Physiological role of CCN5 in pancreatic ß cells: effects on proliferation, survival and insulin secretory function

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ABSTRACT

Type 2 diabetes (T2D) is a complex, progressive disease that results from an interplay between genetic and environmental factors that unfavorably affect β-cell function and tissue insulin sensitivity. The rate of progression of the disease is mainly associated with β-cell abnormalities. A failure in β-cell compensation for prevailing tissue insulin resistance is the main trigger for T2D. Multiple factors lead to β-cell failure and eventual decompensation; those include hyperglycemia, hyperlipidemia, chronic β-cell stimulation and impaired incretin effect. These stressors combined with β-cell limited regenerative capacity will result in β-cell apoptosis, autophagy and dedifferentiation. Novel therapeutic avenues are currently focusing on characterizing regulators or growth factors that target β cells themselves.

Our lab identified CCN5 as a matricellular protein that displays growth promoting effects in pancreatic β cells. We characterized CCN5 in whole genome cDNA microarray analysis of IGF-I overexpressing islets. CCN5 was among the genes that were significantly upregulated in the islets of these mice. Furthermore, overexpression of CCN5 in MIN6 insulinoma cells enhanced the proliferation and survival of these cells. Collectively, these results raised our interest in CCN5's physiological effects in pancreatic β cells.

For my PhD project, I hypothesize that CCN5/WISP2 is a growth factor that stimulates proliferation, survival and function of pancreatic ß cells through the binding to a high-affinity receptor and activation of downstream targets. Thereby, I investigated the physiological effects of CCN5 in pancreatic ß cells using recombinant human CCN5 (rh-CCN5) insulinoma cells INS 832/13, and primary mouse islets.

We found that rh-CCN5 stimulated the proliferation of INS 832/13 and mouse islets via a pathway involving FAK/ERK activation and ultimately leading to the stimulation of cell cycle

regulators CDK4 and Cyclin D1, respectively. Rh-CCN5 also protected INS 832/13 and mouse islets from lipotoxicity, glucolipotoxicity and streptozotocin (STZ) induced-cell death. The expression of key genes associated with ß cells identity and function was also enhanced upon CCN5 addition in culture (Chapter II). We then showed a biphasic regulation of insulin secretion by CCN5 that seems to be dependent on the ER stress/UPR activity in these cells (Chapter III). Using ligand receptor capture technology, we identified neuronal acetylcholine receptor subunit alpha-3 (Chrna3) as a potential receptor for CCN5 on the surface of insulinoma MIN6 cells (Chapter IV). Finally, using microarray gene expression profiling, we identified novel CCN5 targets in primary islets; those are involved in GPCR signaling, wound healing, and immune response (Chapter IV).

These studies have confirmed the growth promoting properties of CCN5 in pancreatic ß cells and characterized the mechanism by which CCN5 exerts such effects. Overall CCN5 represents a potential candidate that could enhance ß cells physiology in vitro and additional studies would be essential to validate its functions in vivo.

Résumé

Le diabète de type 2 (DT2) est une maladie complexe et progressive résultant de l'interaction entre des facteurs génétiques et environnementaux. Cette maladie affecte la fonction des cellules-β ainsi que la sensibilité à l'insuline tissulaire. Le taux de progression de la maladie est principalement lié aux anomalies de la cellule-β. Une défaillance dans la compensation des cellules-β, pour la résistance à l'insuline tissulaire prédominante est le principal déclencheur du DT2. De nombreux facteurs mènent à la défaillance des cellules β et à leurs décompensations éventuelles tels que l'hyperglycémie, l'hyperlipidémie, la stimulation chronique et l'effet altéré de l'incrétine. Ces facteurs de stress ainsi que la capacité limitée de régénération des cellules β, entraînent l'apoptose, l'autophagie et la dédifférenciation. Les nouvelles voies thérapeutiques se concentrent présentement sur la caractérisation des régulateurs ou les facteurs de croissance ciblant les cellules β.

Notre laboratoire a identifié CCN5 comme une protéine matricellulaire qui présente des effets favorisants la croissance des cellules β pancréatiques. Nous avons caractérisé CCN5 dans l'analyse par micropuce d'ADNc du génome entier des îlots surexprimant IGF-I. Nous avons ainsi trouvé que CCN5 faisait partie des gènes régulés positivement dans les îlots de ces souris. De plus, la surexpression de CCN5 dans les cellules d'insulinome MIN6 a amélioré leurs taux de proliférations et survies. Par conséquent, nous avons montré un intérêt pour les effets physiologiques de la CCN5 dans les cellules β pancréatiques.

Pour mon projet de doctorat, j'ai formulé une hypothèse stipulant que la CCN5 / WISP2 est un facteur de croissance qui stimule la prolifération, la survie et la fonction des cellules ß pancréatiques à travers un récepteur spécifique ainsi que l'activation de cibles en aval. Nous avons ainsi étudié les effets physiologiques de la CCN5 dans les cellules ß pancréatiques en utilisant la

protéine recombinante humaine CCN5 (rh-CCN5), les cellules d'insulinome INS 832/13 et des îlots de souris primaires.

Nous avons constaté que le rh-CCN5 stimule la prolifération d'INS 832/13 et d'îlots de souris via une voie impliquant l'activation de FAK/ERK, conduisant finalement à la stimulation des régulateurs du cycle cellulaire CDK4 et Cycline D1 respectivement. Rh-CCN5 a aussi protégé INS 832/13 et les îlots des souris de la mort cellulaire induite par la lipotoxicité, la glucolipotoxicité et la streptozotocine (STZ), en régulant positivement l'expression de gènes clés associés à l'identité et à la fonction des cellules ß (Chapitre II). Nous avons ensuite montré une régulation biphasique de la sécrétion d'insuline par CCN5 qui semble dépendre de l'activité de stress ER/ UPR dans ces cellules (chapitre III). En utilisant la technologie de capture du récepteur de ligand, nous avons identifié la sous-unité alpha-3 (Chrna3) du récepteur de l'acétylcholine neuronale comme récepteur potentiel de CCN5 à la surface des cellules MIN6 de l'insulinome (Chapitre IV). Enfin, en utilisant le profil d'expression des gènes de puces à ADN, nous avons identifié de nouvelles cibles CCN5 dans des îlots primaires, impliquées dans la signalisation du GPCR, la cicatrisation des plaies et la réponse immunitaire (Chapitre IV).

Ces études ont confirmé les propriétés promotrices de la croissance de CCN5 dans les cellules ß pancréatiques et ont permis de caractériser le mécanisme par lequel CCN5 exerce de tels effets. Globalement, CCN5 représente un candidat potentiel qui pourrait améliorer la physiologie des cellules ß in vitro et des études supplémentaires seraient fondamentales pour valider ses fonctions in vivo.

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LIST OF ABBREVIATIONS

ADA	American Diabetes Association
AGE	Advanced glycation end
Ap1	Activator protein
AP-1	Activating protein- 1
ATP	Adenosine triphosphate
ATP	Adenosine triphosphate
BMPs	Bone morphogenic proteins
CK1	Casein kinase 1
CT	Cysteine-knot-containing module
CTGF	Connective tissue growth factor
CREB	Cyclic AMP-responsive element binding protein
Cyr61	Cysteine-rich protein 61
ECM	Extracellular matrix
EGF	Epidermal growth factor
EMT	Epithelial-to-mesenchymal transdifferentiation
ER	Endoplasmic reticulum
ER	Estrogen receptor
ERE	Element response element
ER-α	Estrogen receptor alpha
FFAs	Free Fatty Acids

FOXO1	Forkhead box protein O1
Fzd	Frizzled
G6P	Glucose 6-phosphate
GIP	Gastric Inhibitory Polypeptide
GLP-1	Glucagon-like peptide-1
GLUT2	Glucose transporter
GLUT4	Glucose transporter type 4
GSIS	Glucose-stimulated insulin secretion
GSK-3B	Glycogen synthase kinase
HIF	Hypoxia inducible factor
HSPGs	Heparan Sulfated Proteoglycans
IAPP	Islet amyloid polypeptide
IDF	International Diabetes Federation
IGF-1	Insulin growth factor I
IGFBP	Insulin-like growth factor binding protein-like
IR	Insulin receptors
IRS	Insulin receptor substrates
ISLET1	Insulin Gene Enhancer Proteins 1
LDL	Low density lipoproteins
LRP-1	Lipoprotein receptor-related protein
LRPs	Lipoprotein receptor-related proteins
M6P	Mannose-6-phosphate
MAFA	V-maf musculoaponeurotic fibrosarcoma oncogene homolog A

MMPs	Matrix metalloproteinases
NeuroD1	Neurogenic Differentiation 1
NFκβ	Nuclear factor kappa-light-chain- enhancer of activated B cells
NGN3	Neurogenin-3
NKX6.1	Homeobox Protein
NOV	Nephroblastoma Overexpressed Gene
NTRK1	Neurotrophic tyrosine kinase receptor 1
PAX4	Paired box gene 4
PAX6	Paired Box Gene 6
PDX1	Duodenal Homeobox 1
PKA	Protein Kinase A
PP	pancreatic polypeptide
PPNAD	Primary pigmented nodular adrenocortical disease
PTH	Parathyroid hormone
RER	Rough endoplasmic reticulum
Shc proteins	SHC-transforming protein
SMC	Smooth muscle cells
SNAP-25	Synaptosomal-associated protein
SNAREs	Soluble N-ethylmaleimide-sensitive factor attachment protein receptor proteins
SP1	Specificity proteins 1
T1D	Type 1 diabetes
T2D	Type 2 diabetes
T3	Thyroid hormone

TCF7	Transcription factor 7
TGFβ	Transforming growth factor-β
TGN	Trans-Golgi network
TSP-1	Thrombospondin type-1 repeat module
TSR-1	Thrombospondin type-1 repeats
VDCCs	Voltage-Dependent Ca+channels
VEGF	Vascular Endothelial Growth Factor
VSMCs	Vascular smooth muscle cells
VWC	Von Willebrand factor type C
WISP	Wnt inducible signaling proteins

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CONTRIBUTIONS TO ORIGINAL KNOWLEDGE

Chapter II: I demonstrated that CCN5 exerts growth stimulatory effects on pancreatic β-cells in vitro. Using recombinant human protein CCN5, I established that CCN5 is a secreted protein that enhances β cell proliferation, survival and function through a mechanism involving the activation of FAK and ERK.

Chapter III: I evaluated the effect of CCN5 on the function of β-cells focusing on insulin secretion. I came to the conclusion that CCN5 exerts a biphasic, time-dependent regulation on insulin secretion and content in mouse islets. This regulation was found to be associated with ER stress/UPR status of the β-cell

Chapter IV: I identified potential high affinity receptors for CCN5 on the surface of MIN6 cells using the novel technology LRC-TriCEPS. I also determined novel targets for CCN5 in primary islets; those targets are involved in G-protein coupled receptor signaling, immune response and chemotaxis.

CONTRIBUTIONS OF AUTHORS

Chapter II: "Recombinant protein CCN5/WISP2 promotes islet cell proliferation and survival in vitro" by Nancy Kaddour, Di Zhang, Zu-hua Gao and Jun-Li Liu (Growth Factors, 2019, vol 3: 120-130).

N.K. performed all experiments, analyzed the data, and wrote the manuscript. Z.D helped in some of the experiments and analysis. J.-L.L. designed the study, revised the manuscript, and approved the final revision. Z.G. helped in designing and analysis of the data.

Chapter III: "Recombinant protein CCN5/WISP2 exerts a time-dependent, biphasic effect on insulin secretion in primary mouse islets" by Nancy Kaddour, Zu-Hua Gao and Jun-Li Liu. (In preparation for Molecular and Cellular endocrinology).

N.K. performed all experiments, analyzed the data, and wrote the manuscript. J.-L.L. designed the study, revised the manuscript, and approved the final revision. Z.G. helped in designing and analysis of the data.

Chapter IV: Explorations on the target genes and cell surface receptors that mediate CCN5 effects in pancreatic ß cells by Nancy Kaddour, Larson Grimm, Zu-hua Gao and Jun-Li Liu (In preparation).

N.K. performed all experiments, analyzed the data, and wrote the manuscript. LG assisted in the microarray design and procedure. J.-L.L. designed the study, revised the manuscript, and approved the final revision. Z.G. helped in designing and analysis of the data.

CHAPTER I

GENERAL INTRODUCTION AND LITERATURE REVIEW

1. Regulation of Glucose homeostasis

Glucose is a simple sugar that results from the breakdown of dietary carbohydrates. This simple sugar is considered a key energy source for all animals ranging from bacteria to humans. Interestingly glucose is the primary fuel for the brain, as normal brain function is dependent on a continuous supply of glucose either through carbohydrate intake or through endogenous production.

Normal human body function is dependent on tight control of blood glucose levels. Glycemic control is accomplished by a complex network of hormones and neuropeptides that are released by several interconnected organs, including the brain, pancreas, liver, muscle, and adipose tissues [1]. The pancreas, and more specifically, the α and ß cells within the islets of Langerhans are the key players in this regulation. α-cells produce the catabolic hormone glucagon, which increases blood glucose levels by inducing the breakdown of macronutrients in the liver and adipose tissues. ß cells release the anabolic hormone insulin, which lowers blood glucose levels by promoting its uptake by the muscle, liver, and adipose tissues. Thus, glucose homeostasis is maintained by a reciprocal interplay between insulin and glucagon.

According to the American Diabetes Association (ADA) guidelines, normal blood glucose levels are in the range of 80-130 mg/dL under fasting conditions and less than 180 mg/dl postprandially. Failure to maintain glucose levels within these ranges is associated with serious metabolic diseases, including obesity and diabetes [1]

2. The pancreas

The pancreas is located behind the stomach within the left upper abdominal cavity and is partitioned into head, body, and tail. The pancreas regulates macronutrient digestion and therefore is involved in metabolism and energy homeostasis [1] (Figure I-1).

A. Exocrine partition

The majority of the pancreas consists of exocrine cells known as acinar cells, which regulate the digestive activities of the pancreas. When food enters the stomach, acinar cells release pancreatic enzymes into a system of small ducts that lead to the main pancreatic duct, which runs the length of the pancreas. Pancreatic enzymes include amylase, pancreatic lipase, and trypsinogen. These enzymes form an alkaline fluid known as the pancreatic juice, which is released into the intestine, where it assists in the digestion of fats, carbohydrates, and proteins [1] (Figure I-1). `

B. Endocrine partition

The endocrine cells account for only 1–2% of the entire organ. These cells are clustered together, forming small island-like structures known as the islets of Langerhans, which regulate the endocrine function of the pancreas by releasing various hormones directly into the blood stream [2].

There are five different types of endocrine cells each releasing different hormones: 1) insulin- producing β-cells account for 65–80% of the total cells , 2) glucagon producing α-cells, represent 15–20% of the total islet cells [3], 3) somatostatin- producing δ-cells constitute 3–10% of the total cells, 4) pancreatic polypeptide (PP)-producing γ-cells, comprise 3–5% of the total islet cells [4], and 5) ghrelin-producing ε-cells comprise <1% of the total islet cells [5]. Each of these

hormones has different functions but, overall, they are all implicated in glucose homeostasis in vertebrates (Figure I-1).

3. Pancreatic β-cells

The pancreatic β-cells are the predominant endocrine cells in the islets; their primary function consists of synthesizing, storing, and releasing insulin, that tightly regulates circulating glucose levels. The average diameter of a β cell is 10 μm, and the insulin content per cell and is about 20 pg [6, 7]. In an adult human being, β-cells release about 30–70 U insulin per day, most of which is secreted postprandially [6, 7]. The average β-cell mass in the human pancreas varies from 0.6 to 2.1 g depending mostly on the individual's body mass index and his/her health status. An individual's total β-cell mass is established very early in childhood as the proliferation of β cells is activated shortly after birth, peaks for about one year, and then rapidly declines, to become negligible in the adult stage [8].

β-cells are the first endocrine cells to appear in the human pancreas, which corresponds to 7.5–8 weeks of gestation [9]. Subsequently, at gestational week 8, somatostatin and glucagon secreting cells develop, and then followed by ghrelin-secreting cells around gestational week 9 [9]. The commitment of progenitor cells toward an endocrine cell phenotype is essentially dependent on the expression of several transcription factors, including Neurogenin-3 (NGN3) [10] homeobox protein NKX2.2, paired box gene 4 (PAX4), pancreatic and duodenal homeobox 1 (PDX1), paired box gene 6 (PAX6), homeobox protein NKX6.1, and insulin gene enhancer proteins 1 (ISLET1) [11]. Maintenance of β-cell identity postnatal is as well dependent on the expression of key transcription factors, in particular, V-maf musculoaponeurotic fibrosarcoma oncogene homolog A (MAFA), PDX1, forkhead box protein O1 (FOXO1), and NKX6.1.

4. Insulin

Insulin is the central anabolic hormone of the body, as it regulates the metabolism of most macronutrients, including carbohydrates, fats, and proteins. Insulin is synthesized and released primarily by pancreatic β-cells in response to elevated blood glucose. Once in the bloodstream, insulin promotes the absorption of glucose from the blood into the liver, skeletal muscle, and adipose tissues.

A. Insulin synthesis, processing, and packaging in pancreatic β-cells

The gene encoding preproinsulin displays variabilities among species, as humans have a single gene located on chromosome 11, while rodents such as rats and mice have two copies of the gene, ins1 and ins2 [12, 13]. In humans, the gene is expressed exclusively in the β-cells of the islet of Langerhans, and the primary regulator of its expression is the concentration of blood glucose. The transcription of this gene is regulated by upstream enhancer elements that bind β-cell specific transcription factors such as PDX1, neurogenic differentiation 1 (NeuroD1) and MafA, along with other coregulators [14].

The coding region of the preproinsulin gene consists of three exons. The first exon encodes the signal peptide at the N terminus. The second encodes the B chain in addition to a part from the C peptide and the third encrypts the rest of the C-peptide and the A chain. Transcription and splicing of introns yield a 600 nucleotides mRNA, which once translated, give rise to the precursor preproinsulin. The latter is rapidly discharged into the rough endoplasmic reticulum (RER), where it is processed by peptidases that cleave its signal sequence. The resulting protein is called proinsulin that consists of an amino-terminal B chain, a carboxy-terminal A chain and a connecting C peptide in the middle. The proinsulin is then transported to the Golgi apparatus in microvesicles, where it is packaged in membrane-bound vesicles known as secretory granules. In the Golgi, the

prohormone convertases PC1/3 and PC2 and carboxypeptidase E converts proinsulin into insulin by removing the C peptide [13, 15]. Insulin and C-peptide are stored together in the secretory granules and ultimately produced together by regulated exocytosis [15]. When blood glucose level reaches a threshold (4-6 mM), insulin is secreted from the cell by exocytosis and is diffused into the blood stream [16]. However, not all insulin is released into the bloodstream, 1-2% of the protein remains as proinsulin within mature secretory granules, where insulin couples with Zn^{2+} forming a hexametric crystal (Figure I-2).

B. Insulin secretion mechanism

Glucose entry into the β-cells via the surface membrane facilitative glucose transporter (GLUT2) stimulates insulin release. In the β-cells, glucokinase phosphorylates glucose into glucose 6-phosphate (G6P), which undergoes glycolysis, generating adenosine triphosphate (ATP). An increase in ATP levels leads to closure of ATP-sensitive K^+-channels (K_{ATP}-channels), membrane depolarization, and subsequent opening of voltage-dependent Ca^+channels (VDCCs). Elevated level of intracellular calcium ions induces the fusion of insulin-containing granules with the membrane and the release of their content [17, 18]. A family of proteins known as SNAREs (soluble N-ethylmaleimide-sensitive factor attachment protein receptor proteins) mediates the fusion of the insulin-containing vesicles with the plasma membrane. The key SNAREs proteins involved are SNAP-25 (synaptosomal-associated protein), syntaxin-1, and synaptobrevin 2 [19] (Figure I-3).

C. The fate of insulin after secretion

Insulin is released into the interstitial space of the pancreas, where it readily finds its way into the portal circulation to be delivered to the liver. In its “first pass” in the liver over 50% of insulin is cleared. The remaining insulin is delivered to the heart via the hepatic vein. At this stage,

insulin shifts its route to the arterial circulation, which promotes its delivery to fat cells, skeletal muscles, and the liver for a second pass. In the muscle and fat cells, insulin stimulates GLUT4 translocation to the surface membrane, thereby facilitating glucose uptake and storage. Remaining circulating insulin ultimately reaches the kidney for degradation [13] (Figure I-4).

D. Insulin receptor signaling

Insulin mediates its biological effects via the insulin receptors (IR), which belongs to a family of homologous tyrosine kinase receptors. These receptors are tetrameric proteins that consist of two extracellular subunits and two transmembrane ß subunits that are joined by disulfide bonds. Binding of insulin to its receptor induces a conformational change and autophosphorylation of the ß subunits, leading to the recruitment and phosphorylation of insulin receptor substrates (IRS), and SHC-transforming protein (Shc proteins) [20]. IRS proteins mostly mediate the activation of the PI3K-Akt pathway, while Shc mediates the Ras-MAPK pathway.

The PI3K-AKT pathway mediates most of insulin's metabolic effects, including glucose regulation and transport, lipid synthesis, gluconeogenesis, and glycogen synthesis. The keys events triggered by this pathway are the translocation of glucose transporter type 4 (GLUT4) to the membrane and the inactivation of the transcription factor forkhead box protein O1 (FOXO1)[21]. On the other hand, the Shc-RAS-MAPK pathway regulates cell proliferation and gene transcription [22].

Perturbations in the PI3K-AKT signaling pathway have been linked to a state of insulin resistance [23] that is characterized by a diminished ability of the peripheral tissues (mostly liver, fat cells, and muscle cells) to respond to the metabolic effects of insulin. Consequently, glucose is impeded from entering into the corresponding cells/tissues and a state of hyperglycemia prevails.

Insulin resistance is a hallmark of multiple metabolic diseases [24, 25], particularly diabetes mellitus.

E. Insulin resistance

Insulin resistance is a state where the liver and the muscle, which are the main tissues responsible for glucose disposal, become insensitive to insulin metabolic effects [26]. Interestingly, other tissues seem to be affected by insulin resistance such as the kidney [27], gastrointestinal tract [28] adipose [29, 30], vasculature [31] brain [32] tissues, and pancreatic β-cells [33]. The main causative agents of the state of insulin resistance are obesity, physical inactivity, and genetic predisposition [34]. Altogether, they lead to β-cell stress, and dysfunction and culminate in a progressive decline in insulin secretion [35]. Insulin resistance is the main prerequisite for T2D, and it precedes the diseases by many years [36, 37].

In the muscle, many perturbations lead the resistance state, including defects in glucose transport and phosphorylation, insulin signaling, glycogen synthesis, and mitochondrial oxidative activity [38]. In the liver, insulin resistance is a consequence of insulin deficiency, enhanced glucagon sensitivity, increased rate of gluconeogenesis [39, 40], and fasting hyperglycemia. Finally, at the level of the kidneys, insulin resistance results from augmented gluconeogenesis and glucose reabsorption.

At the molecular level, insulin resistance is associated with perturbations in the PI3K-AKT pathway [23]. In this case, the IRS proteins are subjected to increased serine phosphorylation. The latter inhibits tyrosine phosphorylation, leads to IRS degradation, and result in insulin resistance. [41]. Excessive serine phosphorylation can be attributed to inflammation, endoplasmic reticulum (ER) stress, ectopic lipid accumulation, and mitochondrial dysfunction.

5. Diabetes Mellitus

Diabetes is a metabolic disease that is characterized by a state of chronic hyperglycemia that results from perturbations in insulin secretion, insulin action, or both. It's a heterogeneous disorder with multiple etiologies, either occurring in childhood as type 1 diabetes (T1D) or occurring at a later age in adults as type 2 diabetes (T2D).

A. Diabetes Prevalence: Epidemiology

Diabetes has become one of the leading causes of death worldwide. In 2017, the international diabetes federation (IDF) reported 4 million deaths caused by diabetes. Currently, about 425 million adults are living with diabetes, and this number is expected to rise to 629 million by 2045. The socio-economic status of a country is directly proportional to the disease' prevalence, as 79% of adults with diabetes live in low- and middle-income countries (IDF Diabetes Atlas 2017, 8th edition).

Various pathological complications affecting the heart, eyes, kidneys, and nerves are associated with T2D. Consequently, this ailment places a severe economic burden on governments and individuals. IDF reported an approximate of 727 billion USD dollars in treatment expenditures in 2017 (IDF Diabetes Atlas 2017, 8th edition).

B. Type 1 Diabetes (T1D)

T1D usually presents early in childhood and accounts for 5%–10% of all diabetes cases. It results from a spontaneous or environmentally triggered autoimmune attack on β cells, which leads to marked β-cell deficiency and dysfunction [42]. The presence of autoantibodies in the serum against the pancreatic islet cells is the hallmark of this disease [42]. Possible triggers of TID include genetic predisposition [43, 44], environmental factors such as viruses [45, 46]. Type 1 diabetic children and adolescents usually display several symptoms ranging from polydipsia,

polyuria, lack of energy, polyphagia, sudden weight loss, recurrent infections, blurred vision, to diabetic ketoacidosis [47].

The primary treatments for T1D involve lifelong insulin injections. Other approaches include pancreatic or islets transplantation [48, 49]. However, this approach is not sufficient as it requires organ donors and lifelong treatment of immunosuppression.

C. Type 2 Diabetes

Type 2 diabetes mellitus (T2D) is the most common form of the disease accounting for more than 90% of all cases. T2D is a multifactorial disease involving genetic and environmental factors. The environmental risk factors outweigh the genetic ones, as the manifestation of the disease is tightly associated with the lifestyle of the patients. Increasing adiposity, physical inactivity, cigarette smoking, and abnormal sleeping routine are all risk factors for T2D. Although it might not be that significant, but genetics play an important part in its development as the disease clusters in families and is heritable [50]. Furthermore, mutations in genes associated with β-cell function present high-risk factors [51, 52].

The pathophysiological changes that are associated with T2D include β-cell dysfunction, insulin resistance, and chronic inflammation, all of which progressively hamper control of blood glucose levels. T2D patients as well suffer from reduced β-cell mass caused mainly by apoptosis and dedifferentiation. Besides, insulin resistance is not only confined to the liver and muscle, but other tissues and organs are also affected, such as adipose, kidney, gastrointestinal tract, vasculature, brain tissues, and pancreatic β-cells themselves [53].

Due to its multifactorial nature, T2D management is complicated and encompasses several lines of treatments. Lifestyle adjustment is requisite; this includes an active lifestyle and dietary

restrictions. On the other hand, antidiabetic medications that target insulin resistance and boost β-cell function should be administered regularly to achieve glycemic homeostasis.

6. β-cell state in T2D

β-cells are key parts in the etiology of T2D. During the course of the disease, β-cells undergo severe metabolic stresses that culminates in their dysfunction and ultimate loss. The main trigger of β-cell dysfunction is insulin resistance; a condition that is associated with obesity, physical inactivity, and genetic predisposition. A chronic state of insulin resistance triggers a progressive increase in insulin demand, which ultimately leads to β-cell exhaustion and gradual dysfunction [54]. At this point, the β-cell loses its capacity to meet insulin demand, and T2D occurs. Reduction in β-cell mass, which is another hallmark of the disease, is as well a major contributor to diabetes progression. Examinations of specimens from patients with T2D reveals a 30%-40% reduction in β-cell mass [55], which is mainly attributed to β-cells loss via apoptosis [56] and dysregulated autophagy [57]. Dedifferentiation of β-cells into α and δ-like cells [57, 58] in addition to vascular disarray and amyloid deposition [59] represent other causes of β-cell loss in T2D. On the other hand, diminished β-cell proliferation doesn't seem to be a fundamental causative agent of the disease, as the proliferative capacity of diabetic and normal islets appear to be similar [60, 61].

A. β cell dysfunction

Insulin resistance is indeed a prerequisite for T2D, however chronic hyperglycemia and overt T2D don't occur until β-cells are unable to release sufficient insulin to offset the state of resistance. To overcome insulin resistance, β-cells compensate by increasing their mass and their secretory function. However, they will reach a point where no further compensation can

competently regulate glucose metabolism. At this stage, β-cells experience a state of stress, which leads to their progressive failure and dysfunction. Many factors contribute to β-cell dysfunction, such as aging, genetic abnormalities, hypersecretion of islet amyloid polypeptide (IAPP), resistance and/ or deficiency of (glucagon-like peptide 1 (GLP1) and gastric inhibitory polypeptide (GIP), ER stress, lipotoxicity, glucotoxicity, reactive oxygen stress and activation of inflammatory pathways.

i. Hyperglycemia/glucotoxicity

A large number of in vitro and in vivo studies have reported that chronic elevated glucose concentrations are associated with impaired β-cell function and insulin action [62]. Prolonged exposure of insulinoma INS1 cells to high concentrations of glucose impaired glucose stimulated insulin secretion (GSIS), insulin content, insulin gene expression, insulin promoter activity, and expression of (PDX-1) and MafA [63]. Similar results were obtained in isolated rat islets cultured in high glucose concentrations (16.7 mM) for six weeks [64]. The mechanisms by which chronic high glucose concentrations induce ß-cell dysfunction include: 1) formation of toxic advanced glycation end products (AGE) [65], 2) Oxidative stress [66] 3) Mitochondrial dysfunction and 4) deregulated expression of genes associated with ß-cells function and identity [67].

ii. Dyslipidemia/Lipotoxicity

Lipotoxicity is a state of ß-cell stress and impairment that results from dyslipidemia or chronic exposure to high levels of circulating free fatty acids (FFAs). Studies have shown that prolonged exposure of isolated islets and insulinoma cells to elevated levels of FFAs resulted in impaired glucose-stimulated insulin secretion (GSIS), a decrease in insulin gene expression, and cell death via apoptosis [61]. Nevertheless, FFAs display beneficial effects on ß-cell function under physiological concentrations as they prove to be essential for normal insulin function and GSIS

[68]. Hence, the effects of FFA on ß-cell function are much more complex and are dependent on other factors such as concentration, duration of exposure, and the coexistence with hyperglycemia and insulin resistance [69]. The mechanisms by which chronic FFAs affect GSIS and ß-cell function include: 1) reduced transcription of the Ins gene due to a decreased binding of the ß-cell transcription factors Pdx1 and MafA on the Ins promoter [70] 2) activation of the mediator of apoptosis protein kinase C δ [71], 3) induction of ceramides (Bcl-2 inhibitor) synthesis [72] 4) upregulation of uncoupling protein 2 (UCP2), 5) and activation of oxidative stress [73] and the unfolded protein response pathways [74].

B. Endoplasmic reticulum (ER) stress and unfolded protein response (UPR)

The endoplasmic reticulum (ER) is a dynamic subcellular compartment involved in the regulation of essential activities such as lipid production, calcium storage, and protein biosynthesis. The ER guarantees proper protein folding, assembly, maturation and transportation to final destination [75]. Disturbance in any of these processes, particularly uncontrolled protein misfolding triggers a state known as ER stress. In such conditions, the ER activates a series of signaling events known collectively as the UPR, which aim to restore ER homeostasis. Initiation of UPR is primarily mediated by heat-shock protein/chaperone, GRP78 known as BiP. GRP78 associates with misfolded proteins and simultaneously disassociates from the three major ER-resident UPR pathway initiating molecules, IRE1, PERK and ATF6, triggering their activation and the downstream UPR (Figure I-5) [76]. The activation of UPR result in three outcomes: decreased translation, restoration of protein folding and ER-associated degradation (ERAD) of misfolded proteins. In some cases of prolonged or severe ER stress, the UPR can induce inflammation, autophagy and apoptosis.

As their most fundamental function is to synthesize and secrete insulin, β-cells possess a well-developed ER that enable them to handle a large biosynthetic load. The episodic nature of insulin secretion and biosynthesis suggest a consequent episodic variation in baseline UPR activation. Alterations in the ER homeostasis and dysregulated UPR have been linked to β-cell dysfunction and diabetes [6]. For instance, mutation in the INS2 gene in Akita mice resulted in progressive β-cell apoptosis associated with proinsulin misfolding, β-cell ER stress and CHOP expression [77. Furthermore, β-cell-specific deletion of Xbp1 resulted in ER expansion, decrease in insulin granules formation, impaired proinsulin processing, reduced insulin secretion, and diminished β-cell proliferation . Likewise, mice lacking PERK display a dilated ER, defects in insulin secretion in addition to blunted β-cell proliferation and differentiation [78]. Numerous pathological conditions induce ER stress in diabetes; those include increased insulin biosynthesis [79] as in the condition of insulin resistance, glucolipotoxicity [80]and inflammation [81].

C. ß-cell rescue and therapeutic avenues for T2DM

Due to the heterogeneity of T2D, several lines of treatments have been developed to resolve this disease and its complications. Currently, the most commonly used drugs can be classified into agents that enhance insulin secretion, sensitize the target organs for insulin, or reduce glucose absorption from the gastrointestinal tract. Different therapies address different problems and stages of T2D and may be prescribed in combination to exert synergistic effects [1, 21]. However, the use of these antidiabetic agents is often associated with hypoglycemia and excessive weight gain, which can culminate in myocardial infarction and stroke [82, 83]. Hence, the necessity to find alternative therapeutic avenues.

As it becomes apparent that ß-cell deficiency combined with ß-cell dysfunction have key aetiologic roles in causing T2D, ß-cells themselves have become the target of investigations for

new lines of treatments. Accordingly, researchers are focusing on ß-cell replacement and regeneration strategies to compensate for insulin deficiency. Islet transplantation has been proven to be an effective strategy. However, its clinical application is limited due to a shortage of human cadaveric islets supply in addition to the possibility of immune rejection [8]. Differentiation of human ß-cells from embryonic stem cells or induced pluripotent stem cells represent a promising source of β-cells [84, 85]. Additional supplies involve both the re-differentiation of de-differentiated β-cells and transdifferentiation from lineages of α-cells , δ-cells , or enteroendocrine cells [86] [87].

Systemic β-cell regulators and growth factors

Substantial efforts have been put forward into characterizing systemic regulators and endogenous growth factors that target pancreatic β-cells. Multiple circulating factors with growth-promoting properties have been identified to enhance the regeneration of functional β-cells. Those factors include the intestinal insulinotropic hormone glucagon-like peptide-1 (GLP-1) that stimulates β-cell replication and insulin release [88], the thyroid hormone (T3), which promotes β-cells replication [89] and functional maturation [90], the osteoblast-derived hormone osteocalcin that regulates glucose metabolism, fat mass, and β-cell proliferation [91] and many others [92].

In this scope, we identified CCN5 as a matricellular protein that displays growth-promoting properties in pancreatic β-cells. We first characterized CCN5 in whole-genome cDNA microarray analysis of IGF-I overexpressing islets. CCN5 was among the genes that were significantly upregulated in the islets of these mice. Furthermore, overexpression of CCN5 in MIN6 insulinoma cells enhanced their proliferation and survival [93]. Collectively, these data prompted us to investigate further into the physiological effects of CCN5 in pancreatic β cells.

In the next section, we will discuss key aspects of CCN5 and the CCN family of proteins, including their properties, functions, mode of actions, and relevance to pancreatic β cells and diabetes.

7. CCN protein family

The CCN protein family belongs to a subset of secreted matricellular proteins, which exhibit regulatory rather than structural functions in the matrix [94, 95]. Differential expression screening studies identified the CCN family to include six conserved secreted proteins [96]. The CCN acronym was introduced from the names of the first three members of the family: Cyr61 (cysteine-rich protein 61), CTGF (connective tissue growth factor) and NOV (nephroblastoma overexpressed gene). The other three sets of proteins were discovered later and were known as WNT-inducible signaling proteins: WISP1 (known as CCN4), WISP2 (known as CCN5) WISP3 (known as CCN6) [95]. Several mitogenic signals and environmental stimuli regulate the synthesis of these proteins; those include exposure to steroid hormones, inflammatory cytokines, ultraviolet radiation, and oxygen deprivation [97].

A. Domain structure of CCN proteins

The six CCN proteins share a similar primary structure consisting of a series of 38 cysteine residues that are highly conserved in position and number. These residues are spread across the different domains of the CCN molecule and represent at least 10% of its mass [98]. The CCN proteins share a unique mosaic modular secondary structure consisting of an amino-terminal signal peptide followed by four conserved domains : an insulin-like growth factor binding protein-like module (IGFBP), a von Willebrand factor type C repeat module (VWC), a thrombospondin type-1 repeat module (TSP-1) and a cysteine-knot-containing module (CT) [95, 97]. Exceptionally CCN5 lacks the CT domain. Despite the high structural similarity between the CCN proteins, there

exists a variable hinge region after the VWC domain that connects the first and second half of the molecule. This short sequence varies in length and composition between the different members of the CCN family and is susceptible to cleavage resulting in individual modules with distinct biological properties [99] (Figure I-6).

i. IGFBP domain:

The IGFBP family of proteins consists of six members that bind IGFs with high affinity. This family of proteins regulate essential cellular functions, such as cell proliferation, differentiation, survival, amino acid and glucose uptake in addition to neurotransmitter and hormone secretion [100]. The IGFBP domain of the CCN proteins displays strong sequence homology with the N-terminal domain of conventional IGFBPs [102] However, despite this sequence similarity, the CCN proteins bind IGF with a very low affinity that is 100-fold lower than the traditional IGFBPs [103]. The literature lacks any valuable information regarding the specific role of the IGFBP domain in CCN proteins.

i. The von Willebrand factor C repeat

The von Willebrand factor type C domain (VWC) is a common, occurring motif found in numerous ECM proteins [104]. Its known to regulate many of CCN 's biological functions. Specifically, the VWC regulates TGF-ß signaling and bone morphogenic proteins (BMPs), which are key growth factors in several organs and tissues [105, 106]. CCN2 is among the CCN proteins that has been extensively studied for its interactions with BMPs and TGF-ß [107].

ii. The TSP-1 module

The thrombospondin type-1 repeat module was named after the ECM glycoprotein TSP-1, which consists of repeats of three different domains [108]. It is a common motif found in a wide range of ECM proteins and is known to interact with different targets, including TGF-ß [109], heparan sulfate proteoglycans (HSPGs) [110] collagen V [111], and fibronectin [112]. This module has been implicated in cell adhesion, angiogenesis, binding, and regulating growth factors and other ECM proteins activities [113].

iii. The cysteine knot C-terminal domain

The CT domain is conserved between many ECM proteins and growth factors such as VEGF, TGF-ß, and BMPs [114]. The CT domain is thought to serve as a dimerization module due to its unique structure that consists of a cysteine knot motif of six conserved cysteine residues [102]. This domain interacts with HSPGs, Notch1, and integrins, which are involved in cell proliferation, apoptosis [99], and differentiation [115].

B. CCN proteins mode of action

Early studies supported the notion of CCN proteins as growth factors [116, 117], while other subsequent studies endorsed them as matricellular proteins that modulate cellular activities in response to environmental factors and other stimuli [118, 119]. Following the latter hypothesis, CCNs are currently known to regulate diverse cellular functions. A high-affinity specific receptor for CCN proteins hasn't been identified. However, some specific interactions have been characterized between some of the CCN members and certain surface membrane receptors [120]. Cell surface cross-linking assays have identified a strong interaction between CCN2 and cation-independent mannose-6-phosphate (M6P) receptor in chondrocytes [121], and with lipoprotein receptor-related protein (LRP-1) in bone marrow stromal cells [122].

On the other hand, integrins are known as the primary signaling receptors for these matricellular proteins, with the involvement of co-receptors in some contexts [120]. The most acknowledged CCN co-receptors are heparan sulfate proteoglycans (HSPGs) [123], low- density lipoproteins (LDL) [124], and neurotrophic tyrosine kinase receptor 1 (NTRK1) [124]. Besides, CCN proteins can interact with other ECM proteins, matrix metalloproteinases (MMPs), growth factors, and cytokines [97, 125]. This highly dynamic profile of CCN proteins, contribute to their ability to integrate, modulate multiple signaling pathways, and to elicit cell-specific responses (Figure I-7).

C. CCN proteins functions

The CCN proteins family regulate a common set of cellular activities. The nature of these activities depends on the cellular and environmental context. For instance, one CCN protein can induce positive responses in one cell type and negative responses in other types.

As highly dynamic matricellular proteins, CCN members support cell adhesion [126], migration [127, 128], proliferation [129, 130], survival [131], apoptosis [132], senescence [133], chondrogenesis [134], osteogenesis [135, 136] and stem cell renewal [137]. The involvement of CCN proteins in such a vast array of cellular functions justify their association with multiple human diseases such as wound healing, fibrosis [138, 139], cancer [140, 141], vascular diseases [142, 143], and diabetic nephropathy [144, 145].

8. CCN5

A. CCN5 discovery

CCN5 was originally cloned in the 1990 by Delmolino et al. CCN5 was found to inhibit the proliferation of VSMCs [146]. At least seven independent cDNA clones of CCN5 have indicated that CCN5 is not just a splicing variant that lacks the CT domain but rather an

independent, slightly divergent member of the CCN family [147]. Unlike other CCNs, the CCN5 gene lacks one exon coding for the CT module. Another distinction of CCN5 from the other members is its pattern of gene expression. Most of the other CCN genes are identified as immediate-early genes whose expression is induced, within minutes, by mitogenic growth factors[125] [148]. However, CCN5 expression usually peaks at a later time point. Furthermore, CCN5 expression correlates negatively with cell transformation. Cells in culture, that are transformed by a variety of mechanisms lose CCN5 expression. Accordingly, CCN5 is known to act as a negative regulator of cell transformation and is characterized as a tumor suppressor gene [147].

At the translational level, CCN5 exhibits about 40% identity in amino acid sequence to other members of the CCN family of proteins. About 100 amino acid residues are missing from the CCN5 polypeptide sequence, delineating the missing CT domain. The absence of this domain highlights CCN5 with distinct properties and biological functions from the other members of the family.

Although there is a consensus that CCN proteins are secreted in the ECM, CCN5 [149] and other members [150, 151] were detected in the nucleus of many cells. The mechanism of how CCN5 reaches the nucleus is not well elucidated. However, several hypotheses have been proposed. CCN5 could be translocated: 1) via the assistance of chaperone proteins 2) via post-translational modifications 3) via internalization through endocytic trafficking mechanism and 4) via translation of a splice variant with a nuclear localization signal. The presence of nuclear CCN5 presents the possibility of nuclear as well as surface-mediated functions and emphasizes the complex biology of the CCN family.

B. CCN5 receptor and mode of action

A high affinity specific CCN5 receptor has not yet been identified. Nonetheless, some specific binding has been detected with a few membrane receptors. LRP5/6, a co-receptor for canonical Wnt, represents a potential CCN5 candidate receptor. Typical Wnt proteins require acetylation for their secretion and binding to the heterodimeric receptor complex, consisting of a Frizzled (FZD) and an LRP5/6 protein [152]. However, CCN5 doesn't require acetylation for its secretion and binding to FZD receptors. This implies that CCN5 binds to the LRP5/6 receptor either directly and/or activate it through other signaling pathways. The interaction between CCN5 and LRP5/6 has been investigated in adipocytes [153-155]. CCN5 was found to be an autocrine Wnt ligand that impedes the differentiation of mesenchymal precursor cells into adipocytes. The associated mechanism involve activation of canonical Wnt and a potential binding to LRP5/6 [156].

On the other hand, integrins represent another class of promiscuous receptors for CCN5. There exist about 24 known integrin heterodimers. Each one of them interacts differentially with specific ligands [120]. CCN5 is known to interact with various integrins depending on cell context. On the podosomes of vascular smooth muscle cells (VSMCs), CCN5 binds integrin $\alpha_V\beta_3$ regulating the ability of those organelles to degrade the matrix [157]. In triple-negative breast cancer cells MDA-MB-231, CCN5 interacts with $\alpha 6\beta 1$inhibiting the proliferation of these cells through regulation of cyclin-dependent kinase inhibitor (CKI) $p27^{Kip1}$ [158].

C. Nicotinic acetylcholine receptor (nACHR) as potential receptor candidates for CCN5 on the surface of β-cells

NAChRs are a heterogeneous family of non-selective gated cation (Na^+, k^+, and Ca^{2+}) channels that mediate fast synaptic transmission in neurons [159]. They have a pentameric

structure consisting of combinations of two different types of subunits (**α** and β) or five copies of the same **α** subunit arranged around a central ion pore [159]. Typical ligands/agonists of nAChRs include acetylcholine, nicotine, and other tobacco-related products [160], which upon binding induce a conformational change that opens the ion channel hence allowing signal transmission at neuromuscular junctions in the central and peripheral nervous system (Figure I-8). The expression of nAChRs is not exclusive to the nervous system [161]. nACHRs are present in the pancreas [162], more specifically, β-cells [163, 164] in addition to the liver and muscle [161]. Numerous reports have implicated nicotinic cholinergic signalling in pancreatic islet biology, diabetes and obesity [165] [166]. Furthermore, polymorphisms in human nicotinic receptors were associated with β-cell function and diabetes [166, 167]. RNA-seq and genotyping data from human donor islets revealed an association between CHRNB2, CHRNB4, MAFA, and MAFB transcript levels, insulin secretion, and glucose clearance, hence supporting a critical function for nicotinic receptors in β-cell function [166, 168].

D. Signaling pathways regulating CCN5 gene expression

Several extracellular and environmental stimuli induce the expression of genes encoding CCN proteins; this occurs through the activation of various transcription factors and regulatory elements. In the case of CCN5, the transcription factors and regulatory elements involved in its regulation comprise : hypoxia-inducible factor (HIF), cyclic AMP-responsive element binding protein (CREB), T-cell inducible factor (TCF7), estrogen receptor binding element response element (ERE), activating protein- 1 (AP-1), nuclear factor kappa-light-chain-enhancer of activated B cells (NFκβ) sequences, and binding domains for PPARγ [95, 169, 170]. Accordingly, multiple signaling cascades regulate CCN5's expression; those include canonical Wnt, estrogen, and IGF.

i. Regulation by Wnt signaling

Wnt proteins mediate their signals by interacting with cell surface receptors Frizzled (Fzd) and LRP5/6. The binding initiates a signaling cascade that leads to the inhibition of glycogen synthase kinase (GSK-3B) and casein kinase 1 (CK1). Inhibition of these proteins stabilizes cytoplasmic ß-catenin and promotes its nuclear translocation. In the nucleus, ß-catenin interacts with the transcription factor TCF/LEF thereby activating the expression of a select subset of genes [171] (Figure I-9).

Pennica et al. were the first group to report CCN5 as a Wnt inducible protein [172]. They demonstrated an upregulation of CCN5 at the mRNA and protein level upon overexpression of Wnt-1 protein in the mouse mammary epithelial cell line c57MG. Following this discovery, numerous studies pursued the relationship between Wnt signaling cascade and the expression pattern of CCN5. Constitutive expression of ß-catenin in synovial fibroblasts was associated with a significant increase in CCN5 mRNA level [173]. Furthermore, the addition of either WNT3 or GSK3ß inhibitor to preadipocytes (SB 216763) induce the expression of CCN5 in these cells [174].

Interestingly, non-physiological activators of the Wnt signaling were also found to regulate CCN5 expression. The expression of the hepatitis C virus core protein in the human hepatocellular carcinoma cell line Huh-7 induced Wnt-1 and CCN5 expression [175]. Moreover, MC3T3-E1 osteoblast cells subjected to mechanical loading displayed enhanced activation of the Wnt signaling pathway and an increased expression of CCN5 [176].

The Wnt signaling cascade was also reported to regulate CCN5 expression through the interaction with other signaling molecules. For instance, treatment of the human osteoblast cell line Saos-2 with either forskolin (adenylate cyclase activator) or parathyroid hormone (PTH)

activated the Wnt signaling pathway and induced CCN5 gene transcription [177]. Similar upregulation of CCN5 mRNA has also been reported by the PKA activator CT/IBMX in MCF-7 cells [178]. CCN5 expression was regulated in primary pigmented nodular adrenocortical disease (PPNAD) by a non-canonical pathway of signaling involving miR449. In a PPNAD cell line, CCN5 proved to be a direct target of miR449. The constitutive activation of PKA inhibited miR449 activity which stimulated the Wnt pathway and the sequential upregulation of the CCN5 gene [179].

ii. Regulation by Estrogen signaling

Banerjee et al. were one of the first groups to report the regulation of CCN5/WISP2 by estrogen [180]. Estrogen receptor (ER) positive breast cancer cell lines MCF-7 and human breast cancer cells exhibited elevated levels of CCN5 mRNA and protein [180]. Smooth muscle cells (SMC) derived from human uterine leiomyoma tumors displayed, as well, high expression level of CCN5. Interestingly, CCN5 expression in these cells correlated with the high proliferative phase of the menstrual cycle, where estrogen levels were considerably elevated [181]. Analogous data was demonstrated in rat uterus, where CCN5 levels peaked during the proestrus cycle. Also, ovariectomized rats treated with estrogen showed increased expression of CCN5 protein compared to untreated controls [182].

The estrogen receptor alpha (ER-α) is essential for estrogen's induction of CCN5 expression. Cultured human mammary epithelial cells lacking ER-α were found unresponsive to estrogen stimulation. However, the transfection of ER-α into these cells restored CCN5 upregulation by estrogen [180]. The dependence of CCN5 expression on ER-α stems from the transcription factor-binding site that is found in the 5' promoter region of CCN5. This binding site is identified as a functional estrogen response element that serves as a binding site for ER-α. [170].

Additional regulation of CCN5 by ER-α is detected through transcription cofactors CLIM and RLIM as knockdown of the latter abolished ER-α-mediated upregulation of CCN5. Estrogen was found to stimulate the recruitment of RLIM and CLIM to the CCN5 promoter, hence leading to its activation [183].

A crosstalk between estrogen and CCN5 signaling and other signaling cascades has been reported. The epidermal growth factor (EGF) demonstrates synergistic effects with estrogen in inducing the expression of CCN5 mRNA in MCF-7 cells. The pathways involved in this stimulation are the PI3K and the MAPK signaling pathways. EGF positive regulatory effects in MCF-7 cells are dependent on ER as an ER antagonist blocked EGF-induced upregulation of CCN5 [184]. Another example of crosstalk is through IGF-I, which induced CCN5 mRNA expression in MCF-7 cells through PI3K/AKT signaling. IGF-I mediated proliferative effect in these cells was abrogated upon knockdown of CCN5 [185].

Progesterone has proved, as well, to be involved in CCN5 regulation. MCF-7 cells treated with progesterone displayed a high level of CCN5 mRNA. The pre-treatment with progesterone receptor antagonist, RU486, abolished this increase [180]. On the other hand, when MCF7 cells were treated with both estrogen and progesterone, CCN5 expression levels were not increased as expected [180]. Progesterone effects on CCN5 appear to be cell specific.[182]. In uterine smooth muscle cells, the combined treatment of estrogen and progesterone amplified CCN5 expression as compared to individual treatment with either hormones [182].

iii. Regulation by Insulin-like growth factor I

We were the first to report CCN5 as an integral signaling molecule in the mitogenic action of the IGF-1 axis in pancreatic islets [186]. Mice overexpressing IGF-I in their islets demonstrated higher levels of CCN5 expression as compared to wild type islets. Furthermore, freshly isolated

wild type mouse islets cultured with IGF-I induced CCN5 mRNA and protein levels. Moreover, siRNA mediated knockdown of CCN5 in MIN6 insulinoma cells abrogated IGF-I mediated proliferative effect [186].

A 2-kb region of the CCN5 promoter region has been identified, and interestingly it shares a similar configuration with the IGF-I promoter [170]. As mentioned earlier, the CCN5 promoter includes binding sites for specificity proteins 1(Sp1) and activator protein (Ap1), which are thought to mediate the crosstalk between CCN5 and IGF-I [187].

A signaling crosstalk could occur as well between IGF-I, insulin and Wnt signaling pathways. Together, these signals regulate cat/T cell factor (TCF)-mediated gene transcription via Akt-mediated phosphorylation of GSK-3β, in addition to phosphorylation and stabilization of β-catenin [171, 188] suggesting co-regulation of islet function by WNT, insulin, and IGF-1.

E. The role of CCN5 in human diseases

Similar to other members of the CCN family, CCN5 is thought to be involved in various diseases and pathological conditions. However, the fact that it has a distinct structure from the other members highlight CCN5 with different or even opposing characteristics and biological functions. CCN5 expression was found to be downregulated in a plethora of diseases such as wound healing, fibrosis, cancer, metabolic diseases, cardiovascular diseases, in addition to arthritis and inflammatory diseases [146].

Wound healing is a multiphasic process that starts with inflammation and resolves with matrix deposition and remodeling. However, excessive ECM deposition usually results in fibrosis, scarring, and ultimately loss of tissue function [189]. Many vital organs, such as the liver, kidneys, and the heart, are subjected to fibrosis following chronic injury [190]. Multiple growth factors and inflammatory cells induce the expression of CCN proteins at the site of injury. CCN2 is

overexpressed in most human fibrotic diseases and is known to exert pro-fibrotic effects in vitro and in vivo [191]. On the other hand, CCN5 appears to exert anti-fibrotic effects; transgenic mice overexpressing CCN5 displayed a reduction in cardiac hypertrophy and fibrosis [192].

Aberrant expression of CCN5 has been detected in numerous types of tumors [193]. CCN5 is mostly known for its tumor suppressor activities as it inhibits the growth and proliferation of various tumor cells type [146, 158]. In particular, the role of CCN5 in breast carcinoma has been under a lot of investigations [193]. CCN5 is differentially expressed in breast tumor cell lines; its expression peaks in noninvasive breast cancer cells such as MCF7. On the other hand, it is hardly detectable in invasive/aggressive cell lines such as MDA-MB-231 [194] [158]. CCN5 regulates and promotes its tumor suppressor activates mainly by modulating epithelial-to-mesenchymal transdifferentiation (EMT) [195].

The involvement of CCN5 in metabolic diseases has gained much attention lately [153]. CCN5 is found to be expressed in mesenchymal stem cells, fibroblasts, and preadipocytes, where it regulates their adipogenic commitment through the Wnt pathway[156]. CCN5 stimulates the proliferation of these cells but prevent their commitment to the adipose lineage and subsequent differentiation [155, 196]. On the other hand, we revealed a role for CCN5 in diabetes. CCN5 is found to promote the proliferation and survival of pancreatic ß cells in vitro [93, 197].

F. CCN5 KO and OE models

Given CCN5 impact on osteoblast proliferation and differentiation [13], whole-body CCN5 KO was generated using ES cells in which exons 2-5 were replaced by a lacZ cassette (Knockout Mouse Project (KOMP) [198]. The study revealed no effect of CCN5 on bone formation as no changes in bone mineral density nor bone tissue volume were detected [198]. CCN5 KO mice were used in a distinct study where they assessed the associated metabolic changes

[199]. KO mice displayed mild obesity as they had larger body size at 5-6 months of age. When the mice were fed with a high-fat diet (HFD) they displayed larger subcutaneous WAT (sWAT), perirenal WAT (pWAT), liver, and heart. KO mice displayed as well mild T2D characterized by high blood glucose levels. When the mice were fed with HFD, they exhibited higher fasting blood glucose levels and higher plasma insulin levels, indicating impaired glucose and insulin tolerances [199]. Mice overexpressing CCN5 in their adipose tissue have also been generated [155]. These mice displayed increased lean body mass, expanded brown adipose tissues (BAT), improved insulin sensitivity, and enhanced glucose uptake by adipose cells and skeletal muscle [155].

9. Rationale and objectives of the research

The physiological role of CCN5 has been established in many tissues and pathological conditions. However, the role of CCN5 in pancreatic ß-cells and, more generally, diabetes is not yet well understood. Recently, we were the first to identify CCN5 as a downstream target of insulin growth factor I (IGF-I) in mouse islets [155]. CCN5 expression in the islets was induced by IGF-I overexpression as well by direct stimulation with the exogenous peptide. Similar to IGF-I, CCN5 displayed pro-islet and antidiabetic properties. Overexpression of CCN5 in MIN6 insulinoma cells induced their proliferation and enhanced their survival. Furthermore, differential glucose concentrations were found to influence CCN5's subcellular localization [155]. These findings identify CCN5 as a potent growth factor for ß-cells; however, further investigations are needed to examine its biological effects in vitro directly.

My working hypothesis is that CCN5/WISP2 is a growth factor that stimulates proliferation, survival, and function of pancreatic ß-cells through the binding to a high-affinity specific receptor and activation of downstream targets in those cells. My goals have been to 1) investigate the effects of CCN5 on proliferation, survival and, insulin secretory function of ß-cells

and 2) Identify novel targets for CCN5 in pancreatic ß cells and 3) Characterize a high-affinity receptor for CCN5 on the surface of ß-cells.

The results of my Ph.D. work are presented in the three following chapters that delineate the different inquiries investigated during the progress of my research. Chapter II, which is already published, focused on the effect of CCN5 on the proliferation and survival of pancreatic ß cells in vitro. Chapter III is a manuscript in preparation focused on the effect of CCN5 on the insulin secretory function of ß-cells and the associated mechanism. Chapter IV is a manuscript in preparation, focused on identifying a CCN5 specific cell surface receptor in addition to CCN5 novel targets in ß-cells.

LIST OF FIGURES

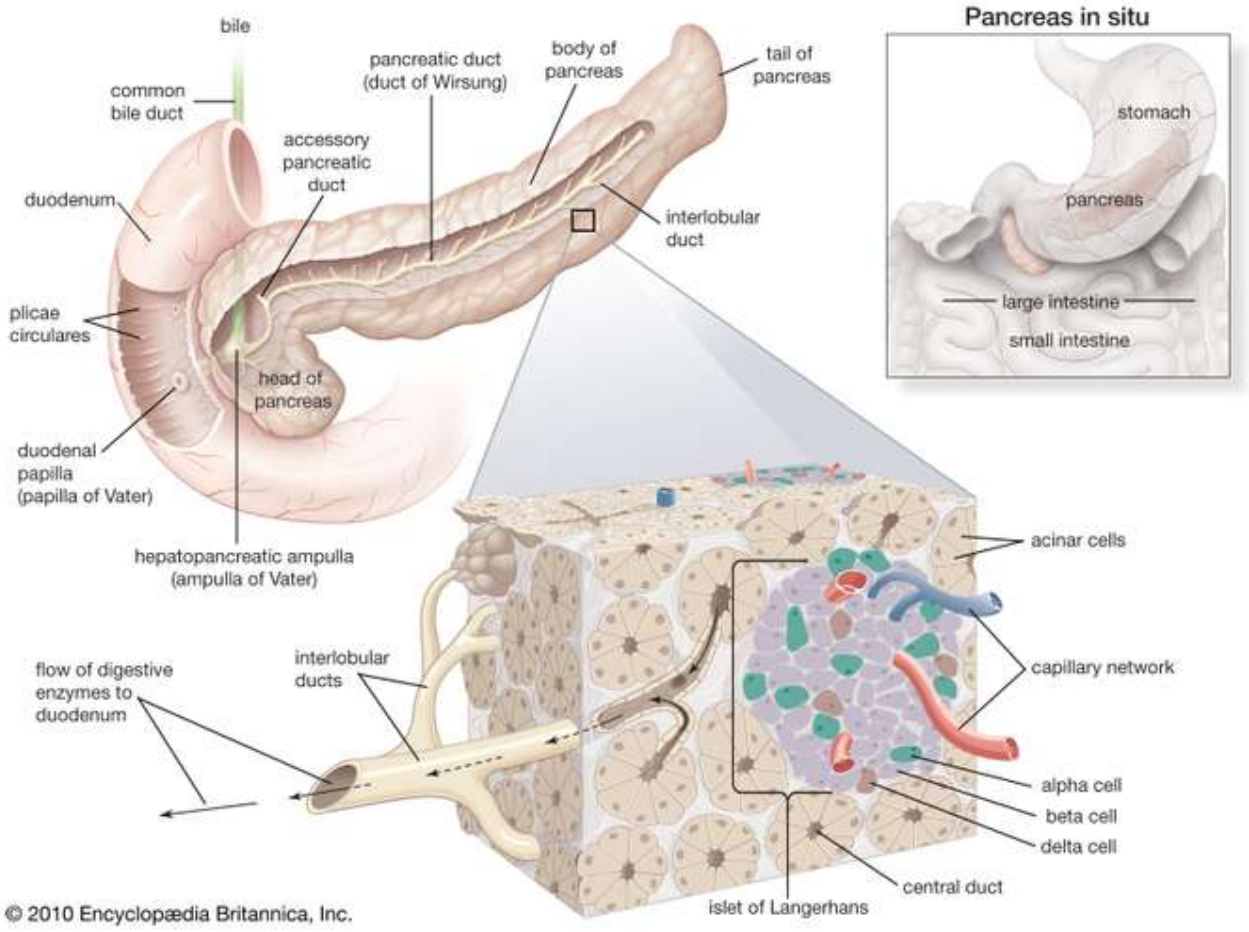

Figure I-1: Anatomical organization of the pancreas.

The exocrine function of the pancreas is mediated by acinar cells that secrete digestive enzymes into the upper small intestine via the pancreatic duct. The endocrine function is mediated by the islets of Langerhans, which consists of alpha, beta, and delta cells that secrete various hormones into a capillary network and also joins the pancreatic duct. (Adapted from Encyclopedia Britannica, Inc. 2010)

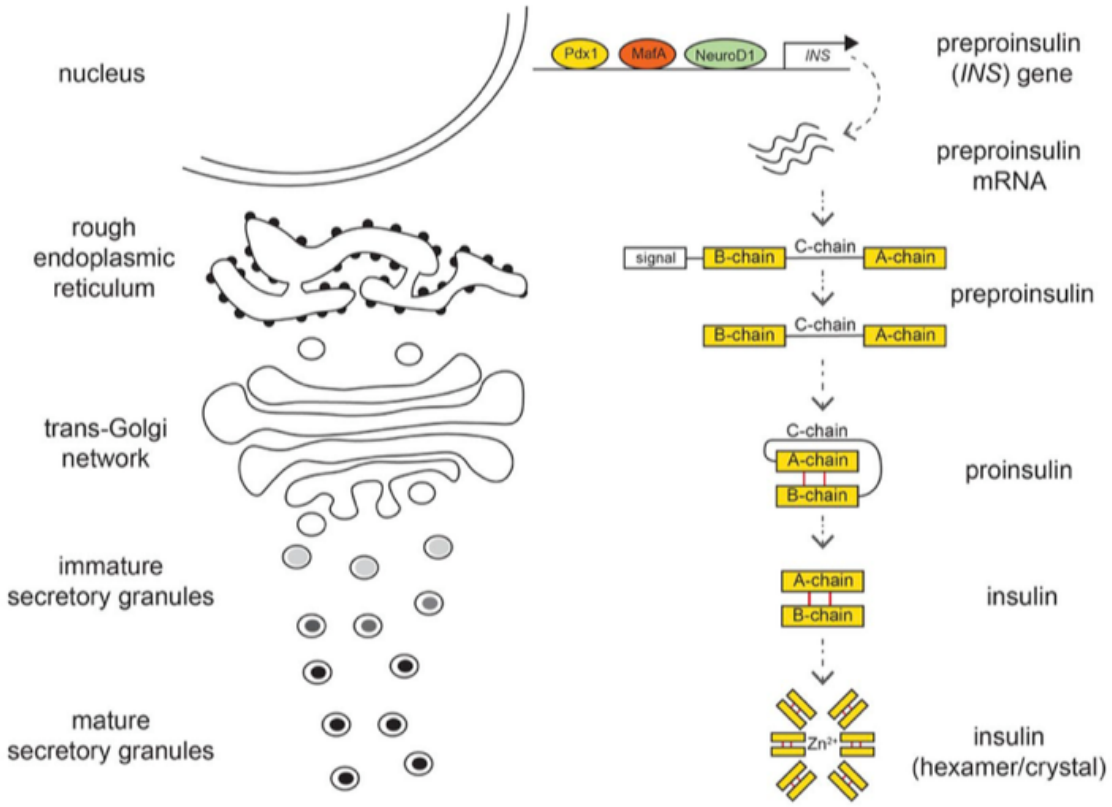

Figure I-2: Insulin maturation along the granule secretory pathway

Preproinsulin mRNA is transcribed from the INS gene and translated to preproinsulin peptide. As this transit through the rough endoplasmic reticulum (RER) and trans-Golgi network (TGN), the prepropeptide is processed to its mature form and ultimately stored as hexameric insulin/Zn^{2+} crystals within mature secretory granules. (Adapted from Victoria L. Tokarz, 2018 [13])

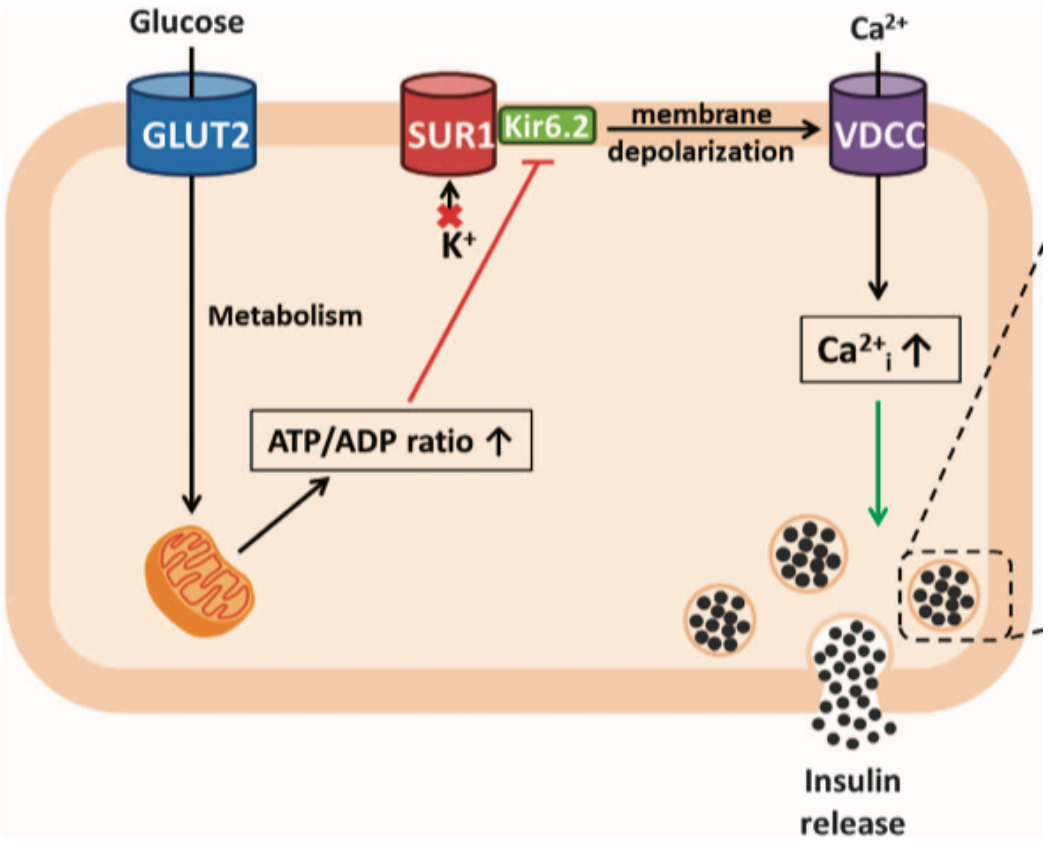

Figure I-3: Glucose-stimulated insulin release from a pancreatic β-cell

Exogenous glucose is taken up by glucose transporter 2 (GLUT2) and undergoes glycolysis inside the cell. Elevated adenosine triphosphate (ATP) levels increase the ATP/ADP ratio, which in turn leads to the closure of ATP-sensitive K^+-channels. The subsequent membrane depolarization opens voltage-dependent Ca^{2+}-channels in causing an increase in intracellular calcium levels, which eventually trigger insulin secretion following vesicle fusion with the membrane. (Adapted from Pia V Röder, 2016 [1])

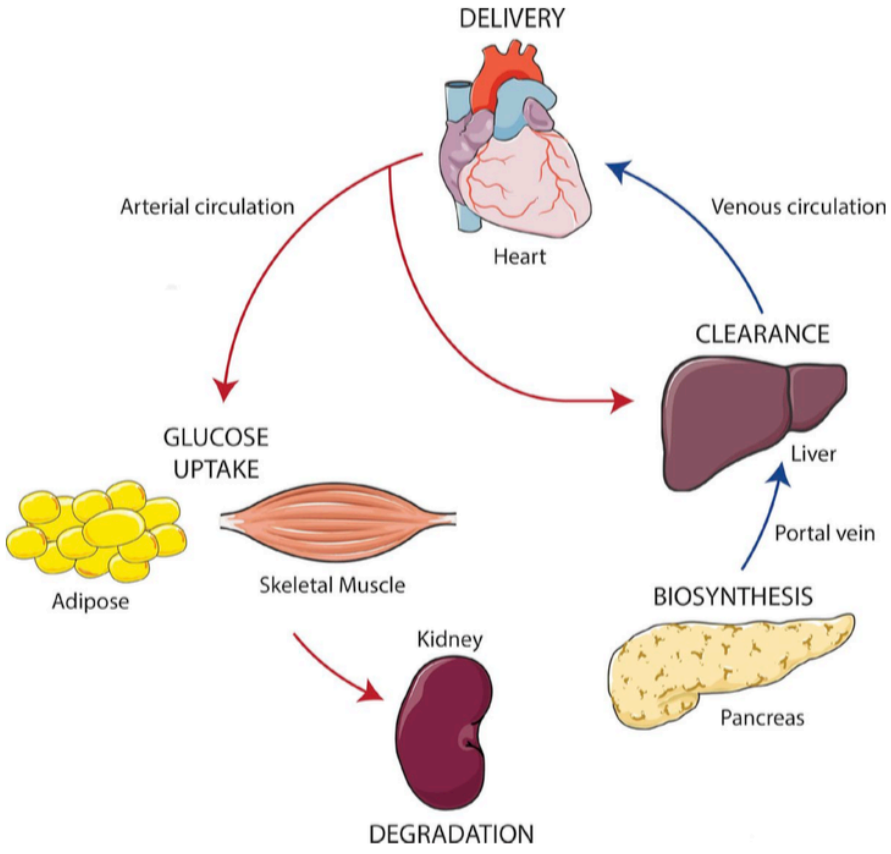

Figure I-4: Journey of insulin in the body

Insulin is transcribed and expressed in the β-cells of the pancreas, from whence, it is exported through the portal circulation to the liver. During this first pass, over 50% of insulin is cleared by the hepatocytes in the liver. The remaining insulin exits the liver via the hepatic vein, where it follows the venous circulation to the heart where is then distributed to the rest of the body through the arterial circulation. Arterially delivered insulin exerts its metabolic actions in the liver where it is further cleared (second pass) and in the muscle and fat cells, where it stimulates GLUT4 translocation and glucose uptake. Remaining circulating insulin is delivered to and finally degraded by the kidney. (Adapted from Victoria L. Tokarz, 2018. [13])

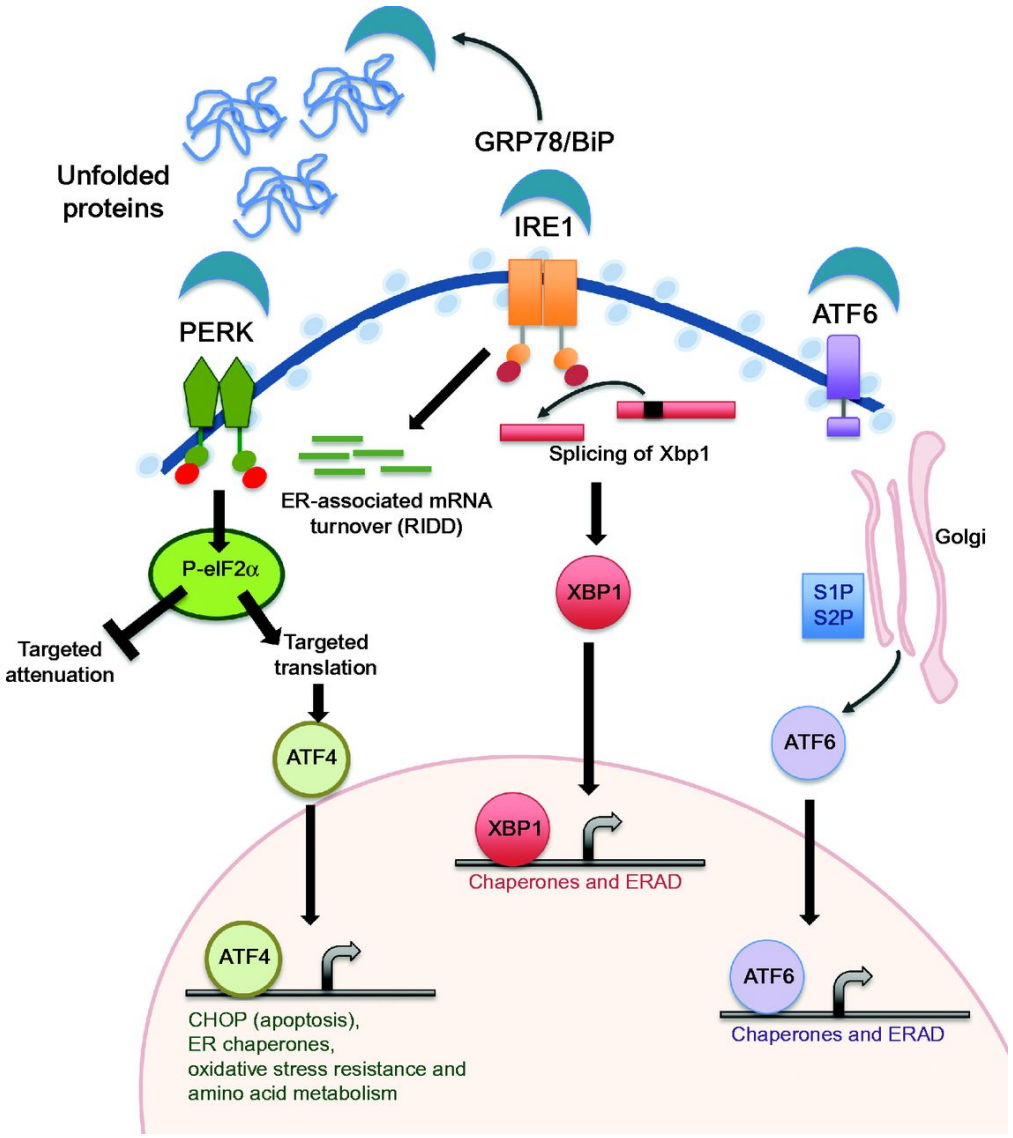

Figure I-5: Key components of the unfolded protein response

The three major arms of the UPR are controlled by regulatory proteins located in the endoplasmic reticulum (ER) membrane: protein kinase-R-like ER kinase (PERK), inositol-requiring enzyme 1 (IRE1α) and activating transcription factor 6 (ATF6). Under homeostatic conditions, these proteins are bound by the chaperone GRP78 (also known as BiP). Protein misfolding and ER stress leads to the sequestration of Grp78 to the exposed hydrophobic domains of misfolded proteins and the

activation of PERK, IRE1α and ATF6. PERK is a kinase that phosphorylates eukaryotic translation initiation factor 2 alpha (eIF2α) resulting in the selective inhibition of the translation of secretory pathway proteins, while promoting the translation of the transcription factor ATF4, that activates genes including C/EBP-homologous protein (CHOP), which drives apoptosis. IRE1α is an endoribonuclease that splices the mRNA transcript for X-box-binding protein 1 (XBP1), which then encodes a transcription factor that induces the expression of genes encoding proteins that restore ER homeostasis, including components of the ER-associated degradation (ERAD) pathway. IRE1α also degrades ribosomal mRNAs encoding secretory proteins in a process known as regulated IRE1-dependent decay (RIDD) and activates the c-Jun N-terminal kinase (JNK), which in turn induces inflammation and apoptosis. ATF6 translocates to the Golgi once activated, where it is cleaved by site 1/2 proteases (S1P and S2P), forming a transcription factor that translocates to the nucleus to activate genes encoding chaperones, ERAD components and XBP1[200].

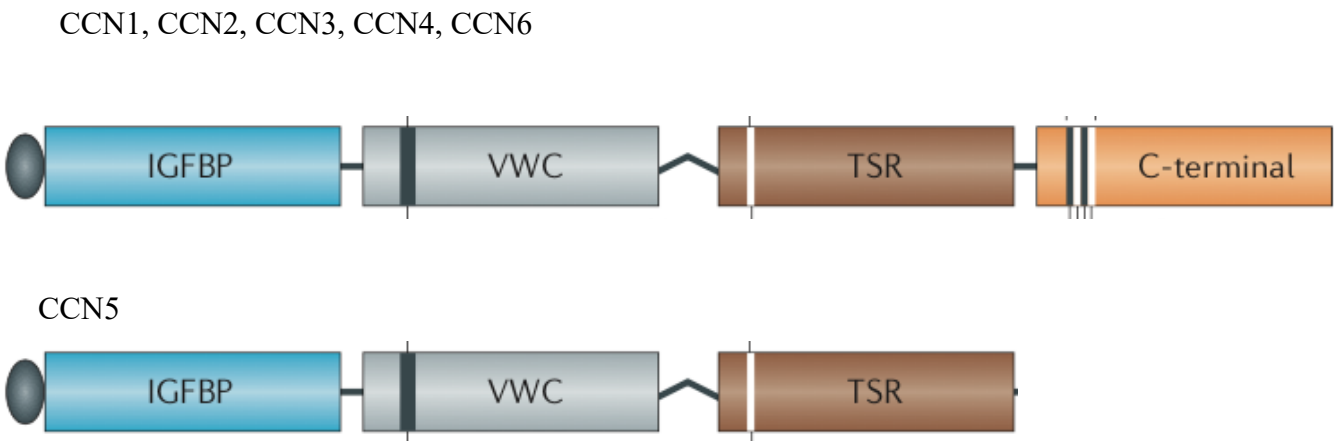

Figure I-6: CCN proteins structure

The CCN matricellular proteins (except for WNT-inducible signaling pathway protein 2 (WISP2; also known as CCN5 which lacks the terminal domain) are composed of an amino-terminal signal peptide and four conserved modular domains: the insulin-like growth factor binding protein (IGFBP) domain, the von Willebrand factor C (VWC) domain, the thrombospondin type 1 repeat (TSR) domain and the carboxy-terminal domain. A non-conserved central hinge region divides the protein into two halves with different binding capabilities for extracellular proteins and cell-surface receptors. (Adapted from Joon-Il Jun, 2011. [95])

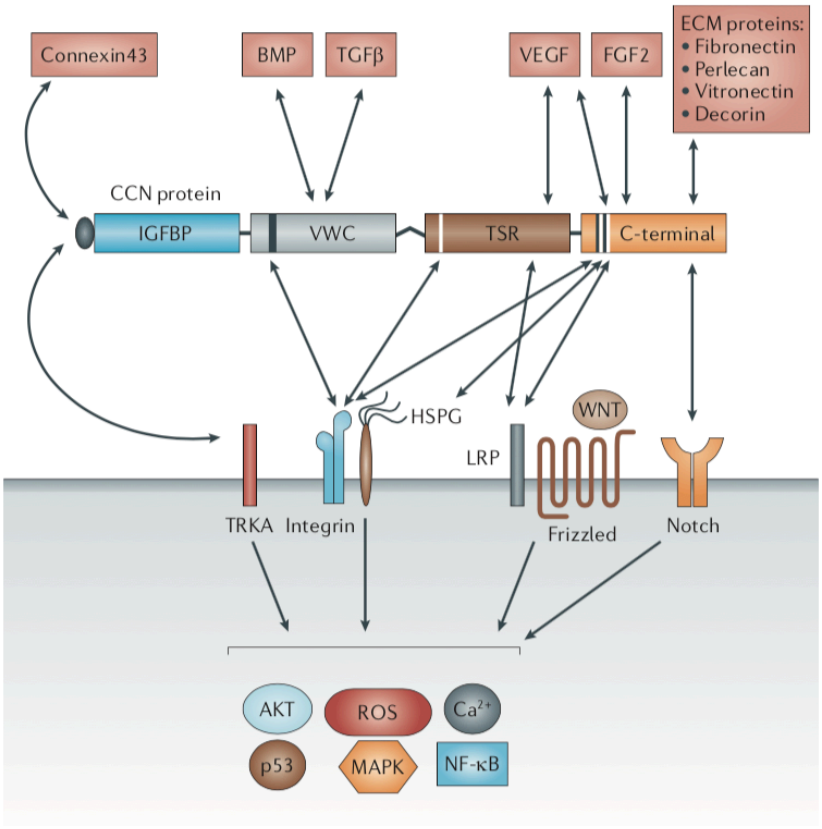

Figure I-7: CCN proteins mode of action

CCN proteins physically interact with several extracellular matrix (ECM) proteins, including fibronectin, perlecan, vitronectin and decorin and growth factors (including vascular endothelial growth factor (VEGF), fibroblast growth factor 2 (FGF2), transforming growth factor-β (TGFβ) and bone morphogenetic proteins (BMPs), as well as the gap junction protein connexin 43. CCN proteins also bind to and signal through several cell-surface receptors, including several integrins, which function in concert with heparan sulfate proteoglycans (HSPGs) or low-density lipoprotein receptor-related proteins (LRPs) as co-receptors in some contexts. CCN proteins can modulate Wnt signaling, in part by binding to the Wnt co-receptor LRP6. The modular domains of CCN proteins may interact in a combinatorial manner to induce unique activities and functions. (Adapted from Joon-Il Jun, 2011. [95])

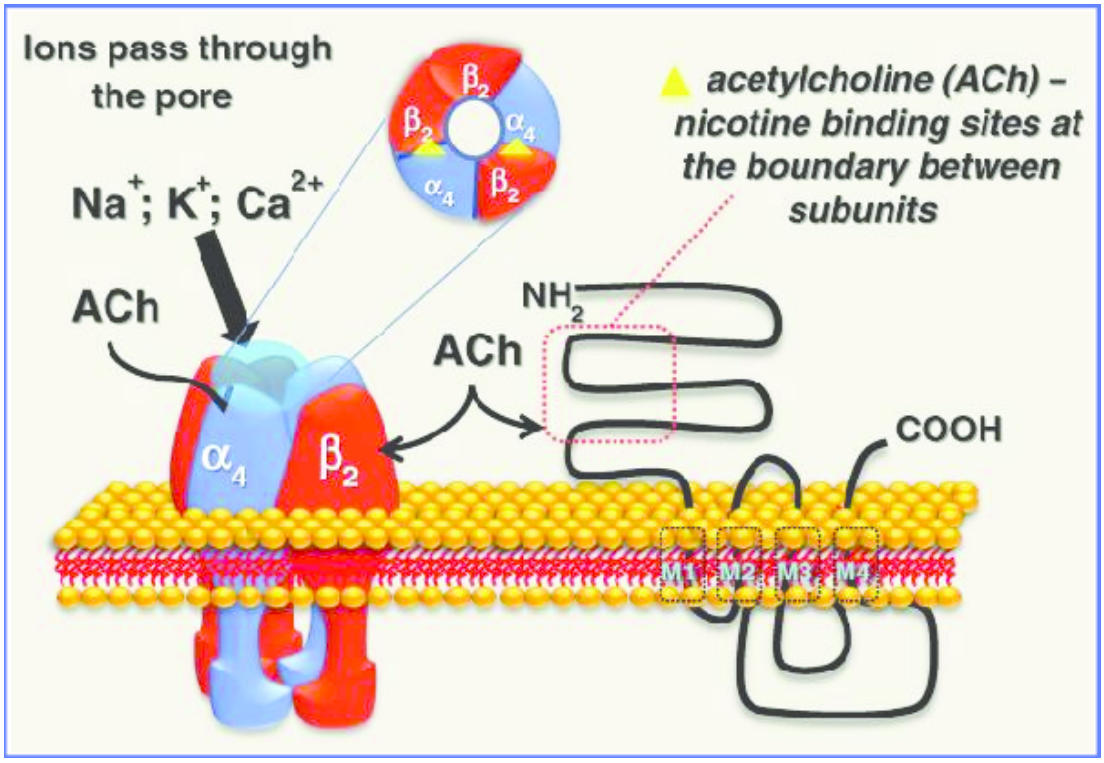

Figure I-8: Schematic representation of nAChRs

The nAChR is a ligand-gated ion channel and is composed of five (α or β) subunits that can be homomeric (all α or all β) or heteromeric (mixture of α and β). Each subunit consists of a large amino-terminal extracellular domain with a transmembrane domain and a variable cytoplasmic domain that include a long cytoplasmic loop between M3 and M4 and other shorter loops connecting the domains. Sites for allosteric modulators are located in four hydrophobic transmembrane domains the transmembrane domains (M1–M4) [201].

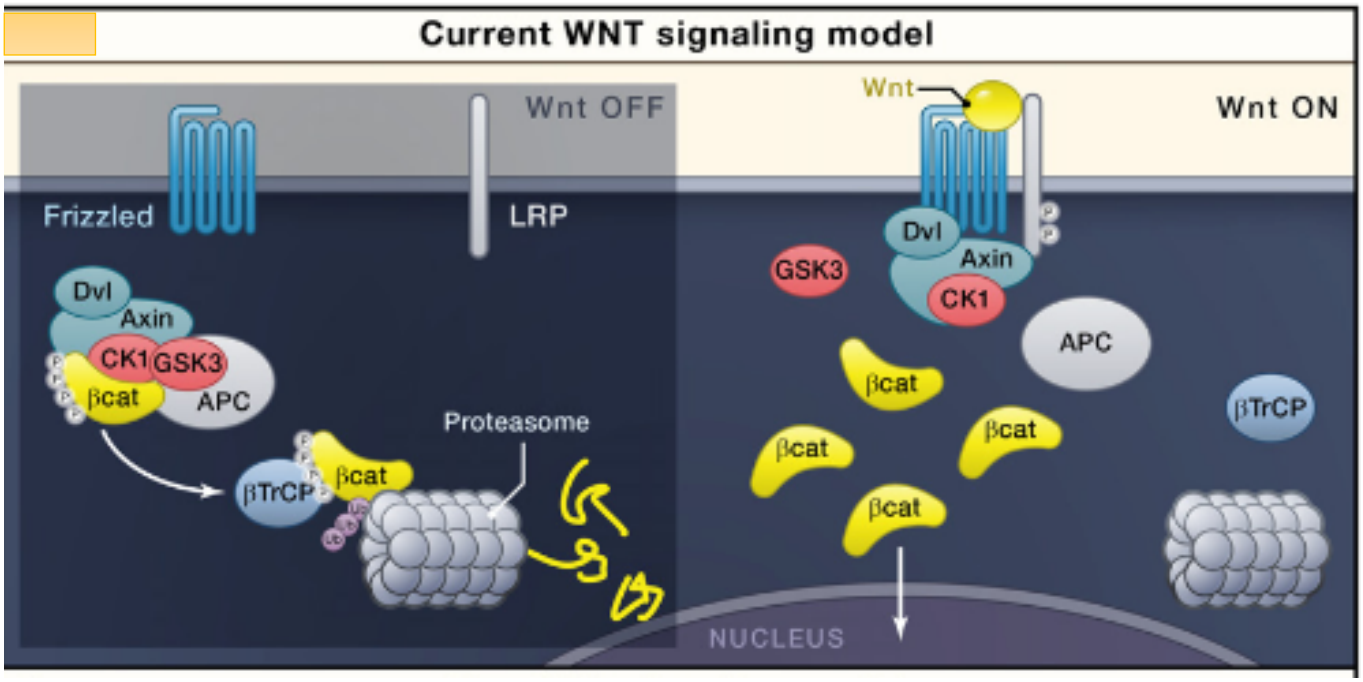

Figure I-9: WNT signalling pathway

In the absence of Wnt, the destruction complex resides in the cytoplasm, where it binds and phosphorylates ß-catenin. The latter then leaves the complex to be ubiquitinated by ß-TrCP, which binds to the phosphorylated ß-catenin and is then degraded by the proteasome. Wnt induces the association of Axin with phosphorylated LRP. The destruction complex falls apart, and ß-catenin is stabilized (Adapted from Clevers, H. and R. Nusse, 2012 [152])

Recombinant protein CCN5/WISP2 promotes islet cell proliferation and survival in vitro

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1. ABSTRACT

Pancreatic ß cell proliferation, survival, and function are key elements that need to be considered in developing novel antidiabetic therapies. We recently identified CCN5/WISP2 to have potential growth promoting properties when overexpressed in ß cells, however further investigations are needed to validate those properties. In this study we demonstrated that exogenous treatment of insulinoma cells and primary islets with recombinant CCN5 enhanced their proliferative capacity and survival rate. CCN5 as well activated cell cycle regulators CDK4 and cyclin D1 and caused an upregulation in the expression of key genes associated with ß cell identity and function such as GLUT-2 and GCK. Finally, CCN5 activated FAK and downstream ERK kinases, which are key regulator in the integrin signalling pathway. Hence, our results validate the growth promoting activities of rh-CCN5 in ß cells and open the door for further investigations in vivo.

Keywords: Diabetes, ß cells, proliferation, survival, insulin secretion

2. INTRODUCTION

Glycemic regulation is vital for normal body function and homeostasis. Blood glucose levels are tightly regulated by various hormones and neuropeptides released by several interconnected organs [1]. Of interest, the pancreas and more specifically ß cells are the key players in this regulation. Any disturbances or failures within these cells is associated with metabolic disorders mainly type 2 diabetes mellitus (T2DM) whose prevalence, comorbidities and medical expenses face a tragic fate [2, 3]. Therefore, the key to potent and efficient therapeutic approaches lies within the ß cells themselves. Anti-diabetic therapies should aim towards 1) Enhancing the regenerative properties of these cells 2) Ameliorating their survival mechanisms, and 3) Upgrading their insulin secreting capacity.

In this scope, we recently identified CCN5 also known as WISP2 (Wnt inducible signalling protein 2) as a potential growth factor for pancreatic ß cells [4]. CCN5 is a member of the cysteine rich CCN family of extracellular matrix–associated proteins [5]. The CCN family consist of six highly conserved members which are implicated in numerous cellular functions and pathological conditions [6]. Unlike other CCN family members, which encompass four structural modules with sequence homologies with insulin-like growth factor binding proteins (IGFBP) , von Willebrand factor (VWC), thrombospondin (TSP-1), and cysteine knot (CT), WISP-2/CCN5 contains only three of these structural modules and lacks the CT domain hence suggesting that its function may be different from that of other members of the family [5, 7].

The physiological role of CCN5 has been established in many tissues and pathological conditions. However, the role of CCN5 in ß cells and more generally diabetes isn't yet well understood. Recently, we were the first to identify CCN5 as a downstream target of insulin growth factor I (IGF-I) in mouse islets [4]. CCN5 expression in the islets was induced by IGF-I

overexpression as well by direct stimulation with the exogenous peptide. Similar to IGF-I, CCN5 displayed pro-islet and antidiabetic properties. Overexpression of CCN5 in insulinoma MIN6 cells induced their proliferation and enhanced their survival. Furthermore CCN5's subcellular localization was influenced by differential glucose concentrations [4].These findings identify CCN5 as a potent growth factor for ß cells, however further investigations are needed to examine its biological effects. We hereby in this study further characterize the biological effects of CCN5 in ß cells by using recombinant CCN5 protein, insulinoma INS832/13 cells and mouse primary islets. We found that exogenous application of recombinant CCN5 to INS832/13 cells and primary islets in vitro enhanced cell proliferation, survival and function.

3. MATERIAL AND METHODS

A. Cell culture

INS832/13 cells were a generous gift from Dr C.B Newgard [8] and they were used for their enhanced responsiveness to glucose compared to other pancreatic β cells [8]. The cells were maintained in RPMI-1640 medium containing 10% fetal bovine serum (FBS),100 U/ml Penicillin, 100 ug/ml streptomycin, 10 mM HEPES, 2 mM L-glutamine, 1 mM sodium pyruvate, and 25 nM ß-mercaptoethanol at 37°C in an atmosphere of humidified air (95%) and 5% CO2.

B. Mouse pancreatic islets isolation

All animal handling procedures were approved by the Research Institute Animal Care Committee of McGill University Health Centre. Pancreatic islets were isolated from 10-12 weeks old male C57BL/6 mice via collagenase perfusion [9]. Briefly, after injecting collagenase XI (0.65 mg/ml) into the bile ducts, inflated pancreas is excised and incubated in a water bath at 37˚C for 15 min. Digestion is then terminated with the addition of cold Hanks' balanced salt solution (Gibco). Islets were then separated from the rest of the pancreatic tissues by using Histopaque

(Sigma Aldrich) gradients followed by several sequential centrifugations. Healthy looking islets were then hand-picked under a stereomicroscope and maintained in RPMI-1640 medium containing 11.1 mM glucose for overnight recovery.

D. Viability of INS832/13 cells

Cell viability was determined using a 3-[4,5-2-yl]-2,5-diphenyltetrazolium bromide (MTT) assay (Sigma Aldrich). INS832/13 cells were seeded in 96-well plates in RPMI-1640 containing 1% serum along with rh-CCN5 or IGF-I for 48 and 72 h. The MTT solution (100 µl/ well; 5 mg/ml in PBS) was added 3 H before the end of the incubation. The medium was then replaced with dimethylsulfoxide (DMSO). Plates were shaken for 20 min and optical densities (OD) were measured at 570 nm using a microplate reader.

E. Proliferation of INS832/13 cells

The rate of INS832/13 cell proliferation was determined using click-it EdU (5-ethynyl-2-deoxyuridine) incorporation assay (Thermo Fisher Scientific). Cells were seeded in 96-well plates in RPMI-1640 containing 1% serum along with rh-CCN5 (12.5 nM) and IGF-I (10 nM) for 72 h. EdU was added in the last 3 h of incubation and its incorporation into DNA was measured using a colorimetric cell proliferation ELISA assay according to the manufacturer's instructions.

F. Proliferation of primary islets

The rate of primary islets proliferation was measured using a BrdU (Bromodeoxyuridine / 5-bromo-2'-deoxyuridine) incorporation assay (Roche). Islets (20-30 per well) were seeded in 96-well plates in RPMI-1640 containing 10% serum along with rh-CCN5 (25 nM) or 16.7 mM glucose for 72 h. BrdU was added in last 18 h of the incubation. BrdU incorporation into DNA was measured using colorimetric cell proliferation ELISA assay according to the manufacturer's instructions (Roche). Optical densities were measured at 450 nM.

G. Proliferation of pancreatic ß cells

The rate of ß cells proliferation was measured by BrdU incorporation and immunofluorescence. Freshy isolated Islets were cultured in chamber slides in RPMI 1640 containing in 10% serum along with rh-CCN5 (25 nM) for 72h. BrdU was added in last 18 h of the incubation. Cells were then fixed with 4% paraformaldehyde, permeabilized with 0.1 % Triton X-100 and blocked by 10% serum before being incubated with fluorescent primary antibodies against insulin and BrdU overnight at 4°C. The corresponding antibodies were conjugated with Alexa 488 or Alexa 594 and DAPI was used as a nuclear counterstain. BrdU incorporation into DNA was assessed using a Zeiss light and fluorescent microscope.

H. Protein extraction and Western blotting

Proteins either from primary islets or INS832/13 cells were extracted using a lysis buffer containing 150 mM NaCl, 10 mM Tris, 1 mM EDTA, 1% Triton X-100, and 0.1% SDS, supplemented with a protease inhibitor tablet (Roche Diagnostics, CA). The samples were then diluted with Lammeli loading buffer, boiled and loaded onto SDS-PAGE gels. The proteins were then transferred to a nitrocellulose membrane. Nonspecific bindings were blocked by 10% skim milk followed by incubation with primary antibodies at 4°C for overnight. Horseradish peroxidase–conjugated secondary antibodies were incubated for 1 h followed by enhanced chemiluminescence substrates. The signals were captured using a Chemidoc Touch Imaging System (Bio-Rad). The antibodies used are : CDK4 (ab38317; Abcam), cyclin D1 (sc753; Santa Cruz), phospho-Erk1/2 and Erk1/2 (9101 and 9102; Cell Signaling Technology), caspase 3 (9662; Cell Signaling), PARP (9542; cell signaling), p-FAK (SC 81493, 2D11); Santa Cruz), t-FAK (SC 1688; Santa Cruz), tubulin (ab 6046; abcam).

I. Apoptosis in INS 832/13 cells

Apoptosis was measured using Annexin V apoptosis detection kit (BD biosciences). INS832/13 cells were seeded in 6-well plates in RPMI-1640 containing 10% serum along with rh-CCN5 (12.5 nM) for 48 h then exposed to palmitate (1 mM) and glucose (25 mM) for 24 h. Cells were then harvested at a density of 2×10^5 cells/ml in the binding buffer and stained with the Annexin V/propidium iodide (PI) conjugates for 15 min. Cells were then analyzed using a BD FACS Calibur flow cytometer.

J. Apoptosis in islets

Apoptosis in pancreatic islets was measured using Cell Death Detection ELISA Plus kit (Roche Diagnostics) which quantify DNA fragmentation. Isolated islets were seeded in a 96 well plate in 10% serum RPMI 1640 supplemented with rh-CCN5 (25 nM) for 48 h then exposed to Streptozotocin for 24 h. Cells were then washed twice with phosphate buffer saline (PBS) and incubated with lysis buffer for the extraction of histone fragment at room temperature. Following centrifugation to remove the nuclei and cellular debris, the supernatants (containing histone fragment) were incubated in microtiter plates that were coated with anti-histone antibody. The plate was then read at 405 nm.

K. RNA isolation and PCR

Total RNA was extracted from primary islets using RNAeasy Micro kit (Qiagen) and reverse transcribed into cDNA using Quantitect reverse transcription kit (Qiagen). Forward and reverse sequence primers were designed by integrated DNA technologies (IDT) and mRNA levels were normalized to GAPDH and HPRT1. Primer sequences were as follow: HPRT1 F: 5'-GCA TGT CAA TAG GAC TCC AG-3' and R: 5'-TTG TTG TAG GAT ATG CCC TTG A-3', GAPDH

F: 5'-TGT AGT TGA GGT CAA ATG AAG GG-3' and R: 5'-ACA TCG CTC AGA CAC CAT G-3', GCK F: 5'-GAT GCA CTC AGA GAT GTA GTC G-3' and GCK R: 5'-TGA AGG TGG GGA GAA GGT GAG-3', SLC2A2 F: 5'-GCT GAT GAA AAG TGC CAG TG-3' and R: 5'-CTG GAG AAG CAT ATC AGG ACT-3', INS F: 5'-CTT CAC GAG CCC AGC CA-3' and R:-5'-ATC AGA AGA GGC CAT CAA GC-3', INS1 F: 5'-TAA CCC CCA GCC CTT AGT GAC CAG CTA TAA-3' and R: 5'-ACA AAG ATG CTG TTT GAC AAA AGC CTG -3', and finally INS2 F: 5'-CCA GCT AAG ACC TCA GGG ACT-3' and R: 5'-CTT GAC AAA AGC CTG GGT GG- 3'. Real-time quantitative PCR were performed using ViiA7 real time PCR system (ThermoFisher Scientific) using PowerUp syber green master mix (ThermoFisher Scientific).

L. Statistical Analysis

All data were presented as Mean ± S.E.M. and analyzed by using Students' t-test, One-Way and Two-Way ANOVA using GraphPad Prism version 5.0. $P<0.05$ was considered as statistically significant.

4. RESULTS

A. Recombinant CCN5 (rh-CCN5) stimulates the proliferation of INS832/13 cells and primary mouse islets

The ability of CCN5 to regulate cell proliferation has been studied in numerous cell lines [10-12]. We found that CCN5 overexpression in insulinoma MIN6 cells stimulate their proliferation [4]. Consequently, we were interested in investigating whether the recombinant form of CCN5 (rh-CCN5) would have a similar stimulatory effect in INS832/13 cell and primary islets. Accordingly, serum starved INS832/13 cells were exposed to rh-CCN5 for different doses (12.5 and 25 nM) and time points (48 and 72 h) in accordance with our previous study [4]. Cell viability and proliferation were assessed using MTT (crystal violet test) (Figure II-1A) and EdU assays

respectively (Figure II-1B). Using MTT, we detected a 15% and 25% increase in cell number after 48 h treatment with 12.5 and 25 nM rh-CCN5 respectively. This effect was further enhanced after 72 h to reach 25% with 12.5 and 40% with 25 nM rh-CCN5 respectively (Figure II-1A and II-1B). Next, we measured DNA synthesis using EdU incorporation assay; we detected 30% increase in EdU incorporation upon treatment with 12.5 nM CCN5 and 50% with 25 nM CCN5 (Figure II-1A and II-1B). In the above assays, IGF-I and 10% FBS were also included as positive controls.

We then addressed the stimulatory effect of CCN5 in primary mouse islets by screening for BrdU incorporation either colorimetrically (Figure II-1C) or via immunofluorescence (Figure II-1D). Treatment of islets with 12.5 and 25 nM rh-CCN5 caused an increase in the level of BrdU incorporation by 20% and 50% respectively. Islets were as well cultured in 16.7 mM glucose [13-15] , a condition considered as positive control, in that case the level of BrdU incorporation reached almost 60%. To confirm that the growth promoting effect of rh-CCN5 was ß cell specific, we checked for BrdU incorporation in partially dispersed mouse islets using triple labeled fluorescence microscopy. In untreated culture of islets (Figure II-1D, upper panel), BrdU incorporation was hardly detectable. On the other hand, in cultures treated with rh-CCN5 (Figure II-1D, lower panel) BrdU incorporation was successfully detectable as displayed by colocalization of the latter with DAPI (blue) in the insulin positive cells (green). In preliminary experiments using human islets, similar stimulatory effects were observed upon CCN treatment (data not shown).

Given that CCN5 displays a mitogenic effect in pancreatic ß cells, we were interested in the cell cycle regulators that might be involved/regulated by CCN5 within the ß cells. Hence, we measured the relative expression of cyclin D1 and other associated CDKs that are known to activate the cell cycle machinery in the ß cells [16] . We found that the level of CDK4 protein was elevated by 1.5 and 2-fold in INS832/13 cells upon treatment with rh-CCN5 for 48h and 72 h

respectively (Figure II-1E). In primary mouse islets, we found that the level of cyclin D1 was significantly elevated by 2-fold upon treatment with rh-CCN5 (Figure II-1F). Several attempts were made; however, we couldn't detect cyclin D1 in INS 832/13 cells. Together these data support a proliferative effect of CCN5 in pancreatic ß cells.

B. Pre-treatment with CCN5 protects INS832/13 cells and primary mouse islets against lipotoxicity, glucolipotoxicity and streptozotocin induced cell death

In our previous published report, we found that CCN5 expression is associated with IGF-I induced islet cell survival [4] . Hence our next step was to investigate the effect of rh-CCN5 on ß cell viability upon exposure to various stressors including high concentration of free fatty acids, glucose and streptozotocin. First, INS832/13 cells were pre-treated with rh-CCN5 for 48 h and then exposed to palmitate, PA (1 mM) for 24 h. Cell death was determined by Annexin V staining which stains apoptotic cells. Palmitate significantly increased cell death by 12-fold, however when the cells were pretreated with rh-CCN5 the ratio of cell death was significantly decreased by 50% (Figure II-2A and II-2B).

To further evaluate the role of CCN5 on ß cell viability, we measured the levels of caspase-3 and PARP cleavage in INS832/13 cells treated with rh-CCN5, PA and PA + glucose as described above. PA slightly induced cell death as compared to PA + glucose which caused a huge increase in cell death as displayed by higher level of cleaved PARP (6-fold) and caspase-3 (11-fold). Interestingly, those cells pre-cultured with CCN5 displayed lower levels of the active form of both caspase 3 (7-fold) and PARP (4-fold) as compared to untreated ones (Figure II-2C and II-2D).
We then addressed the effect of CCN5 pre-treatment on the viability of primary mouse islets. Cells were pre-treated with rh-CCN5 for 48h and then treated with streptozotocin (5mM) for 24h. Cell death was measured by ELISA based apoptosis assay. Streptozotocin significantly induced death

in islet cells by 4-fold, however when the cells were pre-treated with CCN5 cell death was decreased by 2-fold (Figure II-2E).

Together these data establish a protective role of CCN5 against induced cell death in ß cells.

C. CCN5 treatment enhances ß cell identity and function in primary islets cells

Activation of cell proliferation in some systems results in de-differentiation of this system [17]. Accordingly, due to its growth promoting effects in INS832/13 and islet cells, CCN5 is expected to induce their de-differentiation. To gain insight into the effect of CCN5 on ß cell identity and function we probed for changes in expression in key genes associated with ß cell function (Glut2, GCK, Ins1 and Ins2). Gene Expression analysis in islets cells treated with rh-CCN5 for 24h and 48h revealed a 5-fold upregulation of the ß cell glucose transporter (GLUT2) at 24h, this effect decreases to 2.5-fold after 48h (Figure II-3A). The effect of CCN5 on glucokinase (GCK) expression was apparent after 48h of treatment as displayed by a 2-fold upregulation. Interestingly, CCN5 increased the expression of Ins1 and Ins2 genes after 24h of treatment by 3and 6-fold respectively. This effect disappeared after 48h of treatment (Figure II-3B). Treatment of islets with 16.7mM glucose for 24h, which in this case was considered as a positive control [18, 19], increased the expression of all of those key genes significantly. Altogether, these data demonstrate that CCN5 enhances ß cell identity and function.

D. CCN5 regulates the activity of focal adhesion kinase (FAK) and downstream extracellular-signal regulated kinase (ERK)

It has been previously reported that multiple functions of CCN proteins are accomplished through their interaction with integrin receptors [20, 21]. In this report we evaluated indirectly the interaction between CCN5 and integrin by assessing the level of FAK phosphorylation, a key component of the signaling transduction pathways triggered by integrins, in addition to

downstream targets such as ERK phosphorylation in INS832/13 cells treated with rh-CCN5 for different time points. The level of p-FAK was significantly increased by 2-fold at 0.5 h then was diminished by 4 h (Figure II-4A). As for p-ERK, the protein level started increasing at 0.5 h to reach significance at 6 h (3-fold increase) (Figure II-4B). We then tested whether the interaction of CCN5 with integrin was essential for CCN5 triggered proliferative effect in INS832/13 cells. Consistent with our prediction we found that CCN5 mediated growth stimulatory effect on INS832/13 cells was significantly decreased when FAK phosphorylation was inhibited by FAK inhibitor, Y15 (Figure II-4C and D). These data support an indirect interaction between CCN5 and integrins on the surface of ß cells resulting in the activation of signalling pathways involving ERK and contributing to the stimulatory effects in ß cells.

5. DISCUSSION

The role of CCN5 in diabetes and more specifically pancreatic ß cells is not well established. We have previously gathered some insight into the potential relevance of CCN5 in ß cells [4]. We showed that CCN5 is expressed specifically in the ß cells, it's expression at the mRNA and protein level is induced by IGF-I and CCN5 cDNA overexpression in insulinoma MIN6 cells contributes to IGF-I stimulated islet cell growth and survival [4]. In the present study, we benefited from the availability of recombinant protein CCN5 so we can investigate its physiological effects in various pancreatic ß cell lines. We hereby applied exogenous recombinant CCN5 in vitro to mouse primary islets and rat derived transformed INS832/13 cells and assessed its effects on cell proliferation, survival and function.

CCN proteins adopt two mode of actions, either through interactions with cell surface receptors; or/and interactions with receptor ligands. To date, the most credible receptors that are known to interact with CCN proteins are integrins [7]. Other co-receptors might also be involved

depending on the cellular context [22]; a factor that contributes to cell specific effect of CCN proteins. On the other end, CCN proteins act as signal conductors as they modulate the activity of ECM associated proteins such as growth factors, cytokines and matrix MMPs by biding to them and their receptors. CCN5 was reported to interact with different integrins in a cell context dependent manner [23, 24]. Intracellular localization of CCN5 either cytosolic or nuclear was also reported in numerous studies [5]. The present study supports the notion of CCN5 as a secreted extracellular protein that binds to a specific cell surface receptor and ultimately activating cell specific signalling pathways. Hence, our model of using recombinant CCN5.

The mitogenic effect of CCN5 is still controversial, some studies have shown a stimulatory role of CCN5 while others have displayed an inhibitory role. In our case treatment of either INS832/13 cells or primary islets with rh-CCN5 promoted their proliferation as demonstrated by either EdU or BrdU incorporation respectively. Similar growth promoting effect was seen in C3H10T1/2 mesenchymal stem cells that were incubated with serum from animal overexpressing CCN5 [25]. CCN5 knockdown in MCF7 cells inhibited their proliferation indicating the importance of the endogenously expressed CCN5 in promoting their proliferation [12]. On the other hand, inhibitory effects were seen in 3T3-L1 preadipoctyes [6, 26] VSMCs [10] and the highly invasive MDA-MB-231 breast cancer cells [11] whereby overexpression of CCN5 protein abrogated proliferation. These conflicting reports instigate the need for further research on the underlying mechanism behind those cell-specific effects of CCN5.

The growth promoting effects of CCN5 in INS 832/13 cells and primary islets raised our interest in uncovering the cell cycle regulators that could be behind those effects. INS832/13 cells and primary islets treated with rh-CCN5 had higher levels of the cell cycle regulators CDK4 and Cyclin-D1 respectively. CDK4 is a kinase that partner with D-type cyclins to activate cell cycle

progression. The significance of CDK4 lies in its ability to regulate cell cycle specifically within the ß cells. A study by Dubus et al has demonstrated such effect , as a global deletion of CDK4 led to a hypoplastic phenotype specifically in the ß cells [27] [28]. The D type cyclins , mostly cyclin D1 play as well a fundamental role in postnatal pancreatic ß cell growth [29]. A study by Stewart et al has demonstrated that Cyclin D1 can induce ß -cell proliferation in rat and human islets [17].

We have previously reported that CCN5 overexpression in MIN6 cells confers protection against streptozotocin induced cell death [4]. The present study demonstrates a protective role of CCN5 in INS832/13 cells and primary islets cells against glucolipotoxicity and streptozotocin toxicity respectively, whereby pre-treatment with CCN5 decreased the levels of cleaved caspase and PARP in INS 832/13 cells in addition to reduced level of DNA fragmentation in primary islet. CCN5 displays differential responses in regards to apoptosis; some studies report a neutral effect of CCN5 on cell survival [10, 30], other studies support CCN5 as an inducer of apoptosis in cancer cells [31] and myofibroblasts[32]. Hence CCN5 effects on cell survival are rather cell specific and a deeper investigation into the underlying mechanism would help clarify these discrepancies.

Some concerns could be raised on whether the effects of rh-CCN5 on cell proliferation and survival could be related. The settings and the design for the experiments of cell proliferation and cell death were different and independent. Proliferation experiments were conducted in RPMI containing low serum (1%) to allow stimulation by recombinant CCN5 for the allocated period of times. Increased cell viability and DNA synthesis was observed in CCN5 treated cells. On the other hand, apoptosis experiments were performed in a different setting whereby cells were cultured in RPMI containing normal 10% serum along with CCN5, and then challenged with palmitate/glucose/ streptozotocin; an environment that stresses β cell and induces their death.

Decreased Annexin 5 staining, caspase cleavage and DNA fragmentation indicate that CCN5 is rather protecting β cell in that stressful environment.

Conceptionally, however, the effects on proliferation and survival complement each other as they may share some common signaling elements. In the present study, we demonstrated an activation of FAK and ERK kinases by rh-CCN5. FAK is an essential tyrosine kinase in the integrin-mediated signaling pathway which is crucial for cell viability and mobility[33]. FAK is also reported to regulate pancreatic β cell proliferation, survival and function [34, 35]. Upon its activation, FAK triggers a signaling mechanism involving ERK activation which displays dual activities in pancreatic β cell by prompting their proliferation [36-38] and survival[39]. Consistent with these observations, we found that FAK inhibition by the inhibitor Y15 abolished CCN5 mediated stimulatory growth effect in INS832/13 cells. Hence CCN5 could be stimulating both proliferation and survival simultaneously at least in part through integrin receptor by activating FAK and ERK kinases.

Activation of cell proliferation in some systems usually results in their de-differentiation [17, 40]. Accordingly, CCN5 as a growth promoting factor within the ß cells, is expected to alter the identity of the ß cells and hence leading to their de-differentiation. However, this wasn't the case, as the level of expression of ß cell markers such as the glucose transporter GLUT2 and the kinase GCK which are responsible for glucose sensing and metabolism were upregulated hence suggesting that CCN5 might have a potential role in regulating the function of the ß cells. Furthermore, the levels of both INS1 and INS2 genes were increased upon 24h treatment with CCN5 which lead us to suggest that CCN5 might regulate either insulin content or secretion of ß cells. Further experiments will be needed to optimize the role of CCN5 in regulating the function of the ß cells.

In conclusion, we have demonstrated that CCN5 acts a secreted growth factor that enhances the proliferation, survival and function of ß cells in vitro; those effects coincide with activation of FAK and ERK kinases. These findings pave the way towards adding CCN5 to the library of circulating factors / novel molecules that stimulate ß cell mass expansion as a treatment strategy for diabetes [38]. However, further in vivo studies involving CCN5 knockout or overexpression specifically in ß cells are essential to unravel all the unexplored effects of CCN5.

FUNDING

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AUTHORS CONTRIBUTIONS

N.K. performed all experiments, presented the data, and wrote the manuscript. Z.D helped in some of the experiments and corresponding analysis. J.-L.L. designed the study, revised the manuscript, and approved the final revision. Z.G. helped in designing and analysis of the data.

DISCLOSURE OF INTEREST

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

6. REFERENCES

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FIGURE LEGENDS

Figure II-1: Effect of recombinant CCN5 on the proliferation of rat insulinoma cells INS 832/13 and mouse pancreatic islet cells.

(A) INS 832/13 cells were cultured in 1% serum (FBS) and treated with either rh-CCN5 (12.5 or 25 nM), IGF-I (10 nM) for 48 h and 72 h. MTT assay was used to determine cell viability. All quantitative data are presented as mean ± SEM of at least 3 experiments. P-values were generated using t tests. *P<0.05 vs untreated (UT). (B) Cells were cultured either in 1% or 10% FBS and rh-CCN5 (12.5 or 25 nM) for 72 h, EdU was added in the last 3 h of the incubation. EdU incorporation was used to determine DNA synthesis and hence cell proliferation. All quantitative data are presented as mean ± SEM of at least 3 experiments. **P<0.01, ***P<0.001 vs UT (C) Freshly isolated mouse islets were cultured in 10% FBS, rh-CCN5 (12.5 or 25 nM) or glucose (16.7 mM) for 72 h, BrdU was added in the last 18 h of the incubation. BrdU incorporation was measured using ELISA assay. All quantitative data are presented as mean ± SEM of at least three experiments. P value was generated by student t test.*P<0.05, **P<0.01 vs UT (D) Freshly isolated islets were dispersed using dispase II, cultured in 10% serum and treated with rhCCN5 (lower panel), or vehicule (upper panel) for 72 h. BrdU was added in the last 18h. ß-cell specific BrdU incorporation was visualized using triple-labeled immunofluorescence; the cells were fixed and stained for BrdU (red), insulin (green) and DAPI (blue for cell nuclei) consequently. Images were taken at 400X magnification. Representative images of >3 were illustrated. The white arrows (lower panel) indicate BrdU incorporation in the nucleus in CCN5-treated islets. (E) Western blot demonstrating CDK4 protein levels in INS 832/13 cell cultured in 1% serum and treated with rh-CCN5 (12.5 nM) for 48 and 72 h. The blots are a representative of at least 3 experiments. Fold changes were normalised to Tubilin (loading control). All quantitative data are represented as

mean ± SEM. P-value was generated by student t test. **P<0.01 vs UT (F) Western blot demonstrating CyclinD1 protein levels in primary mouse islets cultured in 10% serum and treated with rh-CCN5 (25 nM) for 72 h. The blots are a representative of at least 3 experiments. Fold changes were normalised to β-actin (loading control). All quantitative data are represented as mean ± SEM. P-value was generated by student t test. **P<0.01, ***P<0.001vs UT.

Figure II-2: Effect of rh-CCN5 on lipotoxicity, glucolipotoxicity and streptozotocin induced cell death in INS832/13 cells and primary mouse islets.

(A) (B)INS832/13 cells were pretreated with rh-CCN5 (12.5 nM) for 48 h and then treated with palmitate, PA (1mM) and glucose (GLU) (25 mM) for 24 h. On the Y axis are displayed the percentage of apoptotic cells (Annexin V positive) and on the X axis are displayed the percentage of necrotic cells (propidium iodide positive). Annexin V staining was used to determine cell death. The graphs are representative of at least 3 experiments. All quantitative data are represented as mean ± SEM. P values were generated using student t test. $^{###}$P<0.001 vs UT, *P<0.05 vs PA. Western blots demonstrating relative levels of (C) cleaved caspase, (D) cleaved PARP in INS832/13 lysates. Cells were pre-treated with rh-CCN5 (12.5 nM) for 48 h and then treated with PA (1 mM) and glucose (25 mM) for 24 h. The blots are a representative of at least 3 experiments. Fold changes were normalised to total caspase and total PARP. All quantitative data are represented as mean ± SEM. $^{#}$P<0.05, $^{###}$P<0.001 vs UT, *P<0.05 vs PA+GLU. (E) Islet cells were pretreated with rh-CCN5 (25 nM) for 48 h and then treated with streptozotocin (STZ) (5 mM) for 24 h. Histone associated DNA fragmentation assay was used to evaluate cell death. P-value was generated by student t test. $^{##}$P<0.01 vs UT, *P<0.05 vs STZ.

Figure II-3: Effect of rh-CCN5 on β cell identity and function.

(A)(B) RT–qPCR showing relative level of messenger RNA expression of key genes involved in β cell function. Primary mouse islets were cultured in 10% FBS, rh-CCN5 (25 nM) and 16.7 mM glucose for 24 and 48h. Changes in the levels of GLUT2, GCK, INS1 and INS2 were measured by quantitative RT-PCR. The graphs are representatives of at least 3 experiments. Fold changes were normalised to GAPDH. All quantitative data are represented as mean ± SEM. P-value was generated by t test. ***P<0.001 and *P<0.05 vs UT.

Figure II-4: Regulation of FAK, ERK phosphorylation by rh-CCN5 in INS 832/13 cells and mouse islets.

(A) Western blots demonstrating relative levels of p-FAK in INS832/13 cell extracts cultured in RPMI medium containing 1% FBS and rh-CCN5 (12.5 nM) for 0.5 or 4h. The blots are representatives of at least 3 experiments. Fold changes were normalised to t-FAK. All quantitative data are represented as mean ± SEM. **P<0.01 vs UT. P-value was generated by student t test. (B) Western blots demonstrating relative levels of p-ERK in INS 832/13 cells cultured in RPMI medium containing 10%FBS and rh-CCN5 (12.5 nM) for 0.5, 1, 3, and 6 h. The blots are a representative of at least 3 experiments. Fold changes were normalised to t-ERK. All quantitative data are represented as mean ± SEM. *P<0.05 vs UT, **P<0.01 vs UT, ***P<0.1 vs UT. P-value was generated by student t test. (C) Western blots demonstrating relative levels of p-FAK in INS832/13 cell extracts cultured in RPMI medium containing 2.8 mM glucose and Y15 (FAK inhibitor). T-FAK and tubulin were used as blotting controls. (D) MTT assay representing viability of INS 832/13 cells cultured for 48 h in RPMI medium containing either 1% or 10% FBS in addition to rh-CCN5 (12.5 nM) and Y15 (2 μM). All quantitative data are presented as mean ±

SEM of at least 3 experiments. P-values were generated using t tests. # $P<0.05$ vs UT, @$P<0.01$ vs CCN5.

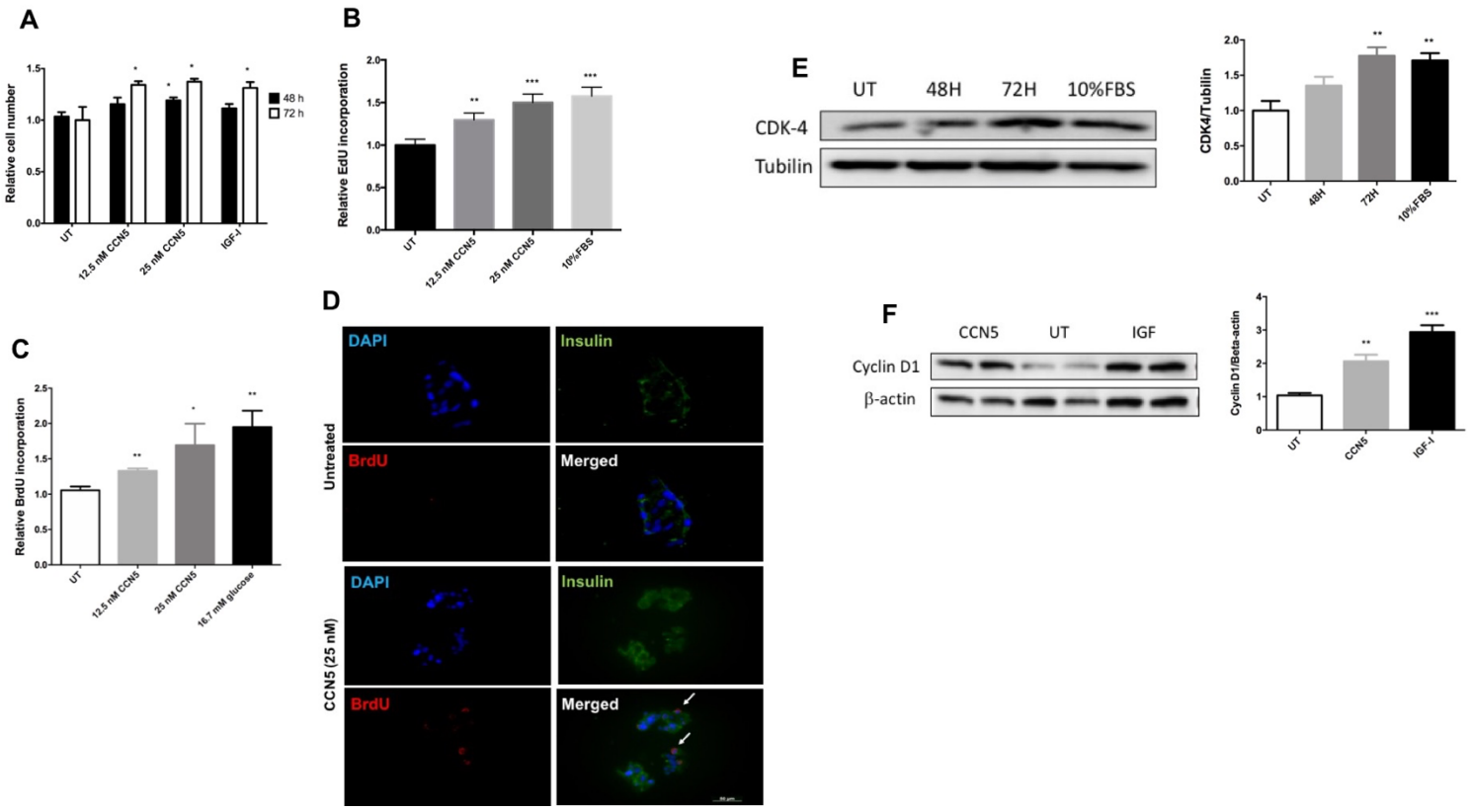

Figure II-1: Effect of recombinant CCN5 on the proliferation of rat insulinoma cells INS 832/13 and mouse pancreatic islet cells

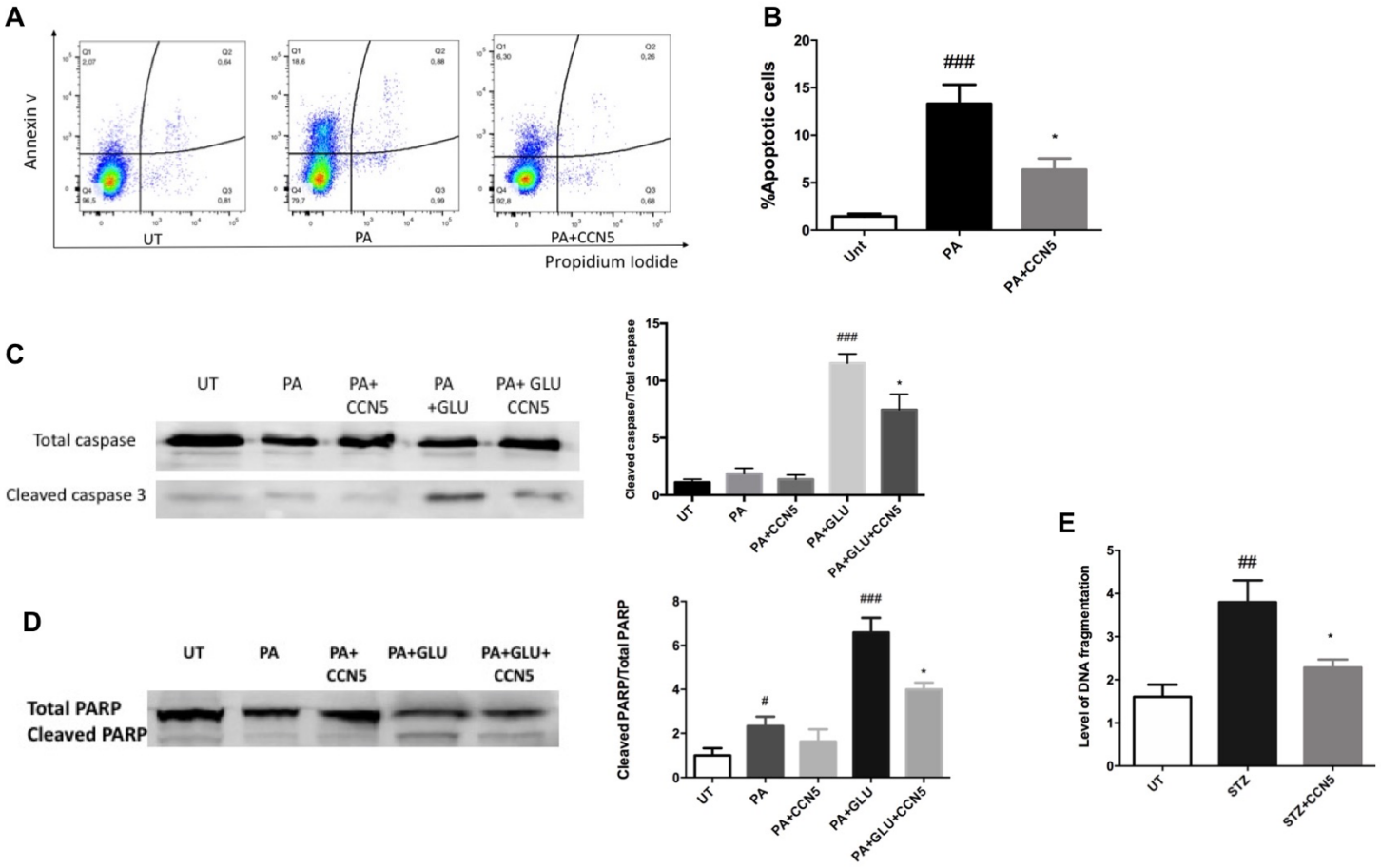

Figure II-2:Effect of rh-CCN5 on lipotoxicity, glucolipotoxicity and streptozotocin induced cell death in INS832/13 cells and primary mouse islets

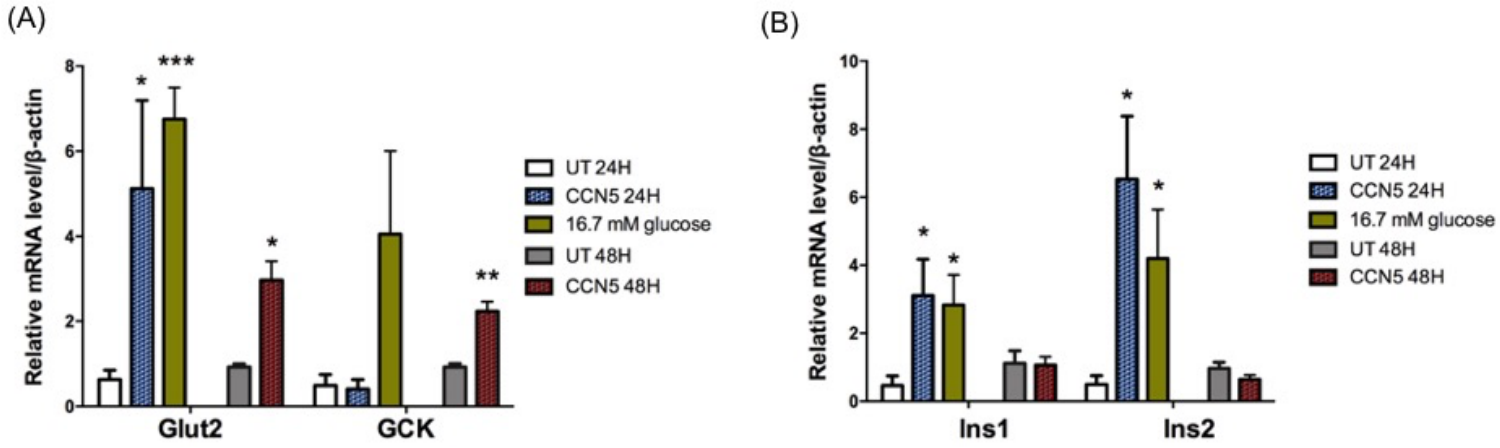

Figure II-3: Effect of rh-CCN5 on β cell identity and function

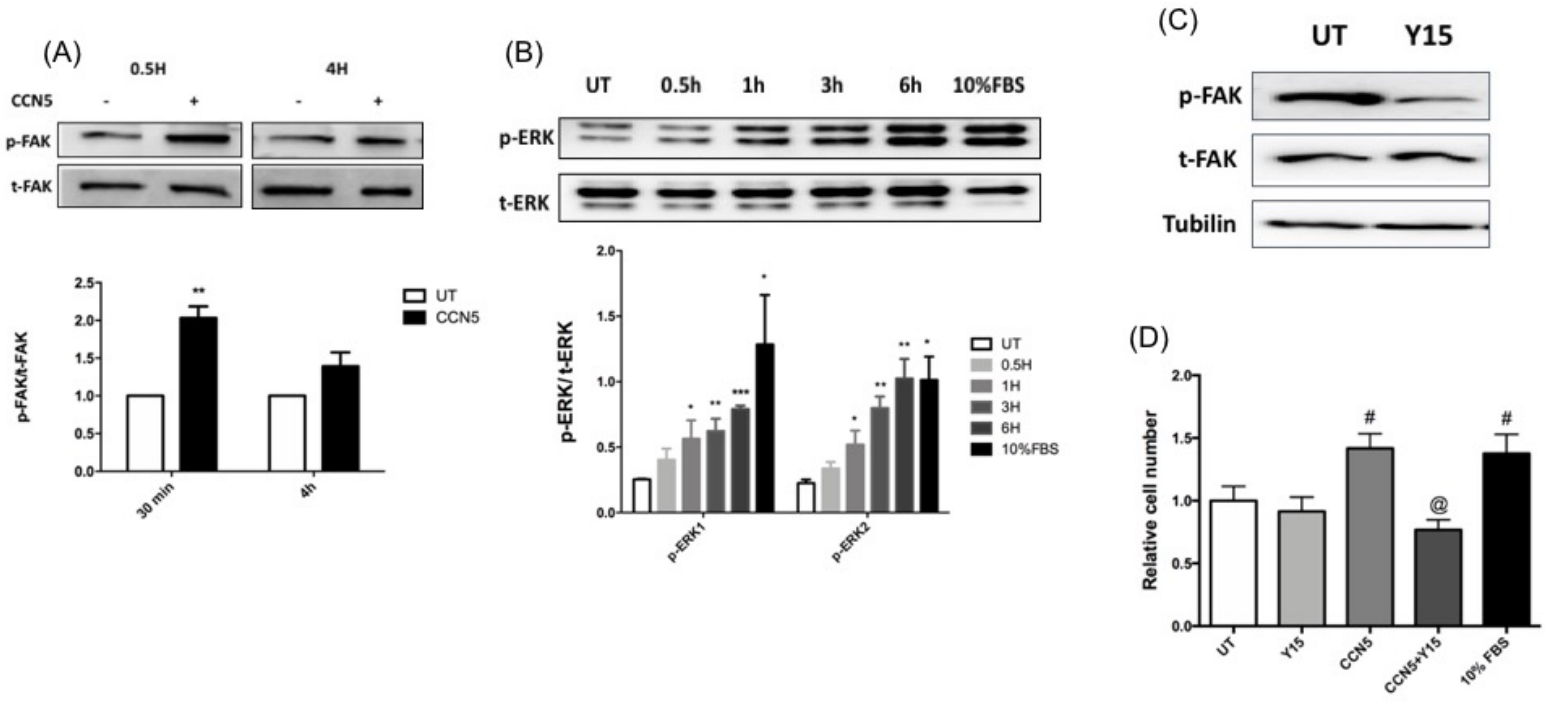

Figure II-4: Regulation of FAK, ERK phosphorylation by rh-CCN5 in INS 832/13 cells and mouse islets

CHAPTER III

In the previous chapter (II), I have established that rh-CCN5 stimulates the proliferation and survival of pancreatic ß-cells. In this chapter, I will examine the effects of rh-CCN5 on the function of ß-cells, focusing mainly on insulin secretion.

(Manuscript in preparation for

Molecular and Cellular Endocrinology)

Recombinant protein CCN5/WISP2 exerts a time-dependent, biphasic effect on insulin secretion in primary mouse islets

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1. ABSTRACT

ß-cell dysfunction is a hallmark of T2D, hence improving ß-cell function appears to be an essential therapeutic route to overcome this disease. We recently identified CCN5/WISP2 to have potential growth and survival-promoting properties when added exogenously to cultures of primary mouse islets and insulinoma cells. This prompted us to initiate further investigations involving the effects of CCN5 on ß-cell function focusing on insulin secretion. Therefore, we evaluated the effects of CCN5 on several parameters of ß-cell function, including mitochondrial ATP generation in addition to insulin biosynthesis and secretion. Treatment of primary islets with rh-CCN5 potentiated a time-dependent, biphasic regulation of insulin content and secretion that correlated with the unfolded protein response (UPR) state of the islets. CCN5 enhanced insulin biosynthesis and secretion after 12 h of treatment, which correlated with an elevation of UPR markers Bip, ATF6, and sXBP1. On the other hand, CCN5 inhibited insulin secretion after 24 hours, which correlated with a decrease in the same UPR markers. Finally, CCN5 potentiated mitochondrial ATP generation independent of its effects on insulin secretion. Hence, our results represent CCN5 as a potential candidate for regulating ß-cells secretory function.

Keywords: ß-cell, insulin secretion, ATP generation, unfolded protein response.

2. INTRODUCTION

Insulin resistance is an essential prerequisite for type 2 diabetes (T2D) [1]; however, chronic hyperglycemia and overt T2D do not occur until β-cells are unable to release sufficient insulin to offset the insulin resistance [2]. To overcome insulin resistance, β-cells compensate by increasing their mass and their secretory function. However, they will reach a point where no further compensation can competently regulate glucose metabolism. At this stage, β-cells experience a state of decompensated endoplasmic reticulum (ER) stress, which leads to their progressive failure and dysfunction [3]. Hence, an effective approach to counteract T2D is to enhance β-cell function and boost insulin release.

In this scope, we recently identified CCN5, a matricellular protein that displays the characteristics of a typical growth factor in pancreatic β-cells. CCN5 belongs to the cysteine-rich family of matricellular proteins, which consists of six members that share a 40% to 60% sequence similarity with one another [4]. Members of this family share a unifying modular structure comprising an amino-terminal signal peptide followed by four conserved domains: the insulin-like growth factor-binding proteins (IGFBP), the von Willebrand factor C (VWC) domain, thrombospondin type 1 repeat (TSR) and a carboxy-terminal domain. However, CCN5 uniquely lacks the CT module. This feature implicates CCN5 in different or perhaps opposing cellular functions from other members [5]. Through the four domains, CCN proteins interact with ECM components, either receptors or other proteins, to mediate essential regulatory activities such as cell proliferation, survival, migration, and differentiation [6].

We have previously investigated the effect of CCN5 on the proliferation and survival of β-cells. Accordingly, we have established that CCN5 either as overexpressed or as a recombinant protein, promotes the proliferation and survival of β-cells [7, 8]. We also evaluated the effect of

CCN5 on the expression of key genes associated with β-cell identity, and function and we detected a time-dependent regulation of the expression of ins1, ins2, Glut2, and GCK. However, we did not examine the effect of CCN5 on β-cell function and, most specifically, insulin secretion. As a result, our objective in this study is to identify whether CCN5 modulates the insulin secretion function of ß-cells and the transduction pathways associated with this modulation. We found that CCN5 stimulates a time-dependent, biphasic regulation of glucose-induced insulin secretion (GSIS) that is associated with the ER stress/UPR state of the ß-cells.

3. MATERIALS AND METHODS:

A. Materials

Culture media RPMI 1640, fetal bovine serum (FBS), and antibiotic supplements were purchased from Wisent (Saint-Jean-Baptiste, QC). Collagenase type Type IV (C5138) was purchased from Sigma-Aldrich (Oakville, ON). PCR primers were purchased from Integrated DNA Technologies (IDT) (Kanata, ON). Mouse insulin ELISA kit (EMINS) was purchased from Thermofisher Scientific (Saint Laurent, QC). Promega 3D Cell Titer-Glo assay was purchased from Promega (Madison, Wisconsin).

B. Mouse islet isolation

All animal handling procedures were approved by the Research Institute Animal Care Committee of McGill University Health Center. Pancreatic islets were isolated from 10–12 weeks old male C57BL/6 mice via collagenase perfusion as described before [7, 9]. Islets were maintained overnight at 37 ^{o}C in RPMI supplemented with 10% fetal bovine serum (FBS), 2 mM glutamine, and 100 U/mL/0.1 mg/mL penicillin/streptomycin. Following overnight (ON) recovery, they were used for subsequent experimental procedures.

C. Quantification of static insulin secretion and content

Recovered islets were pretreated with rh-CCN5 for several time points before being incubated in Krebs-Ringer buffer (KRB) containing 2.8 mM glucose for two periods of 20 min at 37 ^{o}C; islets were washed with the same buffer in between the two periods. Group of 10 islets were then incubated in a 24 well plate in KRB containing either 2.8- or 16.7-mM glucose for 1 h as reported [10]. The buffer surrounding the islets was collected for the measurement of secreted insulin. The remaining islets were picked and lysed with 35% acid ethanol (1.5% HCL, and 75% ethanol) for the measurement of their insulin content. Insulin concentration in islets lysate and surrounding buffer was assessed by ELISA according to the manufacturer's instructions.

D. Quantification of cellular ATP level

ON recovered mouse islets were incubated for 40 min in KRB buffer containing 2 mM glucose, then groups of 10 mouse islets were transferred into white-walled 96-well plates (allow light reflection to maximize light output signal). Islets were incubated for one hour at 37 ^{o}C in the presence of either 2.8 or 16.7 mM glucose, lysed, and ATP levels were determined by chemiluminescence using the 3D-cELL Titer-Glo assay, according to the manufacturer's instructions [11].

E. Gene expression analysis by qRT-PCR

Total RNA was extracted from mouse islets using RNAeasy Micro kit (Qiagen) and reverse transcribed into cDNA using a Quantitect reverse transcription kit (Qiagen). Forward and reverse sequence primers were designed by integrated DNA technologies (IDT), and mRNA levels were normalized to that of ß-actin. Primer sequences were as follow: Bip F: 5'-AGAGTTCTTCAATGGCAAGGAG-3' and R: 5'-ATCAAGCAGT ACCAGATCACC-3', sXBP1 F: 5'-AGTGGTGGATTTGGAAGAAGAG-3' and R: 5'-TCTGGAACCTCGTCAGGAT-

3', ATF6 F: 5'-CCGTGACT AAACCTGTTCTACA-3 and R: 5'-CTTT A TCA TCCGCTGCTGTCT-3'.

Real-time quantitative PCR was performed using the ViiA7 real-time PCR system (ThermoFisher Scientific) and the Power Up SYBR green master mix (ThermoFisher Scientific).

F. Statistical analysis

Numerical values are expressed as mean ± SEM. The numbers of experiments and replicates are included in the figure legends. Statistical analyses were performed using Prism GraphPad software. Differences were considered significant for p values < 0.05.

4. RESULTS

A. Rh-CCN5 regulates insulin secretion in primary islets in a time-dependent, biphasic manner

In our last report, we established the effect of CCN5 on the proliferation and survival of ß-cells [7]. Consequently, our next step was to investigate the effect of CCN5 on the insulin secretory function. Accordingly, primary mouse islets were exposed to rh-CCN5 for different time points (6, 12, 24, and 48 h), then challenged with different concentrations of glucose (2.8 and 16.7 mM) for 1 h. Insulin release and content were assessed by ELISA. As a result, exogenous treatment of primary mouse islets with rh-CCN5 activated a time-dependent biphasic effect on glucose-induced insulin release. Rh-CCN5 had no effect on glucose-stimulated insulin secretion (GSIS) nor insulin content after 6 h of treatment (figure III-1A). However, after 12 h, CCN5 potentiated significant insulin release by 44 %, which was associated with a 60% increase in insulin content (figure III-1B). However, after 24 h of treatment, CCN5 inhibited glucose-induced insulin release by 80% but had no effect on insulin content (figure III-1C). Interestingly, at this time point,

basal insulin secretion was as well decreased by 80 % (figure III-1C). After 48 h, CCN5 lost its effects on both insulin release and content (figure III-1D). Together these data indicate a biphasic regulation of GSIS by CCN5, whereby CCN5 stimulates insulin content and release after 12 h but decreases this effect after 24 h.

B. Rh-CCN5 potentiates ATP generation in primary mouse islets

Once glucose enters into the cytoplasm of ß-cells, it undergoes glycolytic metabolism, generating ATP, which indirectly drives insulin release from the ß-cells [12]. As rh-CCN5 exerted a biphasic regulation of GSIS, we were interested in checking whether CCN5 would similarly regulate the ATP level. Hence, we assessed the effect of CCN5 on the ATP level at 12 and 24 h of CCN5 treatment. Elevation in glucose concentrations from 2.8 to 16.7 mM caused, as expected, an increase in ATP concentration by 60% (Figure III-2A and B). Pre-treatment of islets with CCN5 for 12 h further potentiated glucose-induced ATP production by 50% (Figure III-2A). Treatment with CCN5 for 24 h also enhanced glucose-induced ATP production but not significantly (Figure III-2B). Together these data confirmed a close association between the stimulatory effects of CCN5 on GSIS and ATP after 12 h of treatment. However, the inhibitory effects of CCN5 on GSIS at 24 h wasn't associated with a decrease in ATP concentrations, suggesting an independent mechanism. Additional markers of ß-cell function upstream of insulin release need to be assessed to better understand the prevailing mechanism; those include ongoing efforts to evaluate islet second messenger cAMP and possible changes in intracellular [Ca^{2+}] level as reported [13].

C. Rh-CCN5 regulates the expression of UPR markers in a time-dependent manner

ß-cells are shown to alternate between different states of insulin biosynthesis and UPR activity depending on the body's insulin demand [14, 15]. In a state of high insulin demand, ß-

cells experience a state of elevated ER stress that results from excessive insulin biosynthesis; this state is characterized by high insulin and low UPR. To recover from the stress, ß-cells shift into a state of stress recovery characterized by UPR activation and decrease in insulin biosynthesis [14, 15]. Accordingly, we hypothesize that the observed time-dependent, and biphasic response induced by CCN5 on insulin secretion might be associated with the different states that the ß-cells undergo. For instance, after 12 h, when CCN5 is enhancing insulin synthesis and secretion, ß-cells would be in a state of low UPR, and after 24 h when CCN5 is inhibiting GSIS, ß-cells would be in a state of elevated UPR. We measured the expression of genes associated with UPR (Bip, ATF6, and Xbp1) in mouse islets treated with rh-CCN5 for 12 and 24 h respectively. After 12 h incubation, CCN5 induced a tendency to decrease UPR markers BIP, ATF6, and XBP1 by 22 %, 40%, and 35%, respectively (Figure III-3A). These changes, although evident, didn't reach statistical significance; additional experiments are required for this purpose. After 24 h, CCN5 seems to induce an increase in UPR markers BIP, ATF6, and XBP1 by 94 %, 50 %, and 26 %, respectively (non-significant) (Figure III-3B) as expected. Altogether, these results seem to indicate that a time-dependent, biphasic regulation of GSIS by CCN5 is associated with UPR activation in ß-cells.

5. DISCUSSION

In our previous investigations, we have assessed and demonstrated growth and survival stimulatory effects for CCN5 in pancreatic ß-cells [7, 8]. The present study represents a continuation of our previous inquiries, as we were interested in the effects of CCN5 on the insulin secretory function of ß-cells. As a result of this, we applied exogenous recombinant CCN5 in vitro to primary mouse islets and assessed several markers of ß-cells function and metabolism. Then we tried to develop a mechanism associated with CCN5 regulation for insulin secretion.

Thorough and sophisticated reports have examined the role of CCN5 in the peripheral organs that are responsive to insulin, and those include mostly adipose tissues [16-18]. Mice overexpressing CCN5 in the adipose tissue has been generated, and these mice displayed increased lean body mass, expanded brown adipose tissues (BAT), improved insulin sensitivity, and enhanced glucose uptake by adipose cells and skeletal muscle [19]. The healthy phenotype of CCN5 overexpressing mice highlight CCN5 as a novel and attractive target that could be employed to counteract obesity and insulin resistance, both of which are crucial risk factors in the etiology of type 2 diabetes. Collectively these results link CCN5 effects in the adipose tissues indirectly to pancreatic ß-cells. We, on the other hand, investigated the direct effect of CCN5 on ß-cell physiology [7, 8]. To our knowledge, we were the first to propose a role of CCN5 in ß-cells. We identified CCN5 as a downstream target of IGF-I in primary islets [8]. We demonstrated that CCN5 is a secreted growth factor that stimulates ß-cells proliferation and survival through a mechanism involving FAK/ERK activation [7].

As mentioned previously, CCN5 overexpression enhanced insulin sensitivity in adipose tissues [19], but does it enhance insulin release? In our latest report, we assessed the effect of CCN5 on the expression of the insulin genes ins1 and ins2 in mouse islets. CCN5 upregulated the expression of ins1 and ins2 significantly after 24 h of treatment, but the effect disappeared at 48 h [7]. In the current report, we assessed the direct effect of CCN5 on GSIS in mouse islets. CCN5 triggered an increase in insulin release after 12 h treatment. Interestingly, the content of insulin inside the islets was also increased, signifying that CCN5 enhances not only insulin release but also the rate of insulin biosynthesis.

To our surprise, the stimulatory effect was not only inhibited after 24 h but completely reversed. CCN5 caused a decrease in insulin release after 24 h. The insulin content was not

significantly affected at this time point, indicating the decrease in insulin release might be independent of insulin content/biosynthesis at 24 h. This time-dependent, biphasic regulation of GSIS by CCN5 could be attributed to the dynamic nature and heterogeneity of ß-cells. It has been proposed that ß-cells alternate between states of high activity and stress recovery. The active state is marked by increased insulin biosynthesis and release, then to avoid exhaustion ß-cells shift to a recovery state marked by decreased insulin biosynthesis and secretion [15]. Based on this model, we propose that the treatment of islets with CCN5 for 12h triggers an active mode of ß-cells characterized by insulin synthesis and secretion. On the other hand, the treatment of islets with CCN5 for 24 h push the cell into a resting state characterized by decreased insulin secretion.

Given its regulatory effects on GSIS, we tried to assess the effect of CCN5 on other markers of ß-cells function. Hence, we measured the effect of CCN5 on cellular ATP content, which is a key link between glucose metabolism and insulin release [20]. Once glucose is in the cytoplasm of ß-cells, it undergoes glycolytic metabolism to generate mitochondrial ATP as an end product [21]. An increase in intracellular ATP triggers a sequence of reactions involving Ca^{2+} influx via voltage-gated ion channels, which sequentially triggers insulin exocytosis [11]. Treatment of primary islets with CCN5 for 12 h enhanced ATP generation. This stimulatory effect on ATP correlates with a stimulatory effect on GSIS by CCN5. Hence, we could suggest a direct functional link between CCN5 stimulatory effect on ATP production and GSIS. An additional marker that could strengthen our observation is the changes in Ca^{2+} level. Ca^{2+} influx is a fundamental step between ATP generation, and insulin release, hence quantification of intracellular Ca^{2+} would give us a clear perspective on how CCN5 stimulates GSIS at 1 h. On the other hand, CCN5 also stimulated ATP generation at 24 h. This effect can't explain its inhibitory effect on GSIS at that time. Hence, the inhibitory effect of CCN5 at 24 h is independent of ATP generation. In our latest

report, we assessed the effect of CCN5 on the expression of key genes involved in glucose metabolism: the glucose transporter (Glut2) and the enzyme glucokinase (GCK). CCN5 upregulated the expression of both genes at 24 h [7]. ; this upregulation could be linked to the stimulatory effect of CCN5 on ATP generation at that time point. However, they don't associate with the inhibitory effects on GSIS at 24 h.

As previously reported, ß-cells are highly dynamic as they alternate between states of high activity and stress recovery [22]. Gene expression profiling analyses have revealed that ß-cells display different expression profiles between these states. The genes that display differential expression mainly are those involved in ER stress/UPR [14, 22]. Accordingly, we thought to examine the expression of these genes in primary islets after 12 and 24 h treatment with CCN5. We considered three markers of UPR activation: 1) binding immunoglobulin protein (Bip), which is an essential activator of the UPR pathway, 2) activating transcription factor (ATF) 6 and 3) spliced X-box binding protein (XBP1s),which upon UPR activation, translocate to the nucleus and activate the transcription of ER chaperones. CCN5, after 12 h, induced an active state of ß-cells characterized by enhanced insulin synthesis and release in addition to ATP generation. This state correlates with a gradual increase in ER stress and low UPR as the cells are fueled to synthesize more insulin. Treatment of primary islets with CCN5 for 12 h seems to downregulate the expression of the three UPR markers Bip, ATF6, and spliced XBP-1 as expected. On the other hand, CCN5, after 24 h, induced a recovery state in ß-cells characterized by decreased GSIS. This state would correlate with UPR activation as the cells are trying to counteract and recover from the stress they had faced. Treatment of primary islets with CCN5 for 24 h indeed upregulated the expression of UPR markers Bip, ATF6 and XBP1. Altogether these data confirm a biphasic effect of CCN5 on ß-cells that is most likely associated with the UPR activity in these cells.

In summary, we have demonstrated that CCN5 regulates the insulin secretory function of ß-cells. This regulation appears to be dependent on the ER stress/UPR level in ß-cells, as the ER activity is essential for proper insulin synthesis and folding. High UPR seems to correlate with CCN5 inhibitory effects on insulin secretion, and low UPR correlates with CCN5 stimulatory effects on insulin secretion. Additional investigationS would be required to gather a full insight on how CCN5 regulates UPR and insulin secretion, but overall, CCN5 represents a potential candidate for regulating ß-cells secretory function.

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DISCLOSURE:

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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FIGURE LEGENDS

Figure III-1: Effects of rh-CCN5 on glucose induced insulin secretion in primary mouse islets.

Insulin secretion and insulin content in mouse islets treated with rh-CCN5 for 6 (A), 12 (B), 24 (C) and 48 h (D). Primary islets were allowed to recover overnight in RPMI containing 10% serum then treated with 25 nM rh-CCN5 for 6 to 48 h. Islets were then pre-incubated in Krebs-Ringer buffer (KRB) containing 2.8 mM glucose for 40 min at 37ºC. Group of 10 islets were then incubated in KRB containing either 2.8- or 16.7-mM glucose for 1 h. The buffer surrounding the islets was used for quantification of secreted insulin and remaining islets were lysed for quantification of insulin content. Insulin concentration were determined using ELISA. All quantitative data are presented as mean ± SEM of at least three experiments. p value was generated by Student t-test. $^{*}p<0.05$, $^{***}p<0.001$ vs UT.

Figure III-2: Effects of rh-CCN5 on ATP generation in mouse islets

Cellular ATP level in primary islets treated with rh-CCN5 for 12 (A) and 24 h (B). Islets were allowed to recover overnight in RPMI containing 10% serum then treated with 25 nM rh-CCN5 for 12 or 24 h. Islets were then incubated in Krebs-Ringer buffer (KRB) containing 2.8 mM glucose for 40 min at 37ºC. Group of 10 islets were then incubated in KRB containing either 2.8- or 16.7-mM glucose for 1 h. Islets were picked into a group of 10, then lysed. ATP level was determined by chemiluminescence using the 3D-cELL Titer-Glo assay. All quantitative data are presented as mean ± SEM of at least three experiments. p value was generated by Student t-test. $^{*}p<0.05$ vs UT.

Figure III-3: Effect of rh-CCN5 on the expression of key genes associated with unfolded protein response (UPR)

(A, B) qRT-PCR showing relative level of messenger RNA of key genes involved in UPR in mouse islets treated with rh-CCN5 for 12 or 24 h. Primary islets were allowed to recover overnight in RPMI containing 10% serum before being treated with 25 nM rh-CCN5. Changes in the levels of Bip, ATF6 and spliced XBP1 mRNA were measured by quantitative RT-PCR. The graphs are representatives of two experiments each with n=3. Fold changes were normalized to ß-actin. All quantitative data are represented as mean ± SEM.

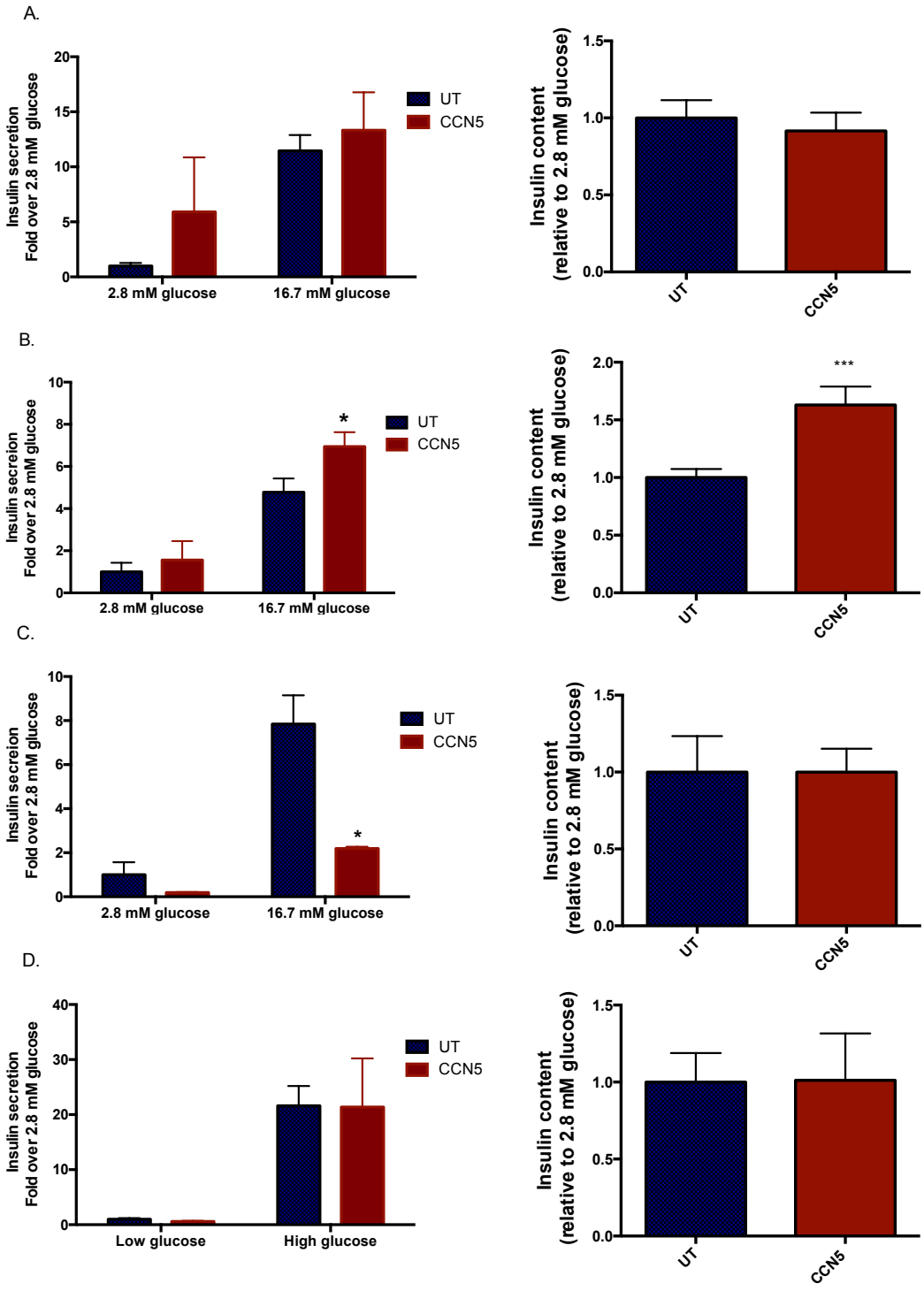

Figure III-1: Effects of rh-CCN5 on glucose induced insulin secretion in primary islets

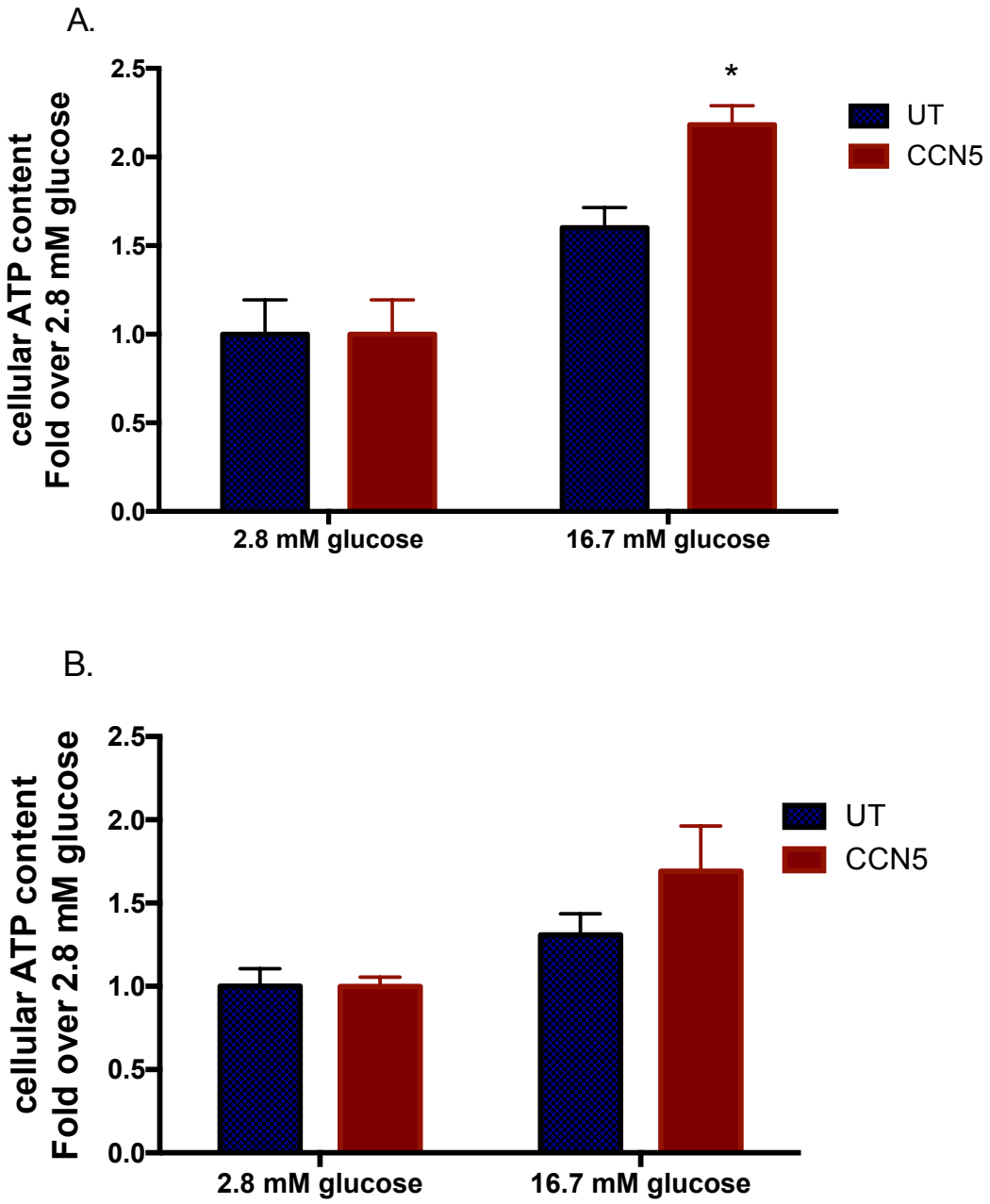

Figure III-2: Effects of rh-CCN5 on ATP generation in primary mouse islets

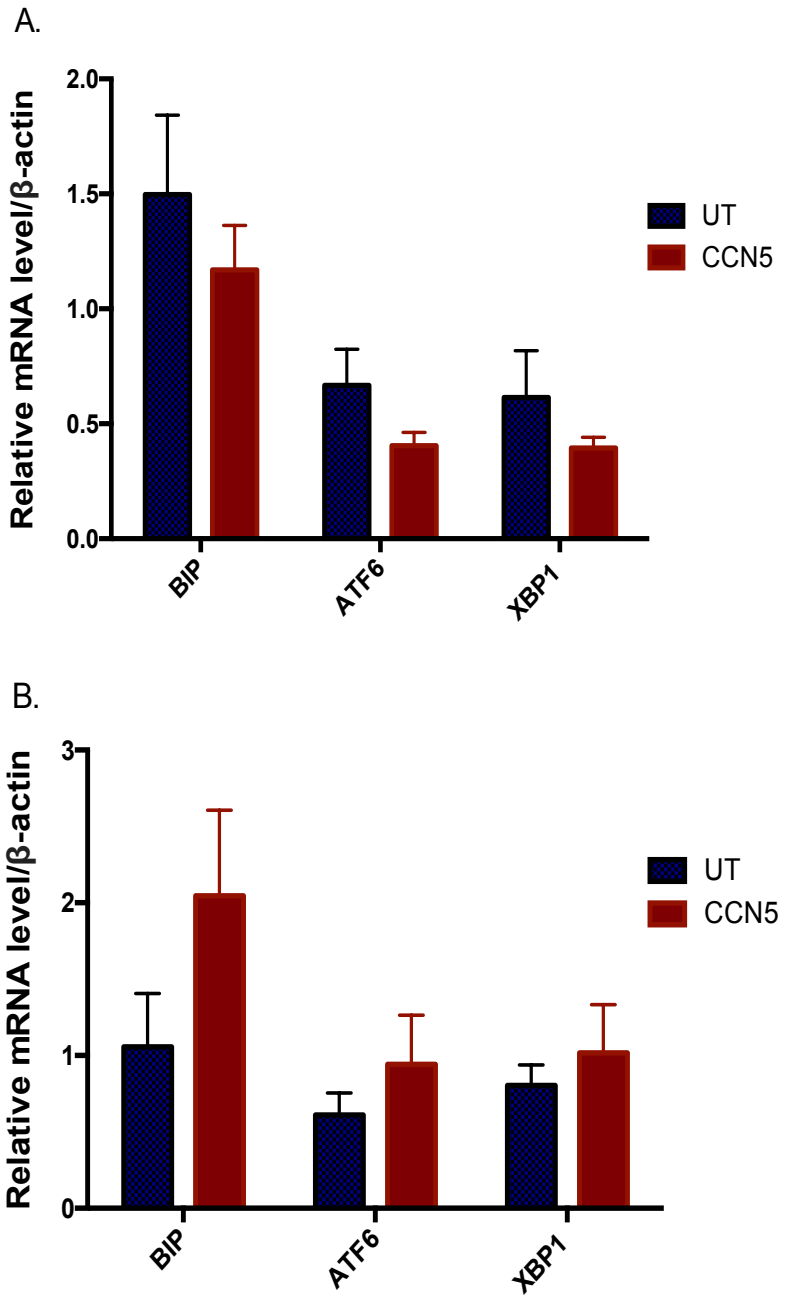

Figure III-3:Effect of rh-CCN5 on the expression of key genes associated with UPR

CHAPTER IV

In the previous chapters (II and III), I have established that rh-CCN5 stimulates the proliferation, survival, and insulin secretory function of pancreatic ß cell. In this chapter, I have examined the mechanism and the targets involved in the stimulatory effects CCN5.

(Manuscript in preparation and to be submitted)

Explorations on the target genes and cell surface receptors that mediate CCN5 effects in pancreatic ß cells

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1. ABSTRACT

We recently identified CCN5/WISP2 as a direct target of insulin-like growth factor I (IGF-I) in primary mouse islets. We further demonstrated that either CCN5 over-expression or the recombinant protein promote the growth, survival, and function of primary islets and insulinoma cells. However, the molecular machinery associated with CCN5 stimulatory effects in pancreatic ß cells is not yet established. Hence, the current study was designed to identify specific and novel targets for CCN5 in pancreatic ß cells in addition to a high-affinity specific receptor. Accordingly, we performed whole-genome cDNA microarray analysis in mouse islets treated with recombinant CCN5 (rh-CCN5) and identified 34 genes mainly involved in inflammation, wound healing, and G-protein coupled receptor signaling. We then performed LRC-TriCEPS (ligand receptor capture) and identified specific interactions with Neuronal acetylcholine receptor subunit alpha-3 (ACHA3). The identifications of particular targets and a high-affinity cell surface receptor for CCN5 in ß cells will help us delineate the mechanism through which CCN5 regulates ß cells function.

Keywords: Diabetes, ß cells, cDNA microarray analysis, LRC-TriCEPS, receptor

2. INTRODUCTION

CCN5, also known as Wnt inducible signaling protein 2 (WISP2), is a 29-kDa secreted cysteine-rich matricellular protein that was first identified in cultures of a heparin treated vascular smooth muscle cell (VSMCs) [1]. CCN5 is a member of the CCN family, which includes five other members recognized as CCN1, 2,3,4, and 6. The members of this family share a 40 to 60 % sequence [2]. Unlike other CCN family members, which encompass four structural modules with sequence homologies with insulin-like growth factor binding proteins (IGFBP), von Willebrand factor (VWC), thrombospondin (TSP-1), and cysteine knot (CT), CCN5/WISP2 contains only first 3 of these structures, but lacks the CT domain [3].

As standard matricellular proteins, CCN members regulate various growth regulatory functions such as mitosis, adhesion, apoptosis, extracellular matrix production, growth arrest, and migration [4]. Typically, CCNs mediate these activities by interacting with multi-ligand receptors, just like other ECM associated proteins. However, in addition to their ECM associated activities, CCNs were found to mediate growth stimulatory functions [3]. Accordingly, CCNs, like typical growth factors, are expected to bind to high-affinity cell surface receptors to mediate their functions [5].CCN5 was shown to interact with typical promiscuous ECM multi-ligand receptors such as integrins. For instance, CCN5 binds $\alpha_v\beta_3$ in the podosomes of vascular smooth muscle cells (VSMC) [6] and α6β1in breast cancer cells MDA-MB-231 [7]. A high-affinity specific receptor for CCN5 is not yet identified. Lipoprotein receptor-related protein 5/6 (LRP 5/6), a co-receptor for canonical Wnt, represents a co-receptor [8] as CCN5 is thought to regulate adipogenesis by activating canonical WNT [9, 10].

Through ligation with integrins and other unknown receptors or binding partners, CCN5 participates/regulates processes such as differentiation [9, 10], adhesion [6], proliferation [7, 11, 12], survival [13], motility, invasiveness, and metastasis [14-16]. The pathways through which

CCN5 mediates most of these activities include PI3K/AKT signaling [17], MAPK/ERK signaling [18], TGF-β signaling [19], and WNT signaling [9]. We identified growth stimulatory effects for CCN5 in pancreatic ß cells through activation of ERK signaling [13]. However, we didn't investigate into the high-affinity receptor and the targets through which CCN5 mediate those effects. A complete understanding of the biological effects of CCN5 on ß cells requires the identification of a specific receptor and the signal transduction pathways. Thereby in this study, we aimed to characterize the targets regulated by CCN5 in pancreatic ß cells in addition to a high-affinity specific receptor that mediates CCN5's actions. Using gene expression profiling, we identified numerous targets regulated by CCN5 in primary islets. Using CaptiREC LRC-TriCEPS technology, we identified a potential receptor for CCN5 on the surface of insulinoma MIN6 cells identified alpha-3 subunit of neuronal acetylcholine receptor (ACHA3).

3. MATERIALS AND METHODS

A. Reagents, cells and tissue culture conditions

Mouse insulinoma MIN6 cells were a generous gift from Dr. Louise Larose. (McGill University). The cells were maintained in DMEM medium containing 10% fetal bovine serum (FBS), 100U/ml penicillin, and 100 μg/ml streptomycin at 37 $^{\circ}$C in an atmosphere of humidified air (95%) and 5% CO2.

Recombinant human CCN5/WISP2 (rh-CCN5) was purchased from PeproTech Inc (Catalog number 120-16). The protein was produced and purified in E. coli with >95% purity by SDS-PAGE and HPLC.

B. Mouse pancreatic islets isolation

All animal handling procedures were approved by the Research Institute Animal Care Committee of McGill University Health Center. Pancreatic islets were isolated from 2-3 months old male

C57BL/6 mice via collagenase perfusion **[20]**. Briefly, collagenase XI (0.65mg/ml) was injected into the bile ducts. The inflated pancreas was then excised and incubated in a water bath at 37 ºC for 15 min. Subsequently, the digestion was stopped with the addition of cold Hanks' balanced salt solution (Gibco). Islets were then separated from the rest of the pancreatic tissues by using Histopaque (Sigma Aldrich) gradients followed by centrifugation at 300 G for 25 min with acceleration 5 and deceleration 1. Healthy looking islets were hand-picked under a stereomicroscope and maintained in RPMI-1640 medium containing 11.1 mM glucose for overnight recovery.

C. cDNA Microarray analysis

Mouse primary islets were cultured in 10% serum RPMI containing vehicle or rh-CCN5 for 48h. Following treatment, islets were lysed using lysis buffer supplemented with 2-Mercaptoethanol, then RNA was isolated using RNAeasy Micro kit (Qiagen) according to the manufacturer's instructions. The quality and integrity of RNA were quantified using Agilent 2100 Bioanalyzer, and concentration was determined via NanoDrop 2000 spectrophotometer. Gene expression profiling was determined using Illumina Whole-Genome Expression BeadChips (MouseRef-8 V2) by McGill University and the Genome Quebec Innovation Centre. To identify and characterize regulated genes, we used the statistical Data analysis software FlexArray software version 1.4. We then uploaded the list of regulated transcripts to the database for annotation, visualization, and integrated discovery (DAVID) v6.8 for functional gene annotation and classification (Table IV-1).

D. TriCEPS-mediated ligand receptor capture (LRC-TriCEPS)

LRC-TriCEPS was performed by Dualsystems Biotech AG (Schlieren, Switzerland). Insulinoma MIN6 cells, CCN5, and insulin were provided by our lab for the experiment. Both

ligands were coupled to TriCEPS reagent. Separately, glycoproteins on the surface of MIN6 cells were selectively oxidized. The cells were incubated with TriCEPS to allow binding and subsequent capture of the complex formed (TriCEPS-ligand-oxidized receptor). Cells were then lysed and passed through affinity columns for purification of ligand-receptor complexes. Once the receptor proteins were purified, they were evaluated and identified by LC-MS analysis using Thermo LTQ Orbitrap XL spectrometer (Figure IV-1). Statistical significance was achieved using an ANOVA model that tests each protein for differential abundance in all pairwise comparisons of ligand and control samples and reports the p-values.

E. Statistical analysis

Numerical data are expressed as mean ± SEM. The numbers of experiments and replicates are included in the figure legends. Statistical analyses were performed using Prism GraphPad software. Differences were considered significant for p values < 0.05.

4. RESULTS:

A. Gene expression profiling identifies novel targets of CCN5 in primary mouse islets

The mechanism and the signaling proteins associated with CCN5 stimulatory activities in pancreatic ß cells [13] are not revealed yet. In this report, we aimed at identifying potential targets regulated by CCN5 in pancreatic ß cells. Accordingly, we performed a microarray gene expression profiling of primary mouse islets treated with vehicle or rh-CCN5 for 48 h. We specifically used 48h due to the significant proliferative effect induced by CCN5 at that time point [13]. Thirty-four genes were found to be significantly regulated by rh-CCN5 (Table IV-1). Among those genes that were significantly induced by CCN5 relative to control were those involved in wound

healing/inflammation (8 genes), immune response (6 genes) G protein-coupled receptor signaling and chemotaxis (5 genes) in addition to several that were unclassified.

Among the genes that were regulated, FPR1 and FPR2 triggered our interest due to their involvement in G protein-coupled receptor signaling (GPCR). Crosstalk between GPCRs, integrin, and ECM proteins has been established in the literature [21]. Hence FPR1 and FPR2 represent crucial CCN5 targets that need be further investigated. Therefore, we further confirmed the CCN5 mediated upregulation of FPR1 and FPR2 mRNA using qRT-PCR. Treatment of mouse islets with CN5 for 48 h upregulated the expression of those genes by 3 and 4-fold, respectively (Figure IV-2). Together these findings identify novel targets for CCN5 in pancreatic ß cells and highlight GPCRs: FPR1 and FPR2 as prospective co-receptors through which CCN5 mediates its regulatory activities in ß cells.

B. LRC-TriCEPS identifies specific interactions between CCN5 and ACHA3 on the surface of MIN6 cells

A complete understanding of the biological effects of CCN5 on any target cell depends on establishing the identity of a specific CCN5 receptor and a signal transduction pathway. Currently, there is limited information on the existence of a CCN5 receptor. On the other hand, CCN2 receptors have been well investigated, and accordingly, few high-affinity receptors have been identified [22, 23]. Hence, in this report, we aim at identifying a specific high-affinity receptor for CCN5 on the surface of insulinoma MIN6 cells using a novel ligand-receptor capture technology known as LRC-TriCEPS or CaptiREC (Dualsystems Biotech AG, Schlieren, Switzerland). Crosslinking of ligand-TriCEPS to the glycans identified a total of 547 enriched surface proteins (Figure IV-3). Three of those surface proteins show a high fold change: 1) Neuronal acetylcholine receptor subunit alpha-3 (ACHA3) with a 12.8 fold change (FC), 2) Multiple epidermal growth

factor-like domains protein 8 (MEGF8; with 12.3 FC and 3) Low-density lipoprotein receptor-related protein 1B (LRP1B) with 7.9 FC (Table IV-2). For the positive control ligand insulin, the receptor peptides of the insulin receptor (INSR) and insulin-like growth factor I receptor (IGF-IR) were identified (Figure IV-3). We then assessed whether CCN5 regulates the expression of ACHA3 in primary islets at the mRNA level. CCN5 treatment downregulated the expression of ACHA3 by 50 % (Figure IV-4). Together these findings bring to table 3 potential high-affinity receptors for CCN5 on the surface of pancreatic ß cells and highlight ACAH3 as the most plausible candidate.

5. DISCUSSION

We have previously demonstrated that CCN5 acts as a secreted growth factor that enhances the proliferation, survival, and function of ß cells [13]. However, the transduction pathway associated with these regulatory functions is still pending. In the present study, we investigated potential targets for CCN5 in primary mouse islets and scanned for a high-affinity receptor on the surface of insulinoma MIN6 cells.

Two prevailing hypotheses depict how CCNs mediate their regulatory activities. The first represents CCN proteins as regular ECM associated proteins that bind to multi-ligand receptors. The second portrays CCN as growth factors that interact with high-affinity receptors specific to them [5]. In our previous report [13], we supported the second notion as we used the recombinant form of CCN5 (rh-CCN5) to test its effects on proliferation, survival, and function of ß cells. Accordingly, we have shown that CCN5 acts as a secreted growth factor in these cells. Hence the necessity to pinpoint a high-affinity specific receptor similar to a typical growth factor receptor.

We first tried to look for general targets regulated by CCN5 in ß cells using microarray gene expression profiling. We identified 34 target genes, some of which are involved in

chemotaxis and G protein-coupled receptor signaling; others are involved in wound healing, inflammation, and immune response. Two targets triggered our attention, formyl peptide receptors 1 and 2 (FPR1 and FPR2), which their regulation was subsequently validated by PCR. These targets are transmembrane proteins that belong to the GPCR family. They regulate similar functions as CCN5 like cell proliferation, wound healing, invasion, migration and apoptosis [24] and are involved in similar signaling pathways as CCN5 such as phosphoinositide 3-kinase (PI3K)/protein kinase B (Akt) pathway [25, 26]and the mitogen-activated protein kinase (MAPK) pathway[27] . The mechanism of how FPR1 and FPR2 contribute to CCN5 effects in pancreatic ß cells is a future direction that we are currently working on. Additionally, other relevant targets within the microarray results will be further assessed based on Gene Enrichment Set Analysis (GESA).

In order to get a better understanding of how CCN5 mediates its regulatory effects on ß cells, our next goal is to identify a membrane receptor. Accordingly, we used a novel technology known as LRC-TriCEPS, which enables the identification of target receptors on living cells without any genetic modification[28]. We identified three potential targets to have specific interactions with CCN5. Those include neuronal acetylcholine receptor subunit alpha-3 (ACHA3), multiple epidermal growth factor-like domains protein 8 (MEGF8), and low-density lipoprotein receptor-related protein 1B (LRP1B). Unlike the two other targets, neuronal acetylcholine receptor subunit alpha-3 (nAChR3) displayed higher statistical significance and hence was our target of interest for further investigations.

NAChRs are a heterogeneous family of non-selective gated cation (Na^+, k^+, and Ca^{2+}) channels that mediate fast synaptic transmission in neurons [29]. They have a pentameric structure consisting of combinations of two different types of subunits (**α** and β) or five copies of the same

α subunit arranged around a central ion pore [29]. Typical ligands/agonists of nAChRs include acetylcholine, nicotine, and other tobacco-related products [30], which upon binding induce a conformational change that opens the ion channel hence allowing signal transmission at neuromuscular junctions in the central and peripheral nervous system. The expression of nAChRs is not exclusive to the nervous system [31]. nACHRs are present in the pancreas [32], more specifically, β-cells [33, 34] in addition to the liver and muscle [31]. Numerous reports have implicated nicotinic cholinergic signalling in pancreatic islet biology, diabetes and obesity [35] [36]. Furthermore, polymorphisms in human nicotinic receptors were associated with β-cell function and diabetes [36, 37]. RNA-seq and genotyping data from human donor islets revealed an association between CHRNB2, CHRNB4, MAFA, and MAFB transcript levels, insulin secretion, and glucose clearance, hence supporting a critical function for nicotinic receptors in β-cell function [36, 38].

We demonstrated a direct regulation of expression of nACHR3 by CCN5 in primary islets. A prolonged exposure of CCN5 caused downregulation of nACHR3 mRNA, thereby suggesting a negative feedback regulation of nACHR3 by CCN5. Similar regulation of nACHR3 was detected in the liver by nicotine as chronic nicotine treatment suppressed the expression of α3 subunit of nAChR [39].

On the other hand, LRP1B, which didn't achieve statistical significance in our screening, represents another potential receptor worth investing. LRP1 showed specific interactions with CCN2 on the surface of bone marrow stromal cells [23]. LRP-1 has also been shown to form a physical complex with CCN1-$\alpha_6\beta_1$ to mediate reactive oxygen species (ROS) production and apoptosis[40]. In this case, LRP served as a co-receptor for CCN1 mediated integrin signaling. Additional experiments are required to identify a specific binding of CCN5 to ACHA3 or LRP1B.

Those include radioactive ligand binding assays, where rh-CCN5 will be radioactively labeled by [125I], and the target receptor will be overexpressed in HEK293 cells.

In conclusion, we have provided new evidence towards a mechanism of how CCN5 mediates its regulatory effects in pancreatic β cells. CCN5 acts as a growth factor that binds a specific receptor on the surface of β cells, thereby activating downstream signaling pathways that lead to the regulation of expression of multiple target genes. Those include transcripts involved in inflammation, wound healing, and G protein-coupled receptors. On-going experiments focus on establishing how those genes are involved in CCN5 mediated activities and on pinpointing chrna3 or LRP1B as a cognate CCN5- high-affinity specific receptor.

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AUTHORS CONTRIBUTIONS

N.K. performed all experiments, presented the data, and wrote the manuscript. L.G. assisted in the microarray experiment. J.-L.L. designed the study, revised the manuscript, and approved the final revision. Z.G. helped in designing and analysis of the data.

DISCLOSURE OF INTEREST

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest

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Table IV-1:Changes in gene expression profiling of mouse primary islets treated with rh-CCN5 for 48h.

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 using (DAVID) v6.8.

Gene symbol	Fold change	P value	Common name
Inflammation, wound healing and signaling			
IL1a	2.25	0.00011	Interleukin 1 alpha
Serpina3n	2.13	3.10E-05	Serine peptidase inhibitor, clade A, member 3N
Saa3	4.1	1.91E-11	Serum amyloid A 3
Ccl5	3.89	2.76E-14	Chemokine (C-C motif) ligand 3
Vnn3	3.65	1.63E-09	Vanin 3
Cxcl3	3.68	1.04E-11	Chemokine (C-X-C motif) ligand 3
Lcn2	2.34	1.04E-11	Lipocalin 2
Immune response, ribonucleotide binding, and nucleotide binding			
Oas2	2.88	8.01E-11	2-5 Oligoadenylate synthetase 2
Gbp10	2.022	3.89E-08	Guanylate-binding protein 10
Gbp8	3.29	9.26E-06	Guanylate-binding protein 8
Oasl2	2.07	3.39E-10	2-5 Oligoadenylate synthetase-like2
Oas1g	2.14	0.0013	2-5 Oligoadenylate synthetase 1G
Gbp3	2.38	9.53E-08	Guanylate binding protein 3
Lfit1	3.17	2.27E-12	Interferon-induced protein with tetratricopeptide repeats 1

C3		2.13	5.35E-08	Complement component 3
Cfb		3.8	8.28E-10	Complement factor B
Chemotaxis, G- protein coupled receptors, and peptide binding				
Rtp4	2.01	1.69E-10	1.69E-10	Receptor transporter protein 4
H2-Q6		2.23	8.73E-07	Histocompatibility 2, Q region locus 6
Emr1		2.04	4.51E-09	EGF-like module containing, mucin like, hormone receptor-like sequence 1
Fpr2		8.97	2.98E-13	Formyl peptide receptor 2
Fpr1		5.04	1.22E-09	Formyl peptide receptor 1
Other unclassified gene targets				
Snord92		2.27	2.71E-05	Small nuclear RNA, C/D box92
Gm26407		2.22	0.0002	Predicted gene,26407
Apol9b		2.94	8.89E-10	Apolipoprotein L9b
Steap4		3.53	1.96E-08	STEAP family member 4
Zbp1		2.56	8.61E-09	Z-DNA binding protein 1
Rhox3f		2.42	0.0002	Reproductive homeobox 3F
IRF7		2.43	2.3E-12	Interferon regulatory factor 7
Gm24162		-2.013	0.0022	Predicted gene 24162
AF357426		-2.03	0.0011	SnoRNA AF357426
Gm24056		-2.07	4.78E-05	Predicted gene 24056

Table IV-2: List of potential receptor candidates for CCN5 on the surface of MIN6 cells (sorted according to P value). N=3.

Entry	Gene name	Protein name	Peptides	Fold change	P value
Q8R4G9	ACHA3	Neuronal acetylcholine receptor subunit alpha 3	1	12.82	0.0032
P60882	MEGF8	Multiple epidermal growth factor-8	1	12.35	0.018
Q9J18	LRP1B	Low-density lipoprotein receptor-related protein 1B	1	7.9	0.145

FIGURE LEGENDS

Figure IV-1: Flow chart of the CaptiRec^{TM} screening procedure. Adopted from Dualsystems Biotech

Figure IV-2: Effect of rh-CCN5 on FPR1 and FPR2 expression. qRT-PCR showing relative level of messenger RNA expression of FPR1 and FPR2. Primary mouse islets were cultured in 10% FBS, and rh-CCN5 (25 nM) for 48 h. Changes in the levels of FPR1 and FPR2 were measured by quantitative RT-PCR. The graphs are representatives of at least three experiments. Fold changes were normalized to HPRT. All quantitative data are represented as mean ± SEM. p-value was generated by t-test. *P < 0.05 vs UT and **P<0.01 vs UT.

Figure IV-3: CaptiRec volcano plot displaying the receptors for Insulin and CCN5 on the surface of MIN6 cells. Data is shown on the protein level. Log2 FC, fold change. N=3. Proteins with an adjusted p-value <0.01 and a fold change > 2 are considered statistically significant and are marked by a dashed line.

Figure IV-4: **Immunohistochemistry of ACAH3 protein in human pancreas, from Human Protein Atlas.** The brown fragmentation, membranous and cytoplasmic staining of acah3 was shown in selected spots throughout acinar pancreas (A), and more enriched in endocrine islets (I), especially in peri-islets membranes.

Figure IV-5: **Effect of rh-CCN5 on ACAH3 expression**. qRT-PCR showing relative level of messenger RNA expression of ACAH3. Primary mouse islets were cultured in 10% FBS, and rh-CCN5 (25 nM) for 48 h. Changes in the levels of ACAH3 were measured by quantitative RT-

PCR. The graphs are representatives of at least three experiments. Fold changes were normalized to ß actin. All quantitative data are represented as mean ± SEM. p-value was generated by t-test. *P < 0.05 vs UT.

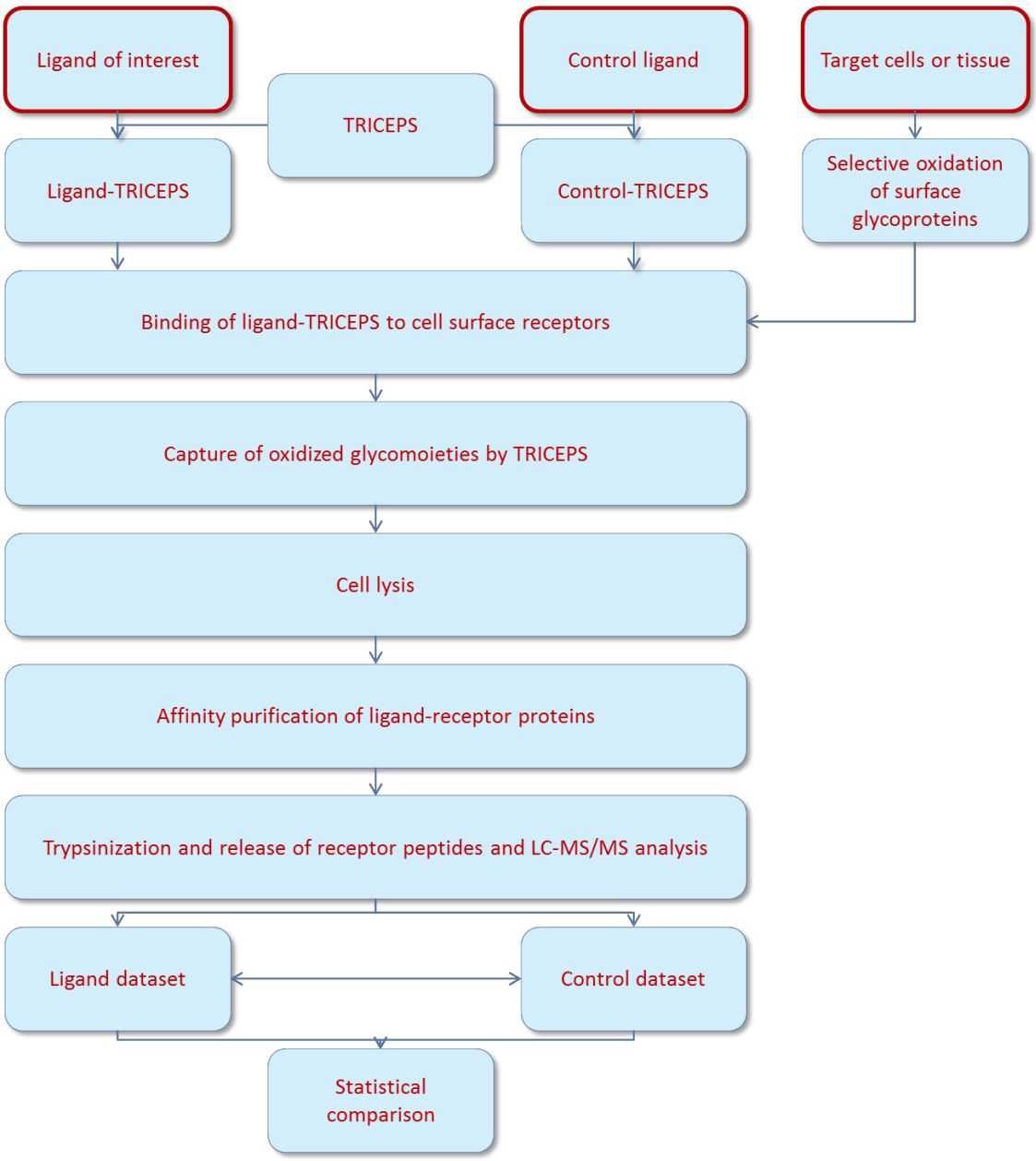

Figure IV-1:Flow chart of the CaptiRec™ screening procedure

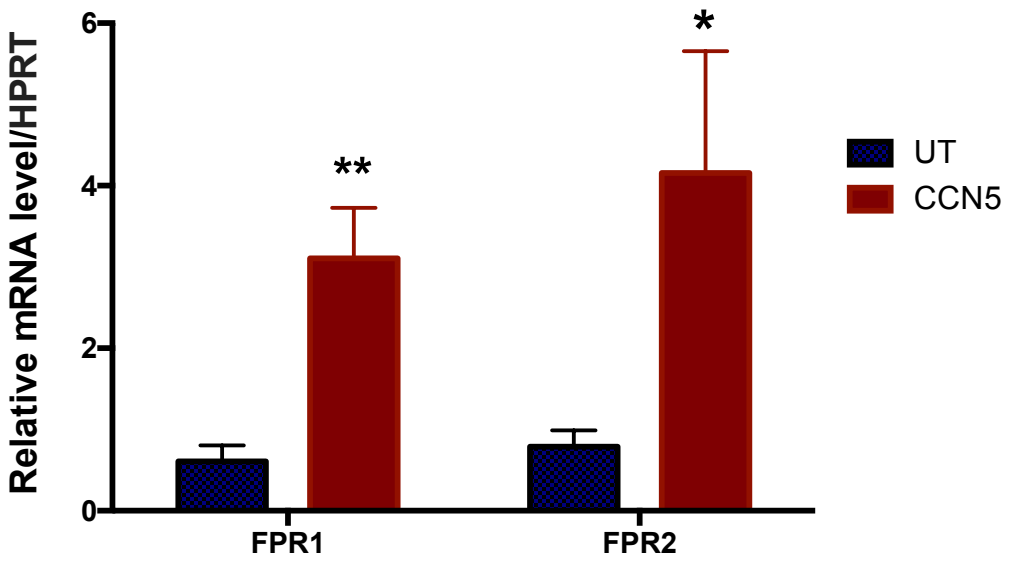

Figure IV-2: Effect of rh-CCN5 on FPR1 and FPR2 expression

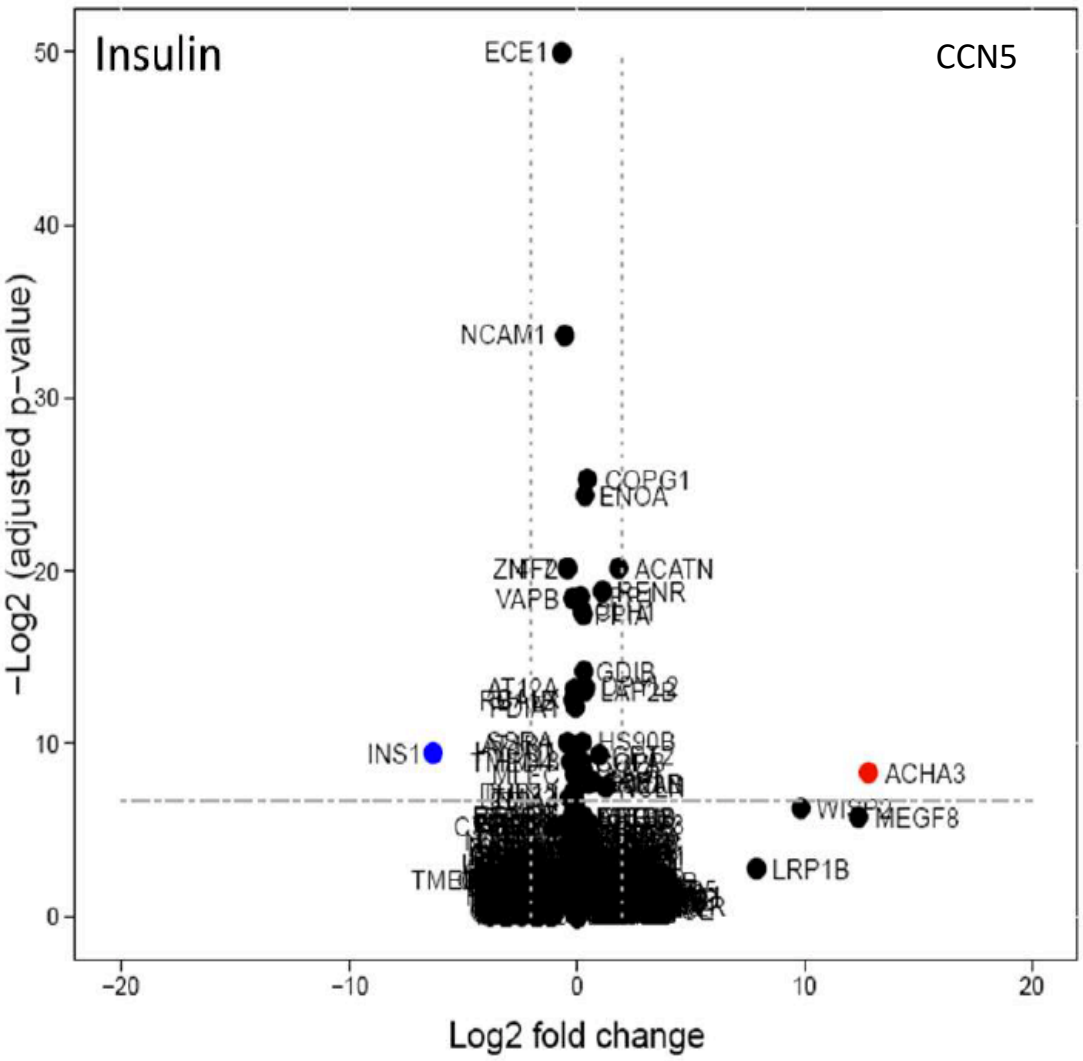

Figure IV-3: CaptiRec volcano plot displaying the receptors for Insulin and CCN5 on the surface of MIN6 cells

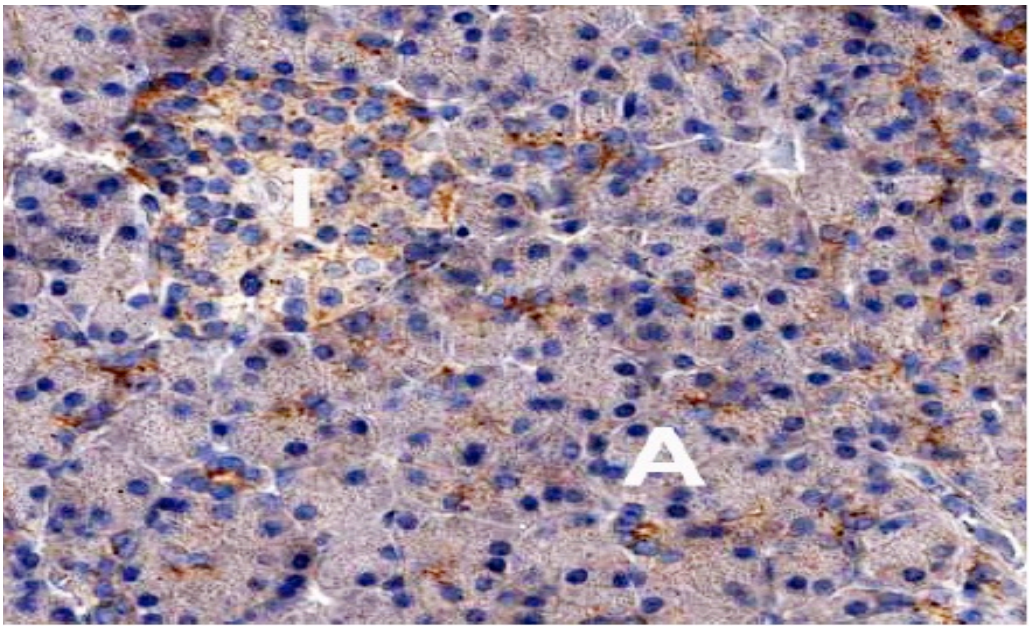

Figure IV-4: Immunohistochemistry of ACAH3 protein in pancreas, from Human Protein Atlas

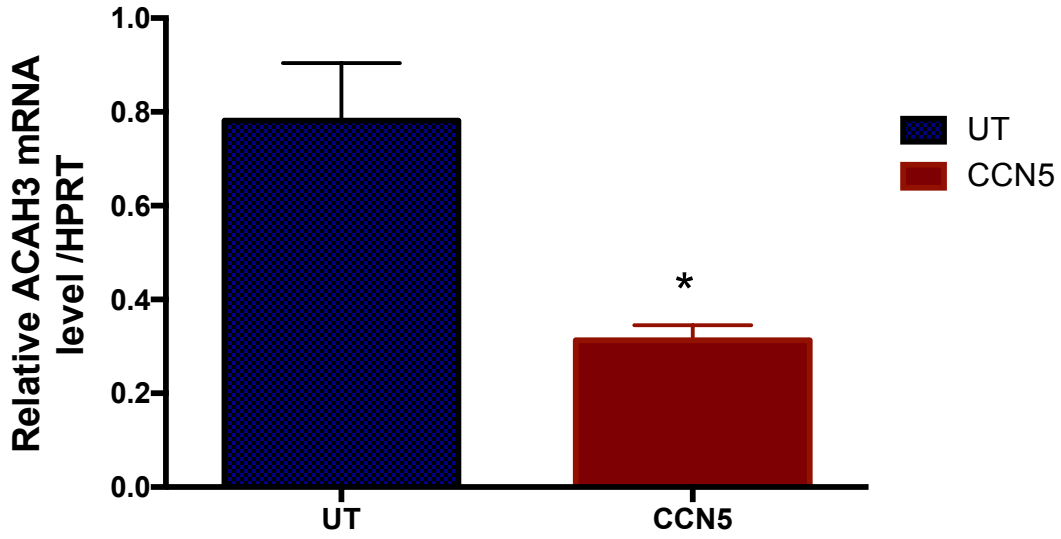

Figure IV-5: Effect of rh-CCN5 on ACAH3 expression

CHAPTER V

GENERAL DISCUSSION AND FUTURE DIRECTIONS

1. Diabetes epidemiology and the need for novel therapeutics

Type 2 diabetes has become a universal public health concern. In 2017, the international diabetes federation (IDF) reported that about 425 million adults are living with diabetes, and this number is expected to rise to 629 million by 2045 (IDF Diabetes Atlas 2017, 8th edition). The accelerated trajectory of the T2D epidemic is mainly attributed to poor nutrition habits either in early life or overnutrition later in life, combined with reduced physical activity [21]. In recent years, a major research focus has been directed on the regeneration and the protection of pancreatic β-cells, as these cells demonstrate plasticity in the context of metabolic challenges such as aging and pregnancy. Growth factors such as hepatocyte growth factor (HGF), glucagon-like peptide (GLP)-1, thyroid hormones and SERPINB1 have proven to enhance β-cell expansion in rodents, but this has yet to be proven in humans. Those growth factors would be beneficial for short term preservation of donor islets that are used for transplantation into T1D hosts. Moreover, they could also be valuable for long term maintenance of β-cell function in-vivo against the development of T2D[202].

2. Discovery of CCN5 as a novel β-cell growth factor

CCN5 was first discovered in 1997 by Delmolino et al as a suppressor of proliferation in VSMCs [203]. Another group in 1998 revealed its negative correlation with transformation in rat embryo fibroblasts [147]. At about the same time, CCN5 was also identified as a Wnt inducible protein in the mouse mammary epithelial cell line C57MG [146].

In the process of looking for novel growth factors targeting β-cells, our lab identified CCN5/WISP2 as a direct target of IGF-I in mouse islets overexpressing IGF-I. Using siRNA-mediated knockdown, we demonstrated that endogenous CCN5 expression is required for the IGF-I-induced proliferation of insulinoma cells. Besides, overexpression of CCN5 in insulinoma MIN6 cells was sufficient to promote their proliferation and to protect them from streptozotocin (STZ) induced cell death [93].

3. Involvement of other CCN proteins in β-cells and diabetes

Some of the CCN proteins have been directly linked to β-cells and diabetes. CCN2 is one of the most highly investigated in β-cell development and maturation. Analysis of the CCN2/ CTGF gene revealed the presence of an enhancer sequence that contains binding sites for neurogenin 3, a key transcription factor associated with pancreatic development, islet morphogenesis, and β cell proliferation [204]. Furthermore, numerous transgenic mouse models revealed the functional role of CTGF/CCN2 in the endocrine pancreas. CCN2 knockout mice are not viable; however during embryonic development, they exhibit distorted islet morphology and contain a higher α/β cell ratio [205]. CTGF/CCN2 heterozygous mice are viable. At the adult stage, they exhibit pancreatic defects characterized by deformed islets that containing a higher α/β cell ratio as compared to their wild type counterparts [206]. Cell-specific inducible transgenic mouse models of CCN2, such as those overexpressing the protein in embryonic β-cells had islets of greater mass and contained a higher α/β cell ratio at postnatal day 1 (P1) than their wild type counterparts [207]. However, overexpression of CCN2 in adult β-cells in mice of 3 and 7 weeks old was found insufficient to drive the proliferation of these cells, nor to affect islet mass and glucose homeostasis [208]. Other CCN family members were also linked to β-cell physiology. For instance, CCN3 was found to be a transcriptional target of FoxO1 in pancreatic β-cells, whereby

it impaired β-cells proliferation and insulin secretory function [209]. Furthermore, differential mRNA expression identified CCN4 to be significantly upregulated during pancreas regeneration [210].

4. Evidence validating growth-promoting properties of CCN5 in β cells

A. Novel findings in the literature

The role of CCN5 in bone tissues has received much attention, given its impact on bone mineralization in addition to osteoblast proliferation and differentiation [211]. Mice with whole-body CCN5 KO revealed no effect of CCN5 on bone formation as no changes in bone mineral density nor bone tissue volume were detected [212]. In a distinct study, the same CCN5 KO mice were assessed for their metabolic profile. Those mice displayed mild obesity as they had larger body size at 5-6 months of age and mild T2D as they had high fasting blood glucose levels[199]. Mice overexpressing CCN5 in their adipose tissue have also been generated. These mice displayed increased lean body mass, expanded brown adipose tissues (BAT), improved insulin sensitivity, and enhanced glucose uptake by adipose cells and skeletal muscle [155]. Although these findings don't examine the direct effect of CCN5 on β cells, they collectively linked CCN5 to metabolism and β cells homeostasis.

B. Summary of our findings

Our previous findings [93] stated above, prompted us to dig deeper into the role of CCN5 in β cells. We benefited from the availability of recombinant human protein (rh-CCN5) to test its physiological effects in β cells. In chapter II, rh-CCN5 has proven to be efficient in: 1) promoting the proliferation of INS832/13 insulinoma cells and mouse islets as demonstrated by increased BrdU incorporation and increased level of cell cycle regulators cyclin D1 and CDK4

2) protecting the cells from lipotoxicity, glucolipotoxicity and, STZ induced cell death as demonstrated by a decreased level of annexin V staining, apoptosis markers caspase 3, PARP, and fragmented DNA.

3) upregulating the expression of key genes associated with β-cell identity and function, including Glut2, GCK, ins1, and ins2. The pathway associated with these stimulatory effects includes the activation of the FAK/ERK pathway.

In chapter III, rh-CCN5 has shown to be efficient in regulating the insulin secretory function of β-cells, as demonstrated by quantification of insulin release/content in mouse islets treated with rh-CCN5. The regulation of GSIS appears to be biphasic, and dependent on ER stress/UPR status of β-cells, as demonstrated by quantification of the expression of genes associated with UPR, including Bip, ATF6, and XBP1s. rh-CCN5 also enhances the level of mitochondrial ATP, which is another marker of β-cell homeostasis and a regulator of insulin release.

In chapter IV, we attempted to decode the mechanism through which CCN5 exerts its physiological effects on β cells. We identified various novel targets regulated by rh-CCN5 in mouse islets, as demonstrated by microarray gene expression profiling. We also identified three potential CCN5 specific receptors, as demonstrated by LRC-TriCEPS technology.

In summary, we established CCN5 as a secreted growth factor that enhances β cells proliferation, survival, and function through a mechanism involving FAK/ERK activation. The effects of CCN5 are mediated through the binding to a high-affinity receptor, potentially known as Cholinergic Receptor Nicotinic Alpha 3 Subunit (CHRNA3) and triggering the expression of multiple downstream targets.

5. Future directions to establish CCN5 as a potent growth factor for β cells

A. Additional in vitro experiments

In my study, the physiological effects of CCN5 on β cells were established using insulinoma cell lines MIN6 and INS832/13 in addition to mouse islets. It would be optimal to use human islets to mimic normal physiology. Now that it's becoming easier to obtain human islets (e.g., University of Alberta Islet Core), it would be ideal for testing the effects of CCN5 on proliferation, survival, and function of those islets in vitro using same techniques that we used in our previous report [197]. An alternative to human islets is the functional human β cell line EndoC-βH, which mimics human β cells (Univercell-Biosolutions) [213] in their low proliferative rate and enhanced insulin production and secretory response [214]. The use of either human islets or human β cell lines will significantly enhance CCN5 clinical potential.

B. CCN5 whole-body knockout

Whole-body CCN5 KO has already been generated [198]. These mice exhibit mild obesity and diabetes, hence highlighting the relevance of CCN5 in metabolism [199]. These mice were already evaluated for their glucose and insulin tolerance using GTT and ITT. Additional experiments that would be of relevance to our study include: 1) evaluation of β-cell mass and architecture using IHC and IF, 2) evaluation of α-cell mass, 3) evaluation of α-cell / β-cell ratio, 4) evaluation of GSIS ex-vivo and 5) evaluation of the expression of key markers related to β-cell identity. We expect whole body CCN5 KO to alter α-cell / β-cell ratio, shifting the balance towards more α-cells and less β-cells. Additional consequences include reduced GSIS and β-cell de-differentiation.

C. Tissue-specific gene overexpression or deletion of CCN5

The role of CCN5 has been well established in adipose tissues, heart tissues and muscle cells [153, 154, 196]. Mice overexpressing CCN5 in their adipose tissues had increased lean body mass and whole-body energy expenditure, hyperplastic brown/white adipose tissues and larger hyperplastic hearts. Furthermore, Obese Tg mice were insulin sensitive, as their adipose cells and skeletal muscle demonstrated enhanced glucose uptake in- and ex-vivo [155]. This study underlines CCN5 as an effective endocrine and paracrine factor secreted by adipose tissues and target peripheral tissues such as heart and muscles. However, the effect on the pancreas was not evaluated. These mice could be of enormous relevance to our study. We could use them to assess α and β-cell mass ratio and architecture, in addition to their insulin secretory function and many other indicators. We expect, effective paracrine effect of adipotic CCN5 such as enhanced β-cell mass and GSIS.

Another alternative, which is a long-term goal, is to establish ß cells specific knockout or overexpression and examine the effect of CCN5 deletion/OE on ß cell mass, blood levels of insulin, glucagon, and glucose, in addition to GTT and ITT. Mice could be as well challenged with HFD, and the same parameters stated above are to be evaluated. We anticipate ß cells specific KO or OE to add great value to our research since it will validate our in vitro results. We expect ß-cell specific KO to diminish ß-cell proliferation, aggravates their survival, and induce their de-differentiation. On the other hand, ß-cells specific OE is expected to enhance their mass and identity in addition to enhancing GSIS and insulin content.

6. Gene expression profiling target validation and future directions

Using microarray gene expression profiling, we identified 34 target genes to be regulated by CCN5 in primary islets. Those genes appear to be involved in chemotaxis, G protein-coupled

receptor signaling, wound healing, inflammation, and immune response. Among those genes, we validated CCN5 regulation of GPCRs, FPR1 and FPR2 by PCR. We will further pursue their role in β-cells by several experiments such as: 1) Validate their regulation by CCN5 at the protein level, 2) Inhibit their activity either buy sing chemical inhibitors or siRNA and then check the effect on CCN5 mediated activities in β-cells such as proliferation, survival and GISIS.

Other targets in the microarray will be taken into consideration. We will apply gene enrichment set analysis to further cluster the genes and narrow down to our focus to the most relevant ones.

7. CCN5 receptor validation

A complete understanding of the biological effects of a protein on any target cell depends on establishing the identity of a specific receptor and a signal transduction pathway. To establish that ChrnA3 is an authentic receptor for CCN5, we will need to perform a radioligand binding assays using HEK293 or CHO-K1 cells overexpressing ChrnA3 and [^{125}I]-labeled CCN5. Those assays will help us characterize the affinity of CCN5 to the receptor in addition to other parameters such as IC50, maximal affinity, and if there is any affinity of these receptors to other CCN proteins.

Once the interaction has been confirmed, the next step would be to determine whether ChrnA3 is required for CCN5 signaling. This could be accomplished by inhibiting ChrnA3 in insulinoma cells or islets using various techniques, such as siRNA mediated ChrnA3 silencing or specific nAChR antagonist (mecamylamine), and check the effects of this inhibition on ß-cell proliferation or survival

Another avenue is to evaluate the binding of CCN5 to the other receptors that were found in our LRC-TriCEPS assay (Table IV-2). Those include MEGF8, a membrane-anchored growth factor , and LRP1B, that is known to interact with other CCNs [215, 216]. We expect those

receptors to act as partners or co-receptors for CCN5.

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