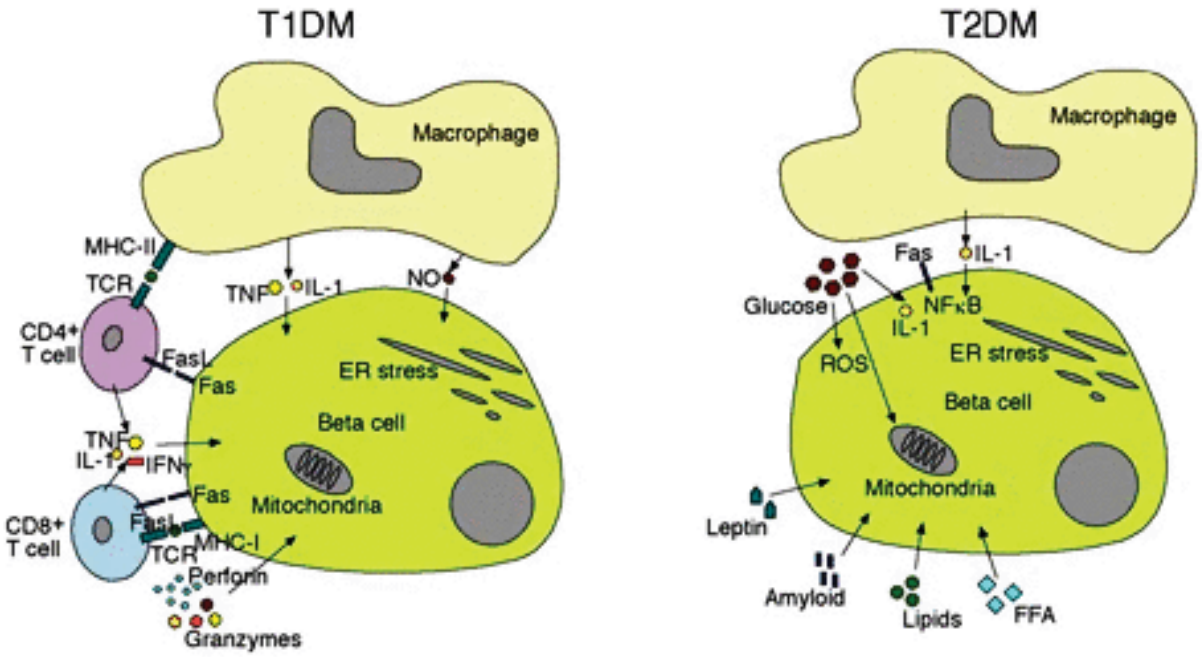

Figure 1.6 β-cell apoptosis in type 1 and type 2 diabetes. The different cellular and molecular mediators of the beta cell apoptosis are shown for T1DM, T2DM. **[Adapted from (615) with permission]**

Recent studies have shown the evidence of a new protein family expression in early pancreatic islet development, named as CCN2, a member of CCN (connective tissue growth factor/cysteine-rich 61/nephroblastoma overexpressed) or WISP (Wnt1 inducible signaling pathway) family proteins. In next section, we will briefly discuss about this protein family together with the regulatory mechanism and potential function in pancreatic islet development, proliferation and survival.

1.9 CCN

CCN is an acronym that is derived from Cyr61 (connective tissue growth factor in human), CTGF (cysteine rich 61, in mouse), and Nov (nephroblastoma overexpressed, in chicken), the names originally given to the first three proteins to be identified in what is now a six member family. The later three proteins, ELM-1/WISP-1, rCop-1/WISP-2/CTGF-L/HICP and WISP-3 were reported to have shared structural identity with Cyr61, CTGF and Nov and therefore belong to the CCN family of proteins (174-177). A consensus was reached to propose a unifying nomenclature for the CCN family, numbering the proteins CCN1-CCN6 (Table: 1) in the order in which they were first described in the literature (178). These proteins comprise a fascinating and important group of homologous proteins that have not received the attention that they deserve from cell and molecular biologists.

1.9.1 Structural aspects of CCN proteins

A prototypical CCN protein contains an N-terminal secretory signal peptide and four functional domains: (i) an insulin-like growth factor binding protein-like module (IGFBP); (ii) a von Willebrand factor type C repeat module (VWC); (iii) a thrombospondin type-1 repeat module (TSP-1); and (iv) a cysteine-knot-containing module (CT) (Figure 1.7).

1.9.1.1 The Cysteine knot (CT):

C-terminal cysteine knot domain. It has a highly conserved pattern of cysteines consistent with a six membered cysteine knot similar to several cytokines (e.g. nerve growth factor, TGF-βs, PDGFs) and hormones (e.g. luteinizing hormone, chorionic gonadotropin, thyroid-stimulating hormone, follicle stimulating hormone). In this unusual structural arrangement (Figure 1.7), two disulfide bridges and their connecting backbone sections that are typically built out of two pairs of antiparallel beta-strands form a loop through which a third disulfide bond passes (179). It occurs in many peptides and proteins among divergent species and provides considerable structural stability.

1.9.1.2 IGFBP domain:

The IGFBP domain of the CCN proteins typically contains 11 of the 12 conserved cysteine residues that are also found in the amino-terminus of IGFBP-1 to -5 suggesting that this sequence might account for an IGF binding activity (357). The characteristic conserved N-terminal cysteine pattern of the IGFBPs (GC(G/S)CCXXCAXXXXXXC) is included in all human CCNs. In line with this hypothesis, affinity-labeling and ligand-blotting studies demonstrated weak IGF-binding affinity for at least two members of the CCN family, i.e. CCN2/CTGF (358) and CCN3/NOV (359).

1.9.1.3 The thrombospondin type I homology domain (TSP):

The thrombospondin repeat domain is a ~55 residue consensus sequence that contains repeats of three distinct domains within a primarily linear structure. The TSP-1 domain binds extracellular targets and important signaling molecules including collagen V, fibronectin, TGF-β and heparin.

They act by bringing together cytokines, growth factors, matrix components, membrane receptors, and extracellular proteases (360). Thrombospondin-1 is a trimeric 420-kDa protein, composed of multiple domains including N- and C-terminal globular domains, a procollagen-like domain, and three types of repeated sequence motifs (type 1, 2, and 3 repeats). Type 1 repeats have a typical three dimensional fold (360) that is also found in many other proteins that mediate cell attachment, glycosaminoglycan binding, are involved in activation of transforming growth factor-beta (TGF-β) and inhibition of matrix metalloproteinases. Typically, proteins of this class are secreted or are transmembrane proteins in which this motif is found in the extracellular portion.

1.9.1.4 Von Willebrand factor C repeat domains (VWFC):

also known to as Chordin-like cysteine-rich (CR) repeats found in >500 extracellular matrix proteins. This repeat typically comprises ~70–100 amino acids with ten conserved cysteine residues and a pair of cysteine-containing motifs located in the sequence. The proteins that contain such domains have the capacity to participate in numerous different biological processes (e.g. cell adhesion, migration, homing, pattern formation, and signal transduction) that require modular features allowing interaction with a large array of different ligands. In the VWF this domain is thought to participate in oligomerization, but not necessarily to be involved in the initial dimerization step (361). Interestingly, in several other proteins (e.g. noggin, follistatin, members of the Dan protein family, chordin-like proteins), this domain is known to bind several bone morphogenetic proteins (BMP) members (362).

Apart from CCN5, all other CCN proteins share a closely related primary structure of 38 cysteine residues in strictly conserved position. The 38 cysteine residues represent almost 10% of the CCN molecule by mass. A short sequence, with varied composition and length among the

CCN family members, is located directly after the VWC domain and acts as hinge between the first and second half of the molecule (363, 364).

Based on the presence of four distinct structural modules covering nearly the whole molecule and the location of exons and introns within the CCN genes, it was suggested that CCN family members are genuine mosaic proteins. The existence of four potentially functional domains in these proteins raised fundamental questions about their contribution to the various biological properties of the CCN proteins (Table 2). It is now accepted that each of the four modules act both independently and interdependently, and that the multi-modular structure of the CCN proteins provide the basis for a wide range of interactions with different partners. Recent data have shown that each of the linker regions that separate the four CCN modules is susceptible to proteolysis (365). Cleavage at these sites might generate truncated molecules and individual modules (366) with distinct biological properties. Moreover, these events might constitute an additional process to regulate the biological activity of the CCN proteins (367, 368).

Table 1. Nomenclature of the CCN family members

CCN nomenclature	Current names
CCN1	CYR61/CEF10/βIG-M1/IGFBP9/IGFBP-rP4
CCN2	CTGF/FISP12/Hcs24/ βIG-M2/HBGF-0.8/ecogenin/ IGFBP8/IGFBP-rP2
CCN3	NOV/IGFBP9/IGFBP-rP3
CCN4	ELM-1/WISP-1
CCN5	rCOP-1/WISP-2/CTGF-L/HICP
CCN6	WISP-3

Abbreviations: Cyr61, cysteine rich 61; CTGF-2, connective tissue growth factor 2; IGFBP-rP2, IGFBP-related protein 2; NOV, nephroblastoma overexpressed gene; HBGF-0.8, heparin-binding growth factor 0.8; Wisp, Wnt-inducible secreted protein; Elm-1, expressed in low metastatic cells; HICP, heparin-induced CCN-like protein; Cop-1 card-only protein 1.

Table 2: Major biological functions of CCN proteins

Function	**Effects of *CCN* expression alterations *in vivo***	**Related activities *in vitro***	**Citation**
Aniogenesis, cardiovascular development	CCN1, CCN2, CCN3 stimulate blood vessel growth in corneal implants, or in ischemic hindlimb; CCN1-null mice suffer embryonic lethality with placental vascular deficiency, Embryonic vessel hemorrhage and atrioventricular septal defects; CCN1+/− mice are viable but exhibit ostium primum atrial septal defect	CCNs promote pro-angiogenic activity in microvascular endothelial cells: support cell adhesion, stimulate cell migration, enhance proliferation and survival, induce endothelial tubule formation	617-619, 638-642
Skeletal Development	*CCN2*-null mice are perinatal lethal, showing severe chondrodisplasia, deficient ECM production in cartilage, impaired endochondrial ossification and reduced growth plate angiogenesis; *CCN3* mutant mice show axial and appendicular skeletal defects and severe joint malformation; *CCN2* and *CCN3* overexpression in osteoblasts leads to osteopenia in mice	CCN1 promotes chondrogenesis in micromass cultures; CCN2 promotes chondrocyte proliferation and differentiation, synthesis of collagen and aggrecan, and osteogenic differentiation; CCN2 binds BMP4 and inhibits its function; CCN3 binds BMP2 and inhibits BMP2-induced osteogenic differentiation	501,620,621, 622-624.
Cell Survival	CCN1-null mice show aberrant apoptosis in vascular cells of largearteries and mesenchymal cells of the cardiac cushion tissue	CCN proteins promote endothelial cell survival	612,613,615, 618,619.
Apoptosis	CCN1 knockin mice expressing an apoptosis-defective allele in place of wild type CCN1 are resistant to TNFα-induced apoptosis	CCN can unmask the cytotoxicity of TNFα by inducing ROS accumulation through 5-lipoxygenase and the mitochondria	625
Cancer	CCN1 overexpression in gastric cancer cells, breast cancer cells and ovarian cancer cells enhances tumorigenicity in nude mice. Ectopic or stromal expression of CCN2 promotes tumorigenicity of esophageal squamous cellcarcinoma or prostate cancer cells, respectively; CCN2 antibody treatment suppresses pancreatic tumor growth and breast cancer osteolytic bone metastasis; expression of CCN1 inhibits tumorigenicity of non-small cell lung carcinoma cells in nude mice, and CCN2 expression suppresses metastasis of human lung adenocarcinoma cells and colorectal cancer cells; expression of CCN4 or CCN5 inhibits tumorigenicity of murine melanoma cells or transformed fibroblasts, respectively	CCNs promote angiogenesis, enhance cancer cell proliferation and resistance to apoptosis	616,626-635.
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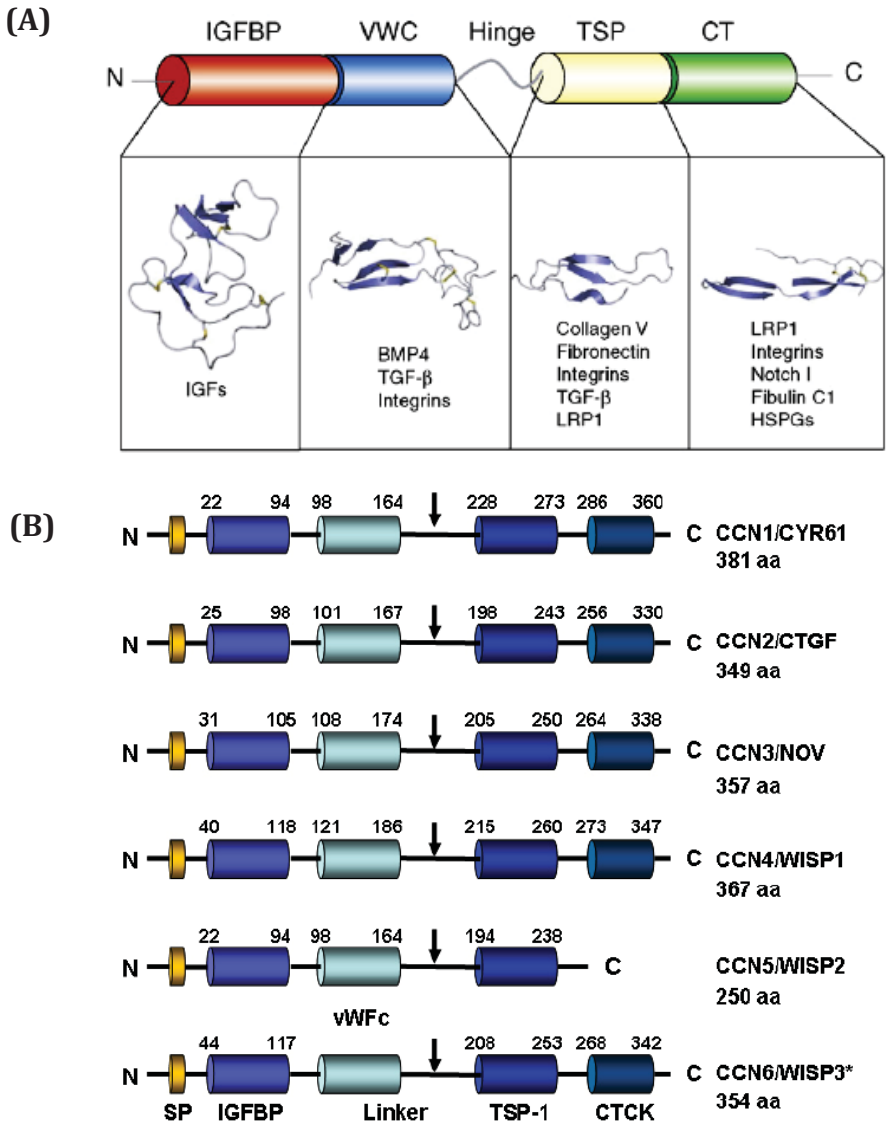

Figure 1.7. (A) Arrangement of CCN domains. A diagram showing the signal peptide (SP), insulin-like growth factor binding domain (IGFBP) in red, Von Willebrand factor C repeat (VWC) in blue, thrombospondin type-1 repeat (TSP-1) in yellow and cysteine knot (CT) in green. The protein is split into two halves separated by a variable 'hinge' region. Some of the known binding partners of each module are also listed: insulin-like growth factors (IGFs); bone morphogenic protein 4 (BMP4); transforming growth factor b (TGF-b); LDL receptor protein 1 (LRP-1); and heparin sulphated proteoglycans (HSPGs). (B) **Structure of human CCN proteins**. In the schematic representation indicated are the locations of individual domains. The identification of individual domains was performed using the PROSITE software (release 20.66) at the ExPASy Proteomics Server. [**Adapted from (605) with permission]**

1.9.2 Functions of CCN family:

The expression of CCN proteins are tightly controlled both temporally and spatially in a number of embryonic and adult tissues. Major sites of expression include the nervous system, the musculoskeletal system and the blood vessels. The structural homology among the CCN family suggested they might have similar or redundant functions. Some recent studies have suggested that functional overlaps result from the presence of common structural motifs among various members. Thus, the CCN proteins are now being defined as a novel family of growth and differentiation regulators acting on a large variety of cell types. Despite their similar structural similarity, the CCN proteins also show significant functional divergence. They exhibit many biological functions that are highly dependent upon the type of cells and the cellular context. Some of the functions depend upon multiple interactions with a variety of regulatory molecules. These proteins are known to be involved in various biological processes such as normal growth and development, wound healing, tissue regeneration and uterine function, fibrosis in various tissues/organs, inflammation such as arthritis and tumor growth (**Table 2**) (352, 353, 354, 355, 374, 375, 376). CCN1 and 2 are involved in the cell cycle process by regulating the immediate early genes, whereas CCN3-5 are putative growth arrest/suppression genes (**Table 2**). CCN proteins are also involved in cell adhesion and migration, cell differentiation, production of ECM components and their degrading enzymes (377), suggesting their involvement in tissue remodeling. Their abilities to bind ECM components, various growth factors, and receptors in a way that can result in modifying their actions (378, 379) suggest that the CCN proteins form multifunctional regulatory complexes as matricellular proteins (matricellular proteins are dynamically expressed non-structural proteins, present in ECM). The multiple biological functions of the CCN proteins are exerted through various signaling pathways involving cell-surface receptors such as integrins (352,

380), 240-kDa protein (353, 388), LRPs (380, 382, 386), Notch (387), as well as connexins and calcium channels (383, 384, 385). The regulation of intracellular calcium concentration by CCN3 and CCN2 has established these proteins as genuine signaling factors. CCN proteins have been proposed to coordinate signaling pathways governing intercellular and intracellular communication needed for efficient control of cell growth, growth arrest and differentiation (375, 381).

1.9.3 History of CCN5:

The same gene has been discovered several times independently. In 1997, Delmolino and Castellot first reported the discovery of CCN5 gene that has features of a growth arrest gene (389) which was first named *hicp*, and then re-named as r*Cop-1*. Through subtractive hybridization, they compared vascular smooth muscle cells treated by heparin to chrondroitin sulfate treated VSMC, which is similar to heparin, but lacks its antiproliferative activity. The screen showed 16 up- and 25 down-regulated genes. One of the up-regulated clones was homologous to members of the CCN family. After the discovery of CCN5 the same group investigated the antiproliferative properties and found that knock down of CCN5 decreased the antiproliferative effect caused by heparin, indicating a role for CCN5 in heparin signaling. Its human ortholog was identified later from the wnt-1 transformed mouse mammary epithelial cell line, C57MG, as encoding a Wnt-induced secreted protein (Wisp-2) (390). The same orthologue was isolated again as a gene encoding a CTGF-L from human osteoblasts (391). Heparin-induced CTGF-like protein isolated from heparin-treated vascular smooth muscle cells is also identical to CCN5 (392).

Independently, another group identified a gene, rCOP-1, as being down-regulated following transformation of rat embryo fibroblasts by inactivation of p53 and concomitant

expression of a constitutively active H-ras (392). rCop-1 (CCN5) was found to be located primarily on the surface and within the cytoplasm of rat embryonic fibroblasts.

A fourth group analyzed a human osteoblast cDNA library and identified an EST with an IGF binding domain and sequence homology to CTGF (CCN2), named the presumed gene product CTGF-like protein or CTGF-L (391). CTGF-L (CCN5) mRNA was found in primary cultures of human osteoblasts, fibroblasts, ovary, testes, and heart. Functional studies showed that the endogenous CTGF-L protein can bind to IGF-1 and IGF-2 and recombinant CTGF-L protein has the ability to promote the adhesion of osteoblasts, inhibit the binding of fibrinogen to purified integrin receptors and inhibit the production of osteocalcin by rat osteoblast-like Ros 17/2.8 cells. These results indicate that CTGF-L plays an important role in regulation of bone turnover (391).

1.9.4 CCN5 Gene location, mRNA structure and Promoter:

The human CCN5 gene is mapped to chromosome 20 at position 20q12-q13.1. The other five known CCN genes are located on different chromosomes (chromosomes 1, 6, and 8). CCN5 has four exons of 96, 216, 254, and 842 bp separated by three introns. Several different CCN5 cDNA clones have been sequenced and taken together strongly suggest that the processed mRNA sequence includes 1708 nucleotides, including a 261 nucleotide 5' UTR, the 750 nucleotide ORF encoding the 250 amino acid residues, a 730 nucleotide 3' UTR beginning with a TAA stop codon at nucleotide 1011, and an mRNA poly-A addition consensus site at nucleotide 1710 (392, 393). The rat and human CCN5 amino acid sequences share ~74% similarity whereas mouse and human CCN5 has 73% similarity (391). The CCN5 polypeptide sequence includes the IGFBP, VWC, and TSP1 modules that are homologous to the same modules in the 4-domain CCN family proteins. Approximately 3kb of genomic sequence upstream from the CCN5 translation start site has been analyzed. This region includes three TATA box motifs, a CCAAT box, AP-1, AP-2, and AP-4 recognition sites; motifs for MyoD, motifs for heat shock factors, and stress response elements

(recognized by p53). The presence of stress response elements supports the designation of CCN5 as a growth arrest gene that is expressed as part of the rescue response of cells subjected to potentially lethal environmental influences.

1.9.5 Analysis of CCN5 Protein:

CCN5 is a secreted protein with 251 amino acids and molecular weight of approximately 28kDa. The reported molecular weights vary from 28 kDa (in growth arrested VSMC conditioned medium and cell lysates made from AdCCN5-transfected VSMC; 394), 26 kDa (in conditioned medium from osteoblasts; 391), 28 kDa (in estrogen treated MCF7 cell lysates, conditioned medium, and ECM; 395), 31 kDa (in MCF7 cell lysates; 396), 28 kDa (HA-tagged CCN5 from AdCCN5 in myometrial uterine SMC using anti-HA; 397), 31 kDa (conditioned medium from VSMC transfected with an expression vector encoded CCN5 with a myc tag; 393) and 31 kDa (conditioned medium from Rat-1 embryonic fibroblast transfected with a CCN5 gene that had no leader peptide and a His6 tag; 392). Recent Western blot analysis of CCN5 in different cells and tissue lysates from both mouse and rat show additional bands of 47 and 45kDa are visible together with a modest 28kDa band. These data suggest that CCN5 is modified *in vivo* by tissue-specific post-translational modification(s) in many, if not all, organs and tissues.

The nucleotide and protein sequence of CCN5 exhibits a 30–40% sequence homology with other family members, largely CCN4 (WISP-1) and CCN6 (WISP-3). The modular architectures of CCN5 are identical with other family members except in their C-terminal Cysteine-knot (CT) domain, which is absent in the CCN5 gene (390). This may render an important biological function to CCN5, as the CT domain is involved in binding matrix proteins, integrins and important signaling molecules (398). Indeed, domain analysis has indicated that each domain may serve a distinct function for the protein, and in certain cell types there are splice variants that create proteins comprised of the individual CCN domains (399). In mouse VSMC, the VWF domain has

been implicated in nuclear localization of CCN5. Disruption of the nuclear localization signal in the VWF domain using site directed mutagenesis abolishes nuclear localization of CCN5 (400). Although other domains have not been investigated for CCN5, other CCN family member proteins have been shown to have domain specific functions. CCN2 has been shown to induce angiogenesis during breast cancer. Removing the CT domain abrogates this activity, suggesting that CCN5 may negatively regulate angiogenesis, since it is missing the CT domain (401). In the retinal angiogenesis model in mice, the CT domain of CCN2 bound to various angiogenic factors, such as VWF, PDGF and VEGF and promoted angiogenesis. Mutant CCN2 (with no CT domain) was injected into the vitreal compartment and decreased angiogenesis (402). The inhibitory effect on angiogenesis, due to CT loss, may implicate CCN5 in negatively regulating angiogenesis. Thus, antibodies against the CT domain could become a candidate for anti-angiogenic therapies. CCN2 CT domain also has been shown to bind fibronectin through α5β1 integrin and enhanced chrondrocyte adhesion to the extracellular matrix (403). CCN5 lacks the CT domain and may play an opposite function in cell adhesion. In CCN2, the VWF and IGFBP domains also bound to the extracellular matrix protein, aggrecan, and increased transcription of aggrecan mRNA (404). Through this research, it is clear that the domain structure plays a role in downstream signaling effects of CCN family members, particularly regarding the CT domain. CCN5 has many physiologic functions, such as proliferation, migration and adhesion. Further domain analysis may link functions to locations on the protein, which will aid in dissecting the CCN5 signaling network.

1.9.6 CCN5 regulation and signaling:

Following the identification, the early studies laid the foundation of nearly 150 publications on CCN5 and its role in cell signaling, migration, proliferation as well as in development and human disease. Based on the recommendations of international CCN society, HICP, rCOP1,

WISP-2 and CTGF-L are identical and called CCN5 (although some cancer literatures are still using the term WISP-2). Potential research areas are expanding with the efforts to focus on well-established signaling cascades and particularly on significant human disease states. More research efforts have been focused on the role of CCN5 in Wnt signaling involving estrogen, p53, mitogenic growth factors, matrix molecules and environmental stimuli.

1.9.6.1 Wnt Signaling

In 1998, Pennica and his group showed that overexpression of Wnt-1 in the mouse mammary epithelial cell line c57MG was responsible for CCN5 up-regulation at both mRNA and protein levels, highlighting the concept that CCN5 is a Wnt regulated gene. Wnt proteins bind to the cell surface receptor, termed frizzled, which initiate a signaling cascade that inhibits glycogen synthase kinase (GSK-3B) and casein kinase 1 (CK1). Inhibition of these proteins stabilized the pool of beta catenin in the cytosol and allowed them to translocate to the nucleus, where β-catenin interacts with the transcription factor TCF/LEF and induces gene expression. Wnt signaling is important in the development and cancer, and can be overexpressed to give cells oncogenic properties. Adenovirus infected mouse pluripotent progenitor cells, C3H10T1/2, overexpressing Wnt3A showed a 2.5 fold increase in CCN5 expression (405). Similarly, synovial fibroblasts which express constitutively downstream Wnt signaling molecule, β-catenin, an up-regulation of CCN by 2.9-fold was observed whereas inactivated (Δβ-catenin-expressing) cells down-regulated the expression. In addition, estrogen was able to enhance CCN5 expression (by 35-fold) synergistically with increased Wnt signaling (406).

Other physiological activators of the Wnt pathway also have been shown to induce CCN5 expression. Under mechanical strain and Wnt3A treatment, MC3T3-E1 osteoblasts cells showed a 7-fold increase in CCN5 transcription. Treatment of these cells with the GSK-3β inhibitor causes

accumulation of β-catenin and activation of canonical Wnt signaling. A combination of both inhibitor and mechanical loading showed a synergistic stimulation on CCN5 expression, (407). Salivary gland development relies on the Wnt signaling pathway. Microarray analysis of gene expression in SGT derived cell lines showed a 4-fold increase in CCN5 expression compared to several other tumor types. However, CCN5 expression was shown to be down regulated compared to normal SG cells, suggesting that loss of CCN5 expression may play a role in SGT progression (408, 409, 410). The Hepatitis C viral (HCV) core protein is known to modulate the Wnt pathway and increases transcriptional activity induced by Wnt3A, through enhancement of Tcf dependent transcription and β-catenin stabilization (406). In the hepatocellular cancer cell-line Huh-7, overexpression of HCV core protein upregulated the expression of Wnt-1 protein and CCN5 expression (407). Childhood adrenocorticoid tumors ACT also involve over activation of the Wnt signaling pathway. ACT tissue samples showed increased CCN5 gene expression compared to control (408).

Small signaling molecules which modulate the Wnt pathway also can induce CCN5 expression. PKA is an important regulatory protein in the Wnt pathway. PKA phosphorylates GSK-3β at Ser_9, inactivating it, allows for stabilization of β- catenin and causes activation of downstream Wnt signaling pathway (409, 410). The effects of PKA signaling to induce CCN5 expression have also been shown in a non-canonical pathway. In PPNAD-derived cell lines, PKA is over activated, resulting in decreased miR449 and increased expression of CCN5. On the other hand, by inhibiting the activity of PKA, levels of miR449 are increased, thereby decreasing CCN5 expression. Treatment of MCF-7 cells with a PKA activator increased CCN5 expression (412). PKC is a negative regulator of β-catenin stability, blocking the Wnt signaling pathway (413). In

MCF-7 cells, PKC activator inhibited estrogen induced CCN5 expression, and activators of PKC , such as

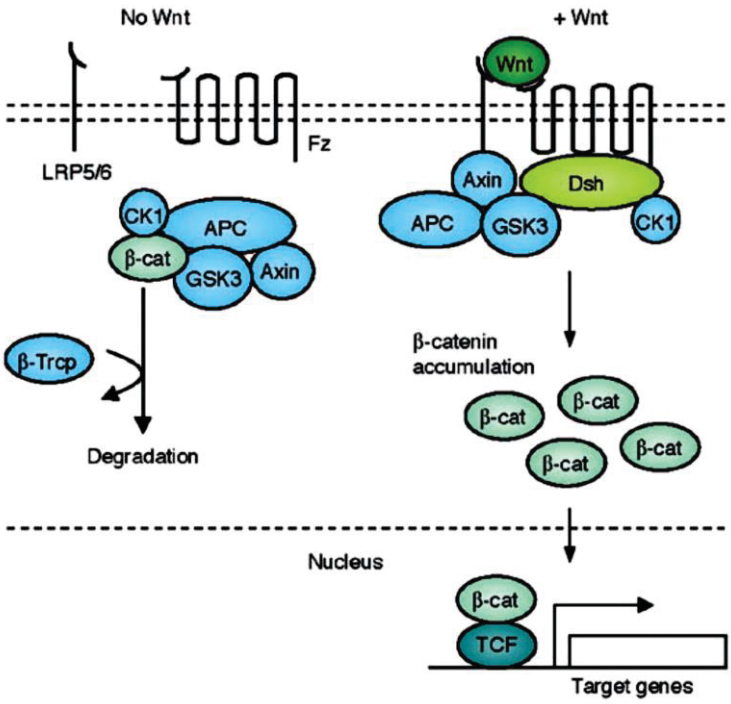

Figure 1. 8. A schematic representation of the canonical Wnt signal transduction cascade. Left, in the absence of Wnt ligand, a complex of Axin, APC, GSK3-β, CK1 and β-catenin is located in the cytosol. β-catenin is dually phosphorylated by CK1 and GSK3-β and targeted degraded by the proteosomal machinery mediated by β-TrCP. Right, with Wnt stimulation, signaling through the Fz receptor and LRP5/6 co-receptor complex induces the dual phosphorylation of LRP6 by CK1 and GSK3-β and this allows for the translocation of a protein complex containing Axin from the cytosol to the plasma membrane. Dsh is also recruited to the membrane and binds to Fz and Axin binds to phosphorylated LRP5/6. This complex formed at the membrane at Fz/LRP5/6 induces the stabilization of β-cat via either sequestration and/ or degradation of Axin. β-catenin translocates into the nucleus where it complexes with Lef/Tcf family members to mediate transcriptional induction of target genes **[Adapted from (612) with permission]**

phorbol esters, increased CCN5 expression, again implicating CCN5 as a downstream target of PKC/wnt signaling (412, 414).

In addition to PKA, large tumor suppressors (LAT1/2) can also modulate CCN5 expression. LAT1/2 are implicated in several types of cancer, including leukemia, lung, prostate and breast cancers. LAT1/2 regulate key proteins in the Wnt pathway and have been shown to down regulate CCN5 in HeLa cells using a whole genome microarray (415).

1.9.6.2 Estrogen Signaling and CCN5

Banerjee and his colleagues first discovered CCN5 regulation through estrogen signaling in breast cancer. They found that both CCN5 mRNA and protein was significantly up regulated in estrogen receptor (ER) positive breast cancer cell lines following the estrogen treatment (416, 417). They observed a strong correlation between CCN5 protein expression and ER-α positivity in human breast cancer samples (416). CCN5 induction by estrogen in breast cancer cells could be mediated through non-genomic pathways by activation of mER-α-MAPK signaling or through a genomic effect (direct transcriptional activation effect) via classical intracellular estrogen receptors that interact (416) with a specific response element (ERE) on promoters or coregulators of the general transcription machinery (419). Interestingly, the non-genomic pathway is also involved in IGF and EGF-induced up regulation of CCN5/WISP-2 in ER positive breast cancers (418, 420).

Extensive studies with the estrogen-positive MCF-7 cell line have been done to establish CCN5 as a target of estrogen signaling in breast cancer cells. The effect of estrogen on CCN5 primarily is attributed to ER-α signaling. ER negative breast cancer epithelium cells fail to respond to estrogen stimulation. However, overexpression of ER-α into these cells restores the ability of

estrogen to induce CCN5 expression (416). Introducing 17β-estradiol to MCF-7 cell culture in a dose-dependent manner increases expression of CCN5 mRNA and protein (416, 421). However, MCF-7 tumor progression was significantly greater in the mice exposed to 17β-estradiol (17β-E2) compared to unexposed mice. WISP-2/CCN5 mRNA expression was correspondingly increased in 17β-E2 exposed MCF-7 tumors compared to unexposed xenografts. Moreover, estrogen exposure followed by anti-estrogen tamoxifen treatment drastically inhibited the tumor growth and WISP-2 expression in nude mice. Therefore, the study suggests that higher WISP-2/CCN5 expression by estrogen may be associated with the estrogen-induced growth of MCF-7 tumors in vivo. During solid tumor formation, CCN5 was increased in MCF7-cell derived tumors. Several xenoestrogens have been reported to increase CCN5 expression through the activation of the signaling cascade (395). Moreover, the ability of estrogen to upregulate CCN5 expression was completely blocked by pure anti-estrogen, ICI 182,780 (416, 417). The partial ER-α antagonist, tamoxifen, inhibited tumor growth and CCN5 expression, implicating CCN5 in the mechanism of tumor formation (422). However, tamoxifen has been shown to be a partial agonist/antagonist for ER-α and a complete antagonist for ER-β.

Estrogen has been shown to regulate CCN5 transcription in MCF-7 cells through an ERE responsive element between -581 and -569 bp upstream of the estrogen inducible transcription start site (423). Chromatin immunoprecipitation showed an estradiol dependent recruitment of ER-A to the estrogen responsive element. CREBP was also found to translocate to the estrogen responsive element in CCN5 (423). This data suggests ER-α and CREB dependent mechanism for CCN5 expression in MCF-7 cells. ER-α has been shown to interact with the transcription co-factors CLIM and RLIM, which regulate the LIM-homeodomain transcription factors (424). Silencing CLIM or RLIM with siRNA in MCF-7 cells decreased estrogen induced expression of

CCN5. In addition, treatment with estrogen induced RLIM and CLIM recruitment to the CCN5 promoter, indicating a direct role for these transcription factors in CCN5 expression (424).

Cross talk between the estrogen and CCN5 signal transduction pathways has been studied by several investigators. Estrogen signaling can be modified by protein kinase A/C activity. Treatment of MCF-7 cells with protein kinase A (PKA) activators induced CCN5 expression. CT/IBMX induced expression of the other estrogen-responsive gene, pS2, more dramatically than maximum stimulation by 17beta-estradiol (E2). Treatment with PKC activator completely prevented CCN5 mRNA induction by E2, whereas it increased pS2 mRNA expression more dramatically than maximum stimulation by E2.

Epidermal growth factor interacts with the estrogen signaling pathway. Activation of EGFR can lead to further activation of ER in a non-estrogen dependent mechanism (425). EGF signaling leads to an increase in proliferation of MCF-7 breast cancer cells, but this can be abrogated by silencing CCN5. In addition, EGF-induced CCN5 expression in a dose- and time-dependent manner and can act synergistically with the addition of estrogen, possibly through PI3K and MAPK signaling pathways. Silencing or inhibiting EGFR, inhibiting PI3K with wortmannin or inhibiting ERs with ICI-182-780 all inhibited EGF induced expression of CCN5 (420). The IGF pathway also activates ER through phosphorylation in a non- estrogen-dependent manner. In the meantime, estrogen signaling can also activate the IGF pathway, leading to bi-directional crosstalk (427). In MCF-7 cells, IGF-I is a potent mitogen and treatment with IGF-I increased CCN5 expression. siRNA knockdown of CCN5 abrogated the mitogen effect of IGF-I. This IGF-I induced CCN5 expression is also regulated by estrogen. The purified anti-estrogen, ICI-182,780 inhibited the IGF induced expression of CCN5 as well as proliferation, implicating membrane ERs as a participant in this signaling pathway (428). Tip30 is a putative tumor suppressor and

transcription factor, which negatively regulates estrogen induced transcription of c-myc (429). Tip-30 also negatively regulates IGF-I and CCN5 expression, possibly through repression of ER-α transcription. Tip30 knock out in mammary epithelial cells promotes proliferation and a 40-fold increase in CCN5 mRNA level. Knock down of either CCN5 or IGF-1 inhibited this effect (430). This data suggests Tip30 may regulate breast cancer progression through estrogen signaling and IGF-1 expression.

Progesterone is also involved in molecular cross-talk with estrogen to stimulate CCN5 expression. In MCF-7 cells progesterone treatment caused a transient increase in CCN5 mRNA which could be blocked by the progesterone antagonist RU38468. However, when treated with progesterone and estrogen together, both induction of CCN5 expression are blocked and CCN5 mRNA remains at basal levels (416).

Estrogen and progesterone are also important regulators of uterine smooth muscle but have a different impact than MCF-7 cells. During the proestrous phase of the reproductive cycle, estrogen levels are high and CCN5 is expressed five-fold higher than in metestrous females with the latter expressing low estrogen (431). Treatment with either estrogen or progesterone increased CCN5 expression in uterine smooth muscle from OVX rats. Interestingly, a combined treatment increased CCN5 regulation much greater than either treatment alone (431). CCN5 signaling appears to be highly dependent on the cell type and state of the tissue. For example, CCN5 expression in response to progesterone differs between uterine smooth muscle and the MCF-7 breast cancer line. Additionally, CCN5 is anti-proliferative in uterine smooth muscle, but mitogenic in breast cancer although in both lines CCN5 is induced by estrogen. In cancer cells, it is possible that signaling pathways including CCN5 expression have been altered.

1.9.7 CCN5 in human Diseases

Since the discovery of CCN5, investigators are focusing on its role in different human diseases. Over last 16 years, many researchers have invested their efforts in studying the role of CCN5 in neoplasia, leiomyoma, hepatic carcinoma, breast cancer, skin carcinoma, pancreatic carcinoma, hyperplasia, atherosclerosis and rheumatoid arthritis.

1.9.7.1 Angiogenesis and cardiovascular development: Angiogenesis is important in multiple physiologic processes, such as vascular development, wound healing, and diabetic retinopathy. On the other hand, angiogenesis and invasion are essential attributes of metastatic cancer cells. Other CCN isoforms, especially CCN2 has been shown to play a key role in developmental and pathologic angiogenesis. CCN2 has been reported to induce angiogenesis in ischemia induced angiogenesis and during lung cancer. In breast carcinoma CCN2 induces angiogenesis, which can be abolished by deleting its CT domain (401). This suggests that CCN5, lacking the CT domain, may play a role as negative regulator in angiogenesis. In human epidermal tissue, CCN5 is localized around blood vessels and is significantly down regulated during wound healing, consistent to a potential negative role on angiogenesis. However, CCN5 can also be implicated in modification of extracellular matrix, invasion, and angiogenesis. In advanced CaP (prostate carcinoma) cells it is highly expressed. Recently it has been reported that the pro-inflammatory chemokine, IL-8 strongly modulates CCN5 expression in CaP cells.

1.9.7.2 Cancer:

CCN5 plays an important role in human breast carcinoma (416, 420, 449). It shows a preventative role in the progression of human breast cancer. As discussed before, CCN5 expression can be induced by various stimulants i.e., estrogen, progesterone and growth factors such as IGF-I, EGF, PMA and serum in ER-positive, noninvasive breast tumor cells (395, 396, 414, 416, 416-418, 420, 422, 423). Moreover, in ER-α positive MCF-7 cell line, CCN5 can be induced by 17β-

estradiol. This induction can reach to its peak by 24 hrs (416). In non-transformed human mammary epithelial cells (HMEC), CCN5 expression remains undetectable, but 17β-estradiol treatment can up-regulate it in the hER-α (human estrogen receptor-α) positive stable cell line. This provides further evidence that in HMEC active ER-α can up-regulate of CCN5. MDA-MB-231 cell line overexpression of CCN5 inhibits of cell proliferation and invasiveness (449, 450) whereas knockdown directly increases the motility and invasiveness of MCF-7 cells. This CCN5 knockdown can downregulate epithelial cell markers while simultaneously upregulating the mesenchymal markers (449, 450). Matrix metalloproteinases are highly expressed in the invasive phenotype due to suppression of CCN5 (449). Finally, CCN5 expression was found to be inversely correlated with the mutational activation of p53 in human breast carcinoma cells lines, and expression of mutated p53 in MCF-7 cells inhibits expression of CCN5 (451). CCN5 is expressed primarily in epithelial keratinocytes, and in the dermis, it is secreted by fibroblasts, blood cells, hair follicles and sweat glands (452). 24hrs after the UV exposure, CCN5 level was down-regulated in human skin and did not return to basal levels until 48hr post irradiation. A similar scenario happened during wound healing in human forearm epidermal tissue leading to a hyper proliferative effect (452, 453).

CCN5 is important in development and is known to control differentiation in multiple cell types. During hepatocellular carcinoma, CCN5 had the opposite effect of increasing cell proliferation through activation of the Wnt pathway in hepatocytes treated with Hepatitis C viral core proteins. The Wnt pathway is also altered in colon carcinoma, but transcript levels were decreased compared to healthy colon tissue (390). CCN5 acts as a tumor suppressor and plays a protective role in the progression of human pancreatic cancer. Overexpression of mutated p53 appeared to be associated with silencing CCN5 (428).

The data to date suggest that CCN5 is multifunctional, mediating many diverse biological functions that vary according to cell type and tissue environment. So far, the data are limited to the functions chosen for study by each investigating group. Most of the investigations began the study of CCN5 with a very specific potential clinical application in mind, or the hope for a simple molecular solution for a long-standing and difficult clinical disease challenge, such as vascular disease and cancer. Unfortunately, several other diseases like diabetes, obesity and pancreatic cancer, the role of CCN5 has not been studied yet. Therefore, it is now necessary to extend and explore the research focus in these areas. Once these studies are completed, it will be much easier to ascertain the role of CCN5 in the pathogenesis and treatment of particular diseases, and to select potential therapeutic modalities for CCN5.

Another factor that has significant impact in diabetic complication is glucocorticoid. We will have a brief discussing on glucocorticoid and glucocorticoid-related enzymes in the final part of our introduction (Section 1.9 & 1.10).

1.10 Glucocorticoids:

Glucocorticoids (GCs) are released from the adrenal gland. The physiological stimulus for glucocorticoid release is corticotropin (adrenocorticotropic hormone) from the anterior pituitary. The primary glucocorticoid in human and most of the mammals is cortisol, whereas in rodents and other lower vertebrates it is corticosterone. The name glucocorticoid (glucose + cortex + steroid) derives from its role in the regulation of the metabolism of glucose, its synthesis in the adrenal cortex, and its steroidal structure.

GCs were first identified by Kendall and colleagues (101) as a potent anti-inflammatory agent in 1949. GCs have pleiotropic effects on physiological functions in the body including those involved in energy balance and metabolism, immune function, cardiovascular function, circadian

rhythmicity, growth and development, cognitive processes and the modulation of various types of behaviour.

1.10.1 Glucocorticoid synthesis, secretion and metabolism

GCs are produced mainly in the adrenal gland from the common precursor cholesterol through a series of enzymatic reactions which are located in either the smooth endoplasmic reticulum or mitochondria. The basic structure of cholesterol is maintained throughout the steroid biosynthetic pathways which contain a cyclopentane ring and 3-cyclohexane rings. Specifically, GCs are synthesized in the adrenal cortex and are secreted into the circulation (Figure 1.8). Cortisol is released under the control of the HPA axis which is activated either by a circadian rhythm or stress stimulus. Under non-stress conditions circulating levels of GCs are released both in an ultradian and diurnal manner. The suprachiasmatic nucleus, which is considered as an endogenous biological 'clock' sets the circadian rhythm by recurring daylight and darkness (102). In mammals, the peak secretion of GCs happens just before the active period, whereas the nadir occurs during the rest (quiescence/sleep) period. The parvocellular cells in hypothalamus rapidly release CRH and AVP into the portal circulation to target the anterior pituitary. CRH and AVP then act synergistically to stimulate the secretion of stored ACTH into the bloodstream (Figure 1.8). Circulating ACTH binds to its receptor at the adrenal cortex to stimulate the synthesis and secretion of GCs (103).

In response to stress, CRH is secreted from the hypothalamus and stimulates the release of ACTH and cortisol from the anterior pituitary and adrenal cortex, respectively. Cortisol then inhibits its own release through a classical feedback process involving the negative regulation of CRH and ACTH secretion. Only 4% of the circulating cortisol is free and biologically active.

Cortisone has a lower binding affinity for corticosteroid-binding globulin, and unbound concentrations are similar to that of free cortisol (106, 107).

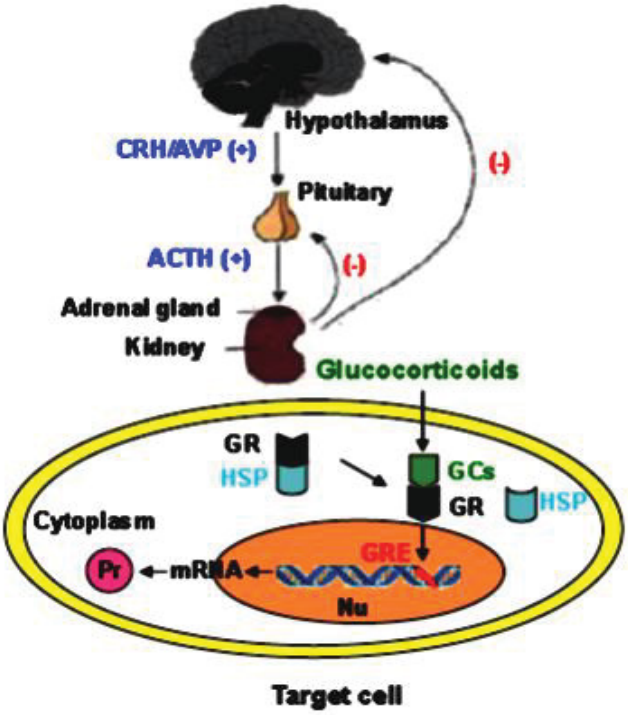

Figure 1.9. Glucocorticoid (GC) secretion is regulated by a negative feed-back loop HPA axis and GCs modulate the target cell gene expression. CRH/AVP is released in the hypothalamus after stimulation, and subsequently stimulates ACTH release from the anterior pituitary which then induces the GC synthesis in the adrenal cortex and release into the circulation. The increased GCs execute feedback inhibition on the HPA axis at both pituitary and hypothalamus, suppressing the synthesis of GC. GCs cross the cell membrane to exert their biological effects by binding to the GR, which exists as a multiprotein complex bound with HSP in the cytoplasm. GC/GR translocates into the nucleus binding to GRE to regulate target gene transcription.
Abbreviations: CRH/AVP, corticotropin-releasing hormone/arginine vasopressin; ACTH, adrenocorticotropin hormone; GCs, glucocorticoids; GR, glucocorticoid receptor; HSP, heart shock protein; GRE, glucocorticoid response element; Nu, nucleus; Pr, protein. **[Adapted from (637) with permission]**

1.10.2 Glucocorticoid receptors (GR)

GCs are lipophilic they can easily cross cell membranes and bind to two structurally related intracellular receptors to exert their effects. The glucocorticoid receptor (GR), which is the main receptor in stress is present in almost all cell types, including pancreatic β-cells (108, 109). In a few cell types, such as epithelial cells of the kidney (110), colon (111), mainly those concerned with fluid or electrolyte balance, GCs bind to the mineralocorticoid receptor (MR) (112). Both receptors act as transcriptional factors that can activate and/or inhibit the transcription of both overlapping and distinct target genes either after binding to DNA or through protein-protein interactions (113). GR is the product of a single gene, NR3C1, located on chromosome 5q31–32 in humans that undergoes alternative processing to yield multiple, functionally distinct subtypes of GR.

1.10.3 Structure of GR

The GR is a modular protein containing an N-terminal transactivation domain (NTD), a central DNA-binding domain (DBD), a C-terminal ligand-binding domain (LBD), and flexible 'hinge region' separating the DBD and the LBD. The NTD has a strong transcriptional activation function (AF1), which allows for the recruitment of co-regulators and transcription machinery. Among the 48 members of the nuclear receptor superfamily, the DBD is the most conserved region. The two zinc-finger motifs present in the DBD recognize and bind specific DNA sequences on target genes (GREs). Upon ligand binding, the second activation function (AF2), located in the LBD interacts with co-regulators. The DBD/hinge region and the LBD each contain a nuclear localization signal that allows translocation to the nucleus via an importin-dependent mechanism (114)

1.10.4 Mechanism of GR action

The classical effects of glucocorticoid signaling are the genomic actions, which depend on GR-mediated transcription and *de novo* protein synthesis. Ligand-bound GR homodimerizes in the nucleus and exerts its transcriptional activation or repression by direct high-affinity binding to GREs found in either the promoters or the intragenic regions of glucocorticoid target genes (Figure 1.9). The gene encoding GILZ (117), SGK1 (118), TTP (119), and MKP-1 (120) are examples of genes up-regulated by activated GR. Examples of genes negatively regulated by GR are β-arrestin 2 (121), osteocalcin (122), and the GR gene NR3C1 itself (123). DNA in the form of GREs modulates GR function by allowing selective regulation of gene expression, whether for gene activation or repression. Similarly, GREs can offer more than just a docking site for GRs in the protein/DNA complex, because the DNA sequence in the GRE can serve as a ligand that can allosterically modulate the structure and transcriptional activity of the GR (124).

Emerging evidence suggests that glucocorticoids can also exert their actions in a more rapid (within minutes), non-genomic signaling mechanism that does not require nuclear GR-mediated transcription or translation (usually takes a few hours). These actions are thought to be mediated by the activation of signal transduction pathways such as the mitogen-activated protein kinase (MAPK) pathway, by the membrane-bound GR or the cytoplasmic GR (125, 126). Additionally, rapid effects, not specific to GRs, also occur as a result of physiochemical interactions of glucocorticoids with the cell membrane (127). These rapid actions of the GR have been reported in various systems, including the cardiovascular, immune and neuroendocrine systems (125, 128, 129).

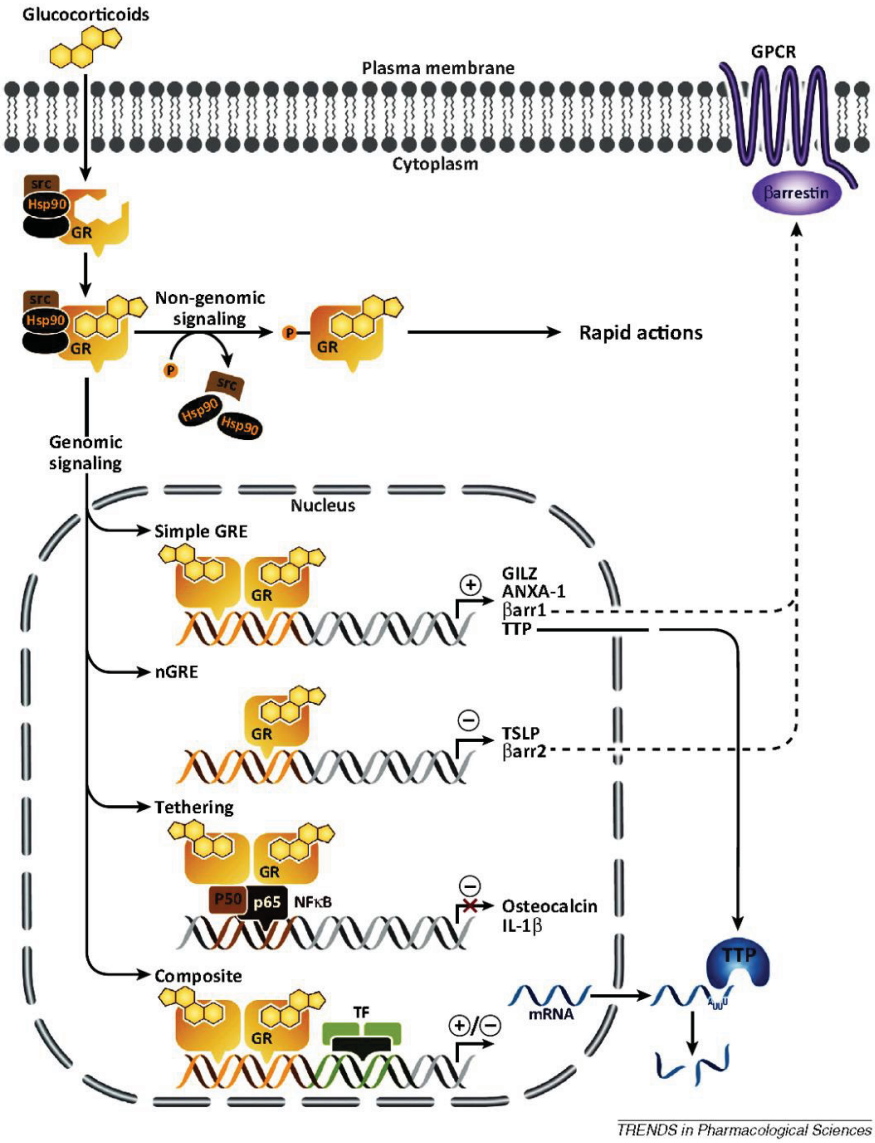

Figure 1.10. Glucocorticoid receptor (GR) signaling. On binding glucocorticoids, the cytoplasmic GR undergoes a conformational change, becomes hyperphosphorylated (P), dissociates from accessory proteins, and translocates into the nucleus, where it can exert its actions through genomic mechanisms. Activated cytoplasmic GR is also known to exert its actions via non-genomic mechanisms. In the nucleus, the GR enhances or represses transcription of target genes by direct binding to 'simple' or negative glucocorticoid-response elements (GREs), respectively, by tethering itself to other transcription factors, or in a composite manner by direct binding to GREs and interacting with other transcription factors. One of the mechanisms by which the GR suppresses inflammation is by inducing expression of tristetraproline (TTP), which in turn binds the mRNA of proinflammatory genes and destabilizes them. The GR modulates gene expression of arrestins 1 and 2 to alter G-protein-coupled receptor (GPCR) signaling. **[Adapted from (474) with permission]**

1.10.5 Glucocorticoids and Pancreas

It is known that elevated levels of glucose and fatty acids decrease β-cell survival by inducing endoplasmic reticulum (ER) stress, by producing reactive oxidative species and reducing insulin synthesis and impaired insulin signalling pathways (193). Indeed, GCs are regulators of glucose and lipid metabolism (195); however, their influence on β-cell function has yet to be fully clarified. It is well known that GCs induce insulin resistance in most tissues, including skeletal muscle, adipose, liver and brain, through impairments in the translocation of the GLUT4 protein (196,197) as well as up-regulating phosphoenolpyruvate carboxykinase and glucose-6-phosphatase (G6P), both *in vitro* and *in vivo* (198,199). However, more recently, studies have been focusing on the role of GCs exert on pancreatic β-cell function by inducing improper folding of the insulin hormone and impaired GSIS (194).

Several studies involving rodent-derived islets have assessed the effects of GCs on insulin secretion *in vitro*. GCs compounds have shown to decrease insulin release in response to both acute (minutes) and more prolonged exposure (hours to days) (202, 203-208). Glucose uptake and its oxidation in beta cells results in an increase in the ATP/ADP ratio, closure of K_{ATP} channels, plasma membrane depolarization, increased cytoplasmic calcium concentrations, and finally exocytosis of insulin granules. In addition, calcium fluxes activate a number of potentiating signal pathways, including PKA and PKC through amplifying insulin secretion (209). GCs were shown to impair β-cell glucose metabolism by reducing the expression levels of GLUT2 (203, 210) and glucokinase (GK) (211), and to increase futile glucose cycling by enhancing G6Pase activity (211, 212). Through these mechanisms, GCs may reduce glucose uptake and thereby decrease ATP synthesis and calcium influx. Furthermore, GCs has been found to be involved with the inhibition of more downstream steps in the insulin pathway. In isolated rat islets, dexamethasone inhibits the

activation of PKC through down-regulating the DAG-phospholipase C (PLC) pathway (208). At the same time, GCs increases the expression of α2-adrenergic receptors (208), leading to reduced PKA activity and decreased insulin release. Together with insulin secretion, GCs may also decrease insulin biosynthesis by reducing the ATP/ADP ratio (205,214) and may reduce beta-cell mass by inducing apoptosis (215). To summarize, GCs interfere with the insulin signaling pathways, but the exact mechanism is yet to be fully understood.

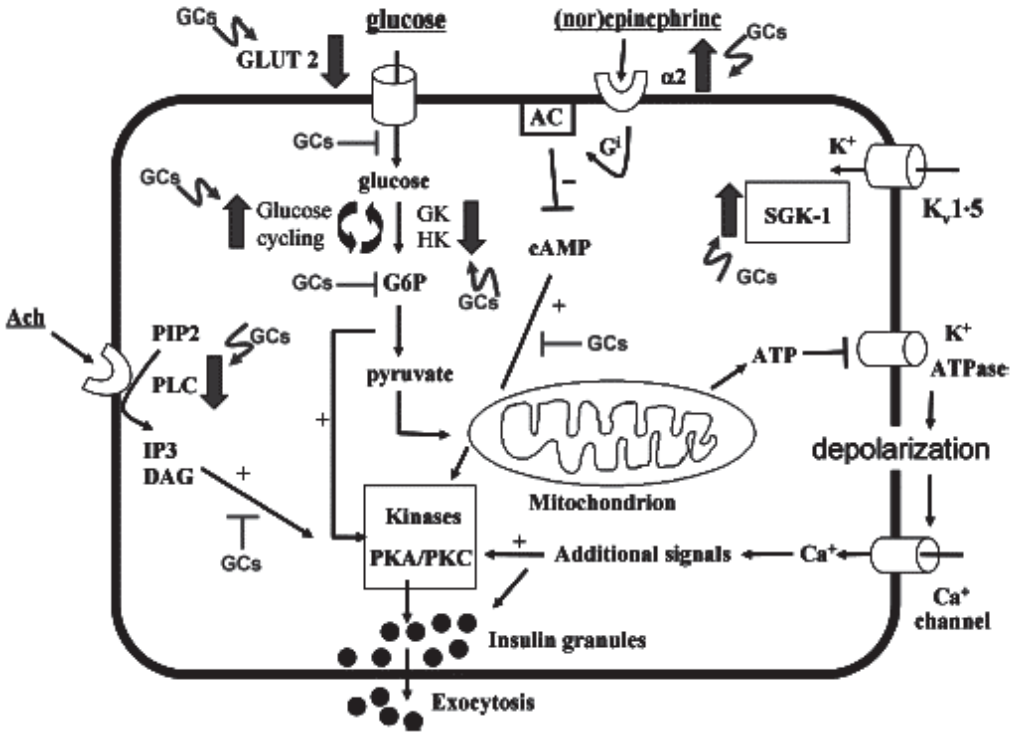

Figure 1.11. The effects of glucocorticoid (GC) treatment on insulin synthesis and secretion in the pancreatic β-cells. GCs decrease glucose uptake into the β-cell as they lower glucose transporter 2 (GLUT2) expressions and content. GCs also decrease glucokinase (GK) and G6Pase (G6P) activity. As glucose uptake is low in the cell, ATP/ADP ratios do not change, thus allowing K+ voltage-gated channels to remain open and permitting K+ to escape the cell. This decreases cell depolarization resulting in hyperpolarization and indirectly limits Ca2+ entry into the cell. GCs also directly impair Ca2+ influx, which creates membrane repolarization and Ca2+ instability, thereby affecting the Ca2+-sensitive endoplasmic reticulum (ER). GCs inhibit protein kinase A/protein kinase C (PKA/PKC) activity, which that would normally signal for insulin synthesis through the ER. Moreover, GCs also induce an 'unfolding' protein response in the ER, which increases ER stress and impairs ER homeostasis, thereby limiting insulin secretion.
Abbreviations: AC, adenyl cyclase; Ach, acetylcholine; ATP, adenosine triphosphate; cAMP, cyclic adenosine monophosphate; DAG, diacylglycerol; G6P, glucose-6-phosphatase; Gi. G-coupled inhibitory protein; GC, glucocorticoid; GK, glucokinase; GLUT2, glucose transporter 2; HK, hexokinase; IP3, inositol triphosphate; Kv1·5, voltage-dependent K channel; PIP2, phosphatidylinositol biphosphate; PKA, protein kinase A; PKC, protein kinase C; PLC, phospholipase C; SGK-1, serum- and glucocorticoid inducible kinase-1. [**Image adapted from (198) with permission]**

1.11 11β-hydroxysteroid dehydrogenases (11β-HSDs)

The physiological GC action depends on several factors- GR receptor and intracellular concentration of the ligand which is regulated by the activity of the HPA axis, by the level of the corticosteroid binding globulin, and by the activity of the intracellular 11β-hydroxysteroid dehydrogenase enzymes. 11β-HSD activity is found in a broad range of cells and tissues such as human placenta, kidney (predominantly dehydrogenase activity) and liver (predominantly reductase activity) (241). This is explained by the existence of two isozymes of 11β-hydroxysteroid dehydrogenase (Figure 1.11), 11β-HSD1 (NADPH dependent type-1 isozyme) that catalyzes the interconversion of hormonally active GC (cortisol in humans and corticosterone in rodents) and 11β-HSD2 (a NAD dependent oxidative type 2) that catalyzes the conversion of inactive GC (cortisone in humans and 11DHC in rodents) to regulate intracellular GCs levels (242). 11β-HSD1 acts as a bi-directional (the reductase usually being predominant) enzyme and it is largely expressed in the tissue and organs associated with high GR expression, such as liver, lung, adipose tissue, and brain (243-248). In contrast, 11β-HSD2 functions solely as a dehydrogenase and is expressed mainly in kidney and other mineralocorticoid target tissues where it prevents inappropriate activation of GCs (249, 250). Notably, MR and GR have indistinguishable affinities for active GCs and as such the 11β-HSD enzymes fulfill a critical pre-receptor ligand access function in these tissues (241).

1.11.1 11β-hydroxysteroid dehydrogenases type 1 (11β-HSD1)

11β-HSD1 is a member of the short chain dehydrogenase/ reductase superfamily with high sequence homology between species, especially the cofactor binding region (GASKGIG) and the catalytic site (YSASK). The *11β-HSD1* expression is postnatal with limited or no expression in

fetal tissues of most mammals. In humans the expression reaches the maximum at adult level with the highest in liver, adipose, gonad and brain. However, it is also expressed in bone, eye, heart, immune system, Gastrointestinal tract, kidney, pancreas and skin.

The *11β-HSD1* gene in rat liver contains 861 bp ORF encoding a protein of 288 amino acids (15-17, 25). Analysis of the mouse cDNA of 14kb shows extensive conservation of nucleotide (91%) and 292 amino acid (86%) sequences with rat 11β-HSD1 (251). The human HSD11B1 gene is over 30 kb in length and consists of 6 exons (182, 130, 111, 185, 143, and 617 bp, respectively) and 5 introns (776, 767, 120, 25 300, 1700 bp, respectively) (20).

Purified 11β-HSD1 enzyme behaves principally as an NADPH dependent dehydrogenase, oxidizing cortisol to cortisone (252). 11β-HSD1 is an NADP(H)-dependent enzyme which is a major regulator of GC metabolism in mammals (252). Perhaps only in tissue homogenates and some transfected cells (248, 254-256) 11β-HSD1 exerts a bi-directional function. However, in intact cells and *in vivo*, 11β-HSD1 mainly acts as a reductase favoring the conversion of cortisone to cortisol (246, 262, 263, 264). 11β-HSD1 is an integral membrane protein of the endoplasmic reticulum (ER) with its N-terminus anchored in the ER membrane and the C-terminus inside the ER lumen (248, 265, 266). 11β-HSD1 activity highly depends on the ratio of NADPH/$NADP^+$ within the ER lumen (248, 256-258). The ER membrane is relatively impermeable to pyridine nucleotides- as a result, the NADPH/$NADP^+$ ratio within the ER is regulated by other enzymes, key among which is hexose-6-phosphate dehydrogenase (H6PDH) that is untethered and resides in the ER lumen (252) (Figure 1.12). The H6PDH is the microsomal counterpart of the cytosolic glucose-6-phosphate dehydrogenase (G6PDH). $NADP^+$ and G6P are the two essential substrates for H6PDH to generate NADPH by catalyzing the first two steps of the endo-luminal pentose-phosphate pathway. Generation of NADPH thereby drives 11β-HSD1 reductase activity (259).

Unlike $NADP^+$, G6P is transported into the ER lumen by the glucose-6-phosphate transporter (G6PT) (260). One recent study showed that G6PT deficiency in liver results in a loss of 11β-HSD1 reductase activity (261).

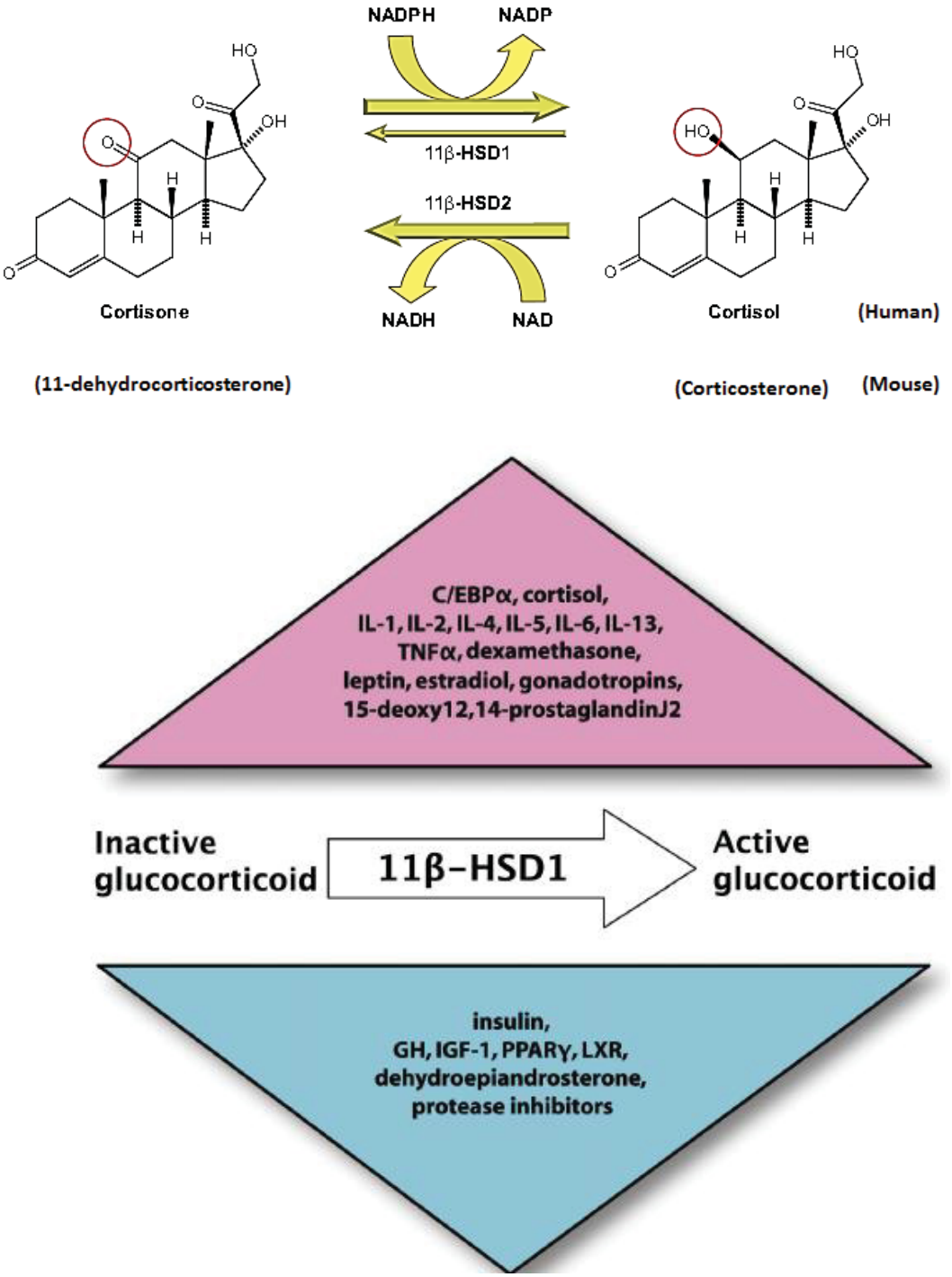

Figure 1.12 Reactions catalyzed by 11β-hydroxysteroid dehydrogenase (11β-HSD) isozymes. A. Interconversion of cortisol and cortisone by 11β-HSD1 and -2 in human (11-dehydrocorticosterone and corticosterone in mouse). **B**. The regulators involving upregulation (pink) and downregulation (blue) of 11ß-HSD1 activity.

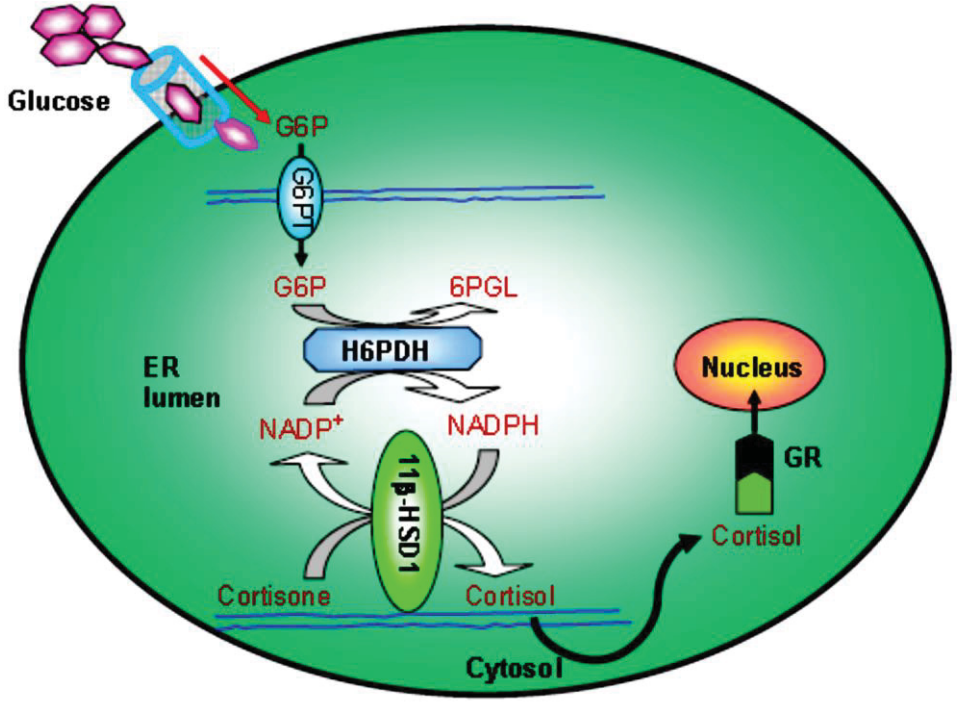

Figure 1.13 A schematic of the interaction between 11β-HSD1 and its cofactor generating enzyme H6PDH in the regulation of glucocorticoid metabolism. G6P from the cytosol is transported into the ER lumen by the G6P transporter (G6PT), where H6PDH oxidizes it to 6PG while reducing $NADP^+$ to NADPH. NADPH availability confers oxoreductase activity (cortisone to cortisol) upon 11β-HSD1.

Abbreviations: ER, endoplasmic reticulum, H6PDH, hexose-6-phosphate dehydrogenase, NADP, Nicotinamide adenine dinucleotide, NADPH, Nicotinamide adenine dinucleotide phosphate, G6P, glucose-6-phosphate; G6PT, Glucose-6-phosphate transporter; 6PG, 6-phosphogluconolactonate **(adapted from 242, 267).**

1.11.2 11β-HSD1 and Metabolism:

The regulation of 11β-HSD1 transcription and activity is highly tissue-specific and influenced potently by hormones GC, insulin as well as cytokines- IL-1 and TNF-α (242, 246). Glucocorticoids play a role in the regulation of carbohydrate metabolism and the development of diabetes (268). They stimulate gluconeogenesis, inhibit glucose utilization, and cause insulin resistance (269, 270, 271). Physiologically, glucocorticoids stimulate of hepatic gluconeogenesis and adipose tissue lipolysis causing increased blood glucose and fatty acid in the fasting state. However, in the fed state they can work together with insulin to promote carbohydrate and lipid storage. Patients with Cushing syndrome develop visceral obesity, hepatic steatosis, dyslipidemia, atherosclerosis, hyperglycemia and insulin resistance, with resultant hyperinsulinemia. All these factors contribute to impaired glucose tolerance or type 2 diabetes mellitus and increased cardiovascular mortality (272).

1.11.3 11β-HSD1 in adipose tissue:

11β-HSD1, with high expression in human adipose tissue (273, 274), acts predominantly as an oxoreductase and is induced by glucocorticoids and pro-inflammatory cytokines (275-277). It is expressed at the same level in sc and visceral adipose tissue depots whereas H6PDH and GR are expressed at higher levels in visceral tissue (278). 11β-HSD1 is involved in the process of differentiation from pre-adipocytes to mature adipocytes in adipose tissue (Figure 1.13) (279). 11β-HSD1 expression and/or activity increased in adipose tissue of obese humans (280-287) and rodents (288-290). The expression of 11β-HSD1 is higher in visceral compared to sc-adipose stromal cells in humans and increases across differentiation (273).

Different rodent obesity models have demonstrated β-HSD1 dysregulation in adipose tissue. 11β-HSD1 overexpression, specifically in adipose tissue under the aP2 promoter (aP2-HSD1), achieved 2-3 fold elevated adipose tissue corticosterone levels in transgenic mice compared to lean mice. This was comparable to that found in obese humans. Crucially, as with idiopathic human obesity, corticosterone levels in the blood were normal but corticosterone was higher in adipose tissue and the portal circulation draining from the visceral fat (289). aP2-HSD1 mice developed glucose intolerance, insulin resistance, elevated fatty acid and triglyceride levels, leptin resistance, hyperphagia (289) as well as hypertension (291), making a causal link between elevated adipose 11β-HSD1 and metabolic syndrome. These mice had decreased sensitivity to insulin and increased glucocorticoid action with decreased expression of adiponectin (292). In obese mice, increased activity of 11β-HSD1 was observed in visceral adipose tissue, compared to lean Zucker and Wistar/obese (WNIN/ob) rats and diabetic (db/db) mice (288, 293, 294, 295). Additionally, obese WNIN/ob and db/db mice showed higher 11β-HSD1 activity in the sc depot (294-295). A large number of studies in sc adipose tissue have shown that 11β-HSD1 expression activity has a positive activity correlate with BMI and insulin resistance (280-282, 284-286, 296, 297).

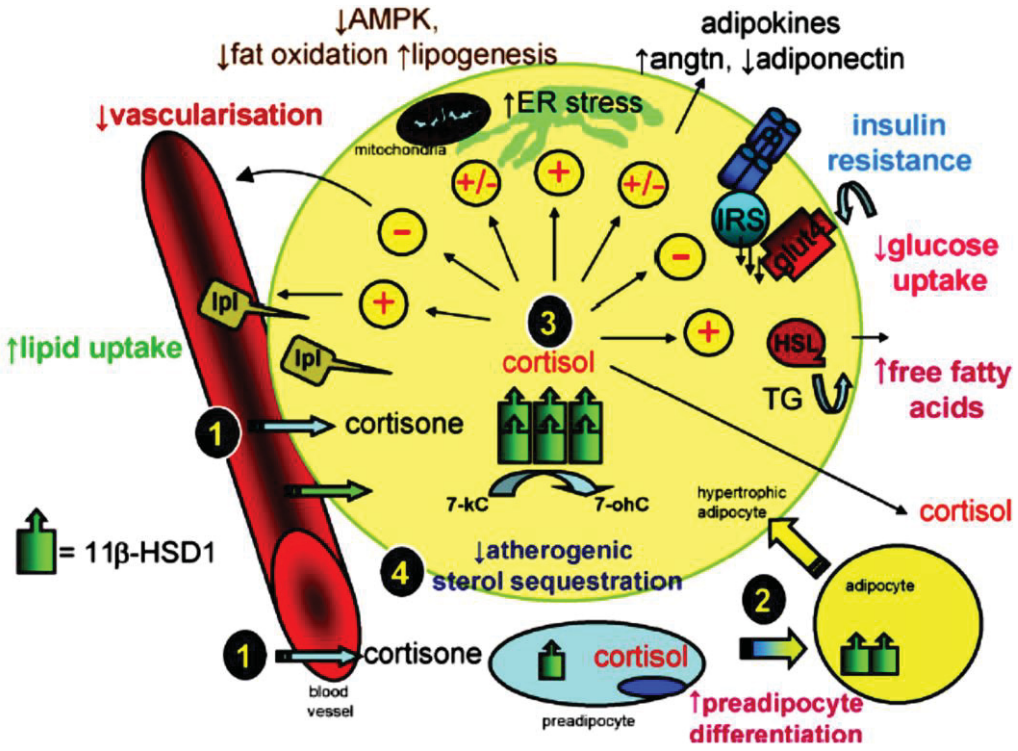

Figure 1.14 Effects of elevated 11β-HSD1 on adipocyte function in obesity. (1) Inactive cortisone from the blood is converted to active cortisol by 11β-HSD1 within the cell. (2) This promotes preadipocyte differentiation, which contributes to (central) obesity during caloric excess. (3) As adipocytes become hypertrophic, 11β-HSD1 levels and intracellular cortisol levels rise causing the following hypothetical effects within the adipocyte (clockwise from far left): increased (+) LPL expression (lpl) and lipid uptake; impaired (−) angiogenic vascularization responses from surrounding blood vessels; suppression (−) of AMPKinase, with a consequent increase in (+) lipogenesis and reduction (−) in fat oxidation; increased (+) ER stress; a variable effect on adipokine secretion including increased (+) angiotensinogen (angtn), which leads to hypertension, and suppression (−) of adiponectin, which exacerbates systemic insulin resistance; direct impairment of the insulin signaling cascade (e.g. IRS 1 or 2) and insulin-responsive glucose uptake (GluT4); increased (+) lipolysis through HSL; spillover of locally produced cortisol which may affect neighboring cells and contribute to insulin resistance in other organs (e.g. portal drainage to the liver). (4) Competitive metabolism of cholesterol metabolites and glucocorticoids may lead to accumulation of atherogenic oxysterols (7-kC = 7-ketocholesterol, 7-ohC = 7-hydroxycholesterol) within the fat/systemically. **Adapted from NM Morton, Molecular and cellular Endocrinology 316 (2010) 154-164 (298)**

1.11.4 11β-HSD1 in liver:

11β-HSD1 is highly expressed in human and rodent liver, more than 10 fold compared to the adipose tissue (299). In human liver, its expression level is highest around the central vein (339), whereas in rat and human hepatocytes primary culture demonstrate exclusively oxoreductase activity (300, 301). Recently a number of human studies showed the relationship of 11β-HSD1 expression and the metabolic syndrome. In obese patients, the expression of GR, 11β-HSD1 and H6PDH in liver were higher with metabolic diseases (302). Although some studies showed hepatic 11β-HSD1 and H6PDH level unaltered with NAFLD (Non-alcoholic fatty liver disease) patients (303, 304). In obese mice, NAFLD was associated with 11β-HSD1 overexpression in visceral adipose tissue with hepatic lipid accumulation due to increased corticosterone delivery to the liver (304). In some obese rodent models (WNIN/ob (294) and obese Zucker rats (107)) the hepatic 11β-HSD1 expression decreased whereas in others such as diabetic db/db mice hepatic 11β-HSD1 and GR expression are increased (305). To explore the role of hepatic 11β-HSD1 in global metabolic homeostasis, mouse models with liver-specific overexpression and knockdown have been developed. The aP2-HSD1 transgenic mice display a high level of corticosterone and FFAs in the hepatic portal vein (289), which suggests overexpression of 11β-HSD1 in visceral adipose tissue could cause a detrimental delivery of elevated GCs to liver, at least in mice. The liver-specific overexpression of 11β-HSD1 was generated by utilizing the ApoE promoter (ApoE-HSD1) (306) to investigate the effects of excess hepatic GCs on metabolic syndrome. ApoE-HSD1 mice exhibited modest insulin resistance, increased liver fat content, and dyslipidaemia in the absence of glucose intolerance, are hypertensive and develop hepatic steatosis due to increased triglyceride accumulation and impaired lipid clearance, obesity or changes of adipose distribution (306). Liver-specific 11β-

HSD1KO mice showed a mild metabolic phenotype, slightly improved glucose tolerance without significant improvement in insulin sensitivity and no changes in hepatic lipid homeostasis, TG accumulation, or serum lipids. Despite unchanged circulating corticosterone levels, these mice showed increased adrenal size, suggesting increased HPA axis activation (307).

1.11.5 Therapeutic inhibition of 11β-HSD1:

The potential use of several 11β-HSD1 inhibitors has been demonstrated in different rodent models for treatment of metabolic syndrome. Although very limited, some of the available clinical data shows improve glucose levels, insulin sensitivity and lipid profiles after these inhibitors treatment (170, 298, 315, 316). Carbenoxolone, a non-selective compound, was the first drug demonstrated in human with improved whole body insulin sensitivity among healthy individuals. Type 2 diabetes patients showed decreased glucose production rates through decreased glycogenolysis with no apparent effect on gluconeogenesis (317-318). In addition, it decreases total circulating cholesterol (318). But Carbenoxolone was able to inhibit local cortisol availability in the sc depot and inhibit glucocorticoid-induced lipolysis (319). Due to its poor selectivity for 11β-HSD1, poor efficacy within adipose tissue highly potent selective 11β-HSD1 inhibitors have now been developed by several pharmaceutical companies. Arylsulfonamidothiazoles (BVT 116429 and BVT 2733: has >200-fold selectivity for 11β-HSD1) were the first reported selective 11β-HSD1 inhibitors and they enhanced insulin action in liver, as well as lowering blood glucose concentrations in diabetic and obese mice (320-322). Compound 531 (Abbott Laboratories, Abbott Park, Illinois) improved insulin sensitivity and decreased hepatic glucose production in dogs through decreased glycogenolysis without any impact on hepatic gluconeogenesis (323). The PF-915275 (Pfizer), is selective for the primate and human 11β-HSD1 and reduced fasting insulin levels (324). The therapeutic benefits of 11β-HSD1 inhibition in rodent models are consistent in

most studies. To date, a number of other 11β-HSD1 inhibitors are under development for potential clinical purposes (311, 314, 322, 325). Recently, the first data using an 11β-HSD1 inhibitor for the clinical treatment of T2D has been reported (326).

Treatment with Incyte (INCB013739), a selective 11β-HSD1 inhibitor, among type 2 diabetes patients completely abolished all conversion of cortisone given orally to cortisol. Metabolically, hepatic glucose production rates decreased without alteration in glucose disposal. In addition, total and LDL cholesterol decreased, with no change in HDL-cholesterol or triglyceride levels (327). Combination treatment with metformin had significantly reduced hemoglobin A1C, fasting glucose, total cholesterol, LDL, cholesterol, and triglycerides, while the basal cortisol level was unchanged. Pfizer has also developed compound PF- 915275, an effective 11β-HSD1 inhibitor which was well tolerated at its maximum oral dose with no effect on the HPA axis (328). Very recently, Amgen developed AMG221 that has been shown to inhibit 11β-HSD1 activity in sc adipose tissue (329).

The prospects for therapeutic inhibition of 11β-HSD1 are, therefore, good, although the downstream effects of these inhibitors in tissues where elevated 11β-HSD1 may play a beneficial functional role (e.g. macrophage inflammation resolution) remains to be clarified.

1.11.6 11β-HSD1 effects in pancreas:

11β-HSD1 mRNA, protein and activity has been demonstrated in rodent (330-333) and human pancreatic islets (330). However, there remains significant controversy on its subcellular localization. Some studies have shown co-localization with glucagon in the periphery of murine and human islets, but not with insulin or somatostatin, suggesting the expression of 11β-HSD1 in the α-cell, not β-cells (333). Others showed its co-localization with insulin, indicating β-cells expression (336). Use of pharmacological inhibitors of 11β-HSD1 can regulate insulin secretion

both *in vitro* (333, 335) and in rodent models (330, 332). Expression is increased from islets from obese ob/ob mice (332) and diabetic Zucker diabetic fatty fa/fa rats, where 11β-HSD1 activity increased with increasing hyperglycemia (331). 11β-HSD1 expression has been found to be higher in pancreatic islets of obese/diabetic rodents and is more sensitive to 11-dehydrocorticosterone (11DHC) mediated suppression of GSIS, an effect reversed by 11β-HSD1 inhibitors (330-333). The high glucose and lipid levels typical of metabolic disease were assessed for their potential regulatory effects on islet 11β-HSD1 *in vitro*, but no clear effect on 11β-HSD1 mRNA levels on isolated islets of prediabetic ZDF rats was found (331). Thus, the increased GC action mediated by elevation of 11β-HSD1 expression may modulate the onset of diabetes by amplifying the inhibitory effect of GCs in β cell. However, there are conflicting reports on the stimulatory or inhibitory effects of glucocorticoids on glucose stimulated insulin secretion (GSIS) *in vitro*. This also raised the possibility that altered β-cell 11β-HSD1 could be secondary to the β-cell failure, or even a mechanism invoked to protect the β-cell from further damage.

In summary, 11β-HSD1 plays a negative role in pancreatic β-cell function, although the complete lack of 11β-HSD1 expression in β-cells has shown to be associated with mild β-cell impairment (334). In addition, moderate β-cell specific over-expression of 11β-HSD1 can protect against the diabetogenic effects of a high-fat diet. Increased number and function of small islets can be a contributing factor for enhanced insulin secretion and β-cell differentiation and survival. This suggests that GCs under certain conditions can promote β-cell function (335, 337).

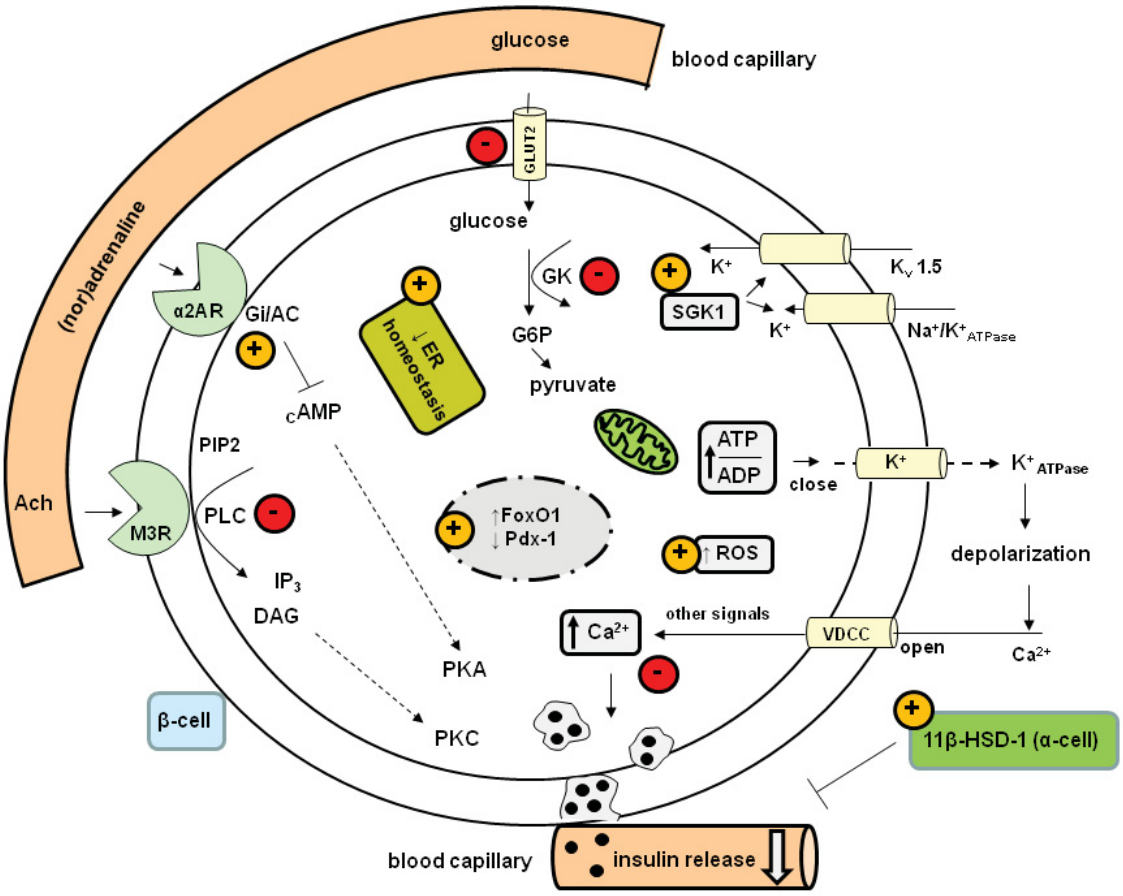

Figure 1.15 Direct GC effects on the insulin secretory process in β-cells. The known components involved in the direct effects of GCs on the β-cell insulin secretory process are highlighted with a positive signal (orange circle – indicates GCs stimulate that action) or negative signal (red circle – indicates GCs inhibit that action). Most notably, GCs impair β-cell glucose metabolism, favor repolarizing K+ currents, decrease PKA and PKC activation, induce ER dyshomeostasis and increased FoxO1 protein content that altogether impair calcium handling and inhibits insulin secretion. Abbreviations: AC, adenylyl cyclase; Ach, acetylcholine; ADP, adenosine diphosphate; ATP, adenosine trisphosphate; cAMP, cyclic adenosine monophosphate; Cx36, connexin 36; DAG, diacylglycerol; ER, endoplasmic reticulum; FFA, free fatty acids; FoxO1, Forkhead box O; G6P, glucose-6-phosphatase; Gs, G-coupled stimulatory protein; GK, glucokinase; GLUT2, glucose transporter 2; IP3, inositol trisphosphate; Kv1·5, voltage dependent K+ channel; M3R, muscarinic receptor type 3; PDX-1, pancreatic duodenal homeobox; PIP2, phosphatidylinositol bisphosphate; PKA, protein kinase A; PKC, protein kinase C; PLC, phospholipase C; SGK-1, serum- and glucocorticoid inducible kinase-1; VDCC, voltage-dependent calcium channel, 11-βHSD-1, 11-β hydroxysteroid dehydrogenase type 1. **Modified from Nussey & Whitehead, 2001 (338) and from van Raalte et al., 2009 (198).**

1.12 Hypothesis and Specific aims

Although IGF-I is known to stimulate protein synthesis, cell survival and proliferation through receptor-mediated activations of PI3K and MAPK, the specific targets within the pancreatic islets have not been studied in a systemic manner. In order to explore novel and previously unknown targets of IGF-I action, we performed a whole genome cDNA microarray analysis on isolated islets from mice overexpressing IGF-I and found 82 genes specifically up- or down-regulated. Prominent among those are intracellular substrates HSD11B1 encoding 11β-hydroxysteroid dehydrogenase 1 (11β-HSD1) and cysteine-rich 61/connective tissue growth factor/nephroblastoma overexpressed (CCN) or Wnt1 inducible signaling pathway (WISP) protein CCN5/WISP-2.

1. To investigate the IGF-I driven expression profiles of these genes at the mRNA, protein and histological levels and assess their function within the islet cells.
2. To determine the role of CCN5 in islet proliferation, regeneration and survival against cell damage and damage and the development of diabetes mellitus, in concert to the actions of IGF-I.
3. To establish the mechanism of CCN5 regulation and to study *in vitro* stimulation of CCN5 protein on the proliferation and survival of pancreatic islet cells.
4. To investigate 11β-HSD1-mediated regulation of islet cell survival and insulin secretion by glucocorticoids.

1.13 Connecting Text

In chapter 1, I made a general discussion about the pancreas development; factors that regulated pancreas regeneration, neogenesis and survival. This chapter also highlighted IGF-I, insulin together with their receptors, their roles and regulation in the different systems including pancreas.

Then I discussed about the role of CCN family and CCN5 in different systems and their regulation in different tissues. At the same time, active glucocorticoid producing enzyme-11β-HSD1 and its function and regulation has been depicted. In our transgenic mice model where IGF-1 is overexpressed, we observe an induction of CCN5 and down regulation of 11β-HSD1. In next two chapters, I have discussed about this IGF-I driven regulation of CCN5 and 11β-HSD1 in details and would try to convince that IGF-I plays important role in islet proliferation, survival as well as GSIS insulin secretion by regulating these two novel targets in pancreas.

Chapter II

IGF-I stimulates CCN5/WISP2 gene expression in pancreatic β-cells

2.1 Abstract

Insulin-like growth factor (IGF)-I is normally produced from hepatocytes and other sources, stimulates protein synthesis, cell survival and proliferation through receptor-mediated activation of PI3K and MAPK and targets specific molecules within the pancreatic islet cells. The current study was designed to identify novel targets that may mediate its pro-islet actions. Whole-genome cDNA microarray analysis in IGF-I overexpressing islets identified 82 genes specifically up- or down-regulated. Prominent among them was CCN5/WISP2 whose expression was increased 3- and 2-fold at the mRNA and protein levels. Dual-labeled immunofluorescence revealed that CCN5 expression was low in the β-cells of wild-type islets but was significantly induced in response to IGF-I overexpression. In vitro treatment of mouse islets with IGF-I increased both CCN5 mRNA and protein levels significantly. To define the role of CCN5 in islet cell biology, we stably overexpressed its cDNA in insulinoma MIN6 cells and detected a 2-fold increase in the proliferation of MIN6-CCN5 compared to control cells that correlated with significant elevations in the levels of cyclin D1 and the phosphorylation of Akt and Erk2. Moreover, MIN6-CCN5 cells were found to be resistant to streptozotocin-induced cell death. Using confocal microscopy and subcellular fractionation overexpressed CCN5 exhibited cytoplasmic accumulation upon stimulation by high glucose. Our results indicate that CCN5, which is minimally expressed in islet β-cells, is strongly and directly induced by IGF-I. CCN5 overexpression stimulates the proliferation of insulinoma cells, activates Akt kinase, and inhibits streptozotocin-induced

apoptosis, suggesting that increased CCN5 expression contributes to IGF-I-stimulated islet cell growth and/or survival.

2.2 Introduction

Insulin-like growth factor (IGF)-I is normally produced from hepatocytes and other sources including the pancreas. Acting through its receptor, IGF-IR, IGF-I promotes embryonic development, postnatal growth, and maturation of major organ systems (501). As a potent mitogenic peptide, it is known to act in endocrine as well as auto- and paracrine manners. Within the pancreatic islets, IGF-I stimulates cell proliferation in the presence of high glucose levels, inhibits insulin secretion and prevents cell apoptosis (502). IGF-I administration in vivo prevents Fas-mediated autoimmune β-cell destruction and delays the onset of diabetes in NOD mice (503). IGF-I treated animals have significantly higher ratio of intact islets and an overall higher β-cell mass relative to untreated mice. To test the effects of increased circulating IGF-I on islet cell growth and glucose homeostasis, an IGF-I transgene was expressed under the metallothionein I promoter (504). We demonstrated previously that the IGF-I overexpression, although widespread, was highly concentrated to the β-cells of the pancreas (505). The transgenic MT-IGF mice were resistant to streptozotocin-induced diabetes, and displayed diminished hyperglycemia and abolished weight loss and mortality. Although IGF-I is known to regulate protein synthesis, cell survival and proliferation through receptor-mediated activation of PI3K and MAPK, the specific targets within the islet cells have not been systematically screened. In order to explore novel targets of IGF-I action, we performed a whole-genome cDNA microarray analysis in isolated islets of MT-IGF vs. wild-type mice and found 82 genes specifically up- or down-regulated. Prominent among them CCN5/WISP2 is a secreted protein that was previously shown to be induced by IGF-

I in breast cancer cells, and either stimulates or inhibits cell proliferation in different systems (506-508). CCN (connective tissue growth factor/cysteine-rich 61/nephroblastoma overexpressed) or WISP (Wnt1 inducible signaling pathway) represents a family of six proteins which regulate cell adhesion and extracellular matrix (ECM) remodelling, skeletal development and chondrogenesis, angiogenesis and wound repair, proliferation and tumorigenesis (509-511). Although the role of another isoform, CCN2/CTGF, in the early development of pancreatic islets has been well established (512-515), CCN5 was not known either to be expressed in the islets or to regulate the islet function. This study was designed to establish CCN5/WISP2 as a novel isoform normally expressed in adult islets that is directly induced by IGF-I, and which plays important roles in pancreatic islet proliferation and/or protection.

2.3 Methods and Materials

2.3.1 MT-IGF mice and pancreatic islet isolation: mice with germline integration of a human IGF-I cDNA driven by mouse metallothionein 1 promoter (MT-IGF) and wild-type littermates on a mixed C57BL/6 background were maintained in 12:12-h dark-light cycles with free access to food and water. As previously reported, zinc supplement in food was not required for the transgene induction; instead of being expressed primarily in the liver and kidney, 31- and 344-fold higher levels of IGF-I mRNA and peptide content were detected in the pancreas (mostly in islet β-cells) vs. the liver (505). Thus MT-IGF mice are a model of islet-enriched overexpression of IGF-I, with minor increases occurring in the liver and kidney. All animal handling procedures were approved

by the Research Institute Animal Care Committee of McGill University Health Centre. Pancreatic islets were isolated by collagenase digestion and allowed to recover for 3 h in DMEM medium containing 11 mM glucose and 10% fetal bovine serum, 10 mM HEPES, 2 mM glutamine, and 1 mM sodium pyruvate as reported (516). They were hand-picked and pooled for RNA or protein isolation.

2.3.2 cDNA microarray and real-time PCR: male MT-IGF mice and wild-type littermates, 3-4 month old, were used to isolate pancreatic islets. Total RNA was prepared from pooled islets from each mouse using Qiagen RNeasy Plus. The RNA quality/integrity was analyzed using Agilent 2100 Bioanalyzer and its concentration determined using NanoDrop 2000 spectrophotometer. The gene expression profile was determined using Illumina Whole-Genome Expression BeadChips (MouseRef-8 V2) by McGill University and Genome Quebec Innovation Centre. The lumi and EB (Wright & Simon) algorithm was applied to the data using the software FlexArray version 1.4 to identify genes that were significantly induced or repressed in transgenic islets. The list of transcripts was uploaded to DAVID (http://david.abcc.ncifcrf.gov), and cell compartment annotation and functional annotation chart were obtained and manually adjusted, including marking the duplicates in Table 1. Real-time PCR was performed using QuantiFast SYBR Green RT-PCR Kit (Qiagen) with LightCycler Systems (Roche Applied Science); primers were purchased from Qiagen. The amount of target RNA was determined by comparing to β-actin as an internal control and expressed as Mean ± S.E.

2.3.3 Western blot analysis: freshly isolated islets from 3-4 month old male mice were sonicated

in 150-200 μl lysis buffer (containing 150 mM NaCl, 10 mM Tris, pH 7.4, 1 mM EDTA, 1% Triton X-100, 0.1% SDS, 100 mM NaF, 10 mM sodium pyrophosphate, 10 mM sodium orthovanadate, and 2 mM PMSF) supplemented with protease inhibitor tablet (Roche Diagnostics). The cell extract was diluted by 1-1.5 volume Laemmli loading buffer (Bio-Rad) containing 5% β-mercaptoethanol (Sigma-Aldrich) and boiled for 5 min before loading on to SDS-PAGE gels. Western blotting was performed using rabbit polyclonal anti-CCN5/WISP2 (ab38317, Abcam), mouse monoclonal anti-HA (G036, ABM), rabbit polyclonal cyclin D1 (sc753, Santa Cruz), phospho-Akt at Ser-473 and total Akt (4058, 9272), phospho- and total Erk1/2 (9101, 9102), cleaved and total Caspase 3 antibodies (9661, 9662) from Cell Signaling, β-actin (MM-0164-P) from Medimabs and USF-2 (sc-862, Santa Cruz) respectively.

2.3.4 In vitro direct stimulation of primary islets by IGF-I: freshly isolated pancreatic islets pooled from 3 wild-type male mice were allowed to recover overnight in culture medium containing 11 mM glucose and 10% fetal bovine serum (517). The islets were randomly distributed in batches of 20 into 24-well plates with 3 replicates for each condition, cultured for 6 to 24 h in the same medium but containing 1% serum with or without 10^{-8} M recombinant human IGF-I, Long R3 (I1271, Sigma-Aldrich). Total RNA was extracted for the measurement of CCN5 mRNA using real-time PCR. To analyze changes in CCN5 protein, 75 isolated islets in 12-well plates were stimulated by IGF-I for 12 or 24 h. Total cell lysates were prepared for Western blots against CCN5 and β-actin.

2.3.5 Dual-labeled immunofluorescence: paraffin sections of the pancreas taken from 3-5 mo

old, male MT-IGF and wild-type littermates were dewaxed, rehydrated, and blocked with 10% donkey serum, followed by overnight incubation with rabbit polyclonal anti-CCN5 at 4ºC. After washing with PBS, sections were stained with guinea pig polyclonal anti-insulin (ab7842, Abcam) followed by Alexa Fluor® 594 conjugated donkey anti-rabbit IgG (H+L) and Alexa Fluor® 488 goat anti-guinea pig IgG (Life technologies) (518, 519). The images were captured and analyzed using Axioshop 2 plus microscope (Carl Zeiss), Retiga 1300 digital camera, and Northern Eclipse software version 8 (Empix Imaging).

2.3.6 Stable overexpression of CCN5 cDNA in MIN6 cells: mouse CCN5 cDNA with a 3'-$(HA)_3$ tag was subcloned into pcDNA3.1 vector between the CMV promoter and BGH poly A sequence and used to transfect MIN6 cells which were selected for resistance against G418 for 45 d (Fig 2A). Following Western blot confirmation, multiple CCN5-overexpressing (MIN6-CCN5) and vector-transfected (MIN6-Vec) clones were subject to the MTT cell viability (Sigma-Aldrich) and 5-bromo-2'-deoxyuridine (BrdU) incorporation assays. The cells were cultured in 1% serum for 1-3 d before the MTT assay. For BrdU incorporation, in the final 18 h of incubation, 10 μM BrdU was added; its incorporation was quantified using ELISA at 450 nm (EMD Millipore). The change in cellular cyclin D1 level was determined in by Western blot. To study CCN5-stimulated signaling mechanisms, we measured changes in the phosphorylation levels of Akt and Erk1/2 kinases in cells cultured for 24 h in either 10% serum or serum-free medium. MTT cell viability assay was also performed in the presence or absence of Akt inhibitor MK-2206 (1 μM) for 24 or 48 h. Finally, to establish CCN5's actions as an extracellular regulator, parental MIN6 cells were

treated with recombinant human CCN5/WISP2 protein (Creative Biomart, Sherley, NY) and its effects on cell proliferation were measured using MTT and Akt phosphorylation.

2.3.7 CCN5 gene silencing using siRNA: the role of endogenous CCN5 on IGF-I-stimulated cell proliferation was investigated by RNA interference mediated gene knockdown. Parental MIN6 cells were transiently transfected with Silencer® Select CCN5-specific (or scrambled control) siRNAs (Ambion/Invitrogen) using Lipofectamine RNAiMAX (Invtrogen) and the decreased level of CCN5 mRNA was confirmed using real-time PCR. CCN5-siRNA transfected cells were allowed to grow in culture medium with or without 5 nM long R3 IGF-I for 3 d and cell viability was assessed by MTT assay.

2.3.8 Streptozotocin-induced apoptosis in MIN6 cells: MIN6-CCN5 and -Vec cells were sub-cultured at a density of 2 x10^4 cells/cm^2 and incubated with 5 mM streptozotocin for 24 h (520). The presence of mono- and oligonucleosomes in apoptosis was measured by histone-associated DNA fragments in the cytoplasm. The degree of cell apoptosis was determined by sandwich enzyme immunoassay cell death detection ELISA plus kit (Roche Cat. No. 11774425001) (521). Briefly, cells were collected by being centrifuged at 200 g for 10 min and lysed for 30 min with the buffer provided. The cell lysate was again centrifuged at 200 g for 10 min. 20 µl aliquots of the supernatant (representing the cytosolic fraction) were transferred to streptavidin-coated wells, incubated with anti-histone-biotin and anti-DNA-peroxidase antibody for 2 h, followed by the 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) substrate for 10 min, and then measured as a ratio of absorbance at 405 and 490 nm using Perkin Elmer Enspire multiplate reader. The

occurrence of apoptosis was correlated with the amount of streptozotocin-induced caspase 3 cleavage in MIN6 cells which is quantified by Western blot.

2.3.9 Confocal microscopy of CCN5-overexpressing cells: in order to monitor intracellular CCN5 expression and distribution in response to high glucose stimulation, MIN6-CCN5 cells were seeded into 8-well Lab-Teck chamber slides and cultured for 24 h. The cells were then cultured in fresh media containing either 2.8 mM or 16.7 mM glucose for 60 min before being fixed with 3% paraformaldehyde and permeablized with 0.1% Triton X-100. After blocking with 0.5% BSA, cells were incubated with mouse monoclonal anti-HA and rabbit polyclonal anti-insulin antibodies for 1 h. After washing with PBS the cells were incubated with Alexa Fluor® 594 donkey anti-mouse (H+L) and Alexa Fluor® 488 donkey anti-rabbit IgGs (Life technologies). Nuclei were stained with 4',6'-diamino-2-phenylindole (DAPI) at 1 µg/ml. Images were obtained using a 63× (NA 1.40) oil Plan-Apochromat objective and detected with GaSaP detector using LSM780 laser scanning confocal microscope (Carl Zeiss). DAPI was excited with 405 Diode, anti-insulin with 488 nm argon, and anti-HA with 561 nm DPASS lasers, respectively.

2.3.10 Subcellular fractionation: to confirm the confocal result, MIN6-CCN5 cells in confluent culture dishes were incubated for 60 min with 2.8 or 16.7 mM glucose, washed in ice-cold PBS, scraped from the dishes and resuspended in lysis buffer containing 20 mM Tris-HCl, pH 8.0, 1% Triton X-100, 150 mM NaCl and protease inhibitor cocktail. Cell lysates were centrifuged twice at 2,600 x g for 7 min to pellet nuclear enriched fraction. The supernatant was cleared at 20,000 x g to obtain the cytosolic faction. Protein concentration was determined using BCA assay.

2.3.11 Statistical analysis: data were expressed as Mean ± S.E. and plotted using Sigma Plot version 11 (Systat Software), which was also used to perform ANOVA and pot-hoc Holm-Sidak test. Unpaired Student's t-test was performed using InStat software version 3 (GraphPad Software, San Diego, CA). P values <0.05 were considered to be significant.

2.4 Results

2.4.1 Changes in gene expression profile induced by IGF-I overexpression in pancreatic islets

To explore novel targets that are involved in IGF-I regulated β-cell function, we performed a whole-genome microarray analysis on total RNA prepared from freshly isolated islets, and found 82 genes either up- or down regulated significantly in MT-IGF vs. wild-type mice, with the threshold of >1.5 or <0.7-fold and P≤0.01 (**Table 2.1**). Many of them, including ECM proteins (fibulin-2, COL14A1), ion channels (KCNF1, CATSPER2), intracellular substrates (11β-HSD1, PI3K-C2γ), and CCN5/WISP2 are novel targets of IGF-I whose impact on β-cell function has not been previously known. Among them, expression of CCN5/WISP2, a gene not known to be expressed in the islets, exhibited a 2.7-fold increase as a result of IGF-I overexpression.

To avoid false positives in the microarray result, changes in the expression of nine target genes were independently confirmed using real-time PCR in fresh batches of isolated islets (**Table 2.2**). Notably, the level of CCN5/WISP2 mRNA was significantly increased 3.3-fold in MT-IGF vs. wild-type mice, consistent with the microarray result. CCN5/WISP2 is a secreted protein of 29 kDa and has been reported to either stimulate or inhibit cell proliferation in various systems (506-

508, 522). Although its expression in pancreatic acinar and ductal cells has been reported (523), its presence in the islet cells was not known.

2.4.2 Islet β-cell specific CCN5/WISP2 expression and its direct stimulation by IGF-I

To confirm CCN5 gene expression at the protein level, we performed Western blot analysis on the extract of freshly isolated islets. As shown in **Figure 2.1A**, CCN5 was found to be expressed at low level in the islets of wild-type mice and at a 2-fold higher level in those of MT-IGF mice. Using dual-labeled immunofluorescence, we further confirmed the presence of CCN5 in wild-type islets, and its induction in MT-IGF mice (**Figure 2.1B**). In sections co-stained for insulin (green, top panels) and CCN5 (red, middle panels), the colocalization of insulin and CCN5 in islet β-cells was evident from the merged images (bottom panels) and confirmed also the increase in β-cell-specific expression in MT-IGF mice. In parallel experiments we found that CCN5 does not colocalize with glucagon (data not shown). To further demonstrate an in vitro direct stimulation, freshly isolated islets of C57BL/6 mice were cultured with 10^{-8} M IGF-I that elicited a significant stimulation on CCN5 mRNA level after 12 and 24 h (**Figure 2.1C**). The stimulation at protein level was more striking, exceeding 6.6-fold after 12 h treatment (**Figure 2.1D**). Thus, we demonstrated increased CCN5 protein in islet β-cells of MT-IGF mice using IHC and Western blots and a direct stimulation of CCN5 gene expression by IGF-I in primary islets.

2.4.3 Increased proliferation of insulinoma cells caused by CCN5/WISP2 overexpression

To define the role of CCN5 in islet function, we stably transfected its cDNA with a 3'-triple HA tag into MIN6 cells using the pcDNA3.1 vector (**Figure 2.2A**). Using Western blots, we confirmed

significantly overexpressed CCN5 protein in MIN6-CCN5 cells. Consequently, the cell number increase representing cell proliferation in three independent lines of MIN6-CCN5 cells was significantly higher than those of MIN6-Vec cells using MTT assay (**Figure 2.2B**). To directly measure the change in DNA synthesis, we demonstrated ~1.7- and ~2.7-fold significantly increased BrdU-incorporation in two lines of MIN6-CCN5 compared to MIN6-Vec cells cultured either for 48 or 72 h in 1% serum (**Figure 2.2C**). Replication of β-cell replication is known to be associated with increased cyclin D1 and CDK4 levels (524, 525). In MIN6-CCN5 cells, we detected a 3-fold increase in the level of cyclin D1 (**Figure 2.2D**), further supporting a proliferative effect of CCN5.

2.4.4 CCN5/WISP2 overexpression increased Akt and Erk2 phosphorylation

The proliferation of islet cells is known to be controlled by PI3K, Akt and Erk1/2 pathways and stimulated by IGF-I. In order to determine if Akt and Erk1/2 are regulated by CCN5, we assessed and detected a 2.4-fold increase in Akt phosphorylation at Ser-473 in MIN6-CCN5 compared to MIN6-Vec cells incubated in serum-free medium (**Figure 2.2E**); even in cells stimulated with 10% serum, CCN5 overexpression caused a 2-fold increase in Akt phosphorylation. The effect on Erk1/2 was moderate, i.e. under serum-free condition, CCN5 overexpression only caused a 1.6-fold increase Erk2 phosphorylation ($P<0.05$, **Figure 2.2F**); under 10% serum, however, it had no significant effect. As shown in these blots, the total levels of Akt, Erk1 and Erk2 remained unaltered (**Figure 2.2E, 2.2F**). We confirmed the role of Akt activation in CCN5-induced proliferative signaling by comparing the relative number of MIN6-CCN5 cells incubated in the

presence or absence of Akt inhibitor MK-2206 and found that inhibition of Akt decreased cell proliferation by 61% and 53% after 24 and 48 h incubation respectively (**Figure 2G**). At the same time, Akt inhibition caused a 50% reduction in cyclin D1 level (**Figure 2H**). Our results indicate that CCN5 stimulates β-cell replication, at least in part by activating Akt kinase and cyclin D1.

2.4.5 CCN5 overexpression in MIN6 cells confers resistance to streptozotocin-induced apoptosis

Since IGF-I has a potent effect on β-cell survival against various types of damage, we anticipated that CCN5 may also exert prosurvival effects. We tested the effect of CCN5 overexpression on streptozotocin-induced cell death in stably transfected MIN6 lines. Streptozotocin treatment for 24 h caused significant cell loss showing condensation and debris in MIN6-Vec cells (**Figure 2.3A** bottom-left vs. top-left panels) which was largely avoided in MIN6-CCN5 cells (**Figure 2.3A** bottom-right panel). This was confirmed by a 3.6-fold increase in cleaved caspase-3 in MIN6-Vec cells after 12 h exposure to streptozotocin (**Figure 2.3B**). CCN5 overexpression significantly diminished caspase-3 activation and blunted streptozotocin-induced cell apoptosis. Histone-associated DNA fragmentation in the cytoplasm is the hallmark of cell apoptosis (521). Streptozotocin treatment caused a significant 6.5-fold increase in the level of DNA fragmentation in MIN6-Vec cells (**Figure 2.3C**, bar 2 vs. 1), compared to a mere 1.6-fold increase in MIN6-CCN5 cells (bar 4 vs. 3). The 4-fold reduction in DNA fragmentation (compare bars 2 and 4) confirmed the ability of overexpressed CCN5 to attenuate streptozotocin-induced apoptosis. As streptozotocin-induced cellular toxicity is dependent on the normal expression of GLUT2, the

possibility that CCN5-induced decrease in GLUT2 expression may limit the uptake of streptozotocin thereby indirectly diminishing its cytotoxicity was excluded as CCN5 overexpression did not influence GLUT2 level in MIN6 cells (data not shown).

2.4.6 Glucose stimulation causes cytoplasmic accumulation of CCN5/WISP2

As a secreted protein, CCN5 is expected to be localized in cytoplasmic vesicles (526); as a matricellular protein it might be associated with cell membrane and ECM; its presence in the nucleus has also been described (527, 528). To help understand the secretion and subcellular localization of CCN5 protein, we performed confocal microscopy on MIN6 cells overexpressing the protein under basal (low glucose) condition and after being stimulated by high glucose. As shown in **Figure 2.4A**, under low glucose condition, HA-tagged CCN5 protein was present at a low level in the cytoplasm (**Figure 2.4A**, top panels). Upon high glucose treatment for 60 min, significant cytoplasmic accumulation of CCN5-HA protein was found in close association with the staining of insulin (**Figure 2.4A**, bottom panels), which is consistent with early observation of perinuclear and vesicular distribution (529). In addition, the cellular content of both CCN5 and insulin was dramatically enhanced by high level glucose, compared to cells cultured under low glucose. To further quantify the change, in a separate experiment we prepared nuclear and cytosolic fractions from low and high glucose treated cells and determined the relative levels of CCN5-HA protein per β-actin or USF-2 respectively (**Figure 2.4B, C**). Compared to the cells cultured in low glucose, we confirmed a 4.3-fold increase in cytosolic CCN5-HA protein upon high glucose stimulation but no significant decrease in nuclear protein, consistent to our finding in

confocal and the notion that CCN5 is a glucose responsive secretory protein. However, we have not been able to detect CCN5 secretion using Western blot, unlike being reported (526).

2.4.7 Recombinant CCN5 protein directly stimulated cell replication and Akt phosphorylation

As we were studying the effect of CCN5 overexpression, recombinant and highly purified human protein became available and proved effective in inducing mesenchymal–epithelial transition (MET) in pancreatic adenocarcinoma cells (523). In order to establish if it exerts an endocrine/paracrine effect on insulin producing cells, we treated parental MIN6 cells with recombinant CCN5 protein for 3 d, together with IGF-I as positive control. In untreated cells, the proliferation rate was low as measured using MTT assay (**Figure 2.5A**); treatment with low doses of CCN5 protein, 6.25 and 12.5 nM, significantly increased the proliferation activity upto 3 d; a higher dose of 25 nM led to early stimulation but became somewhat toxic after 48 h, and the stimulatory effect of exogenous CCN5 protein was somewhat less than that of 10 nM IGF-I. This result was further supported by the increased BrdU incorporation in cells stimulated for 3 d by IGF-I (1.7-fold at 10 nM) or CCN5 (1.4-fold at 12.5 nM; **Figure 2.5B**). Treatment of MIN6 cells by exogenous CCN5 protein for 6 h led to a 1.8-fold increase in Akt phosphorylation at Ser-473 (**Figure 2.5C**). This effect seemed specific as Erk1/2 activity was not significantly affected (data not shown), consistent with the moderate changes in Erk1/2 phosphorylation upon CCN5 overexpression (**Figure 2.2F**). Thus, exogenously recombinant protein was found to replicate two key effects of CCN5 overexpression in MIN6 cells, i.e. proliferation and Akt phosphorylation.

To further assess the role of endogenous CCN5 expression on IGF-I-stimulated cell proliferation, we studied the effect of its knockdown using RNA interference. Basal CCN5 expression in parental MIN6 cells was abolished by CCN5-specific, but not a non-specific scrambled siRNA as determined by qRT-PCR (**Figure 2.5D**, compare bars 1 and 2). Likewise, siRNA transfection blunted CCN5 induction by 5 nM IGF-I (**Figure 2.5D**, compare bars 3 and 4). CCN5 knockdown did not affect the basal level of cell proliferation after 3-d culture, as determined by MTT assay (**Figure 2.5E**); however, it clearly reduced IGF-I stimulated effect from 2.3- to 1.5-fold stimulation ($^{@}P<0.05$), supporting a role of endogenous CCN5 in IGF-I stimulated cell proliferation.

2.5 Discussion

In this study we identified CCN5 as a novel target of IGF-I in islet β-cells that is induced not only by IGF-I overexpression, but also by direct stimulation with exogenously added peptide. We demonstrated that IGF-I induces CCN5 gene expression both at the mRNA and protein level. In order to define its role in islet cell biology, we stably overexpressed CCN5 cDNA in MIN6 insulinoma cells and demonstrated that it induces phosphorylation of Akt and Erk2, cyclin D1 expression and cell proliferation measured by MTT and BrdU assays. IGF-I stimulated cell proliferation is known to involve Akt and Erk1/2 signaling leading to cyclin D1 activation (524). Our result also supports previous findings in neuroblastoma Neuro2a cells that CCN5 induces Akt and Erk2 phosphorylation (530). We then showed that treatment of MIN6 cells using recombinant CCN5 protein promotes Akt phosphorylation and cell proliferation. Finally, MIN6-CCN5 cells

were found to be resistant to streptozotocin-induced caspase-3 activation and apoptosis. Together the present findings demonstrate that IGF-I-induced CCN5 in islet β-cells may contribute not only to mitogenic signaling, but also promote cell survival by inhibiting apoptosis.

Among the IGF-I targets revealed in this study, 16 genes encode ECM-related proteins, including fibulin-2, which belongs to a family of five that shares a distinctive C-terminal globular domain and a tandem array of calcium-binding EGF-like modules (531). The expression of PI3K-C2γ, a new member of the PI3K family, was specifically *decreased* at the mRNA level in IGF-I overexpressing islets (532, 533). It should be noted that not all those affected genes can be considered direct targets of IGF-I action on the islets because our model of overexpression also saw considerable levels of IGF-I expression in the liver and kidney, and 50% increase in serum IGF-I concentration (505).

Most CCN/WISP proteins contain four functional domains, i.e. the IGF-BP domain with sequence homology to IGF-BPs but confers less than 1% affinity to IGFs (534), the von Willebrand factor C (VWC) repeat often seen in oligomerization of ECM proteins, the thrombospondin type I repeat (TSP-1) interacting with integrins, and cysteine-rich carboxyl-terminal repeat (CT; which is missing from CCN5) associated with dimerization and receptor binding (509, 510, 535). Overall, they share ~50% amino acid sequence, including 38 cysteines at homologous sites of the proteins (535). As matricellular proteins, they contain binding sites for ECM and putative cell surface receptors; most of their biologic activities are thought to be mediated by cell adhesion receptors,

including integrins (e.g. α2β1, α5β1, etc.), and heparin sulfate proteoglycans (HSPGs).

Amongst the CCN5 family members, CCN2/CTGF has clearly been shown to regulate pancreatic islet function (512- 515). CCN2 promotes the formation of new islets from pancreatic ducts, the expansion of immature β-cells, and islet vascular development (512, 514). Islet morphogenesis and function are exquisitely regulated by CCN2, which is both required and sufficient to induce proliferation of immature β-cells. On the other hand, β-cells in more mature, postnatal animals do not express CCN2 and appear refractory to its presence in other cells (512-515). The novel role of CCN5 in promoting β-cell proliferation in this study and the reported induction of CCN4/WISP1 expression during islet regeneration after 90% pancreatectomy (536) suggest that CCN2 is not the only member of the family that regulates islet cell biology. Interestingly the expression of CCN2 and CCN5 appear to be interdependent since knockdown of CCN5 gene expression in MCF-7 cells resulted in 12-fold compensatory increase of CCN2 mRNA level (527). CCN5 has a unique age-dependent and tissue-specific expression pattern, its expression in β-cells is selectively induced by IGF-I, and it has a unique structure missing the CT domain. All these characteristics, together with its regulation by glucocorticoids (537), estrogen and progesterone (538, 539), EGF (40), PMA and phorbol esters (541), and IGF-I (506) in other type of cells, may contribute to its unique role in islet function.

CCN5 is expressed as early as the 4-cell stage and seems to be important for embryonic development. In early-mid embryonic stages, E9-11, it is expressed in the cells of ectodermal, mesodermal, and endodermal origins and is essential for development as CCN5 knockout caused

early embryonic lethality (522). From early embryonic upto adult stage, CCN5 is highly expressed in the layers of endothelium and smooth muscle of blood vessels, as well as in the myocardium of the heart, skeletal muscle, colon and ovary (522). As a direct target of Wnt signaling, CCN5 is a potential link between insulin and IGF-I in regulating the islet function. Overexpression of Wnt protein increased CCN5 mRNA and protein levels (542). Interestingly, insulin and IGF-I also stimulate cat/TCF-mediated gene transcription, independent of Wnt; stimulate PKB/Akt and the phosphorylation of GSK-3β at Ser21/9; and stabilize β-catenin (543). While IGF-I, Wnt and TCF are clearly involved in pancreatic islet function (544), the role of their common target protein CCN5 has never been studied. Supporting a role of CCN5 in islet function and/or diabetes, both CCN5 mRNA level from micro dissected β-cells and blood CCN5 concentration were doubled in T2D subjects according to data available on Gene Expression Omnibus (profile ID GDS3782 and 3963; http://www.ncbi.nlm.nih.gov/geo/).

Our islet-proliferative findings support previously reported ability of IGF-I to induce CCN5 expression which enhances the proliferation of breast cancer cells through PI3K/Akt pathway (506), and its involvement in the proliferation of 3T3-L1 preadipocytes (526). In non-islet cells, the proliferative role of CCN5 has been studied in great length, e.g. knockdown of CCN5 expression inhibited the proliferation of MCF-7 cells induced by either serum, EGF or PMA (538, 540, 541). Nevertheless, CCN5 has also been reported to *inhibit* proliferation of smooth muscle, uterine myometrial, ER-negative breast cancer and MCF-7 cells (507, 508, 522, 545). These seemingly conflicting reports warrant further studies to decipher the underlying mechanisms

for those cell-specific effects and to establish its role in islet cell biology.

In summary, we have shown that CCN5/WISP2 is normally expressed in mouse islet β-cells and that IGF-I directly stimulate its expression. CCN5 overexpression increases the proliferation of insulinoma cells, activates Akt and Erk2 kinases, and inhibits streptozotocin-induced caspase-3 activation and apoptosis. Recombinant CCN5 protein seems to reproduce the proliferative effect and the stimulation on Akt phosphorylation. These findings suggest that CCN5 is capable of regulating islet cell proliferation and survival and that increased CCN5 expression may contribute to IGF-I-stimulated islet cell growth and/or survival.

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2.7 Author's contribution: S.C. performed all experiments, presented the data and revised the manuscript, X.W. performed microarray and Table 1, C.B.S. and G.N. contributed to discussion and revision, M.F. performed confocal microscopy and data analysis, Q.L. and Y.J.G. participated part of the experiments and their analysis, J.L.L. designed the study, wrote the manuscript and approved the final revision.

Table 2.1. The effects of IGF-I overexpression on pancreatic islet gene expression by cDNA microarray. N=3. Listed were transcript ID (gene symbol), average fold change, P value based on two-tailed t-tests, and common gene name, divided into functional clusters. Repeat: when the same gene has been classified into more than one gene groups, the fold change and P value in the table were not repeated.

Gene symbol	Fold change	P value	Common name
Extracellular matrix			
ADAMTS2	2.23	0.007	a disintegrin and metalloproteinase with thrombospondin repeats; procollagen N-proteinase
BGN	1.96	0.003	biglycan
COL14A1	2.14	0.000	collagen, type xiv, alpha 1
COL16A1	2.29	0.006	collagen, type XVI, alpha 1
COL20A1	2.21	0.001	collagen, type xx, alpha 1
COL3A1	2.35	0.004	collagen, type iii, alpha 1
COL8A1	1.91	0.009	collagen, type viii, alpha 1
DCN	2.47	0.008	decorin
ELN	1.98	0.002	elastin
FBLN2	3.24	0.008	fibulin 2
MMP2	3.24	0.009	matrix metallopeptidase 2
OLFM4	1.59	0.004	olfactomedin 4
SPARCL1	1.62	0.004	sparc-like 1 (mast9, hevin)
TGM2	1.93	0.005	transglutaminase 2, c polypeptide
THSD4	1.53	0.007	thrombospondin, type i, domain containing 4
VWF	3.37	0.001	von willebrand factor homolog
Cell morphogenesis			
GALR2	0.60	0.009	galanin receptor 2
GAS6	1.53	0.009	growth arrest specific 6
HTRA1	1.73	0.007	htra serine peptidase 1
IGFBP4	2.09	0.009	insulin-like growth factor binding protein 4
IGFBP5	1.76	0.006	insulin-like growth factor binding protein 5
NRN1	2.92	0.008	neuritin 1
RDX	1.82	0.008	radixin
WISP2/CCN 5	**2.70**	**0.006**	**wnt1 inducible signaling pathway protein 2**
Endoplasmic reticulum			
APH1A	0.69	0.008	anterior pharynx defective 1a homolog (c. elegans)

CYP1B1	2.36	0.010	cytochrome p450, family 1, subfamily b, polypeptide 1
HSD11B1	2.32	0.009	11ß-hydroxysteroid dehydrogenase 1 (11ß-HSD1)
KDELR3	0.69	0.008	kdel (lys-asp-glu-leu) ER protein retention receptor 3
SCD2	1.66	0.004	stearoyl-coenzyme a desaturase 2
VWF	(Repeat)		
Cell signal, proliferation and transcription			
2310047A01 RIK	1.70	0.001	zinc finger, CCHC domain containing 24
AXL	1.54	0.008	axl receptor tyrosine kinase
BATF	0.51	0.007	basic leucine zipper transcription factor, atf-like
EFS	2.60	0.003	embryonal Fyn-associated substrate, Sin, SH3 adaptor protein
ERRFI1	1.72	0.003	ERBB receptor feedback inhibitor 1
GDF10	2.81	0.005	growth differentiation factor 10
HAVCR2	1.70	0.001	hepatitis a virus cellular receptor 2
HMGB1	1.52	0.004	high-mobility group box 1
HOXA5	1.91	0.002	homeo box a5
JDP2	1.64	0.007	Jun dimerization protein 2
LGALS12	0.67	0.002	lectin, galactose binding, soluble 12
MDK	1.89	0.005	midkine
NNMT	1.89	0.008	nicotinamide N-methyltransferase
PAK6	0.61	0.001	p21 (cdkn1a)-activated kinase 6
PEBP1	0.32	0.000	phosphatidylethanolamine binding protein 1
PHLDB2	1.53	0.001	pleckstrin homology-like domain, family B, member 2
PHLPPL	0.68	0.000	PH domain and leucine rich repeat protein phosphatase-like
PIK3C2G	0.65	0.005	Class II PI3K, C2γ subunit (PI3K-C2γ)
PLEKHK1	1.51	0.008	pleckstrin homology domain containing, family k member 1
PPP1R14A	1.63	0.004	protein phosphatase 1, regulatory (inhibitor) subunit 14A
RMND5B	0.44	0.005	required for meiotic nuclear division 5 homolog B
RTKN2	0.44	0.004	rhotekin 2, PH Domain
TGFA	0.66	0.003	transforming growth factor alpha
ZFP365	3.18	0.005	zinc finger protein 365
Peptidase activity			
ADAMTS2	(Repeat)		
APH1A	(Repeat)		
DHH	1.79	0.004	desert hedgehog
HTRA1	(Repeat)		
KLK12	0.70	0.000	kallikrein 12
MMP2	(Repeat)		
PAPPA	0.66	0.006	pregnancy-associated plasma protein a

TGM2	(Repeat)		
THSD4	(Repeat)		
	Ion transport		
ATP2B3	0.65	0.006	atpase, ca++ transporting, plasma membrane 3
CATSPER2	2.10	0.010	cation channel, sperm associated 2
CP	1.76	0.005	ceruloplasmin
KCNF1	2.25	0.001	potassium voltage-gated channel, subfamily f, member 1
	GPCR signaling pathway		
CSPRS	0.59	0.000	component of Sp100-rs
GALR2	(Repeat)		
OLFR1123	1.64	0.001	olfactory receptor 1123
OLFR584	1.50	0.000	olfactory receptor 584
OLFR61	0.69	0.008	olfactory receptor 61
OLFR691	1.88	0.008	olfactory receptor 691
TGM2	(Repeat)		
UTS2R	1.91	0.008	urotensin 2 receptor
V1RI1	1.57	0.008	vomeronasal 1 receptor, i1
	Other unclassified		
2010110I21RIK	1.68	0.008	atlastin GTPase 2
ABCA8A	2.69	0.008	ATP-binding cassette, sub-family A (ABC1), member 8a
ADFP	1.85	0.001	adipose differentiation related protein
C1QTNF5	1.54	0.008	c1q and tumor necrosis factor related protein 5
C730026E21RIK	0.61	0.008	methionine-tRNA synthetase 2 (mitochondrial) (Mars2),
CAR15	1.91	0.009	carbonic anhydrase 15
CCL2	1.58	0.001	chemokine (C-C motif) ligand 2
CDH6	0.69	0.009	cadherin 6, type 2, K-cadherin (fetal kidney)
GAST	0.44	0.009	Gastrin
GPT1	0.59	0.003	glucose-6-phosphate transporter 1
LHFP	1.97	0.001	lipoma HMGIC fusion partner
LRRC38	4.72	0.002	leucine rich repeat containing 38
PLXDC2	1.51	0.009	plexin domain containing 2
RNU65	1.60	0.004	small nucleolar RNA, H/ACA box 65
TMEM208	0.44	0.002	transmembrane protein 208

Table 2.2. **The effects of IGF-I overexpression on pancreatic islet gene expression determined by real-time PCR.** Total RNA was prepared from isolated islets from 4-5 month old MT-IGF vs. wild-type male mice. Real-time PCR was performed using QuantiFast SYBR Green RT-PCR Kit (Qiagen) and Roche LightCycler System. The relative expression level in arbitrary unit (mean ± S.E.) was based on an experiment of N=6.

Target gene	Wild-type	MT-IGF	P value
KCNF1 (Kv5.1 potassium channel)	13.1 ± 1.1	23.9 ± 2.6	0.0064
HSD11B1 (11β-HSD1)	7.5 ± 1.7	16.3 ± 2.3	0.0072
FBLN2 (Fibulin-2)	9.9 ± 2.0	23.3 ± 4.1	0.0149
COL14A1 (Collagen 14A1)	8.6 ± 1.3	12.7 ± 1.2	0.035
VWF1 (von willebrand factor homolog)	6.9 ± 1.9	14.3 ± 2.5	0.046
PIK3C2G (PI3K-C2γ)	16.8 ± 1.7	11.3 ± 1.1	0.006
CCN5/WISP2 (wnt1 inducible signaling pathway protein)	2.5 ± 2.7	8.4 ± 1.3	0.008
MMP2 (Matrix metallopeptidase 2)	9.3 ± 3.3	16.0 ± 2.8	NS
Catsperm2 (Cation channel, sperm associated 2)	8.0 ± 2.9	9.9 ± 1.8	NS

Figure Legends:

Figure 2.1

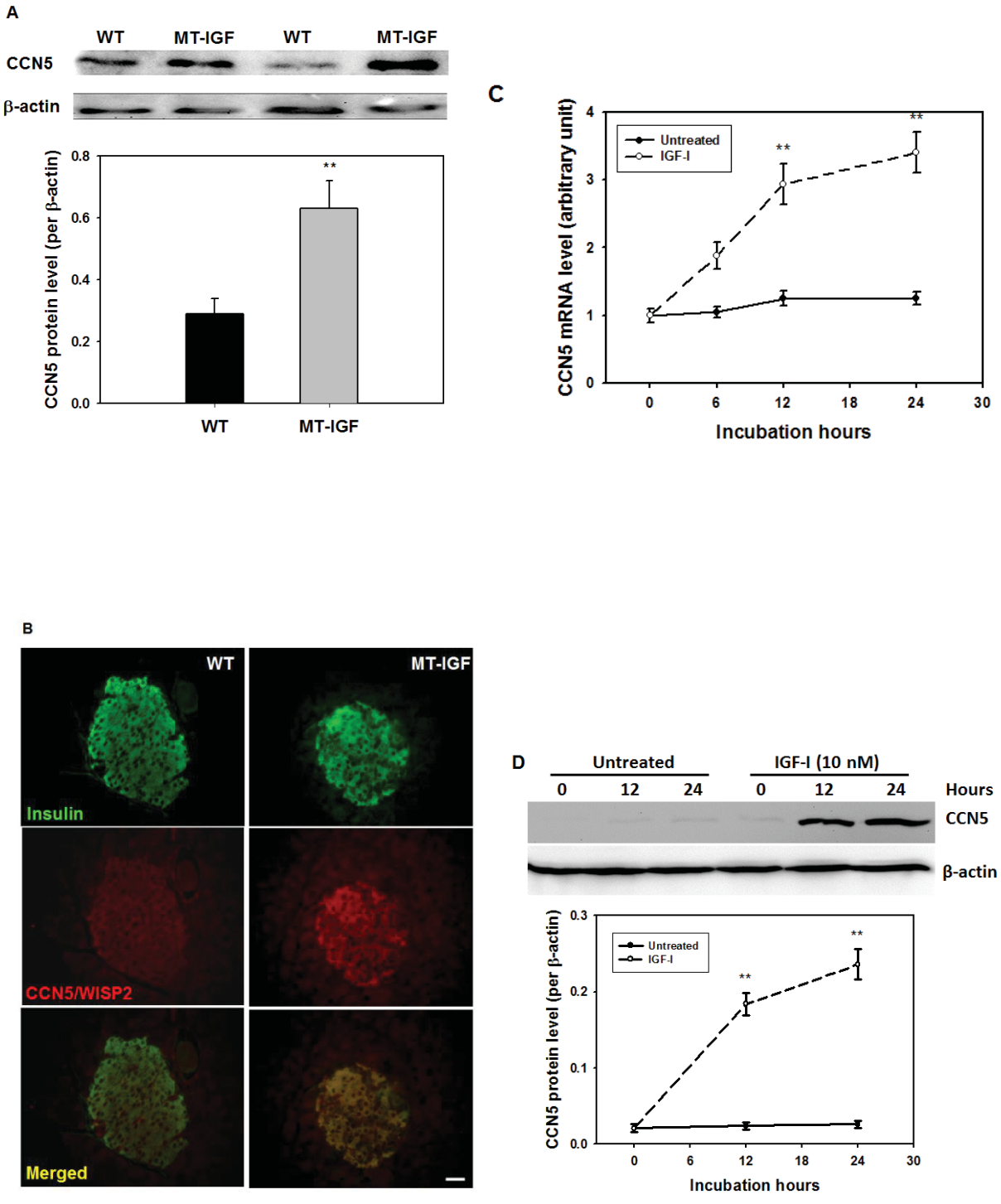


Figure 2.1. Islet β-cell-specific expression of CCN5/WISP2 and its significant induction by IGF-I. **A.** Representative Western blots using freshly isolated islets of 3-4-mo-old wild-type (WT) or MT-IGF mice and specific antibodies against CCN5 and β-actin. The result of densitometry was shown below. N=3, P<0.01 using unpaired t-test; the experiment was repeated twice with at least three independent data points each time. **B.** Immunofluorescence was performed using paraffin sections of the pancreata and primary antibodies against insulin (green) and CCN5/WISP2 (red). Merged image showed co-localization of CCN5 and insulin in ß-cells of the islets (orange). The scale bar was 60 microns. Representative images taken from 3 mice in each genotype and >10 images. **C.** The effect of in vitro direct stimulation by IGF-I. Freshly isolated islets from 3-4 mo-old male C57B/L6 mice were treated with 10^{-8} M recombinant human IGF-I, Long R3 or vehicle for 6-24 h. Changes in the level of CCN5 mRNA in arbitrary unit were measured by qRT-PCR. N=4, **P<0.01 vs. untreated islets using unpaired t-test. **D.** Changes in the level of CCN5 protein determined by Western blot and quantified using densitometry. N=5, **P<0.01 vs. untreated using unpaired t-test.

Figure 2.2

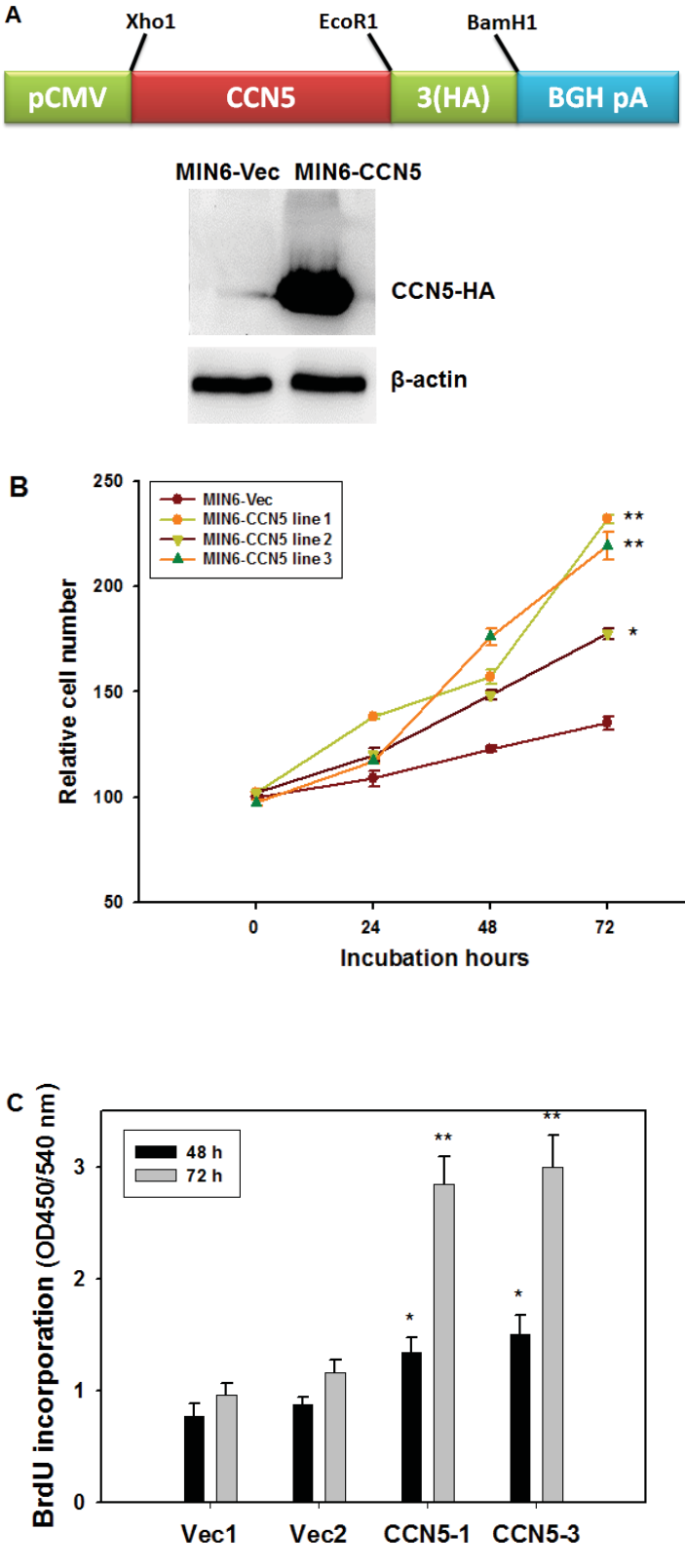
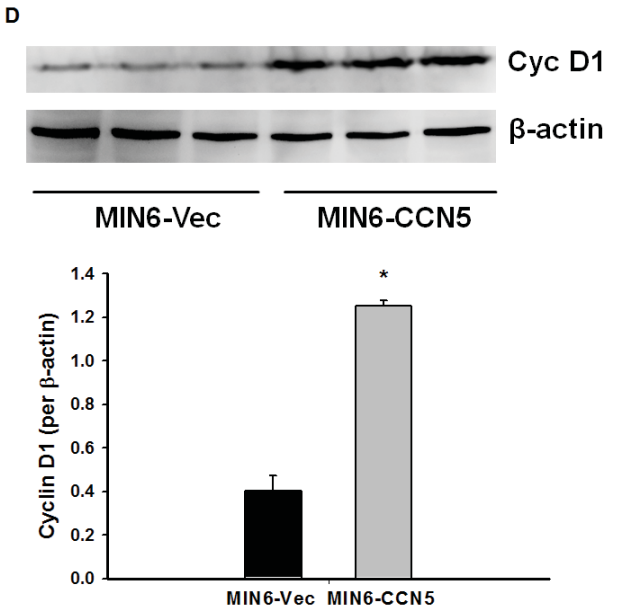

A
Xho1
EcoR1
BamH1
pCMV
CCN5
3(HA)
BGH pA
MIN6-Vec
MIN6-CCN5
CCN5-HA
β-actin
B
MIN6-Vec
MIN6-CCN5 line 1
MIN6-CCN5 line 2
MIN6-CCN5 line 3
Relative cell number
Incubation hours
C
48 h
72 h
BrdU incorporation (OD450/540 nm)
Vec1
Vec2
CCN5-1
CCN5-3

Figure 2.2


D
Cyc D1
β-actin
MIN6-Vec
MIN6-CCN5
Cyclin D1 (per β-actin)
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
*
MIN6-Vec
MIN6-CCN5

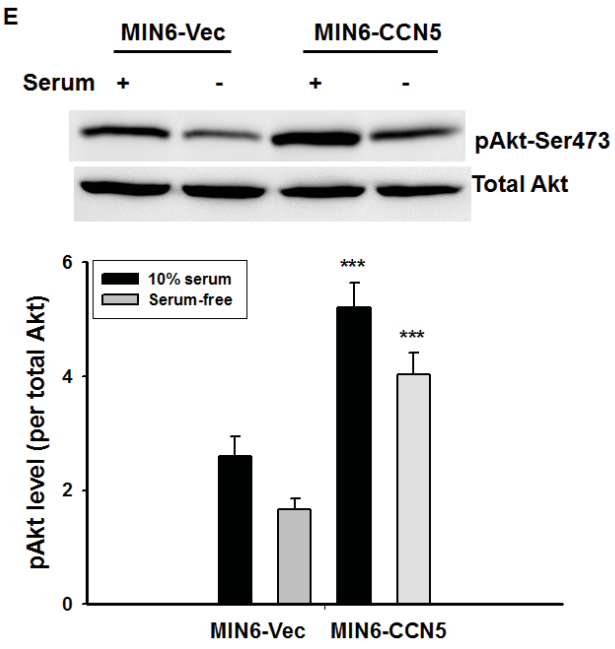

E
MIN6-Vec
MIN6-CCN5
Serum + - + -
pAkt-Ser473
Total Akt
10% serum
Serum-free
pAkt level (per total Akt)
6
4
2
0
***
***
MIN6-Vec
MIN6-CCN5

Figure 2-2

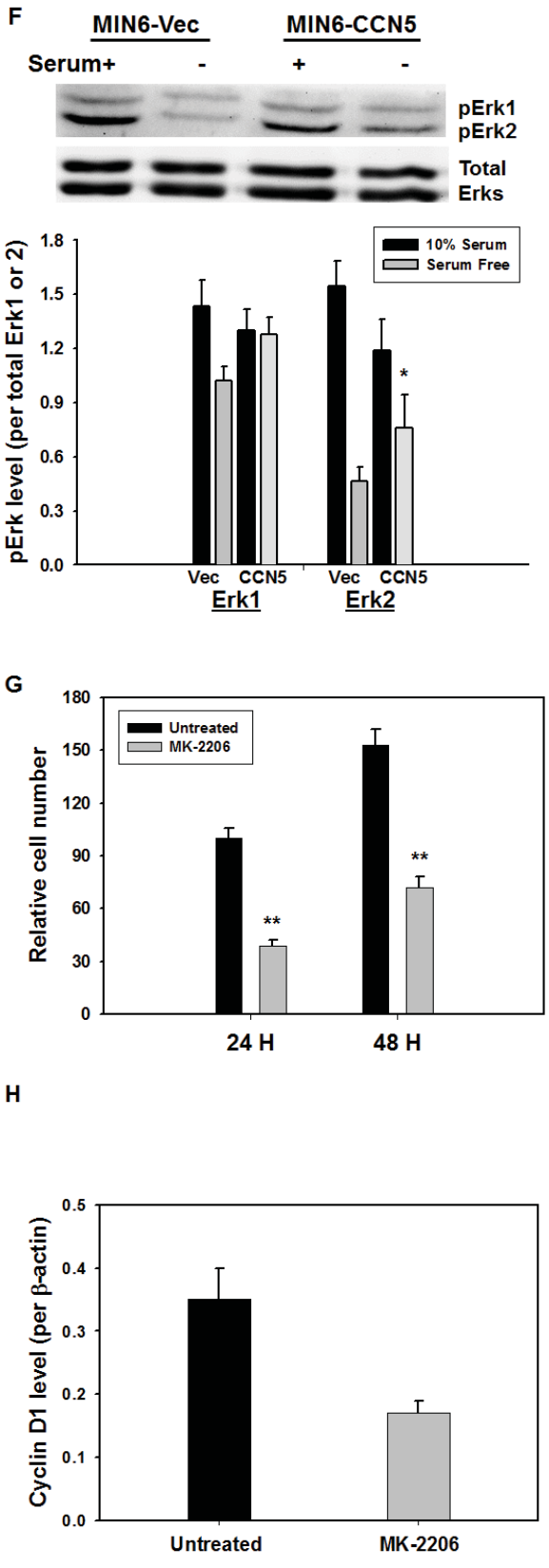

F
MIN6-Vec
MIN6-CCN5
Serum+
-
+
-
pErk1
pErk2
Total
Erks
pErk level (per total Erk1 or 2)
1.8
1.5
1.2
0.9
0.6
0.3
0.0
10% Serum
Serum Free
*
Vec
CCN5
Vec
CCN5
Erk1
Erk2
G
Relative cell number
180
150
120
90
60
30
0
Untreated
MK-2206
**
**
24 H
48 H
H
Cyclin D1 level (per β-actin)
0.5
0.4
0.3
0.2
0.1
0.0
Untreated
MK-2206

Figure 2.2 Increased cell proliferation and Akt phosphorylation in CCN5-overexpressing MIN6 cells. A. Stable overexpression of CCN5 cDNA with a 3'-HA tag in MIN6 cells using pcDNA3.1 vector. The construct was illustrated and CCN5 overexpression was confirmed using Western blot and HA antibody. **B.** Higher increase in the cell numbers measured by MTT assay. CCN5-overexpressing cell lines cultured with 1% serum showed increased cell proliferation at 48-72 h. N=5, *P<0.05, **P<0.01 vs. MIN6-Vec cells using one-way ANOVA. **C**. Increased cell proliferation measured by the ratio of BrdU incorporation. CCN5-overexpressing vs. vector-control cell lines (two each) cultured with 1% serum showed increased cell proliferation at 48 and 72 h. The relative ratio of BrdU incorporation was measured by ELISA. N=4, *P<0.05, **P<0.01 vs. either MIN6-Vec cells, using one-way ANOVA and post-hoc Holm-Sidak test. **D.** CCN5 overexpression caused increased cyclin D1 level. The cells were cultured in 5.5 mM glucose and 1% serum for 3 d. Western blots were performed in multiple lines of MIN6-CCN5 vs. MIN6-Vec cells. The result of densitometry was illustrated in bar graph. N=3; *P<0.05 vs. MIN6-Vec cells using unpaired t-test. **E.** CCN5 overexpression caused increased phosphorylation of Akt kinase. MIN6-Vec or –CCN5 cells were cultured for 24 h under zero or 10% serum as indicated. Representative Western blots for Akt and results of densitometry were illustrated. **F.** Representative Western blots for Erk1/2 and results of densitometry. Mean ± S.E. Representatives of three experiments. For E and F, N=3. *P<0.05, ***P<0.001 vs. MIN6-Vec cells under the same condition using unpaired t-test. **G.** Effect of Akt inhibitor MK-2206 (1 μM) on the viability of MIN6-CCN5 cells measured using MTT assay after 24 and 48 h incubation as in Fig 2B. N=3, **P<0.01 vs. untreated cells using unpaired t-test. **H.** Effect of Akt inhibitor MK-2206 on cyclin D1 level in MIN6-CCN5 cells after 24 h incubation. Western blots and the result of densitometry were illustrated. N=2.

Figure 2.3

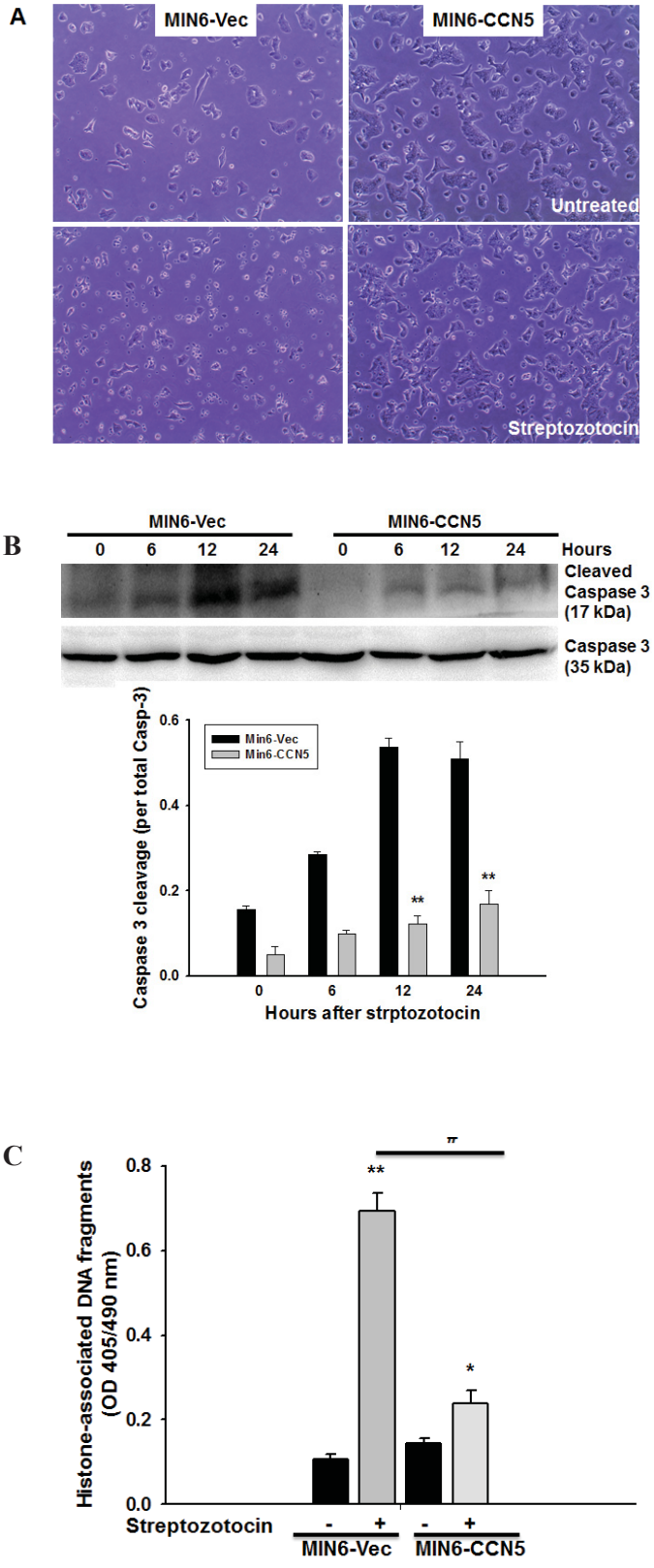
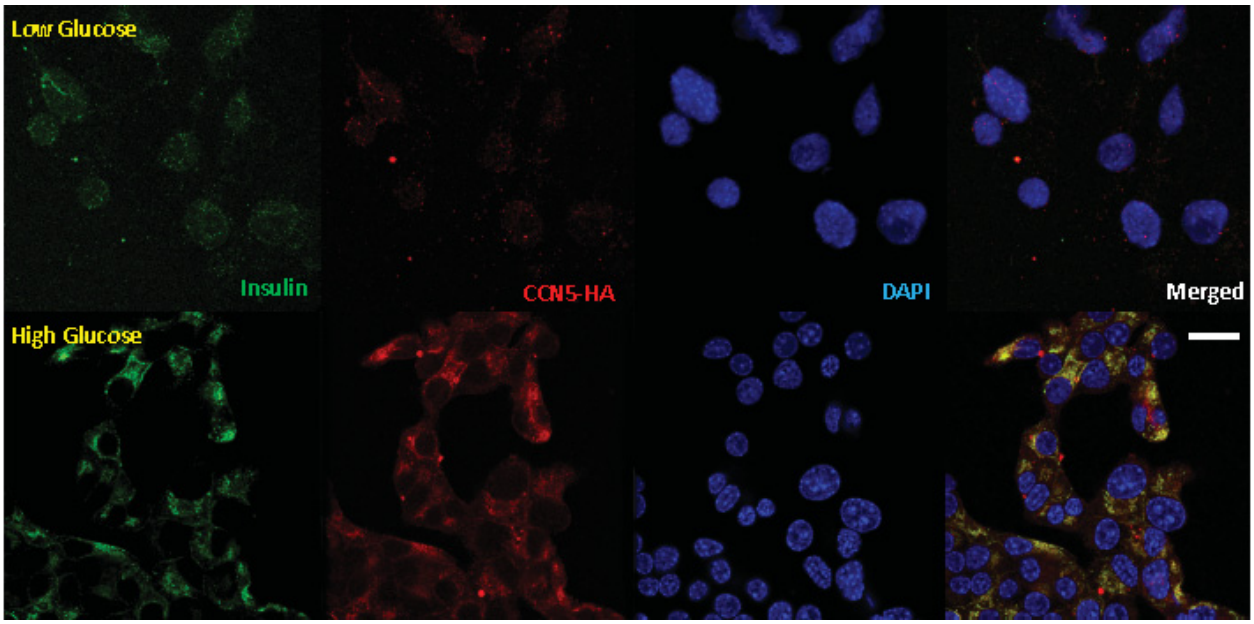

A
MIN6-Vec
MIN6-CCN5
Untreated
Streptozotocin
B
MIN6-Vec
MIN6-CCN5
0 6 12 24 0 6 12 24
Hours
Cleaved Caspase 3 (17 kDa)
Caspase 3 (35 kDa)
Caspase 3 cleavage (per total Casp-3)
Min6-Vec
Min6-CCN5
0.0
0.2
0.4
0.6
0 6 12 24
Hours after strptozotocin
C
Histone-associated DNA fragments (OD 405/490 nm)
0.0
0.2
0.4
0.6
0.8
Streptozotocin
- + - +
MIN6-Vec
MIN6-CCN5

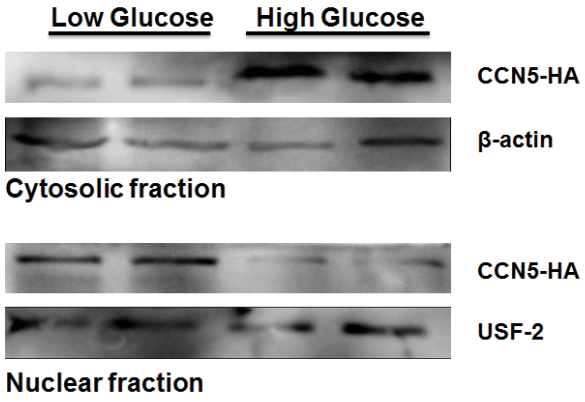
Figure 2.3 CCN5 overexpression enabled MIN6 cells resistant to streptozotocin-induced apoptosis. **A.** Cell morphology after 24 h treatment with or without streptozotocin in MIN6-CCN5 vs. MIN6-Vec cells in 100X magnification, showing condensation, cell loss and debris (bottom left, streptozotocin on vector-transfected cells) vs. high density, normally stretched cells in other three panels. **B.** Changes in the level of caspase 3 cleavage induced by streptozotocin. N=3, **P<0.01 vs. MIN6-Vec cells using one way ANOVA. The experiment was repeated once. **C**. Changes in cytoplasmic level of histone-associated DNA fragments, quantified using ELISA. Absorbance reading at 490 nm was subtracted from that of 405 nm, following the manufacturer's instruction. N=5, *P<0.05, **P<0.01 vs. untreated cells; $^{\#}P < 0.05$ vs. streptozotocin-treated MIN6-Vec cells using one-way ANOVA.

Figure 2.4

A



B



C

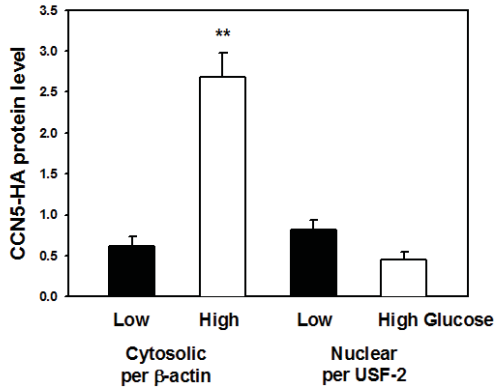
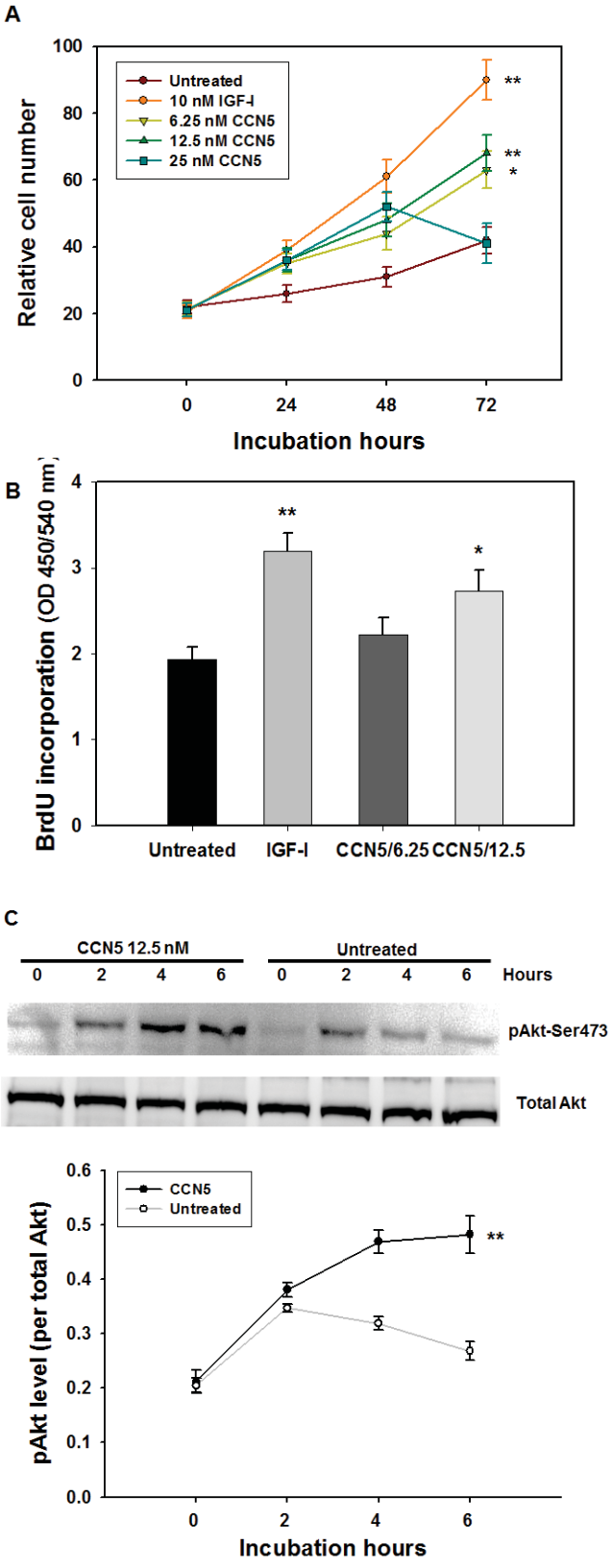


Figure 2.4 High glucose-induced subcellular distribution of CCN5-HA protein in MIN6-CCN5 cells. A. Cells were cultured in chamber slides and at low (2.8 mM) or high (16.7 mM) glucose for 60 min. Confocal microscopy images were developed against insulin (green), HA-tag (for CCN5, red), DAPI (nucleus, blue) and merged. Representative images from triplicated chambers and >10 sets of images were illustrated. The scale bar was 20 micron. **B.** Changes in subcellular distribution of CCN5 confirmed by Western blots. From a separate experiment cultured by low and high glucose, cytosolic and nuclear fractions were blotted using antibodies against CCN5-HA, β-actin, and USF-2. Negative signals of USF-2 in cytosolic and β-actin in nuclear fractions were confirmed to exclude cross contamination, but not shown. Representatives of N=4. **C.** Quantification of result by densitometry. The level of cytosolic protein was corrected by β-actin, and nuclear protein by USF-2. N=4, **P<0.01 vs. low glucose using unpaired t-test.

Figure 2.5


A
Relative cell number
Incubation hours
Untreated
10 nM IGF-I
6.25 nM CCN5
12.5 nM CCN5
25 nM CCN5
B
BrdU incorporation (OD 450/540 nm)
Untreated
IGF-I
CCN5/6.25
CCN5/12.5
C
CCN5 12.5 nM
Untreated
Hours
pAkt-Ser473
Total Akt
pAkt level (per total Akt)
Incubation hours
CCN5
Untreated

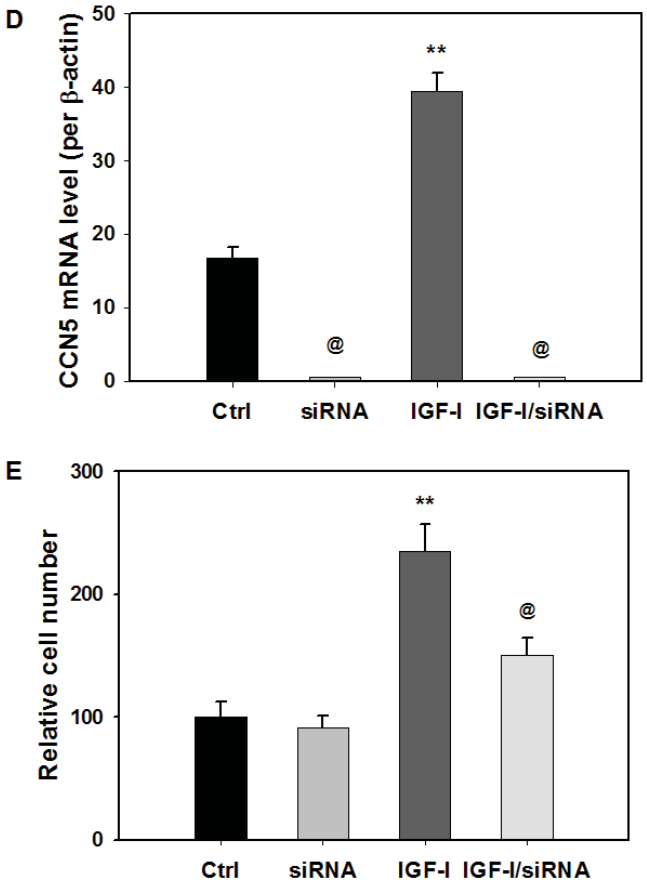


Figure 2.5 Direct effect of recombinant CCN5 protein on islet cell proliferation and Akt phosphorylation. A. Parental MIN6 cells (20,000 per well) were cultured for 1-3 d with IGF-I or increasing doses of CCN5 in DMEM containing 1% serum and 11.1 mM glucose, cell numbers were measured using MTT assay. N=6, *P<0.05, **P<0.01 vs. untreated using one-way ANOVA. **B**. Changes in the rate of cell proliferation measured by BrdU incorporation. Parental MIN6 cells were cultured with or without 10 nM IGF-I, or 6.25 and 12.5 nM CCN5 for 3 d in 1% serum. N=3-4, *P<0.05, **P<0.01 vs. untreated cells using unpaired t-test. **C.** CCN5 stimulated Akt phosphorylation. Under similar conditions as in Fig 5A, MIN6 cells were cultured for 2-6 h with or without 12.5 nM CCN5. Cell lysate was probed for pAkt at Ser-473 or total Akt using Western blots. A representative blot was illustrated on top panel from three experiments. Result of densitometry was depicted at lower panel. N=3, **P<0.01 vs. untreated cells using one-way ANOVA. **D.** siRNA-mediated transient knockdown of basal and IGF-I-stimulated (5 nM, 48 h) CCN5 gene expression in parental MIN6 cells measured by qRT-PCR. **E.** The effect of CCN5 gene knockdown on basal and IGF-I stimulated (5 nM, 72 h) change in cell viability measured by MTT assay. For D and E, N=4, **P<0.01 vs. scrambled vector-transfected untreated cells (1st columns; Ctrl), $^{@}$P<0.01 vs. scrambled vector-transfected cells treated with or without IGF-I using unpaired t-test.

Chapter III

IGF-I inhibits 11β-HSD1 level and activity in pancreatic β-cells

3.1 Abstract

We have previously reported high expression of IGF-I in pancreatic islet β-cells of transgenic mice expressing IGF-I under the metallothionein I promoter. cDNA microarray analysis of the islets isolated from the transgenic mice revealed 82 genes whose expression was significantly altered in comparison to that of wild-type littermates. Of these, 11β-hydroxysteroid dehydrogenase 1 (11β-HSD1), which is responsible for the conversion of hormonally inactive cortisone (11-dehydrocorticosterone, DHC in rodents) to active cortisol (corticosterone) in the liver and adipose tissues, has not previously been identified as a molecular target of IGF-I in pancreatic islets. Here we characterized the changes in 11β-HSD1 protein level, enzyme activity and glucose-stimulated insulin secretion (GSIS). In freshly isolated islets, the level of 11β-HSD1 protein was significantly lower in MT-IGF mice; using dual-labeled immunofluorescence, 11β-HSD1 was observed exclusively in islet α-cells at a diminished level in transgenic vs. wild-type animals. Decreased expression of 11β-HSD1 in MT-IGF mice correlated with decreased enzymatic activities as measured by the interconversion of 11-dehydrocorticosterone (DHC) and corticosterone. Dexamethasone (DEX) and DHC inhibited GSIS from freshly isolated islets of wild-type mice; in the islets of MT-IGF mice, 48-h pre incubation of DEX caused a significant decrease in GSIS; the effect of DHC was largely blunted, however, indicating the result of diminished 11β-HSD1 activity. In order to establish the function of intracrine glucocorticoids, we overexpressed 11β-HSD1 cDNA in insulinoma MIN6 cells, which together with DHC caused apoptosis and a significant decrease in proliferation, both of which can be abolished by the treatment of 11β-HSD1 inhibitor. Our results demonstrated an inhibitory effect of IGF-I on 11β-HSD1 expression and activity within the pancreatic islets, which may mediate at least part of the IGF-I effects on cell proliferation, survival and insulin secretion.

3.2 Introduction

Insulin-like growth factor I (IGF-I) stimulates proliferation of pancreatic islet cells in a glucose-dependent manner, protects the β-cells against diabetes, and exerts insulin-like effects (502, 581). It activates IGF-I receptor through tyrosine phosphorylation and recruits intracellular substrates such as insulin receptor substrates (IRS) 1-4 and Shc, which trigger activations of PI3K/Akt, Ras/MAPK (Erk) and c-Jun N-terminal kinase (JNK) pathways (584), as well as enabling stimulation on protein synthesis, cell survival and proliferation. We have previously reported MT-IGF mice exhibit both highly concentrated IGF-I overexpression in the β-cells of the pancreas and significant resistant to streptozotocin-induced diabetes (505). In order to seek novel molecular targets that mediate IGF-I action, we recently performed a whole-genome cDNA microarray analysis on total RNA prepared from isolated islets of MT-IGF mice and found 82 genes specifically up- or down-regulated (604). HSD11B1, encoding 11β-hydroxysteroid dehydrogenase 1 (11β-HSD1), was one of the prominent targets previously not shown to be normally expressed in the pancreatic islets nor regulated by IGF-I.

It has been known that glucocorticoids at pharmacological concentrations directly affect β-cell integrity and function (330, 502, 598). Either decreased or increased insulin secretion has been reported in response to both acute (in minutes) and prolonged (hours to days) exposure. However, the role of intracrine production of active glucocorticoids within the islets is poorly understood. As a reductase, 11β-HSD1 catalyzes the conversion of hormonally inactive cortisone (11-dehydrocorticosterone, DHC in rodents) to active cortisol (corticosterone) in the liver and adipose tissues, while the isozyme 11β-HSD2 (and 11β-HSD1 *in vitro*) catalyzes the reversed process of dehydrogenation (inactivating human cortisol into cortisone) (591). The level of 11β-HSD1 was found to be increased in the islets of diabetic rodents (330, 331) which is a suspected cause of β-

cell failure (330). In order to establish 11β-HSD1 as a novel target of IGF-I action and its physiological function, we first confirmed the localization of 11β-HSD1 in pancreatic islets at the protein level, characterized the changes in 11β-HSD1 level and activity regulated by IGF-I, and assessed its functional relevance to glucose-stimulated insulin secretion (GSIS). Our results seem to support an IGF-I-induced inhibition of this intracrine production of glucocorticoids, the latter of which serves as a molecular target in anti-obesity and anti-diabetic interventions, many of which are currently in advanced stages of drug development (562, 588).

3.3 Methods and Materials

3.3.1 Transgenic mice, pancreatic islet isolation and insulin secretion

Previously we reported MT-IGF mice (505, 604); the Research Institute Animal Care Committee of McGill University Health Centre approved all animal handling procedures. Pancreatic islets were isolated from MT-IGF and wild-type mice by collagenase digestion and allowed to recover for 3 h in DMEM medium containing 11 mM glucose and 10% fetal bovine serum, 10 mM HEPES, 2 mM glutamine, and 1 mM sodium pyruvate as reported (516). They were hand-picked either for pooled RNA and protein isolations, or to test for acute insulin secretion. Otherwise, they were divided into a 12-well plate (20 islets/ea) and cultured overnight at 37^{o}C in a humidified atmosphere containing 5% CO_2. Where indicated, the islets were pre-treated with or without 100 nM dexamethasone (DEX) and DHC for 48 h. For GSIS, the islets were pre-incubated with low glucose (3.3 mM) in Krebs-Ringer bicarbonate buffer for 1 h, and switched to those containing 16.7 mM glucose for another hour. The supernatant and disrupted islets in lysis buffer (70% Ethanol, 0.18 N HCl) were stored at -80^{o}C until measurement of insulin using ELISA (Alpco).

3.3.2 Western blot analysis

Freshly isolated islets from 3-4 month old male mice were sonicated in 150-200 □l lysis buffer supplemented with protease inhibitor tablet (Roche Diagnostics). The cell extract was diluted by 1-1.5 volume Laemmli loading buffer containing 5% β-mercaptoethanol (Sigma-Aldrich) and boiled for 5 min before loading onto SDS-PAGE gels. Together with liver and visceral fat extracts, Western blotting was performed to quantify protein levels of 11β-HSD1 (1:250, H-10 sc-20175, Santa Cruz) and β-actin (Santa Cruz or Medimabs). MIN6 cells were used to probe for total and cleaved caspase-3 (1:1000, Cell Signaling).

3.3.3 *In vitro* direct stimulation by IGF-I

Similar to our previous report (604), freshly isolated pancreatic islets pooled from 3 wild-type mice were allowed to recover overnight in culture medium containing 11 mM glucose and 10% fetal bovine serum (517). Distributed in triplicates of 50 into 24-well plates for each condition, the islets were cultured for 0 to 72 h in the same medium but containing 1% fetal bovine serum with or without 10^{-8} M of recombinant human IGF-I, Long R3 (I1271, Sigma-Aldrich). Total protein lysate was used for Western blots against 11β-HSD1 and β-actin.

3.3.4 Dual-labeled immunofluorescence

Paraffin sections of the pancreas taken from 3-5 month old, male MT-IGF and wild-type littermates were dewaxed, rehydrated, and blocked with 10% donkey serum, followed by overnight incubation with rabbit anti-11β-HSD1 antibodies (1:100; H-10 sc-20175, Santa Cruz and ab83522, Abcam) at 4ºC. After washing with PBS, sections were independently stained with anti-glucagon (C-18 sc-7779, Santa Cruz) and guinea pig polyclonal anti-insulin (ab7842, Abcam) followed by Alexa Fluor® 594 conjugated donkey anti-rabbit IgG (H+L) and Alexa Fluor® 488 goat anti-guinea pig IgG (Life technologies) (518, 519). The images were analyzed using Axioshop

2 plus microscope (Carl Zeiss), Retiga 1300 digital camera, and Northern Eclipse software (Empix Imaging).

3.3.5 Liver microsomal preparation

Microsomes were prepared from livers of male C57BL/6 mice, homogenized in 0.5 ml/mg tissue TED buffer (10 mM Tricine, 1.5 mM EDTA, and 1 mM dithiothreitol, pH 7.4) using a glass homogenizer and a loose-fitting Teflon piston with intermittent cooling. The homogenate was first cleared by being microcentrifuged at 13,000 g for 30 min at 4ºC, the supernatant was further centrifuged at 105,000 g for 60 min at 4ºC. The pellet was washed in TED buffer, centrifuged again at 105,000 g for 30 min at 4ºC, and resuspended in TED buffer (331). The protein concentration was determined by the Bradford method using Bio-Rad protein assay.

3.3.6 *In vitro* dehydrogenase and reductase assays of 11β-HSD1 activity

11β-HSD1 is a bidirectional enzyme with both reductase and dehydrogenase activities (572). To measure dehydrogenase activity, the conversion of corticosterone to DHC was measured by incubating 150 freshly isolated islets from each mouse for 24 h with 1.5 nM [1,2,6,7-^{3}H]-corticosterone (Perkin-Elmer). The steroids were extracted from the medium with ethyl acetate by vigorously vortexing and being centrifuged at 2,000 g for 10 min at 4ºC. The upper phase extract was evaporated under nitrogen. The steroids were then dissolved in 450 µl methanol, spotted on a thin layer chromatography (TLC) plate with unlabeled DHC and corticosterone as reference markers, and resolved with chloroform-methanol (95:5 v/v). Liver microsomes were also used as a positive control in comparison to the changes in pancreatic islets. TLC plates were first dried and placed in an enclosure containing iodine resublimed crystals (Fisher). Yellow-brownish spots appearing in regions containing DHC and corticosterone were scraped off, eluted with ethyl acetate, dried under nitrogen and mixed with LSC-cocktail (ScientiSafe Gel, Fisher). The

fractional conversion of corticosterone to DHC was calculated by analyzing LSC-cocktail mixture in liquid scintillation analyzer Ti-Carb 2801TR (Perkin Elmer). The dehydrogenase activity was expressed as percentage radioactivity of the fractions (587).

Alternatively, the established *in vivo* role of 11β-HSD1 is reductase-directed conversion of DHC into corticosterone in rodents, the activity of which was measured by the conversion of human cortisone to cortisol from freshly isolated islets and liver microsome. Batches of 300 islets from each mouse were incubated with 500 nM cortisone (Sigma-Aldrich) for 3 h. The steroids were extracted from the medium and the cortisol concentration was measured using ELISA (Enzo Life Science) as reported (585).

3.3.7 Stable overexpression of 11β-HSD1 cDNA and proliferation assay in MIN6 cells

To test the function of 11β-HSD1 *in vitro*, mouse cDNA with a 3'-$(HA)_3$ tag was sub-cloned into pcDNA3.1 vector (Invitrogen) between the cytomegalovirus promoter and bovine growth hormone polyadenylation sequence and used to transfect murine insulinoma MIN6 cells, which were selected using G418 (Wisent) for 60 d as reported (604). After Western blot confirmation (using Anti-HA Tag Antibody, Cat# G036 Abm), 11β-HSD1-overexpressing (MIN6-HSD1) and vector transfected (MIN6-Vec) clones were subject to 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium (MTT) cell viability (Sigma-Aldrich) and 5-bromo-2'-deoxyuridine (BrdU) incorporation assays. The cells were cultured in 10% serum for 1 to 3 d in the presence or absence of 11β-HSD1 inhibitor 10j (Cat# 385581, Calbiochem) before the assays were performed. For BrdU incorporation, in the final 18 h of incubation, 10 μM BrdU was added; its incorporation was quantified using ELISA at 450 nm (EMD Millipore).

3.3.8 Dehydrocorticosterone (DHC)-induced apoptosis in MIN6-HSD1 cells

MIN6-HSD1 cells were sub-cultured at a density of 2 $x10^4$ cells/cm^2 and incubated with

or without 100 nM DHC and 1 μM of 11β-HSD1 inhibitor for 72 h. DEX (100 nM) was used as a positive control to cause apoptosis. The occurrence of apoptosis was represented by the amount of DHC/DEX-induced caspase-3 cleavage which is quantified by Western blot using cleaved caspase-3 antibody (9661, Cell Signaling). In separate experiments, the change of cleaved caspase-3 level was also determined using immunofluorescence staining in MIN6-HSD1 cells treated with DHC alone, DHC and 11β-HSD1 inhibitor, and vehicle. 4% Paraformaldehyde fixed cells were permeabilized with 0.1% Triton X-100 in TBS for 10 min, probed with DAPI (blue) and cleaved caspase-3 antibody followed by Alexa 488 conjugated goat anti-rabbit IgG secondary antibody (green). Images were taken on an Axioskop 2 Plus microscope (Carl Zeiss) at 200X magnification.

The presence of mono- and oligonucleosomes in apoptosis were measured by histone-associated DNA fragments in the cytoplasm, using sandwich enzyme immunoassay cell death detection ELISA plus kit (Roche, Cat#11774425001) (600). Both MIN6-HSD1 and MIN6-Vec cells, after being treated for 72 h with or without DHC and 11β-HSD1 inhibitor, were collected by being centrifuged at 200 g for 10 min and lysed for 30 min with the buffer provided. The cell lysate was cleared by being centrifuged again at 200 g for 10 min. Aliquots of the supernatant (20 μl, representing the cytosolic fraction) were transferred to streptavidin-coated wells and incubated with anti-histone-biotin and anti-DNA-peroxidase antibodies for 2 h, followed by the 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) substrate for 10 min, and measured as a ratio of absorbance at 405 and 490 nm using Perkin Elmer Enspire multiplate reader.

3.3.9 Statistical analysis

Data were expressed as Mean ± S.E. and plotted using Sigma Plot version 11 (Systat Software), which was also used to perform ANOVA and pot-hoc Holm-Sidak test. Unpaired Student's t-test was performed using GraphPad InStat version 3. *P* values <0.05 were considered

to be significant.

3.4 Results

3.4.1 Decreased 11β-HSD1 protein level in the islets of IGF-I overexpressing mice

In a recent report, we performed whole-genome microarray analysis on freshly isolated pancreatic islets of MT-IGF and wild-type mice in order to identify novel targets of IGF-I action. Amongst those was CCN5/WISP2 which was functionally evaluated and reported by us (604). In this study, we report the elucidation of 11β-HSD1, another IGF-I regulated target which catalyzes the intracellular conversion and activation of glucocorticoids as previously reported in the liver, skeletal muscles and adipose tissues but not in pancreatic islets.

In order to establish functional relevance of the microarray screening, we compared the levels of 11β-HSD1 protein in freshly isolated islets from wild-type and MT-IGF mice. Although the microarray screening showed a significant increase in 11β-HSD1 mRNA level (604), we observed a 35% *decrease* in the protein level in the islets of MT-IGF vs. wild-type littermates (Fig 3.1A and 3.1B). The finding of 11β-HSD1 regulation by IGF-I in the islets is novel, although the inhibitory effects have been established in the liver and adipose tissues (275, 591); the latter was clearly confirmed in Fig 3.1A and 3.1B. In order to determine if these findings arise from direct or indirect effects of IGF-I, we treated freshly isolated wild-type islets directly with 10 nM IGF-I for 1-3 d, which revealed a late-onset inhibition on 11β-HSD1 protein level (Fig 3.1C). At 48 and 72 h, the protein level was significantly decreased to 55% and 30% of untreated islets.

3.4.2 Islet α-cell specific 11β-HSD1 expression and its reduction by IGF-I overexpression

To determine the localization of 11β-HSD1 in specific cell types within the islets, we

performed dual-labeled immunofluorescence in reference to both glucagon-producing α-cells (Fig 3.2A) and insulin-labeled β-cells (Fig 3.2B). In wild-type islets, 11β–HSD1 was only detected in islet α-cells and in most α-cells as reported (587). In contrast to two other reports of β-cell expression (384, 585), we didn't detect significant 11β–HSD1 expression in the β-cells; in the islets of MT-IGF mice, there was no change in the α-cell localization but a clear reduction in the protein level per cell, and some islet α-cells become devoid of 11β-HSD1 (yellow arrow). As a positive control for antibody specificity, we confirmed extensive cytosolic staining of 11β-HSD1 in hepatocytes (data not shown). Highly consistent results were obtained using two specific antibodies against 11β-HSD1 from Abcam and Santa Cruz.

3.4.3 Decreased 11β-HSD1 reductase and dehydrogenase activities in the islets of MT-IGF mice

Following the demonstrations *in vivo* using immunohistochemistry, Western blotting on isolated islets, and *in vitro* IGF-I treatment, we sought to further validate the decreased 11β-HSD1 enzymatic activity. The notion this enzyme was not present in the islet β-cells but mainly expressed by the α-cells also requires further experiments to confirm its functional relevance. While normally 11β-HSD1 is an NADPH-dependent reductase, it can also act as an NAD-dependent dehydrogenase *in vitro*, thus catalyzing the interchangeable conversion of DHC and corticosterone in rodents. We first isolated fresh islets from MT-IGF and wild-type mice and performed an *in vitro* dehydrogenase assay on the conversion of [1,2,6,7-^{3}H]-corticosterone to DHC. The separation of different molecular forms was achieved by thin layer chromatography (TLC) and the radioactivity measured by liquid scintillation. On the TLC plates, in addition to the radioactive substrate and conversion product, we detected a third nonspecific intermediate which had very

little radioactivity and no significant variation among MT-IGF and wild-type mice, in both the liver and pancreatic islet samples (Fig 3.3A). We observed a significant 44% decrease in the rate of corticosterone dehydrogenation in the islets of MT-IGF vs. wild-type littermates (Fig 3.3B, first two bars). This reduction was consistent with the decrease of 11β-HSD1 protein level (Fig 3.1) and immunohistochemistry (Fig 3.2A and 3.2B). We further confirmed a 45% decrease in 11β-HSD1 activity in liver microsomes of MT-IGF vs. wild-type mice (Fig 3.3C, first two bars) (575).

Alternatively, the established *in vivo* role of 11β-HSD1 in rodents is reductase-directed conversion of DHC into corticosterone, the activity of which was measured by the production of (human) cortisol from cortisone in isolated islets of wild-type and MT-IGF mice (Fig 3.3D, first two bars). In the islets of MT-IGF mice it demonstrated a more significant 64% reduction; together with the 44% decrease in dehydrogenase activity (Fig 3.3B), they support a net reduction in 11β-HSD1 protein level. The decrease in liver 11β-HSD1 reductase activity was also confirmed (Fig 3.3D, right panels).

3.4.4 Differential effects of DEX and DHC on glucose-stimulated insulin secretion

Although excess glucocorticoids inhibit insulin secretion and cause β-cell death (582), the role of locally generated glucocorticoids within the islets by the action of 11β-HSD1 has not been established. We speculate that significantly decreased 11β-HSD1 level in the pancreatic islets and the resulting decrease in intracrine glucocorticoid concentration constitute part of the activity. In order to examine the effect on GSIS, we pre-incubated the islets for 48 h with 100 nM DEX or DHC, the latter requiring 11β-HSD1-mediated intracrine activation. As shown in Fig 3.4A, our islets exhibited a robust response in insulin release upon high glucose stimulation; MT-IGF islets seemed to have an elevated basal release (4^{th} vs 1^{st} bars), both DEX and DHC caused significant

25-39% decreases in GSIS from wild-type islets (compare bars 8 and 9 vs. 7). In MT-IGF islets, DEX had an even stronger inhibition on GSIS (50% inhibition of 11^{th} vs. 10^{th} bar); however, the inhibition by DHC was significantly weakened (only 26% inhibition of 12^{th} vs. 10^{th} bar). We suggest the latter effect was related to the diminished 11β-HSD1 level and intra-islet conversion of glucocorticoids. The relative fold changes were illustrated in Fig 3.4B, which showed a significant decrease from 11.8 fold in wild-type islets to 4.5 fold in MT-IGF islets due to elevated basal insulin release (Fig 3.4A, bars 1 and 4). Although DEX still inhibited GSIS (from 4.5 to 2.3 fold), the effect of DHC was largely blunted (from 4.5 to 3.6 fold; Fig 3.4B, bars 5 and 6 vs. 4), which seems to suggest that IGF-I overexpression cause diminished 11β-HSD1 level, decrease the activation of DHC in the islet cells, and partially rescue the inhibition of GSIS by intracrine glucocortiocids.

3.4.5 Decreased proliferation of insulinoma cells caused by 11β-HSD1 overexpression

To further define the effect of increased 11β-HSD1 in islet function, we transfected its cDNA into MIN6 cells using the pcDNA3.1 vector and generated three independent stable cell lines and confirmed significant overexpression of 11β-HSD1 protein in MIN6-HSD1 cells through Western blot. Immediately we noticed that MIN6-HSD1 cells were somehow unhealthy and grew slower which prompted us to assess cell viability using MTT. As shown in Fig 3.5A, MIN6-Vec cells with only low and endogenous 11β-HSD1 expression grew normally from 0 to 3 d, and relative cell numbers increased 11-fold (purple color line). The addition of DHC (100 nM, orange line) did not cause much change, consistent to that they only have low level endogenous 11β-HSD1. Consequently, 11β-HSD1 inhibitor (1 μM, light and dark green lines) had no effect on cells with/out DHC. However, MIN6-HSD1 cells grew much slower, reached only 7-fold increase in 3

d [vs. MIN6-Vec cells; ANOVA P<0.01] (Fig 3.5B, purple line). The addition of DHC further delayed cell number acceleration to only 2-fold (orange line); which was completely rescued by 11β-HSD1 inhibitor (dark green line back to 7-fold). Thus, simple overexpression of 11β-HSD1 in MIN6 cells (DHC is available in culture medium) either caused cell death or slowed proliferation, while increased DHC further deteriorated the situation; 11β-HSD1 inhibitor was sufficient to rescue the effect of added DHC but not enough to rescue the negative effect caused by 11β-HSD1 overexpression alone.

To confirm decreased proliferation caused by 11β-HSD1 overexpression and/or added substrate DHC, we assessed BrdU incorporation. As shown in Fig 3.5C, there was a 4.8-fold increase in BrdU incorporation in MIN6-Vec cells after 3 d culture (purple line). Addition of DHC and/or 11β-HSD1 inhibitor had no effect, supporting again that endogenous 11β-HSD1 level was low. In Fig 3.5D, overexpression of 11β-HSD1 itself had no effect on the proliferation of MIN6-HSD1 cells (purple line, same 4.8-fold). Addition of extra DHC decreased BrdU incorporation from 4.8 to 3-fold (orange line; ANOVA P<0.01 between two curves), an effect mostly rescued by the further addition of 11β-HSD1 inhibitor (dark green line); however the inhibitor itself had no effect (light green line). Clearly in the presence of added DHC, 11β-HSD1 overexpression inhibited cell proliferation but it cannot explain the entire decrease revealed using MTT assay (Fig 3.5A, B), which called for a study on 11β-HSD1 and intracrine glucocorticoids mediated cell death.

3.4.6 11β-HSD1 overexpression conferred DHC-induced apoptosis in insulinoma cells

Glucocorticoids are known to induce cell apoptosis by directly regulating typical apoptotic or survival genes, or by inducing cellular distress that triggers the apoptotic cascade (585). We

investigated the effect of 11β-HSD1 overexpression and DHC incubation on cell death in stably transfected MIN6 lines, first measured by histone associated DNA fragmentation in the cytoplasm (604). As shown in Figure 3.6A, first four bars, DEX treatment (4^{th} vs. 1^{st} untreated) in MIN6-Vec cells caused more than a 5.1-fold increase in the amount of DNA fragmentation; under the same condition, DHC (2^{nd} vs. 1^{st}) was without effect, because MIN6 cells have very little endogenous 11β-HSD1 (data not shown) to convert and activate DHC. In MIN6-HSD1 cells, both DHC and DEX caused 3.4- and 4.4-fold increases in DNA fragmentation respectively (6^{th} and 8^{th} vs. 5^{th} bars); addition of the 11β-HSD1 inhibitor largely abolished the effect of DHC (7^{th} vs. 6^{th} bars), supporting the notion that 11β-HSD1 mediated DHC activation causes apoptotic cell death.

Caspase-3 activation and cleavage is another independent indicator of cell apoptosis. In Fig 3.6B and 3.6C and MIN6-HSD1 cells, we demonstrated a 2.2-fold increase in caspase-3 cleavage after 72 h treatment with DHC, compared to 3-fold increase with DEX (Fig 3.6C, 2^{nd} and 4^{th} vs. 1^{st} bars). The effect of DHC was again abolished by the incubation with 11β-HSD1 inhibitor (3^{rd} vs. 2^{nd} bars), closely confirming our finding using DNA fragmentation in Fig 3.6A. In Fig 3.6C, using immunofluorescence, we further confirmed significantly increased caspase-3 cleavage in MIN6-HSD1 cells after being treated with DHC (middle panels, green spots highlighted by yellow arrows) which was largely abolished by the treatment with 11β-HSD1 inhibitor (bottom panels). Our results in Fig 3.5 and 3.6 support the notion that intracrine activation of DHC by the action of 11β-HSD1 not only inhibits cell proliferation, but perhaps more significantly causes apoptotic cell death.

3.5 Discussion

Following our recent report and from the same screening system (604), this study

characterized yet another target of IGF-I action in pancreatic islets. We demonstrate that 11β-HSD1 is normally expressed in pancreatic islets, more specifically in the □-cells; IGF-I overexpression decreased the protein level and enzymatic activity; and direct treatment of IGF-I in isolated islets caused a late onset reduction of the protein level. The decrease in the islets of MT-IGF mice has functional consequences, as it was associated with a blunted response to the substrate of the enzyme (DHC) which normally inhibits insulin secretion in wild types islets. To directly assess the function of this enzyme, in stably transfected insulinoma MIN6 cells, 11β-HSD1 overexpression decreased cell proliferation and caused cell apoptosis in the presence of DHC. Thus, the activity of 11β-HSD1 seems to affect β-cell survival, proliferation and insulin secretion. Together with established roles played in liver and adipose tissues, 11β-HSD1 expression in pancreatic islets constitutes a putative, novel target of diabetic intervention.

It is well established that excess glucocorticoids lead to obesity, insulin resistance and even T2D because glucocorticoids increase hepatic gluconeogenesis, promote central obesity with peripheral lipolysis, and cause β-cell apoptosis (330, 582). In the islet β-cells, glucocorticoids decreased Glut2 level, calcium influx and cellular cAMP generation by increasing α2-adrenergic receptor, all contributing to a general inhibition of insulin secretion 202, 205, 213). However, not all those effects are mediated by the classic endocrine mechanism. The last decade has seen an exponential increase in the research on 11β-HSD1, principally because of its role in human obesity and insulin resistance. This enzyme boosts glucocorticoid concentration in key tissues and has now been recognized as an important pre-receptor regulator. Targeted inhibition of 11β-HSD1 has the advantage of reducing the level of cortisol (corticosterone in rodents) in local tissues without causing a generalized glucocorticoid deficiency (591). Indeed, several 11β-HSD1 inhibitors are in advanced stage of development as candidate drugs against various metabolic syndromes (562).

In rodents, the active hormone corticosterone is predominantly bound to corticosteroid binding globulin, which is highly abundant and limits the hormonal effects (264). In contrast, the substrate DHC is freely available in circulation and in excess, which is readily activated in local tissues by the action of 11β-HSD1. Gene targeting experiments have clearly demonstrated that intracellular generation of corticosterone in the liver and adipose tissues from inert DHC constitutes a major component of glucocorticoid activities. 11β-HSD1 knockout caused a deficiency in DHC reductase, hyperandrogenism, precocious puberty and obesity in mice (264, 591). These animals exhibited significant hypoglycemia upon starvation and after diet-induced obesity, supporting a physiological role of 11β-HSD1 in maintaining normal glucose production and/or inhibiting insulin secretion (264). Our finding of islet-specific 11β-HSD1 expression is consistent to the overall model of intracrine secretion and that the expression and activity of 11β-HSD1 were both increased in diabetic islets (331). It has been reported that DEX, DHC and corticosterone all inhibit insulin and glucagon secretion; the effect of DHC on insulin secretion can be abolished by 11β-HSD1 inhibitors (330, 580,587). In obese diabetic KKAy mice, isosteviol, a diterpene molecule, improved insulin sensitivity by decreasing the expression of 11β-HSD1 in the pancreatic islets (577).

Early 11β-HSD1 was detected in the pancreatic islets of humans and ob/ob mice using RT-PCR (330, 580); in the pancreas as a double band of 25 and 34-kDa by Western blotting, with an extensive acinar cell staining by immunohistochemistry (247); and it was expressed specifically in the α- and PP-cells (587). Consequently, our observation of islet α-cell expression was based on the use of two specific antibodies from Abcam (ab83522) and Santa Cruz. However, a recent report indicated its expression in *human and rat β-cells* based on immunohistochemistry; although we can only detect a faint, non-specific staining in some *mouse* islets using the same antibody (Abcam

ab39364; data not shown) (568, 585). Another report using a polyclonal sheep antibody provided by Dr. Scott Webster (University of Edinburgh) also indicated 11β-HSD1 staining throughout the islets (151, 334), both of which have been criticized by us and need to be reconciled with other findings and using independent techniques such as *in situ* hybridization.

In MT-IGF mice, ectopically overexpressed IGF-I could influence neighboring α-cells and the expression of 11β-HSD1 through intra-islet paracrine or increased endocrine action (505). We propose that corticosterone produced in the α-cells by the action of 11β-HSD1 could either inhibit insulin release directly, in a paracrine fashion, or indirectly by inhibiting the secretion of glucagon (587), which stimulates insulin release. This would be consistent with 11β-HSD1-/- mice showing increased insulin but decreased glucose levels (264), and with the emerging importance of glucagon and α-cells in the etiology of diabetes mellitus (593). Unexpectedly, a moderate, β-cell-specific overexpression of 11β-HSD1 was reported to boost islet compensation against high-fat diet through islet neogenesis and diminished inflammation (334). We have reservations to accepting the "U-shaped" dose response because it directly challenged the diabetogenic role of glucocorticoids, and there was insufficient evidence to establish β-cell failure (151). Although a 58% fat diet was implemented for 12 weeks, wild-type mice did not gain any weight, there was no elevation in fasting blood glucose and insulin levels (Table 1 of the report) and no significant decrease in GSIS (Figure 3.2C, first two columns) (334). Islet compensation cannot be substantiated if the high-fat diet did not cause β-cell failure, unlike the data we have published (518).

It has been known that IGF-I inhibits 11β-HSD1 expression and its activity in adipocytes, stromal cells and hepatocytes (275, 575, 594). Conversely, glucocorticoids attenuate IGF-I action (558). However, in IGF-I knockout mice, the levels of 11β-HSD1 mRNA, protein and enzymatic

activity were significantly *decreased* in the liver, suggesting rather a stimulatory role for IGF-I (561). Through a whole genome screening, we first discovered a significant increase in 11β-HSD1 mRNA in the islets of MT-IGF mice (604), consequently decreased 11β-HSD1 protein level and enzyme activity *in vivo* in this study. The latter was further supported when primary islets were treated directly with IGF-I. The differential changes in mRNA and protein/activity levels may not be unreasonable as it has been found in yeast that for genes with equal mRNA levels, protein levels varied by more than 20-fold; for proteins with equal abundance, mRNA levels could vary by as much as 30-fold (556). A recently report investigated the discrepancy highlighting the facts that protein abundance seems to be predominantly regulated at the ribosome and the importance of translational control (601).

Glucocorticoids counteract IGF-I actions on islet β-cells in Akt phosphorylation, nuclear exclusion of FoxO1 and PDX1 activation and resulted in cell death and diminished proliferation (596). Assuming IGF-I decreases 11β-HSD1 level and its activity on the α-cells, the result would explain why MT-IGF islets have elevated basal insulin secretion *in vitro* (Figure 3.4) and how islet β-cells might be protected from streptozotocin-induced apoptosis as we have reported (505). Regardless of the exact mechanism, our observation is interesting because IGF-I was not known to regulate its level or activity in the pancreatic islets; and various 11β-HSD1 inhibitors decrease hepatic glucose production and fat mass, and are potential drugs against insulin resistance, obesity and T2D.

To further verify its functional relevance in islet β-cells, we evaluated possible changes in GSIS and the role of IGF-I and 11β-HSD1. As it has been well known, DEX inhibits GSIS from isolated islets of wild-type mice; a similar effect was seen using DHC (Figure 3.4). In isolated islets of MT-IGF mice, DEX caused a significant reduction in glucose-stimulated insulin release;

the same dose of DHC was not as effective, however, indicating the consequence of diminished 11β-HSD1 activity. There have been two earlier reports that insulin secretion was suppressed by 11β-HSD1-mediated intracrine production of corticosterone (580, 581). Incubation of the β-cells with DHC led to a dose-dependent inhibition of insulin release, which was reversed by 11β-HSD1 inhibitor carbenoxolone (330). The ability of DHC to reduce both the early and late phases of GSIS relies on 11β-HSD1 activity and the glucocorticoid receptor. Either elevated circulating level or increased intra-islet level of 11β-HSD1 or both, may increase the level of glucocorticoids and lead to inhibition of GSIS (580). As 11β-HSD1 activity regulates insulin secretion and IGF-I decreases 11β-HSD1 level/activity, we believe IGF-I regulates insulin secretion at least in part by inhibiting 11β-HSD1. The situation *in vivo* however is more complex in that decreased blood glucose level in MT-IGF mice, caused by increased serum IGF-I level, resulted in a 50% diminished insulin level (505). This systemic inhibition on insulin release seems to dominate a local suppression on glucocorticoid conversion within the islets by IGF-I overexpression.

To directly define the role of 11β-HSD1 on pancreatic islets, we stably overexpressed its cDNA in MIN6 cells and observed a decreased cell proliferation in the presence of substrate DHC, as measured by MTT assay and BrdU incorporation. The effect seemed to involve an inhibition of AKT phosphorylation (data not shown). In the meantime, the overexpression of 11β-HSD1 in the presence of DHC also triggered apoptosis of MIN6 cells as measured by caspase 3 activation and DNA fragmentation. The effects 11β-HSD1 overexpression on both cell proliferation and apoptosis were reversed by specific inhibitor of this enzyme.

In summary, our results support islet □-cell-specific expression of 11β-HSD1, and demonstrated an inhibitory effect of IGF-I on its expression and activity within the pancreatic islets, which may explain at least in part the decreased insulin secretion and/or previously reported

islet protective effects of overexpressed IGF-I. Increased expression of 11β-HSD1 in mouse insulinoma cells render DHC-induced apoptosis and inhibition on proliferation. An inhibitory effect of IGF-I in MT-IGF mice on 11β-HSD1 expression and activity exerted a positive impact on pancreatic islet through islet preservation.

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Disclosure: The authors have no conflict of interest to disclose.

Figures with legends

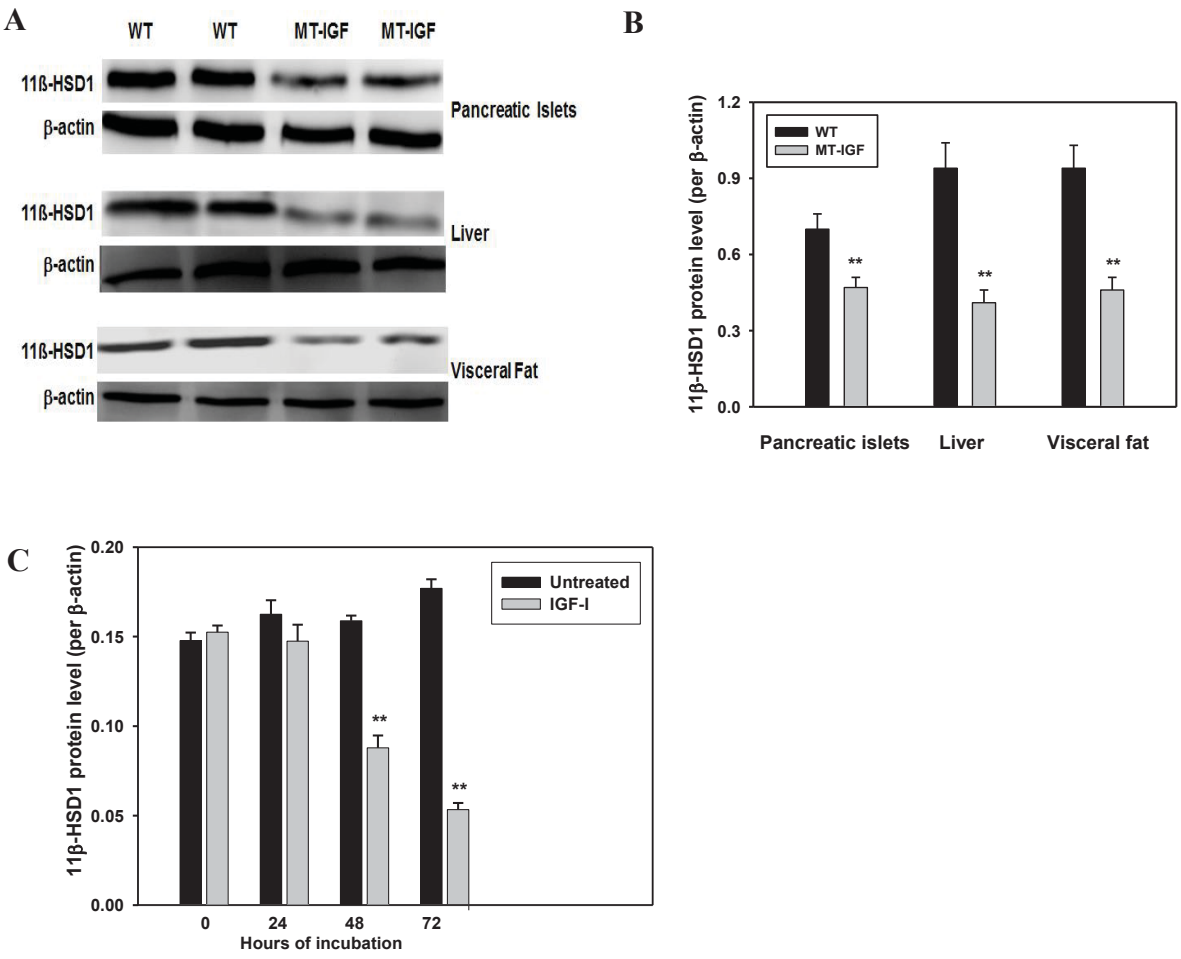
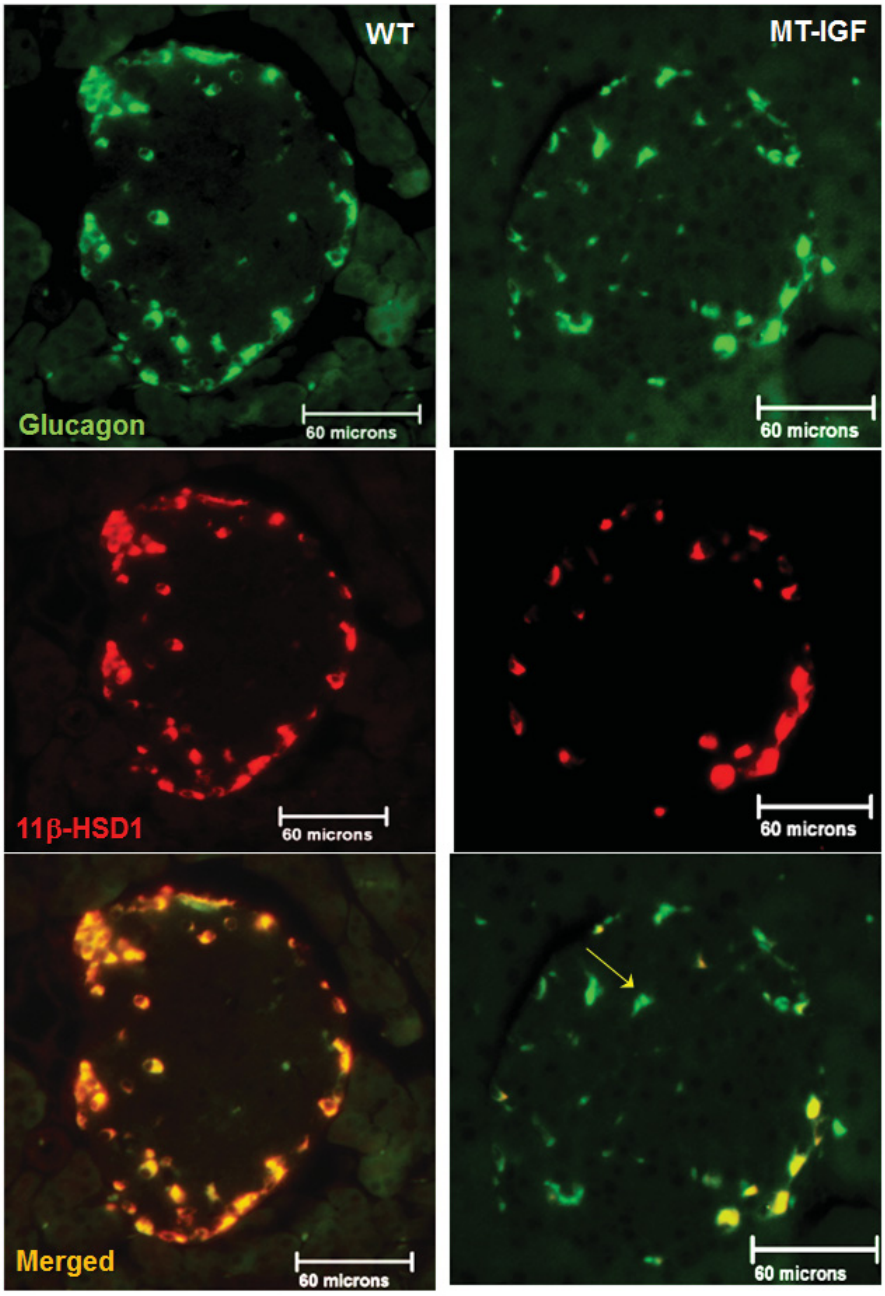


Figure 3.1 Decreased 11ß-HSD1 protein level by IGF-I overexpression *in vivo* and a direct treatment with IGF-I. A. IGF-I overexpression decreased the levels of 11ß-HSD1 in pancreatic islets, liver and visceral fat, shown in Western blots. Wild-type and MT-IGF littermates, 3-4 months old, were sacrificed to isolate pancreatic islets; liver and visceral fat; cell lysates were loaded on blots which were probed against 11β-HSD1 and β-actin. **B**. The result of Western blot densitometry. Mean ± SE, N=6, **P<0.01 vs. wild-type islets. **C.** Decreased 11β-HSD1 protein level caused by direct IGF-I treatment in freshly isolated islets from wild-type mice. Long R3 IGF-I, Sigma-Aldrich, 10 nM or vehicle was added; the islets cells were harvested after 1, 2, or 3 d. The result of Western blotting was corrected with that of β-actin. N=4, **P<0.05 vs. untreated.

A.


WT
MT-IGF
Glucagon
60 microns
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11β-HSD1
60 microns
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60 microns
60 microns

B.

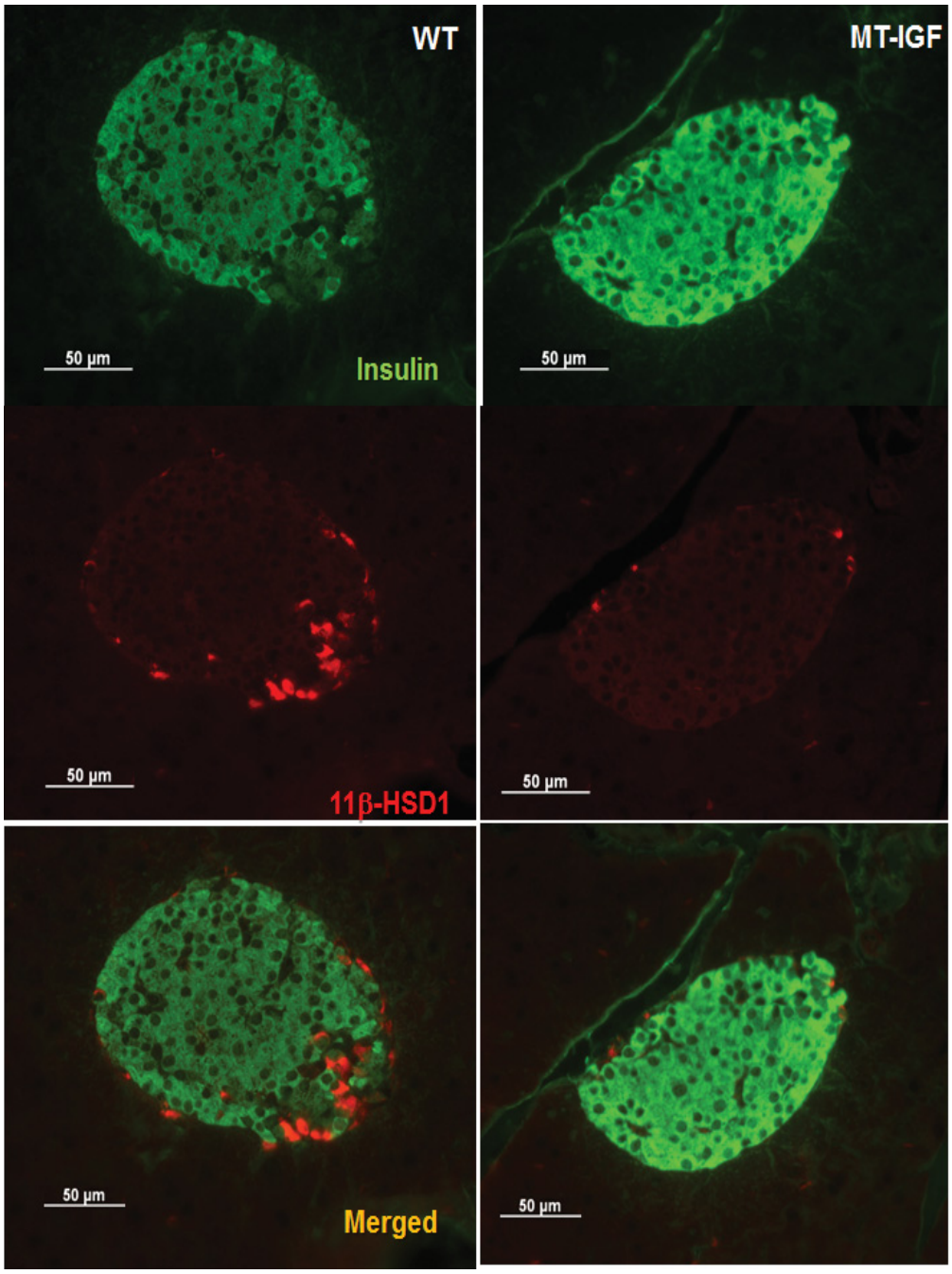
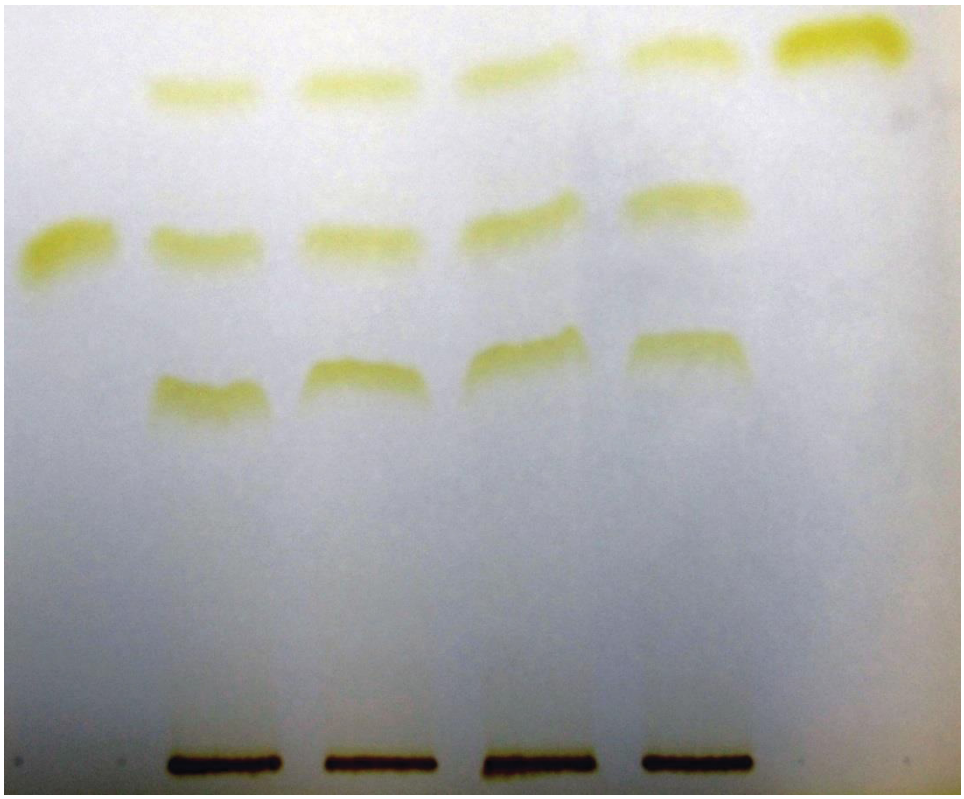

WT
MT-IGF
50 µm
Insulin
50 µm
50 µm
11β-HSD1
50 µm
50 µm
Merged
50 µm

Figure 3.2 Pancreatic islet α-cell-specific expression of 11β-HSD1 and its inhibition by IGF-I overexpression. A. Paraffin sections of the pancreas taken from 3-month-old wild-type (WT) or MT-IGF mice were stained for glucagon using DY488- (upper panels) and 11β-HSD1 using rhodamine-conjugated secondary antibodies (middle panels). Consequently, the images were merged into the bottom panels using Northern Eclipse software. 11β-HSD1 staining was distributed in the cytosol of most α-cells (average 68%) but not in other endocrine or acinar cells in wild-type mice (left panels); diminished staining per cell, some totally devoid of 11β-HSD1 (yellow arrow), was revealed in MT-IGF mice (right panels, only 21% of α-cells were positive for 11β-HSD1). Representative islets of ten from each genotype were illustrated. The scale bar was 60 microns.
B. The same sections were stained for insulin using DY488-(upper panels) and11β-HSD1 using rhodamine-conjugated secondary antibodies (middle panels). The scale bar was 50 microns.

A



1. DHC

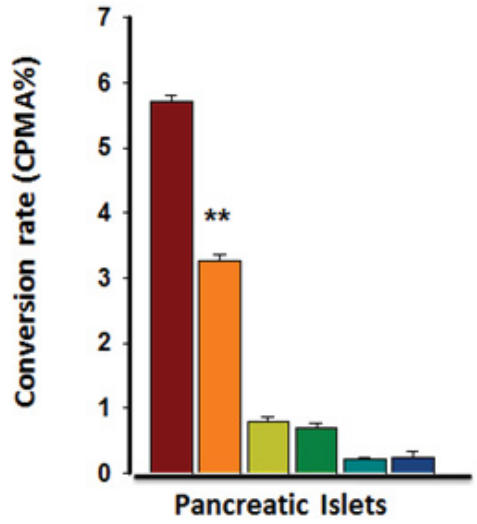
2. Corticosterone

3. Non-specific intermediate

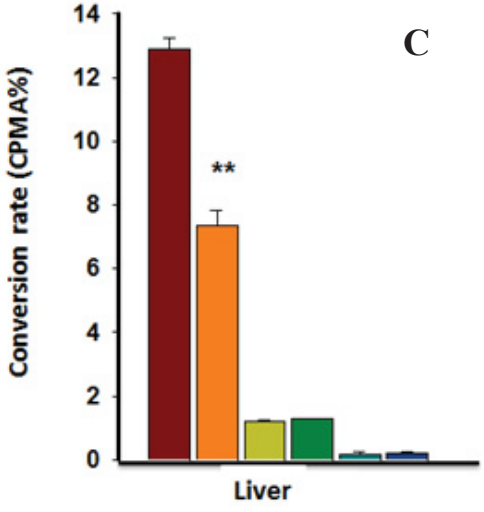
4. Origin

A B C D E F

B



C



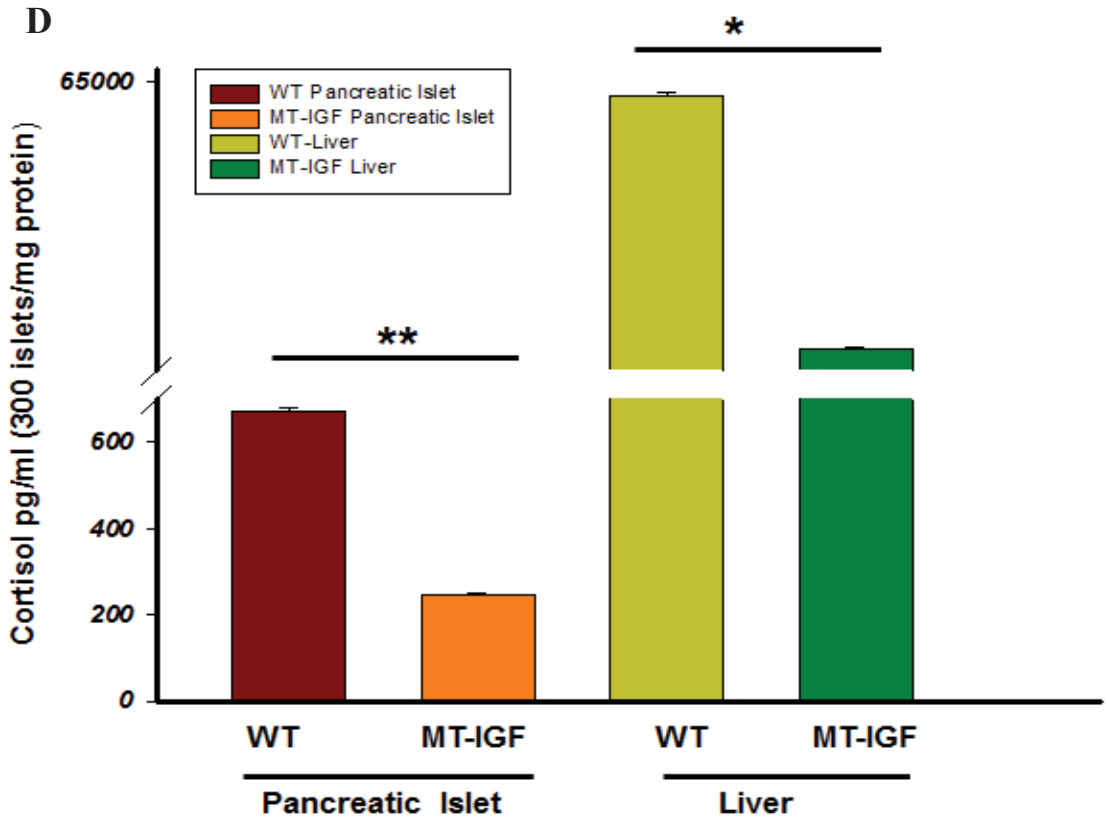


Figure 3.3 Decreased 11β-HSD1 activity in the liver and pancreatic islets of MT-IGF mice. **A.** Conversion of [1,2,6,7-^{3}H] corticosterone to DHC (bands 2 to 1) illustrated by an image of TLC plate showing the position of various molecular forms. 11β-HSD1 activity in homogenized liver microsome was determined using [^{3}H] corticosterone as substrate at a final concentration of 12 μM and 5 mM of $NADP^{+}$ as cofactor. In lane A and F, only standard corticosterone and DHC were loaded respectively. In lanes B to E, liver microsomes were loaded. The positions of four products or substrates were marked as 1 to 4. **B.** Changes in DHC conversion rate in pancreatic islets. Radioactivity corresponding to [1,2,6,7-^{3}H] DHC was expressed as percentage of the total radioactivity pooled from all four bands illustrated in Fig 3A. Also illustrated were the radioactivities of the non-specific band (3. NS) and those remained at the loading spots (4. Origin). **C.** Changes in DHC conversion rate in the liver. Mean ± SE. N=3. **P<0.01 vs. wild-type islets or liver. A representative assay was illustrated from three experiments. **D.** Decreased conversion of human cortisone to cortisol in the islets and liver of MT-IGF mice. Freshly isolated islets and liver microsomes from wild-type and MT-IGF mice were incubated with 500 nM cortisone for 3 h. Steroids were extracted and the concentration of newly converted cortisol was measured using immunoassay EIA. N=3; **P<0.01.

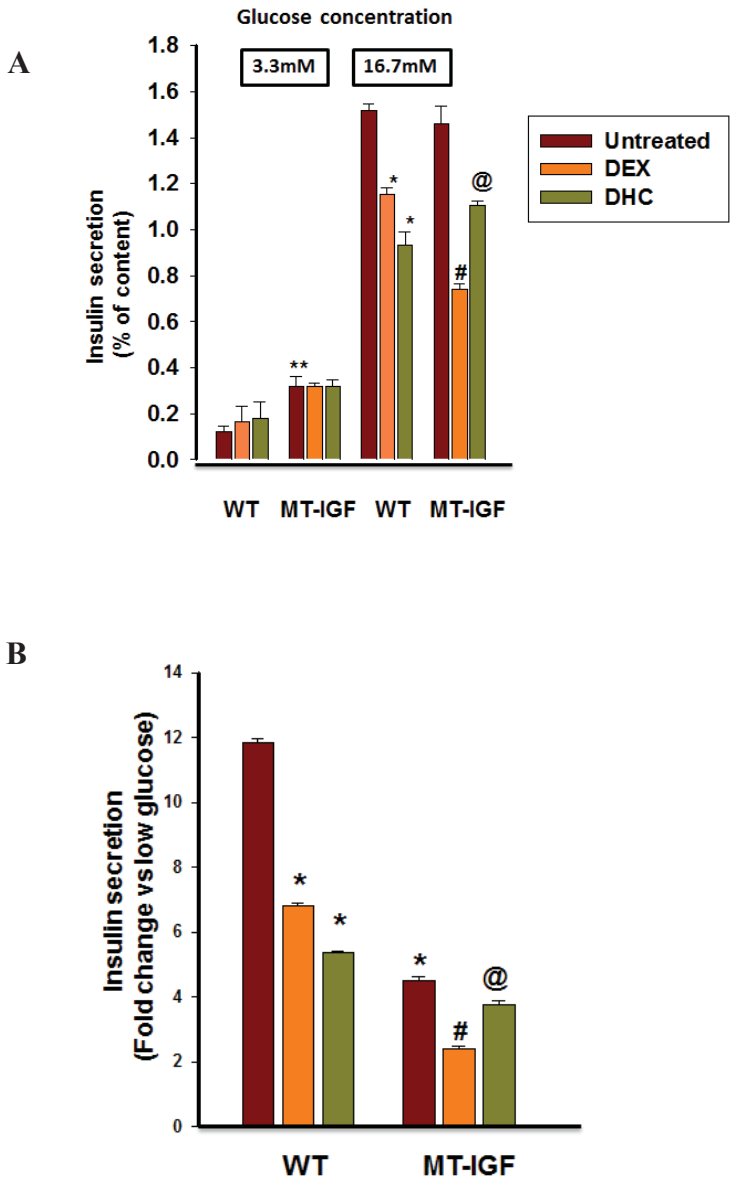
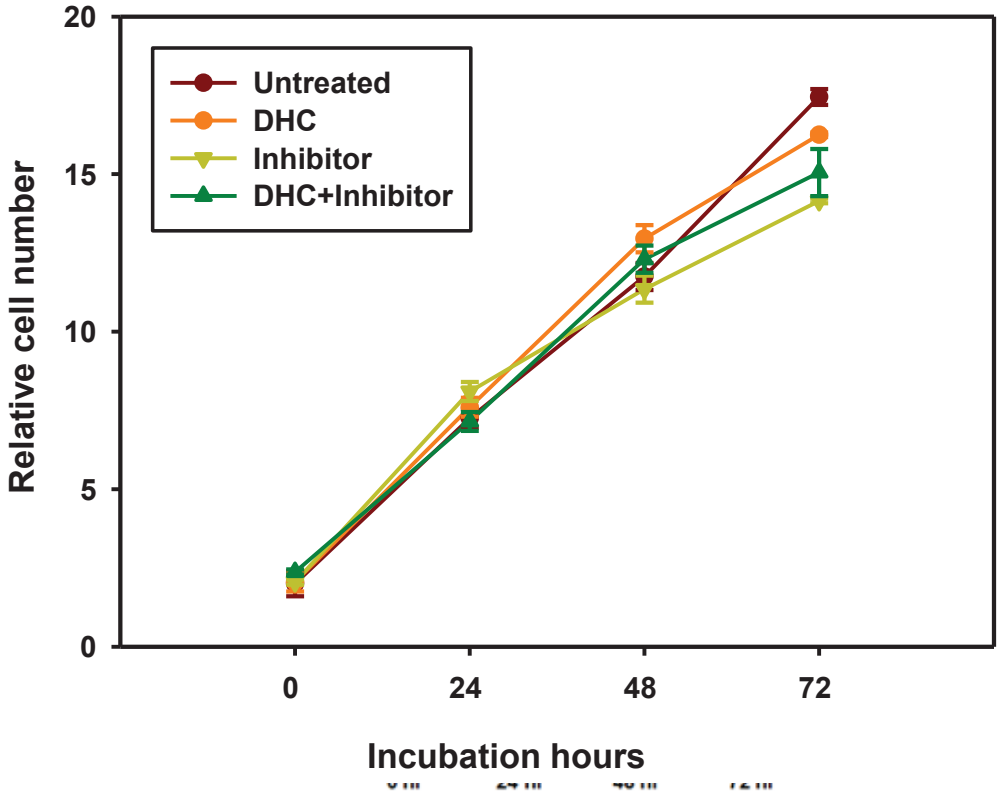
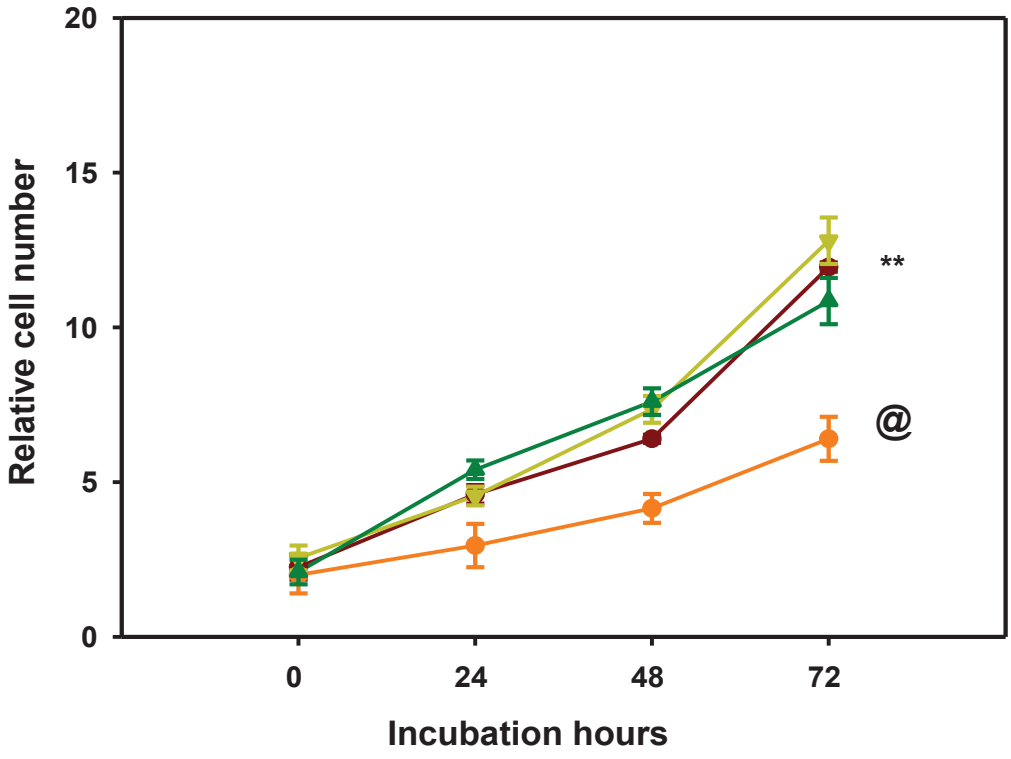


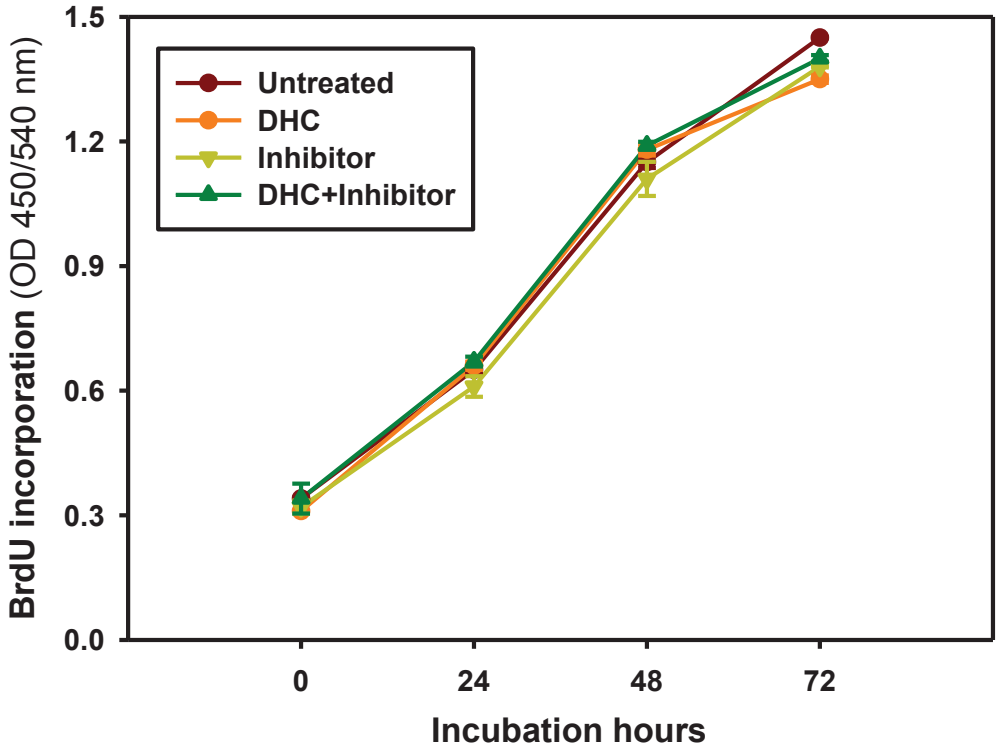
Figure 3.4 Effects of dexamethasone (DEX) and 11-dehydrocorticosterone (DHC) preincubations on glucose-stimulated insulin secretion. Freshly isolated islets from wild-type mice were pre-incubated in regular medium with 100 nM DEX or DHC for 48 h, first kept at 3.3 mM glucose for 60 min before being stimulated with 16.7 mM glucose for another 60 min. Insulin concentrations in the incubation buffer were measured using ELISA and expressed as % insulin content directly (A) or as fold stimulation vs. untreated (low glucose) islets (B). The experiment was repeated three times; a representative assay was illustrated. Mean ± S.E. N=3. Results of 1-way ANOVA: (A) among the other 6 columns of 3.3 mM glucose, $P<0.01$; among the first 6 columns of 16.7 mM glucose, $P<0.001$. (B) Among the first 6 columns $P<0.001$. *$P<0.05$, **$P<0.01$ vs. untreated wild-type islets; #$P<0.01$ vs. untreated MT-IGF islets; @$P<0.05$ vs. DEX-treated MT-IGF islets.



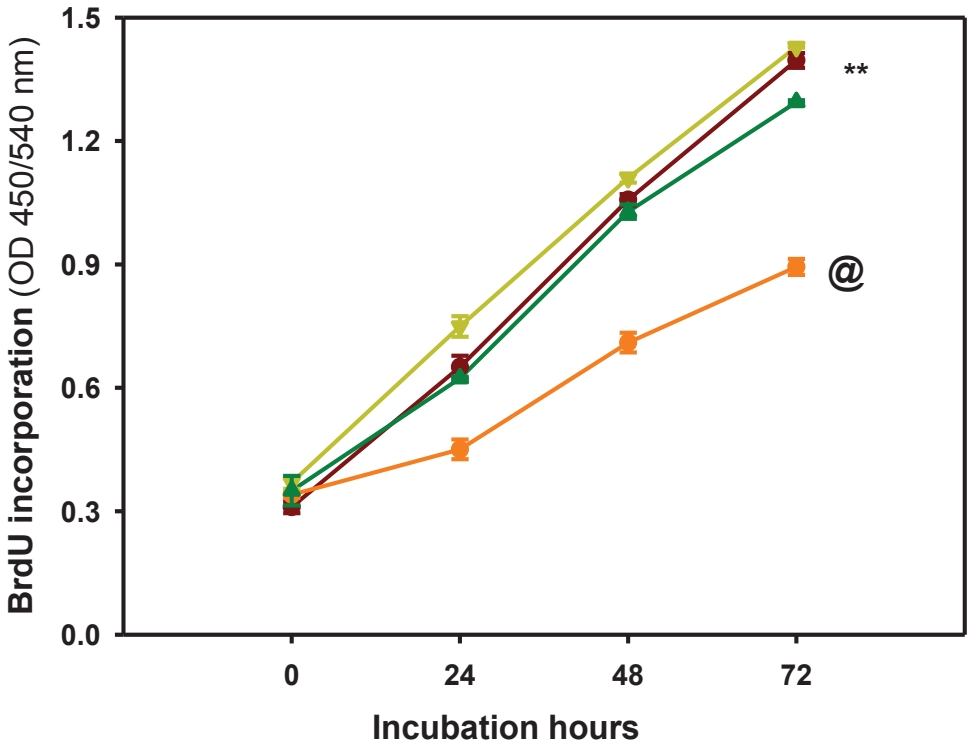
A.
MIN6-Vec



B.
MIN6-HSD1

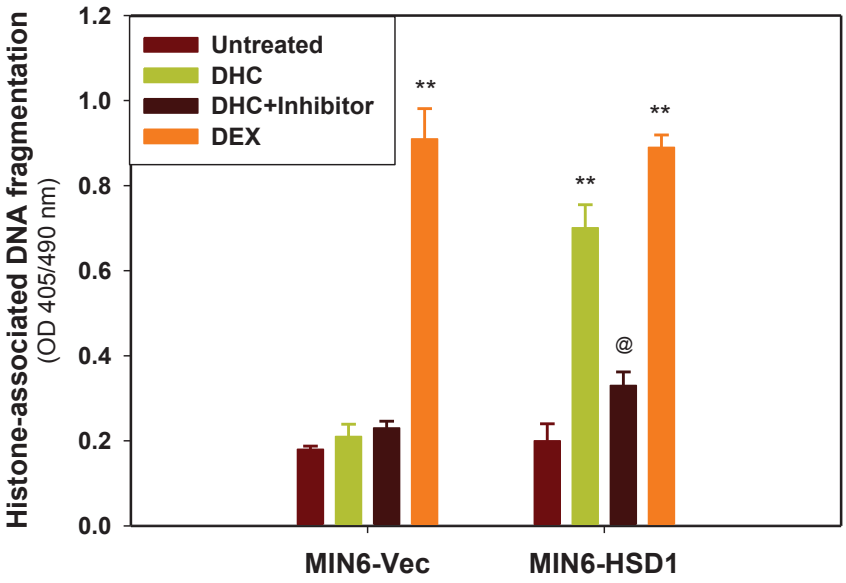


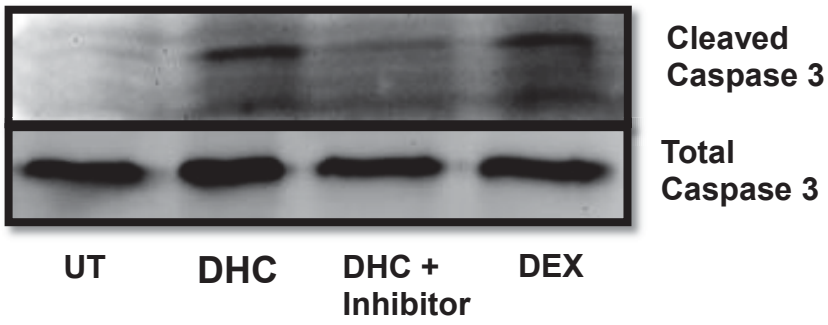
C.
MIN6-Vec

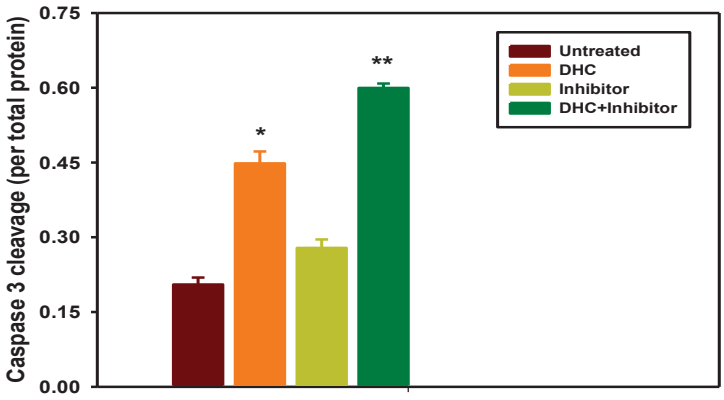


D.
MIN6-HSD1

Figure 3.5 Decreased cell proliferation in MIN6 cells overexpressing 11β-HSD1 in the presence of DHC. In A and B, MIN6-Vec and MIN6-HSD1 cells were cultured for 3 d with/out DHC (100 nM) and 11β-HSD1 inhibitor (1 □M). Relative cell numbers were determined using **MTT assay**. N=10, **P<0.01 in untreated MIN6-HSD1 vs. MIN6-Vec cells using one-way ANOVA. @P<0.01 in DHC-treated vs. untreated MIN6-HSD1 cells using one-way ANOVA. In C and D, cells were cultured for 3 d with/out DHC and 11β-HSD1 inhibitor. Relative rate of cell proliferation was determined using **BrdU incorporation** into newly synthesized DNA. N=10, **P<0.01 in DHC-treated vs. untreated MIN6-HSD1 cells using one-way ANOVA.


Histone-associated DNA fragmentation
(OD 405/490 nm)
1.2
1.0
0.8
0.6
0.4
0.2
0.0
Untreated
DHC
DHC+Inhibitor
DEX
**
**
**
@
MIN6-Vec
MIN6-HSD1


Cleaved Caspase 3
Total Caspase 3
UT
DHC
DHC + Inhibitor
DEX


Caspase 3 cleavage (per total protein)
0.75
0.60
0.45
0.30
0.15
0.00
Untreated
DHC
Inhibitor
DHC+Inhibitor
*
**

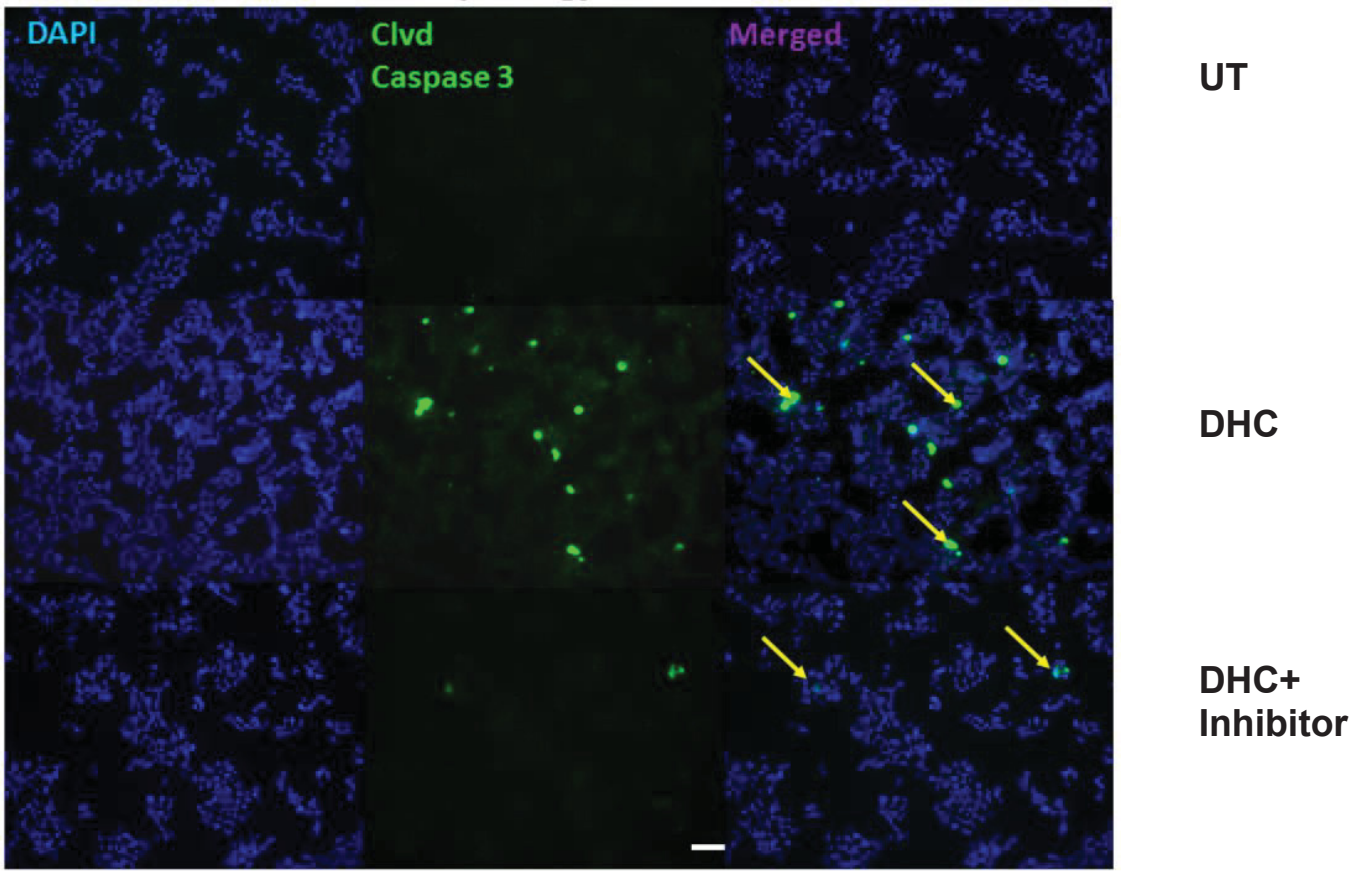


Figure 3.6. 11β-HSD1 dependent, DHC-induced apoptosis in MIN6 cells. A. MIN6-Vec and MIN6-HSD1 cells were cultured for 3 d with/out the presence of DHC (100 nM) and 11β-HSD1 inhibitor (1 μM). The relative rate of cell apoptosis was measured by cytoplasmic histone-associated DNA fragmentation using ELISA. N=5, **P<0.01 vs. untreated MIN6 cells; @P<0.05 vs. DHC-treated MIN6-HSD1 cells. **B and C**. In a separate experiment but under the same condition as in A, caspase 3 cleavage in MIN6-HSD1 cells alone was quantified using Western blots and densitometry. N=5, *P<0.05, **P<0.01 vs. untreated cells. **D.** In another experiment under the same condition as in A to C, MIN6-HSD1 cells were cultured in chamber slides, treated and representative fluorescence images of cleaved caspase 3 were illustrated. Arrows indicate active caspase 3 cleavage which was diminished by 11β-HSD1 inhibitor. Representative fields were illustrated from N=5.

Chapter IV

GENERAL DISCUSSION

4.1 General Discussion

IGF-I is an important mitogenic hormone that not only regulates development and maturation of major organ systems but also plays a role in the maintenance and function of several organs including the pancreas. Its effects on pancreatic islets include stimulation of cell proliferation in a glucose dependent manner, and contributing to delaying the on-set of diabetes. IGF-I-stimulated islet cell growth is signaled by IRS-2-mediated phosphorylation of PI3K, Grb2/mSOS, Erk1/2 and p70 S6K (163, 502, 518). It promotes β-cell survival by inhibiting cell apoptosis through PI3K-mediated phosphorylation of PKB, GSK 3β, BAD and p70 S6K (154). It inhibits insulin secretion by activating phosphodiesterase 3B and PKB (597). This lab generated a transgenic mouse model that overexpresses IGF-I under metallothionine I promoter. These mice were found to express IGF-I highly in β-cells. IGF-I overexpression led to a significant hypoglycemia in fasted animals, hypoinsulinemia and improved glucose tolerance. Furthermore, due to a partial prevention of β-cell death and the insulin-like effects, MT-IGF mice were significantly resistant to streptozotocin-induced diabetes (151). Although the signal transduction pathway of IGF-I has been studied for past 20-25 years, we have limited knowledge about the targets of IGF-I inside pancreatic islets. In order to study the islet specific novel molecular mediators of IGF-1 action in a systematic manner, we performed a whole-genome DNA microarray analysis on isolated islets and found 82 genes specifically up- or down-regulated in MT-IGF vs. wild-type mice. Many of them including extracellular matrix proteins (FBLN2 encoding fibulin-2, COL14A1), ion channels (KCNF1, CATSPER2) and intracellular substrates [HSD11B1 encoding 11 β-hydroxysteroid dehydrogenase 1 (11β-HSD1), PI3K-C2γ, CCN5/WISP2] have never been known to be regulated by IGF-I nor involved in β -cell function.

In this thesis, I presented studies elucidating the roles of two of these genes- 11β-HSD1 and CCN5/WISP2 in β-cell proliferation, islet cell survival and insulin secretion.

In Chapter II, I illustrated localization and the expression pattern of CCN5/WISP2 in pancreatic islets, followed by its role in islet cell proliferation and regeneration. CCN5/WISP2 expression in MT-IGF mice was increased 3- and 2-fold at the mRNA and protein levels respectively in the pancreatic islets. I demonstrated the localization of CCN5 in MT-IGF pancreatic β-cells with significantly higher expression level. Both the over-expressed CCN5 cell-line and recombinant CCN5 showed positive impact on β-cell proliferation. The β-cell expression provides additional importance of the gene in many ways- it would be interesting to see whether CCN5 can help the islet proliferate faster or even survive for longer time in the presence of inflammation and stress. As IGF-I is known to exert mitogenic effects, it would be relevant to see whether 1) its effects are mediated in party by CCN5/WISP2 or 2) IGF-I and CCN5/WISP2 exert synergistic/additive effects on cell growth, proliferation and survival. In my data, when CCN5 was knocked down IGF-I still showed positive effect but its effect was markedly decreased. This may mean that they might have a synergistic effect or at least CCN5-mediate part of the IGF-I effects in the cell proliferation. The other important factor is the effect of CCN5 in GSIS or basal insulin secretion. Although IGF-I is known to inhibit insulin secretion, we demonstrated higher basal insulin in IGF-I expressing transgenic mice model. I believe that CCN5 has an important role in insulin secretion. Even when I did the GSIS from islets of MT-IGF-I or CCN5-over expressing MIN6, I observed higher insulin secretion in both cases. This may suggest that CCN5 can play a pivotal role in pancreatic islets that promotes increased insulin secretion as well as β-cell proliferation and survival.

In Chapter III, I presented studies on 11β-HSD1, an enzyme, responsible for converting hormonally inactive glucocorticoid cortisone (11-dehydrocorticosterone, DHC in rodents) to active cortisol (corticosterone; as reductase). Active glucocorticoids, in excess, lead to insulin resistance and Type 2 diabetes. In freshly isolated islets, the level of 11β-HSD1 protein was significantly lower in MT-IGF mice. 11β-HSD1 was observed exclusively in islet α-cells at a diminished level in MT-IGF mice vs. wild-type animals using dual-labeled immunofluorescence independently with both glucagon and insulin. This pattern of 11β-HSD1 localization, demonstrating its presence in α- but not β-cells, is at odds with its previously reported expression in β-cells. Regardless, in order to define the role of 11β-HSD1 on pancreatic β-cells, I generated 11β-HSD1 overexpressing MIN6 cells (MIN6-HSD1) and observed a decreased cell-proliferation in presence of substrate DHC through possible inhibition of AKT phosphorylation. Min6-HSD1 cells in presence of DHC also undergo apoptosis measured by change of caspase 3 level in both western blot and immunohistochemistry and apoptotic ELISA. 11β-HSD1 specific inhibitor can significantly delay this apoptosis together with improvement in cell-proliferation. Finally, Min6-HSD1 cells showed decreased glucose stimulated insulin secretion. 11β-HSD1 in islet α-cells may cause a direct paracrine inhibition in insulin secretion.

It will be essential to confirm the relative abundance of 11β-HSD1 in α- and β-cells of the pancreas, and to delineate whether it exerts its direct and/or indirect effects to regulate β-cell survival and function. This aspect is beyond the scope of the present thesis.

4.2 Summary

My studies described in this thesis demonstrate clearly the roles of two IGF-I regulated genes, CCN5/WISP2 and 11β-HSD1, in the control of islet β-cell survival, proliferation and insulin secretion. Further, I showed that CCN5 promotes islet cell proliferation and regeneration, provides

protection against cell damage leading to diabetes mellitus. Secondly, I demonstrated that 11β-HSD1 renders the opposite effect on islet cell proliferation and survival. Increased 11β-HSD1 can cause more production of active cortisol which leads to cell apoptosis. Although one recent paper mentioned (334) complete lack of 11β-HSD1 can lead to mild β-cell impairment and moderate overexpression on β-cell can provide protection against high fat diet induced type 2 diabetes. The general notion is that 11β-HSD1 plays a negative role in β-cell function. MT-IGF mice showed increased levels of CCN5 but decreased 11β-HSD1 compared to wild type mice. Both of these can contribute to islet- more specifically β-cell- proliferation and survival.

The preliminary data indicated an increased trend of insulin secretion in CCN5-MIN6 cell-line which might be correlated with the survival effect and increased proliferation rate of β-cell. Moreover, decreased level of 11β-HSD1 activity in presence of 11β-HSD1 inhibitor in Min6-HSD1 cell-line impacts on increased basal insulin level and GSIS. This observation is further supported by the notion that MT-IGF mice have higher basal insulin secretion, resulting from less availability of active glucocorticoid. Increased MT-IGF mice are also known to show protection against STZ-induced diabetes. Overexpression of 11β-HSD1 in MIN6 cells also showed increased apoptosis which contributes to the relevance of global IGF-I induced islet proliferation and survival.

The results describing the roles of CCN5/WISP2 and 11β-HSD1 in mediating some of the effects of IGF-I opens up an opportunity to expand our understanding of new mechanisms of β-cell protection. Studies on the regulations of islet cell growth and/or β-cell secretion of insulin are ultimately crucial for us to combat type 1 diabetes by rescuing the β-cell mass and type 2 diabetes by preserving β-cell function. To this goal, the study of IGF-I targets envisaged in this thesis is

one of relevance, and hopefully leads to development of novel strategies aimed at improved islet β-cell survival and function.

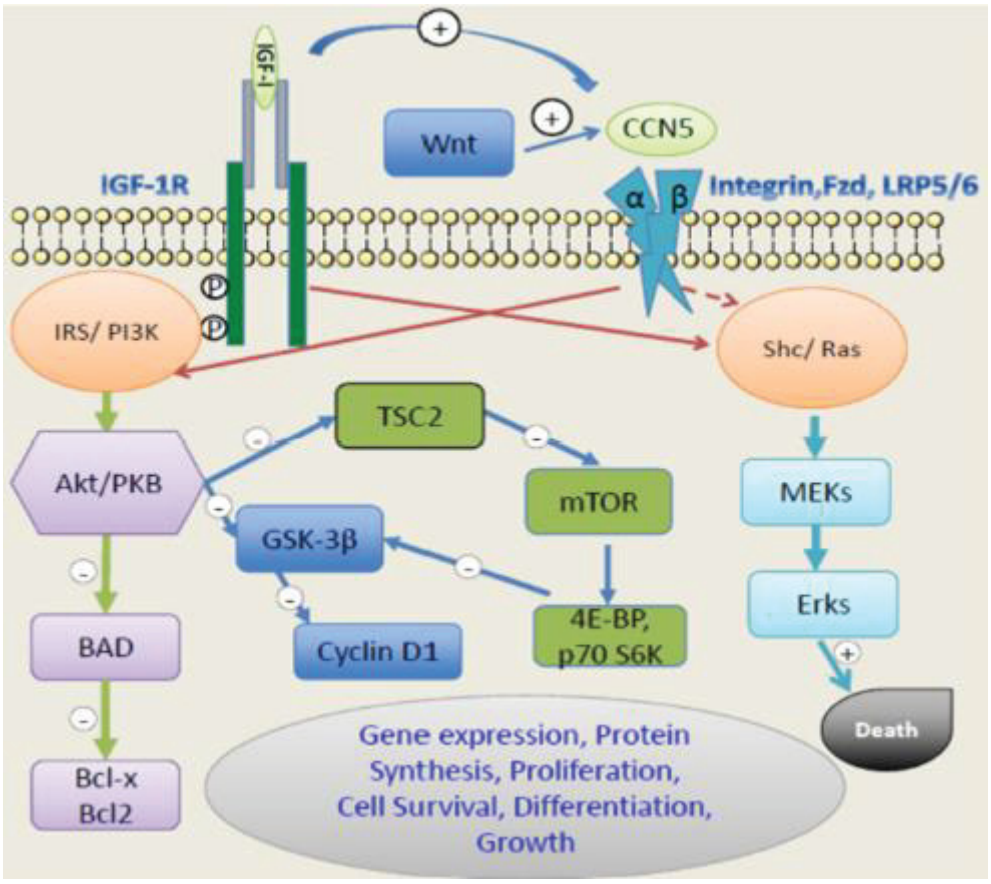


Figure 4.1 Proposed interaction of CCN5 and IGF-I signaling mechanism. (1) CCN5 as an extracellular ligand interacts with integrins and LRP5/6 which are known to interact with IGF-IR/IRS-1 and Wnt signaling; or with other cell membrane receptors. (2) It may activate PI3K/Akt/mTOR to stimulate gene expression, protein synthesis, proliferation, survival and differentiation. (3) It may also activate Ras/MEKs/Erks pathway to achieve similar functions. (4) Now, CCN5 expression is stimulated by IGF-I; CCN5 may mediate at least part of IGF-I effects. The IGF-I signaling pathway is reviewed by (584, 609). (5) Sustained ERK activation leads to apoptosis (611), by promoting either intrinsic or extrinsic apoptotic pathways, induction of mitochondrial cytochrome C release or caspase-8 activation. (6) Akt/PKB Ser/Thr kinase plays a central role in increasing cyclin D1 level through two major mechanisms. (i) GSK-3β-mediated, e.g. GSK-3β Ser/Thr kinase causes cyclin D1 phosphorylation and its degradation(607); Akt-mediated phosphorylation of GSK-3β inhibits its catalytic activity, resulting in the stabilization of cyclin D1(608). (ii) mTORC1-mediated, the phosphorylation and inactivation of TSC2 by Akt leads to activation of mTOR, which forms part of the mTORC1 complex and directly activates 4E-BP and ribosomal S6 kinases (S6K)(609). The latter phosphorylates GSK-3 β and inhibits its activity, again contributing to the stabilization of cyclin D1 (610).

4.3 Future Perspectives

Our study on 11β-HSD1 also raised several controversy that should be addreseds in the future. We need to reconcile the differences between mRNA and protein levels. There are increased evidences of this type of discrepancy. Possible decrease in post-translational efficiency of 11β-HSD1 may also be a contributing factor to differential changes in mRNA. In addition, genes with stable mRNAs but unstable proteins can be regulated quickly at the translational level (607). Since IGF-I inhibited 11β-HSD1 expression or its activity in most other cell/tissues, I believe the effect on islet cells is still an inhibition, which might cause delayed and compensatory increase in 11ß-HSD1 gene expression and partially explain the discrepancy of our preliminary findings. The other debate is on the localization of 11β-HSD1 expression on pancreatic islet. My finding (α-cell specific expression) is consistent with several groups but differs from two other (β-cell specific) recent papers. To address the issue, we can perform independent experiments such as *in situ* hybridization to check mRNA expression, localization and convincing IHC/dual labelled IF with different antibodies.

To further characterize its pro-islet and anti-diabetic properties, by using recombinant protein and gain-of-function overexpression, **future prospective will be to** 1) further establish the mechanism of regulation on endogenous CCN5 gene expression; 2) establish the potential link between CCN5 and Wnt signaling pathway; 3) study *in vitro* stimulation of CCN5 protein on the proliferation and survival of the pancreatic islet cells; 3) test the effect of *in vivo* administration of CCN5 on the rate of pancreatic regeneration after partial pancreatectomy; and 4) characterize *in vivo* protection of CCN5 against streptozotocin-induced β-cell damage and type 1 diabetes.

Also, we can investigate the roles some other potential IGF-I targets from the micro array data (later verified through qRT-PCR) such as extracellular matrix proteins (FBLN2 encoding

fibulin-2, COL14A1), ion channels (KCNF1, CATSPER2) and intracellular substrates PI3K-C2γ, which is a new member of PI3K family.

References

References

1. Standards of medical care in diabetes. 2014. Diabetes Care 2014, 37 Suppl 1:S14-80.
2. Rozance PJ, Hay WW, Jr. Describing hypoglycemia--definition or operational threshold? Early human development 2010, 86(5):275-280.
3. Desouza CV, Bolli GB, Fonseca V: Hypoglycemia, diabetes, and cardiovascular events. Diabetes Care 2010, 33(6):1389-1394.
4. Donath MY, Halban PA (2004). Decreased beta-cell mass in diabetes: significance, mechanisms and therapeutic implications. Diabetologia. 47(3):581-589.
5. Langer, R. M. (2010). "Islet transplantation: lessons learned since the Edmonton breakthrough." Transplant Proc .42(5): 1421-1424.
6. Ludwig, B., et al. (2010). "Islet versus pancreas transplantation in type 1 diabetes: competitive or complementary?" Curr Diab Rep.10(6): 506-511.
7. Lingohr MK, Buettner R, Rhodes CJ (2002). Pancreatic beta-cell growth and survival--a role in obesity-linked type 2 diabetes. Trends in molecular medicine 8(8):375-384.
8. Maximilian, C. (1990). The genetics of diabetes mellitus. Endocrinologie 28(3-4):81-94.
9. Williams, A. J., et al. (1990). "Raised temperature reduces the incidence of diabetes in the NOD mouse." Diabetologia 33(10): 635-637.
10. Ader, D. N., et al. (1991). "Group size, cage shelf level, and emotionality in non obese diabetic mice: impact on onset and incidence of IDDM." Psychosom Med 53(3):313-321.
11. Surwit, R. S., et al. (1992). "Stress and diabetes mellitus." Diabetes Care 15(10): 1413-1422.
12. Durant, S., et al. (1993). "Effects of various environmental stress paradigms and adrenalectomy on the expression of autoimmune type 1 diabetes in the non-obese diabetic (NOD) mouse." J Autoimmun 6(6): 735-751.
13. Yoon, J. W. (1990). "The role of viruses and environmental factors in the induction of diabetes." Curr Top Microbiol Immunol 164: 95-123.
14. Sadelain, M. W., et al. (1990). "Prevention of type I diabetes in NOD mice by adjuvant immunotherapy." Diabetes 39(5): 583-589.
15. Elliott, R. B. and J. M. Martin (1984). "Dietary protein: a trigger of insulin-dependent diabetes in the BB rat?" Diabetologia 26(4): 297-299.
16. Daneman, D., et al. (1987). "Dietary triggers of insulin-dependent diabetes in the BB rat." Diabetes Res 5(2): 93-97.
17. Elliott, R. B., et al. (1988). "Dietary prevention of diabetes in the non-obese diabetic mouse." Diabetologia 31(1): 62-64.
18. Issa-Chergui, B., et al. (1988). "The effect of diet on the spontaneous insulin dependent diabetic syndrome in the rat." Diabetes Res 9(2): 81-86.

19. Robertson, R. P. (2010). "Update on transplanting beta cells for reversing type 1 diabetes." Endocrinol Metab Clin North Am 39(3): 655-667.
20. Kort, H., et al. (2011). "Islet transplantation in type 1 diabetes." Bmj 342: d217.
21. Opara, E. C., et al. (2010). "Design of a bioartificial pancreas(+)." J Investig Med 58(7): 831-837.
22. DeFronzo, R. A., et al. (1992). "Pathogenesis of NIDDM. A balanced overview." Diabetes Care 15(3): 318-368.
23. Kahn, C. R. (1996). "New concepts in the pathogenesis of diabetes mellitus." Adv Intern Med 41: 285-321.
24. Hu, F. B. (2003). "Sedentary lifestyle and risk of obesity and type 2 diabetes." Lipids 38(2): 103-108.
25. Dupuis, J., et al. (2010). "New genetic loci implicated in fasting glucose homeostasis and their impacton type 2 diabetes risk." Nat Genet 42(2): 105-116.
26. Jack, L., Jr., et al. (2004). "Aging Americans and diabetes. A public health and clinical response." Geriatrics 59(4): 14-17.
27. Lovejoy, J. C., et al. (2002). "Effect of diets enriched in almonds on insulin action and serum lipids in adults with normal glucose tolerance or type 2 diabetes." Am J Clin Nutr 76(5): 1000-1006.
28. Eberhart (2004). "Prevalence of overweight and obesity among adults with diagnosed diabetes-United States, 1988-1994 and 1999-2002." MMWR Morb Mortal Wkly Rep 53(45): 1066-1068.
29. Clark, A., et al. Islet amyloid, increased A-cells, reduced B-cells and exocrine fibrosis: quantitative changes in the pancreas in type 2 diabetes. Diabetes Res, 1988. 9(4): p. 151-9.
30. Butler, A.E., et al., Beta-cell deficit and increased beta-cell apoptosis in humans with type 2 diabetes. Diabetes, 2003. 52(1): p. 102-10.
31. Donath, M.Y. and P.A. Halban. Decreased beta-cell mass in diabetes: significance, mechanisms and therapeutic implications. Diabetologia, 2004. 47(3): p. 581-9.
32. Kaiser, N., G. Leibowitz, and R. Nesher. Glucotoxicity and beta-cell failure in type 2 diabetes mellitus. J Pediatr Endocrinol Metab, 2003. 16(1): p. 5-22.
33. Paolisso, G., et al. A high concentration of fasting plasma non-esterified fatty acids is a risk factor for the development of NIDDM. Diabetologia, 1995. 38(10): p. 1213-7. Rhodes, C.J., Type 2 diabetes-a matter of beta-cell life and death? Science, 2005. 307(5708): p. 380-4.
34. Schroder, M. and R.J. Kaufman. ER stress and the unfolded protein response. Mutat Res, 2005. 569(1-2): p. 29-63.
35. Robertson, R.P., et al. Glucose toxicity in beta-cells: type 2 diabetes, good radicals gone bad, and the glutathione connection. Diabetes, 2003. 52(3): p. 581-7.
36. Malaisse, W.J., et al. Interference of glycogenolysis with glycolysis in pancreatic islets from glucose-infused rats. J Clin Invest, 1993. 91(2): p. 432-6.
37. Carlson, B. (2004). Human embryology and developmental biology., St. Louis: Mosby.
38. Pictet RL, Clark WR, Williams RH, Rutter WJ.(1972). An ultrastructural analysis of the developing embryonic pancreas. Dev Biol. Dec; 29(4):436-67.
39. Rutter, W. J., et al. (1968). "Regulation of specific protein synthesis in cytodifferentiation." J Cell Physiol 72(2): Suppl 1:1-18.

40. Jorgensen, M. C., et al. (2007). An illustrated review of early pancreas development in the mouse. Endocr Rev 28(6): 685-705.
41. Bonner-Weir S. Regulation of pancreatic β-cell mass in vivo. Recent Prog Horm Res 1994;49:91–104
42. Bonner-Weir S. (2000). Perspective: postnatal pancreatic beta cell growth. Endocrinology. 141:1926–1929
43. Bouwens L, Rooman I. (2005). Regulation of pancreatic beta-cell mass. Physiol Rev 85:1255–1270
44. Finegood DT, Scaglia L, Bonner-Weir S. Dynamics of β-cell mass in the growing rat pancreas: estimation with a simple mathematical model. Diabetes. 44:249–256
45. Scaglia L, Cahill CJ, Finegood DT, Bonner-Weir S. Apoptosis participates in the remodeling of the endocrine pancreas in the neonatal rat. Endocrinol. 138:1736–1741
46. Montanya E, Nacher V, Biarnes M, Soler J. (2000) Linear correlation between beta cell mass and body weight throughout life in Lewis rats: role of β-cell hyperplasia and hypertrophy. Diabetes.49:1341–1346.
47. Xu G, Stoffers DA, Habener JF, Bonner-Weir S. (1999) Exendin-4 stimulates both β-cell replication and neogenesis, resulting in increased β-cell mass and improved glucose tolerance in diabetic rats. Diabetes. 48:2270–2276
48. Kaiser, N., G. Leibowitz, and R. Nesher. Glucotoxicity and beta-cell failure in type 2 diabetes mellitus. J Pediatr Endocrinol Metab, 2003. 16(1): p. 5-22.
49. Scaglia L, Smith FE, Bonner-Weir S.(1995) Apoptosis contributes to the involution of β- cell mass in the post partum rat pancreas. Endocrinol. 136: 5461–5468
50. Bonner-Weir S, Deery D, Leahy JL, Weir GC. (1989) Compensatory growth of pancreatic β-cells in adult rats after short-term glucose infusion. Diabetes. 38:49–53
51. Yamamoto H, Uchigata Y, Okamoto H. (1981) Streptozotocin and alloxan induce DNA strand breaks and poly(ADP- ribose) synthetase in pancreatic islets. Nature. 294: 284–286.
52. Yamamoto K, Miyagawa J, Waguri M, et al. (2000) Recombinant human betacellulin promotes the neogenesis of beta-cells and ameliorates glucose intolerance in mice with diabetes induced by selective alloxan perfusion. Diabetes. 49: 2021–2027
53. Li L, Seno M, Yamada H, et al. (2001) Promotion of beta-cell regeneration by beta cellulin in ninety percentpancreatectomized rats. Endocrinology. 142: 5379–5385.
54. Terazono K, Uchiyama Y, Ide M, et al. (1990) Expression of reg protein in rat regenerating islets and its co-localization with insulin in the beta cell secretory granules. Diabetologia. 33: 250–252.
55. Gu D, Sarvetnick N. (1993). Epithelial cell proliferation and islet neogenesis in IFN-g transgenic mice. Development. 118: 33–46.
56. Gu D, Lee MS, Krahl T, et al. (1994) Transitional cells in the regenerating pancreas. Development. 120: 1873–1881.
57. Gu D, Arnush M, Sarvetnick N. (1997) Endocrine/exocrine intermediate cells in streptozotocin-treated Ins-IFN-gamma transgenic mice. Pancreas .15: 246–250.

58. Dor, Y., et al. (2004). Adult pancreatic beta-cells are formed by self-duplication rather than stem-cell differentiation. Nature 429(6987): 41-46.
59. Bonner-Weir, S. (2000). "Islet growth and development in the adult." J Mol Endocrinol 24(3): 297-302.
60. Xu G, Stoffers DA, Habener JF, et al. Exendin-4 stimulates both beta-cell replication and neogenesis, resulting in increased beta-cell mass and improved glucose tolerance in diabetic rats. Diabetes 1999; 48: 2270–2276.
61. Gedulin, B.R., S.E. Nikoulina, P.A. Smith, G.Gedulin, L.L. Nielsen, A.D. Baron, D.G. Parkes, And A.A. Young. 2005. Exenatide (Exendin-4) Improves Insulin Sensitivity and OE≤Cell Mass In Insulin-Resistant Obese fa/fa Zucker Rats Independent Of Glycemia And BodyWeight. Endocrinology. 146:2069--- 2076.
62. Tourrel C1, Bailbé D, Meile MJ, Kergoat M, Portha B. (2001). Glucagon-like peptide-1 and exendin-4 stimulate beta-cell neogenesis in streptozotocin-treated newborn rats resulting in persistently improved glucose homeostasis at adult age. Diabetes. Jul;50(7):1562-70.
63. German, M., et al. (1995). The insulin gene promoter. A simplified nomenclature. Diabetes 44 (8):1002-1004.
64. Davies, P. O., et al. (1994). Genetic reassignment of the insulin-1 (Ins1) gene to distal mouse chromosome 19. Genomics 21 (3): 665-667.
65. Duvillie, B., et al. (1998). Imprinting at the mouse Ins2 locus: evidence for cis- and trans-allelic interactions. Genomics 47 (1): 52-57.
66. Gerozissis, K. (2003). Brain insulin: regulation, mechanisms of action and functions. Cell Mol Neurobiol 23 (1): 1-25.
67. Poitout, V., et al. (2006). Regulation of the insulin gene by glucose and fatty acids. J Nutr 136 (4): 873-876.
68. Billestrup N, Nielsen JH. The stimulatory effect of growth hormone, prolactin, and placental lactogen on beta-cell proliferation is not mediated by insulin-like growth factor-I. Endocrinology 1991; 129: 883–888.
69. Hayek A, Beattie GM, Cirulli V, et al. Growth factor/matrixinduced proliferation of human adult beta-cells. Diabetes 1995; 44: 1458–1460.
70. Beattie GM, Cirulli V, Lopez AD, et al. Ex vivo expansion of human pancreatic endocrine cells. J Clin Endocrinol Metab 1997; 82: 1852–1856.
71. Gershengorn MC, Hardikar AA, Wei C, et al. Epithelialto-mesenchymal transition generates proliferative human islet precursor cells. Science 2004; 306: 2261–2264.
72. Russ HA, Ravassard P, Kerr-Conte J, et al. Epithelialmesenchymal transition in cells expanded in vitro from lineage-traced adult human pancreatic beta cells. PLoS ONE 2009; 4: e6417.
73. Minami K, Miyawaki K, Hara M, et al. Tracing phenotypic reversibility of pancreatic b-cells in vitro. J Diabetes Invest 2010; 1: 242–251
75. Atouf F, Park CH, Pechhold K, et al. No evidence for mouse pancreatic beta-cell epithelial-mesenchymal transition in vitro. Diabetes 2007; 56: 699–702.
76. Morton RA, Geras-Raaka E, Wilson LM, et al. Endocrine precursor cells from mouse islets are not generated by epithelial-to-mesenchymal transition of mature beta cells. Mol Cell Endocrinol 2007; 270: 87–93.
77. Swenne, I. The role of glucose in the in vitro regulation of cell cycle kinetics and proliferation of fetal pancreatic B-cells. Diabetes, 1982. 31(9): p. 754-60.

78. Egan JM, Bulotta A, Hui H, Perfetti R. GLP-1 receptor agonists are growth and differentiation factors for pancreatic islet beta cells. Diabetes/metabolism research and reviews 2003, 19(2):115-123.
79. Takasawa S, Okamoto H. Pancreatic beta-cell death, regeneration and insulin secretion: roles of poly(ADP-ribose) polymerase and cyclic ADP-ribose. International journal of experimental diabetes research 2002, 3(2):79-96.
80. Soares, M. B., et al. . RNA-mediated gene duplication: the rat preproinsulin I gene is a functional retroposon. Mol Cell Biol 1985; 5 (8): 2090-2103.
81. Newgard, C. B. and J. D. McGarry. Metabolic coupling factors in pancreatic beta-cell signal transduction. Annu Rev Biochem 1995;64 : 689-719.
82. Maechler, P., et al. In beta-cells, mitochondria integrate and generate metabolic signals controlling insulin secretion. Int J Biochem Cell Biol . 2006; 38 (5-6): 696-709.
83. Prentki, M. New insights into pancreatic beta-cell metabolic signaling in insulin secretion. Eur J Endocrinol. 1996 134 (3): 272-286.
84. Prentki, M. and C. J. Nolan (2006). "Islet beta cell failure in type 2 diabetes. J Clin Invest 116 (7): 1802-1812.
85. Schuit, F., et al. (1997). "Metabolic fate of glucose in purified islet cells. Glucose-regulated anaplerosis in beta cells." J Biol Chem 272 (30): 18572-18579.
86. Ashcroft, F. M. and P. Rorsman (1990). "ATP-sensitive K+ channels: a link between B-cell metabolism and insulin secretion." Biochem Soc Trans 18 (1): 109-111.
87. MacDonald, P. E. and M. B. Wheeler (2003). "Voltage-dependent K(+) channels in pancreatic beta cells: role, regulation and potential as therapeutic targets." Diabetologia 46 (8): 1046-1062.
88. Brennan, L., et al. (2002). "A nuclear magnetic resonance-based demonstration of substantial oxidative L-alanine metabolism and L-alanine-enhanced glucose metabolism in a clonal pancreatic beta-cell line: metabolism of L-alanine is important to the regulation of insulin secretion." Diabetes 51 (6): 1714-1721.
89. Liu, Y. Q., et al. (2002). "beta-Cell adaptation to insulin resistance. Increased pyruvate carboxylase and malate-pyruvate shuttle activity in islets of nondiabetic Zucker fatty rats." J Biol Chem 277 (42): 39163-39168.
90. Ogawa, W.; Matozaki, T.; Kasuga, M. Role of binding proteins to IRS-1 in insulin signalling. Mol. Cell. Biochem. 182:13–22; 1998.
91. Bloch-Damti, A.; Bashan, N. Proposed mechanisms for the induction of insulin resistance by oxidative stress. Antioxid. Redox Signaling 7:1553–1567; 2005.
92. Saltiel, A. R.; Kahn, C. R. Insulin signalling and the regulation of glucose and lipid metabolism. Nature 414:799–806; 2001.
93. Kriauciunas, K. M.; Myers Jr., M. G.; Kahn, C. R. Cellular compartmentalization in insulin action: altered signaling by a lipid-modified IRS-1. Mol. Cell. Biol. 20.: 6849–6859; 2000.
94. Anai, M.; Ono, H.; Funaki, M.; Fukushima, Y.; Inukai, K.; Ogihara, T.; Sakoda, H.; Onishi, Y.; Yazaki, Y.; Kikuchi, M.; Oka, Y.; Asano, T. Different subcellular distribution and regulation of expression of insulin receptor substrate (IRS)-3 from those of IRS-1 and IRS-2. J. Biol. Chem. 273:29686–29692; 1998.

95. Slack, J.M. Developmental biology of the pancreas. Development, 1995. 121(6): p. 1569-80
96. Tannenbaum GS, Ling N. The interrelationship of growth hormone (GH)-releasing factor and somatostatin in generation of the ultradian rhythm of GH secretion. Endocrinology 1984;115:1952–7.
97. Ghigo E, Boghen M, Casanueva FF, et al., eds. Growth hormone secretagogues. Basic findings and clinical implications. Amsterdam: Elsevier, 1994.
98. Jaffe CA, Ho PJ, Demott-Friberg R, et al. Effects of a prolonged growth hormone (GH)-releasing peptide infusion on pulsatile GH secretion in normal men. J Clin Endocrinol Metab 1993;77:1641–7.
99. Laron Z. The somatostatin-GHRH-GH-IGF-I axis. In: Merimee T, Laron Z, eds. Growth hormone, IGF-I and growth: new views of old concepts. Modern endocrinology and diabetes, Vol. 4. London-Tel Aviv: Freund Publishing House Ltd, 1996:5–10.
100. Daughaday WH, Hall K, Raben MS, et al. Somatomedin: a proposed designation for the sulfation factor. Nature 1972; 235:107.
101. Laron Z. Somatomedin-1 (recombinant insulin-like growth factor-I). Clinical pharmacology and potential treatment of endocrine and metabolic disorders. Biodrugs 1999;11:55–70.
102. Rinderknecht E, Humbel RE. The amino acid sequence of human insulin like growth factor I and its structural homology, with proinsulin. J Biol Chem 1978;253:2769–76.
103. Jansen, E., et al. Identification of multiple transcription start sites in the human insulin-like growth factor-I gene. Mol Cell Endocrinol, 1991. 78(1-2): p. 115-25.
104. Rotwein, P. Two insulin-like growth factor I messenger RNAs are expressed in human liver. Proc Natl Acad Sci U S A, 1986. 83(1): p. 77-81.
105. Roberts, C.T., Jr., et al. Molecular cloning of rat insulin-like growth factor I complementary deoxyribonucleic acids: differential messenger ribonucleic acid processing and regulation by growth hormone in extrahepatic tissues. Mol Endocrinol, 1987. 1(3): p. 243-8.
106. Bach, L. and N. Parker. IGFBPs - Gene and Protein Structure, in Insulin-Like Growth Factors. D. Le Roith, W. Zumkeller, and R. Baxter, Editors. 2003, Kluwer Academic/Plenum Publishers: New York. p. 48-63.
107. Hampton, B., et al. Purification and characterization of an insulin-like growth factor II variant from human plasma. J Biol Chem, 1989. 264(32): p. 19155-60.
108. Matsuguchi, T., et al. Functional analysis of multiple promoters of the rat insulin-like factor IIgene. Biochim Biophys Acta, 1990. 1048(2-3): p. 165-70.
109. Gray, A., et al. Tissue-specific and developmentally regulated transcription of the insulin-like growth factor 2 gene. DNA, 1987. 6(4): p. 283-95.
110. Sussenbach, J.S., P.H. Steenbergh, and P. Holthuizen. Structure and expression of the human insulin-like growth factor genes. Growth Regul, 1992. 2(1): p. 1-9.
111. Hwa V, Oh Y, Rosenfeld RG. The insulin-like growth factor binding protein (IGFBP) superfamily. Endocr Rev 1999;20: 761–87
112. Hwa, V., Y. Oh, and R.G. Rosenfeld. The insulin-like growth factor-binding protein (IGFBP) superfamily. Endocr Rev, 1999. 20(6): p. 761-87.

113. Bach, L. and N. Parker. IGFBPs - Gene and Protein Structure, in Insulin-Like Growth Factors, D. Le Roith, W. Zumkeller, and R. Baxter, Editors. 2003, Kluwer Academic/Plenum Publishers: New York. p. 48-63.
114. Povoa, G., et al. Isolation and characterization of a somatomedin-binding protein from mid-term human amniotic fluid. Eur J Biochem, 1984. 144(2): p. 199-204.
115. Bang, P., et al. Ontogeny of insulin-like growth factor-binding protein-1, -2, and -3: quantitative measurements by radioimmunoassay in human fetal serum. Pediatr Res, 1994. 36(4): p. 528-36.
116. Jones, J.I. and D.R. Clemmons. Insulin-like growth factors and their binding proteins: biological actions. Endocr Rev, 1995. 16(1): p. 3-34.
117. Jones, J.I. and D.R. Clemmons. Insulin-like growth factors and their binding proteins: biological actions. Endocr Rev, 1995. 16(1): p. 3-34.
118. Rechler MM (1993). Insulin-like growth factor binding proteins Vitam Horm 47: 1-114
119. Firth, S.M., Baxter, R.C. (2002). Cellular actions of the insulin-like growth factor binding proteins. Endocr. Rev. 23, 824–854.
120. Jones, J.I., Clemmons, D.R. 1995. Insulin-like growth factors and their binding proteins: biological actions. Endocr. Rev. 16, 3–34.
121. Yin, P., Xu, Q., Duan, C. (2004). Paradoxical actions of endogenous and exogenous insulin-like growth factor binding protein (IGFBP)-5 revealed by RNA interference analysis. J. Biol. Chem. 279, 32660–32666.
122. Conover, C.A., Ronk, M., Lombana, F., Powell, D.R. (1990). Structural and biological characterization of bovine insulin-like growth factor binding protein-3. Endocrinology 127, 2795–2803.
123. DeMellow, J.S.M., Baxter, R.C. (1988). Growth hormone-dependent insulin-like growth factor (IGF) binding protein both inhibits and potentiates IGF-I-stimulated DNA-synthesis in human-skin Wbroblasts. Biochem. Biophys. Res. Commun. 156, 199–204.
124. Jones, J.I., Gockerman, A., Busby Jr., W.H., Camacho-Hubner, C., Clemmons, D.R. (1993). Extracellular matrix contains insulin-like growth factor binding protein-5: potentiation of the eVects of IGF-1 J. Cell Biol. 121, 679–687.
125. Duan, C., Clemmons, D.R. (1998). DiVerential expression and biological effects of insulin-like growth factor-binding protein-4 and -5 in vascular smooth muscle cells. J. Biol. Chem. 273, 16836–16842.
126. Abrass, C.K., BerWeld, A.K., Andress, D.L. (1997). Heparin binding domain of insulin-like growth factor binding protein-5 stimulates mesangial cell migration. Am. J. Physiol. 273, F899–F906.
127. James, P.L., Stewart, C.E.H., Rotwein, P. (1996). Insulin-like growth factor binding protein-5 modulates muscle diVerentiation through an insulin-like growth factor-dependent mechanism. J. Cell Biol. 133, 683–693.
128. Xu, Q., Yan, B., Li, S., Duan, C. (2004). Fibronectin binds insulin-like growth factor binding protein (IGFBP)-5 and abolishes its ligand-dependent action on cell migration. J. Biol. Chem. 279, 4269–4277.
129. Werner H. Molecular biology of the type I IGF receptor. In: Rosenfeld RG, Roberts CT, Jr, eds. The IGF system— molecular biology, physiology and clinical applications. Totowa, NJ: Humana Press, 1999:63–88.

130. Seino S, Seino M, Nishi S, et al. Structure of the human insulin receptor gene and characterization of its promoter. Proc Natl Acad Sci U S A 1989;86:114–18.
131. Nakae, J., Y. Kido, and D. Accili. Distinct and overlapping functions of insulin and IGF-I receptors. Endocr Rev, 2001. 22(6): p. 818-35.
132. Khandwala HM, McCutcheon IE, Flyvbjerg A, Friend KE. The effects of insulin-like growth factors on tumorigenesis and neoplastic growth. Endocr Rev 2000;21:215–44.
133. Pollak MN, Schernhammer ES, Hankinson SE. Insulin-like growth factors and neoplasia. Nat Rev Cancer 2004;4:505–18.
134. Nakae J, Kido Y, Accili D. Distinct and overlapping functions of insulin and IGF-I receptors. Endocr Rev 2001;22:818–35.
135. D enley A,Wallace JC, Cosgrove LJ, Forbes BE. The insulin receptor isoform exon 11_ (IR-A) in cancer and other diseases: a review. Horm Metab Res 2003;35:778–85.
136. Sesti G. Insulin receptor variant forms and type 2 diabetes mellitus. Pharmacogenomics 2000;1:49–61.
137. Pandini G, Vigneri R, Costantino A, Frasca F, Ippolito A, Fujita- Yamaguchi Y, et al. Insulin and insulin-like growth factor-I (IGF-I) receptor overexpression in breast cancers leads to insulin/IGF-I hybrid receptor overexpression: evidence for a second mechanism of IGF-I signaling. Clin Cancer Res 1999;5:1935–44.
138. Adams TE, Epa VC, Garrett TP,Ward CW. Structure and function of the type 1 insulin-like growth factor receptor. Cell Mol Life Sci 2000;57:1050–93.
139. Czech MP. Structural and functional homologies in the receptors for insulin and the insulin-like growth factors. Cell 1982;31:8–10.
140. Bhaumick B, Bala RM, Hollenberg MD. Somatomedin receptor ohuman placenta: solubilization, photolabeling, partial purification, and comparison with insulin receptor. Proc Natl Acad Sci USA 1981;78:4279–83.
141. Chernausek SD, Jacobs S, Van Wyk JJ. Structural similarities between human receptors for somatomedin C and insulin: analysis by affinity labeling. Biochemistry 1981;20:7345–50.
142. Kasuga M, Karlsson FA, Kahn CR. Insulin stimulates the phosphorylation of the 95,000-dalton subunit of its own receptor. Science 1982;215:185–7.
143. Rubin JB, Shia MA, Pilch PF. Stimulation of tyrosine-specific phosphorylation in vitro by insulin-like growth factor I. Nature 1983;305:438–40.
144. Dimitriadis, G., et al. Effects of insulin-like growth factor I on the rates of glucose transport and utilization in rat skeletal muscle in vitro. Biochem J, 1992. 285 (Pt 1): p. 269-74.
145. Di Cola, G., M.H. Cool, and D. Accili. Hypoglycemic effect of insulin-like growth factor-1 in mice lacking insulin receptors. J Clin Invest, 1997. 99(10): p. 2538-44.
146. Clemmons, D.R. Involvement of insulin-like growth factor-I in the control of glucose homeostasis. Current Opinion in Pharmacology, 2006. In Press, Corrected Proof.
147. Jacob, R., et al. Acute effects of insulin-like growth factor I on glucose and amino acid metabolism in the awake fasted rat. Comparison with insulin. J Clin Invest, 1989. 83(5): p. 1717-23.

148. Zenobi, P.D., et al. Effects of insulin-like growth factor-I on glucose tolerance, insulin levels, and insulin secretion. J Clin Invest, 1992. 89(6): p. 1908-13.
149. Yakar, S., et al. Liver-specific igf-1 gene deletion leads to muscle insulin insensitivity. Diabetes, 2001. 50(5): p. 1110-8.
150. Sjogren, K., et al. Liver-derived IGF-I is of importance for normal carbohydrate and lipid metabolism. Diabetes, 2001. 50(7): p. 1539-45.
151. Liu J-L, Srikant CB, and Chowdhury S. Comment on: Turban et al. Optimal Elevation of β-Cell 11β-Hydroxysteroid Dehydrogenase Type 1 Is a Compensatory Mechanism That Prevents High-Fat Diet–Induced β-Cell Failure. Diabetes 2012;61:642–652. *Diabetes* 61: e13, 2012.
152. Hill, D.J., et al. Cellular distribution and ontogeny of insulin-like growth factors (IGFs) and IGF binding protein messenger RNAs and peptides in developing rat pancreas. J Endocrinol, 1999. 160(2): p. 305-17.
153. Rabinovitch, A., et al. Insulin and multiplication stimulating activity (an insulin-like growth factor)stimulate islet (beta-cell replication in neonatal rat pancreatic monolayer cultures. Diabetes, 1982. 31(2)p. 160-4.
154. Bergerot, I., et al. Insulin-like growth factor-1 (IGF-1) protects NOD mice from insulitis and diabetes. Clin Exp Immunol, 1995. 102(2): p. 335-40.
155. Harrison, M., et al. Growth factor protection against cytokine-induced apoptosis in neonatal rat islets of Langerhans: role of Fas. FEBS Lett, 1998. 435(2-3): p. 207-10.
156. Vasavada, R.C., et al. Growth factors and beta cell replication. The International Journal of Biochemistry & Cell Biology, 2006. 38(5-6): p. 931-950.
157. White, M.F. IRS proteins and the common path to diabetes. Am J Physiol Endocrinol Metab, 2002. 283(3): p. E413-22.
158. Tuttle, R.L., et al. Regulation of pancreatic [beta]-cell growth and survival by the serine/threonine protein kinase Akt1/PKB[alpha]. Nat Med, 2001. 7(10): p. 1133-1137.
159. Dickson, L.M. and C.J. Rhodes. Pancreatic beta-cell growth and survival in the onset of type 2 diabetes: a role for protein kinase B in the Akt? Am J Physiol Endocrinol Metab, 2004. 287(2): p. E192-8.
160. Hennige, A.M., et al. Upregulation of insulin receptor substrate-2 in pancreatic {beta} cells prevents diabetes. J Clin Invest, 2003. 112(10): p. 1521-1532.
161. George, M., et al. Beta cell expression of IGF-I leads to recovery from type 1 diabetes. Journal of Clinical Investigation, 2002. 109(9): p. 1153-1163.
162. Kulkarni, R.N., et al. Beta-cell-specific deletion of the Igf1 receptor leads to hyperinsulinemia and glucose intolerance but does not alter beta-cell mass. Nat Genet, 2002. 31(1): p. 111-5.
163. Xuan, S., et al. Defective insulin secretion in pancreatic beta cells lacking type 1 IGF receptor. J Clin Invest, 2002. 110(7): p. 1011-9.
164. Kaino, Y., et al. Insulin-like growth factor I (IGF-I) delays the onset of diabetes in non-obese diabetic (NOD) mice. Diabetes Res Clin Pract, 1996. 34(1): p. 7-11.
165. Hill, D.J., et al. Insulin-like growth factors prevent cytokine-mediated cell death in isolated islets of Langerhans from pre-diabetic non-obese diabetic mice. J Endocrinol, 1999. 161(1): p. 153-65.

166. Liu, W., et al. Activation of Phosphatidylinositol 3-Kinase Contributes to Insulin-Like Growth Factor I-Mediated Inhibition of Pancreatic {beta}-Cell Death. Endocrinology, 2002. 143(10): p. 3802-3812.
167. Davidson, H. W., Rhodes, C. J., and Hutton, J. C. Intraorganellar calcium and pH control proinsulin cleavage in the pancreatic beta cell via two distinct site-specific endopeptidases. *Nature.* 1988; 333, 93–96.
168. George, M., et al. Beta cell expression of IGF-I leads to recovery from type 1 diabetes. Journal of Clinical Investigation, 2002. 109(9): p. 1153-1163.
169. Mathews, L.S., et al. Growth enhancement of transgenic mice expressing human insulin-like growth factor I. Endocrinology, 1988. 123(6): p. 2827-33.
170. Sjogren K, Liu JL, Blad K, Skrtic S, Vidal O, Wallenius V, LeRoith D, Tornell J, Isaksson OG, Jansson JO, Ohlsson C (1999). Liver-derived insulin-like growth factor I (IGF-I) is the principal source of IGF-I in blood but is not required for postnatal body growth in mice. Proc Natl Acad Sci USA 96:7088–7092
171. Behringer RR, Lewin TM, Quaife CJ, Palmiter RD, Brinster RL, D'Ercole AJ (1990). Expression of insulin-like growth factor I stimulates normal somatic growth in growth hormone-deficient transgenic mice. Endocrinology 127:1033–1040.
172. Augstein P, Elefanty AG, Allison J et al (1998). Apoptosis and beta-cell destruction in pancreatic islets of NOD mice withspontaneous and cyclophosphamide-accelerated diabetes. Diabetologia 41:1381–1388.
173. Butler AE, Janson J, Bonner-Weir S et al (2003). Beta-cell deficit and increased beta-cell apoptosis in humans with type 2 diabetes. Diabetes 52:102–110. 3. Kurrer MO, Pakala SV, Hanson HL et al (1997) Beta cell apoptosis in T cell-mediated autoimmune diabetes. Proc Natl Acad Sci USA 94:213–218.
174. O'Brien BA, Harmon BV, Cameron DP et al (1997). Apoptosis is the mode of beta-cell death responsible for the development of IDDM in the nonobese diabetic (NOD) mouse. Diabetes 46:750–757.
175. Cnop M, Welsh N, Jonas JC et al (2005). Mechanisms of pancreatic beta-cell death in type 1 and type 2 diabetes: many differences, few similarities. Diabetes 54(Suppl 2):S97–S107.
176. Finegood DT, Scaglia L, Bonner-Weir S. Dynamics of beta-cell mass in the growing rat pancreas Estimation with a simple mathematical model. Diabetes 44: 249–256, 1995.
177. Bonner-Weir S. Life and death of the pancreatic beta cells. Trends Endocrinol Metab 11: 375–378, 2000.
178. Kassem SA, Ariel I, Thornton PS, Scheimberg I, Glaser B. Beta-cell proliferation and apoptosis in the developing normal human pancreas and in hypinsulinemia of infancy. Diabetes 49: 1325–1333, 2000.
179. Bonner-Weir S. Perspective: Postnatal pancreatic beta cell growth. Endocrinology 141: 1926–1929, 2000.
180. Mathis D, Vence L, Benoist C. Cell death during progression to diabetes. Nature 414: 792–798, 2001.
181. Notkins AL, Lernmark A. Autoimmune type 1 diabetes: resolved and unresolved issues. J Clin Invest 108: 1247–1252, 2001.

182. Hoffman EP, Nader GA. Balancing muscle hypertrophy and atrophy. Nat Med 2004; 10:584.
183. Sandri M, Sandri C, Gilbert A, et al. Foxo transcription factors induce the atrophy-related ubiquitin ligase atrogin-1 and cause skeletal muscle atrophy. Cell 2004; 117:399.
184. Stitt TN, Drujan D, Clarke BA, et al. The IGF-1/PI3K/Akt pathway prevents expression of muscle atrophy-induced ubiquitin ligases by inhibiting FOXO transcription factors. Mol Cell 2004; 14:395.
185. Bodine SC, Latres E, Baumhueter S, et al. Identification of ubiquitin ligases required for skeletal muscle atrophy. Science 2001; 294:1704.
186. Sun L, Trausch-Azar JS, Muglia LJ, Schwartz AL. Glucocorticoids differentially regulate degradation of MyoD and Id1 by N-terminal ubiquitination to promote muscle protein catabolism. Proc Natl Acad Sci U S A 2008; 105:3339.
188. Horner, H. C., et al. (1987) "Dexamethasone causes translocation of glucose transporters from the plasma membrane to an intracellular site in human fibroblasts." J Biol Chem 262(36): 17696-17702.
189. Oda, N., et al. (1995) "Dexamethasone-induced changes in glucose transporter 4 in rat heart muscle,
skeletal muscle and adipocytes." Eur J Endocrinol 133(1): 121-126.
190. Weinstein, S. P., et al. (1995). "Glucocorticoid-induced insulin resistance: dexamethasone inhibits the activation of glucose transport in rat skeletal muscle by both insulin- and non-insulin-related stimuli." Diabetes 44(4): 441-445.
191. Coderre, L., et al. (1996) "In vivo effects of dexamethasone and sucrose on glucose transport (GLUT-4) protein tissue distribution." Am J Physiol 271(4 Pt 1): E643-648.
192. Dimitriadis, G., et al. (1997) "Effects of glucocorticoid excess on the sensitivity of glucose transport and metabolism to insulin in rat skeletal muscle." Biochem J 321 (Pt 3): 707-712.
193. Kahn SE. Clinical review 135: The importance of beta-cell failure in the development and progression of type 2 diabetes. J Clin Endocrinol Metab 2001; 86(9): 4047–4058.
194. Jeschke MG, Boehning D. Endoplasmic reticulum stress and insulin resistance post-trauma: similarities to type 2 diabetes. J Cell Mol Med 2012; 16(3): 437–444.
195. McMahon M, Gerich J, Rizza R. Effects of glucocorticoids on carbohydrate metabolism. Diabetes Metab Rev 1988; 4(1): 17–30.
196. Piroli GG, Grillo CA, Reznikov LR, et al. Corticosterone impairs insulin-stimulated translocation of GLUT4 in the rat hippocampus. Neuroendocrinology 2007; 85(2): 71–80.
197. Schacke H, Rehwinkel H, Asadullah K, Cato AC. Insight into the molecular mechanisms of glucocorticoid receptor action promotes identification of novel ligands with an improved therapeutic index. Exp Dermatol 2006; 15(8): 565–573.
198. van Raalte DH, Ouwens DM, Diamant M. Novel insights into glucocorticoidmediated diabetogenic effects: towards expansion of therapeutic options? Eur J Clin Invest 2009; 39(2): 81–93.

199. Vander Kooi BT, Onuma H, Oeser JK, et al. The glucose-6-phosphatase catalytic subunit gene promoter contains both positive and negative glucocorticoid response elements. Mol Endocrinol 2005; 19(12): 3001–3022.
200. Barseghian G, Levine R. Effect of corticosterone on insulin and glucagon secretion by the isolated perfused rat pancreas. Endocrinology 1980;106:547–52.

201. Billaudel B, Mathias PC, Sutter BC, Malaisse WJ. Inhibition by corticosterone of calcium inflow and insulin release in rat pancreatic islets. J Endocrinol 1984;100:227–33.
202. Lambillotte C, Gilon P, Henquin JC. Direct glucocorticoid inhibition of insulin secretion. An in vitro study of dexamethasone effects in mouse islets. J Clin Invest 1997;99:414–23.
203. Brunstedt J, Nielsen JH. Direct long-term effect of hydrocortisone on insulin and glucagon release from mouse pancreatic islets in tissue culture. Acta Endocrinol (Copenh) 1981;96:498–504.
204. Gremlich S, Roduit R, Thorens B. Dexamethasone induces posttranslational degradation of GLUT2 and inhibition of insulin secretion in isolated pancreatic beta cells. Comparison with the effects of fatty acids. J Biol Chem 1997;272:3216–22.
205. Jeong IK, Oh SH, Kim BJ, Chung JH, Min YK, Lee MS et al. The effects of dexamethasone on insulin release and biosynthesis are dependent on the dose and duration of treatment. Diabetes Res Clin Pract 2001;51:163–71.
206. Pierluissi J, Navas FO, Ashcroft SJ. Effect of adrenal steroids on insulin release from cultured rat islets of Langerhans. Diabetologia 1986;29:119–21.
207. Ullrich S, Berchtold S, Ranta F, Seebohm G, Henke G, Lupescu A et al. Serum- and glucocorticoid-inducible kinase 1 (SGK1) mediates glucocorticoid-induced inhibition of insulin secretion. Diabetes 2005;54:1090–9.
208. Zawalich WS, Tesz GJ, Yamazaki H, Zawalich KC, Philbrick W. Dexamethasone suppresses phospholipase C activation and insulin secretion from isolated rat islets. Metabolism 2006;55:35–42.
209. Nesher R, Anteby E, Yedovizky M, Warwar N, Kaiser N, Cerasi E. Beta-cell protein kinases and the dynamics of the insulin response to glucose. Diabetes 2002;51 (Suppl. 1):S68–73.
210. Ogawa A, Johnson JH, Ohneda M, Mcallister CT, Inman L, Alam T et al. Roles of insulin resistance and beta-cell dysfunction in dexamethasone-induced diabetes. J Clin Invest 1992;90:497–504.
211. Borboni P, Porzio O, Magnaterra R, Fusco A, Sesti G, Lauro R et al. Quantitative analysis of pancreatic glucokinase gene expression in cultured beta cells by competitive polymerase chain reaction. Mol Cell Endocrinol 1996;117:175–81.
212. Khan A, Ostenson CG, Berggren PO, Efendic S. Glucocorticoid increases glucose cycling and inhibits insulin release in pancreatic islets of ob/ob mice. Am J Physiol 1992;263:E663–6.
213. Hamamdzic D, Duzic E, Sherlock JD, Lanier SM. Regulation of alpha 2-adrenergic receptor expression and signaling in pancreatic beta-cells. Am J Physiol 1995;269:E162–71.

214. Philippe J, Missotten M. Dexamethasone inhibits insulin biosynthesis by destabilizing insulin messenger ribonucleic acid in hamster insulinoma cells. Endocrinology 1990;127:1640–5.
215. Ranta F, Avram D, Berchtold S, Dufer M, Drews G, Lang F et al. Dexamethasone induces cell death in insulin-secreting cells, an effect reversed by exendin-4. Diabetes 2006;55:1380–90.
216. Longano CA, Fletcher HP. Insulin release after acute hydrocortisone treatment in mice. Metabolism 1983; 32:603–8.
217. Wang ZL, Bennet WM, Wang RM, Ghatei MA, Bloom SR. Evidence of a paracrine role of neuropeptide-Y in the regulation of insulin release from pancreatic islets of normal and dexamethasone-treated rats. Endocrinology 1994;135:200–6.
218. et amyloid polypeptide (amylin) and insulin gene expression in rats islets following dexamethasone treatment. Diabetologia 1995;38:395–402.
219. Ohneda M, Johnson JH, Inman LR, Unger RH. GLUT-2 function in glucose-unresponsive beta cells of dexamethasone-induced diabetes in rats. J Clin Invest 1993;92:1950–6.
220. Grill V, Rundfeldt M. Abnormalities of insulin responses after ambient and previous exposure to glucose in streptozocin-diabetic and dexamethasone-treated rats. Role of hyperglycemia and increased B-cell demands. Diabetes 1986;35:44–51.
221. Delaunay F, Khan A, Cintra A, Davani B, Ling ZC, Andersson A et al. Pancreatic beta cells are important targets for the diabetogenic effects of glucocorticoids. J Clin Invest 1997;100:2094–8.
222. Davani B, Portwood N, Bryzgalova G, Reimer MK, Heiden T, Ostenson CG et al. Aged transgenic mice with increased glucocorticoid sensitivity in pancreatic beta-cells develop diabetes. Diabetes 2004;53 (Suppl. 1):S51–9.
223. Kalhan SC, Adam PA. Inhibitory effect of prednisone on insulin secretion in man: model for duplication of blood glucose concentration. J Clin Endocrinol Metab 1975;41:600–10.
224. Van Raalte DH, Mari A, Bunck MC, Nofrate V, Dokter W, Nassander U et al. Acute and subacute exposure to glucocorticoids differentially impair various aspects of beta-cell function in healthy males (1468-P). 68th Sessions American Diabetes Association. San Francisco, 2008.
225. Shamoon H, Soman V, Sherwin RS. The influence of acute physiological increments of cortisol on fuel metabolism and insulin binding to monocytes in normal humans. J Clin Endocrinol Metab 1980;50:495–501.
226. Beard JC, Halter JB, Best JD, Pfeifer MA, Porte D Jr. Dexamethasoneinduced insulin resistance enhances B cell responsiveness to glucose level in normal men. Am J Physiol 1984;247:E592–6.
227. Grill V, Pigon J, Hartling SG, Binder C, Efendic S. Effects of dexamethasone on glucose-induced insulin and proinsulin release in low and high insulin responders. Metabolism 1990;39:251–8.
228. Matsumoto K, Yamasaki H, Akazawa S, Sakamaki H, Ishibashi M, Abiru N et al. High-dose but not low-dose dexamethasone impairs glucose tolerance by inducing compensatory failure of pancreatic beta-cells in normal men. J Clin Endocrinol Metab 1996;81:2621–6.

229. Besse C, Nicod N, Tappy L. Changes in insulin secretion and glucose metabolism induced by dexamethasone in lean and obese females. Obes Res 2005;13:306–11.
230. Cobelli C, Toffolo GM, Dalla MC, Campioni M, Denti P, Caumo A et al. Assessment of beta-cell function in humans, simultaneously with insulin sensitivity and hepatic extraction, from intravenous and oral glucose tests. Am J Physiol Endocrinol Metab 2007;293:E1–15.
231. Lambillotte C, Gilon P, Henquin JC. Direct glucocorticoid inhibition of insulin secretion. An in vitro study of dexamethasone effects in mouse islets. J Clin Invest 1997;99:414–23.
232. Henriksen JE, Alford F, Ward GM, Beck-Nielsen H. Risk and mechanism of dexamethasone-induced deterioration of glucose tolerance in non-diabetic first-degree relatives of NIDDM patients. Diabetologia 1997;40:1439–48.
233. Wajngot A, Giacca A, Grill V, Vranic M, Efendic S. The diabetogenic effects of glucocorticoids are more pronounced in low- than in high-insulin responders. Proc Natl Acad Sci U S A 1992;89:6035–9.
234. Dessein PH, Joffe BI. Insulin resistance and impaired beta cell function in rheumatoid arthritis. Arthritis Rheum 2006;54:2765–75.
235. Stumvoll M, Goldstein BJ, van Haeften TW. Type 2 diabetes: principles of pathogenesis and therapy. Lancet 2005;365:1333–46.
236. Taskinen MR, Nikkila EA, Pelkonen R, Sane T. Plasma lipoproteins, lipolytic enzymes, and very low density lipoprotein triglyceride turnover in Cushing's syndrome. J Clin Endocrinol Metab 1983;57:619–26.
237. Wajchenberg BL. Subcutaneous and visceral adipose tissue: their relation to the metabolic syndrome. Endocr Rev 2000;21:697–738.
238. Shao S, Fang Z, Yu X, Zhang M. Transcription factors involved in glucosestimulated insulin secretion of pancreatic beta cells. Biochem Biophys Res Commun 2009; 384(4): 401–404.
239. Gesina E, Tronche F, Herrera P, et al. Dissecting the role of glucocorticoids on pancreas development. Diabetes 2004; 53(9): 2322–2329.
240. Lantz KA, Kaestner KH. Winged-helix transcription factors and pancreatic development. Clin Sci (Lond) 2005; 108(3): 195–204.
241. Shen CN, Seckl JR, Slack JM, Tosh D. Glucocorticoids suppress beta-cell development and induce hepatic metaplasia in embryonic pancreas. Biochem J 2003; 375(Pt 1): 41–50.
241. Seckl, J. R. and B. R. Walker (2004). "11beta-hydroxysteroid dehydrogenase type 1 as a modulator of glucocorticoid action: from metabolism to memory." Trends Endocrinol Metab 15(9): 418-424.
242. Tomlinson, J. W., et al. (2004). "11beta-hydroxysteroid dehydrogenase type 1: a tissue-specific regulator of glucocorticoid response." Endocr Rev 25(5): 831-866.
243. Moisan MP, Seckl JR, Edwards CR. 11β-Hydroxysteroiddehydrogenase bioactivity and messenger RNAexpression in rat forebrain: localization in hypothalamus, hippocampus, and cortex. Endocrinology. 1990;127:150–1455.
244. Tannin GM, Agarwal AK, Monder C, New MI, White PC. The human gene for 11 hydroxysteroid dehydrogenase. Structure, tissue distribution, and chromosomal localization. J Biol Chem. 1991;266:16653–16658.

245. Ricketts ML, Verhaeg JM, Bujalska I, Howie AJ, RaineyWE, Stewart PM. Immunohistochemical localization of type 1 11β-hydroxysteroid dehydrogenase in human tissues. J Clin Endocrinol Metab. 1998;83:1325–1335.
246. Jamieson, P. M., et al. (2000). "11 beta-hydroxysteroid dehydrogenase type 1 is a predominant 11 beta-reductase in the intact perfused rat liver." J Endocrinol 165(3): 685-692.
247. Brereton, P. S., et al. (2001). "Light and electron microscopy localization of the 11beta-hydroxysteroid dehydrogenase type I enzyme in the rat." Endocrinology 142(4): 1644-1651.
248. Dzyakanchuk, A. A., et al. (2009). "11beta-Hydroxysteroid dehydrogenase 1 reductase activity is dependent on a high ratio of NADPH/NADP(+) and is stimulated by extracellular glucose." Mol Cell Endocrinol 301(1-2): 137-141.
249. Cook, P. W., et al. (1988). Glucocorticoid receptor-dependent inhibition of cellular proliferation in dexamethasone-resistant and hypersensitive rat hepatoma cell variants. Mol Cell Biol 8(4): 1449-1459.
250. Van Uum, S. H., et al. (2004). "Cortisol, 11beta-hydroxysteroid dehydrogenases, and hypertension" Semin Vasc Med 4(2): 121-128.
251. Rajan, V., et al. (1995). "Cloning, sequencing and tissue-distribution of mouse 11 beta-hydroxysteroid dehydrogenase-1 cDNA." J Steroid Biochem Mol Biol 52(2): 141-147.
252. Agarwal, A. K. and R. J. Auchus (2005). "Minireview: cellular redox state regulates hydroxysteroid dehydrogenase activity and intracellular hormone potency." Endocrinology 146(6): 2531-2538.
253. Lakshmi, V. and C. Monder (1988). "Purification and characterization of the corticosteroid 11 beta-dehydrogenase component of the rat liver 11 beta-hydroxysteroid dehydrogenase complex." Endocrinology 123(5): 2390-2398.
254. Agarwal, A. K., et al. (1989). "Cloning and expression of rat cDNA encoding corticosteroid 11-beta-dehydrogenase." J Biol Chem 264(32): 18939-18943.
255. Bujalska, I., et al. (1997). "Human 11®-hydroxysteroid dehydrogenase: studies on the stably transfected isoforms and localization of the type 2 isozyme within renal tissue." Steroids 62(1): 77-82.
256. Atanasov, A. G., et al. (2004). "Hexose-6-phosphate dehydrogenase determines the reaction direction of 11®-hydroxysteroid dehydrogenase type 1 as an oxoreductase." FEBS Lett 571(1-3): 129-133.
257. Banhegyi, G., et al. (2004). "Cooperativity between 11beta-hydroxysteroid dehydrogenase type 1 and hexose-6-phosphate dehydrogenase in the lumen of the endoplasmic reticulum." J Biol Chem 279(26): 27017-27021.
258. Bujalska, I. J., et al. (2005). "Hexose-6-phosphate dehydrogenase confers oxo-reductase activity upon 11 beta-hydroxysteroid dehydrogenase type 1." J Mol Endocrinol 34(3): 675-684.
259. Lavery, G. G., et al. (2006). "Hexose-6-phosphate dehydrogenase knock-out mice lack 11 beta-hydroxysteroid dehydrogenase type 1-mediated glucocorticoid generation." J Biol Chem 281(10): 6546-6551.
260. Chou, J. Y., et al. (2002). "Type I glycogen storage diseases: disorders of the glucose-6-phosphatase complex." Curr Mol Med 2(2): 121-143.

261. Walker, E. A., et al. (2007). "11beta-Hydroxysteroid Dehydrogenase Type 1 Regulation by Intracellular Glucose 6-Phosphate Provides Evidence for a Novel Link between Glucose Metabolism and Hypothalamo-Pituitary-Adrenal Axis Function." J Biol Chem 282(37): 27030-27036.
262. Low, S. C., et al. (1994). "'Liver-type' 11 beta-hydroxysteroid dehydrogenase cDNA encodes reductase but not dehydrogenase activity in intact mammalian COS-7 cells. J Mol Endocrinol 13(2): 167-174.
263. Jamieson, P. M., et al. (1995). "11 beta-hydroxysteroid dehydrogenase is an exclusive 11-beta reductase in primary cultures of rat hepatocytes: effect of physicochemical and hormonal manipulations." Endocrinology 136(11): 4754-4761.
264. Kotelevtsev, Y., et al. (1997). "11beta-hydroxysteroid dehydrogenase type 1 knockout mice show attenuated glucocorticoid-inducible responses and resist hyperglycemia on obesity or stress." Proc Natl Acad Sci U S A 94(26): 14924-14929.
265. Ozols, J. (1993). "Isolation and the complete amino acid sequence of lumenal endoplasmic reticulum glucose-6-phosphate dehydrogenase." Proc Natl Acad Sci U S A 90(11): 5302-5306.
266. Mziaut, H., et al. (1999). "Targeting proteins to the lumen of endoplasmic reticulum using N-terminal domains of 11beta-hydroxysteroid dehydrogenase and the 50-kDa esterase." J Biol Chem 274(20): 14122-14129.
267. Zielinska, A. E., et al. (2011). "Biochemistry and physiology of hexose-6-phosphate knockout mice." Mol Cell Endocrinol 336(1-2): 213-218.
268. Long CN, Lukens FD. The effects of adrenalectomy and hypophysectomy upon experimental diabetes in the cat. J Exp Med. 1936;63:465–490.
269. Ingle DJ, Meeks RC. Comparison of some metabolic and morphologic effects of cortisone and hydrocortisone given by continuous injection to rats. Am J Physiol. 1952;170: 77–80.
270. Ingle DJ. Some further studies on the relationship of adrenal cortical hormones to experimental diabetes. Diabetes. 1952;1:345–350.
271. Long CN. The endocrine control of the blood-sugar. Lancet. 1952;262:325–329.
272. Gathercole LL, Stewart PM. Targeting the pre-receptor metabolism of cortisol as a novel therapy in obesity and diabetes. J Steroid Biochem Mol Biol. 2010;122:21–27.
273. Bujalska IJ, Kumar S, Stewart PM (1997). Does central obesity reflect “Cushing’s disease of the omentum”? Lancet; 349:1210–1213.
274. Quirk SJ, Slattery JA, Funder JW. Epithelial and adipose cells isolated from mammary glands of pregnant and lactating rats differ in 11 _-hydroxysteroid dehydrogenase activity. J Steroid Biochem Mol Biol. 1990;37:529–534.
275. Tomlinson JW, Moore J, Cooper MS, et al. Regulation of expression of 11β-hydroxysteroid dehydrogenase type 1 in adipose tissue: tissue-specific induction by cytokines. Endocrinology. 2001;142:1982–1989.
276. Handoko K, Yang K, Strutt B, Khalil W, Killinger D. Insulin attenuates the stimulatory effects of tumor necrosis factor on 11β-hydroxysteroid dehydrogenase 1 in human adipose stromal cells. J Steroid Biochem Mol Biol. 2000;72:163–168.

277. Friedberg M, Zoumakis E, Hiroi N, Bader T, Chrousos GP, Hochberg Z. Modulation of 11 _-hydroxysteroid dehydrogenase type 1 in mature human subcutaneous adipocytes by hypothalamic messengers. J Clin Endocrinol Metab. 2003;88:385–393.
278. Veilleux A, Rheaume C, Daris M, Luu-The V, Tchernof A. Omental adipose tissue type 1 11 _-hydroxysteroid dehydrogenase oxoreductase activity, body fat distribution, and metabolic alterations in women. J Clin Endocrinol Metab. 2009;94:3550–3557.
279. Napolitano, A., Voice, M., Edwards, C.R.W., Seckl, J.R., Chapman, K.E. (1998). 11β-Hydroxysteroid dehydrogenase type 1 in adipocytes: expression is differentiation-dependent and hormonally-regulated. J. Steroid Biochem. Mol. Biol. 64, 251–260.
280. Rask, E., Walker, B.R., Soderberg, S., Livingstone, D.E., Eliasson, M., Johnson, O., Andrew, R., Olsson, T. (2002). Tissue-specific changes in peripheral cortisol metabolism in obese women: increased adipose 11 beta-hydroxysteroid dehydrogenase type 1 activity. J. Clin. Endocrinol. Metab. 87 (7), 3330–3336.
281. Rask, E., Olsson, T., Soderberg, S., Andrew, R., Livingstone, D.E.W., Johnson, O., Walker, B.R.(2001). Tissue-specific dysregulation of cortisol metabolism in human obesity. J. Clin. Endocrinol. Metab. 86, 1418–1421.
282. Lindsay, R.S., Wake, D.J., Nair, S., Bunt, J., Livingstone, D.E.W., Permana, P.A., Tataranni, P.A., Walker, B.R., 2003. Subcutaneous adipose 11 betahydroxysteroid dehydrogenase type 1 activity and messenger ribonucleic acid levels are associated with adiposity and insulinemia in Pima Indians and Caucasians. J. Clin. Endocrinol. Metab. 88 (6), 2738–2744.
283. Wake, D.J., Rask, E., Livingstone, D.E.W., Soderberg, S., Olsson, T., Walker, B.R., 2003. Local and systemic impact of transcriptional up-regulation of 11 betahydroxysteroid dehydrogenase type 1 in adipose tissue in human obesity. J. Clin. Endocrinol. Metab. 88 (8), 3983–3988.
284. Kannisto, K., Pietiläinen, K.H., Ehrenborg, E., Rissanen, A., Kaprio, J., Hamsten, A., Yki- Järvinen, H. 2004. Overexpression of 11beta-hydroxysteroid dehydrogenase- 1 in adipose tissue is associated with acquired obesity and features of insulin resistance: studies in young adult monozygotic twins. J. Clin. Endocrinol. Metab. 89 (9), 4414–4421.
285. Sandeep, T.C., Andrew, R., Homer, N.Z.M., Andrews, R.C., Smith, K., Walker, B.R., 2005. Increased in vivo regeneration of cortisol in adipose tissue in human obesity and effects of the 11 beta-hydroxysteroid dehydrogenase type 1 inhibitor carbenoxolone. Diabetes 54, 872–879.
286. Goedecke, J.H., Wake, D.J., Levitt, N.S., Lambert, E.V., Collins, M.R., Morton, N.M., Andrew, R., Seckl, J.R., Walker, B.R. (2006). Glucocorticoid metabolism within superficial subcutaneous rather than visceral adipose tisuue is a ssociated with features of the metabolic syndrome in South African women. Clin. Endocrinol. 65, 81–87.
287. Michailidou, Z., Jensen, M., Dumesic, D., Seckl, J.R., Walker, B.R., Morton, N.M. (2007). Omental fat 11 beta-HSD1 is correlated with fat cell size independently of obesity. Obesity 15, 1155–1163.

288. Livingstone, D.E.W., Jones, G., Smith, K., Jamieson, P.M., Andrew, R., Kenyon, C.J., Walker, B.R. (2000). Understanding the role of glucocorticoids in obesity: tissue-specific alterations of corticosterone metabolism in obese Zucker rats. Endocrinology 141, 560–563.
289. Masuzaki, H., Paterson, J., Shinyama, H., Morton, N.M., Mullins, J.J., Seckl, J.R., Flier, J.S., 2001. A transgenic model of visceral obesity and the Metabolic Syndrome. Science 294, 2166–2170.
290. Morton, N., Ramage, L., Seckl, J. (2004). Down-regulation of adipose 11β-hydroxysteroid dehydrogenase type 1 by high fat feeding in mice: a potential adaptive mechanism counteracting metabolic disease. Endocrinology 145, 2707–2712.
291. Masuzaki H, Yamamoto H, Kenyon CJ, et al. Transgenic amplification of glucocorticoid action in adipose tissue causes high blood pressure in mice. J Clin Invest. 2003; 112:83–90.
292. Weyer C, Funahashi T, Tanaka S, Hotta K, Matsuzawa Y, Pratley RE, Tataranni PA (2001). Hypoadiponectinemia in obesity and hyperinsulinemia. J Clin Endocrinol Metab, 86: 1930-1935.
293. Livingstone DE, Kenyon CJ, Walker BR. Mechanisms of dysregulation of 11 β-hydroxysteroid dehydrogenase type 1 in obese Zucker rats. J Endocrinol. 2000;167:533–539.
294. Prasad SS, Prashanth A, Kumar CP, Reddy SJ, Giridharan NV, Vajreswari A. A novel genetically-obese rat model with elevated 11β-hydroxysteroid dehydrogenase type 1 activity in subcutaneous adipose tissue. Lipids Health Dis. 2010;9:132.
295. Nakano S, Inada Y, Masuzaki H, et al. Bezafibrate regulates the expression and enzyme activity of 11β-hydroxysteroid dehydrogenase type 1 in murine adipose tissue and 3T3–L1 adipocytes. Am J Physiol Endocrinol Metab. 2007;292:E1213–E1222.
296. Engeli S, Bohnke J, Feldpausch M, etnak al. Regulation of 11β-HSD genes in human adipose tissue: influence of central obesity and weight loss. Obes Res. 2004;12:9–17.
297. Paulmyer-Lacroix O, Boullu S, Oliver C, AlessiMC,Grino M. Expression of the mRNA coding for 11β-hydroxysteroid dehydrogenase type 1 in adipose tissue from obese patients: an in situ hybridization study. J Clin Endocrinol Metab. 2002;87: 2701–2705.
298. Nicholas Michael Morton. Obesity and corticosteroids (2010): 11β-Hydroxysteroid type 1 as a cause and therapeutic target in metabolic disease. Molecular and Cellular Endocrinology 316 154–164.
299. Tannin GM, Agarwal AK, Monder C, New MI, White PC. The human gene for 1β-hydroxysteroid dehydrogenase.Structure, tissue distribution, and chromosomal localization. J Biol Chem. 1991;266:16653–16658.
300. Jamieson PM, Chapman KE, Edwards CR, Seckl JR. 11β-Hydroxysteroid dehydrogenase is an exclusive 11 β-eductase in primary cultures of rat hepatocytes: effect of physicochemical and hormonal manipulations. Endocrinology.1995;136:4754–4761.

301. Ricketts ML, Shoesmith KJ, Hewison M, Strain A, Eggo MC, Stewart PM. Regulation of 11β-hydroxysteroid dehydrogenase type 1 in primary cultures of rat and human hepatocytes. J Endocrinol. 1998;156:159–168.
302. Torrecilla E, Fernandez-Vazquez G, Vicent D, et al. Liver upregulation of genes involved in cortisol production and action is associated with metabolic syndrome in morbidly obese patients. Obes Surg. 2012;22:478–486.
303. Konopelska S, Kienitz T, Hughes B, et al. Hepatic 11β- HSD1 mRNA expression in fatty liver and non-alcoholic steatohepatitis. Clin Endocrinol (Oxf). 2009;70:554–560.
304. Candia R, Riquelme A, Baudrand R, et al. Overexpression of 11β-hydroxysteroid dehydrogenase type 1 in visceral adipose tissue and portal hypercortisolism in non-alcoholic fatty liver disease. Liver Int. 2012;32:392–399.
305. Liu Y, Nakagawa Y, Wang Y, et al. Increased glucocorticoid receptor and 11β-hydroxysteroid dehydrogenase type1 expression in hepatocytes may contribute to the phenotype of type 2 diabetes in db/db mice. Diabetes. 2005;54: 32–40.
306. Paterson, J. M., et al (2004). "Metabolic syndrome without obesity: Hepatic overexpression of 11beta-hydroxysteroid dehydrogenase type 1 in transgenic mice." Proc Natl Acad Sci U S A 101(18): 7088-7093.
307. Lavery GG, Zielinska AE, Gathercole LL, et al. Lack of significant metabolic abnormalities in mice with liverspecific disruption of 11βhydroxysteroid dehydrogenase type 1. Endocrinology. 2012;153:3236–3248.
308. Zhang, M., et al. (2009). "Alteration of 11beta-hydroxysteroid dehydrogenase type 1 in skeletal muscle in a rat model of type 2 diabetes." Mol Cell Biochem 324(1-2): 147-155.
309. Dimitriadis G, Leighton B, Parry-Billings M, et al. Effects of glucocorticoid excess on the sensitivity of glucose transport and metabolism to insulin in rat skeletal muscle. Biochem J. 1997;321:707–712.
310. Morgan SA, Sherlock M, Gathercole LL, et al. 11β- Hydroxysteroid dehydrogenase type 1 regulates glucocorticoid-induced insulin resistance in skeletal muscle. Diabetes. 2009;58:2506–2015.
311. Hermanowski-Vosatka, A., et al. (2005). 11beta-HSD1 inhibition ameliorates metabolic syndrome and prevents progression of atherosclerosis in mice. J Exp Med 202(4): 517-527.
312. Abdallah BM, Beck-Nielsen H, Gaster M. Increased expression of 11β-hydroxysteroid dehydrogenase type 1 in type 2 diabetic myotubes. Eur J Clin Invest. 2005;35:627–634.
313. Whorwood CB, Donovan SJ, Wood PJ, Phillips DI. Regulation of glucocorticoid receptor _ and _ isoforms and type I 11β-hydroxysteroid dehydrogenase expression in human skeletal muscle cells: a key role in the pathogenesis of insulin resistance? J Clin Endocrinol Metab. 2001;6: 2296–2308.
314. Webster, S. P., et al. (2007). Discovery and biological evaluation of adamantyl amide 11beta-HSD1inhibitors. Bioorg Med Chem Lett 17(10): 2838-2843.
315. Boyle, C. D. (2008). "Recent advances in the discovery of 11beta-HSD1 inhibitors." Curr Opin Drug Discov Devel 11(4): 495-511.

316. Tomlinson, J. W., et al. (2010). "The role of 11beta-hydroxysteroid dehydrogenase 1 in adipogenesis in thyroid-associated ophthalmopathy." J Clin Endocrinol Metab 95(1): 398-406.
317. Latif SA, Pardo HA, Hardy MP, Morris DJ. Endogenous selective inhibitors of 11 β-hydroxysteroid dehydrogenase isoforms 1 and 2 of adrenal origin. Mol Cell Endocrinol. 2005;243:43–50.
318. Andrews RC, Rooyackers O, Walker BR. Effects of the 11β-hydroxysteroid dehydrogenase inhibitor carbenoxolone on insulin sensitivity in men with type 2 diabetes. J Clin Endocrinol Metab. 2003;88:285–291.
319. Tomlinson JW, Sherlock M, Hughes B, et al. Inhibition of 11β-hydroxysteroid dehydrogenase type 1 activity in vivo limits glucocorticoid exposure to human adipose tissue and decreases lipolysis. J Clin Endocrinol Metab. 2007; 92:857–864.
320. Alberts, P., et al. (2002). "Selective inhibition of 11beta-hydroxysteroid dehydrogenase type 1 decreases blood glucose concentrations in hyperglycaemic mice." Diabetologia 45(11): 1528-1532.
321. Alberts, P., et al. (2003). "Selective inhibition of 11 beta-hydroxysteroid dehydrogenase type 1 improves hepatic insulin sensitivity in hyperglycemic mice strains." Endocrinology 144(11): 4755-4762.
322. Barf, T., et al. (2002). "Arylsulfonamidothiazoles as a new class of potential antidiabetic drugs. Discovery of potent and selective inhibitors of the 11beta-hydroxysteroid dehydrogenase type 1." J Med Chem 45(18): 3813 3815.
323. Edgerton DS, Basu R, Ramnanan CJ, et al. Effect of 11 β-hydroxysteroid dehydrogenase-1 inhibition on hepatic glucose metabolism in the conscious dog. Am J Physiol Endocrinol Metab. 2010;298:E1019–E1026.
324. Bhat BG, Hosea N, Fanjul A, et al. Demonstration of proof of mechanism and pharmacokinetics and pharmacodynamics relationship with 4_-cyano-biphenyl-4-sulfonic acid (6-amino-pyridin-2-yl)-amide (PF-915275), an inhibitor of 11 β-hydroxysteroid dehydrogenase type 1, in cynomolgus monkeys. J Pharmacol Exp Ther. 2008;324:299–305.
325. Bujalska, I. J., et al. (2008). A novel selective 11beta-hydroxysteroid dehydrogenase type 1 inhibitor prevents human adipogenesis. J Endocrinol 197(2): 297-307.
326. Rosenstock, J., et al. (2010). The 11-beta-hydroxysteroid dehydrogenase type 1 inhibitor INCB13739 improves hyperglycemia in patients with type 2 diabetes inadequately controlled by metformin monotherapy. Diabetes Care 33(7): 1516-1522.
327. National Institutes of Health (2012). A study of the effect of INCB013739 on cortisone reducing enzyme activity in obese people predisposed to diabetes. http:www. clinicaltrials.gov/ct2/show/ NCT00398619.
328. Courtney R, Stewart PM, Toh M, Ndongo MN, Calle RA, Hirshberg B. Modulation of 11β-hydroxysteroid dehydrogenase (11β- HSD) activity biomarkers and pharmacokinetics of PF-00915275, a selective 11β- HSD1 inhibitor. J Clin Endocrinol Metab. 2008;93:550–556.
329. Gibbs JP, Emery MG, McCaffery I, et al. Population pharmacokinetic/ pharmacodynamic model of subcutaneous adipose 11β-hydroxysteroid

dehydrogenase type 1 (11β- HSD1) activity after oral administration of AMG 221, a selective 11β-HSD1 inhibitor. J Clin Pharmacol. 2011;51: 830–841.
330. Davani B, Khan A, Hult M, et al (2000). "Type 1 11beta -hydroxysteroid dehydrogenase mediates glucocorticoid activation and insulin release in pancreatic islets." J Biol Chem 275(45): 34841-34844.
331. Duplomb L, Lee Y, Wang MY, et al. (2004). "Increased expression and activity of 11beta-HSD-1 in diabetic islets and prevention with troglitazone." Biochem Biophys Res Commun 313(3): 594-599.
332. Ortsater H, Alberts P, Warpman U, Engblom LO,Abrahmsen L, Bergsten P. (2005). "Regulation of 11beta-hydroxysteroid dehydrogenase type 1 and glucose-stimulated insulin secretion in pancreatic islets of Langerhans." Diabetes Metab Res Rev 21(4): 359-366.
333. Swali A, Walker EA, Lavery GG, Tomlinson JW, Stewart PM. (2008). "11beta-Hydroxysteroid dehydrogenase type 1 regulates insulin and glucagon secretion in pancreatic islets." Diabetologia 51(11): 2003-2011.
334. Turban S, Liu X, Ramage L, et al. Optimal elevation of β-cell 11β-hydroxysteroid dehydrogenase type 1 is a compensatory mechanism that prevents high fat diet-induced β-cell failure. Diabetes. 2012;61:642–652.
335. Hult M, Ortsater H, Schuster G, et al. Short-term glucocorticoid treatment increases insulin secretion in islets derived from lean mice through multiple pathways and mechanisms. Mol Cell Endocrinol. 2009;301:109–116.
336. Schmid J, Ludwig B, Schally AV, et al. Modulation of pancreatic islets-stress axis by hypothalamic releasing hormones and 11β-hydroxysteroid dehydrogenase. Proc Natl Acad Sci USA. 2011;108:13722–13727.
337. Rafacho, A., et al. (2009). "High doses of dexamethasone induce increased beta-cell proliferation in ancreatic rat islets." Am J Physiol Endocrinol Metab 296(4): E681-689.
338. Nussey, S., & Whitehead, S. (2001). Endocrinology: An Integrated Approach. Oxford, BIOS Scientific Publishers Limited.
339. Ricketts ML, Verhaeg JM, Bujalska I, Howie AJ, Rainey WE, Stewart PM. Immunohistochemical localization of type-1 11β-hydroxysteroid dehydrogenase in human tissues. J Clin Endocrinol Metab. 1998;83:1325–1335.
340. Bo Hellman and L Gervall (1961).Thefrequency distribution of the number and volume of the islets of langerhans in man. Acta Pathologica Microbiologica Scandinavica 53(3): 230-236 .
341. Nielsen, J.H., A. Moldrup, and N. Billestrup, Expression of the growth hormone receptor gene in insulin producing cells. Biomed Biochim Acta, 1990. 49(12): p. 1151-5.
342. Soares, M.B., et al., RNA-mediated gene duplication: the rat preproinsulin I gene is a functional retroposon. Mol Cell Biol, 1985. 5(8): p. 2090-103.
343. Sander, M. and M.S. German. The beta cell transcription factors and development of the pancreas. J Mol Med, 1997. 75(5): p. 327-40.
344. Slack, J.M. Developmental biology of the pancreas. Development, 1995. 121(6): p. 1569-80
345. Nakae J, Kido Y & Accili D (2001) Distinct and overlapping functions of insulin and IGF-I. Endocrine Reviews 22818–835

346. Nelson JD, LeBoeuf RC & Bomsztyk K (2011). Direct recruitment of insulin receptor and ERK signaling cascade to insulin-inducible gene loci. Diabetes 60127–137
347. Pollak M (2008). Insulin and insulin-like growth factor signalling in neoplasia.Nature Reviews. Cancer 8 915–928.
348. Dumont JE, Dremier S,Pirson I & Maenhaut C. (2002) Cross signaling, cell specificity, and physiology. American Journal of Physiology. Cell Physiology283 C2–C28.
349. Belfiore A, Frasca F, Pandini G, Sciacca L & Vigneri R (2009). Insulin receptor isoforms and insulin receptor/insulin-like growth factor receptor hybrids in physiology and disease. Endocrine Reviews 30586–623.
350. Leibiger IB, Brismar K & Berggren. PO2010a Novel aspects on pancreatic beta-cell signal transduction. Biochemical and Biophysical Research Communications 396 111–115.
351. Brigstock DR(1999). The connective tissue growth factor/cysteine-rich 61/nephroblastoma overexpressed (CCN) family. Endocr Rev 20:189–206.
352. Lau LF and Lam SC (1999). The CCN family of angiogenic regulators: the integrin connection. Exp Cell Res 248: 44–57.
353. Perbal B (2001) . NOV (nephroblastoma overexpressed) and the CCN family of genes: structural and functional issues. Mol Pathol 54:57–79.
354. Takigawa M (2003). CTGF/Hcs24 as a multifunctional growth factor for fibroblasts, chondrocytes and vascular endothelial cells. Drug News Perspect 16:11–21.
355. Brigstock DR (2003). The CCN family: a new stimulus package. J Endocrinol 178:169–175
356. N. Q. McDonald and W. A. Hendrickson. A structural superfamily of growth factors containing a cystine knot motif. Cell 73, 421-424 (1993).
357. V. Hwa, Y. Oh and R. G. Rosenfeld. Insulin-like growth factor binding proteins: a proposed superfamily. Acta Paediatrica Suppl 428, 37-45 (1999).
358. H. S. Kim, S. R. Nagalla, Y. Oh, E. Wilson, C. T Roberts Jr and R. G. Rosenfeld. Identification of a family of low affinity insulin-like growth factor binding proteins (IGFBPs): characterization of connective tissue growth factor as a member of the IGFBP superfamily. Proc Natl Acad Sci USA 94, 12981–12986 (1997).
359. C. P. Burren, E. M. Wilson, V. Hwa, Y. Oh and R. G. Rosenfeld. Binding properties and distribution of insulinlike growth factor binding protein-related protein 3 (IGFBP-rP3/NovH), an additional member of the IGFBP superfamily. J Clin Endocrinol Metab 84, 1096–1103 (1999).
360. K. Tan, M. Duquette, J. H. Liu, Y. Dong, R. Zhang, A. Joachimiak, J. Lawler and J. H. Wang. Crystal structure of the TSP-1 type 1 repeats: a novel layered fold and its biological implication. J Cell Biol 159, 373-382 (2002).
361. J. Voorberg, R. Fontijn, J. Calafat, H. Janssen, J. A. van Mourik and H. Pannekoek. Assembly and routing of von Willebrand factor variants: the requirements for disulfidelinked dimerization reside within the carboxy-terminal 151 amino acids. J Cell Biol 113, 195-205 (1991.)

362. J. L. Zhang, Y. Huang, L. Y. Qiu, J. Nickel and W. Sebald. von Willebrand factor type C domain-containing proteins regulate bone morphogenetic protein signalling through different recognition mechanisms. J Biol Chem 282, 20002-20014 (2007).
363. P. Bork. The modular architecture of a new family of growth regulators related to connective tissue growth factor. FEBS Lett 327, 125-130 (1993).
364. L. F. Lau and D. Nathans. Expression of a set of growthrelated immediate early genes in BALB/c 3T3 cells: coordinate regulation with c-fos or c-myc. Proc Natl Acad Sci U S A 84, 1182-1186 (1987).
365. P. Jay, J. L. Bergé-Lefranc, C. Marsollier, C. Méjean, S. Taviaux and P. Berta. The human growth factor-inducible immediate early gene, CYR61, maps to chromosome 1p. Oncogene 14, 1753-1757 (1997).
366. D. L. Simmons, D. B. Levy, Y. Yannoni and R. L. Erikson.Identification of a phorbol ester-repressible v-srcinducible gene. Proc Natl Acad Sci U S A 86, 1178-1182 (1989).
367. T. P. O'Brien, G. P. Yang, L. Sanders and L. F. Lau. Expression of cyr61, a growth factor-inducible immediateearly gene. Mol Cell Biol 10, 3569-3577 (1990).
368. D. M. Bradham, A. Igarashi, R. L. Potter and G. R. Grotendorst. Connective tissue growth factor: a cysteinerich mitogen secreted by human vascular endothelial cells is related to the SRC-induced immediate early gene product CEF-10. J Cell Biol 114, 1285-1294 (1991).
369. Chen, N. et al. (2004) Identification of a novel integrin alphavbeta3 binding site in CCN1 (CYR61) critical for pro-angiogenic activities in vascular endothelial cells. J. Biol. Chem. 279, 44166–44176.
370. Leu, S.J. et al. (2003) Identification of a novel integrin alpha 6 beta 1 binding site in the angiogenic inducer CCN1 (CYR61). J. Biol. Chem. 278, 33801–33808 .
371. Leu, S.J. et al. (2004) Targeted mutagenesis of the angiogenic protein CCN1 (CYR61). Selective inactivation of integrin alpha6beta1- heparan sulfate proteoglycan coreceptor-mediated cellular functions. J. Biol. Chem. 279, 44177–4418.
372. Notredame, C. et al. (2000) T-Coffee: A novel method for fastand accurate multiple sequence alignment. J. Mol. Biol. 302, 205–217.
373. M. Carson: Ribbons. Methods Enzymol.277, 493-505 (1997).
374. Brigstock DR. (1999) The connective tissue growth factor/cysteine-rich 61/nephroblastoma overexpressed (CCN) family. Endocr Rev 20:189–206.
375. Perbal B. (2004) CCN proteins: multifunctional signalling regulators. Lancet 363: 62–64.
376. Takigawa M, Nakanishi T, Kubota S and Nishida T. (2003) Role of CTGF/ HCS24/ecogenin in skeletal growth control. J Cell Physiol 194:256–266.
377. HashimotoG, Inoki I, FujiiY, Aoki T, Ikeda E and OkadaY.(2002)Matrix metalloproteinases cleave connective tissue growth factor and reactivate angiogenic activity of vascular endothelial growth factor 165. J Biol Chem 277:36288–36295.
378. Abreu JG, Ketpura NI, Reversade B and De Robertis EM. (2002) Connective-tissue growth factor (CTGF) modulates cell signalling by BMP and TGF-beta. Nat Cell Biol 4:599–604.

379. Inoki I, Shiomi T, Hashimoto G, Enomoto H, Nakamura H, Makino K, Ikeda E, Takata S, Kobayashi K and Okada Y. (2002) Connective tissue growth factor binds vascular endothelial growth factor (VEGF) and inhibits VEGF-induced angiogenesis. Faseb J 16:219–221.
380. Gao R and Brigstock DR. (2003) Low density lipoprotein receptor-related protein (LRP) is a heparin-dependent adhesion receptor for connective tissue growth factor (CTGF) in rat activated hepatic stellate cells. Hepatol Res 27:214–220.
381. Lombet A, Planque N, Bleau AM, Li CL and Perbal B. (2003) CCN3 and calcium signaling. Cell Commun Signal 1:1.
382. Mercurio S, Latinkic B, Itasaki N, Krumlauf R and Smith JC. (2004) Connective-tissue growth factor modulates WNT signalling and interacts with the WNT receptor complex. Development 131:2137–2147.
383. Gellhaus A, Dong X, Propson S, Maass K, Klein–Hitpass L, Kibschull M, Traub O, Willecke K, Perbal B, Lye SJ andWinterhager E .(2004) Connexin43 interacts with NOV: a possible mechanism for negative regulation of cell growth in choriocarcinoma cells. J Biol Chem 279:36931–36942.
384. Gellhaus A, Dong X, Propson S, Maass K, Klein–Hitpass L, Kibschull M, Traub O, Willecke K, Perbal B, Lye SJ andWinterhager E. (2004) Connexin43 interacts with NOV: a possible mechanism for negative regulation of cell growth in choriocarcinoma cells. J Biol Chem 279:36931–36942.
385. FuCT, Bechberger JF, OzogMA, Perbal B and NausCC. (2004) CCN3(NOV) interacts with connexin43 in C6 glioma cells: possible mechanism of connexin-mediated growth suppression. J Biol Chem 279:36943–36950.
386. Segarini PR, Nesbitt JE, Li D, Hays LG, Yates JR, 3rd and Carmichael DF. (2001) The low density lipoprotein receptor-related protein/alpha2-macroglobulin receptor is a receptor for connective tissue growth factor. J Biol Chem 276:40659–40667.
387. Sakamoto K, Yamaguchi S, Ando R, Miyawaki A, Kabasawa Y, Takagi M, Li CL, Perbal B and Katsube K. (2002) The nephroblastoma overexpressed gene (NOV/ ccn3) protein associates with Notch1 extracellular domain and inhibits myoblast differentiation via Notch signaling pathway. J Biol Chem 277:29399–29405.
388. Nishida T, Nakanishi T, Shimo T, Asano M, Hattori T, Tamatani T, Tezuka K and Takigawa M. (1998) Demonstration of receptors specific for connective tissue growth factor on a human chondrocytic cell line (HCS-2/8). Biochem Biophys Res Commun 247:905–909.
389. Delmolino LM, Stearns NA and Castellot JJ Jr. (1997) Heparin induces a member of the CCN family which has characteristics of a growth arrest gene. Mol Biol Cell 8:287a.
390. Pennica D, Swanson TA, Welsh JW, Roy MA, Lawrence DA, Lee J, Brush J, Taneyhill LA, Deuel B, Lew M, Watanabe C, Cohen RL, MelhemMF, Finley GG, Quirke P, Goddard AD, Hillan KJ, Gurney AL, Botstein D and Levine AJ. (1998) WISP genes are members of the connective tissue growth factor family that are up-regulated in wnt-1-transformed cells and aberrantly expressed in human colon tumors. Proc Natl Acad Sci USA 95:14717–14722.
391. Kumar S, Hand AT, Connor JR, Dodds RA, Ryan PJ, Trill JJ, Fisher SM, NuttallME, Lipshutz DB, Zou C, Hwang SM, Votta BJ, James IE, Rieman DJ,

Gowen Mand Lee JC. (1999) Identification and cloning of a connective tissue growth factor-like cDNA from human osteoblasts encoding a novel regulator of osteoblast functions. J Biol Chem 274:17123–17131.
392. Zhang R, Averboukh L, Zhu W, Zhang H, Jo H, Dempsey PJ, Coffey RJ, Pardee AB and Liang P. (1998) Identification of rCop-1, a new member of the CCN protein family, as a negative regulator for cell transformation. Mol Cell Biol 18:6131–6141.
393. Delmolino LM, Stearns NA and Castellot JJ Jr. (2001) COP-1, a member of the CCN family, is a heparin-induced growth arrest specific gene in vascular smooth muscle cells. J Cell Physiol 188:45–55.
394. Lake AC, Bialik A, Walsh K and Castellot JJ Jr. (2003) CCN5 is a growth arrestspecific gene that regulates smooth muscle cell proliferation and motility. Am J Pathol 162:219–231.
395. Inadera H, Dong H-Y and Matsushima K. (2002) WISP-2 is a secreted protein and can be a marker of estrogen exposure in MCF-7 cells. Biochem Biophys Res Commun 294:602–608.
396. ZoubineMN, Banerjee S, Saxena NK, CampbellDR and Banerjee SK . (2001) WISP-2: a serum-inducible gene differentially expressed in human normal breast epithelial cells and in MCF-7 breast tumor cells. Biochem Biophys Res Commun 282:421–425.
397. Mason HR, Nowak RA, 4c y CC and Castellot JJ Jr. (2003) Heparin inhibits the motility and proliferation of human myometrial and leiomyoma smooth muscle cells. Am J Pathol 162:1895–1904.
398. Holbourn KP, Perbal B, Ravi Acharya K. (2009) Proteins on the catwalk: modelling the structural domains of the CCN family of proteins. J Cell Commun Signal 3:25–41.
399. . Wei L, Mckeon F, Russo Jw, Lemire J, and Castellot J. (2010) CCN Proteins in Health and Disease. 77-95.
400. Wiesman Kc, Wei L, Baughman C, Russo J, Gray Mr, and Castellot Jj (2010). CCN5, a secreted protein, localizes to the nucleus. Journal of Cell Communication & Signaling. 4(2): 91-8.
401. Chien W, O'kelly J, Lu D, Leiter A, Sohn J, Yin D, Karlan B, Vadgama J, Lyons Km, and Koeffler Hp (2011). Expression of connective tissue growth factor (CTGF/CCN2) in breast cancer cells is associated with increased migration and angiogenesis. Int J Oncology. 36(6): 1741-7.
402. Pi L, Shenoy Ak, Liu J, Kim S, Nelson N, Xia H, Hauswirth Ww, Petersen Be, Schultz Gs, and Scott Ew (2012). CCN2/CTGF regulates neovessel formation via targeting structurally conserved cystine knot motifs in multiple angiogenic regulators. The FASEB Journal. Epub ahead of print.
403. Hoshijima M, Hattori T, Inoue M, Araki D, Hanagata H, Miyauchi A, and Takigawa M (2006). CT domain of CCN2/CTGF directly interacts with fibronectin and enhances cell adhesion of chondrocytes through integrin α5β1. FEBS letters. 580(5): 1376-82.
404. Aoyama E, Hattori T, Hoshijima M, Araki D, Nishida T, Kubota S, and Takigawa M (2009). N-terminal domains of CCN family 2/connective tissue growth factor bind to aggrecan. Biochem J. 420 (3): 413-20.

405. Si W, Kang Q, Luu Hh, Park Jk, Luo Q, Song W-X, Jiang W, Luo X, Li X, Yin H, et al. (2006). CCN1/Cyr61 is regulated by the canonical Wnt signal and plays an important role in Wnt3A-induced osteoblast differentiation of mesenchymal stem cells. Molecular and Cellular Biology. 26(8): 2955-64.
406. Tanaka I, Morikawa M, Okuse T, Shirakawa M, and Imai K (2005). Expression and regulation of WISP2 in rheumatoid arthritic synovium. Biochemical and Biophysical Research Communications. 334(4): 973-8.
407. Fukutomi T, Zhou Y, Kawai S, Eguchi H, Wands Jr, and Li J (2005). Hepatitis C virus core protein stimulates hepatocyte growth: correlation with upregulation of wnt-1 expression. Hepatology. 41(5): 1096-105.
408. Letica L, Mermejo Lm, Ramalho Lz, Martinelli Ce, Yunes Ja, Seidinger Al, Mastellaro Mj, Cardinalli Ia, Brandalise Sr, Moreira Ac, et al. (2011) Wnt/ -Catenin Pathway Deregulation in Childhood Adrenocortical Tumors. Journal of Clinical Endocrinology & Metabolism. 96(10): 3106-14.
409. McManus EJ, Sakamoto K, Armit LJ, Ronaldson L, Shpiro N, Marquez R, Alessi DR. (2005) Role that phosphorylation of GSK3 plays in insulin and Wnt signalling defined by knockin analysis. EMBO J 24:1571–1583.
410. Suzuki A, Ozono K, Kubota T, Kondou H, Tachikawa K, Michigami T. (2008) PTH/cAMP/PKA signaling facilitates canonical Wnt signaling via inactivation of glycogen synthase kinase-3beta in osteoblastic Saos-2 cells. J Cell Biochem 104:304–317.
411. Iliopoulos D, Bimpaki EI, Nesterova M, Stratakis CA. (2009) Micro- RNA signature of primary pigmented nodular adrenocortical disease: clinical correlations and regulation of Wnt signaling. Cancer Res 69(8):3278–3782.
412. Inadera H. (2003) Estrogen-induced genes, WISP-2 and pS2, respond divergently to protein kinase pathway. Biochem Biophys Res Commun 309:272–278.
413. Gwak J, Cho M, Gong S-J, Won J, Kim D-E, Kim E-Y, Lee Ss, Kim M, Kim Tk, Shin J-G, et al. (2006) Protein-kinase-C-mediated -catenin phosphorylation negatively regulates the Wnt/ -catenin pathway. Journal of Cell Science. 119(22): 4702-9.
414. Sengupta K, Banerjee S, Dhar K, Saxena Nk, Mehta S, Campbell Dr, and Banerjee Sk (2006). WISP-2/CCN5 is involved as a novel signaling intermediate in phorbol esterprotein kinase C alpha-mediated breast tumor cell proliferation. Biochemistry. 45(35): 10698-709.
415. Visser S, Yang X (2010). Identification of LATS transcriptional targets in HeLa cells using whole human genome oligonucleotide microarray. Gene 449:22–29.
416. Banerjee S, Saxena N, Sengupta K, Tawfik O, Mayo Ms, and Banerjee Sk (2003). WISP-2 gene in human breast cancer: estrogen and progesterone inducible expression and regulation of tumor cell proliferation. Neoplasia. 5(1): 63-73.
417. Inadera H, Hashimoto S, Dong HY, Suzuki T, Nagai S, Yamashita T, Toyoda N, Matsushima K (2000) WISP-2 as a novel estrogenresponsive gene in human breast cancer cells. Biochem Biophys Res Commun 275:108–114.
418. Dhar K, Banerjee S, Dhar G, Sengupta K, Banerjee SK. (2007b) Insulin-like growth factor-1 (IGF-1) induces WISP-2/CCN5 via multiple molecular cross-talks and is essential for mitogenic switch by IGF-1 axis in estrogen receptor-positive breast tumor cells. Cancer Res 67:1520–1526.

419. Beato M, Chalepakis G, Schauer M, Slater EP. (1989) DNA regulatory elements for steroid hormones. J Steroid Biochem 32:737–747.
420. Banerjee S, Sengupta K, Saxena NK, Dhar K, Banerjee SK (2005) Epidermal growth factor induces WISP-2/CCN5 expression in estrogen receptor-{alpha}-positive breast tumor cells through multiple molecular cross-talks. Mol Cancer Res 3:151–162.
421. Inadera H, Dong HY, Matsushima K (2002). WISP-2 is a secreted protein and can be a marker of estrogen exposure in MCF-7 cells. Biochem Biophys Res Commun 294:602–608.
422. Ray G, Banerjee S, Saxena NK, Campbell DR, Van VP, Banerjee SK (2005). Stimulation of MCF-7 tumor progression in athymic nude mice by 17beta-estradiol induces WISP-2/CCN5 expression in xenografts: a novel signaling molecule in hormonal carcinogenesis. Oncol Rep 13:445–448.
423. Fritah A, Redeuilh G, Sabbah M (2006). Molecular cloning and characterization of the human WISP-2/CCN5 gene promoter reveal its upregulation by oestrogens. J Endocrinol 191:613–624.
424. Johnsen SA, Gungor C, Prenzel T, Riethdorf S, Riethdorf L, Taniguchi-Ishigaki N, Rau T, Tursun B, Furlow JD, Sauter G, Scheffner M, Pantel K, Gannon F, Bach I (2009). Regulation of estrogen-dependent transcription by the LIM cofactors CLIM and RLIM in breast cancer. Cancer Res 69: 128–136.
425. Levin Er (2003). Bidirectional Signaling between the Estrogen Receptor and the Epidermal Growth Factor Receptor. Molecular Endocrinology. 17(3): 309-17.
426. Joshua W. Russo & John J. Castellot Jr. (2011). CCN5: Biology and pathophysiology. J. Cell Commun. Signal. 4:119–130.
427. Bartella V, Marco Pd, Malaguarnera R, Belfiore A, and Maggiolini M (2012). New advances on the functional cross-talk between insulin-like growth factor-I and estrogen signaling in cancer. Cellular Signaling. 24(8): 1515-21.
428. Dhar G, Mehta S, Banerjee S, Gardner A, McCarty BM, Mathur SC, Campbell DR, Kambhampati S, Banerjee SK (2007). Loss of WISP-2/CCN5 signaling in human pancreatic cancer: a potential mechanism for epithelial-mesenchymal-transition. Cancer Lett 254:63–70.
429. Jiang C, Ito M, Peining V, Bruck K, Roeder G, Xiao H. (2004) TIP30 Interacts with an Estrogen Receptor-interacting Coactivator CIA and Regulates c-myc Transcription. Journal of Biological Chemistry. 279(26): 27781-9.
430. Pecha J, Ankrapp D, Jiang C, Tang W, Hoshino I, Bruck K, Wagner K-U, and Xiao H (2007). Deletion of Tip30 leads to rapid immortalization of murine mammary epithelial cells and ductal hyperplasia in the mammary gland. Oncogene. 26(53): 7423-31.
431. Mason Hr, Lake Ac, Wubben Je, Nowak Ra, and Castellot Jj (2004). The growth arrest-specific gene CCN5 is deficient in human leiomyomas and inhibits the proliferation and motility of cultured human uterine smooth muscle cells. Molecular human reproduction. 10(3): 181-7.
432. Russo JW, and Castellot JJ (2010). CCN5: biology and pathophysiology. Journal of Cell Communication & Signaling. 4(3): 119-30.

433. Ronald B. Myers & Kibibi Rwayitare & Lauren Richey & Janis Lem & John J. Castellot Jr. (2012). CCN5 Expression in mammals. III. Early embryonic mouse development. J. Cell Commun. Signal 6:217–223.
434. Surveyor GA, Wilson AK, Brigstock DR (1998). Localization of connective tissue growth factor during the period of embryo implantation in the mouse. Biol Reprod 59:1207–13.
435. Jones JA, Gray MR, Oliveira BE, Koch M, Castellot JJ Jr (2007). CCN5 expression in mammals: I. Embryonic and fetal tissues of mouse and human. J Cell Commun Signal 1:127–43.
436. Gray MR, Malmquist JA, Sullivan M, Blea M, Castellot JJ Jr (2007). CCN5 Expression in mammals. II. Adult rodent tissues. J Cell Commun Signal 1:145–58.
437. Inadera H, Shimomura A, Tachibana S (2009). Effect of Wnt-1 inducible signaling pathway protein-2 (WISP-2/CCN5), a downstream protein of Wnt signaling, on adipocyte differentiation. Biochem Biophys Res Commun 379:969–974.
438. Van den Brule S, Heymans J, Havaux X, Renauld JC, Lison D, Huaux F, Denis O (2007). Profibrotic effect of IL-9 overexpression in a model of airway remodeling. Am J Respir Cell Mol Biol 37:202–9.
439. Mason HR, Lake AC, Wubben JE, Nowak RA, Castellot JJ Jr (2004). The growth arrest-specific gene CCN5 is deficient in human leiomyomas and inhibits the proliferation and motility of cultured human uterine smooth muscle cells. Mol Hum Reprod 10:181–7.
440. Cervello M, Giannitrapani L, Labbozzetta M, Notarbartolo M, D'Alessandro N, Lampiasi N, Azzolina A, Montalto G (2004). Expression of WISPs and of their novel alternative variants in human hepatocellular carcinoma cells. Ann N YAcad Sci 1028:432–9.
441. Hirasaki S, Koide N, Ujike K, Shinji T, Tsuji T (2001). Expression of Nov, CYR61 and CTGF genes in human hepatocellular carcinoma. Hepatol Res 19:294–305.
442. Ando H, Fukuda N, Kotani M, Yokoyama S, Kunimoto S, Matsumoto K, Saito S, Kanmatsuse K, Mugishima H (2004). Chimeric DNARNA hammerhead ribozyme targeting transforming growth factor-beta 1 mRNA inhibits neointima formation in rat carotid artery after balloon injury. Eur J Pharmacol 483:207–14.
443. Fan WH, Karnovsky MJ (2002). Increased MMP-2 expression in connective tissue growth factor over-expression vascular smooth muscle cells. J Biol Chem 277:9800–5.
444. Fan WH, Pech M, Karnovsky MJ (2000). Connective tissue growth factor (CTGF) stimulates vascular smooth muscle cell growth and migration in vitro. Eur J Cell Biol 79:915–23.
445. Lake AC, Castellot JJ Jr (2003). CCN5 modulates the antiproliferative effect of heparin and regulates cell motility in vascular smooth muscle cells. Cell Commun Signal 1:5.
446. Manns JM, Uknis AB, Rico MC, Agelan A, Castaneda J, Arango I, Barbe MF, Safadi FF, Popoff SN, DeLa Cadena RA (2006). A peptide from thrombospondin 1 modulates experimental erosive arthritis by regulating connective tissue growth factor. Arthritis Rheum 54:2415–22.

447. Shin JY, Hur W, Wang JS, Jang JW, Kim CW, Bae SH, Jang SK, Yang SH, Sung YC, Kwon OJ, Yoon SK (2005). HCV core protein promotes liver fibrogenesis via up-regulation of CTGF with TGF-β. Exp Mol Med 37:138–45.
448. Saxena N, Banerjee S, Sengupta K, Zoubine MN, Banerjee SK (2001). Differential expression of WISP-1 and WISP-2 genes in normal and transformed human breast cell lines. Mol Cell Biochem 228:99–104.
449. Banerjee S, Dhar G, Haque I, Kambhampati S, Mehta S, Sengupta K, Tawfik O, Phillips TA, Banerjee SK (2008). CCN5/WISP-2 expression in breast adenocarcinoma is associated with less frequent progression of the disease and suppresses the invasive phenotypes of tumor cells. Cancer Res 68:7606–7612.
450. Fritah A, Saucier C, De WO, Bracke M, Bieche I, Lidereau R, Gespach C, Drouot S, Redeuilh G, SabbahM (2008). Role ofWISP-2/CCN5 in the maintenance of a differentiated and noninvasive phenotype in human breast cancer cells. Mol Cell Biol 28:1114–1123.
451. Dhar G, Banerjee S, Dhar K, Tawfik O, Mayo MS, Vanveldhuizen PJ, Banerjee SK Gain of oncogenic function of p53 mutants induces invasive phenotypes in human breast cancer cells by silencing CCN5/WISP-2. Cancer Res. 2008; 68:4580–4587.
452. Rittié L, Perbal B, Castellot JJ, Orringer JS, Voorhees JJ, and Fisher GJ. Spatial-temporal modulation of CCN proteins during wound healing in human skin in vivo. Journal of Cell Communication & Signaling. 2011 5(1): 69-80.
453. Quan T, Shin S, Qin Z, and Fisher Gj. Expression of CCN family of genes in human skin in vivo and alterations by solar-simulated ultraviolet irradiation. Journal of Cell Communication & Signaling. 2009. 3(1): 19-23.
461. Kendell EC. Cortisone: Memoirs of a hormone hunter. New York, NY: Charles Scribner's sons. 1971;121–137.
462. Takahashi, J. S. and M. Zatz (1982). "Regulation of circadian rhythmicity." Science 217(4565): 1104-1111.
463. Axelrod, J. and T. D. Reisine (1984). "Stress hormones: their interaction and regulation." Science 224(4648): 452-459.
464. Cope CL, Black EG. The behaviour of 14c-cortisol and estimation of cortisol production rate in man. Clin Sci (Lond). 1958; 17:147–163.
465. Esteban NV, Loughlin T, Yergey AL, et al. Daily cortisol production rate in man determined by stable isotope dilution/mass spectrometry. J Clin Endocrinol Metab. 1991;72:39–45.
466. Siiteri PK, Murai JT, Hammond GL, Nisker JA, Raymoure WJ, Kuhn RW. The serum transport of steroid hormones.Recent Prog Horm Res. 1982; 38:457–510.
467. Meulenberg PM, Hofman JA. Differences between concentrations of salivary cortisol and cortisone and of free cortisol and cortisone in plasma during pregnancy and postpartum. Clin Chem. 1990; 36:70–75.
468. Fischer, B., et al. (1990). "Immunohistochemical localization of the glucocorticoid receptor in pancreatic beta-cells of the rat." Endocrinology 126(5): 2635-2641.
469. Matthes, H., et al. (1994). "Glucocorticoid receptor gene expression in the exocrine and endocrine rat pancreas." Endocrinology 135(1): 476-479.
470. Krozowski, Z. S., et al. (1989). "Immunolocalization of renal mineralocorticoid receptors with an antiserum against a peptide deduced from the complementary deoxyribonucleic acid sequence. Endocrinology 125(1): 192-198.

471. Rafestin-Oblin, M. E., et al. (1984). "Mineralocorticoid receptors in the epithelial cells of human colon and ileum." J Steroid Biochem 20(1): 311-315.
472. de Kloet, E. R. (2003). "Hormones, brain and stress." Endocr Regul 37(2): 51-68
473. Datson, N. A., et al. (2008). "Central corticosteroid actions: Search for gene targets." Eur J Pharmacol 583(2-3): 272-289.
474. Oakley, R.H. and Cidlowski, J.A. (2011). Cellular processing of the glucocorticoid receptor gene and protein: new mechanisms for generating tissue-specific actions of glucocorticoids. J. Biol. Chem.286, 3177–3184.
475. Kino, T. et al. (2009). Human glucocorticoid receptor isoform beta: recent understanding of its potential implications in physiology and pathophysiology. Cell. Mol. Life Sci. 66, 3435–3448
476. Anbalagan, M. et al. (2012). Post-translational modifications of nuclear receptors and human disease. Nucl. Recept. Signal. 10, e001
477. Wang, J.C. et al. (2004). Chromatin immunoprecipitation (ChIP) scanning identifies primary glucocorticoid receptor target genes. Proc. Natl. Acad. Sci. U.S.A. 101, 15603–15608
478. Itani, O.A. et al. (2002). Glucocorticoids stimulate human sgk1 gene expression by activation of a GRE in its 50-flanking region. Am. J. Physiol. Endocrinol. Metab. 283, E971–E979
479. Smoak, K. and Cidlowski, J.A. (2006). Glucocorticoids regulate tristetraprolin synthesis and posttranscriptionally regulate tumor necrosis factor alpha inflammatory signaling. Mol. Cell. Biol. 26, 9126–9135
480. Barnes, P.J. (2011). Glucocorticosteroids: current and future directions. Br. J. Pharmacol. 163, 29–43
481. Oakley, R.H. et al. (2012). Glucocorticoids regulate arrestin gene expression and redirect the signaling profile of G protein-coupled receptors. Proc. Natl. Acad. Sci. U.S.A. 109, 17591–17596.
482. Ramamoorthy, S. and Cidlowski, J.A. (2013). Ligand-induced repression of the glucocorticoid receptor gene is mediated by an NCoR1 repression complex formed by long-range chromatin interactions with intragenic glucocorticoid response elements. Mol. Cell. Biol. 33, 1711–1722
483. Beck, I.M. et al. (2011) Glucocorticoid receptor mutants: man-made tools for functional research. Trends Endocrinol. Metab. 22, 295–310
484. Meijsing, S.H. et al. (2009) DNA binding site sequence directs glucocorticoid receptor structure and activity. Science 324, 407–410
485. Ayroldi, E. et al. (2012) Mechanisms of the anti-inflammatory effects of glucocorticoids: genomic and nongenomic interference with MAPK signaling pathways. FASEB J. 26, 4805–4820
486. Busillo, J.M. and Cidlowski, J.A. (2013) The five Rs of glucocorticoid action during inflammation: ready, reinforce, repress, resolve, and restore. Trends Endocrinol. Metab. 24, 109–119
487. Song, I.H. and Buttgereit, F. (2006) Non-genomic glucocorticoid effects to provide the basis for new drug developments. Mol. Cell. Endocrinol. 246, 142–146
488. Lee, S.R. et al. (2012) Non-genomic effect of glucocorticoids on cardiovascular system. Pflugers Arch. 464, 549–559

489. Bellavance, M.A. and Rivest, S. (2012) The neuroendocrine control of the innate immune system in health and brain diseases. Immunol. Rev. 248, 36–55.
490. Weinstein, S. P., et al. (1995). "Glucocorticoid-induced insulin resistance: dexamethasone inhibits the activation of glucose transport in rat skeletal muscle by both insulin- and non-insulin-related stimuli." Diabetes 44(4): 441-445.
491. Coderre, L., et al. (1996) "In vivo effects of dexamethasone and sucrose on glucose transport (GLUT-4) protein tissue distribution." Am J Physiol 271(4 Pt 1): E643-648.
492. Dimitriadis, G., et al. (1997) "Effects of glucocorticoid excess on the sensitivity of glucose transport and metabolism to insulin in rat skeletal muscle." Biochem J 321 (Pt 3): 707-712.
493. Kahn SE. Clinical review 135: The importance of beta-cell failure in the development and progression of type 2 diabetes. J Clin Endocrinol Metab 2001; 86(9): 4047–4058.
494. Jeschke MG, Boehning D. Endoplasmic reticulum stress and insulin resistance post-trauma: similarities to type 2 diabetes. J Cell Mol Med 2012; 16(3): 437–444.
495. McMahon M, Gerich J, Rizza R. Effects of glucocorticoids on carbohydrate metabolism. Diabetes Metab Rev 1988; 4(1): 17–30.
496. Piroli GG, Grillo CA, Reznikov LR, et al. Corticosterone impairs insulin-stimulated translocation of GLUT4 in the rat hippocampus. Neuroendocrinology 2007; 85(2): 71–80.
497. Schacke H, Rehwinkel H, Asadullah K, Cato AC. Insight into the molecular mechanisms of glucocorticoid receptor action promotes identification of novel ligands with an improved therapeutic index. Exp Dermatol 2006; 15(8): 565–573.
498. van Raalte DH, Ouwens DM, Diamant M. Novel insights into glucocorticoidmediated diabetogenic effects: towards expansion of therapeutic options? Eur J Clin Invest 2009; 39(2): 81–93.
499. Vander Kooi BT, Onuma H, Oeser JK, et al. The glucose-6-phosphatase catalytic subunit gene promoter contains both positive and negative glucocorticoid response elements. Mol Endocrinol 2005; 19(12): 3001–3022.
500. Barseghian G, Levine R. Effect of corticosterone on insulin and glucagon secretion by the isolated perfused rat pancreas. Endocrinology 1980;106:547–52.
501. LeRoith D, Bondy C, Yakar S, Liu J-L, Butler A. The Somatomedin Hypothesis: 2001. Endocr Rev. 2001;22(1):53-74.
502. Liu JL. Does IGF-I stimulate pancreatic islet cell growth? Cell biochemistry and biophysics. 2007;48(2-3):115-125.
503. Bergerot I, Fabien N, Maguer V, Thivolet C. IGF-I protects NOD mice from insulitis and diabetes. Clin Exp Immunol. 1995;102(2):335-340.
504. Ohneda K, Ulshen MH, Fuller CR, D'Ercole AJ, Lund PK. Enhanced growth of small bowel in transgenic mice expressing human insulin-like growth factor I. Gastroenterology. 1997;112(2):444-454.
505. Robertson K, Lu Y, De Jesus K, Li B, Su Q, Lund PK, Liu JL. A general and islet cell-enriched overexpression of IGF-I results in normal islet cell growth, hypoglycemia, and significant resistance to experimental diabetes. Am J Physiol Endocrinol Metab. 2008;294(5):E928-938.

506. Dhar K, Banerjee S, Dhar G, Sengupta K, Banerjee SK. IGF-I induces WISP-2/CCN5 via multiple molecular cross-talks and is essential for mitogenic switch by IGF-I axis in estrogen receptor–positive breast tumor cells. Cancer research. 2007;67(4):1520-1526.
507. Delmolino LM, Stearns NA, Castellot JJ. COP-1, a member of the CCN family, is a heparin-induced growth arrest specific gene in vascular smooth muscle cells. Journal of Cellular Physiology. 2001;188(1):45-55.
508. Fritah A, Saucier C, De Wever O, Bracke M, Bièche I, Lidereau R, Gespach C, Drouot S, Redeuilh G, Sabbah M. Role of WISP-2/CCN5 in the Maintenance of a Differentiated and Noninvasive Phenotype in Human Breast Cancer Cells. Mol Cell Biol. 2008;28(3):1114-1123.
509. Holbourn KP, Malfois M, Acharya KR. First Structural Glimpse of CCN3 and CCN5 Multifunctional Signaling Regulators Elucidated by Small Angle X-ray Scattering. J Biol Chem. 2011;286(25):22243-22249.
510. Brigstock DR, Goldschmeding R, Katsube K-i, Lam SC-T, Lau LF, Lyons K, Naus C, Perbal B, Riser B, Takigawa M, Yeger H. Proposal for a unified CCN nomenclature. Molecular Pathology. 2003;56(2):127-128.
511. Holbourn KP, Acharya KR, Perbal B. The CCN family of proteins: structure–function relationships. Trends Biochem Sci. 2008;33(10):461-473.
512. Charrier A, Brigstock DR. Regulation of pancreatic function by connective tissue growth factor (CTGF, CCN2). Cytokine & growth factor reviews. 2012.
513. Henley KD, Gooding KA, Economides AN, Gannon M. Inactivation of the dual Bmp/Wnt inhibitor Sostdc1 enhances pancreatic islet function. Am J Physiol Endocrinol Metab. 2012;303(6):E752-761.
514. Guney MA, Petersen CP, Boustani A, Duncan MR, Gunasekaran U, Menon R, Warfield C, Grotendorst GR, Means AL, Economides AN, Gannon M. Connective tissue growth factor acts within both endothelial cells and beta cells to promote proliferation of developing beta cells. Proc Natl Acad Sci U S A. 2011;108(37):15242-15247.
515. Crawford LA, Guney MA, Oh YA, Deyoung RA, Valenzuela DM, Murphy AJ, Yancopoulos GD, Lyons KM, Brigstock DR, Economides A, Gannon M. Connective tissue growth factor (CTGF) inactivation leads to defects in islet cell lineage allocation and beta-cell proliferation during embryogenesis. Mol Endocrinol. 2009;23(3):324-336.
516. Xiong X, Wang X, Li B, Chowdhury S, Lu Y, Srikant CB, Ning N, Liu J-L. Pancreatic islet-specific overexpression of Reg3β protein induced the expression of pro-islet genes and protected mice against streptozotocin-induced diabetes. Am J Physiol Endocrinol Metab. 2011;300:E669-E680.
517. Getty-Kaushik L, Richard AM, Deeney JT, Krawczyk S, Shirihai O, Corkey BE. The CB1 antagonist rimonabant decreases insulin hypersecretion in rat pancreatic islets. Obesity (Silver Spring). 2009;17(10):1856-1860.
518. Lu Y, Herrera PL, Guo Y, Sun D, Tang Z, LeRoith D, Liu JL. Pancreatic-specific inactivation of IGF-I gene causes enlarged pancreatic islets and significant resistance to diabetes. Diabetes. 2004;53(12):3131-3141.
519. Lu Y, Ponton A, Okamoto H, Takasawa S, Herrera PL, Liu J-L. Activation of the Reg family genes by pancreatic-specific IGF-I gene deficiency and after

streptozotocin-induced diabetes in mouse pancreas. Am J Physiol Endocrinol Metab. 2006;291(1):E50-58.
520. Liu L, Liu JL, Srikant CB. Reg2 protects mouse insulinoma cells from streptozotocin-induced mitochondrial disruption and apoptosis. Growth Factors. 2010;28(5):370-378.
521. Terui Y, Furukawa Y, Kikuchi J, Saito M. Apoptosis during HL-60 cell differentiation is closely related to a G0/G1 cell cycle arrest. J Cell Physiol. 1995;164(1):74-84.
522. Russo JW, Castellot JJ. CCN5: biology and pathophysiology. Journal of cell communication and signaling. 2010;4(3):119-130.
523. Dhar G, Mehta S, Banerjee S, Gardner A, McCarty BM, Mathur SC, Campbell DR, Kambhampati S, Banerjee SK. Loss of WISP-2/CCN5 signaling in human pancreatic cancer: a potential mechanism for epithelial-mesenchymal-transition. Cancer Lett. 2007;254(1):63-70.
524. Cozar-Castellano I, Takane KK, Bottino R, Balamurugan AN, Stewart AF. Induction of b-cell proliferation and retinoblastoma protein phosphorylation in rat and human islets using adenovirus-mediated transfer of CDK4 and cyclin D1. Diabetes. 2004;53(1):149-159.
525. Fatrai S, Elghazi L, Balcazar N, Cras-Méneur C, Krits I, Kiyokawa H, Bernal-Mizrachi E. Akt Induces β-Cell Proliferation by Regulating Cyclin D1, Cyclin D2, and p21 Levels and Cyclin-Dependent Kinase-4 Activity. Diabetes. 2006;55(2):318-325.
526. Hammarstedt A, Hedjazifar S, Jenndahl L, Gogg S, Grünberg J, Gustafson B, Klimcakova E, Stich V, Langin D, Laakso M, Smith U. WISP2 regulates preadipocyte commitment and PPARγ activation by BMP4. Proc Natl Acad Sci USA. 2013;110(7):2563-2568.
527. Sabbah M, Prunier C, Ferrand N, Megalophonos V, Lambein K, De Wever O, Nazaret N, Lachuer J, Dumont S, Redeuilh G. CCN5, a novel transcriptional repressor of the transforming growth factor beta signaling pathway. Mol Cell Biol. 2011;31(7):1459-1469.
528. Wiesman KC, Wei L, Baughman C, Russo J, Gray MR, Castellot JJ. CCN5, a secreted protein, localizes to the nucleus. Journal of cell communication and signaling. 2010;4(2):91-98.
529. Zhang R, Averboukh L, Zhu W, Zhang H, Jo H, Dempsey PJ, Coffey RJ, Pardee AB, Liang P. Identification of rCop-1, a New Member of the CCN Protein Family, as a Negative Regulator for Cell Transformation. Mol Cell Biol. 1998;18(10):6131-6141.
530. Ohkawa Y, Ohmi Y, Tajima O, Yamauchi Y, Furukawa K, Furukawa K. Wisp2/CCN5 up-regulated in the central nervous system of GM3-only mice facilitates neurite formation in Neuro2a cells via integrin-Akt signaling. Biochem Biophys Res Commun. 2011;411(3):483-489.
531. Timpl R, Sasaki T, Kostka G, Chu M-L. Fibulins: a versatile family of extracellular matrix proteins. Nat Rev Mol Cell Biol. 2003;4(6):479-489.
532. Kok K, Geering B, Vanhaesebroeck B. Regulation of phosphoinositide 3-kinase expression in health and disease. Trends in Biochemical Sciences. 2009;34(3):115-127.

533. Ono F, Nakagawa T, Saito S, Owada Y, Sakagami H, Goto K, Suzuki M, Matsuno S, Kondo H. A Novel Class II Phosphoinositide 3-Kinase Predominantly Expressed in the Liver and Its Enhanced Expression during Liver Regeneration. J Biol Chem. 1998;273(13):7731-7736.
534. Grotendorst GR, Lau LF, Perbal B. CCN Proteins Are Distinct from and Should Not Be Considered Members of the Insulin-Like Growth Factor-Binding Protein Superfamily. Endocrinology. 2000;141(6):2254-2256.
535. Rachfal AW, Brigstock DR. Structural and functional properties of CCN proteins. Vitamins and hormones. 2005;70:69-103.
536. Lim HW, Lee JE, Shin SJ, Lee YE, Oh SH, Park JY, Seong JK, Park JS. Identification of differentially expressed mRNA during pancreas regeneration of rat by mRNA differential display. Biochem Biophys Res Commun. 2002;299(5):806-812.
537. Ferrand N, Stragier E, Redeuilh G, Sabbah M. Glucocorticoids induce CCN5/WISP-2 expression and attenuate invasion in oestrogen receptor-negative human breast cancer cells. The Biochemical journal. 2012;447(1):71-79.
538. Banerjee S, Saxena N, Sengupta K, Tawfik O, Mayo MS, Banerjee SK. WISP-2 gene in human breast cancer: estrogen and progesterone inducible expression and regulation of tumor cell proliferation. Neoplasia. 2003;5(1):63-73.
539. Fritah A, Redeuilh G, Sabbah M. Molecular cloning and characterization of the human WISP-2/CCN5 gene promoter reveal its upregulation by oestrogens. J Endocrinol. 2006;191(3):613-624.
540. Banerjee S, Sengupta K, Saxena NK, Dhar K, Banerjee SK. Epidermal Growth Factor Induces WISP-2/CCN5 Expression in Estrogen Receptor-α-Positive Breast Tumor Cells through Multiple Molecular Cross-talks. Molecular Cancer Research. 2005;3(3):151-162.
541. Sengupta K, Banerjee S, Dhar K, Saxena NK, Mehta S, Campbell DR, Banerjee SK. WISP-2/CCN5 Is Involved As a Novel Signaling Intermediate in Phorbol Ester-Protein Kinase Cα-Mediated Breast Tumor Cell Proliferation. Biochemistry. 2006;45(35):10698-10709.
542. Pennica D, Swanson TA, Welsh JW, Roy MA, Lawrence DA, Lee J, Brush J, Taneyhill LA, Deuel B, Lew M, Watanabe C, Cohen RL, Melhem MF, Finley GG, Quirke P, Goddard AD, Hillan KJ, Gurney AL, Botstein D, Levine AJ. WISP genes are members of the connective tissue growth factor family that are up-regulated in Wnt-1-transformed cells and aberrantly expressed in human colon tumors. Proc Natl Acad Sci. 1998;95(25):14717-14722.
543. Jin T, George Fantus I, Sun J. Wnt and beyond Wnt: Multiple mechanisms control the transcriptional property of b-catenin. Cell Signal. 2008;20(10):1697-1704.
544. Ip W, Chiang YT, Jin T. The involvement of the wnt signaling pathway and TCF7L2 in diabetes mellitus: The current understanding, dispute, and perspective. Cell & bioscience. 2012;2(1):28.
545. Lake AC, Bialik A, Walsh K, Castellot Jr JJ. CCN5 Is a Growth Arrest-Specific Gene That Regulates Smooth Muscle Cell Proliferation and Motility. Am J Pathol. 2003;162(1):219-231.

551. Brereton PS, van Driel RR, Suhaimi FbH, Koyama K, Dilley R, and Krozowski Z. Light and Electron Microscopy Localization of the 11{beta}-Hydroxysteroid Dehydrogenase Type I Enzyme in the Rat. Endocrinology 142: 1644-1651, 2001.
552. Davani B, Khan A, Hult M, Martensson E, Okret S, Efendic S, Jornvall H, and Oppermann UCT. Type 1 11beta -Hydroxysteroid Dehydrogenase Mediates Glucocorticoid Activation and Insulin Release in Pancreatic Islets. J Biol Chem 275: 34841-34844, 2000.
553. Duplomb L, Lee Y, Wang M-Y, Park BH, Takaishi K, Agarwal AK, and Unger RH. Increased expression and activity of 11[beta]-HSD-1 in diabetic islets and prevention with troglitazone. Biochemical and Biophysical Research Communications 313: 594-599, 2004.
554. Escribano O, Guillen C, Nevado C, Gomez-Hernandez A, Kahn CR, and Benito M. Beta-Cell hyperplasia induced by hepatic insulin resistance: role of a liver-pancreas endocrine axis through insulin receptor A isoform. Diabetes 58: 820-828, 2009.
555. Getty-Kaushik L, Richard AM, Deeney JT, Krawczyk S, Shirihai O, and Corkey BE. The CB1 antagonist rimonabant decreases insulin hypersecretion in rat pancreatic islets. Obesity (Silver Spring) 17: 1856-1860, 2009.
556. Gygi SP, Rochon Y, Franza BR, and Aebersold R. Correlation between protein and mRNA abundance in yeast. Mol Cell Biol 19: 1720-1730, 1999.
557. Hamamdzic D, Duzic E, Sherlock JD, and Lanier SM. Regulation of alpha 2-adrenergic receptor expression and signaling in pancreatic beta-cells. Am J Physiol Endocrinol Metab 269: E162-171, 1995.
558. Hansson A, Hehenberger K, and Thoren M. Long-term treatment of Swiss 3T3 fibroblasts with dexamethasone attenuates MAP kinase activation induced by insulin-like growth factor- I (IGF-I). Cell Biochem Funct 14: 121-129, 1996.
559. Hazelgrove KB, Flynn RS, Qiao L-Y, Grider JR, and Kuemmerle JF. Endogenous IGF-I and avb3 integrin ligands regulate increased smooth muscle growth in TNBS-induced colitis. Am J Physiol Gastrointest Liver Physiol 296: G1230-1237, 2009.
560. Hill DJ, Sedran RJ, Brenner SL, and McDonald TJ. IGF-I has a dual effect on insulin release from isolated, perifused adult rat islets of Langerhans. J Endocrinol 153: 15-25., 1997.
561. Huang Y, Li X, Lin H, Chu Y, Chen B, Lian Q, and Ge R-S. Regulation of 11[beta]-hydroxysteroid dehydrogenase 1 and 2 by IGF-1 in mice. Biochemical and Biophysical Research Communications 391: 1752-1756, 2010.
562. Hughes KA, Webster SP, and Walker BR. 11-Beta-hydroxysteroid dehydrogenase type 1 (11beta-HSD1) inhibitors in Type 2 diabetes mellitus and obesity. Expert Opinion on Investigational Drugs 17: 481-496, 2008.
563. Babic AM, Chen C-C, Lau LF. Fisp12/mouse connective tissue growth factor mediatesendothelial cell adhesion and migration through integrin αvβ3, promotes endothelial cell survival, and induces angiogenesis in vivo. Mol Cell Biol1999;19:2958–66.
564. Jeong I-K, Oh S-H, Kim B-J, Chung J-H, Min Y-K, Lee M-S, Lee M-K, and Kim K-W. The effects of dexamethasone on insulin release and biosynthesis are dependent on the dose and duration of treatment. Diabetes Research and Clinical Practice 51: 163-171, 2001.

565. Kok K, Geering B, and Vanhaesebroeck B. Regulation of phosphoinositide 3-kinase expression in health and disease. Trends in Biochemical Sciences 34: 115-127, 2009.
566. Kotelevtsev Y, Holmes MC, Burchell A, Houston PM, Schmoll D, Jamieson P, Best R, Brown R, Edwards CRW, Seckl JR, and Mullins JJ. 11beta-Hydroxysteroid dehydrogenase type 1 knockout mice show attenuated glucocorticoid-inducible responses and resist hyperglycemia on obesity or stress. Proc Natl Acad Sci U S A 94: 14924-14929, 1997.
567. Lambillotte C, Gilon P, and Henquin JC. Direct glucocorticoid inhibition of insulin secretion. An in vitro study of dexamethasone effects in mouse islets. J Clin Invest 99: 414-423, 1997.
568. Liu J-L, Srikant CB, and Chowdhury S. Is 11β-HSD1 expressed in islet β-cells and regulated by corticotropin-releasing hormone? Proceedings of the National Academy of Sciences 108: E1390, 2011.
569. Babic AM, Kireeva ML, Kolesnikova TV, Lau LF. CYR61, product of a growth factorinducibleimmediate-early gene, promotes angiogenesis and tumor growth. Proc Natl Acad Sci USA 1998;95:6355–60.
570. Cool DR, Loh YP. Carboxypeptidase E is a sorting receptor for prohormones: binding and kinetic studies. Mol Cell Endocrinol 1998; 139:7–13.
571. Irminger JC, Verchere CB, Meyer K, Halban PA. Proinsulin targeting to the regulated pathway is not impaired in carboxypeptidase E-deficient Cpefat/Cpefat mice. J Biol Chem 1997;272:27532– 27534.
572. Man TY, Michailidou Z, Gokcel A, Ramage L, Chapman KE, Kenyon CJ, Seckl JR, and Morton NM. Dietary manipulation reveals an unexpected inverse relationship between fat mass and adipose 11{beta}-hydroxysteroid dehydrogenase type 1. American journal of physiology - Endocrinology and Metabolism 300: E1076-1084, 2011.
573. Moore JS, Monson JP, Kaltsas G, Putignano P, Wood PJ, Sheppard MC, Besser GM, Taylor NF, and Stewart PM. Modulation of 11{beta}-Hydroxysteroid Dehydrogenase Isozymes by Growth Hormone and Insulin-Like Growth Factor: In Vivo and In Vitro Studies. J Clin Endocrinol Metab 84: 4172-4177, 1999.
574. Morgan SA, Sherlock M, Gathercole LL, Lavery GG, Lenaghan C, Bujalska IJ, Laber D, Yu A, Convey G, Mayers R, Hegyi K, Sethi JK, Stewart PM, Smith DM, and Tomlinson JW. 11β-Hydroxysteroid Dehydrogenase Type 1 Regulates Glucocorticoid-Induced Insulin Resistance in Skeletal Muscle. Diabetes 58: 2506-2515, 2009.
575. Morita J, Hakuno F, Hizuka N, Takahashi S-i, and Takano K. Growth Hormone (GH) or Insulin-like Growth Factor (IGF)-I Represses 11beta-Hydroxysteroid Dehydrogenase Type 1 (HSD1) mRNA Expression in 3T3-L1 Cells and Its Activity in Their Homogenates. Endocrine Journal 56: 561-570, 2009.
576. Morton NM. Obesity and corticosteroids: 11[beta]-Hydroxysteroid type 1 as a cause and therapeutic target in metabolic disease. Molecular and Cellular Endocrinology 316: 154-164, 2010.
577. Nordentoft I, Jeppesen PB, Hong J, Abudula R, and Hermansen K. Isosteviol increases insulin sensitivity and changes gene expression of key insulin regulatory

genes and transcription factors in islets of the diabetic KKAy mouse. Diabetes, Obesity and Metabolism 10: 939-949, 2008.
578. Okada T, Liew CW, Hu J, Hinault C, Michael MD, Krutzfeldt J, Yin C, Holzenberger M, Stoffel M, and Kulkarni RN. Insulin receptors in {beta}-cells are critical for islet compensatory growth response to insulin resistance. Proc Natl Acad Sci U S A 104: 8977-8982, 2007.
579. Ono F, Nakagawa T, Saito S, Owada Y, Sakagami H, Goto K, Suzuki M, Matsuno S, and Kondo H. A Novel Class II Phosphoinositide 3-Kinase Predominantly Expressed in the Liver and Its Enhanced Expression during Liver Regeneration. J Biol Chem 273: 7731-7736, 1998.
580. Ortsater H, Alberts P, Warpman U, Engblom LOM, Abrahmsen L, and Bergsten P. Regulation of 11beta-hydroxysteroid dehydrogenase type 1 and glucose-stimulated insulin secretion in pancreatic islets of Langerhans. Diabetes/Metabolism Research and Reviews 21: 359-366, 2005.
581. Rajpathak SN, Gunter MJ, Wylie-Rosett J, Ho GYF, Kaplan RC, Mazumdar R, Rohan TE, and Strickler HD. The role of insulin-like growth factor-I and its binding proteins in glucose homeostasis and type 2 diabetes. Diabetes-Metab Res Rev 25: 3-12, 2009.
582. Ranta F, Avram D, Berchtold S, Dufer M, Drews G, Lang F, and Ullrich S. Dexamethasone Induces Cell Death in Insulin-Secreting Cells, an Effect Reversed by Exendin-4. Diabetes 55: 1380-1390, 2006.
583. Varlamov O, Fricker LD, Furukawa H, et al. Beta-cell lines derived from transgenic Cpe(fat)/Cpe(fat) mice are defective in carboxypeptidase E and proinsulin processing. Endocrinology 1997;138:4883– 4892.
584. Samani AA, Yakar S, LeRoith D, and Brodt P. The Role of the IGF System in Cancer Growth and Metastasis: Overview and Recent Insights. Endocr Rev 28: 20-47, 2007.
585. Schmid J, Ludwig B, Schally AV, Steffen A, Ziegler CG, Block NL, Koutmani Y, Brendel MD, Karalis KP, Simeonovic CJ, Licinio J, Ehrhart-Bornstein M, and Bornstein SR. Modulation of pancreatic islets-stress axis by hypothalamic releasing hormones and 11beta-hydroxysteroid dehydrogenase. Proc Natl Acad Sci U S A 108: 13722-13727, 2011.
586. Stulnig TM, and Waldhäusl W. 11ß-Hydroxysteroid dehydrogenase Type 1 in obesity and Type 2 diabetes. Diabetologia 47: 1-11, 2004.
587. Swali A, Walker EA, Lavery GG, Tomlinson JW, and Stewart PM. 11beta-Hydroxysteroid dehydrogenase type 1 regulates insulin and glucagon secretion in pancreatic islets. Diabetologia 51: 2003-2011, 2008.
588. Tahrani AA, Bailey CJ, Del Prato S, and Barnett AH. Management of type 2 diabetes: new and future developments in treatment. Lancet 378: 182-197, 2011.
589. Timpl R, Sasaki T, Kostka G, and Chu M-L. Fibulins: a versatile family of extracellular matrix proteins. Nat Rev Mol Cell Biol 4: 479-489, 2003.
590. Tomlinson JW, Moore J, Cooper MS, Bujalska I, Shahmanesh M, Burt C, Strain A, Hewison M, and Stewart PM. Regulation of Expression of 11{beta}-Hydroxysteroid Dehydrogenase Type 1 in Adipose Tissue: Tissue-Specific Induction by Cytokines. Endocrinology 142: 1982-1989, 2001.

591. Tomlinson JW, Walker EA, Bujalska IJ, Draper N, Lavery GG, Cooper MS, Hewison M, and Stewart PM. 11{beta}-Hydroxysteroid Dehydrogenase Type 1: A Tissue-Specific Regulator of Glucocorticoid Response. Endocr Rev 25: 831-866, 2004.
592. Turban S, Liu X, Ramage L, Webster SP, Walker BR, Dunbar DR, Mullins JJ, Seckl JR, and Morton NM. Optimal elevation of beta-cell 11beta-hydroxysteroid dehydrogenase type 1 is a compensatory mechanism that prevents high-fat diet-induced beta-cell failure. Diabetes 61: 642-652, 2012.
593. Unger RH, and Cherrington AD. Glucagonocentric restructuring of diabetes: a pathophysiologic and therapeutic makeover. The Journal of Clinical Investigation 122: 4-12, 2012.
594. Voice MW, Seckl JR, Edwards CR, and Chapman KE. 11 beta-hydroxysteroid dehydrogenase type 1 expression in 2S FAZA hepatoma cells is hormonally regulated: a model system for the study of hepatic glucocorticoid metabolism. Biochem J 317: 621-625, 1996.
595. Orci, L. Macro- and micro-domains in the endocrine pancreas. *Diabet*es. 1982; 31, 538–565.
596. Zhang X, Yong W, Lv J, Zhu Y, Zhang J, Chen F, Zhang R, Yang T, Sun Y, and Han X. Inhibition of FoxO1 (Forkhead Box O1) Protects Pancreatic {beta} Cells Against Dexamethasone-induced Dysfunction. Endocrinology 150: 4065-4073, 2009.
597. Zhao AZ, Zhao H, Teague J, Fujimoto W, and Beavo JA. Attenuation of insulin secretion by insulin-like growth factor 1 is mediated through activation of phosphodiesterase 3B. Proc Natl Acad Sci U S A 94: 3223-3228, 1997.
598. Gremlich S, Roduit R, Thorens B. Dexamethasone induces posttranslational degradation of GLUT2 and inhibition of insulin secretion in isolated pancreatic beta cells comparison with the effects of fatty acids. J Biol Chem 272: 3216–3222, 1997.
599. Dosiou C, Giudice LC. Natural killer cells in pregnancy and recurrent pregnancy loss: endocrine and immunologic perspectives. Endocr Rev 26: 44–62, 2005.
600. Terui Y, Furukawa Y, Kikuchi J, Saito M. Apoptosis during HL-60 cell differentiation is closely related to aG0/G1 cell cycle arrest. J Cell Physiol. 1995;164:74–84.
601. Schwanhäusser B1, Busse D, Li N, Dittmar G, Schuchhardt J, Wolf J, Chen W, Selbach M. Global quantification of mammalian gene expression control. Nature. 2011 473(7347):337-42.
602. Koizumi, M., & Yada, T. (2008). Sub-chronic stimulation of glucocorticoid receptor impairs and mineralocorticoid receptor protects cytosolic Ca2+ responses to glucose in pancreatic beta-cells. J Endocrinol Vol. 197, No. 2, pp. 221-229
603. Linssen, M.M., van Raalte, D.H., Toonen, E.J., Alkema, W., van der Zon, G.C., Dokter, W.H., Diamant, M., Guigas, B., & Ouwens, D.M. (2011). Prednisolone-induced beta cell dysfunction is associated with impaired endoplasmic reticulum homeostasis in INS-1E cells. Cell Signal Vol. 23, No. 11, pp. 1708-1715
604. 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: 1629-42

605. Chen CC, Lau LF**.** Functions and mechanisms of action of CCN matricellular proteins. *Int J Biochem Cell Biol*. 2009; 41(4):771-83.
606. Orci, L. The insulin factory: a tour of the plant surroundings and a visit to the assembly line. The Minkowski lecture 1973 revisited. *Diabetologia*. 1985; 28, 528–546.
607. Guo Y, Yang K, Harwalkar J, Nye JM, Mason DR, Garrett MD, Hitomi M, Stacey DW: Phosphorylation of cyclin D1 at Thr 286 during S[thinsp]phase leads to its proteasomal degradation and allows efficient DNA synthesis. Oncogene 2005;24:2599-2612
608. Guo X, Li W, Wang Q, Yang H-S: AKT Activation by Pdcd4 Knockdown Up-Regulates Cyclin D1 Expression and Promotes Cell Proliferation. Genes & Cancer 2011;2:818-828
609. Kulkarni RN, Mizrachi E-B, Ocana AG, Stewart AF: Human β-Cell Proliferation and Intracellular Signaling. Diabetes 2012;61:2205-2213
610. Lawlor MA, Alessi DR: PKB/Akt: a key mediator of cell proliferation, survival and insulin responses? Journal of Cell Science 2001;114:2903-2910
611. Cagnol S, Chambard J-C: ERK and cell death: Mechanisms of ERK-induced cell death– apoptosis, autophagy and senescence. FEBS Journal 2010;277:2-21
612. Komiya, Y., & Habas, R. Wnt signal transduction pathways. *Organogenesis*, 2008; *4*(2), 68–75.
613. Saltiel, A. R.; Kahn, C. R. The molecular mechanism of Insulin signalling and the regulation of glucose and lipid metabolism. *Joslin's Diabetes Mellitus* 2012; Chap 9:145-168.
614. Orci, L., Ravazzola, M., Amherdt, M., Madsen, O., Perrelet, A., Vassalli, J. D., and Anderson, R. G. Conversion of proinsulin to insulin occurs coordinately with acidification of maturing secretory vesicles. *J. Cell Biol.* 1986;103, 2273–2281.
615. Helen E. Thomas, Mark D. McKenzie, Eveline Angstetra, Peter D. Campbell, Thomas W. Kay. Beta Cell Apoptosis in diabetes. Apoptosis 2009; 14: 1389-1404
616. Adam Denley, Leah J. Cosgrove, Grant W. Booker, John Wallace, Briony Forbes. Molecular interactions of the IGF system. *Cytokines and Growth Factor Reviews* 2005; 16: 421-439.
617. Fataccioli V,AbergelV,Wingertsmann L,Neuville P, Spitz E,Adnot S, et al. Stimulationof angiogenesis by cyr61 gene: a new therapeutic candidate. Hum Gene Ther 2002;13:1461–70.
618. Leu S-J, Lam SCT, Lau LF. Proangiogenic activities of CYR61 (CCN1) mediated through integrins αv β3 and α6 β1 in human umbilical vein endothelial cells. J Biol Chem 2002;277:46248–55.
619. Lin CG, Leu SJ, Chen N, Tebeau CM, Lin SX, Yeung CY, et al. CCN3 (NOV) is a novel angiogenic regulator of the CCN protein family. J Biol Chem 2003;278:24200–8.
620. Ivkovic S, Yoon BS, Popoff SN, Safadi FF, Libuda DE, Stephenson RC, et al. Connective tissue growth factor coordinates chondrogenesis and angiogenesis during skeletal development. Development 2003;130:2779–91.
621. Rydziel S, Stadmeyer L, Zanotti S, Durant D, Smerdel-Ramoya A, Canalis E. Nephroblastomaoverexpressed (Nov) inhibits osteoblastogenesis and causes osteopenia. J Biol Chem 2007;282:19762–72.

622. Smerdel-Ramoya A, Zanotti S, Stadmeyer L, Durant D, Canalis E. Skeletal overexpression of connective tissue growth factor (ctgf) impairs bone formation and causes osteopenia. Endocrinology 2008;149:4374–81.
623. Abreu JG, Ketpura NI, Reversade B, De Robertis EM. Connective-tissue growth factor (CTGF) modulates cell signalling by BMP and TGF-β. Nat Cell Biol 2002;4:599–604.
624. Kubota S, Takigawa M. Role of CCN2/CTGF/Hcs24 in bone growth. Int Rev Cytol 2007b;257:1–41.
625. Chen CC, Young JL, Monzon RI, Chen N, Todorovic V, Lau LF. Cytotoxicity of TNFalpha is regulated by integrin-mediated matrix signaling. EMBO J 2007;26:1257–67.
626. Hashimoto Y, Shindo-Okada N, Tani M, Nagamachi Y, Takeuchi K, Shiroishi T, et al. Expression of the Elm1 gene, a nove gene of the CCN (connective tissue growth .factor, Cyr61/Cef10, and neuroblastoma overexpressed gene) family, suppresses in vivo tumor growth and metastasis of K- 1735 murine melanoma cells. J Exp Med 1998;187:289–96.
627. Zhang R, Averboukh L, Zhu W, Zhang H, Jo H, Dempsey PJ, et al. Identification of Cop-1, a new member of the CCN protein family, as a negative regulator for cell transformation. Mol Cell Biol 1998;18:6131–41.
628. Xie D, Miller CW, O'Kelly J, Nakachi K, Sakashita A, Said JW, et al. Breast cancer. Cyr61 is overexpressed, estrogen-inducible, and associated with more advanced disease. J Biol Chem 2001;276:14187–94.
629. Tsai MS, Bogart DF, Castaneda JM, Li P, Lupu R. Cyr61 promotes breast tumorigenesis and cancer progression. Oncogene 2002;21:8178–85.
630. Gery S, Xie D, Yin D, Gabra H,Miller C,Wang H, et al. Ovarian carcinomas: CCN genesare aberrantly expressed and CCN1 promotes proliferation of these cells. Clin Cancer Res 2005;11:7243–54.
631. Aikawa T, Gunn J, Spong SM, Klaus SJ, Korc M. Connective tissue growth factorspecific antibody attenuates tumor growth, metastasis, and angiogenesis in an orthotopic mouse model of pancreatic cancer.Mol Cancer Ther 2006;5:1108–16.
632. Dornhofer N, Spong S, Bennewith K, Salim A, Klaus S, Kambham N, et al. Connective tissue growth factor-specific monoclonal antibody therapy inhibits pancreatic tumor growth and metastasis. Cancer Res 2006;66:5816–27.
633. Shimo T, Kubota S, Yoshioka N, Ibaragi S, Isowa S, Eguchi T, et al. Pathogenic role of connective tissue growth factor (CTGF/CCN2) in osteolytic metastasis of breast cancer. J Bone Miner Res 2006;21:1045–59.
634. Yang F, Tuxhorn JA, Ressler SJ, McAlhany SJ, Dang TD, Rowley DR. Stromal expression of connective tissue growth factor promotes angiogenesis and prostate cancer tumorigenesis. Cancer Res 2005;65:8887–95.
635. Deng YZ, Chen PP, Wang Y, Yin D, Koeffler HP, Li B, et al. Connective tissue growth factor is overexpressed in esophageal squamous cell carcinoma and promotes tumorigenicity through beta-catenin-T-cell factor/Lef signaling. J Biol Chem 2007;282:36571–81.
636. Johansson, K. A., et al. . "Temporal control of neurogenin3 activity in pancreas progenitors reveals competence windows for the generation of different endocrine cell types." Dev Cell 2007. 12(3): 457-465.

637. Mahita Kadmiel, John A. Cidlowski. Glucocorticoid receptor signaling in health and disease. Trends in Pharmacological Science; (2013). 34(9): 518-530
638. Mo FE, Muntean AG, Chen CC, Stolz DB, Watkins SC, Lau LF. CYR61 (CCN1) is essential for placental development and vascular integrity. Mol Cell Biol2002;22:8709–20.
639. Mo F-E, Lau LF. The matricellular protein CCN1 is essential for cardiac development. Circ Res 2006;99:961–9.
640. Heath E, Tahri D, Andermarcher E, Schofield P, Fleming S, Boulter CA.Abnormal skeletal and cardiac development, cardiomyopathy, muscle atrophy and cataracts in mice with a targeted disruption of theNov (Ccn3) gene.BMCDev Biol 2008;8:18.

IGF-I Stimulates CCN5/WISP2 Gene Expression in Pancreatic β-Cells, Which Promotes Cell Proliferation and Survival Against Streptozotocin

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IGF-I is normally produced from hepatocytes and other sources, stimulates protein synthesis, cell survival, and proliferation through receptor-mediated activation of phosphatidylinositol 3-kinase and MAPK, and targets specific molecules within the pancreatic islet cells. The current study was designed to identify novel targets that may mediate its pro-islet actions. Whole-genome cDNA microarray analysis in IGF-I–overexpressing islets identified 82 genes specifically up- or down-regulated. Prominent among them was CCN5/WISP2 whose expression was increased 3- and 2-fold at the mRNA and protein levels. Dual-labeled immunofluorescence revealed that CCN5 expression was low in the β-cells of wild-type islets but was significantly induced in response to IGF-I overexpression. In vitro treatment of mouse islets with IGF-I increased both CCN5 mRNA and protein levels significantly. To define the role of CCN5 in islet cell biology, we stably overexpressed its cDNA in insulinoma MIN6 cells and detected a 2-fold increase in the proliferation of MIN6-CCN5 compared with that in control cells, which correlated with significant elevations in the levels of cyclin D1 and the phosphorylation of Akt and Erk2. Moreover, MIN6-CCN5 cells were found to be resistant to streptozotocin-induced cell death. Using confocal microscopy and subcellular fractionation, we found that overexpressed CCN5 exhibited cytoplasmic accumulation upon stimulation by high glucose. Our results indicate that CCN5, which is minimally expressed in islet β-cells, is strongly and directly induced by IGF-I. CCN5 overexpression stimulates the proliferation of insulinoma cells, activates Akt kinase, and inhibits streptozotocin-induced apoptosis, suggesting that increased CCN5 expression contributes to IGF-I–stimulated islet cell growth and/or survival. (***Endocrinology* 155: 1629–1642, 2014**)

IGF-I is normally produced from hepatocytes and other sources including the pancreas. Acting through its receptor, IGF-IR, IGF-I promotes embryonic development, postnatal growth, and maturation of major organ systems (1). As a potent mitogenic peptide, it is known to act in endocrine as well as autocrine and paracrine manners. Within the pancreatic islets, IGF-I stimulates cell proliferation in the presence of high glucose levels, inhibits insulin secretion, and prevents cell apoptosis (2). IGF-I administration in vivo prevents Fas-mediated autoimmune β-cell destruction and delays the onset of diabetes in NOD mice (3). IGF-I–treated animals have a significantly higher ratio of intact islets and an overall higher β-cell mass relative to those of untreated mice. To test the effects of increased circulating IGF-I on islet cell growth and glucose homeostasis, an IGF-I transgene was expressed under the

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Abbreviations: BrdU, 5-bromo-2′-deoxyuridine; CCN, connective tissue growth/cysteine-rich 61/nephroblastoma overexpressed; DAPI, 4′,6′-diamino-2-phenylindole; ECM, extracellular matrix; EGF, epidermal growth factor; GLUT2, glucose transporter 2; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium; rh, recombinant human; RNAi, RNA interference; PI3K, phosphatidylinositol 3-kinase; siRNA, small interfering RNA; USF-2, upstream stimulatory factor 2; WISP, Wnt1 inducible signaling pathway.

metallothionein I promoter (4). We demonstrated previously that the IGF-I overexpression, although widespread, was highly concentrated in the β-cells of the pancreas (5). The transgenic MT-IGF mice were resistant to streptozotocin-induced diabetes and displayed diminished hyperglycemia and abolished weight loss and mortality. Although IGF-I is known to regulate protein synthesis, cell survival, and proliferation through receptor-mediated activation of phosphatidylinositol 3-kinase (PI3K) and MAPK, the specific targets within the islet cells have not been systematically screened. To explore the novel targets of IGF-I action, we performed a whole-genome cDNA microarray analysis in isolated islets of MT-IGF vs wild-type mice and found 82 genes specifically up- or down-regulated. Prominent among them, connective tissue growth/cysteine-rich 61/nephroblastoma overexpressed (CCN) 5/Wnt1 inducible signaling pathway (WISP)2 is a secreted protein that was previously shown to be induced by IGF-I in breast cancer cells and either stimulates or inhibits cell proliferation in different systems (6–8). CCN (connective tissue growth factor/cysteine-rich 61/nephroblastoma overexpressed) or WISP (Wnt1 inducible signaling pathway) represents a family of 6 proteins that regulate cell adhesion and extracellular matrix (ECM) remodeling, skeletal development and chondrogenesis, angiogenesis and wound repair, proliferation, and tumorigenesis (9–11). Although the role of another isoform, CCN2/CTGF, in the early development of pancreatic islets has been well established (12–15), CCN5 was not known either to be expressed in the islets or to regulate the islet function. This study was designed to establish CCN5/WISP2 as a novel isoform normally expressed in adult islets that is directly induced by IGF-I and that plays important roles in pancreatic islet proliferation and/or protection.

Materials and Methods

MT-IGF mice and pancreatic islet isolation

Mice with germline integration of a human IGF-I cDNA driven by mouse metallothionein 1 promoter (MT-IGF) and wild-type littermates on a mixed C57BL/6 background were maintained in 12:12-hour dark-light cycles with free access to food and water. As reported previously, a zinc supplement in food was not required for the transgene induction; instead of being expressed primarily in the liver and kidney, 31- and 344-fold higher levels of IGF-I mRNA and peptide content were detected in the pancreas (mostly in islet β-cells) vs the liver (5). Thus, MT-IGF mice are a model of islet-enriched overexpression of IGF-I, with minor increases occurring in the liver and kidney. All animal handling procedures were approved by the Research Institute Animal Care Committee of McGill University Health Centre. Pancreatic islets were isolated by collagenase digestion and allowed to recover for 3 hours in DMEM containing 11 mM glucose and 10% fetal bovine serum, 10 mM HEPES, 2 mM glutamine, and 1 mM sodium pyruvate as reported previously (16). They were hand-picked and pooled for RNA or protein isolation.

cDNA microarray and real-time PCR

Male MT-IGF mice and wild-type littermates, 3 to 4 months old, were used to isolate pancreatic islets. Total RNA was prepared from pooled islets from each mouse using QIAGEN RNeasy Plus. The RNA quality and integrity were analyzed using an Agilent 2100 Bioanalyzer, and its concentration was determined using a NanoDrop 2000 spectrophotometer. The gene expression profile was determined using Illumina Whole-Genome Expression BeadChips (MouseRef-8 V2) by McGill University and the Genome Quebec Innovation Centre. The lumi and EB (Wright and Simon) algorithm was applied to the data using FlexArray version 1.4 software to identify genes that were significantly induced or repressed in transgenic islets. The list of transcripts was uploaded to DAVID (http://david.abcc.ncifcrf.gov), and cell compartment annotation and functional annotation chart were obtained and manually adjusted, including marking the duplicates in Table 1. Real-time PCR was performed using a QuantiFast SYBR Green RT-PCR Kit (QIAGEN) with LightCycler systems (Roche Applied Science); primers were purchased from QIAGEN. The amount of target RNA was determined by a comparison to β-actin as an internal control, and results are expressed as means ± SE.

Western blot analysis

Freshly isolated islets from 3- to 4-month-old male mice were sonicated in 150 to 200 μL of lysis buffer (containing 150 mM NaCl, 10 mM Tris [pH 7.4], 1 mM EDTA, 1% Triton X-100, 0.1% SDS, 100 mM NaF, 10 mM sodium pyrophosphate, 10 mM sodium orthovanadate, and 2 mM phenylmethylsulfonylfluoride) supplemented with a protease inhibitor tablet (Roche Diagnostics). The cell extract was diluted by 1 to 1.5 volumes of Laemmli loading buffer (Bio-Rad Laboratories) containing 5% β-mercaptoethanol (Sigma-Aldrich) and boiled for 5 minutes before loading onto SDS-PAGE gels. Western blotting was performed using rabbit polyclonal anti-CCN5/WISP2 (ab38317; Abcam), mouse monoclonal anti-hemagglutinin (HA) (G036; ABM), rabbit polyclonal cyclin D1 (sc753; Santa Cruz Biotechnology), phospho-Akt at Ser-473 and total Akt (4058 and 9272; Cell Signaling Technology), phospho-Erk1/2 and total Erk1/2 (9101 and 9102; Cell Signaling Technology), and cleaved and total caspase 3 antibodies (9661and 9662; Cell Signaling Technology), β-actin (MM-0164-P; Medimabs), and upstream stimulatory factor 2 (USF-2) (sc-862; Santa Cruz Biotechnology).

In vitro direct stimulation of primary islets by IGF-I

Freshly isolated pancreatic islets pooled from 3 wild-type male mice were allowed to recover overnight in culture medium containing 11 mM glucose and 10% fetal bovine serum (17). The islets were randomly distributed in batches of 20 into 24-well plates with 3 replicates for each condition, cultured for 6 to 24 hours in the same medium but containing 1% serum with or without 10^{-8} M recombinant human (rh) IGF-I (LONG R3, I1271; Sigma-Aldrich). Total RNA was extracted for the measurement of CCN5 mRNA using real-time PCR. To analyze changes in CCN5 protein, 75 isolated islets in 12-well plates were

Table 1. Effects of IGF-I Overexpression on Pancreatic Islet Gene Expression by cDNA Microarray

Gene Symbol	Fold Change	*P* Value	Common Name
Extracellular matrix			
ADAMTS2	2.23	.007	A disintegrin and metalloproteinase with thrombospondin repeats; procollagen N-proteinase
BGN	1.96	.003	Biglycan
COL14A1	2.14	.000	Collagen, type XIV, α 1
COL16A1	2.29	.006	Collagen, type XVI, α 1
COL20A1	2.21	.001	Collagen, type XX, α 1
COL3A1	2.35	.004	Collagen, type III, α 1
COL8A1	1.91	.009	Collagen, type VIII, α 1
DCN	2.47	.008	Decorin
ELN	1.98	.002	Elastin
FBLN2	3.24	.008	Fibulin 2
MMP2	3.24	.009	Matrix metallopeptidase 2
OLFM4	1.59	.004	Olfactomedin 4
SPARCL1	1.62	.004	SPARC-like1 (mast9, hevin)
TGM2	1.93	.005	Transglutaminase 2, C polypeptide
THSD4	1.53	.007	Thrombospondin, type I, domain containing 4
VWF	3.37	.001	von Willebrand factor homolog
Cell morphogenesis			
GALR2	0.60	.009	Galanin receptor 2
GAS6	1.53	.009	Growth arrest specific 6
HTRA1	1.73	.007	HTRA serine peptidase 1
IGFBP4	2.09	.009	Insulin-like growth factor binding protein 4
IGFBP5	1.76	.006	Insulin-like growth factor binding protein 5
NRN1	2.92	.008	Neuritin 1
RDX	1.82	.008	Radixin
WISP2/CCN5	2.70	.006	WNT1 inducible signaling pathway protein 2
Endoplasmic reticulum			
APH1A	0.69	.008	Anterior pharynx defective 1a homolog (*Caenorhabditis elegans*)
CYP1B1	2.36	.010	Cytochrome P450, family 1, subfamily B, polypeptide 1
HSD11B1	2.32	.009	11β-Hydroxysteroid dehydrogenase1 (11β-HSD1)
KDELR3	0.69	.008	KDEL (Lys-Asp-Glu-Leu) ER protein retention receptor 3
SCD2	1.66	.004	Stearoyl-coenzyme A desaturase 2
VWF	(Repeat)[a]		
Cell signal, proliferation, and transcription			
2310047A01RIK	1.70	.001	Zinc finger, CCHC domain containing 24
AXL	1.54	.008	AXL receptor tyrosine kinase
BATF	0.51	.007	Basic leucine zipper transcription factor, ATF-like
EFS	2.60	.003	Embryonal Fyn-associated substrate, Sin, SH3 adaptor protein
ERRFI1	1.72	.003	ERBB receptor feedback inhibitor 1
GDF10	2.81	.005	Growth differentiation factor 10
HAVCR2	1.70	.001	Hepatitis A virus cellular receptor 2
HMGB1	1.52	.004	High-mobility group box 1
HOXA5	1.91	.002	Homeo box A5
JDP2	1.64	.007	Jun dimerization protein 2
LGALS12	0.67	.002	Lectin, galactose binding, soluble 12
MDK	1.89	.005	Midkine
NNMT	1.89	.008	Nicotinamide *N*-methyltransferase
PAK6	0.61	.001	p21 (Cdkn1a)-activated kinase 6
PEBP1	0.32	.000	Phosphatidylethanolamine binding protein 1
PHLDB2	1.53	.001	Pleckstrin homology-like domain, family B, member 2
PHLPPL	0.68	.000	PH domain and leucine rich repeat protein phosphatase-like
PIK3C2G	0.65	.005	Class II PI3K, C2γ subunit (PI3K-C2γ)
PLEKHK1	1.51	.008	Pleckstrin homology domain containing, family k member 1
PPP1R14A	1.63	.004	Protein phosphatase 1, regulatory (inhibitor) subunit 14A
RMND5B	0.44	.005	Required for meiotic nuclear division 5 homolog B
RTKN2	0.44	.004	Rhotekin 2, PH domain
TGFA	0.66	.003	Transforming growth factor α
ZFP365	3.18	.005	Zinc finger protein 365

(*Continued*)

Table 1. Continued

Gene Symbol	Fold Change	*P* Value	Common Name
Peptidase activity			
ADAMTS2	(Repeat)		
APH1A	(Repeat)		
DHH	1.79	.004	Desert hedgehog
HTRA1	(Repeat)		
KLK12	0.70	.000	Kallikrein 12
MMP2	(Repeat)		
PAPPA	0.66	.006	Pregnancy-associated plasma protein A
TGM2	(Repeat)		
THSD4	(Repeat)		
Ion transport			
ATP2B3	0.65	.006	ATPase, Ca^{2+} transporting, plasma membrane 3
CATSPER2	2.10	.010	Cation channel, sperm associated 2
CP	1.76	.005	Ceruloplasmin
KCNF1	2.25	.001	Potassium voltage-gated channel, subfamily F, member 1
G protein–coupled receptor signaling pathway			
CSPRS	0.59	.000	Component of Sp100-rs
GALR2	(Repeat)		
OLFR1123	1.64	.001	Olfactory receptor 1123
OLFR584	1.50	.000	Olfactory receptor 584
OLFR61	0.69	.008	Olfactory receptor 61
OLFR691	1.88	.008	Olfactory receptor 691
TGM2	(Repeat)		
UTS2R	1.91	.008	Urotensin 2 receptor
V1RI1	1.57	.008	Vomeronasal 1 receptor, I1
Other unclassified			
2010110I21RIK	1.68	.008	Atlastin GTPase 2
ABCA8A	2.69	.008	ATP-binding cassette, subfamilyA (ABC1), member 8a
ADFP	1.85	.001	Adipose differentiation related protein
C1QTNF5	1.54	.008	C1q and tumor necrosis factor related protein 5
C730026E21RIK	0.61	.008	Methionine-tRNA synthetase 2 (mitochondrial) (Mars2)
CAR15	1.91	.009	Carbonic anhydrase 15
CCL2	1.58	.001	Chemokine (C-C motif) ligand 2
CDH6	0.69	.009	Cadherin 6, type 2, K-cadherin (fetal kidney)
GAST	0.44	.009	Gastrin
GPT1	0.59	.003	Glucose-6-phosphate transporter 1
LHFP	1.97	.001	Lipoma HMGIC fusion partner
LRRC38	4.72	.002	Leucine rich repeat containing 38
PLXDC2	1.51	.009	Plexin domain containing 2
RNU65	1.60	.004	Small nucleolar RNA, H/ACA box 65
TMEM208	0.44	.002	Transmembrane protein 208

n = 3. Listed are transcript identification (gene symbol), average fold change, *P* value based on two-tailed *t* tests, and common gene name, divided into functional clusters.

[a] (Repeat): when the same gene was classified into more than 1 gene group, the fold change and *P* value were not repeated in the table.

stimulated by IGF-I for 12 or 24 hours. Total cell lysates were prepared for Western blots against CCN5 and β-actin.

Dual-labeled immunofluorescence

Paraffin sections of the pancreas taken from 3- to 5-month old, male MT-IGF and wild-type littermates were dewaxed, rehydrated, and blocked with 10% donkey serum, followed by overnight incubation with rabbit polyclonal anti-CCN5 at 4°C. After washing with PBS, sections were stained with guinea pig polyclonal anti-insulin (ab7842; Abcam) followed by Alexa Fluor 594 conjugated donkey anti-rabbit IgG (H+L) and Alexa Fluor 488 goat anti-guinea pig IgG (Life Technologies) (18, 19). The images were captured and analyzed using an Axioskop 2 Plus microscope (Carl Zeiss), Retiga 1300 digital camera, and Northern Eclipse version 8 software (EMPIX Imaging).

Stable overexpression of CCN5 cDNA in MIN6 cells

Mouse CCN5 cDNA with a 3′-$(HA)_3$ tag was subcloned into a pcDNA3.1 vector between the cytomegalovirus promoter and bovine growth hormone polyadenylation sequence and used to transfect MIN6 cells, which were selected for resistance against G418 for 45 days (Figure 2A). After Western blot confirmation, multiple CCN5-overexpressing (MIN6-CCN5) and vector-transfected (MIN6-Vec) clones were subject to the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium (MTT) cell viability (Sigma-Aldrich) and 5-bromo-2′-deoxyuridine (BrdU) incorporation assays. The cells were cultured in 1% serum for 1 to 3 days

before the MTT assay. For BrdU incorporation, in the final 18 hours of incubation, 10 μM BrdU was added; its incorporation was quantified using an ELISA at 450 nm (EMD Millipore). The change in the cellular cyclin D1 level was determined by Western blotting. To study CCN5-stimulated signaling mechanisms, we measured changes in the phosphorylation levels of Akt and Erk1/2 kinases in cells cultured for 24 hours in either 10% serum or serum-free medium. An MTT cell viability assay was also performed in the presence or absence of the Akt inhibitor MK-2206 (1 μM) for 24 or 48 hours. Finally, to establish the actions of CCN5 as an extracellular regulator, parental MIN6 cells were treated with rh CCN5/WISP2 protein (Creative Biomart), and its effects on cell proliferation were measured using an MTT assay and Akt phosphorylation.

CCN5 gene silencing using small interfering RNA (siRNA)

The role of endogenous CCN5 on IGF-I–stimulated cell proliferation was investigated by RNA interference (RNAi)–mediated gene knockdown. Parental MIN6 cells were transiently transfected with Silencer Select CCN5-specific (or scrambled control) siRNAs (Ambion/Invitrogen) using Lipofectamine RNAiMAX (Invitrogen), and the decreased level of CCN5 mRNA was confirmed using real-time PCR. CCN5-siRNA transfected cells were allowed to grow in culture medium with or without 5 nM LONG R3 IGF-I for 3 days, and cell viability was assessed by an MTT assay.

Streptozotocin-induced apoptosis in MIN6 cells

MIN6-CCN5 and MIN6-Vec cells were subcultured at a density of 2×10^4 cells/cm^2 and incubated with 5 mM streptozotocin for 24 hours (20). The presence of mononucleosomes and oligonucleosomes in apoptosis was measured by histone-associated DNA fragments in the cytoplasm. The degree of cell apoptosis was determined by a sandwich enzyme immunoassay cell death detection ELISA Plus kit (11774425001; Roche Applied Science) (21). In brief, cells were collected by being centrifuged at 200 $\times$ *g* for 10 minutes and lysed for 30 minutes with the buffer provided. The cell lysate was again centrifuged at 200 $\times$ *g* for 10 minutes. Then 20-μL aliquots of the supernatant (representing the cytosolic fraction) were transferred to streptavidin-coated wells, incubated with anti-histone–biotin and anti-DNA-peroxidase antibody for 2 hours, followed by the 2,2′-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) substrate for 10 minutes, and then measured as a ratio of absorbance at 405 and 490 nm using a PerkinElmer EnSpire multiplate reader. The occurrence of apoptosis was correlated with the amount of streptozotocin-induced caspase 3 cleavage in MIN6 cells, which was quantified by Western blots.

Confocal microscopy of CCN5-overexpressing cells

To monitor intracellular CCN5 expression and distribution in response to high glucose stimulation, MIN6-CCN5 cells were seeded into 8-well Lab-Tek chamber slides and cultured for 24 hours. The cells were then cultured in fresh medium containing either 2.8 or 16.7 mM glucose for 60 minutes before being fixed with 3% paraformaldehyde and permeabilized with 0.1% Triton X-100. After blocking with 0.5% BSA, cells were incubated with mouse monoclonal anti-HA and rabbit polyclonal anti-insulin antibodies for 1 hour. After washing with PBS, the cells were incubated with Alexa Fluor 594 donkey anti-mouse IgG (H+L) and Alexa Fluor 488 donkey anti-rabbit IgG (Life Technologies). Nuclei were stained with 4′,6′-diamino-2-phenylindole (DAPI) at 1 μg/mL. Images were obtained using a 63$\times$ (numerical aperture 1.40) oil Plan-Apochromat objective and detected with a GaSaP detector using an LSM780 laser scanning confocal microscope (Carl Zeiss). DAPI was excited with a 405-nm diode laser, anti-insulin with a 488-nm argon laser, and anti-HA with a 561-nm DPASS laser.

Subcellular fractionation

To confirm the confocal result, MIN6-CCN5 cells in confluent culture dishes were incubated for 60 minutes with 2.8 or 16.7 mM glucose, washed in ice-cold PBS, scraped from the dishes, and resuspended in lysis buffer containing 20 mM Tris-HCl (pH 8.0), 1% Triton X-100, 150 mM NaCl, and protease inhibitor cocktail. Cell lysates were centrifuged twice at 2600 $\times$ *g* for 7 minutes to pellet the nuclear enriched fraction. The supernatant was cleared at 20 000 $\times$ *g* to obtain the cytosolic faction. The protein concentration was determined using a BCA assay.

Statistical analysis

Data are expressed as means $\pm$ SE and plotted using Sigma Plot version 11 (Systat Software), which was also used to perform an ANOVA and post hoc Holm-Sidak test. An unpaired Student *t* test was performed using InStat version 3 software (GraphPad Software). Values of $P < .05$ were considered to be significant.

Results

Changes in gene expression profile induced by IGF-I overexpression in pancreatic islets

To explore novel targets that are involved in IGF-I–regulated β-cell function, we performed a whole-genome microarray analysis on total RNA prepared from freshly isolated islets and found 82 genes either up- or down-regulated significantly in MT-IGF vs wild-type mice, with the threshold of $>$1.5- or $<$0.7-fold and $P \leq .01$ (Table 1). Many of them, including ECM proteins (fibulin-2 and COL14A1), ion channels (KCNF1 and CATSPER2), intracellular substrates (11β-HSD1 and PI3K-C2γ), and CCN5/WISP2 are novel targets of IGF-I whose impact on β-cell function had not been previously known. Among them, expression of CCN5/WISP2, a gene not known to be expressed in the islets, exhibited a 2.7-fold increase as a result of IGF-I overexpression.

To avoid false-positive results in the microarray result, changes in the expression of nine target genes were independently confirmed using real-time PCR in fresh batches of isolated islets (Table 2). Notably, the level of CCN5/WISP2 mRNA was significantly increased 3.3-fold in MT-IGF vs that in wild-type mice, consistent with the microarray result. CCN5/WISP2 is a secreted protein of 29 kDa and has been reported to either stimulate or inhibit cell

Table 2. Effects of IGF-I Overexpression on Pancreatic Islet Gene Expression Determined by Real-Time PCR

Target Gene	Relative Expression Level, Arbitrary Units[a]		*P* Value
	Wild-Type	MT-IGF	
KCNF1 (Kv5.1 potassium channel)	13.1 ± 1.1	23.9 ± 2.6	.0064
HSD11B1 (11β-HSD1)	7.5 ± 1.7	16.3 ± 2.3	.0072
FBLN2 (fibulin-2)	9.9 ± 2.0	23.3 ± 4.1	.0149
COL14A1 (collagen 14A1)	8.6 ± 1.3	12.7 ± 1.2	.035
VWF1 (von Willebrand factor homolog)	6.9 ± 1.9	14.3 ± 2.5	.046
PIK3C2G (PI3K-C2γ)	16.8 ± 1.7	11.3 ± 1.1	.006
CCN5/WISP2 (WNT1 inducible signaling pathway protein)	2.5 ± 2.7	8.4 ± 1.3	.008
MMP2 (matrix metallopeptidase 2)	9.3 ± 3.3	16.0 ± 2.8	NS
Catsperm2 (cation channel, sperm associated 2)	8.0 ± 2.9	9.9 ± 1.8	NS

Abbreviation: NS, not significant. Total RNA was prepared from isolated islets from 4- to 5-month-old MT-IGF vs wild-type male mice. Real-time PCR was performed using a QuantiFast SYBR Green RT-PCR Kit (QIAGEN) and Roche LightCycler system.

[a] Data are means ± SE, based on experiments of n = 6.

proliferation in various systems (6–8, 22). Although its expression in pancreatic acinar and ductal cells has been reported (23), its presence in the islet cells was not known.

Islet β-cell–specific CCN5/WISP2 expression and its direct stimulation by IGF-I

To confirm CCN5 gene expression at the protein level, we performed Western blot analysis on the extract of freshly isolated islets. As shown in Figure 1A, CCN5 was found to be expressed at low level in the islets of wild-type mice and at a 2-fold higher level in those of MT-IGF mice. Using dual-labeled immunofluorescence, we further confirmed the presence of CCN5 in wild-type islets, and its induction in MT-IGF mice (Figure 1B). In sections costained for insulin (green, top panels) and CCN5 (red, middle panels), the colocalization of insulin and CCN5 in islet β-cells was evident from the merged images (bottom panels) and confirmed also the increase in β-cell–specific expression in MT-IGF mice. In parallel experiments, we found that CCN5 does not colocalize with glucagon (data not shown). To further demonstrate an in vitro direct stimulation, freshly isolated islets of C57BL/6 mice were cultured with 10^{-8} M IGF-I that elicited significant stimulation of the CCN5 mRNA level after 12 and 24 hours (Figure 1C). The stimulation at the protein level was more striking, exceeding 6.6-fold after 12 hours of treatment (Figure 1D). Thus, we demonstrated increased CCN5 protein in islet β-cells of MT-IGF mice using immunohistochemistry and Western blots and direct stimulation of CCN5 gene expression by IGF-I in primary islets.

Increased proliferation of insulinoma cells caused by CCN5/WISP2 overexpression

To define the role of CCN5 in islet function, we stably transfected its cDNA with a 3′-$(HA)_3$ tag into MIN6 cells using the pcDNA3.1 vector (Figure 2A). Using Western blots, we confirmed significantly overexpressed CCN5 protein in MIN6-CCN5 cells. Consequently, the cell number increase representing cell proliferation in 3 independent lines of MIN6-CCN5 cells was significantly higher than those of MIN6-Vec cells using an MTT assay (Figure 2B). To directly measure the change in DNA synthesis, we demonstrated ~1.7- and ~2.7-fold significantly increased BrdU incorporation in 2 lines of MIN6-CCN5 cells compared with that in MIN6-Vec cells cultured either for 48 or 72 hours in 1% serum (Figure 2C). β-Cell replication is known to be associated with increased cyclin D1 and CDK4 levels (24, 25). In MIN6-CCN5 cells, we detected a 3-fold increase in the level of cyclin D1 (Figure 2D), further supporting a proliferative effect of CCN5.

CCN5/WISP2 overexpression increased Akt and Erk2 phosphorylation

The proliferation of islet cells is known to be controlled by PI3K, Akt, and Erk1/2 pathways and stimulated by IGF-I. To determine whether Akt and Erk1/2 are regulated by CCN5, we assessed and detected a 2.4-fold increase in Akt phosphorylation at Ser-473 in MIN6-CCN5 compared with that in MIN6-Vec cells incubated in serum-free medium (Figure 2E); even in cells stimulated with 10% serum, CCN5 overexpression caused a 2-fold increase in Akt phosphorylation. The effect on Erk1/2 was moderate; ie, under serum-free condition, CCN5 overexpression caused only a 1.6-fold increase Erk2 phosphorylation ($P < .05$) (Figure 2F), but under 10% serum it had no significant effect. As shown in these blots, the total levels of Akt, Erk1, and Erk2 remained unaltered (Figure 2, E and F). We confirmed the role of Akt activation in CCN5-induced proliferative signaling by comparing the relative number of MIN6-CCN5 cells incubated in the presence or absence of Akt inhibitor MK-2206 and found that inhibition of

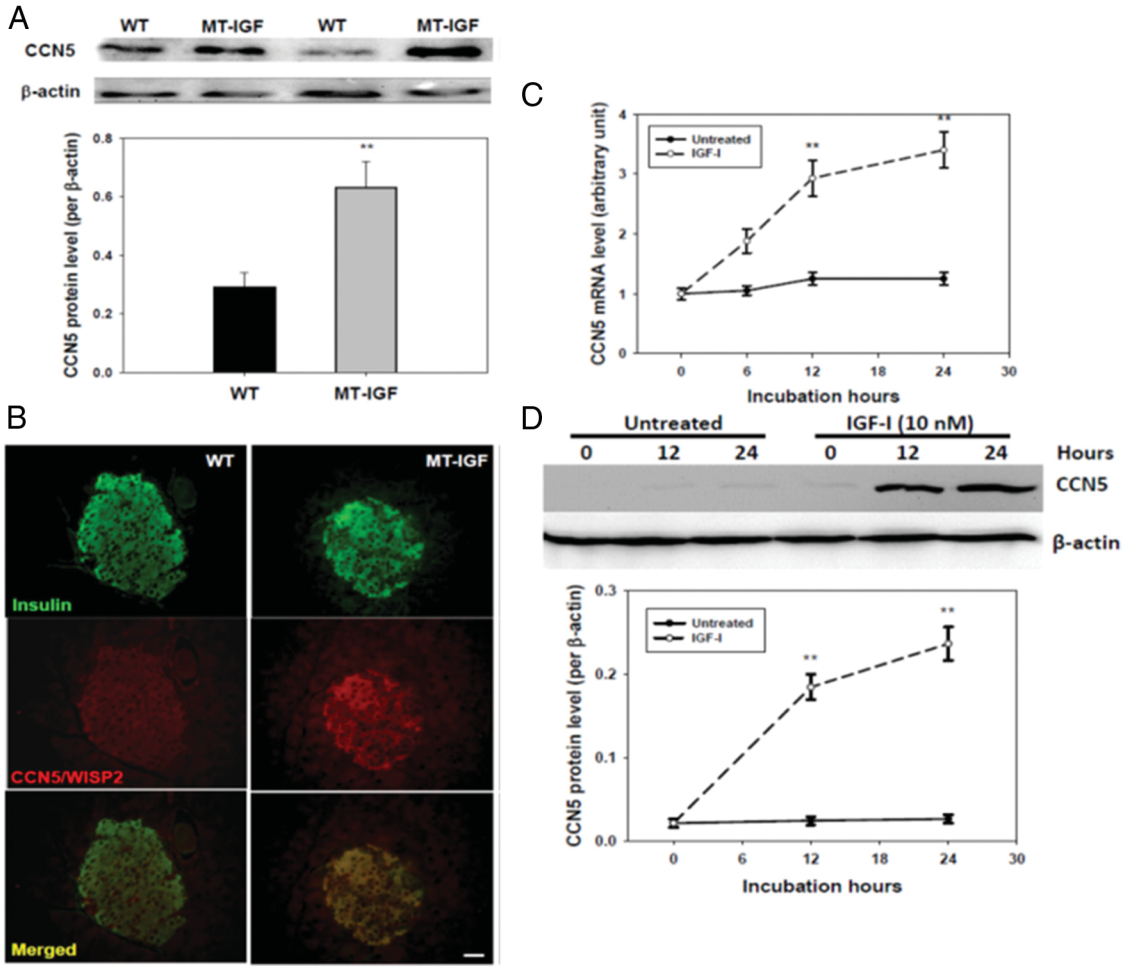

Figure 1. Islet β-cell–specific expression of CCN5/WISP2 and its significant induction by IGF-I. A, Representative Western blots using freshly isolated islets of 3- to 4-month-old wild-type (WT) or MT-IGF mice and specific antibodies against CCN5 and β-actin. The result of densitometry analysis is shown below. n = 3. **, $P < .01$ using an unpaired *t* test. The experiment was repeated twice with at least 3 independent data points each time. B, Immunofluorescence was determined using paraffin sections of the pancreata and primary antibodies against insulin (green) and CCN5/WISP2 (red). The merged image shows colocalization of CCN5 and insulin in β-cells of the islets (orange). The scale bar corresponds to 60 μm. Representative images were taken from 3 mice in each genotype and >10 images. C, Effect of in vitro direct stimulation by IGF-I. Freshly isolated islets from 3- to 4-month-old male C57B/L6 mice were treated with 10^{-8} M rh IGF-I, LONG R3, or vehicle for 6 to 24 hours. Changes in the level of CCN5 mRNA in arbitrary units were measured by quantitative RT-PCR. n = 4. **, $P < .01$ vs untreated islets using an unpaired *t* test. D, Changes in the level of CCN5 protein determined by Western blots and quantified using densitometry. n = 5. **, $P < .01$ vs untreated using unpaired *t* test.

Akt decreased cell proliferation by 61% and 53% after 24 and 48 hours of incubation, respectively (Figure 2G). At the same time, Akt inhibition caused a 50% reduction in the cyclin D1 level (Figure 2H). Our results indicate that CCN5 stimulates β-cell replication, at least in part by activating Akt kinase and cyclin D1.

CCN5 overexpression in MIN6 cells confers resistance to streptozotocin-induced apoptosis

Because IGF-I has a potent effect on β-cell survival against various types of damage, we anticipated that CCN5 may also exert prosurvival effects. We tested the effect of CCN5 overexpression on streptozotocin-induced cell death in stably transfected MIN6 lines. Streptozotocin treatment for 24 hours caused significant cell loss, showing condensation and debris in MIN6-Vec cells (Figure 3A, bottom left vs top left panels), which was largely avoided in MIN6-CCN5 cells (Figure 3A, bottom right panel). This was confirmed by a 3.6-fold increase in cleaved caspase-3 in MIN6-Vec cells after 12 hours of exposure to streptozotocin (Figure 3B). CCN5 overexpression significantly diminished caspase-3 activation and blunted streptozotocin-induced cell apoptosis. Histone-associated DNA fragmentation in the cytoplasm is the hallmark of cell apoptosis (21). Streptozotocin treatment caused a significant 6.5-fold increase in the level of DNA fragmentation in MIN6-Vec cells (Figure 3C, bar 2 vs 1), compared with a mere 1.6-fold increase in MIN6-CCN5 cells (Figure 3C, bar 4 vs 3). The 4-fold reduction in DNA

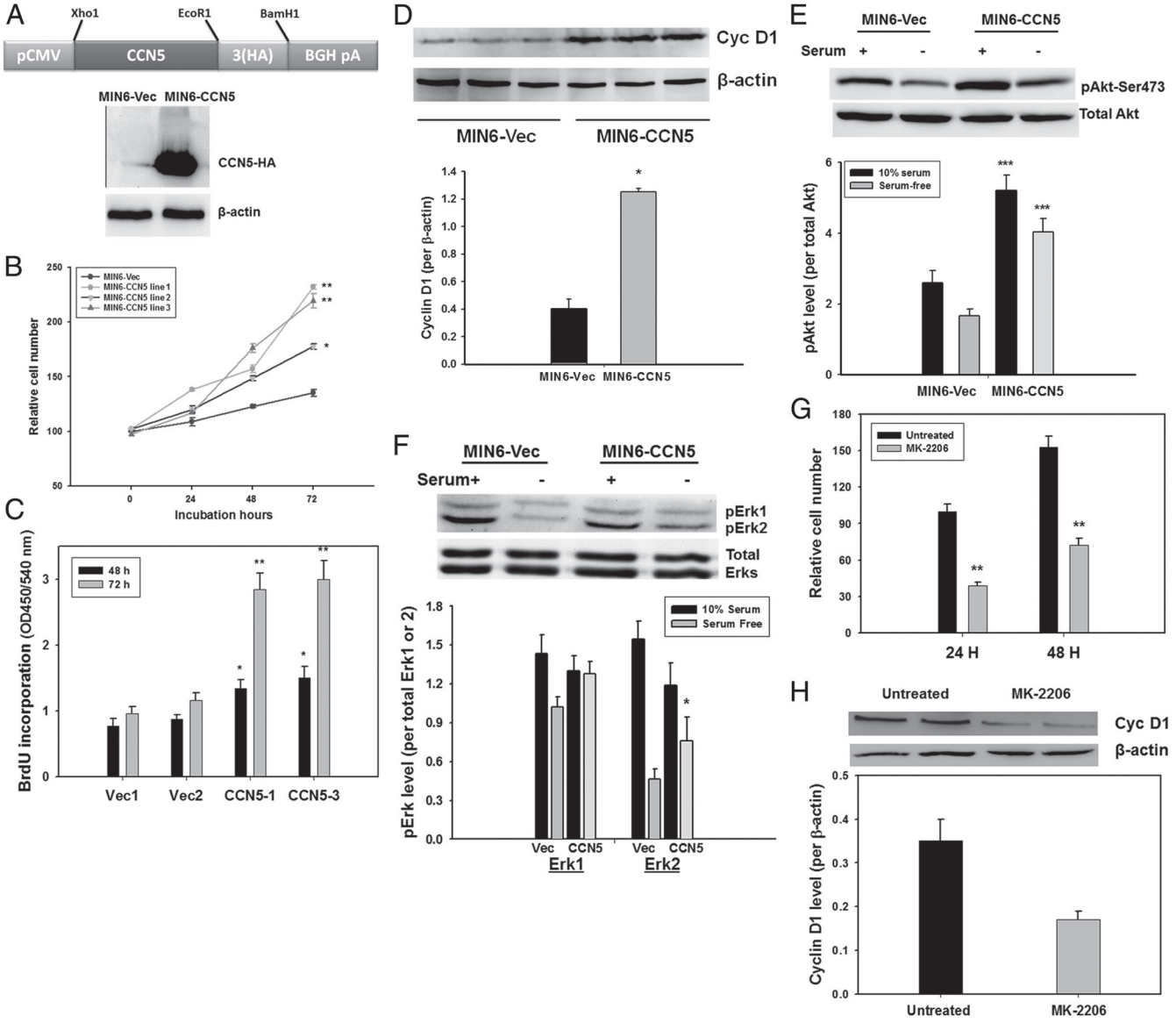

Figure 2. Increased cell proliferation and Akt phosphorylation in CCN5-overexpressing MIN6 cells. A, Stable overexpression of CCN5 cDNA with a 3′-$(HA)_3$ tag in MIN6 cells using a pcDNA3.1 vector. The construct is illustrated, and CCN5 overexpression was confirmed using Western blotting and HA antibody. B, Higher increases in the cell numbers measured by an MTT assay. CCN5-overexpressing cell lines cultured with 1% serum showed increased cell proliferation at 48 to 72 hours. n = 5. *, $P < .05$; **, $P < .01$ vs MIN6-Vec cells using a one-way ANOVA. C, Increased cell proliferation measured by the ratio of BrdU incorporation. CCN5-overexpressing vs vector control cell lines (2 each) cultured with 1% serum showed increased cell proliferation at 48 and 72 hours. The relative ratio of BrdU incorporation was measured by an ELISA. n = 4. *, $P < .05$; **, $P < .01$ vs MIN6-Vec cells, using a one-way ANOVA and post hoc Holm-Sidak test. D, CCN5 overexpression caused an increased cyclin D1 (Cyc D1) level. The cells were cultured in 5.5 mM glucose and 1% serum for 3 days. Western blots were obtained for multiple lines of MIN6-CCN5 vs MIN6-Vec cells. The result of densitometry analysis is illustrated in the bar graph. n = 3. *, $P < .05$ vs MIN6-Vec cells using an unpaired *t* test. E, CCN5 overexpression caused increased phosphorylation of Akt kinase. MIN6-Vec or MIN6-CCN5 cells were cultured for 24 hours under 0% or 10% serum as indicated. Representative Western blots for Akt and results of densitometry are illustrated. F, Representative Western blots for Erk1/2 and results of densitometry. Data are means ± SE representative of 3 experiments. For E and F, n = 3. *, $P < .05$; ***, $P < .001$ vs MIN6-Vec cells under the same condition using an unpaired *t* test. G, Effect of the Akt inhibitor MK-2206 (1 μM) on the viability of MIN6-CCN5 cells measured using an MTT assay after 24 and 48 hours of incubation as in Figure 2B. n = 3. **, $P < .01$ vs untreated cells using an unpaired *t* test. H, Effect of the Akt inhibitor MK-2206 on the cyclin D1 level in MIN6-CCN5 cells after 24 hours of incubation. Western blots and the result of densitometry analysis are illustrated. n = 2.

fragmentation (compare bars 2 and 4) confirmed the ability of overexpressed CCN5 to attenuate streptozotocin-induced apoptosis. Because streptozotocin-induced cellular toxicity is dependent on the normal expression of glucose transporter 2 (GLUT2), the possibility that the CCN5-induced decrease in GLUT2 expression may limit the uptake of streptozotocin thereby indirectly diminishing its cytotoxicity was excluded because CCN5 overexpression did not influence the GLUT2 level in MIN6 cells (data not shown).

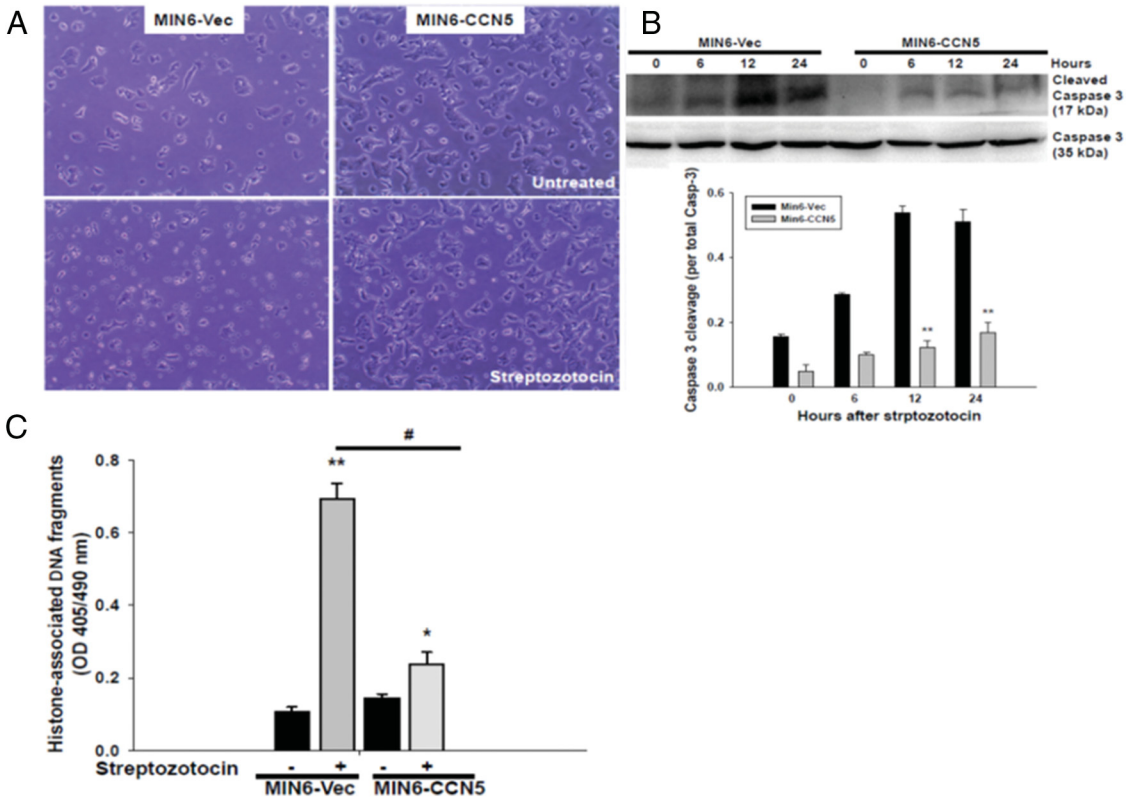

Figure 3. CCN5 overexpression enabled MIN6 cells resistant to streptozotocin-induced apoptosis. A, Cell morphology after 24 hours of treatment with or without streptozotocin in MIN6-CCN5 vs MIN6-Vec cells at 100× magnification, showing condensation, cell loss, and debris (bottom left, streptozotocin on vector-transfected cells) vs high-density, normally stretched cells in the other 3 panels. B, Changes in the level of caspase 3 cleavage induced by streptozotocin. n = 3, **, $P < .01$ vs MIN6-Vec cells using a one-way ANOVA. The experiment was repeated once. C, Changes in cytoplasmic levels of histone-associated DNA fragments, quantified using an ELISA. The absorbance reading at 490 nm was subtracted from that at 405 nm, following the manufacturer's instruction. n = 5. *, $P < .05$; **, $P < .01$ vs untreated cells; #, $P < .05$ vs streptozotocin-treated MIN6-Vec cells using a one-way ANOVA.

Glucose stimulation causes cytoplasmic accumulation of CCN5/WISP2

As a secreted protein, CCN5 is expected to be localized in cytoplasmic vesicles (26); as a matricellular protein, it might be associated with cell membrane and ECM; and its presence in the nucleus has also been described (27, 28). To help understand the secretion and subcellular localization of CCN5 protein, we performed confocal microscopy on MIN6 cells overexpressing the protein under the basal (low-glucose) condition and after being stimulated by high glucose. As shown in Figure 4A, under a low-glucose condition, HA-tagged CCN5 protein was present at a low level in the cytoplasm (Figure 4A, top panels). Upon high-glucose treatment for 60 minutes, significant cytoplasmic accumulation of CCN5-HA protein was found in close association with the staining of insulin (Figure 4A, bottom panels), which is consistent with the early observation of perinuclear and vesicular distribution (29). In addition, the cellular contents of both CCN5 and insulin were dramatically enhanced by the high level of glucose, compared with cells cultured under low glucose. To further quantify the change, in a separate experiment we prepared nuclear and cytosolic fractions from low- and high-glucose–treated cells and determined the relative levels of CCN5-HA protein compared with those for β-actin or USF-2, respectively (Figure 4, B and C). Compared with the cells cultured in low glucose, we confirmed a 4.3-fold increase in cytosolic CCN5-HA protein upon high-glucose stimulation but no significant decrease in nuclear protein, consistent to our finding in confocal and the notion that CCN5 is a glucose-responsive secretory protein. However, we have not been able to detect CCN5 secretion using Western blotting, unlike previous report (26).

Recombinant CCN5 protein directly stimulated cell replication and Akt phosphorylation

As we were studying the effect of CCN5 overexpression, recombinant and highly purified human protein became available and proved effective in inducing mesenchymal-epithelial transition in pancreatic adenocarcinoma cells (23). To establish whether it exerts an endocrine/paracrine effect on insulin-producing cells, we treated parental MIN6 cells with recombinant CCN5 protein for 3 days, together with IGF-I as a positive con-

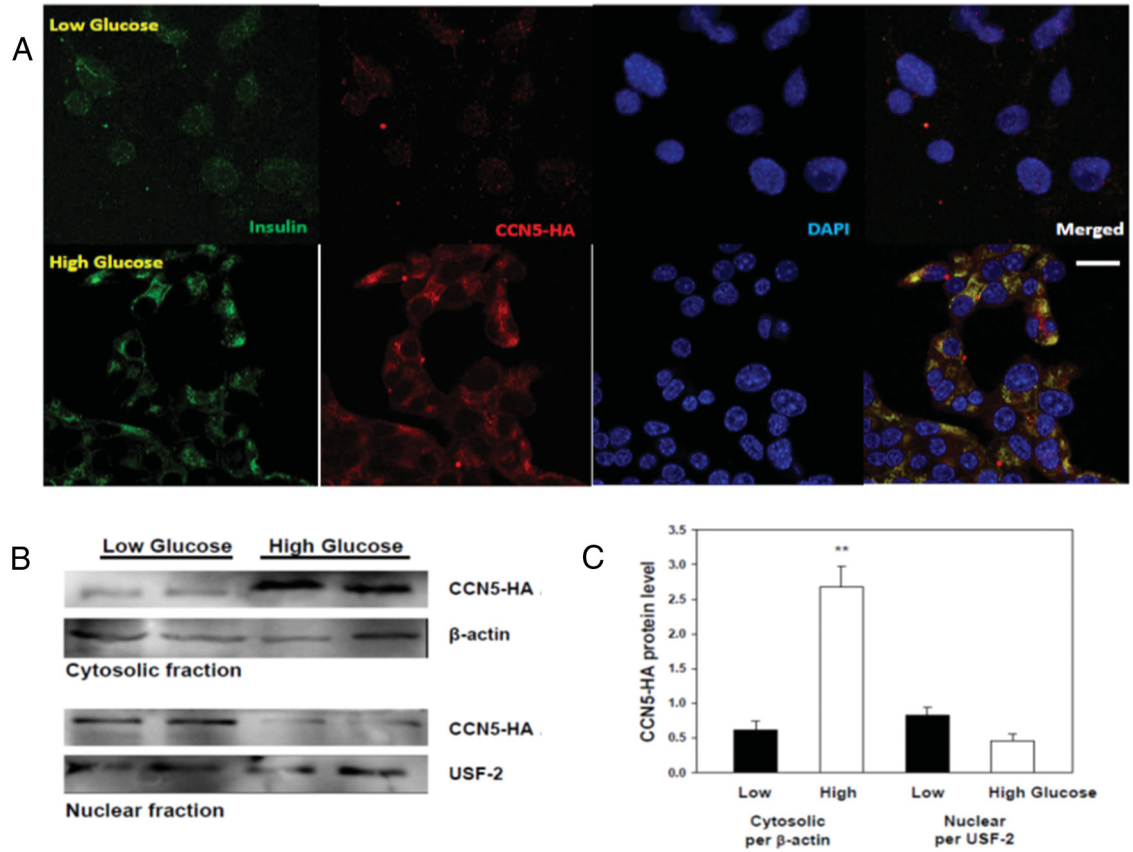

Figure 4. High-glucose–induced subcellular distribution of CCN5-HA protein in MIN6-CCN5 cells. A, Cells were cultured in chamber slides and at low (2.8 mM) or high (16.7 mM) glucose for 60 minutes. Confocal microscopy images were developed against insulin (green), the HA tag (for CCN5, red), DAPI (nucleus, blue), and merged. Representative images from triplicate chambers and >10 sets of images are illustrated. The scale bar corresponds to 20 μm. B, Changes in the subcellular distribution of CCN5 confirmed by Western blots. From a separate experiment cultured with low and high glucose, cytosolic and nuclear fractions were blotted using antibodies against CCN5-HA, β-actin, and USF-2. Negative signals of USF-2 in cytosolic fractions and of β-actin in nuclear fractions were confirmed to exclude cross-contamination (not shown). n = 4. C, Quantification of results by densitometry. The level of cytosolic protein was corrected by β-actin and that nuclear protein by USF-2. n = 4; **, $P < .01$ vs low glucose using an unpaired *t* test.

trol. In untreated cells, the proliferation rate was low as measured using an MTT assay (Figure 5A). Treatment with low doses of CCN5 protein (6.25 and 12.5 nM) significantly increased the proliferation activity up to 3 days; a higher dose of 25 nM led to early stimulation but became somewhat toxic after 48 hours, and the stimulatory effect of exogenous CCN5 protein was somewhat less than that of 10 nM IGF-I. This result was further supported by the increased BrdU incorporation in cells stimulated for 3 days by IGF-I (1.7-fold at 10 nM) or CCN5 (1.4-fold at 12.5 nM) (Figure 5B). Treatment of MIN6 cells with exogenous CCN5 protein for 6 hours led to a 1.8-fold increase in Akt phosphorylation at Ser-473 (Figure 5C). This effect seemed specific because Erk1/2 activity was not significantly affected (data not shown), consistent with the moderate changes in Erk1/2 phosphorylation upon CCN5 overexpression (Figure 2F). Thus, exogenously recombinant protein was found to replicate 2 key effects of CCN5 overexpression in MIN6 cells, ie, proliferation and Akt phosphorylation.

To further assess the role of endogenous CCN5 expression on IGF-I–stimulated cell proliferation, we studied the effect of its knockdown using RNAi. Basal CCN5 expression in parental MIN6 cells was abolished by CCN5-specific but not a nonspecific scrambled siRNA as determined by quantitative RT-PCR (Figure 5D, compare bars 1 and 2). Likewise, siRNA transfection blunted CCN5 induction by 5 nM IGF-I (Figure 5D, compare bars 3 and 4). CCN5 knockdown did not affect the basal level of cell proliferation after 3 days of culture, as determined by an MTT assay (Figure 5E); however, it clearly reduced the IGF-I–stimulated effect from 2.3- to 1.5-fold stimulation (@, $P < .05$), supporting a role for endogenous CCN5 in IGF-I–stimulated cell proliferation.

Discussion

In this study, we identified CCN5 as a novel target of IGF-I in islet β-cells that is induced not only by IGF-I overex-

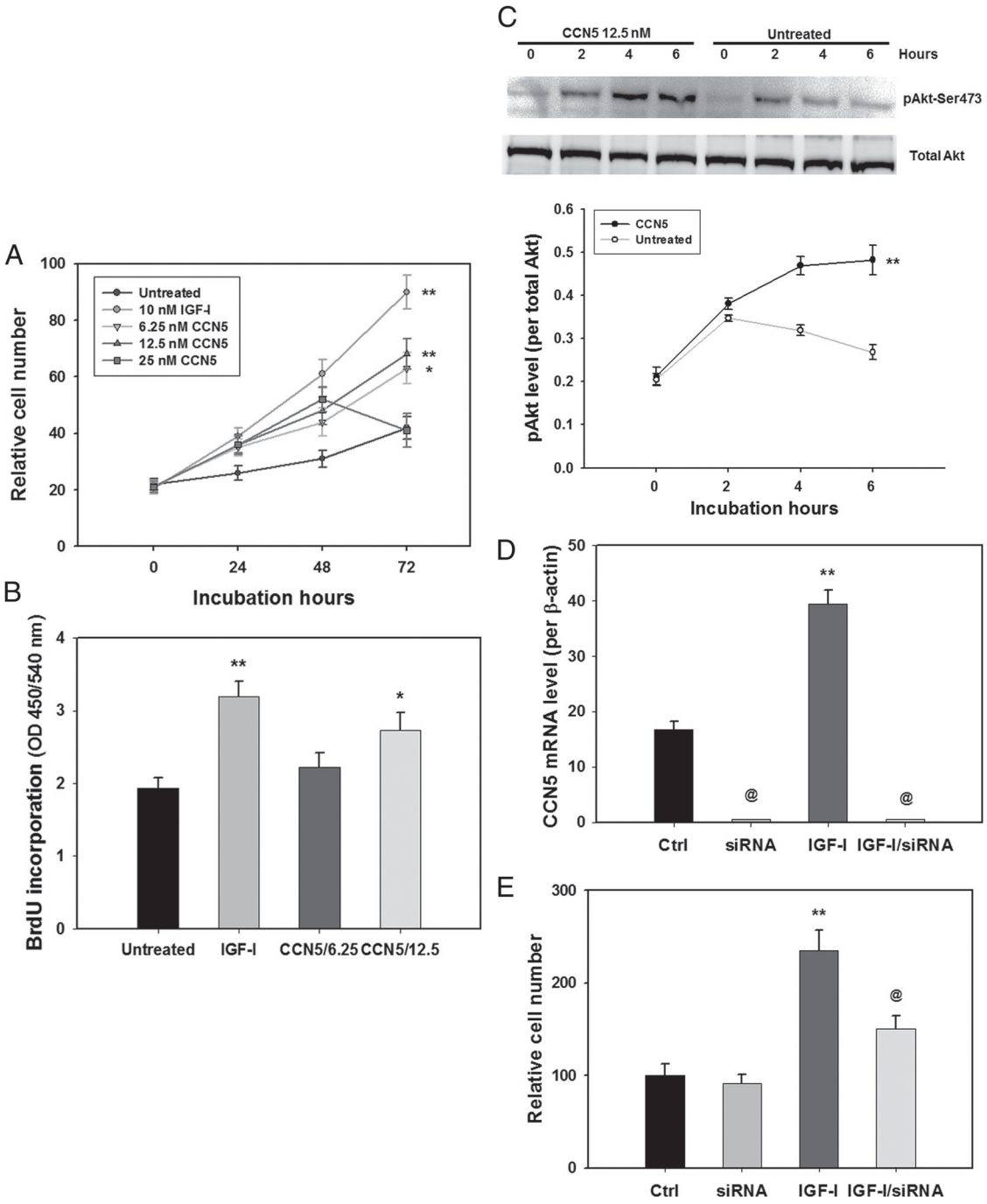

Figure 5. Direct effect of recombinant CCN5 protein on islet cell proliferation and Akt phosphorylation. A, Parental MIN6 cells (20 000/well) were cultured for 1 to 3 days with IGF-I or increasing doses of CCN5 in DMEM containing 1% serum and 11.1 mM glucose. Cell numbers were measured using an MTT assay. n = 6. *, $P < .05$; **, $P < .01$ vs untreated using a one-way ANOVA. B, Changes in the rate of cell proliferation measured by BrdU incorporation. Parental MIN6 cells were cultured with or without 10 nM IGF-I or 6.25 and 12.5 nM CCN5 for 3 days in 1% serum. n = 3 to 4. *, $P < .05$; **, $P < .01$ vs untreated cells using an unpaired *t* test. C, CCN5 stimulated Akt phosphorylation. Under similar conditions as in Figure 5A, MIN6 cells were cultured for 2 to 6 hours with or without 12.5 nM CCN5. The cell lysate was probed for pAkt at Ser-473 or total Akt using Western blots. A representative blot from 3 experiments is illustrated in the top panel. The result of densitometry analysis is depicted in the lower panel. n = 3. **, $P < .01$ vs untreated cells using one-way ANOVA. D, siRNA-mediated transient knockdown of basal and IGF-I–stimulated (5 nM, 48 hours) CCN5 gene expression in parental MIN6 cells measured by quantitative RT-PCR. E, Effect of CCN5 gene knockdown on the basal and IGF-I–stimulated (5 nM, 72 hours) change in cell viability measured by an MTT assay. For D and E, n = 4. **, $P < .01$ vs scrambled vector-transfected untreated cells (first columns; Ctrl), @, $P < .01$ vs scrambled vector-transfected cells treated with or without IGF-I using an unpaired *t* test.

pression but also by direct stimulation with exogenously added peptide. We demonstrated that IGF-I induces CCN5 gene expression both at the mRNA and protein levels. To define its role in islet cell biology, we stably overexpressed CCN5 cDNA in MIN6 insulinoma cells and demonstrated that it induces phosphorylation of Akt

and Erk2, cyclin D1 expression, and cell proliferation as measured by MTT and BrdU assays. IGF-I–stimulated cell proliferation is known to involve Akt and Erk1/2 signaling, leading to cyclin D1 activation (24). Our result also supports previous findings in neuroblastoma Neuro2a cells that CCN5 induces Akt and Erk2 phosphorylation (30). We then showed that treatment of MIN6 cells using recombinant CCN5 protein promotes Akt phosphorylation and cell proliferation. Finally, MIN6-CCN5 cells were found to be resistant to streptozotocin-induced caspase-3 activation and apoptosis. Taken together, the present findings demonstrate that IGF-I–induced CCN5 in islet β-cells may not only contribute to mitogenic signaling but also promote cell survival by inhibiting apoptosis.

Among the IGF-I targets revealed in this study, 16 genes encode ECM-related proteins, including fibulin-2, which belongs to a family of 5 that shares a distinctive C-terminal globular domain and a tandem array of calcium-binding epidermal growth factor (EGF)–like modules (31). The expression of PI3K-C2γ, a new member of the PI3K family, was specifically *decreased* at the mRNA level in IGF-I–overexpressing islets (32, 33). It should be noted that not all of the affected genes can be considered direct targets of IGF-I action on the islets because our model of overexpression also exhibited considerable levels of IGF-I expression in the liver and kidney and a 50% increase in the serum IGF-I concentration (5).

Most CCN/WISP proteins contain 4 functional domains, ie, the IGF binding protein domain with sequence homology to IGF binding proteins but confers less than 1% affinity to IGFs (34), the von Willebrand factor C repeat often seen in oligomerization of ECM proteins, the thrombospondin type I repeat interacting with integrins, and the cysteine-rich carboxyl-terminal repeat (which is missing from CCN5) associated with dimerization and receptor binding (9, 10, 35). Overall, they share an ~50% amino acid sequence, including 38 cysteines at homologous sites of the proteins (35). As matricellular proteins, they contain binding sites for ECM and putative cell surface receptors; most of their biological activities are thought to be mediated by cell adhesion receptors, including integrins (eg, α2β1, α5β1,and others) and heparin sulfate proteoglycans.

Among the CCN5 family members, CCN2/CTGF has clearly been shown to regulate pancreatic islet function (12–15). CCN2 promotes the formation of new islets from pancreatic ducts, the expansion of immature β-cells, and islet vascular development (12, 14). Islet morphogenesis and function are exquisitely regulated by CCN2, which is both required and sufficient to induce proliferation of immature β-cells. On the other hand, β-cells in more mature, postnatal animals do not express CCN2 and appear refractory to its presence in other cells (12–15). The novel role of CCN5 in promoting β-cell proliferation in this study and the reported induction of CCN4/WISP1 expression during islet regeneration after 90% pancreatectomy (36) suggest that CCN2 is not the only member of the family that regulates islet cell biology. Interestingly, the expressions of CCN2 and CCN5 appear to be interdependent because knockdown of CCN5 gene expression in MCF-7 cells resulted in a 12-fold compensatory increase in the CCN2 mRNA level (27). CCN5 has a unique age-dependent and tissue-specific expression pattern, its expression in β-cells is selectively induced by IGF-I, and it has a unique structure, which is missing the carboxyl-terminal domain. All of these characteristics, together with its regulation by glucocorticoids (37), estrogen and progesterone (38, 39), EGF (40), phorbol 12-myristate 13-acetate and phorbol esters (41), and IGF-I (6) in other types of cells, may contribute to its unique role in islet function.

CCN5 is expressed as early as the 4-cell stage and seems to be important for embryonic development. In early to mid embryonic stages (embryonic days 9 to 11), it is expressed in the cells of ectodermal, mesodermal, and endodermal origins and is essential for development because CCN5 knockout caused early embryonic lethality (22). From the early embryonic up to the adult stage, CCN5 is highly expressed in the layers of endothelium and smooth muscle of blood vessels, as well as in the myocardium of the heart, skeletal muscle, colon, and ovary (22). As a direct target of Wnt signaling, CCN5 is a potential link between insulin and IGF-I in regulating the islet function. Overexpression of Wnt protein increased CCN5 mRNA and protein levels (42). Interestingly, insulin and IGF-I also stimulate cat/T-cell factor–mediated gene transcription, independent of Wnt; stimulate protein kinase B/Akt and the phosphorylation of glycogen synthase kinase 3β at Ser-21/9; and stabilize β-catenin (43). Whereas IGF-I, Wnt, and T-cell factor are clearly involved in pancreatic islet function (44), the role of their common target protein CCN5 has never been studied. Supporting a role of CCN5 in islet function and/or diabetes, both the CCN5 mRNA levels from microdissected β-cells and the blood CCN5 concentration were doubled in subjects with type 2 diabetes according to data available on Gene Expression Omnibus (profiles GDS3782 and GDS3963; http://www.ncbi.nlm.nih.gov/geo/).

Our islet proliferative findings support the previously reported ability of IGF-I to induce CCN5 expression, which enhances the proliferation of breast cancer cells through the PI3K/Akt pathway (6) and its involvement in the proliferation of 3T3-L1 preadipocytes (26). In nonislet cells, the proliferative role of CCN5 has been studied in

great length; eg, knockdown of CCN5 expression inhibited the proliferation of MCF-7 cells induced by either serum, EGF, or phorbol 12-myristate 13-acetate (38, 40, 41). Nevertheless, CCN5 has also been reported to *inhibit* proliferation of smooth muscle, uterine myometrial, estrogen receptor–negative breast cancer, and MCF-7 cells (7, 8, 22, 45). These seemingly conflicting reports warrant further studies to decipher the underlying mechanisms for those cell-specific effects and to establish its role in islet cell biology.

In summary, we have shown that CCN5/WISP2 is normally expressed in mouse islet β-cells and that IGF-I directly stimulates its expression. CCN5 overexpression increases the proliferation of insulinoma cells, activates Akt and Erk2 kinases, and inhibits streptozotocin-induced caspase-3 activation and apoptosis. Recombinant CCN5 protein seems to reproduce the proliferative effect and the stimulation on Akt phosphorylation. These findings suggest that CCN5 is capable of regulating islet cell proliferation and survival and that increased CCN5 expression may contribute to IGF-I–stimulated islet cell growth and/or survival.

Acknowledgments

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S.C. performed all experiments, presented the data, and revised the manuscript. X.W. performed microarray and Table 1. C.B.S. and G.N. contributed to the discussion and revision. M.F. performed confocal microscopy and data analysis. Q.L. and Y.J.G. participated part of the experiments and their analysis. J.-L.L. designed the study, wrote the manuscript, and approved the final revision.

Disclosure Summary: The authors have nothing to disclose.

References

1. **Le Roith D, Bondy C, Yakar S, Liu JL, Butler A.** The somatomedin hypothesis: 2001. *Endocr Rev*. 2001;22:53–74.
2. **Liu JL.** Does IGF-I stimulate pancreatic islet cell growth? *Cell Biochem Biophys*. 2007;48:115–125.
3. **Bergerot I, Fabien N, Maguer V, Thivolet C.** Insulin-like growth factor-1 (IGF-1) protects NOD mice from insulitis and diabetes. *Clin Exp Immunol*. 1995;102:335–340.
4. **Ohneda K, Ulshen MH, Fuller CR, D'Ercole AJ, Lund PK.** Enhanced growth of small bowel in transgenic mice expressing human insulin-like growth factor I. *Gastroenterology*. 1997;112:444–454.
5. **Robertson K, Lu Y, De Jesus K, et al.** A general and islet cell-enriched overexpression of IGF-I results in normal islet cell growth, hypoglycemia, and significant resistance to experimental diabetes. *Am J Physiol Endocrinol Metab*. 2008;294:E928–E938.
6. **Dhar K, Banerjee S, Dhar G, Sengupta K, Banerjee SK.** Insulin-like growth factor-1 (IGF-1) induces WISP-2/CCN5 via multiple molecular cross-talks and is essential for mitogenic switch by IGF-1 axis in estrogen receptor-positive breast tumor cells. *Cancer Res*. 2007;67:1520–1526.
7. **Delmolino LM, Stearns NA, Castellot JJ.** COP-1, a member of the CCN family, is a heparin-induced growth arrest specific gene in vascular smooth muscle cells. *J Cell Physiol*. 2001;188:45–55.
8. **Fritah A, Saucier C, De Wever O, et al.** Role of WISP-2/CCN5 in the maintenance of a differentiated and noninvasive phenotype in human breast cancer cells. *Mol Cell Biol*. 2008;28:1114–1123.
9. **Holbourn KP, Malfois M, Acharya KR.** First structural glimpse of CCN3 and CCN5 multifunctional signaling regulators elucidated by small angle X-ray scattering. *J Biol Chem*. 2011;286:22243–22249.
10. **Brigstock DR, Goldschmeding R, Katsube KI, et al.** Proposal for a unified CCN nomenclature. *Mol Pathol*. 2003;56:127–128.
11. **Holbourn KP, Acharya KR, Perbal B.** The CCN family of proteins: structure-function relationships. *Trends Biochem Sci*. 2008;33:461–473.
12. **Charrier A, Brigstock DR.** Regulation of pancreatic function by connective tissue growth factor (CTGF, CCN2). *Cytokine Growth Factor Rev*. 2013;24:59–68.
13. **Henley KD, Gooding KA, Economides AN, Gannon M.** Inactivation of the dual Bmp/Wnt inhibitor Sostdc1 enhances pancreatic islet function. *Am J Physiol Endocrinol Metab*. 2012;303:E752–E761.
14. **Guney MA, Petersen CP, Boustani A, et al.** Connective tissue growth factor acts within both endothelial cells and β cells to promote proliferation of developing β cells. *Proc Natl Acad Sci USA*. 2011;108:15242–15247.
15. **Crawford LA, Guney MA, Oh YA, et al.** Connective tissue growth factor (CTGF) inactivation leads to defects in islet cell lineage allocation and β-cell proliferation during embryogenesis. *Mol Endocrinol*. 2009;23:324–336.
16. **Xiong X, Wang X, Li B, et al.** Pancreatic islet-specific overexpression of Reg3β protein induced the expression of pro-islet genes and protected mice against streptozotocin-induced diabetes mellitus. *Am J Physiol Endocrinol Metab*. 2011;300:E669–E680.
17. **Getty-Kaushik L, Richard AM, Deeney JT, Krawczyk S, Shirihai O, Corkey BE.** The CB1 antagonist rimonabant decreases insulin hypersecretion in rat pancreatic islets. *Obesity (Silver Spring)*. 2009;17:1856–1860.
18. **Lu Y, Herrera PL, Guo Y, et al.** Pancreatic-specific inactivation of IGF-I gene causes enlarged pancreatic islets and significant resistance to diabetes. *Diabetes*. 2004;53:3131–3141.
19. **Lu Y, Ponton A, Okamoto H, Takasawa S, Herrera PL, Liu JL.** Activation of the Reg family genes by pancreatic-specific IGF-I gene deficiency and after streptozotocin-induced diabetes in mouse pancreas. *Am J Physiol Endocrinol Metab*. 2006;291:E50–E58.
20. **Liu L, Liu JL, Srikant CB.** Reg2 protects mouse insulinoma cells from streptozotocin-induced mitochondrial disruption and apoptosis. *Growth Factors*. 2010;28:370–378.
21. **Terui Y, Furukawa Y, Kikuchi J, Saito M.** Apoptosis during HL-60 cell differentiation is closely related to a G_0/G_1 cell cycle arrest. *J Cell Physiol*. 1995;164:74–84.

22. **Russo JW, Castellot JJ.** CCN5: biology and pathophysiology. *J Cell Commun Signal*. 2010;4:119–130.
23. **Dhar G, Mehta S, Banerjee S, et al.** Loss of WISP-2/CCN5 signaling in human pancreatic cancer: a potential mechanism for epithelial-mesenchymal-transition. *Cancer Lett*. 2007;254:63–70.
24. **Cozar-Castellano I, Takane KK, Bottino R, Balamurugan AN, Stewart AF.** Induction of β-cell proliferation and retinoblastoma protein phosphorylation in rat and human islets using adenovirus-mediated transfer of cyclin-dependent kinase-4 and cyclin D1. *Diabetes*. 2004;53:149–159.
25. **Fatrai S, Elghazi L, Balcazar N, et al.** Akt induces β-cell proliferation by regulating cyclin D1, cyclin D2, and p21 levels and cyclin-dependent kinase-4 activity. *Diabetes*. 2006;55:318–325.
26. **Hammarstedt A, Hedjazifar S, Jenndahl L, et al.** WISP2 regulates preadipocyte commitment and PPARγ activation by BMP4. *Proc Natl Acad Sci USA*. 2013;110:2563–2568.
27. **Sabbah M, Prunier C, Ferrand N, et al.** CCN5, a novel transcriptional repressor of the transforming growth factor β signaling pathway. *Mol Cell Biol*. 2011;31:1459–1469.
28. **Wiesman KC, Wei L, Baughman C, Russo J, Gray MR, Castellot JJ.** CCN5, a secreted protein, localizes to the nucleus. *J Cell Commun Signal*. 2010;4:91–98.
29. **Zhang R, Averboukh L, Zhu W, et al.** Identification of rCop-1, a new member of the CCN protein family, as a negative regulator for cell transformation. *Mol Cell Biol*. 1998;18:6131–6141.
30. **Ohkawa Y, Ohmi Y, Tajima O, Yamauchi Y, Furukawa K, Furukawa K.** Wisp2/CCN5 up-regulated in the central nervous system of GM3-only mice facilitates neurite formation in Neuro2a cells via integrin-Akt signaling. *Biochem Biophys Res Commun*. 2011;411:483–489.
31. **Timpl R, Sasaki T, Kostka G, Chu ML.** Fibulins: a versatile family of extracellular matrix proteins. *Nat Rev Mol Cell Biol*. 2003;4:479–489.
32. **Kok K, Geering B, Vanhaesebroeck B.** Regulation of phosphoinositide 3-kinase expression in health and disease. *Trends Biochem Sci*. 2009;34:115–127.
33. **Ono F, Nakagawa T, Saito S, et al.** A novel class II phosphoinositide 3-kinase predominantly expressed in the liver and its enhanced expression during liver regeneration. *J Biol Chem*. 1998;273:7731–7736.
34. **Grotendorst GR, Lau LF, Perbal B.** CCN proteins are distinct from and should not be considered members of the insulin-like growth factor-binding protein superfamily. *Endocrinology*. 2000;141:2254–2256.
35. **Rachfal AW, Brigstock DR.** Structural and functional properties of CCN proteins. *Vitam Horm*. 2005;70:69–103.
36. **Lim HW, Lee JE, Shin SJ, et al.** Identification of differentially expressed mRNA during pancreas regeneration of rat by mRNA differential display. *Biochem Biophys Res Commun*. 2002;299:806–812.
37. **Ferrand N, Stragier E, Redeuilh G, Sabbah M.** Glucocorticoids induce CCN5/WISP-2 expression and attenuate invasion in oestrogen receptor-negative human breast cancer cells. *Biochem J*. 2012;447:71–79.
38. **Banerjee S, Saxena N, Sengupta K, Tawfik O, Mayo MS, Banerjee SK.** WISP-2 gene in human breast cancer: estrogen and progesterone inducible expression and regulation of tumor cell proliferation. *Neoplasia*. 2003;5:63–73.
39. **Fritah A, Redeuilh G, Sabbah M.** Molecular cloning and characterization of the human WISP-2/CCN5 gene promoter reveal its up-regulation by oestrogens. *J Endocrinol*. 2006;191:613–624.
40. **Banerjee S, Sengupta K, Saxena NK, Dhar K, Banerjee SK.** Epidermal growth factor induces WISP-2/CCN5 expression in estrogen receptor-α-positive breast tumor cells through multiple molecular cross-talks. *Mol Cancer Res*. 2005;3:151–162.
41. **Sengupta K, Banerjee S, Dhar K, et al.** WISP-2/CCN5 is involved as a novel signaling intermediate in phorbol ester-protein kinase Cα-mediated breast tumor cell proliferation. *Biochemistry*. 2006;45:10698–10709.
42. **Pennica D, Swanson TA, Welsh JW, et al.** WISP genes are members of the connective tissue growth factor family that are up-regulated in Wnt-1-transformed cells and aberrantly expressed in human colon tumors. *Proc Natl Acad Sci*. 1998;95:14717–14722.
43. **Jin T, George Fantus I, Sun J.** Wnt and beyond Wnt: multiple mechanisms control the transcriptional property of beta-catenin. *Cell Signal*. 2008;20:1697–1704.
44. **Ip W, Chiang YT, Jin T.** The involvement of the wnt signaling pathway and TCF7L2 in diabetes mellitus: the current understanding, dispute, and perspective. *Cell Biosci*. 2012;2:28.
45. **Lake AC, Bialik A, Walsh K, Castellot JJ Jr.** CCN5 is a growth arrest-specific gene that regulates smooth muscle cell proliferation and motility. *Am J Pathol*. 2003;162:219–231.

Comment on: Turban et al. Optimal Elevation of β-Cell 11β-Hydroxysteroid Dehydrogenase Type 1 Is a Compensatory Mechanism That Prevents High-Fat Diet–Induced β-Cell Failure. Diabetes 2012;61:642–652

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Turban et al. (1) recently reported a surprise finding that moderately elevated 11β-hydroxysteroid dehydrogenase type 1 (11β-HSD1) expression in the pancreatic β-cells promoted a compensation against high-fat (HF) diet–induced β-cell failure because glucocorticoids are well established to impair insulin secretion and cause β-cell death and insulin resistance in key insulin targets. Rather than accepting the U-shaped dose response, nongenomic (glucocorticoid receptor–independent) mechanism, or even mineralocorticoid receptor–mediated effect, a simpler approach is to question the model. While we have no reason to doubt the data integrity, the evidence used to establish HF diet–induced β-cell failure was far from convincing. Although an established procedure of using a 58% fat diet to feed mice for 12 weeks was used, wild-type mice did not become obese by gaining any weight, there was no elevation in fasting blood glucose and serum insulin levels (Table 1), and no significant decrease in glucose-stimulated insulin secretion caused by HF diet and measured by area under the curve (Fig. 2*C*, first two columns in ref. 1), leading one to question the authors' statement that "HF-fed KsJ mice … showed markedly attenuated GSIS [glucose-stimulated insulin secretion] indicative of β-cell failure and … (Fig. 2*B* and *C*)" (1). The HF feeding experiment has clearly failed to cause obesity and insulin resistance versus our previously published findings (2); there was no obvious sign of β-cell failure. How could one compensate a nonfailure?

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REFERENCES

1. Turban S, Liu X, Ramage L, et al. Optimal elevation of β-cell 11β-hydroxysteroid dehydrogenase type 1 is a compensatory mechanism that prevents high-fat diet–induced β-cell failure. Diabetes 2012;61:642–652
2. Lu Y, Herrera PL, Guo Y, et al. Pancreatic-specific inactivation of IGF-I gene causes enlarged pancreatic islets and significant resistance to diabetes. Diabetes 2004;53:3131–3141

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LETTER

Is 11β-HSD1 expressed in islet β-cells and regulated by corticotropin-releasing hormone?

We read with interest the PNAS article by Schmid et al. entitled "Modulation of pancreatic islets-stress axis by hypothalamic releasing hormones and 11β-hydroxysteroid dehydrogenase" (11β-HSD) (1). Although we welcome most of the findings and interpretation, several points raised our concern.

i) After analyzing the enlarged digital versions of Fig. 5*A* and *B*, we do not agree with the authors' conclusion that INS-1 cells had positive expression of 11β-HSD1 mRNA and protein; there was hardly any detectable protein/mRNA band in the Western blot and RT-PCR gel image. More troubling evidence was from Fig. 6*A* vs. Fig. 6*B* showing an approximately 27-fold higher level of 11β-HSD2 vs. -HSD1, adding to our doubt whether 11β-HSD1 was expressed in these cells. We therefore disagree with the following italicized phrases in their Results (1): "RT-PCR analysis revealed mRNA expression of *11β-HSD-1* and 11β-HSD-2 in *rat INS-1 cells*, primary rat islets and primary human islets [Fig. 5*A*]. Western blot analysis documented the *protein expression of 11β-HSD-1 (35 kDa) in INS-1 cells*, rat islets, and human islets [Fig. 5*B*]."

ii) The left panels in Fig. 5 *C* and *D* seemed to indicate 11β-HSD1 staining in most islet cells or at least many β-cells, and in cell nuclei, if one compares them with the cytosolic staining of insulin on the right panels. They contradict current consensus that 11β-HSD1 is expressed by α- and PP- rather than β-cells of the pancreas and mostly in microsome (cytosol), which should not have been ignored by the authors (2). In fact, we have observed similar nonspecific staining, as extensive as that of insulin, when using presumably the same anti-11β-HSD1 antibody marketed by Abcam (cat. no. ab39364). By contrast, the anti–11β-HSD1 antibody procured from Santa Cruz Biotechnology (cat. no. sz20175) provided more specific, α-cell–specific signal in our study.* Therefore, we believe that the authors' statement (1) that, "In addition, the 11β-HSD-1 protein was detected by immunohistochemical staining in human pancreatic islets [Fig. 5*C*], rat pancreatic islets [Fig. 5*D*], and INS-1 cells [Fig. 5*E*]" is misleading and inaccurate.

iii) To measure 11β-HSD enzyme activity, enzyme immunoassay was used, as in the supplemental data. We believe the current standard is to use radiolabeled substrates ([^{3}H]dehydrocorticosterone) and separate it from the conversion product (corticosterone) using TLC followed by scintillation counting of radioactivity on the corresponding bands (2, 3). We thus doubt the specificity and sensitivity of the assay result in Fig. 6*C*. For instance, concerning the assay sensitivity, the result difference was 240 vs. 300 pg/mL, whereas the detection limit is at 57 pg/mL (cat. no. ADI-900-071; Enzo Life Sciences) (1). If so, especially if INS-1 cells hardly express 11β-HSD1, the result contained in Fig. 6*C* may not, in our opinion, be accurate.

Thus, we have reservations on the interpretation of Figs. 5 and 6 and the findings concerning 11β-HSD1. Specifically, we disagree with the authors' conclusion that 11β-HSD1 was expressed in normal β-cells and INS-1 cells based on Fig. 5 (1). If 11β-HSD1 was not supposed to be expressed in INS-1 cells, especially if a nonconventional enzyme assay has been used, the result of Fig. 6*C* showing corticotropin-releasing hormone-induced inhibition also becomes questionable.

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1. Schmid J, et al. (2011) Modulation of pancreatic islets-stress axis by hypothalamic releasing hormones and 11β-hydroxysteroid dehydrogenase. *Proc Natl Acad Sci USA* 108: 13722–13727.
2. Swali A, Walker EA, Lavery GG, Tomlinson JW, Stewart PM (2008) 11beta-Hydroxysteroid dehydrogenase type 1 regulates insulin and glucagon secretion in pancreatic islets. *Diabetologia* 51:2003–2011.
3. Man TY, et al. (2011) Dietary manipulation reveals an unexpected inverse relationship between fat mass and adipose 11β-hydroxysteroid dehydrogenase type 1. *Am J Physiol Endocrinol Metab* 300:E1076–E1084.

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