# CONTEXT-DEPENDENT EFFECTS OF CCN2 ON β-CELL MASS EXPANSION AND INDICATORS OF CELL STRESS IN THE SETTING OF ACUTE AND CHRONIC STRESS

By

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## CHAPTER I: INTRODUCTION

### General significance

The pancreas is an organ with two primary functional roles. One role, governed by the acinar cells of the exocrine pancreas, is to secrete digestive enzymes that aid in food digestion. In humans, the exocrine pancreas comprises approximately 90 percent of the pancreas, while about six percent is comprised of the endocrine pancreas [1]. The endocrine pancreas consists of mini organs known as *islets of Langerhans*, which contain five distinct hormone-producing cell types: β (insulin), α (glucagon), δ (somatostatin), ε (ghrelin), and PP (pancreatic polypeptide) cells. Insulin is the main hormone produced by the islet, and it functions to stimulate glucose uptake by insulin-sensitive tissues in the body such as the muscle and liver. This uptake of glucose by these tissues partly contributes to the maintenance of glucose homeostasis specifically by preventing hyperglycemia, which is a hallmark of diabetes mellitus.

Diabetes mellitus is a group of diseases characterized by insufficient insulin output due to either autoimmune destruction of β cells (Type 1 Diabetes), or loss and/or dysfunction of β cells (Type 2 Diabetes). Current treatments of diabetes focus on managing glucose homeostasis. However, there is currently no cure for these diseases. With loss and/or dysfunction of β cells being characteristic of diabetes, there is therefore therapeutic potential in increasing or restoring functional β-cell mass. However, islet transplantation is not a feasible cure due to a lack of donor tissue. Researchers are currently examining methods to increase β-cell number through *in vivo* expansion by identifying factors that can stimulate *in vivo* β-cell proliferation, with the priority being to characterize the signaling molecules activated by these factors. By understanding the

pathways activated to stimulate β-cell proliferation, manipulation of these pathways could further advance efforts to create a cure for diabetes.

## Pancreas development

Pancreas organogenesis can be described as an ordered continuum of molecular and morphological events that include regionalization of the endoderm and early ventral and dorsal bud formation, bud outgrowth and cell specification, endocrine lineage allocation, endocrine proliferation and maturation, and finally islet morphogenesis. Each process will be described in more detail below.

## Endoderm specification and initiation of pancreas development

The exocrine compartment consists of acinar cell clusters and the ductal network which functions to transport digestive enzymes into the rostral duodenum. The endocrine compartment is composed of the *islets of Langerhans*, which are spherical cell clusters that produce hormones that work in synchrony to regulate glucose homeostasis. Insulin secretion stimulates glucose uptake in glucose-sensitive tissues to reduce blood glucose levels, while glucagon secretion stimulates glycogenolysis to raise blood glucose in times of fasting. Acinar, ductal and endocrine cells are all derived from the endodermal germ layer during embryogenesis [2].

Mammalian development is unique in that cells with an endodermal identity are present at two distinct times during development. Primitive or so-called extra-embryonic endoderm arises in the pre-implantation embryo. Primitive endoderm predominantly gives rise to the endoderm layers of yolk sacs, which are crucial for nutrient transport from mother to embryo [3,4]. Definitive endoderm, or the embryonic endoderm, gives

rise to all digestive organs including the pancreas and liver [5]. The endodermal gut tube is compartmentalized based on expression of transcription factors and their arrangement along the anterior-posterior axis of the digestive tract. The posterior foregut region encompasses the anterior stomach, liver, and the prepancreatic endoderm. These regions aside from the liver are marked by expression of the transcription factor pancreatic and duodenal homeobox 1 (Pdx1) [6]. Pdx1 is expressed throughout the endoderm of pancreatic buds and in the antral stomach, rostral duodenum, and common bile duct [7]. Pdx1 is necessary for development of each of these organs. All cells within the pancreatic epithelium derive from Pdx1-positive cells. Inactivating mutations in Pdx1 lead to pancreas agenesis in both mice and humans [8,9], while subtler mutations in Pdx1 can lead to a monogenic form of diabetes known as maturity onset diabetes of the young (MODY) [9,10-12]. Pdx1 heterozygosity in mice and humans leads to an increased risk for Type 2 diabetes [10,14]. The initial presumptive pancreatic area is identified by overlapping expression of Pdx1 and pancreas transcription factor 1a, or Ptf1a [6,15,16,17]. Ptf1a was originally identified as a regulator of exocrine-specific genes, but it has since been established to be very important for pancreas development as it is necessary for the development of multipotent pancreatic progenitors (MPCs) which are cells that can give rise to all pancreatic cell types [18-20]. Similar to Pdx1, loss of Ptf1a leads to pancreatic agenesis [15]. Linear tracing analysis has shown that acinar, ductal and endocrine cells are all derived from a Ptf1a-expressing cell [15,21].

The vascular endothelium has been demonstrated to be important for the maintenance of Pdx1 expression, initiation of *Ptf1a*, dorsal pancreatic bud outgrowth

and *insulin* gene expression [22,23]. This was discovered by co-culture experiments utilizing the vascular endothelium and endoderm, which established that factors from the endothelium support pancreas development. One endothelium-derived factor known to play a role in pancreas development is vascular endothelial growth factor A (VEGF-A). Overexpression of VEGF-A under the control of the *Pdx1* promoter leads to an increase in pancreatic blood vessels, as well as an increase in pancreatic islets and ectopic insulin-positive cells in the posterior stomach [24]. Because Pdx1 is expressed in the posterior stomach during this time in development, it is suggested that the endothelium can induce the β-cell fate in competent regions of the endoderm. In contrast, loss of VEGF signaling utilizing a mouse model with a null mutation in VEGF receptor type 2 (VEGFR-2) leads to failure of early insulin- and glucagon-positive cells to develop. Despite this, mice in this model express most pancreatic/endocrine transcription factors except for Ptf1a, implicating VEGF-A signaling in the initiation of *Ptf1a* [23].

**Pancreatic bud formation, outgrowth, and branching morphogenesis**

Ventral and dorsal pancreatic bud outgrowth occurs in the initial stages of pancreatic development. The dorsal pancreatic bud forms at mouse embryonic day 9.5 (e9.5) and human gestational day 26 (hd26), while the ventral bud forms at e10 and hd30 (however, humans have two ventral buds) [25,26]. The period following bud evagination (e8.5-e12.5 in mouse; hd42-49 in human) from the endoderm is known as the primary transition period, and the first known hormone-positive cells scattered among the MPCs can be detected at this time [27]. Early MPCs are marked by expression of *Pdx1, Ptf1a, SRY-box transcription factor 9 (Sox9)* and *Onecut1* (*Oc1).*

Early MPCs have the potential to differentiate into acinar, ductal or endocrine cells [28]. Oc1 (formerly known as hepatic nuclear factor 6 or Hnf6) is a transcription factor that directly activates the pro-endocrine transcription factor Neurogenin-3 (Ngn3) [29], which will be discussed later in this chapter. Thus, Oc1 is required for endocrine differentiation but is also needed for embryonic pancreatic duct development and later branching morphogenesis [30]. Inactivation of *Oc1* leads to a delay in *Pdx1* expression, pancreatic hypoplasia, and impaired endocrine and duct cell differentiation [31]. Similarly, inactivation of *Sox9* in the developing pancreas leads to pancreatic hypoplasia and premature depletion of the MPC pool [32].

At ~e12, after growth and elongation of the dorsal and ventral pancreatic buds occurs, the two pancreatic buds fuse. At this time, the pancreas undergoes morphological and gene expression changes to establish a tip and trunk domain structure. Molecular marker analyses and lineage tracing studies suggest that the tip and trunk domains are both molecularly and functionally different. The tip of the branching pancreatic epithelium is where most of the outgrowth and elongation occurs. It is the most outward projecting domain and is composed of secondary MPCs that express Ptf1a, c-Myc, carboxypeptidase A (Cpa1), and Pdx1 and Oc1 at lower levels. The secondary MPCs in this domain have the capability of giving rise to all three pancreatic cell types (endocrine, duct, and acinar) until e14.5 [28]. However, the majority of these secondary MPCs in the tip domain differentiate into acinar cells [Figure 1-1]. In the trunk domain, differentiation of endocrine progenitor cells is regulated by juxtacrine Notch-Delta signaling [33,34]. Initially, endocrine/ductal bipotent cells in the trunk express low levels of the Notch receptor and its ligand Delta. The cells with

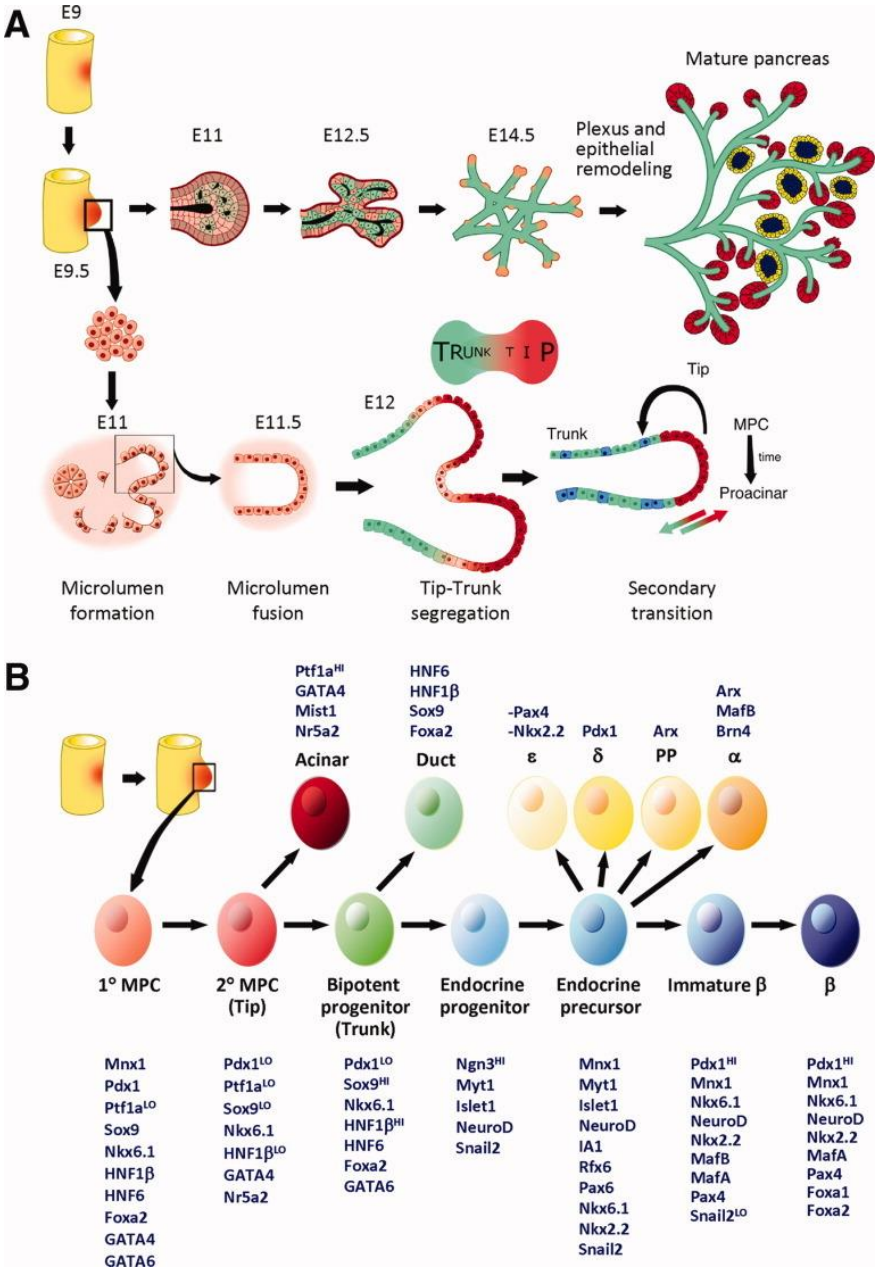



**Figure 1-1. Overview models of mouse pancreas organogenesis.** A: The upper diagrams depict the branching morphogenesis that occurs during pancreas development, as well as the tip-trunk segregation depicting organization of pancreatic cell lineage. B: The bottom diagram depicts the important transcriptional regulators that are expressed during each stage of pancreas development. Adapted from Pan and Wright (2011). Pancreas organogenesis: From bud to plexus to gland. *Dev Dyn* 240(3):530-565.

increased Delta expression give rise to the endocrine population and leave the epithelium, while cells with increased Notch signaling remain in the epithelium. The cells that leave the epithelium are endocrine progenitor cells, give rise to all five endocrine cell types, and express Ngn3+ [35,36]. Cells remaining in the epithelium repress expression of Ngn3, and eventually mature into ductal epithelial cells [37].

**Endocrine cell development and differentiation – transcription factors**

The earliest known marker of an endocrine progenitor is Ngn3, and all endocrine cell types arise from an Ngn3-expressing progenitor cell [35,36]. Bipotential trunk cells with low levels of Ngn3 expression diverge toward the ductal cell fate. In mice, complete inactivation of *Ngn3* during development leads to a lack of all endocrine cell types, resulting in neonatal death due to diabetes [35]. These findings support the fact that Ngn3 is vital for the differentiation of all pancreatic hormone-producing endocrine cells. Interestingly, transgenic Ngn3 overexpression throughout the pancreatic epithelium results in increased differentiation of α cells without a change to the number of other islet endocrine cell types [38,39]. When investigating this phenomenon, it was found that the time of activation of *Ngn3* is an important factor in determining endocrine cell fate. For example, early activation of *Ngn3* yields α cells, while later activation induces differentiation of β, δ, and PP cells. It has been further demonstrated that a single Ngn3+ progenitor cell is unipotent and can only give rise to one specific endocrine cell type rather than to all [40].

The secondary transition is the time period between e12.5 and e16, and during this time there is a significant increase in the number of endocrine cells budding from the ductal epithelium. The endocrine cells formed during this developmental window

eventually contribute to the mature islet. *Ngn3* expression peaks during the secondary transition [35]. Because *Ngn3* expression is induced by cooperation between Pdx1 and Oc1, it is crucial that proper levels of Oc1 expression are achieved. Loss of Oc1 during development in mice leads to a dramatic decrease in Ngn3 expression, subsequently leading to a significant reduction in insulin and glucagon expression [29]. After cells are committed to the endocrine fate by activation of *Ngn3*, Oc1 expression is decreased. Bipotent duct/endocrine progenitor cells with high expression of Oc1 are fated to become ductal cells and Oc1 expression remains in these cells throughout adulthood [41]. If Oc1 expression is experimentally maintained in islets, there is impairment of migration of endocrine cells from the ductal epithelium, disrupted organization of endocrine cell types within the islet, and severely compromised β-cell maturation and function which subsequently leads to overt diabetes [42,43].

The formation of α and β cells is thought to be regulated by the opposing actions of the transcription factors Paired Box 4 (Pax4) and Aristaless-related homeobox (Arx). *Pax4* can be detected by mRNA within the pancreas from e9.5 [44]. It is specifically expressed in first and second wave insulin-producing cells but becomes downregulated soon after birth and is expressed at low levels in adult β cells [45]. Embryonic loss of *Pax4* does not result in loss of first wave insulin-producing cells; rather, there is loss of mature β cells, indicating that Pax4 is required during the secondary transition for β-cell differentiation [44,46]. Furthermore, *Pax4* null embryos have an increase in the numbers of glucagon-expressing cells [47]. Pax4-expressing cells can give rise to α, β and ε cells, suggesting that Pax4 is expressed in pluripotent endocrine progenitors [48].

However, ectopic expression of Pax4 in α cells or pancreatic progenitor cells induces re-specification toward the β-cell fate [49].

While Pax4 acts to direct endocrine progenitors toward the β-cell fate, Arx expression directs endocrine progenitors toward the α-cell fate. Arx is first expressed between e10.5 and e12.5 in scattered cells throughout the pancreatic buds and is later co-expressed with glucagon at e14.5 [49]. Arx acts downstream of Ngn3, and embryonic loss of *Arx* results in complete loss of second wave α cells with a concomitant increase in both β and δ cells [49,50]. Inactivation of both Pax4 and Arx leads to a loss of both β- and α-cell lineages and a concomitant increase in δ cells [50]. Paired box 6 or Pax6 is another transcription factor important for the α-cell lineage. Pax6 is expressed at e9.5-10.5 in a population of cells in the pancreatic epithelium. Later in embryogenesis, Pax6 is expressed in cells committed to the endocrine lineage [33,51,52]. Although Pax6 is expressed in both glucagon- and insulin-expressing cells, it is thought to be only essential for α-cell formation. Inactivation of *Pax6* causes a dramatic loss of glucagon-producing cells along with a smaller decrease in other endocrine cell types [53]. This finding suggests that Pax6 is important for the allocation of endocrine progenitor cells to the α-cell lineage, and for the expansion of the endocrine population as a whole [54,55].

The large Musculoaponeurotic fibrosarcoma (Maf) family of proteins are transcription factors that were first identified in an avian retrovirus. Two members of the Maf family, MafA and MafB, are crucial for β-cell differentiation and maturation. The expression pattern of both transcription factors differs between human and mouse. MafA was identified by several groups as an activator of *insulin* gene expression [56-

60]. Expression of MafA begins at e13.5 and continues into adulthood, making it a marker of mature murine β cells [61,62]. In humans, similar to mice, MAFA expression increases in an age-dependent manner and high expression is maintained in β cells throughout adulthood [63]. Although indicated as a critical β-cell maturation factor, global deletion of *MafA* in mice has no effect on the number of insulin-producing cells generated during embryogenesis. However, loss of *MafA* in mice leads to defects in β-cell gene expression and postnatal β-cell function, ultimately leading to diabetes [64]. Importantly, reduced MafA/MAFA expression is associated with diabetes progression in both mice and humans. Islets isolated from *db/db* mice, a model of T2D, have reduced MafA levels and “rescue” of the transcription factor improves GSIS and β-cell mass [65-68]. Single cell RNA-seq analysis (scRNA-seq) of human β cells demonstrated that β cells that were metabolically inflexible expressed MAFA at a lower level than healthy β cells, solidifying the importance of MAFA in proper β-cell function [69].

The lack of a developmental islet phenotype in *MafA* knockout animals may be due to compensation by another related Maf family member, MafB [62,70]. MafB is also expressed in developing endocrine cells and is capable of activating *insulin* reporter gene transcription in tissue culture. During mouse embryogenesis, MafB is expressed in first- and second-wave insulin- and glucagon-producing cells but becomes restricted to α cells soon after birth. In β cells, MafB promotes activation of differentiation genes including *Nkx6.1*, *Glut2, Pdx1*, and *MafA* [62]. Loss of MafB results in a decrease of insulin- and glucagon-positive cells [64]. However, in adult mouse islets, MafB is only expressed in α cells and regulates expression of the *glucagon* gene [70]. The

expression of MAFB differs in humans from mice. In adult humans, in addition to being expressed in α cells, MAFB is co-expressed in β cells with MAFA [70-73].

Other transcription factors involved in specifying the pancreatic endocrine lineage are the members of the NKX class of homeodomain proteins Nkx2.2 and Nkx6.1. IN mice, both Nkx2.2 and Nkx6.1 are expressed in most pancreatic epithelial cells during early embryogenesis. By e15.5, however, Nkx2.2 is restricted to the endocrine cell population and Nkx6.1 is expressed exclusively in insulin-producing cells and other scattered cells in the ductal epithelium [74-76]. Late in gestation, Nkx2.2 can be detected in nearly all hormone-positive cells except for somatostatin-expressing cells. After birth, both genes are exclusively expressed in the β-cell population. The primary function of Nkx2.2 is as a transcriptional repressor which regulates endocrine differentiation [77]. Loss of *Nkx2.2* leads to loss of insulin-positive cells at any stage examined, with a significant reduction in glucagon-expressing cells and a more modest reduction in pancreatic polypeptide (PP)-positive cells [74]. This occurs without a loss of total endocrine cells due to increased numbers of ghrelin-producing ε cells [47]. These findings demonstrate that Nkx2.2 is required to generate β cells, maintain and expand α- and PP cells, and repress ε-cell fate.

Loss of *Nkx6.1* results in dramatic loss of second-wave insulin-positive cells (after e13.5), with no change in the number of other islet endocrine cell types [75]. Therefore, in the absence of Nkx6.1, endocrine cells delegated to be β cells do not adopt an alternate endocrine cell fate. Genetic studies have demonstrated that *Nkx6.1* functions downstream of *Nkx2.2* to expand and terminally differentiate the β-cell lineage [74]. Transgenic overexpression of *Nkx6.1* in β cells using the *Pdx1* promoter does not

enhance β-cell mass or function [78]. Conversely, loss of both *Nkx2.2* and *Nkx6.1* results in a decrease of both α- and β cells, indicating a role for both transcription factors in endocrine cell development [79,80].

In addition to its role in pancreas organogenesis, Pdx1 plays a crucial role in later β-cell differentiation and mature function. By late gestation, *Pdx1* expression is maintained at high levels in β cells, with minimal expression in acinar cells [81,82]. While Pdx1 is not required to generate the endocrine cells present during the primary transition, it is specifically required for differentiation of endocrine and exocrine cells between e11.5 and e13.5 [83]. Studies conducted using mice with β-cell-specific *Pdx1* inactivation demonstrated that loss of *Pdx1* at e11.5 resulted in very early onset diabetes with plasma glucose being significantly higher in Pdx1-mutant pups than control pups at P1 [84]. Examination of β-cell proliferation at e18.5 found that β-cell proliferation was significantly impaired in Pdx1-mutant animals compared to controls, which translated into a decreased number of overall β cells. Interestingly, α-cell proliferation was significantly increased in Pdx1-mutant mice. Analysis of glucose tolerance via IP-GTT in adult mice revealed that male Pdx1-mutant mice had significantly impaired glucose tolerance compared to controls indicating β-cell dysfunction [84]. This study demonstrated that Pdx1 is required during late gestation for sufficient β-cell mass expansion to occur and for proper β-cell function throughout life.

Other studies have revealed that Pdx1 promotes expression of proinsulin, *Glut2* and *glucokinase* in adult mice which mediates proper glucose-stimulated insulin secretion. The role of Pdx1 in maintaining expression of these genes is critical, as loss of Pdx1 during adulthood utilizing tamoxifen-inducible Cre recombinase resulted in loss

of the key β-cell identity transcription factors *Nkx6.1, Ins1, Glut2* and *MafA.* Furthermore, loss of Pdx1 in adult β cells results in acquisition of α-cell-like features, demonstrating the importance of Pdx1 in maintaining adult β-cell identity [85].

**Embryonic endocrine cell proliferation**

There is little proliferation of endocrine cells during early to mid-gestation. However, the percentage of proliferating endocrine cells dramatically increases in late gestation through the early neonatal period [86-89]. For example, during late embryogenesis, β-cell proliferation proceeds at approximately 10% per day in mice [90]. Adequate proliferation is required for sufficient numbers of endocrine cells in adults, but few factors *in vivo* have been identified that affect embryonic endocrine proliferation.

The Protein kinase R-like endoplasmic reticulum kinase (PERK) is required for both exocrine and endocrine proliferation and function. PERK localizes to the endoplasmic reticulum (ER) membrane and is hyperactivated in response to ER stress caused by the unfolded protein response (UPR) [91]. Global *Eif2ak3* (PERK) inactivation in mice results in insufficient β-cell mass leading to diabetes, and a progressive loss of exocrine tissue after four weeks of age. Inactivation of *Eif2ak3* during embryogenesis leads to decreased β-cell proliferation, leading to decreased β-cell mass in the adult. Gene expression profiling of postnatal day 2 (P2) control and PERK knockout islets revealed that this decreased β-cell proliferation may be due to decreased expression of genes important for progression of the G2 and M phases of the cell cycle such as *CyclinA* and cyclin dependent kinase 1 (*Cdk1*) [92]. PERK may also play a role in regulating β-cell maturation or function, as both global and pancreas-wide inactivation of PERK leads to decreased *MafA, Pdx-1,* and *insulin* gene

expression. Furthermore, inactivation of PERK causes impaired glucose-stimulated insulin secretion (GSIS) [93,94].

β cells may also provide signals that regulate the population of other cell types, specifically the α cell. Removal of *Pdx1* in embryonic β cells leads to a significant decrease in β-cell proliferation with a concomitant increase in α-cell proliferation at late gestation [84]. Reciprocal alteration in β- and α-cell proliferation when *Pdx1* is inactivated suggests that embryonic β cells normally provide an inhibitory signal to the α-cell population. Glucagon-positive cells may also send signals to regulate β-cell proliferation as models of glucagon inactivation, such as embryonic global deletion of the glucagon receptor or pro-hormone convertase-2 (PC2), result in increased postnatal β-cell proliferation [95,96].

**Islet morphogenesis**

At e18.5 and continuing after birth, endocrine cells organize into islets within the acinar parenchyma [27]. Mouse islets have a characteristic architecture with β cells in the core of the islet, with the other cell types such as α and δ cells forming a mantle around the islet. This organization is thought to promote proper cell-cell communication and enhance islet function [97,98]. Although the process of islet morphogenesis is not completely understood, changes in expression of cell adhesion molecules, modifications in extracellular matrix (ECM) proteins, and paracrine and juxtacrine cell-cell communication events likely play a role in this process. Real-time imaging in pancreatic explants has demonstrated that β cells migrate using extended cytoplasmic filapodia [99], suggesting that migration is an active process. However, it is still not clear whether islets are formed by individual cells which lose connections with neighboring cells,

migrate to form clusters, and then reestablish cell adhesions to form islets. One feasible alternative to this process would be groups of cells that migrate in clusters while still maintaining cell-cell contact.

Cell migration requires dynamic regulation of cell-cell, cell-ECM adhesion, and intracellular signaling that regulates the actin cytoskeleton. Islets in mice lacking the epidermal growth factor receptor (EGFR) are elongated and closely opposed to the ductal epithelium [100]. This suggests that EGFR may act to modulate activity of Rac1, a Rho-GTPase involved in migration and adhesion in many cell types. Islets expressing dominant negative Rac1 fail to spread on ECM when treated with EGFR ligands, indicating that Rac1 may function downstream of EGFR [101]. Integrins, transmembrane heterodimer receptors that interact with the ECM to affect migration, also play a role in endocrine cell migration. Inhibition of αv integrins in fetal pancreas explants block the emergence of endocrine cells from the ductal epithelium [102]. Furthermore, EGFR and integrin signaling both regulate the activity of matrix metalloproteinases (MMPs), molecules that can alter the structure of the ECM, which can thus affect the migration of cells [103].

Cell-cell contacts are required for the formation of the typical islet architecture. One molecule, E-cadherin, is necessary for endocrine cell clustering. β cells that express a dominant-negative form of E-cadherin remain dispersed throughout the pancreas as individual cells [104]. Other adhesion molecules, such as neural cell adhesion molecule (N-CAM), are involved in endocrine cell type segregation [105]. It has been hypothesized that segregation of endocrine cell types, such as α and β cells, is due to differential expression of adhesion molecules [105,106]. This can be observed

in the MIN6 and INS-1 immortalized β cell lines, where β cell-only clusters are substantially more cohesive than clusters of the α-TC α cell line. [106].

**Postnatal β-cell mass expansion**

After birth, β-cell mass increases rapidly and eventually plateaus when an organism reaches adulthood. There are multiple mechanisms by which β-cell mass can increase postnatally, with the most common being proliferation and hypertrophy [107] [Figure 1-2]. Proliferation involves an increase in β cells due to replication of existing β cells and has been demonstrated to be the main mechanism by which postnatal β-cell mass increases [108]. Hypertrophy is characterized by an increase in the size of existing β cells. In rats, β-cell proliferation proceeds at a rate of 1-4% per day between the age of 30 to 100 days old [109]. In mature adult mice, β-cell proliferation proceeds at a rate of <1% per day demonstrating the dynamic property of β-cell mass expansion [110]. While these previously described studies were conducted in rodents, it has also been demonstrated that β-cell mass dynamics are similar in humans. For example, human β-cell proliferation peaks around 4% per day during the early neonatal period and decreases to ~0.2% per day in adults under normal physiological conditions [111].

For many years, it was believed that β cells were terminally differentiated cells existing in a G0 state, which is a state in which a terminally differentiated cell is dormant. However, it is now known that adult β-cell proliferation does occur, albeit at low rates, and can be induced as a response to physiological and pathophysiological conditions as well as a multitude of other factors.

Cell proliferation in general is highly regulated through the interaction of a diverse

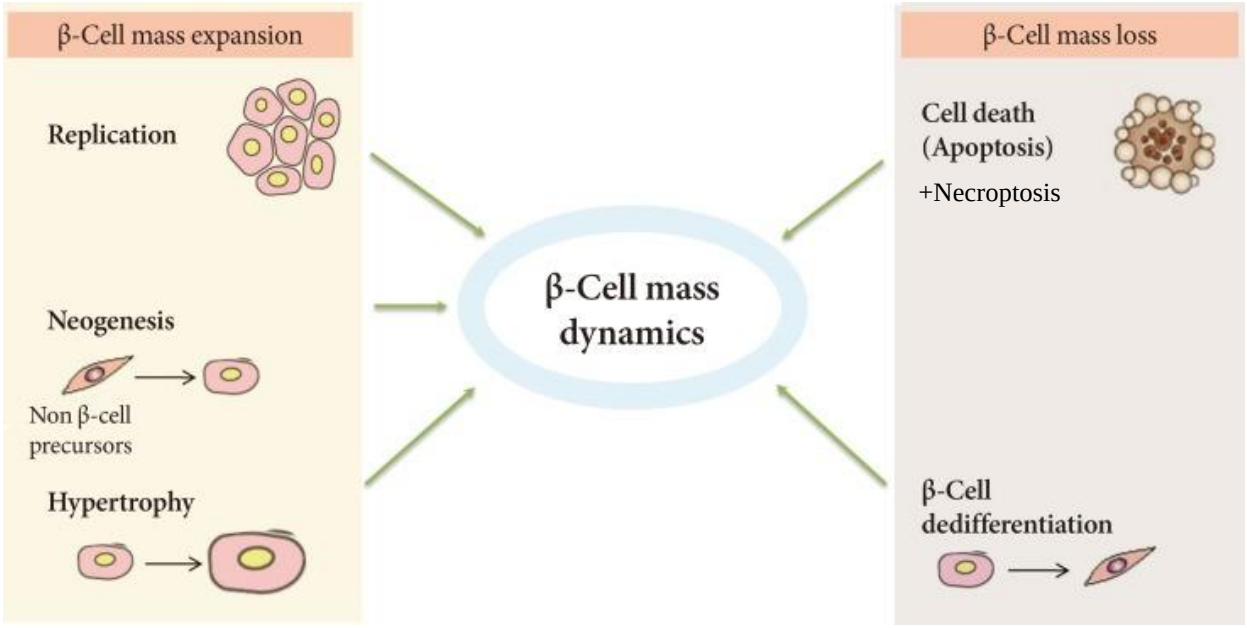



**Figure 1-2. Factors that induce changes in β-cell mass.** β-cell mass can expand via replication or proliferation, neogenesis, and hypertrophy. β-cell mass can be lost by apoptosis and necroptosis (not shown) and dedifferentiation. Adapted from Jung et al. (2014). *Diabetes Metab J* 38(6):426-436.

set of proteins. Briefly, the cell cycle is a four-stage process that consists of Gap 1 (G1), synthesis (S), Gap 2 (G2) and mitosis (M). Active eukaryotic cells will pass through these different phases during proliferation. During G1, the cell increases in size and duplicates its contents. During S phase, DNA is replicated. During G2, the cell grows and prepares for mitosis. Finally, mitosis occurs, and the result can be two identical daughter cells [112]. In some cases, asymmetric division occurs whereby a stem or progenitor cell generates a daughter cell which has different characteristics [113]. The cell cycle is highly controlled by checkpoints at different stages, which regulate which cells will enter and complete the cell cycle [114]. The main restriction point is located at G1, and cells that pass this point will end up undergoing and completing the cell cycle. There are many regulators that function at different stages of the cell cycle, with the most important being cyclins and cyclin-dependent kinases (Cdks) [115]. Cyclins are a group of related proteins whose levels fluctuate during the cell cycle, and expression is regulated at the level of protein degradation. Four basic types of cyclins are found during the cell cycle: G1 cyclins, G1/S cyclins, S cyclins and M cyclins [116]. Cyclins form complexes with Cdk serine-threonine kinases, which phosphorylate their substrates to regulate cell cycle progression [115,116]. The consensus in mammalian cells is that Cdk4 and Cdk6, after transcriptional induction of D-type cyclins in response to mitogenic stimuli, promote entry into the cell cycle [115].

Like most cells, the major restriction checkpoint for both murine and human β cells is the G1 checkpoint [117,118]. Although sharing the same restriction checkpoint, the expression of different cell cycle regulators differs slightly between murine and human β cells. In murine islets, cell cycle progression is controlled by three D cyclins

(D1, D2, D3) which bind and activate Cdk4 to permit cell cycle entry. Global deletion of Cdk4 in mice causes abnormalities in β cells including cell hypoplasia, which resulted in diabetes and ketoacidosis [119,120]. Cyclins D1 and D2 are the most highly expressed of the three, and although cyclin D2 is not required for neonatal development, it still plays a role in controlling β-cell growth and replication [121]. Cyclin D1 can compensate for loss of cyclin D2, but loss of both cyclins D1 and D2 leads to uncontrollable diabetes and eventual death in young mice [121]. Conversely, overexpression of cyclin D1 causes increased β-cell proliferation both *in vitro* and *in vivo* [122]. It is known that β-cell proliferation declines with age, and the expression levels of cyclin D1 and D2 decline in the same manner, providing evidence for the importance of these cyclins in murine β-cell proliferation. Human β cells express both Cdk4 (at very low levels) and Cdk6 as well as cyclins D1 and D3. While the mouse expresses cyclin D2 in β cells, cyclin D2 is not observed in human β cells [123]. Adenoviral overexpression of Cdk4 in human β cells increases proliferation rate and like mice, adenoviral overexpression of cyclin D1 in human β cells increases β-cell proliferation [123,124].

## Factors that can induce adult β-cell proliferation

### *Glucose*

Glucose is one of the most potent mitogens for β cells both *in vivo* and *in vitro* [125-127]. Studies have shown that both long- and short-term glucose infusion promotes β-cell proliferation in mice. For example, one study in mice utilized a 50% glucose infusion for 96 hours to maintain elevated blood glucose and found that there was an almost five-fold increase in β-cell proliferation compared to saline infusion [127]. In *ex vivo* studies, it was demonstrated that culturing human islets in high glucose for 96

hours increased β-cell proliferation [128]. Another study utilized streptozotocin (STZ) to deplete β cells in the non-obese diabetic/severe combined immunodeficiency (NOD/SCID) mouse model, leading to hyperglycemia. Human islets transplanted in this hyperglycemic environment had increased proliferation, again illustrating that glucose acts as a mitogen for β cells [129].

*Extracellular matrix proteins*

The vascular endothelium, while being important for secreting factors that stimulate pancreas development during embryogenesis, is also involved in β-cell proliferation postnatally. The intra-islet endothelial cells (ECs) are responsible for depositing the components of the islet vascular basement membrane such as collagen, laminin, fibronectin. Intra-islet ECs also secrete many molecules that affect β-cell proliferation and survival [130]. For example, ECs secrete hepatocyte growth factor (HGF) and cellular communication network 2 (CCN2; formerly known as CTGF) that each enhance β-cell proliferation and survival [131-133]. Intra-islet ECs can also affect β-cell proliferation and survival by modulating the availability of growth factors in the ECM. They secrete matrix metalloproteinases (MMPs) that alter ECM integrity and release sequestered growth factors that affect β-cell proliferation and survival such as fibroblast growth factor (FGF) and VEGF [134].

Collagen, laminin, and fibronectin are the three most abundant and well-studied proteins in the ECM [134]. In mammals, collagen makes up a major portion of the basement membrane and provides structural stiffness and cohesiveness to tissues [135,136]. A study using the INS-1 line, a rat-derived immortalized β-cell line, demonstrated that cell proliferation was significantly increased after being plated on

type IV collagen compared to control [137]. A similar trend is seen when cells are cultured on laminin. Studies using immortalized cell lines and primary islets from both rodents and humans have demonstrated that culturing cells on laminin increases β-cell proliferation and decreases β-cell death [138-142]. Finally, studies have shown that culturing MIN6 cells, a mouse-derived immortalized β-cell line, on fibronectin induces DNA synthesis indicative of cells entering the cell cycle. Furthermore, fibronectin contains multiple protein-binding domains where growth factors such as FGF and VEGF can bind, suggesting that fibronectin also plays a critical role in sequestering and regulating accessibility of critical β-cell mitogens [143,144].

Integrins, transmembrane heterodimer receptors for ECM components, are expressed on virtually all cell types. Integrins are composed of one α and β subunit. In the islet, the specific α and β subunits expressed on the different cell types differs among species [145]. Integrins can act synergistically with growth factor receptors including VEGFR, c-Met and EGFR, all of which have been implicated in β-cell proliferation. One specific integrin subunit, the β1 subunit, is the most studied in general and regarding β-cell proliferation. The β1 subunit is the most promiscuous subunit in that it is capable of binding multiple α subunits, and loss of β1 integrin is embryonically lethal [146]. β1 integrin is highly expressed on β cells and promotes β-cell proliferation through binding of multiple ECM components including laminin, collagen and fibronectin. Both *ex vivo* and *in vivo* studies have demonstrated that β1 integrin inactivation affects β-cell proliferation. For example, blockade of β1 integrin using neutralizing antibodies attenuates the beneficial effects of ECM components on β-cell proliferation and survival [137,139]. *In vivo* β-cell-specific inactivation of β1 integrin in

mice during embryogenesis resulted in significantly decreased β-cell area in adulthood due to decreased embryonic and postnatal β-cell proliferation [147]. Furthermore, tamoxifen-inducible β-cell-specific inactivation of β1 integrin at four weeks of age in mice led to reduced β-cell mass due in part to a decrease in β-proliferation when assessed eight weeks later [148].

*Other secreted factors*

Fibroblast growth factors (FGFs) have been mentioned previously in the context of pancreas development, but FGFs also play a role in postnatal β-cell proliferation [150]. Many FGF ligands are expressed in adult mouse β cells including FGF1, FGF2, FGF4, FGF5, FGF7 and FGF10. Furthermore, FGF receptor 1 (FGFR1) and FGF receptor 2 (FGFR2) are also expressed in adult mouse β cells [149]. One study sought to examine the roles of FGF1 and FGF2 in β-cell proliferation. In this study, two mouse models were generated with a β-cell-specific inactivation of either FGF1R or FGF2R during embryogenesis. It was revealed that decreased FGF2R signaling did not have detrimental effects on postnatal β-cell mass expansion. However, while FGF1R-deficient mice had the same number of β cells compared to control at birth, loss of FGF1R resulted in a gradual ~25% decrease in the number of β cells by postnatal day (P)-27, demonstrating a role for FGF signaling in postnatal β-cell expansion [149].

VEGF-A is the prototypical member of the VEGF family and has been demonstrated to be important for β-cell proliferation during embryogenesis. VEGF-A can also induce β-cell proliferation postnatally into adulthood. Loss of VEGF-A during embryogenesis causes decreased β-cell proliferation and overall reduced β-cell mass when assessed at later in life [150]. In some cases, overexpression of VEGF-A leads to

β-cell proliferation. For example, overexpression of VEGF-A utilizing the *Pdx1* promoter led to islet hyperplasia when assessed in two-month-old mice [24]. Furthermore, another study demonstrated that VEGF-A overexpression in adult β cells for two weeks promoted β-cell proliferation [150].

HGF, or hepatocyte growth factor, was previously mentioned as a factor made and secreted by intra-islet ECs. HGF signaling has been implicated in β-cell proliferation and regeneration in many mouse models. β-cell-specific overexpression of HGF in mice resulted in increased β-cell proliferation [152]. Furthermore, HGF plays a role in maternal β-cell mass expansion. Loss of c-Met, the HGF receptor, in pregnant dams resulted in decreased β-cell proliferation, reduced β-cell mass and an increase in β-cell apoptosis. This ultimately led to gestational diabetes in this model [153]. HGF/c-Met signaling also plays a role in β-cell regeneration. Following partial pancreatectomy, there is an upregulation of c-Met and exogenous intraperitoneal injection of HGF further increases β-cell proliferation. Conversely, inactivation of c-Met in the setting of partial pancreatectomy impaired β-cell proliferation and regeneration [154].

Another factor secreted by intra-islet ECs is platelet derived growth factor (Pdgf). Pdgf binds to its tyrosine kinase receptors (PDGFα αnd PDGFβ) to control the growth of connective tissue cells including smooth muscle cells and fibroblasts [155,156]. PDGF signaling also promotes proliferation, survival and migration in a multitude of cell types [157]. Classic studies have elucidated that PDGF signaling in cultured islets stimulated DNA synthesis [158,159]. More recent studies have demonstrated an age-dependent expression pattern of Pdgf, with expression of both the receptor and ligand being markedly reduced in murine β cells at 6 weeks and 6 months of age compared to

neonatal β cells [160]. β-cell-specific loss of Pdgf during embryogenesis led to a three-fold reduction in β-cell proliferation and a 50% reduction in β-cell mass when assessed at two to three weeks of age in mice [160]. Conversely, β-cell-specific Pdgf overexpression resulted in significantly increased β-cell proliferation in 14-month-old mice compared to controls, demonstrating that Pdgf signaling activation can sustain adult β-cell expansion *in vivo* [160].

Pdgf signaling is also implicated in β-cell regeneration – loss of Pdgf in the setting of β-cell loss by STZ treatment resulted in a failure of β-cell mass restoration due to impairment in β-cell proliferation. Studies conducted using juvenile and adult human islets discovered the same age-dependent expression of PDGF that was observed in mice where PDGF and PDGF receptor expression drastically decreases as age increases. Furthermore, exogenous PDGF treatment was able to induce β-cell proliferation only in juvenile human islets which contrasts with what was observed in mouse studies [160]. However, differences in proliferative capacity and responsiveness to mitogens exist between murine and human β cells, and the disparity between the β-cell response to PDGF may simply be a species difference.

**Diabetes Mellitus**

Diabetes mellitus is a very complex cluster of diseases and was described as long ago as the ancient Greeks. Multiple forms of diabetes exist, with the two most common being Type 1 diabetes (T1D) and Type 2 diabetes (T2D). T1D is characterized by insufficient insulin output due to the autoimmune destruction of β cells and accounts for 5-10% of diabetes cases. T2D accounts for 90-95% of diabetes cases [161] and is characterized by loss or dysfunction of β-cell mass in the setting of insulin resistance,

leading to insufficient insulin output and prolonged hyperglycemia. Another form of diabetes, maturity-onset diabetes of the young (MODY), is caused by genetic autosomal dominant inheritance of mutations in at least 14 identified genes. MODY accounts for 1-5% of diabetes cases [162,163]. Diabetes is a global health burden with an estimation of 537 million adults (20-79 years) being diagnosed with the disease [164]. As of 2022, in the United States alone there were 37.3 million people estimated to have diabetes, with 8.5 million of those people being undiagnosed [161]. By 2060, the number of adult Americans with diabetes is expected to nearly triple, with the percent prevalence nearly doubling [165]. Diabetes was the seventh leading cause of death in 2019 in the United States, although the number of deaths attributed to diabetes may be underreported [166]. Complications of the disease include blindness, limb amputation, cardiovascular disease, neuropathy, and kidney failure [167]. Thus, it is crucial for effective therapeutics to be developed while investigation of a cure is ongoing.

*Type 1 Diabetes*

T1D has been long considered a chronic immune condition characterized by autoimmune destruction of β cells, but as more information comes to light about the disease, it is now considered a disorder that results from complex interactions between genetic and environmental factors, microbiome, metabolism and the immune system. While T1D has historically been considered a childhood-onset disease, it is now known that up to 50% of T1D cases are diagnosed in adulthood [168].

In 1984, George Eisenbarth developed a model that illustrates the progression of of T1D relating genetic and environmental factors to progressive loss of β-cell mass and

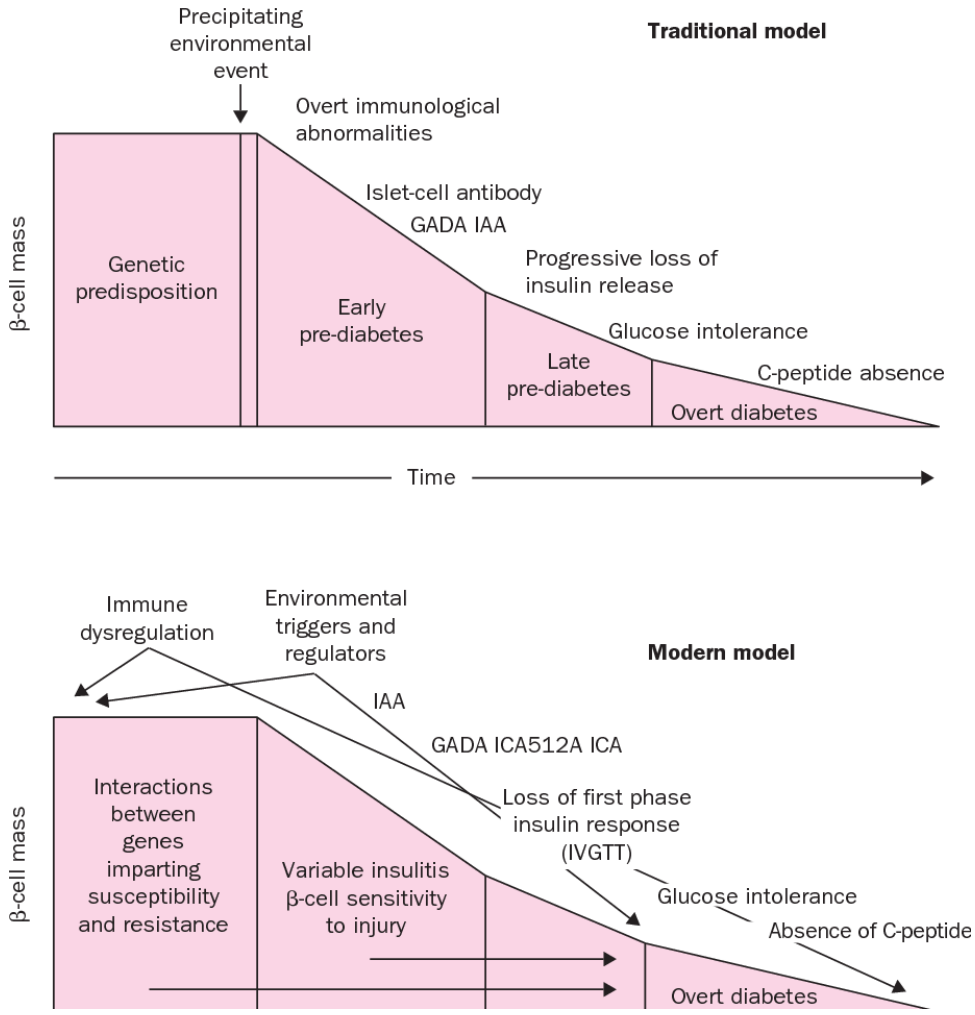



**Figure 1-3. Schematic of the traditional and modern models of the original Eisenbarth of T1D model created in 1984.** As more information has come to light about the disease, parameters of the disease and how it progresses has become more understood. Adapted from Atkinson M. A., and Eisenbarth G. S. (2001). Type 1 diabetes: new perspectives on disease pathogenesis and treatment. *The Lancet* **358**, 221–229.

eventual T1D onset [169] [Figure 1-3]. In this since updated model, those with a genetic predisposition to the disease undergo a precipitating event that leads to immunological dysfunction and the eventual loss of β cells due to autoimmune attack. This precipitating event may be different for different people. Overt diabetes is observed when a significant portion of functional β-cell mass is lost, leading to insufficient insulin output and uncontrolled hyperglycemia [170]. The current standard of care for T1D is exogenous insulin replacement therapy via multiple daily injections of insulin, or by use of insulin pumps [171]. The use of continuous glucose monitors with insulin pumps, which is referred to in the literature as a closed-loop system, allows for only sufficient amounts of insulin to be injected to maintain euglycemia. The specificity in amount of insulin injected at a given time reduces the hypoglycemic episodes experienced by people with T1D [172]. Precise maintenance of glycemic control still bears a challenge due to the varying pharmacokinetics and absorption rates of currently used insulin analogues. Thus, other therapeutics are being developed to allow for more accurate glycemic control in T1D patients. Some of the therapeutics currently being developed involve immune system modulation, use of stem cell-derived β-like cells, and islet transplantation.

As mentioned previously, T1D is a heritable polygenic disease with risk for the disease varying from 30-70% in identical twins, 6-7% in siblings, and 1-9% for children with a parent with the disease [173,174]. As of 2019, genome-wide association studies (GWAS) have identified more than 50 loci that contribute to the genetic susceptibility of the disease [175]. However, the strongest association with T1D involves variations in classic histocompatibility leukocyte antigens (HLA) class II molecules, which are

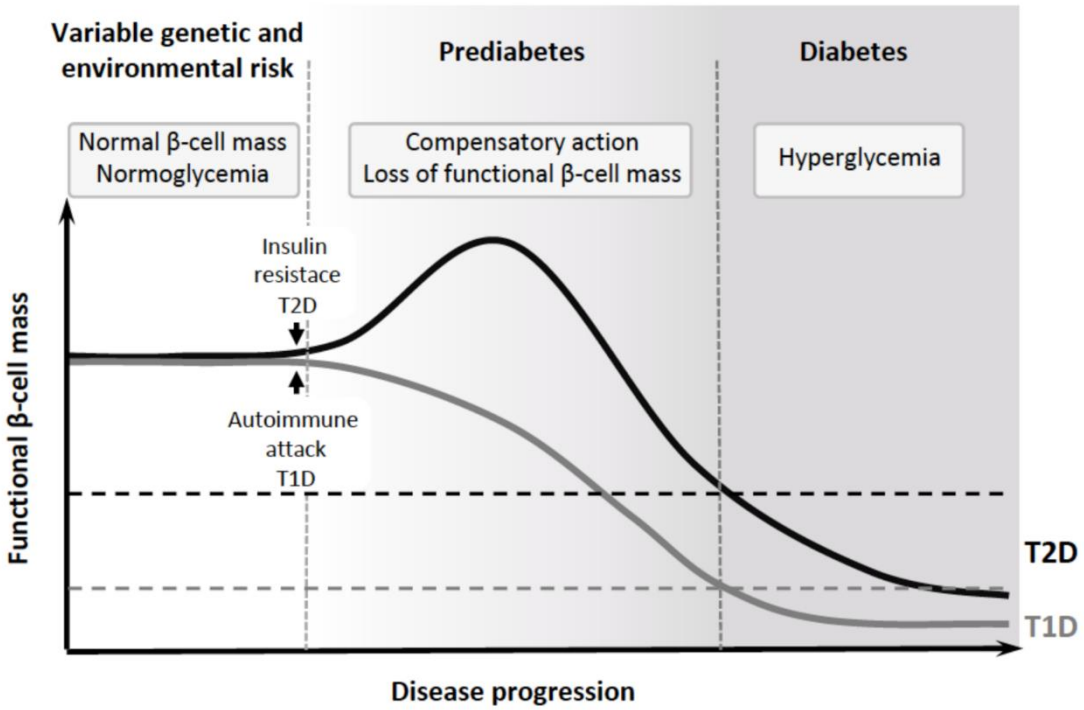



**Figure 1-4. Comparison between the etiology of Type 1 and Type 2 Diabetes.** In Type 1 Diabetes, autoimmune attack results in a significant loss of functional β-cell mass which subsequently causes overt hyperglycemia and the onset of T1D due to insufficient insulin output. In Type 2 Diabetes progression, in the presence of insulin resistance, functional β-cell mass increases. However, eventually β-cell dysfunction and loss occur resulting in insufficient insulin output, hyperglycemia, and overt diabetes. Compared to T1D, hyperglycemia in T2D may be present at a higher functional β-cell mass due to the increased presence of insulin resistance in T2D patients. Adapted from Lorenzo et al. (2021). *Int J Mol Sci* 22(8), 4239.

cell-surface molecules that bind and present antigen to T cell receptors. HLA genes are known to be the most polymorphic in the human genome – classical HLA genes can have thousands of sequences [176]. Class I molecules have also been implicated in the autoimmune destruction of β cells, as it has been demonstrated that these molecules present antigens to CD8+ T cells, which in turn cause β-cell death by the initiation of cytotoxic T cell killing [177]. Animal models have illustrated the role of class I molecules in T1D. In the NOD mouse model, a mouse model for T1D, loss of expression of MHC class I molecules renders NOD mice resistant to autoimmune diabetes [178].

Outside of genetic susceptibility, another risk factor for the disease in at-risk populations is the presence of islet autoantibodies. The presence of islet autoantibodies is also considered a marker of the disease. As of 2019, there were over 30 identified autoantigens for T1D [179]. There are four major autoantigens associated with T1D – insulin, GAD65, IA-2 and ZnT8 [180]. GAD65, the 65-kD isoform of glutamic acid decarboxylase (GAD), is expressed in neuroendocrine cells and it has been demonstrated that higher levels of GAD65 autoantibodies are associated with earlier onset of T1D in genetically susceptible individuals [181]. A study evaluating the diagnostic value of examining Anti-GAD65 autoantibodies found that these autoantibodies were present in 80% of newly diagnosed children with T1D [182]. IA-2, or islet tyrosine phosphatase-like protein, is a neuroendocrine molecule that is localized to the insulin secretory granules of β cells [183]. ZnT8, or zinc transporter 8, is necessary for zinc flux into β-cell insulin granules [184,185]. Zinc is co-secreted with insulin and plays both autocrine and paracrine roles in the islet [186]. In genetically

susceptible patients, as the number of autoantibodies present increases, so does the risk of developing the disease.

While T1D is thought of as a disease due to malfunction of the immune system, several studies have demonstrated that the β cell plays a role in its own demise. β cells can secrete chemokines, a group of small secreted molecules, which contributes to immune infiltration of the islet. Some examples of chemokines secreted by β cells are CCL2, CCL5, and CXCL10 [187]. All of these chemokines are induced by inflammation which is heavily present in the setting of T1D. CCL2 is involved with monocyte, natural killer (NK) cell, and T-cell recruitment during inflammation [188-190]. CCL2 may be responsible for the influx in macrophages in the islet as studies have demonstrated that increased monocyte recruitment, insulitis and islet destruction occurs during transgenic overexpression of *Ccl2* in murine β cells [189]. CCL5 is a chemoattractant for monocytes, eosinophils and T cells, and blockade of one of the cognate receptors for CCL5 inhibits future immune infiltration and prevents development of diabetes [191]. Finally, CXCL10 mediates chemotaxis in lymphocytes. CXCL10 binds the CXC receptor 4 (CXCR3), and it has been demonstrated that inactivation of CXCR3 delays insulitis in a T1D mouse model. Conversely, overexpression of CXCL10 in mouse islets accelerated diabetes in a T1D mouse model. [192-194]. CXCL10 is directly toxic to β cells due to signaling through Toll-like receptor-4 (TLR) which leads to pro-apoptotic signaling within the cells [195,196].

There is still much to learn about T1D, and the NOD mouse model has been utilized in efforts to identify therapeutics and a potential cure for T1D [180]. The autoimmune diabetes phenotype in this mouse model is similar to the course of disease

in humans. One strength of the model is that autoimmunity is spontaneous, and mice develop autoantibodies, many of which are present in human T1D, along with circulating autoreactive T cells prior to the onset of disease. One difference between the model and human disease, however, is that the initial autoantigen in the NOD model is insulin while in humans it is thought that disease results from multiple initial autoantigens. However, progressive loss of β-cell function and mass resembles that of the human disease, and the degree of insulitis increases as disease progresses, although this is not readily observed in humans with long-standing disease [197]. Another difference between the NOD model and human T1D is that overt diabetes occurs primarily in female NOD mice while T1D does not skew towards one sex in humans. Similar to other mouse models of human disease, there has been a failure to translate some basic science findings discovered in the NOD mouse model to humans. Moving forward, along with increasing understanding about T1D in the human population, more understanding needs to be gained about the NOD model in order to bridge the gap between basic science findings and the potential for these findings to be applied in the clinic.

*Type 2 Diabetes*

Unlike T1D, T2D is not caused by autoimmune attack of the β cells. However, in both of these diseases, loss of functional β-cell mass is key to progression of disease [Figure 1-4]. While T2D does have a stronger link to family history and lineage than T1D, development of the disease depends largely on environmental factors [198]. T2D is primarily linked to insulin resistance and prolonged hyperinsulinemia, with both being associated with poor dietary choices and a sedentary lifestyle. Obesity is heavily linked

to T2D as well, with almost 80-90% of T2D patients being overweight or obese [199]. It has been demonstrated that β-cell mass is significantly lower in pancreata from human cadaveric donors who had T2D compared to body mass index (BMI)-matched donors who did not have T2D [200,201]. Furthermore, islets from donors with T2D are smaller and have less than half the islet equivalents of non-diabetic donors [202].

There are multiple mechanisms by which functional β-cell mass can be insufficient to meet metabolic demand in the setting of T2D [Figure 1-5]. There is great heterogeneity in the human population, and as such people are born with varying amounts of β-cell mass. Someone born with a low β-cell mass may undergo β-cell mass compensation in the face of metabolic demand, but their increased functional β-cell mass may still be insufficient to meet the increased need for insulin. A lack of functional β-cell mass could also arise if β-cell mass expansion simply fails in the face of increased metabolic demand. Finally, in the presence of increased metabolic demand, β-cell mass expansion may successfully happen. However, due to the prolonged hyperinsulinemia and the increase of β-cell stress due to increased demand for insulin, β cells may fail, die or lose their identity, all of which can result in loss of functional β-cell mass. In all of these situations, prolonged hyperinsulinemia and insulin resistance would occur, leading to development of T2D [Figure 1-4].

**β-cell loss and dysfunction**

β-cell loss occurs primarily through dedifferentiation and cell death. Dedifferentiation, also referred to as loss of β-cell identity, is the process by which β cells lose their β-cell-defining transcription factors and occurs due to prolonged exposure to hyperglycemic

conditions [203]. Transcription factors expressed by mature adult β cells include Pdx1, Pax6, Nkx6.1, Nkx2.2, and MafA [203-206]. β cells from human T2D donors and T2D mouse models show loss of some of these transcription factors, especially those involved in mature β-cell function such as Pdx1 and MafA [207-209]. Loss of MafA, the last β-cell identity transcription factor to be expressed during β-cell maturation, causes impairments in glucose-stimulated insulin secretion leading to eventual hyperglycemia [210]. Furthermore, in the early stages of human T2D and in the T2D-like db/db mouse model, a model of leptin insufficiency, loss of nuclear MafA is one of the earliest changes observed in β cells [211]. Another phenomenon that occurs during dedifferentiation is the induction of so-called disallowed genes, which are genes that are normally actively repressed or not expressed highly in β cells. Increased expression of these disallowed genes is associated with decreased β-cell function. Currently, there have been 60 β-cell disallowed genes identified, and many of them are expressed in immature β cells [212-214]. Therefore, during dedifferentiation, both loss of key identity transcription factors and induction of disallowed genes leads to β-cell dysfunction and thus insufficient insulin output. The processes that induce dedifferentiation are still being elucidated; however, at this point it is known that chronic hyperglycemia and hyperlipidemia can lead to β-cell dedifferentiation and β-cell mass loss [Figure 1-6].

β-cell death can be induced by different mechanisms such as apoptosis and necroptosis, two forms of programmed cell death. Apoptosis results in clearance of cells with minimal damage to the surrounding tissues. However, necroptosis is a type of programmed cell death that combines features of apoptosis and necrosis [215]. Recent studies utilizing a zebrafish model of overnutrition demonstrated that islet inflammation

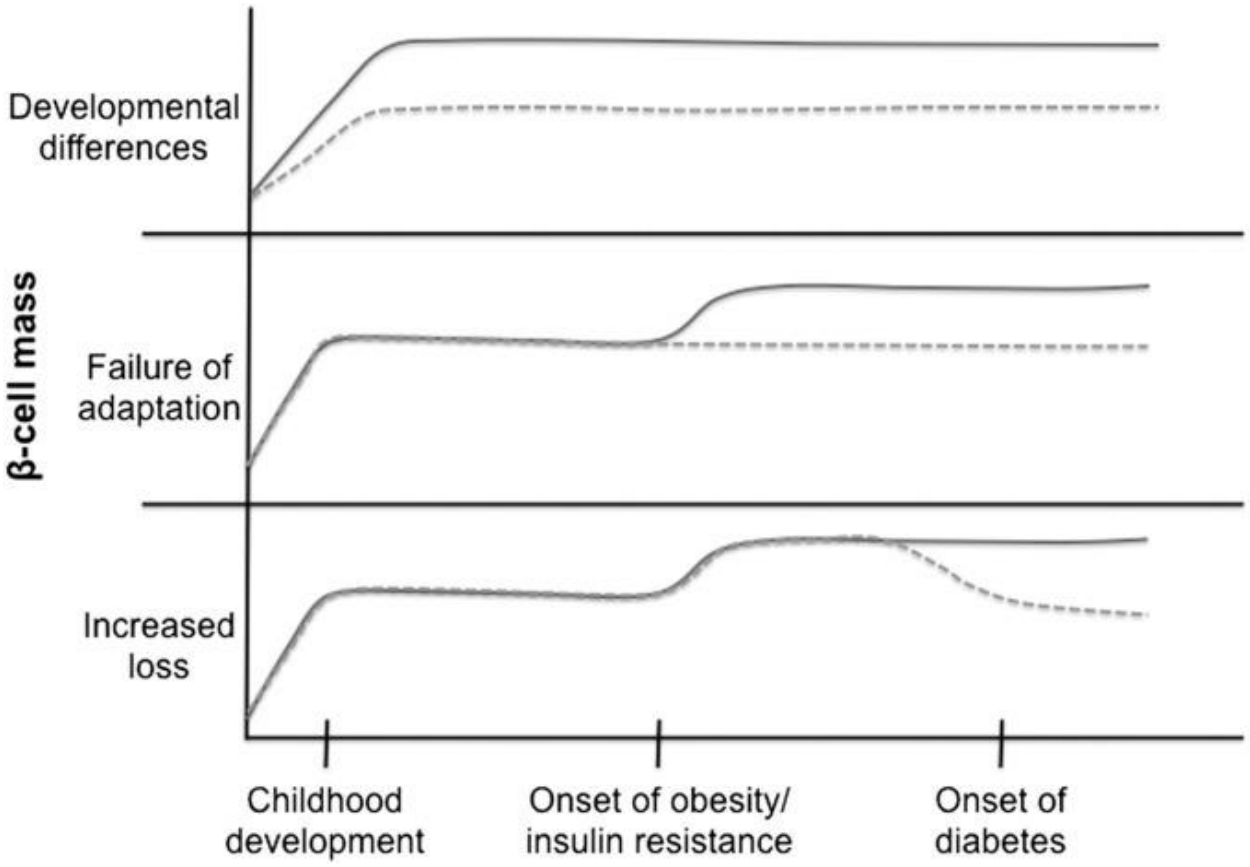



**Figure 1-5. Etiologies of Type 2 Diabetes.** Diabetes is a disease characterized by loss/dysfunction of β-cell mass, eventually leading to insufficient insulin output and hyperglycemia. This illustration details three etiologies by which functional β-cell mass can be insufficient. Firstly, a person may simply be born with too little functional β-cell mass to meet the demand for insulin when insulin resistance becomes present. Second, a person may have enough β-cell mass to maintain euglycemia for a time, but once a metabolic demand requires more insulin output, there is a failure of β-cell adaptation. Finally, successful β-cell mass adaptation may occur in the face of increased metabolic demand; however, eventually β cells become stressed and die, and β-cell mass is lost. Adapted from Linnemann A. K., Baan M., and Davis D. B. (2014). Pancreatic β-cell proliferation in obesity. *Adv Nutri* **5**, 278-288

and eventual β-cell loss were mediated by receptor-interacting protein kinase 3 (Ripk3), a known mediator of necroptosis [216]. Additionally, prolonged oxidative and ER stress can also induce β-cell death. In diabetes, chronic hyperglycemia leads to excess accumulation of glucose intracellularly in β cells, which leads to mitochondrial dysfunction and ER stress [217]. Furthermore, lipotoxic conditions can result in increased oxidative stress due to increased lipid peroxidation and hydrogen peroxide generation [218]. Compared to other cell types, β cells have low levels of antioxidant enzymes making them particularly sensitive to ROS [219]. One study in INS-1 cells found that chronic exposure to high glucose and/or palmitate (to mimic glucotoxicity and lipotoxicity respectively) led to a 2.5-fold increase in ROS, leading to severely reduced insulin secretion. It was postulated that this was due to defects in MafA expression and activity since MafA is particularly sensitive to oxidative stress [211]. Another study *in vivo* using the db/db mouse line found that overexpression of the antioxidant enzyme glutathione peroxidase (Gpx) in situations of oxidative stress preserved nuclear MafA expression and reversed diabetes [220].

β cells undergo ER stress in the face of chronic hyperglycemia due to increased demand for insulin, leading to increased insulin synthesis and processing. Furthermore, proinsulin is very susceptible to misfolding, and it has been estimated that around 20% of proinsulin misfolds in healthy β cells, which can further aggravate ER stress [221]. ER stress leads activation of the unfolded protein response (UPR), a compensatory response that involves the proteins X-box binding protein 1 (XBP1), inositol-requiring enzyme 1 (IRE1), and PERK. These proteins work together to slow protein synthesis which allows for refolding or degradation of improperly folded proteins, leading to the

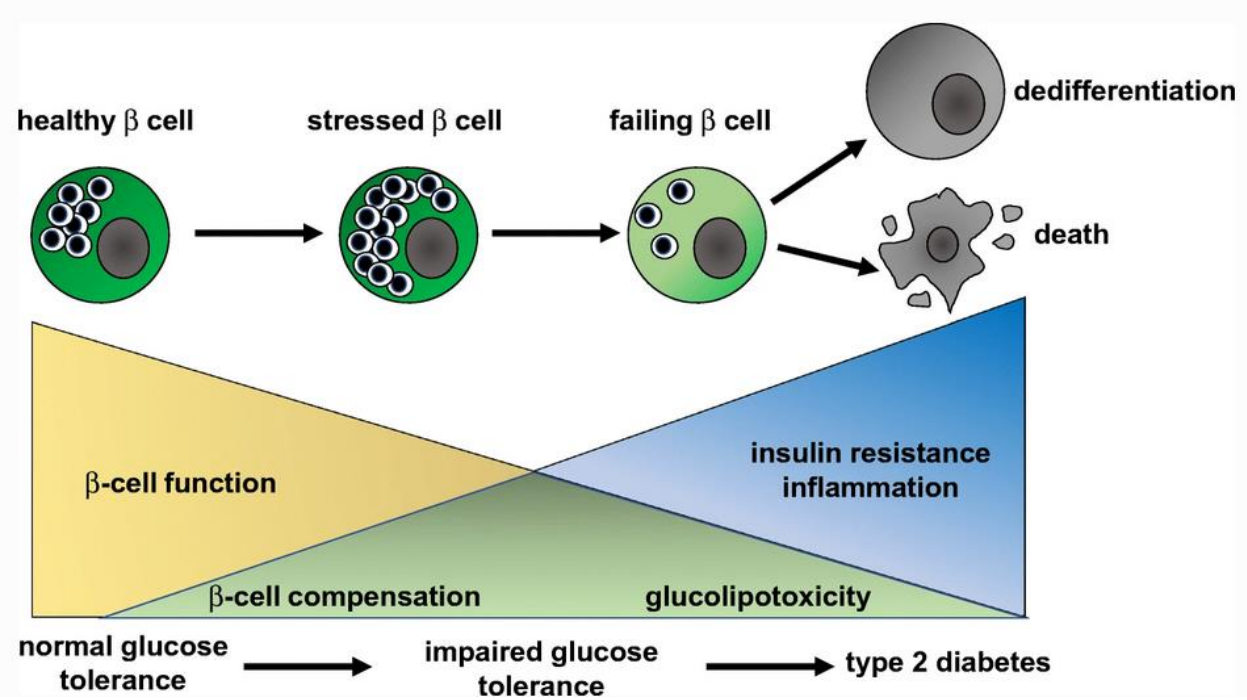



**Figure 1-6. Mechanistic timeline of Type 2 Diabetes onset and progression.** As impaired glucose tolerance occurs, β cells become stressed due to the increased demand for insulin to maintain euglycemia. Overwork of β cells in the presence of glucolipotoxicity, insulin resistance and low-grade inflammation leads to eventual failure of β cells. This can result in dedifferentiation or death, both of which contribute to loss of functional β-cell mass. Adapted from Christensen and Gannon. (2019). *Curr Diab Rep* 19, 81.

resolution of ER stress [222]. Prolonged UPR can lead to upregulation of C/EBP homologous protein (CHOP) however, which is a protein heavily involved in apoptosis [223]. Another diabetes mouse model, the Akita mouse, has a phenotype of rapid-onset diabetes due to prolonged UPR. This mouse model results from a spontaneous mutation in the *Ins2* gene, causing misfolding of proinsulin, ER stress, subsequent activation of the UPR, and ultimately β-cell apoptosis leading to rapid onset of diabetes [224].

**Endoplasmic reticulum stress – an overview**

Studies in this dissertation examine the effects of CCN2 induction on ER stress and oxidative stress. In eukaryotes, the UPR is governed by activation of three key transmembrane ER-resident sensors: PERK, activating transcription factor 6 (ATF6), and IRE1 [222] [Figure 1-7].

PERK is a kinase that possesses both cytoplasmic and kinase domains. During ER stress-free conditions, PERK exists as a homodimer and is bound to glucose-regulated protein 78 (GRP78)/binding immunoglobulin protein (BiP), which keeps it inactive. Upon ER stress conditions, such as during the presence of misfolded proteins, BiP disassociates from PERK and allows for activation of the kinase by autophosphorylation of its kinase domain [225-227]. Activation leads to full catalytic activity which subsequently phosphorylates eIF2α [228-230]. eIF2α in its non-phosphorylated condition functions as a component of the 43S pre-initiation complex that is necessary for the formation of 80S ribosomal complex, which is vital for proper protein translation [231]. Upon phosphorylation by PERK, eIF2α prevents reformation of the 43S pre-initiation complex and subsequently prevents formation of the 80S

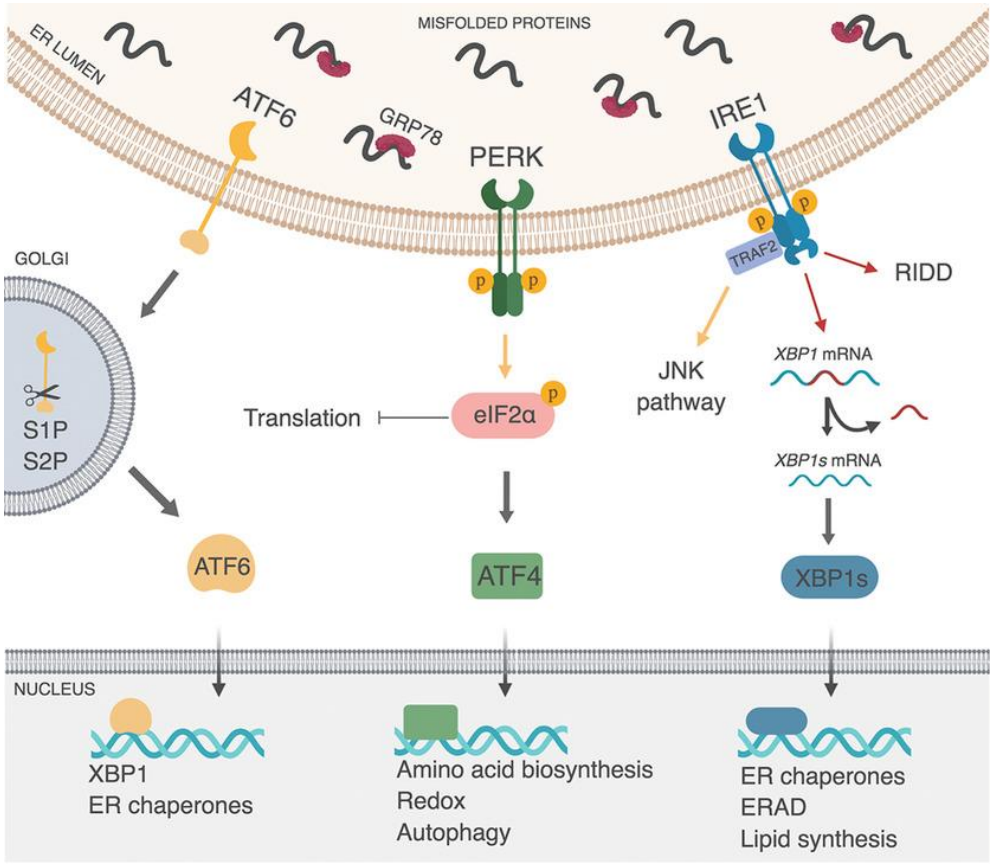



**Figure 1-7. Schematic of the three unfolded protein response (UPR) pathways.** ATF6 is a protein that is cleaved in the Golgi apparatus to produce the ATF6 transcription factor. ATF6 induces expression of XBP1 and BiP upon binding to ER-stress responsive elements. PERK phosphorylates eIF2α, which subsequently results in activation of ATF4. ATF4 induces expression of molecules involved in amino acid biosynthesis, redox and autophagy. IRE1 can function to modulate posttranscriptional modifications via regulated IRE-1-dependent decay (RIDD). IRE1 splices XBP1, which can then translocate to the nucleus and induce expression of ER chaperones and ERAD components. Adapted from Madden et al. (2018). *Biol Cell* 111(1): 1-17.

ribosomal complex, leading to inhibition of protein translation. Furthermore, eIF2α in its phosphorylated form directly regulates expression of a multitude of cell-fate determining genes [232,233].

One gene that eIF2α regulates is activating transcription factor 4 (ATF4) [234,235]. ATF4 has multiple functions – it can induce expression of BiP to increase folding capacity, increase resistance to oxidative stress and activate various inhibitors of apoptosis [236-238]. However, ATF4 can also induce expression of the pro-apoptotic protein CHOP [239]. CHOP acts to negatively regulate members of the pro-survival Bcl-2 family, while also activating Death Receptor 5 (DR5) [240-243]. DR5 binds to the Fas-activated death domain (FADD) to activate caspase 8, which is a cysteine protease that functions to dismantle multiple cellular structures such as mitochondria, ER, cytoskeleton and nucleus itself [244-246]. PERK can also activate mechanistic target of rapamycin (mTOR) signaling through its lipid kinase activity, leading to activation of protein kinase B (Akt). Akt can function to modulate both pro-survival and pro-apoptotic signaling [247,248]. Overall, while PERK has canonically been referred to in a negative light, it has the capability of modulating both cell survival and apoptosis through activation of multiple pathways.

ATF6 is a transmembrane glycoprotein, and mammals have two homologous proteins of ATF6: ATF6α (which is better characterized) and ATF6β [249,250]. ATF6α possesses a cytoplasmic domain that encompasses both a basic leucine zipper DNA binding domain and a transcriptional activation domain [251]. Under ER stress-free conditions, ATF6α is bound to BiP. However, when ER stress is present, BiP dissociates from ATF6α, revealing two Golgi-localization sequences. ATF6α then

translocates to the Golgi apparatus where proteolytic cleavage occurs to create the amino-terminal cytoplasmic fragment of the protein, ATF6f [252,253]. ATF6f, now a transcription factor, translocates to the nucleus where it binds to ER-stress responsive elements [254,255]. Expression of BiP and Xbp1 is induced – expression of Xbp1 by ATF6f also functions to augment the regulatory output of IRE1 signaling which will be described below [256,257].

IRE1 is a transmembrane protein that possesses both kinase and endonuclease activity. In the presence of ER stress IRE1, like PERK, has the capability of autophosphorylation, subsequently causing activation of its RNAse domain [258-260]. One method of regulation of ER stress by IRE1 involves modulating posttranscriptional modifications via regulated IRE1-dependent decay (RIDD). Due to its endonuclease activity, IRE1 can degrade ER-located mRNAs to attenuate protein translation by the ER [261]. IRE1 also unconventionally splices Xbp1 to generate spliced Xbp1 (Xbp1s) which can translocate to the nucleus and affect transcription of many target genes including those encoding ER chaperones and ERAD components [256,262]. Furthermore, Xbp1s has been demonstrated to be indispensable for proteostasis through its regulation of genes involved in ER expression, protein folding glycosylation, protein entry and vesicular trafficking [263].

**ER stress and β cells**

Since β cells secrete insulin at a high rate, ER homeostasis is tightly controlled to ensure that insulin processing and folding occurs correctly. Several studies have investigated the roles of various members of the UPR to elucidate how they affect β-cell health. It has been demonstrated that loss of components of the UPR can be

detrimental, solidifying the importance of the UPR in maintaining ideal β-cell health and function. Studies investigating embryonic β-cell-specific PERK deletion in mice have demonstrated that embryonic loss of PERK lead to defects in fetal/neonatal β-cell proliferation and differentiation which in turn causes decreased β-cell mass. Furthermore, there are defects in proinsulin trafficking and permanent neonatal diabetes due to a reduction in GSIS [92]. Another study demonstrated that loss of PERK lead to reduced endocrine and exocrine tissue and increased β-cell death [264]. In humans, mutations in PERK cause neonatal diabetes [265]. β-cell-specific loss of IRE1α leads to hyperglycemia and hypoinsulinemia due to reduced proinsulin and insulin content, decreased insulin biosynthesis and defective GSIS [266]. Deficiency of ATF6 accelerates diabetes due to blunted insulin secretion in HFD-fed mice [267], and it has been reported that polymorphisms of ATF6 are associated with prediabetes in humans [268]. β-cell-specific deletion of *Xbp1* in mice decreased pancreatic insulin content, and impaired GSIS leading to glucose intolerance [269].

While the UPR is necessary for proper functioning of β cells, chronic ER stress eventually leads to β-cell dysfunction and apoptosis. Nutrient overload, such as high glucose and saturated free fatty acids, can induce sustained activation of PERK, ATF6 and IRE1 pathways leading to β-cell apoptosis [270]. In cases of increased metabolic stress, such as in *ob/ob*, a genetically obese mouse model, and *db/db* mice where increased demand for insulin is present, there is upregulation of various UPR modulators which can be both beneficial and maladaptive [271,272]. The relationship between ER stress and human T2D is a bit unclear; studies have shown that there is decreased immunostaining of XBP1s, ATF6 and p-eIF2α in islets from donors with T2D

[273]. However, other studies have shown an increase in immunostaining for BiP and CHOP in islets from donors with T2D compared to islets from non-diabetic donors, with some of the immunostaining being β-cell-specific [272]. With the current knowledge in the field, it is not fully understood if these increases in UPR modulators are beneficial or implicated in the pathophysiology of diabetes. *In vitro*, the UPR has been referred to as a double-edged sword where chronic signaling leads to apoptosis. However, there is not much evidence to support this concept *in vivo*. Regardless, it is generally agreed upon that sustained ER stress response leads to increased transcriptional expression of CHOP, which leads to β-cell apoptosis [274].

**Oxidative stress – an overview**

Oxidative stress is a phenomenon characterized by the production and build-up of ROS, which are byproducts of cellular metabolism and biochemical processes, in cells and tissues. Mitochondrial aerobic respiration is the primary source of ROS production in cells [275]. During unstressed conditions, ROS are signaling molecules that mediate many physiological responses such as differentiation, migration and proliferation. However, the prolonged presence of ROS without mechanisms of proper detoxification can cause detrimental effects to cells and tissues. The best characterized ROS are superoxide radicals, hydrogen peroxide, hydroxyl radicals, and singlet oxygen [276,277]. These molecules, if unchecked, can cause damage to lipids, proteins and nucleic acids leading to the onset and progression of several diseases including diabetes and other metabolic disorders [278,279].

There are multiple mechanisms by which oxidative stress is resolved to maintain redox homeostasis. One of the main mechanisms of antioxidant action is activation of

nuclear factor erythroid 2-related factor 2 (Nrf2), which is considered the transcriptional master regulator of the cellular antioxidant response [280]. Under basal unstressed conditions, Nrf2 is bound to the substrate adaptor protein Kelch-like ECH-associated protein (Keap1) [281]. Keap1 facilitates polyubiquitination of Nrf2, leading to its degradation during unstressed conditions. In the presence of oxidative stress, Keap1 becomes oxidized and undergoes a conformational change that prevents it from binding to Nrf2 [282-284]. Dissociation of Keap1 from Nrf2 allows Nrf2 to be stabilized and translocate to the nucleus where it can bind to antioxidant response elements (AREs) upstream of antioxidant target genes, leading to increased transcription of genes that facilitate redox homeostasis [285] [Figure 1-8]. Some genes that are upregulated upon Nrf2 binding to AREs are *Txn1, Gpx2, Nqo1,* and *Hmox1* [286-288]. Studies in this dissertation examine expression of the aforementioned genes as well as another antioxidant enzyme *Sod1*.

*Antioxidants and enzymes that facilitate redox homeostasis*

*Txn1* is the gene that encodes cytosolic thioredoxin 1 (Trx1), a 12kDA reductase, in mammalian cells. There also exists another isoform of thioredoxin, thioredoxin 2 (Trx2), which is the mitochondrial isoform [289,290]. Trx1, Trx2 and thioredoxin reductases (TrxRs) are part of the thioredoxin family of antioxidant proteins that are present in all organisms from bacteria to mammals. Trx1 functions to transfer electrons to other members of the thioredoxin family including peroxidases, which aid in removal of hydrogen peroxide and peryoxynitrite, as well as some redox-sensitive transcription factors [291,292]. Trx1 can also function as a growth factor, enzyme cofactor, and acts as a reducer of thiols that are critical for DNA binding [293]. Upon oxidative stress, Trx1

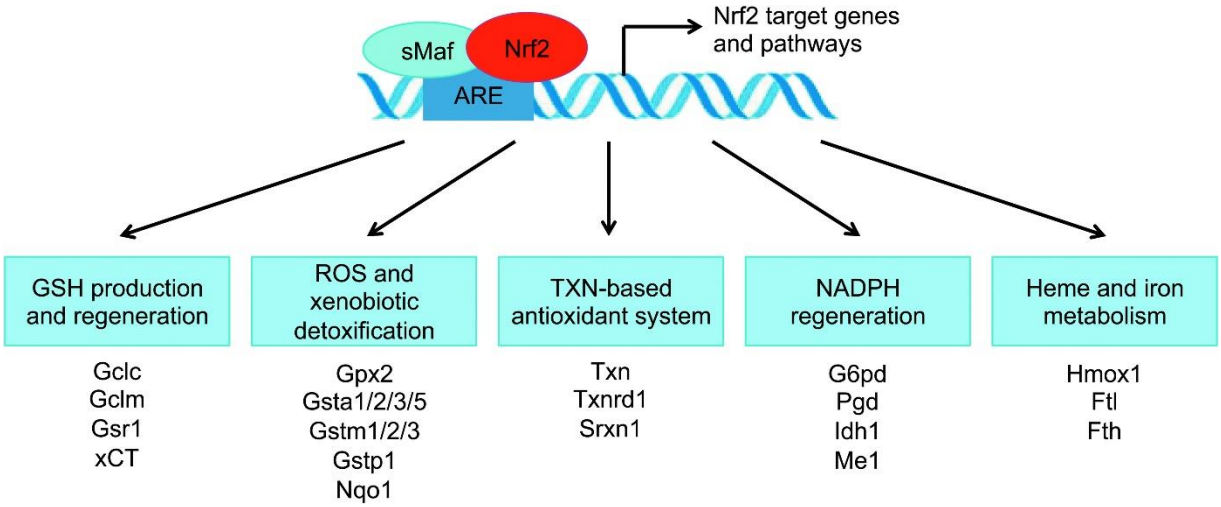



**Figure 1-8. Nrf2, known as the master antioxidant regulator, binds to antioxidant response elements (AREs) in the nucleus to induce expression of various antioxidant genes including *Gpx2, Txn, Txnrd1, Nqo1* and *Hmox1*.** The proteins listed in the illustration aid in ameliorating cellular oxidative stress. In this dissertation, expression of *Gpx2, Nqo1, Txn*, and *Hmox1* are utilized as readouts of Nrf2 activation and overall presence of cellular oxidative stress. Adapted from Tonelli, Chio and Tuveson. (2018). *Antioxid Redox Signal* 29(17): 1727-1745.

expression is upregulated allowing it to migrate into the nucleus and activate multiple transcription factors related to cell proliferation and survival including NF-κB and p53 [294,295]. Furthermore, Trx1 can activate ERK1/2 MAP kinases to aid in cell survival [296,297]. Trx1 is regulated by TrxRs and thioredoxin-interacting protein (TXNIP), with the former essentially “resetting” Trxs by reducing the protein into its active state [298]. The latter suppresses Trx action by binding to its catalytic active center, leading to inhibition of its expression and activity [299-302]. In studies described in this dissertation, upregulation of *Txn1* is utilized as a measure of oxidative stress.

*Gpx2* encodes glutathione peroxidase 2, which is a target of Nrf2. Gpx2 is a member of the glutathione peroxidase family, which are antioxidant enzymes that scavenge hydrogen peroxide and thus protect cell membranes and other components from oxidative stress [303-305]. Gpx2, while known to be predominantly expressed in the cytosol of cells in the gastrointestinal tract, has been demonstrated to be upregulated in islets upon oxidative stress [306]. *Nqo1* encodes a member of the NAD(P)H:quinone acceptor oxidoreductases (NQO). Unlike Trx1 and Gpx2, Nqo1 is a member of the plasma membrane redox system (PMRS), which is a mechanism by which cells are defended against oxidative stress [307,308]. Nqo1 functions to catalyze quinones, reactive intermediate molecules that can interact with antioxidant enzymes to reduce their activity, into hydroquinones as a method of cellular detoxification [309]. Nqo1 also has superoxide reductase activity although the rate of reaction is several magnitudes lower than that of superoxide dismutase (Sod) [310].

*Hmox1*, or heme oxygenase-1, is the rate-limiting enzyme in heme degradation and is also an essential membrane protein of the smooth ER [311]. Hmox1 is induced in

response to different types of chemical and physical stress, such as oxidative stress, heat shock and iron starvation. Hmox1 has also been demonstrated to localize to other organelles including the nucleus and mitochondria, with the capability of shuttling between organelles [312,313]. While the protein is anchored to the ER via a transmembrane sequence, it can be truncated in response to stress, allowing for translocation into the nucleus [311]. Outside of its activity involving heme degradation, nuclear-located Hmox1 can activate various transcription factors to alleviate oxidative stress. One study demonstrated that nuclear translocation of Hmox1 led to increased expression of various antioxidant enzymes including Gpx and catalase (CAT) independent of its enzymatic activity [314].

**Oxidative stress and β cells**

β cells are very sensitive to oxidative stress due to low expression of the antioxidant enzymes catalase, Sod1 and Gpx2 compared to other cell types [315,316]. Thus, presence of increased antioxidant enzymes may be beneficial to the health of β cells. One of the main drivers of oxidative stress during diabetes is glucolipotoxicity [317]. When excess glucose and saturated fatty acids are present, ROS and reactive nitrogen species (RNS) are generated by several pathways, including mitochondrial pathways. Of note, it has been demonstrated that prolonged elevations in ROS generated by mitochondrial metabolism can trigger apoptosis in both INS-1 and murine β cells, and mitochondrial-mediated apoptosis is closely associated with the diabetic condition [318,319]. Recent studies have reported that hyperlipidemic conditions may be the major driving force for ROS production. In one study, treatment with palmitic acid, which is the most common saturated fatty acid found in the human body, resulted

in induction of nitric oxide and lipid peroxidation which are two molecules known to be major contributors to oxidative stress [217]. Hyperlipidemia can also aggravate oxidative stress by reducing antioxidant capability, with studies demonstrating an increase in both reduced SOD1 and total SOD1 expression during hyperlipidemic conditions in human and rat β cells [320,321]. Another study reported similar results, where treatment of Rin-5F cells, an insulin-secreting pancreatic β-cell line, with palmitate significantly induced nitric oxide (NO) production as well as lipid peroxidation, which are two major contributors of oxidative stress. Furthermore, this study found that hyperglycemia had no effect (217).

Pathways that are altered by oxidative stress include the AMP-activated protein kinase (AMPK), mTOR, and c-Jun N-terminal kinase (JNK) pathways. The AMPK pathway regulates insulin secretion, proliferation and survival. In healthy non-diabetic conditions in human and mouse β cells, AMPK phosphorylation is reduced leading to its inactivation. However, under pathological conditions where oxidative stress is present, there is reduced inactivation of AMPK [322,323]. Short-term increased activation of AMPK can play both protective and harmful roles in β cells. For instance, several studies have demonstrated that AMPK activation is beneficial for β cells as it promotes autophagy, aids in maintaining mature β-cell identity, represses expression of disallowed genes, and increases insulin secretion through increased uptake of calcium in the cytosol [324-326]. However, other studies have demonstrated that ROS-mediated upregulation of AMPK resulted in an increase in phosphorylated extracellular-signal-regulated kinase (p-ERK), which is known to impair β-cell proliferation [327].

Additionally, continued activation of AMPK increased β-cell apoptosis and reduced insulin secretion in mouse islets [328].

Increased oxidative stress can also cause inhibition of mTOR signaling potentially through the activation of AMPK. Downstream targets of mTORC1 and mTORC2 primarily function to stimulate growth and increase proliferation and survival. In healthy β cells, mTOR plays an essential role in preserving β-cell mass through the regulation of proliferation, apoptosis, autophagy and the cell cycle [329-331]. Inactivation of mTORC1 leads to increased expression of TXNIP, which negatively regulates the Txn antioxidant system potentiating the toxicity of cytosolic ROS [332,333]. Evidence of this phenomenon comes from a study demonstrating that mouse islets and β-cell lines with mTORC1 inactivation display mitochondrial dysfunction and oxidative stress that is associated with an increase in TXNIP [334]. TXNIP can cause induction of apoptosis under oxidative stress by inducing the NLR family pyrin domain containing 3 (NLRP3) inflammasome which causes induction of cell death through the formation of micropores in the plasma membrane [335-337]. Interestingly, TXNIP has been found to be upregulated in pancreatic sections from donor patients with T2D, suggesting that activation of this pathway may possibly be a major contributor of β-cell dysfunction in diabetes [333]. Finally, because of the role of mTOR in modulating β-cell proliferation, autophagy, apoptosis and insulin secretion, β-cell-specific loss of mTORC1 causes diabetes and β-cell failure in mice [338].

Finally, another pathway activated by oxidative stress is the JNK pathway. JNK is a mitogen-activated protein kinase (MAPK) that is activated in the presence of extracellular stress. In both humans and rodent β cells, it has been demonstrated that

treatment of β cells with ROS leads to activation of JNK [339-341]. JNK impairs insulin signaling through inactivation of insulin receptor substrate 1/2 (IRS1/2) which subsequently leads to inactivation of the PI3K/AKT pathway [340,342,343]. Inactivation of this pathway leads to reduced activation of mTOR which causes detrimental effects as previously mentioned. Another consequence of inactivation of the PI3K/AKT pathway is nuclear translocation of forkhead box protein O1 (FOXO1) which competes with the Pdx1 promoter, leading to a decrease in Pdx1 which contributes to both β-cell dedifferentiation and impaired insulin secretion [344-348]. Finally, JNK activation may induce apoptosis in human β cells through the p38MAPK, another MAPK activated by oxidative stress and implicated in β-cell apoptosis, and the p53 tumor suppressor protein which acts to induce cell cycle arrest and apoptosis in response to cellular stresses [349,350].

**Current treatments for Type 2 Diabetes**

While there is no cure for T2D, there are many treatments available. While insulin therapy is utilized in some patients with long-standing T2D, a wide range of pharmacological agents exist to treat the disease. These include insulin secretagogues, biguanides, insulin sensitizers, incretin mimetics, and sodium-glucose co-transporter 2 (SGLT2) inhibitors [350]. Some of these pharmacological agents are used either as monotherapy or in combination with others to achieve glycemic control in patients with T2D.

Insulin secretagogues are a category of drugs that include sulfonylureas which are commonly prescribed drugs for the treatment of diabetes. This category of drugs functions to increase insulin secretion by binding to and inhibiting the sulfonylurea

receptor (SUR) component of ATP-sensitive potassium ($K_{ATP}$) channels on β cells [351]. Inhibition of $K_{ATP}$ channels leads to β-cell plasma membrane depolarization, and subsequent insulin secretion. Biguanides such as metformin function to reduce hepatic glucose output by decreasing gluconeogenesis and stimulating glycolysis. Biguanides also increase insulin sensitivity and improve glucose uptake by the muscle [352]. Outside of these actions, biguanides block the breakdown of fatty acids by activating AMPK, which gives it anti-hypertriglyceridemic properties [353]. Insulin sensitizers such as thiazolidinediones are also known as Peroxisome Proliferator Activated Receptor agonists (PPARs). PPARs are a nuclear hormone receptor superfamily of ligand-activated transcription factors [354]. PPARγ plays a central role in glucose homeostasis with activation of this transcription factor increasing insulin sensitivity, decreasing systemic fatty acid uptake and production, slowing gluconeogenesis, and improving glucose uptake by skeletal muscle [355]. Recently, dual activity of PPARα/γ has been discovered to be antidiabetic, with combination of the two producing synergistic actions to maintain insulin sensitivity and lipid metabolism [356].

Incretins are endogenously-produced gut peptide hormones that are secreted from intestinal enteroendocrine cells after a meal is consumed. There are two major incretins: glucose-dependent insulinotropic peptide (GIP) and glucagon-like peptide-1 (GLP-1) [357]. The more studied incretin is GLP-1, and pharmacological agents have been developed to target the receptor by mimicking GLP-1. Binding of GLP-1 to its receptor on β cells results in activation of adenylyl cyclase, increased production of cyclic AMP (cAMP) and subsequent activation of PKA. Activation of PKA results in closure of $K_{ATP}$ channels on the β cell, leading to depolarization of the cell membrane

and eventual insulin secretion [358]. The effect of incretins on insulin secretion is deemed the incretin effect [359]. Incretins are rapidly degraded in the circulation by dipeptidyl peptidase IV (DPP-IV), causing the half-life of GLP-1 to only be about one or two minutes [330]. GLP-1 analogs or agonists are used in the treatment of T2D. GLP-1 analogs are usually injectable, and have been generated to be more resistant to DPP-IV [360-362]. Agonists of the GLP-1 receptor function to increase signaling through the receptor, resulting in insulin secretion, similar to the action of GLP-1 agonists [363,364]. DPP-IV inhibitors are another class of drugs used for treatment of T2D. Inhibition of the protease increases the half-life of GLP-1, leading to increased insulin secretion and lowering of blood glucose [365]. Finally, the more recently developed SGLT2 inhibitors have become commonly prescribed for the treatment of T2D. Glucose is reabsorbed in the proximal convoluted tubule, a part of the renal cortex, by the active sodium glucose co-transporter (SGLT) [366]. Inhibition of SGLT2 prevents reabsorption of glucose. Glucose is then excreted in the urine which allows for better glycemic control [367].

Like most pharmacological agents, there are side effects associated with the aforementioned drugs used for treatment of T2D. Some of these side effects include bloating, gastrointestinal irritation and distress, weight gain, edema and headaches [350]. Both clinical and basic science research is ongoing to develop new therapeutics for the disease. Since T2D results from a loss of functional β-cell mass, there is therapeutic potential in finding agents that can increase β-cell proliferation and thus cause β-cell mass expansion. As such, there has been discovery of molecules that can induce β-cell proliferation such as dual-specificity tyrosine-related kinases 1A (DYRK1A) inhibitors [368]. DYRKs have four subtypes (1A, 1B, 2, 3 and 4), with DYRK1A being

| Type of Treatment | Mechanism of Action |
|---|---|
| Insulin secretagogues (sulfonylureas) | -Increase insulin secretion<br>-Bind to and inhibit the sulfonylurea receptor (SUR) component of the $K_{ATP}$ channel on β cells, leading to depolarization of the β-cell membrane and insulin secretion |
| Biguanides | -Increase insulin sensitivity<br>-Reduce plasma glucose concentrations by increasing glucose uptake by glucose-sensitive tissues<br>-Decrease gluconeogenesis by the liver |
| Insulin sensitizers (Peroxisome Proliferator Activated Receptor agonists – PPARs) | -Increase insulin sensitivity<br>-Decrease systemic fatty acid uptake and production, slowing gluconeogenesis<br>-Improve glucose uptake by skeletal muscle |
| Incretins and DPP-IV inhibitors | -Incretin analogs and incretin receptor agonists increase insulin secretion<br>-DPP-IV inhibitors decrease degradation of incretins, leading to increased insulin secretion |
| SGLT2 inhibitors | -Reabsorb glucose in the proximal convoluted tubule, a part of the renal cortex, by the active sodium glucose co-transporter (SGLT)<br>-Prevent reabsorption of glucose to decrease blood glucose |

**Table 1-1. Types of treatment for Type 2 Diabetes (T2D).** Sulfonylureas, biguanides, insulin sensitizers, incretins and DPP-IV inhibitors and SGLT2 inhibitors are commonly used therapeutics for the treatment of T2D. The mechanism of each of these treatments is described in this table.

the most extensively studied of the four [369,370]. DYRK1A is ubiquitously expressed and is involved in multiple pathways relevant to cell proliferation [369,371]. Harmine is a DYRK1A inhibitor that has been demonstrated to induce mouse and human β-cell proliferation. However, while increased β-cell mass is the goal, harmine also potently induces α-cell proliferation. Since T2D is also a disease of α-cell dysfunction, an increase in the α-cell population may be detrimental to patients with the disease [371,372-374]. Thus, it is of utmost importance to find proliferative agents that can induce β-cell proliferation without affecting other islet cells.

**Cellular communication network factor 2 (CCN2)**

One proliferative agent that our lab has extensively studied is cellular communication network factor 2 (CCN2). The Gannon lab identified CCN2 as a gene that is downregulated in response to Hnf6/Oc1 overexpression in islets [375]. CCN2, formally known as connective tissue growth factor (CTGF), is the second member of the CCN family of matricellular proteins, of which there are six in total [376]. The *Ccn2* gene consists of five exons that encode the full-length 38-kDa CCN2 protein. Full-length CCN2 is multi-modular with four distinct modules, each capable of interacting with distinct molecular partners. The four modules (in order from N- to C-terminus) have homology with: 1. insulin-like growth factor binding protein-like domain (IGFBP), 2. von Willebrand factor type C repeat (VWC), 3. thrombospondin type 1 repeat (TSP-1), and 4. C-terminal cysteine-knot (CT). There is a flexible hinge-like domain between the two N-terminal domains (IGFBP, VWC) and the two C-terminal domains (TSP-1, CT) [377-379]. Enzymatic cleavage by MMPs at this hinge region creates di-modular N- and C-terminal fragments respectively, each of which can modulate cellular processes on their

own [380]. Although CCN2 has been heavily studied by many groups, no specific receptor for the protein has been identified.

The first domain (IGFBP) has sequence homology to insulin-like growth factor binding protein but interacts with insulin-like growth factor (IGF) at a much lower affinity than the canonical IGF binding proteins [381], and the functional significance of this domain has not been fully elucidated. The VWC domain has similarities to the cysteine repeats in Chordin, a BMP antagonist, and interacts with both TGF-β and BMP ligands [382,383]. CCN2 and TGF-β exist in a positive feedback loop in that TGF-β promotes CCN2 gene expression, while CCN2 enhances TGF-β binding to its receptor leading to increased TGF-β signaling. The VWC domain also inhibits BMP signaling by suppressing BMP interaction with its receptors. The third module (TSP-1) allows for ECM interactions through integrins. It also acts as an extracellular VEGF sink and binds to and modulates the function of VEGF [384,385]. Finally, the CT domain contains a cysteine knot motif which binds the Wnt co-receptor LRP5/6, subsequently preventing Wnt ligand binding and inhibiting Wnt signaling. The CT domain mediates most of CCN2 interactions with ECM components such as integrins, heparan sulfate proteoglycans, laminin, and fibronectin [379,386,387].

Because of the variety of molecules that CCN2 can interact with and signal through, it is no surprise that CCN2 is involved with various cellular processes such as adhesion, angiogenesis, migration and proliferation depending on the cellular microenvironment. Many studies have been conducted to uncover how genetic modulation of *Ccn2 in vivo* affects overall organism homeostasis. The Lyons group generated mice with a global *Ccn2* null allele to characterize the role of CCN2 *in vivo*.

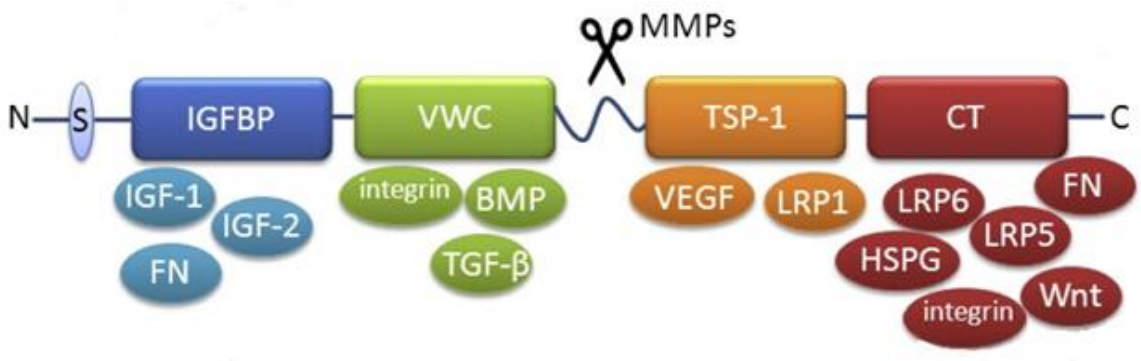
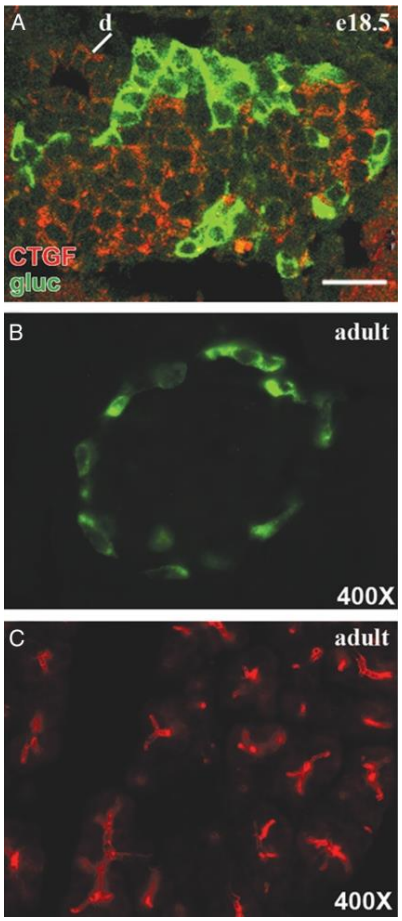



**Figure 1-9. Structure of CCN2.** CCN2 is a 38 kDa protein composed of four distinct domains, and it is readily cleaved by MMPs at the hinge region between the first two and last two modules to create an N- and C-terminal fragment, respectively. Each module has the capability of interacting with a variety of signaling molecules and receptors, and that governs the ability of CCN2 to be implicated in multiple biological processes including adhesion, migration, proliferation and angiogenesis. Adapted from Klaassen I., van Geest R. J., Kuiper E. J., van Noorden C. J. F., and Schlingemann R. O. (2015). The role of CTGF in diabetic retinopathy. *Exp Eye Res* **133**, 37-48.

*Ccn2* null mice have a skeletal phenotype including deformation of craniofacial bones, kinked ribs, and bent long bones [388]. Another persistent phenotype is observed in the lung, where proper proliferation and differentiation of lung epithelial cells is lost resulting in lung hypoplasia [389]. The combination of these phenotypes leads to respiratory failure and subsequent death of *Ccn2* null mice soon after birth. Due to this, only embryonic studies of global *Ccn2* null mice are possible. These findings demonstrate the importance of CCN2 in the requirement for proper proliferation and differentiation *in vivo.*

Since loss of CCN2 during development is neonatally lethal, conditional *Ccn2* gene inactivation methods have been utilized to analyze its function postnatally. One method is the conditional by inversion (COIN) strategy which has been utilized to elucidate the roles of CCN2 in specific cell types in the adult mouse [390]. The CCN2 COIN allele was generated by inserting a COIN intron (an inverted GFP cDNA cassette with splice donor and acceptor sites allowing for the complete exon 2 to be incorporated into the mature mRNA) into exon 2 of the endogenous *Ccn2* gene, thereby splitting exon 2 into two halves (2a and 2b). The first half-exon encodes the signal peptide, while the second half-exon encodes the first module of the protein, the IGFBP module. The loxP sites are located on either side of the COIN intron in opposite orientation. In the absence of Cre recombinase, normal *Ccn2* mRNA and protein are generated. In the presence of Cre recombinase, the COIN allele undergoes recombination leading to irreversible inversion of the COIN intron, placing the GFP cDNA and its 3' untranslated region in-frame with the Ccn2 gene after exon 2a. The truncated CCN2 protein translated from this mRNA is not functional, thus CCN2 is inactivated in this situation



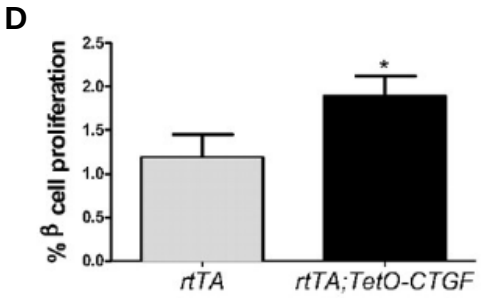




**Figure 1-10. CCN2 has differential expression in embryonic and adult pancreata.** A) CCN2 (red) is expressed in the islets, ductal epithelium and vasculature during embryogenesis. B) CCN2 is not localized to the endocrine compartment of the pancreas during adulthood. C) CCN2 expression is maintained in the ductal network in the adult pancreas. D) Overexpression of CCN2 during embryogenesis using the RIP-rtTA;TetO-CTGF system results in a significant increase in β-cell proliferation. A-C: Adapted from Crawford et al. (2009). *Mol Endocrinol* 23(3):324-336. D: Adapted from Guney et al. (2011). *PNAS* 108(37):15242-15247.

[390].

*The role of CCN2 in the embryonic pancreas*

*Ccn2* was found to be downregulated in a transgenic mouse model of islet dysmorphogenesis and diabetes in which the Hnf6/Oc1 transcription factor was overexpressed in pancreatic endocrine cells during pancreas development [375]**.** This finding suggested that CCN2 plays a role in pancreas development. Immunolabeling for CCN2 proves difficult; thus, using a lacZ knock-in *Ccn2* allele, it was observed that CCN2 is expressed in insulin-positive cells, blood vessels, and ducts as early as e12.5 during pancreas development. However, by P3, CCN2 expression is restricted to ducts and blood vessels, with this expression pattern remaining the same through adulthood [391]. Global inactivation of *Ccn2* results in perturbed endocrine cell allocation, with an increase in α-cell differentiation at the expense of β-cell number by the beginning of the secondary transition (e12.5). Furthermore, embryos with global inactivation of *Ccn2* displayed altered islet morphogenesis evidenced by the intermingling of α and β cells in the islet core, which is normally restricted to β cells in mice. At e18.5, there was significant impairment in β-cell proliferation, but this did not result in decreased overall endocrine area or impaired β-cell function (under normal basal conditions) due in part to compensatory β-cell hypertrophy [391]. Loss of CCN2 had no effect on α-cell proliferation.

Given that CCN2 is expressed in insulin-positive cells, blood vessels and ducts during pancreas development, it was initially unclear which cellular source of CCN2 acted to promote proper β-cell development. Using tissue-specific models of *Ccn2* inactivation, it was found that loss of CCN2 from any one cell source in which it is

expressed in the embryonic pancreas results in significantly decreased β-cell proliferation. These findings illustrate that CCN2 works in both an autocrine and paracrine manner to induce β-cell proliferation during development [392]. Using a tissue-specific overexpression system, the inducible transgenic Tet-On system, it was found that overexpression of CCN2 in developing β cells led to an overall increase of endocrine mass by 25 percent. This increase was due to increased proliferation of both α and β cells [392]. The conclusions of these embryonic studies showed that CCN2 plays a critical role in β-cell proliferation and β-cell mass expansion during embryogenesis.

*The role of CCN2 in the adult endocrine pancreas*

As mentioned previously, CCN2 is expressed in the insulin-positive cells, vasculature and ductal epithelium during development. By P7 however, CCN2 expression is restricted to the vascular endothelium and ductal network [391] [Figure 1-10]. While overexpression of CCN2 during embryogenesis results in an increase in overall endocrine area due to significant increases in both α- and β-cell proliferation, overexpression of CCN2 in adult β cells under basal conditions does not affect β-cell proliferation [87]. Further studies in the lab have tried to elucidate the role, if any, of CCN2 during adulthood. One study conducted in our lab utilized the *Ccn2* lacZ knock-in allele in a model of CCN2 haploinsufficiency during pregnancy. During pregnancy, there is increased metabolic demand for insulin due to increased insulin resistance associated with pregnancy [393]. This increase in metabolic demand typically leads to an overall increase in β-cell mass to provide sufficient insulin to maintain glucose homeostasis. When comparing wild-type pregnant dams to CCN2 haploinsufficient

pregnant dams, there was an impairment in maternal β-cell proliferation in pregnant dams with CCN2 haploinsufficiency [393]. This suggests that CCN2 plays a role in promoting maternal β-cell proliferation.

In another study from the Gannon lab, the effects of CCN2 overexpression on adult β-cell proliferation were examined using a mouse model of 50% β-cell ablation. In this model developed by the Herrera lab, the Rat Insulin Promoter (RIP) drives expression of a diptheria toxin receptor (DTR) transgene specifically in β cells. The DTR transgene was inserted via homologous recombination into the *Hprt* locus on the X chromosome [394]. In males, which have only one X chromosome, all β cells are ablated after diphtheria toxin administration. In females, only 50% of β cells are ablated, since their genome undergoes inactivation of one X chromosome. Importantly, glucose homeostasis remains normal after 50% β-cell ablation and spontaneous β-cell proliferation and regeneration does not occur [133]. The Gannon lab combined this model of 50% β-cell ablation with a bi-transgenic mouse model in which the RIP drove expression of the Reverse Tetracycline Transactivator (rtTA) transcription factor and CCN2 expression is driven by the tetracycline operator (Tet-O). The ability of the rtTA protein to bind the Tet-O DNA sequence is doxycycline (DOX)-dependent. Thus, DOX administration in the drinking water activates rtTA, leading to induction of the TetO-CCN2 transgene only in β cells [133]. After partial β-cell ablation, CCN2 was induced in the remaining β cells via DOX administration in the drinking water for different lengths of time. Two days post-ablation with concurrent CCN2 overexpression, β-cell proliferation was significantly increased compared to control and by four weeks post-ablation with concurrent CCN2 overexpression, β-cell mass had regenerated to approximately 75%

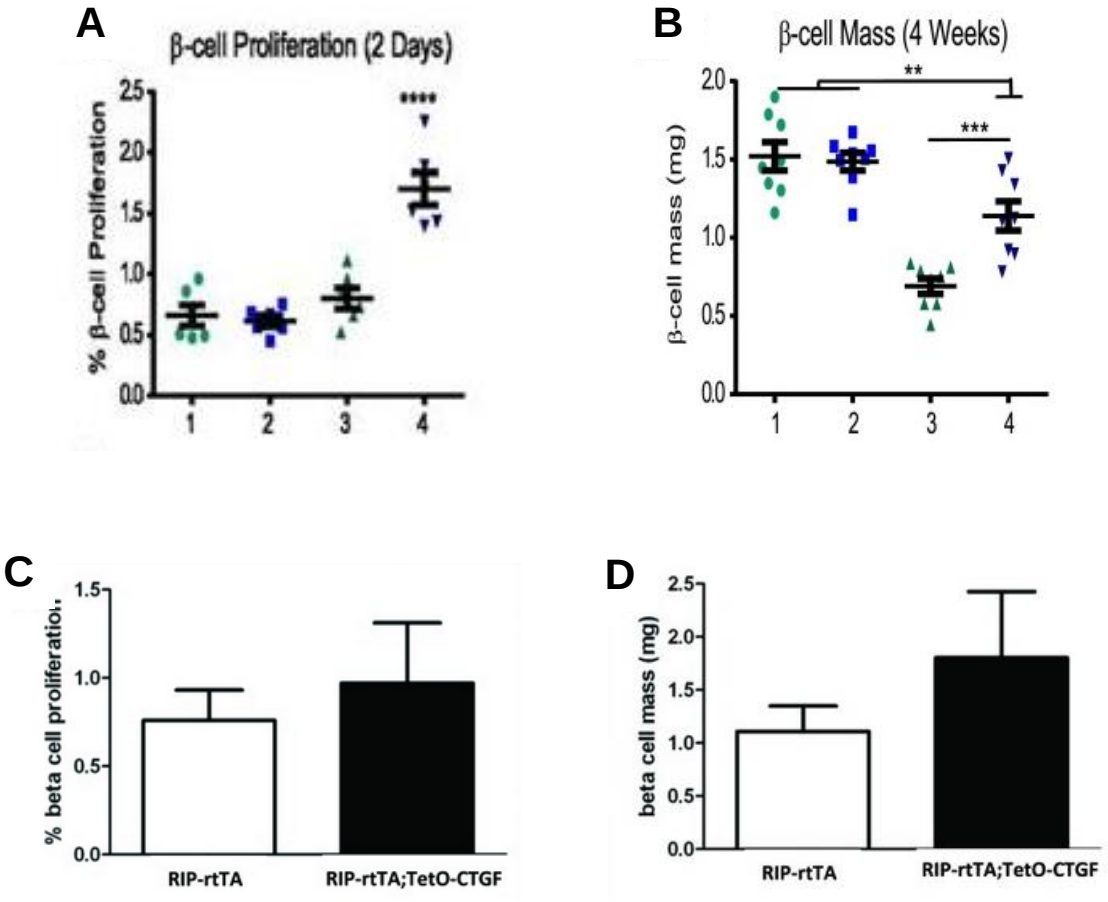



**Figure 1-11. CCN2 induces β-cell proliferation in settings of β-cell stress.** A) β-cell proliferation was significantly increased after two days of β-cell-specific CCN2 induction in the setting of 50% β-cell ablation (Condition 4). B) β-cell-specific CCN2 induction in the setting of 50% ablation for four weeks partially restores β-cell mass (Condition 4). C) β-cell-specific CCN2 induction utilizing the RIP-rtTA;TetO-CTGF model during euglycemic unstressed conditions in adulthood does not alter β-cell proliferation or D) β-cell mass. A and B: Adapted from Riley et al. (2015). *Diabetes* 64(4):1284-1298. C and D: Adapted from Gunasekaran et al. (2012) *Cell Cycle* 11(13):2431-2442.

of control [Figure 1-11A and B]. Gene expression analysis was conducted on islets after two days post-ablation with concurrent CCN2 overexpression and the results demonstrated that there was upregulation of cell cycle markers such as *Ccnd1*, *Ccnd2, Ccnd3* and and *Mki67*. Furthermore, genes in pathways known to promote β-cell proliferation were also upregulated including *Met*, *Hgf* and *Tph1* [133].

While overexpression of CCN2 has no effect on β-cell proliferation under normal, euglycemic conditions during adulthood [87] [Figure 1-11C and D], it is evident that under certain situations, CCN2 can induce β-cell proliferation. Taken together, the Gannon lab concluded that CCN2 can only stimulate β-cell proliferation under situations of suboptimal functional β-cell mass and metabolic stress such as occurs during pregnancy and β-cell injury. Since islet isolation and culture also induce β-cell stress, the lab examined whether exogenous, recombinant human CCN2 (rhCCN2) could induce β-cell proliferation in isolated mouse islets *ex vivo*. Multiple experiments in the lab have now demonstrated that *ex vivo* treatment of wild-type C57BL/6J adult mouse islets with rhCCN2 does stimulate β-cell proliferation [133]. Gene expression analyses conducted on wild-type islets treated with rhCCN2 demonstrated that upregulation of *Ccnd1* occurred after rhCCN2 treatment, demonstrating that CCN2 stimulates β-cell proliferation through upregulation of cyclin D1 [133]. Taking all these studies together, it can be concluded that CCN2 can induce β-cell proliferation under situations of β-cell stress such as pregnancy, β-cell injury, and the stress associated with islet isolation procedures.

**Thesis overview**

Loss of functional β-cell mass is an etiology by which T2D develops. Thus, discovering factors that can stimulate β-cell proliferation and ultimately lead to functional β-cell mass expansion may have therapeutic potential for the disease. The Gannon lab has demonstrated through many studies that CCN2 can stimulate β-cell proliferation in many different settings. For example, induction of CCN2 after partial β-cell ablation induces β-cell regeneration, overexpression of CCN2 during embryogenesis results in an overall increase in endocrine area, treatment of isolated islets *ex vivo* with recombinant human CCN2 results in an increase in β-cell proliferation, and *CCN2* haploinsufficiency attenuates pregnancy-induced β-cell mass expansion [133,392,393]. While the action of CCN2 on β-cell proliferation has been elucidated in these studies, many details are still unknown. First, the signaling pathways activated by CCN2 to affect β-cell proliferation have not been elucidated. Second, induction of CCN2 during euglycemic, unstressed conditions fails to induce β-cell proliferation [87], suggesting that β-cell stress might be necessary for β cells to be responsive to the stimulatory effects of CCN2 and increase β-cell proliferation. Thus, the main hypothesis of this thesis is that β-cell stress is required for CCN2 to be able to induce β-cell proliferation. It was also hypothesized that CCN2 acts to alleviate β-cell stress during situations of acute and chronic cellular stress.

Chapter II of this thesis describes the methods utilized to investigate these hypotheses, and Chapter III details findings regarding signaling mechanisms activated by CCN2. Chapter IV through VII discusses the role of CCN2 in modulating β-cell stress and describes how CCN2 promotes β-cell mass expansion in situations of acute and chronic β-cell stress. Finally, Chapter VIII includes a summary of studies described in

this thesis, the overarching conclusions about CCN2 action as it relates to β-cell stress, and the future direction of these studies. Included appendices detail other potential signaling mechanisms by which CCN2 can stimulate β-cell mass expansion. Overall, the results of this thesis contribute knowledge to the field about the signaling mechanisms activated by CCN2 to induce β-cell proliferation and provide more details about the cellular microenvironment that is required for CCN2 to induce β-cell mass expansion. The knowledge gained furthers understanding of a proliferative factor that could potentially be utilized as a therapeutic for diabetes.

## CHAPTER II: MATERIALS AND METHODS

### Animals

*Generation of mouse models.*

RIP-rtTA;TetO-CTGF: Generation of mice harboring the rat insulin promoter (RIP) driving expression of the reverse tetracycline transactivator protein (RIP-rtTA), and the Tet-On operator upstream of the CCN2 transgene (TetO-CTGF) was described previously [389,392]. RIP-rtTA;TetO-CTGF mice were maintained on a mixed C57BL6/J background. To induce CCN2 expression, mice were administered doxycycline (Sigma-Aldrich D9891; Darmstadt, Germany) in the drinking water at 2 mg/ml concentration with 2% Splenda (referred to as DOX). DOX volume was checked every three days and replaced as needed. Only male mice were used for the HFD studies while islets from both sexes were pooled and used for *ex vivo* assays. Mice were housed in a 12-hour light/dark cycle with *ad libitum* access to food (11% kcal from fat; 5LJ5, Purina, St. Louis, MO) and water.

*db*/+;RIP-rtTA;TetO-CTGF: In-house bred *Lepr*$^{db/+}$ (db/+) on the C57BL/KsJ genetic background (Jax #000697) were bred with RIP-rtTA;TetO-CTGF mice to obtain experimental db/+;RIP-rtTA;TetO-CTGF mice and littermate controls. Mice were housed in a 12-hour light/dark cycle and had *ad libitum* access to food (11% kcal from fat; 5LJ5, Purina, St. Louis, MO) and water. Four- or six-week-old male mice were administered DOX and euthanized two weeks later at six or eight weeks of age.

All mice were housed in the Vanderbilt University Medical Center (Nashville, TN) animal care facility. This facility is accredited by the American Association for

Accreditation of Laboratory Animal Care. All mouse experiments obtained approval by the Vanderbilt University Medical Center Institutional Animal Care and Use Committee.

*PCR and Genotyping*

Genotyping of RIP-rtTA;TetO-CTGF, and db/+;RIP-rtTA;TetO-CTGF adult mice was performed by PCR on DNA isolated from ear punches using the primers listed in Table 3-1.

*Intraperitoneal glucose tolerance tests (IPGTT)*

IPGTTs were performed on RIP-rtTA;TetO-CTGF and monogenic control mice (RIP-rtTA or TetO-CTGF alone) in HFD and control groups at one week, four weeks, seven weeks, and ten weeks after diet was started. IPGTTs were performed on db/+;RIP-rtTA;TetO-CTGF mice and monogenic controls (db/+;RIP-rtTA or db/+;TetO-CTGF) at either six weeks or eight weeks of age. Mice were fasted for 16 hours and given intraperitoneal (IP) injections of filter-sterilized glucose in phosphate buffered saline (PBS) (2 mg dextrose/g body weight). Glucose concentrations were measured via tail vein blood sampling at 0, 15, 30, 60, 90 and 120 minutes post-glucose injection using an Accuchek Aviva glucometer and test strips (Roche Pharmaceuticals).

*High-fat diet studies*.

Male RIP-rtTA;TetO-CTGF and monogenic RIP-rtTA and TetO-CTGF control mice aged eight- to ten-weeks of age were weighed and randomly assigned to one of two groups: 1) control diet (CD - BioServ F4031, Flemington, NJ) and 2) HFD (60% kcal from fat; BioServ F3282, Flemington, NJ). Mice were maintained on a mixed genetic background (C57BL/6J). Mice were administered either CD or HFD for one week or ten

weeks. Mice were housed in a controlled-temperature environment on a 12-hour light/dark cycle with *ad libitum* access to food and DOX. Euthanasia was performed at time of pancreatic dissection using isoflurane until mice were unresponsive, followed by cervical dislocation.

*Islet isolations.*

Mouse islets were isolated from male and female mice by the Islet and Pancreas Analysis Core. Briefly, after euthanasia, pancreata were perfused with 0.5 mg/mL type IV collagenase that was dissolved in Hanks Balanced Salt Solution as described previously [395]. Wild-type C57BL/6J islets were handpicked and cultured overnight in 11 mM glucose RPMI-1640 with 10% horse serum and 1% penicillin/streptomycin (P/S) in preparation for *ex vivo* assays and RNA isolation. Islets isolated from RIP-rtTA;TetO-CTGF mice and monogenic controls were cultured in 11 mM glucose RPMI-1640 with 10% horse serum, 1% penicillin/streptomycin (P/S) and DOX (to maintain CCN2 expression) in preparation for *ex vivo* assays and RNA isolation.

*Ex vivo islet proliferation assays.*

Forty isolated mouse islets per replicate were treated for four days with recombinant human N-terminal CCN2, recombinant human C-terminal CCN2, or a combination of both proteins diluted in culture media (11 mM glucose RPMI-1640 with 10% horse serum and 1% P/S) to varying concentrations ranging from 100 ng/mL to 500 ng/mL. PBS was utilized as a vehicle control. Islets were treated in the presence of 0.1 mM EGTA, a compound that mildly loosens cell-cell contacts and allows better permeability for compounds to reach the core of the islet. After the treatment period,

islets were dispersed with 0.025% trypsin in PBS and cytospun onto charged slides for immunolabeling. Dispersed cells were immunolabeled with guinea pig anti-insulin (Dako #10564, Carpinteria, CA), and rabbit anti-Ki67 (1:400; Abcam). Primary antibodies were detected with the appropriate species-specific secondary antibodies (Jackson ImmunoResearch Laboratories, West Grove, PA): Cy2-conjugated anti-guinea pig IgG (1:400; #GP 706-225-148), Cy3-conjugated anti-rabbit IgG (1:300; #Rb 711-165-152), Cy3-conjugated anti-mouse IgG (1:400; #Ms 715-165-150), and Cy3-conjugated anti-goat IgG (1:400; #705-165-003). Nuclei were visualized with 4'6'-diamidino-2-phenylindole (DAPI, 1 µg/mL; Molecular Probes). A ScanScope FL slide scanner (Aperio Technologies, Inc.) was utilized to obtain images. β-cell proliferation was determined by quantifying the number of insulin-Ki67 double-positive cells using a macro generated with the CytoNuclearFL algorithm in eSlide Manager (Aperio Technologies, Inc.). Data are represented as fold change in proliferation compared to vehicle groups.

*Ex vivo islet stress assays.*

Isolated islets from RIP-rtTA;TetO-CTGF and monogenic control mice were treated for 24h with 10 nM thapsigargin (Sigma-Aldrich T9003, Darmstadt, Germany) diluted in PBS. After the 24h treatment period, islets were harvested, washed in cold PBS and prepared for RNA extraction in 1 mL Trizol reagent (ThermoFisher, Waltham, MA) and stored at -80 $^{o}$C prior to RNA isolation.

*Islet gene expression analysis.*

RNA isolation was performed via phenol-chloroform extraction. RNA integrity and concentration were assessed with a ND-1000 spectrophotometer (NanoDrop). A total of ≥50 ng cDNA was prepared from mouse islets using the High-Capacity cDNA Reverse Transcription Kit (ThermoFisher #4368814, Waltham, MA). Quantitative RT-PCR (qRT-PCR) was performed as described previously [396]. Data was normalized to *Actb*. Data are represented as $2^{-\Delta\Delta Ct}$ compared to the respective control groups [397].

*Kinexus phospho-protein microarray.*

Islets were isolated from eight-to-ten-week-old male wild-type C57BL/6J, pooled and allowed to recover overnight in culture media (11 mM RPMI-1640 with 10% horse serum and 1% P/S) in a 37C, 5% $CO_2$ incubator. Islets were treated with 250 ng/mL recombinant human CCN2 (rhCCN2) or vehicle PBS for 12h. After 12h, islets were collected in a 1.5 mL centrifuge tube, washed three times in cold PBS, pelleted and stored at -80C until sample preparation for the Kinex™ KAM-900P antibody microarray kit (Kinexus Bioinformatics Corporation). Protein lysate was prepared by adding 50 µL of Kinexus Cell Lysis Buffer (supplied in kit) to each islet pellet. Samples were then sonicated using a microprobe sonicator (Virtis Virsonic) before centrifugation for 30 minutes at 4C at maximum speed to obtain the protein lysate. Protein concentration was determined by Bradford assay using the Protein Assay Dye Reagent Concentrate (Bio-Rad).

Protein labeling and purification with chemical cleavage was performed using the maximum concentration of protein possible. Equal amounts of protein for each experimental group were loaded on the microarrays. Protein labeling, purification with

**Table 2-1 – Genotyping and qRT-PCR primers utilized in studies.**

| Gene | Primer sequences |
|---|---|
| RIP-rtTA - genotyping | FW: CGC TGT GGG GCA TTT TAC TTT AG<br>RV: CAT GTC CAG ATC GAA ATC GTC |
| TetO-CTGF - genotyping | FW: AAG TGA AAG TCG AGC TCG GTA<br>RV: TCC CGG TGT CTT CTA TGG AG |
| db/db - genotyping | FW: AAC CAT AGT TTA GGT TTG TTT C<br>RV: CAA TTC AGT GTA AAC CAT AGT TTA GGT TTG TTT A |
| β-actin – qRT-PCR | FW: ATG ACG ATA TCG CTG CGC TGG T<br>RV: ATA GGA GTC CTT CTG ACC CAT TCC |
| BiP – qRT-PCR | FW: TGC AGC AGG ACA TCA AGT TC<br>RV: TTT CTT CTG GGG CAA ATG TC |
| CHOP – qRT-PCR | FW: CTG CAC CAA GCA TGA ACA GT<br>RV: CTA CCC TCA GTC CCC TCC TC |
| sXBP1 – qRT-PCR | FW: CTG AGT CCG CAG CAG GTG<br>RV: GGC AAC AGT GTC AGA GTC CA |
| tXBP1 – qRT-PCR | FW: TCA AAT GTC CTT CCC CAG AG<br>RV: AAA GGG AGG CTG GTA AGG AA |
| Nrf2 – qRT-PCR | FW: CTG AAC TCC TGG ACG GGA CTA<br>RV: CGG TGG GTC TCC GTA AAT GG |
| Sod1 – qRT-PCR | FW: GCC CGG CGG ATG AAG<br>RV: CCT TTC CAG CAG TCA CAT TGC |
| Txn1 – qRT-PCR | FW: GTC GTG GTG GAC TTC TCT GCT A<br>RV: TTG TCA CAG AGG GAA TGG AGG |
| Eif2s1 (Eif2a) – qRT-PCR | FW: GCC TTT CTT GAA CTC TCA CC<br>RV: CCG TGC TTT CTG TGA AGT GT |
| Atf6 – qRT-PCR | FW: GCG GAT GAT AAA GAA CCG AGA G<br>RV: ACA GAC AGC TCT TCG CTT TG |
| Gpx2 – qRT-PCR | FW: GAG GAA CAA CTA CCC GGG ACT A<br>RV: ACC CCC AGG TCG GAC ATA CT |
| Hmox1 – qRT-PCR | FW: GAA CCC AGT CTA TGC CCC AC<br>RV: GGC GTG CAA GGG ATG ATT TC |
| Nqo1 – qRT-PCR | FW: AGG ATG GGA GGT ACT CGA ATC<br>RV: TGC TAG AGA TGA CTC GGA AGG |
| Ccn2 – qRT-PCR | FW: TTC TGC GAT TTC GGC TCC<br>RV: ACC ATC TTT GGC AGT GCA CA |
| Pdx1 – qRT-PCR | FW: TGT TCG CGG GCA CCT AA<br>RV: CCA TCA TTG GCC TGG AGG |
| Ins1 – qRT-PCR | FW: CCC TGC TTG CCC TCT GG<br>RV: TGC TGT TTG ACA AAA GCC TGG |
| Glut2 – qRT-PCR | FW: ACG GAT GCC AAT TAC CGA CA<br>RV: TGC TGG GCC ATG TGC A |
| Nkx6.1 – qRT-PCR | FW: AGA AAT TGT GGC GGG AAG G<br>RV: TTC TCC TCA TCA GCG CAC C |

| Gene | Primer sequences |
|---|---|
| Bad – qRT-PCR | FW: GAA GGG CTG GAG GAC TTA TCA<br>RV: ATA CTC TGG GCT GCT GGT CTC |
| Bax – qRT-PCR | FW: CAA GAA GCT GAG CGA GTG TCT<br>RV: GCA AAG TAG AAG AGG GCA ACC |
| Bcl-xL – qRT-PCR | FW: TGA CCA CCT AGA GCC TTG GA<br>RV: TGT TCC CGT AGA GAT CCA CAA |
| Casp3 – qRT-PCR | FW: GAC TGA TGA GGA GAT GGC TTG<br>RV: CTT CCT GTT AAC GCG AGT GAG |

chemical cleavage, and microarray incubation were performed as detailed by the supplied protocol. Microarrays were stored at 4C in the dark until shipped to Kinexus Bioinformatics Corporation for analysis. KiNetscape maps were generated by Kinexus Bioinformatics Corporation from the leads identified in the microarray analysis. To qualify as a lead, experimental conditions had to produce percent changes from control (%CFC) values that were at least 1000 counts and at least 45% higher or lower with fluorescent signals. Kinase-substrate relationships identified with these leads were used for pathway mapping with the Cytoscape 3.4.0 program (The Cytoscape Consortium).

*Assessment of islet function by perifusion*

Islets were isolated from RIP-rtTA;TetO-CTGF and monogenic control islets. The function of RIP-rtTA;TetO-CTGF and control islets was studied in a dynamic cell perifusion system at a perifusate flow rate of 1 mL/min as described previously [398,399] using approximately 50 IEQs/chamber. Secretagogues utilized in this study were 5.6 mM glucose, 16.7 mM glucose, 16.7 mM glucose with 100 μM IBMX, and 5.6 mM glucose with 20 mM KCl. The effluent was collected at 3-minute intervals using an automatic fraction collector. Insulin in each perifusion fraction and islet extracts were measured by radioimmunoassay (insulin, RI-13K; MilliporeSigma).

*Immunolabeling.*

Pancreata were dissected, fixed, and processed as described previously [400]. Primary antibodies used were: guinea pig anti-insulin (Dako #10564, Carpinteria, CA), mouse anti-glucagon (details), rabbit anti-Ki67 (1:400; Abcam #ab15580, Cambridge, MA), mouse anti-8-hydroxy-3-deoxyguanosine (8-OhDG) (1:100; Abcam #ab62623,

Cambridge, MA), rabbit anti-Grp78 (also known as BiP) (1:500; Abcam #ab21685, Cambridge, MA), goat anti-Pdx1 (Beta Cell Biology Consortium Cat#AB2027), and rabbit anti-phospho-Nrf2 (1:200; Abcam #ab76026, Cambridge, MA).. Primary antibodies were detected with the appropriate species-specific secondary antibodies (Jackson ImmunoResearch Laboratories, West Grove, PA): Cy2-conjugated anti-guinea pig IgG (1:400; #GP 706-225-148), Cy3-conjugated anti-rabbit IgG (1:400; #Rb 711-165-152) and Cy3-conjugated anti-mouse IgG (1:400; #Ms 715-165-150). Nuclei were visualized using 4',6'-diamidino-2-phenylindole (DAPI, 1 ug/mL; Molecular Probes D1306, Eugene, OR). All sections immunolabeled were subject to sodium citrate (pH6) antigen retrieval. For Insulin/8-OHdG, Insulin/Pdx1 and Insulin/phospho-Nrf2, the antibodies used were diluted in 0.2% Triton X-100 in PBS with 5% normal donkey serum and 1% BSA. For all other immunolabeling, antibodies used were diluted in 0.2% Triton X-100 in PBS with 5% normal donkey serum. A ScanScope FL scanner (Aperio Technologies, Inc.) was used for imaging Insulin/Ki67, Insulin/BiP, and Insulin/Pdx1 immunolabeling and Stochastic Optical Reconstruction Microscopy (STORM; Nikon) was used for imaging Insulin/8-OHdG immunolabeling. Images were quantified using ImageScope and ImageJ software (Aperio Technologies, Inc.;).

*β-cell mass.*

At least five slides per animal spaced at least 250 μm apart (spanning 1 to 2% of the entire pancreas) were immunolabeled for insulin and visualized using a DAB Peroxidase Substrate Kit (Vector Laboratories, Inc), then counterstained with eosin. Quantification of β-cell mass was performed as described in Golson et al. [401].

*β-cell proliferation.*

Three slides (at least 250 μm apart) per animal were immunolabeled for insulin and Ki67. A minimum of 3000 cells were counted using ImageScope software (Aperio Technologies, Inc). β-cell proliferation was determined by quantifying the number of dual-positive insulin/Ki67 cells using a macro generated with the CytoNuclear FL algorithm in eSlide Manager (Aperio Technologies, Inc.).

*β-cell size.*

Three slides (at least 250 μm apart) per animal were immunolabeled for insulin. Insulin-positive area was determined using a macro generated with the CytoNuclear FL algorithm in eSlide Manager (Aperio Technologies, Inc). β-cell size was determined by dividing the insulin-positive area by the number of nuclei. A minimum of 1000 β cells per animal were counted to calculate β-cell size.

*Assessing oxidative and endoplasmic reticulum stress.*

To quantify β-cell 8-OHdG, three slides (at least 250 μm apart) per animal were immunolabeled for 8-OHdG and insulin and imaged by STORM (Nikon). A macro generated using NIS-Elements Imaging Software (Nikon) was utilized to quantify the percentage of β cells with nuclear localization of 8-OHdG. To quantify BiP levels, three slides (at least 250 μm apart) per animal were immunolabeled for BiP and insulin, and imaged by a ScanScope FL scanner (Aperio Technologies, Inc.). Individual islets from each section were imaged using ImageScope software (Aperio Technologies, Inc.), and mean grey value was calculated using ImageJ software [402]. At least 20 islets per

animal were analyzed. To examine nuclear localization of phospho-Nrf2, three slides (at least 250 μm apart) per animal were immunolabeled for phospho-Nrf2 and insulin and imaged by STORM (Nikon). A macro generated using NIS-Elements Imaging Software (Nikon) was utilized to quantify the percentage of β cells with nuclear localization of phospho-Nrf2.

*Statistics.*

All data were plotted as mean ± SEM. Student's *t* test, one-way ANOVA, and two-way ANOVA were used to analyze data. The Tukey post-hoc analysis was performed where appropriate. Statistical significance was set at $p<0.05$ (denoted by one symbol), $p<0.01$ (denoted by two symbols), $p<0.001$ (denoted by three symbols), and $p<0.0001$ (denoted by four symbols). Statistical analysis was conducted using Prism 8.0 and 9.5.0 (GraphPad software).

## CHAPTER III: Full-length recombinant human CCN2 induces β-cell proliferation *ex vivo* and stimulates activating phosphorylation events on RSK1 and PLCγ1.

### Introduction

Previous studies in our laboratory have demonstrated that treatment of wild-type C57BL/6J isolated islets with recombinant human CCN2 (rhCCN2) stimulates β-cell proliferation *ex vivo* [133]. However, it is unknown whether there are specific modules of the protein responsible for the action of CCN2 on β-cell proliferation. The CCN2 protein is 38 kDa and has four distinct domains, and each domain can interact with unique proteins including but not limited to ECM molecules and growth factors. CCN2 is readily cleaved between the first two modules (IGF-BP and VWC domains) and the last two modules (TSP1 and CT domains) to create two ~20 kDa fragments: an N-terminal and C-terminal fragment of the protein, respectively. In the literature, it has been established that the N-terminal portion of the protein interacts with various signaling molecules including VEGF, BMP, and TGF-β, while the C-terminal portion is capable of binding to various extracellular matrix molecules and receptors including fibronectin, integrins, and heparan sulfate proteoglycans (HSPGs) [376-379].

Studies investigating the actions of the N- and C-terminal domains of CCN2 found that, when comparing the actions of the full-length protein and the C terminal domain, the most potent biological activity was observed when only the C terminal was present [380]. This was found through multiple assays: while full-length CCN2 induced AKT activity, C-terminal CCN2 alone led to prolonged AKT activity when compared to the full-length protein. Furthermore, when examining activation of cell migration in Rat2 immortalized fibroblasts, it was discovered that C-terminal CCN2 alone was more

effective compared to the full-length protein in stimulating cell migration. Finally, using the same Rat2 fibroblast cell line, it was demonstrated that C-terminal CCN2 alone induced cell proliferation at a much lower concentration than the full-length protein [380]. These studies suggest that the physiological effects of CCN2 are heavily governed by the action of the C-terminal of the protein.

No studies have been conducted to determine what portion of the protein induces β-cell proliferation. Thus, *ex vivo* assays in wild-type C57BL/6J mouse islets were conducted by treating the islets with rhN- and C-terminal CCN2 both separately and combined. When treating islets with each domain separately, there was no induction of β-cell proliferation. However, when combining the two domains to mirror a full-length version of the protein, there was a significant increase in β-cell proliferation when comparing both vehicle and treatment with each individual domain to the treatment with the combination of both domains. This suggests that, contrary to previously published studies, both the N-terminal and C-terminal of the protein are required for CCN2 to stimulate β-cell proliferation.

Currently, there is no consensus on which signaling mechanisms are activated by CCN2 to induce β-cell proliferation. Previous studies in the laboratory have demonstrated that CCN2 stimulates β-cell mass regeneration after partial β-cell ablation [133]. Gene expression analysis following two days of CCN2 overexpression in the setting of β-cell ablation revealed increased expression of various β-cell mitogens, including *Met* and its ligand *Hgf*, and *Tph1* [133]. However, outside of this study, the signaling mechanisms by which CCN2 stimulates β-cell proliferation are unknown. Thus, a Kinexus phosphoarray was utilized to probe the pathways activated after 12

hours of rhCCN2 treatment in isolated wild-type C57BL/6J islets *ex vivo*. The Kinexus phosphoarray is an assay used to analyze changes in levels and phosphorylation of various proteins and utilizes ~900 antibodies (a combination of pan-specific and phospho-site specific) to examine potential changes. The results of this phosphoarray demonstrated that rhCCN2 treatment for 12h in isolated islets *ex vivo* caused increased RSK1 total protein expression. Furthermore, rhCCN2 treatment led to increased phosphorylation at activating residues on RSK1 and PLCγ1, implicating these two signaling molecules in CCN2-mediated β-cell proliferation.

**Results**

Islets were isolated from wild-type C57BL6/J mice aged eight-to-ten weeks old and treated with rhN-terminal and C-terminal CCN2 separately at concentrations ranging from 100 ng/mL to 500 ng/mL for four days. It was found that treatment with either fragment of the protein alone did not induce β-cell proliferation at any concentration assessed [Figure 3-1A and B]. In further experiments, islets were treated with a combination of both rhN-terminal and C-terminal CCN2 at a concentration of 250 ng/mL for four days. This concentration was chosen because previous studies in the lab examining the effect of rhCCN2 on β-cell proliferation found that β-cell proliferation was significantly increased after treatment with 250 ng/mL rhCCN2 [133]. After four days of treatment, islets were harvested, cytospun onto charged slides, and immunolabeled for insulin and Ki67 to examine β-cell proliferation. Significant β-cell proliferation was induced when islets were treated with a combination of rhN- and C-terminal CCN2 at a concentration of 250 ng/mL, suggesting that the full-length protein is required for CCN2-induced β-cell proliferation [Figure 3-1C].

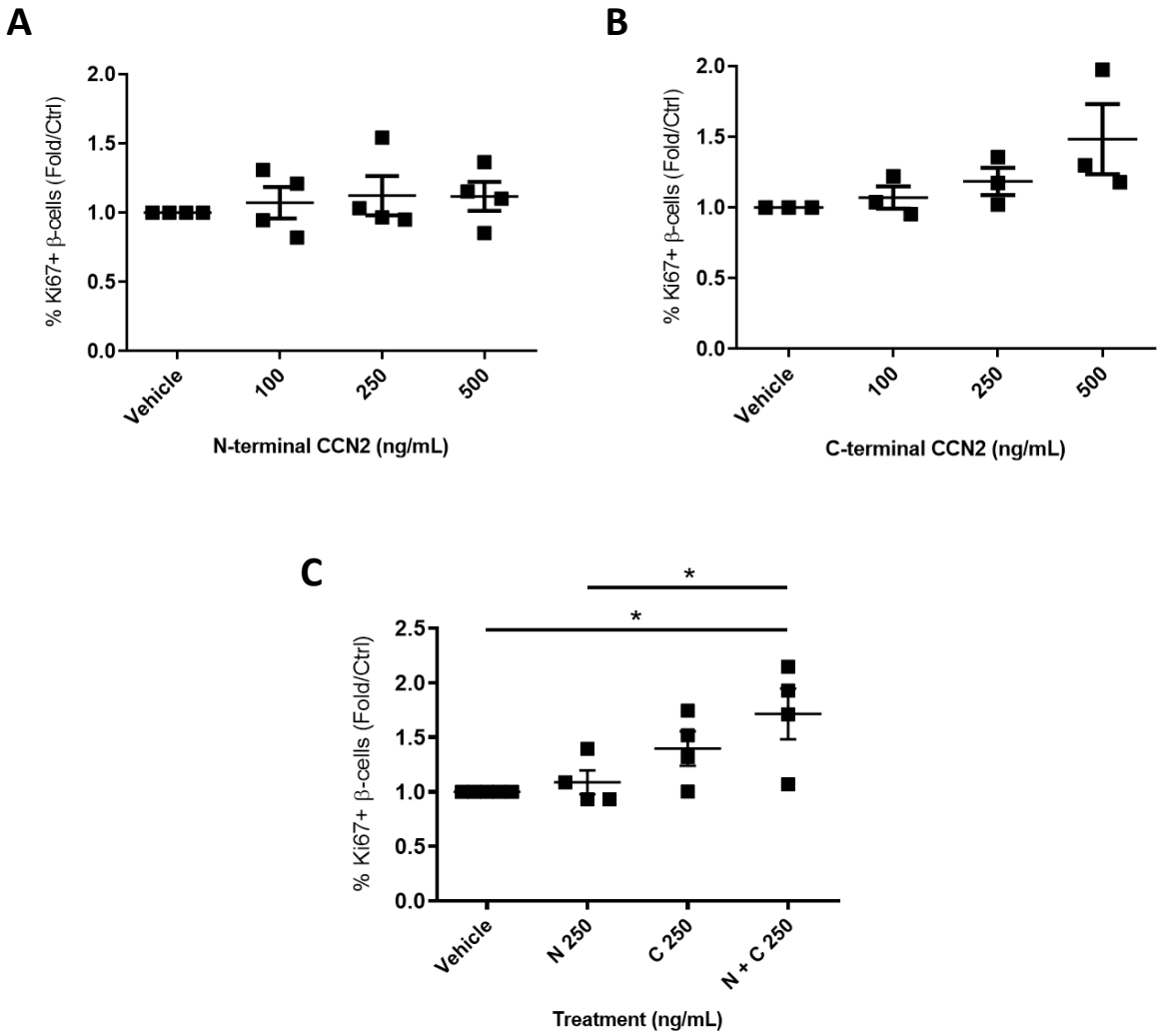



**Figure 3-1. CCN2 stimulates β-cell proliferation when both domains are present.** β-cell proliferation is not induced when islets are treated with **A)** N-terminal and **B)** C-terminal CCN2 alone. **C)** Treatment of islets with a combination of N- and C-terminal CCN2 at 250 ng/mL stimulates β-cell proliferation. *n*=3-4 biological replicates. Statistics: A) One-way ANOVA – F=0.2981, *p*=0.8261. B) One-way ANOVA -- F=2.372, *p*=.1463. C) One-way ANOVA – F=5.364, *p*<0.05. Tukey *post-hoc* analysis: **p*<0.05.

To elucidate the signaling pathways activated by rhCCN2 treatment, isolated wild-type C57BL/6J mouse islets were pooled and treated with either vehicle PBS (control) or rhCCN2 at 250 ng/mL, a concentration that has been established to induce β-cell proliferation [133]. After 12h of treatment, islets were harvested, lysed, and equal amounts of protein lysate for each condition were applied to a Kinexus protein phosphoarray. The level of protein expression and phosphorylation in rhCCN2-treated islets was compared to that of vehicle-treated islets to directly examine the effects of rhCCN2 treatment on these parameters. The results of the phosphoarray demonstrated that rhCCN2 treatment caused an increase in total expression of Ribosomal Protein S6 Kinase A1 (RSK1) and caused an increase in activating phosphorylation events of RSK1 and Phospholipase C Gamma 1 (PLCγ1) [Figure 3-2].

**Discussion**

To date, there have been no studies examining which domain of CCN2 induces β-cell proliferation. While it has been stated in the literature that the C-terminal of CCN2 harbors much of the ability of the protein [380], my study suggests that the N-terminus of CCN2 has biological activity only in the presence of the C-terminus of the protein, at least in pancreatic β cells. Previous studies have suggested that the N-terminal of CCN2 inhibits the activity of the protein [380]. One study assessed the activity of CCN2 and found that full-length CCN2 is an inactive precursor, and cleavage by matrix metalloproteinases activates the protein. In this same study, it was found that the C-terminal domain of CCN2 can form homodimers that are approximately 20-fold more potent than the monomer in activating various phosphokinase cascades [380]. It is

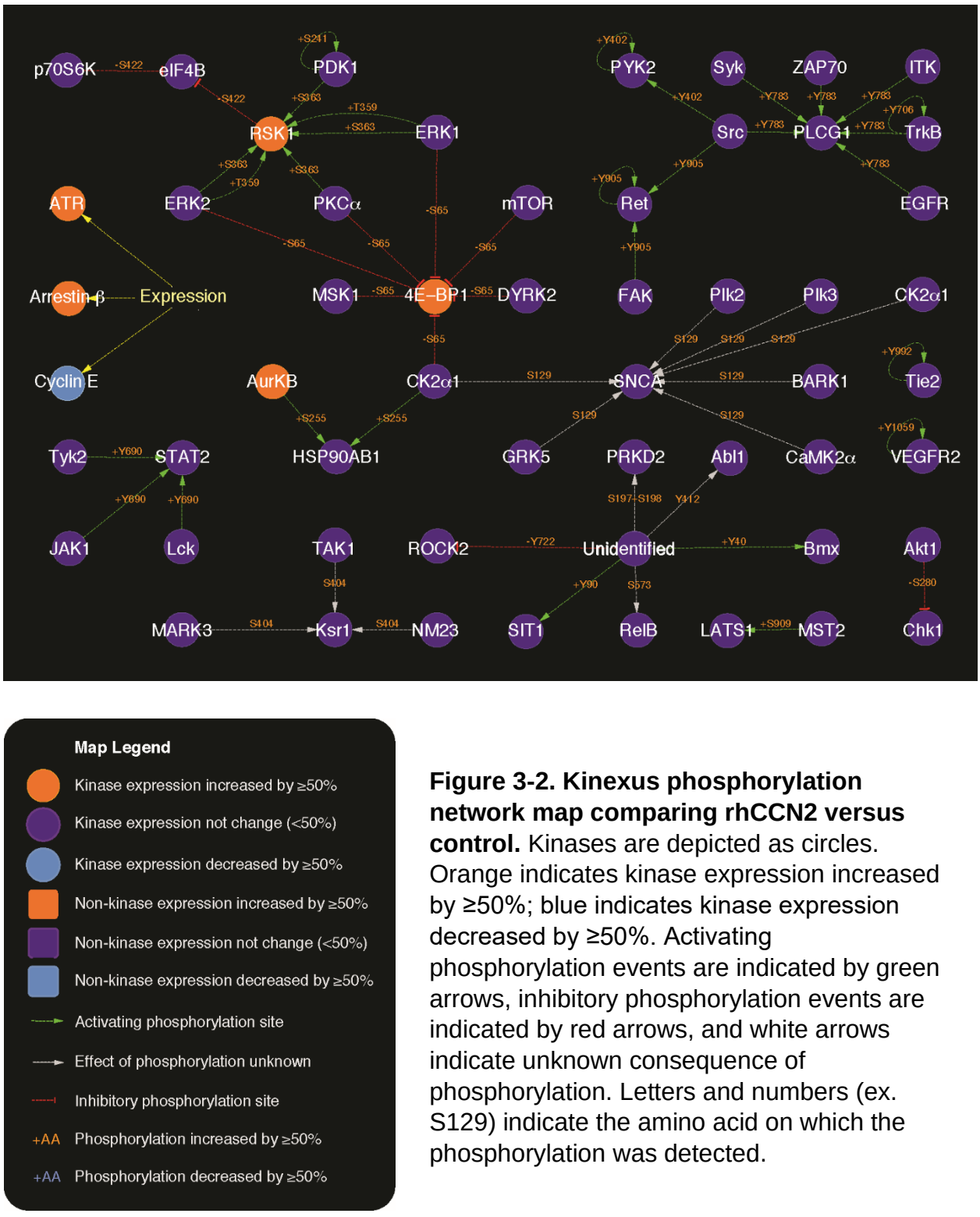



**Figure 3-2. Kinexus phosphorylation network map comparing rhCCN2 versus control.** Kinases are depicted as circles. Orange indicates kinase expression increased by ≥50%; blue indicates kinase expression decreased by ≥50%. Activating phosphorylation events are indicated by green arrows, inhibitory phosphorylation events are indicated by red arrows, and white arrows indicate unknown consequence of phosphorylation. Letters and numbers (ex. S129) indicate the amino acid on which the phosphorylation was detected.

possible that our full-length CCN2 does act as an inactive precursor, and that cleavage into N- and C-terminal fragments activates the protein. However, this finding does not change the result that both the N- and C-terminal of CCN2 is required for CCN2 to induce β-cell proliferation.

Based on the structure of CCN2, studies have demonstrated that the C-terminal domain binds to integrins on the surface of osteoblasts and chondrocytes to induce proliferation [403]. However, the action of N-terminal CCN2 alone has not been explored in terms of its effect on cell proliferation. N-terminal CCN2 interacts with various growth factors such as BMP, TGF-β and FGF [403]. However, studies have established that the interaction of CCN2 with these growth factors inhibits proliferation in multiple cell types. Thus, it is unlikely that the action of N-terminal CCN2 on β-cell proliferation is mediated by interactions with growth factors. While some studies have been published specifying the action of CCN2 based on the domain of the protein, there are not many that have defined a specific role for N-terminal CCN2. Thus, it is difficult to pinpoint the role N-terminal CCN2 plays in promoting β-cell proliferation.

The results of the Kinexus phosphoarray demonstrated that there was an increase in total expression of RSK1, along with an increase in activating phosphorylation events of RSK1 and PLCγ1. RSK1 is a member of the RSK family of proteins and is downstream of the Ras-extracellular signal-related kinase (ERK)/mitogen-activated protein kinase (MAPK) signaling cascade which is canonically involved in cell proliferation and survival [404]. While the mechanism of increase in total expression of RSK1 is unknown, several studies have demonstrated that CCN2 can induce activation of the ERK/MAPK signaling pathway. For instance, in a model of

hypertrophic scarring, it was found that treatment with CCN2 activated the MAPK pathway [405]. In another study, exogenous addition of CCN2 to human keratinocyte cultures resulted in phosphorylation of ERK [406]. Finally, it was demonstrated that CCN2 signaling in lymphatic endothelial cells resulted in phosphorylation of ERK in an integrin-dependent manner, which also has implications for a potential mechanism by which CCN2 stimulates β-cell proliferation [407].

PLCγ1 is a protein that is involved in cell migration, growth, apoptosis, calcium signaling, brain development and proliferation. It is ubiquitously expressed and highly regulated by many factors including AMPK and focal adhesion kinase (FAK) [408]. PLCγ1 is typically activated by signaling through receptor tyrosine kinases (RTKs), such as epidermal growth factor receptor (EGFR), PDGFR, c-Met, and VEGFR2 but it can also be activated by non-receptor tyrosine kinases such as members of the Src family of kinases [409]. In regard to cell proliferation, multiple studies have demonstrated that PLCγ1 is implicated in the stimulation of cell proliferation. Interestingly, studies from our group examining prostaglandin signaling as it relates to β-cell proliferation demonstrated that activity of PLCγ1 was required for stimulation of β-cell proliferation in that experimental model [409].

Taken together, these studies demonstrate that full-length CCN2 is required for stimulation of β-cell proliferation, and that the signaling mechanisms by which CCN2 induces proliferation involve activation of RSK1 and PLCγ1, two molecules that have been elucidated to be involved in cell proliferation. This provides more information into how signaling molecules could be manipulated to induce β-cell mass expansion when CCN2 is present.

## CHAPTER IV: Context-dependent effects of CCN2 on β-cell mass expansion and indicators of cell stress in the setting of acute and chronic stress.

### Introduction

As mentioned in Chapter I, our lab has demonstrated that CCN2 does not induce β-cell proliferation in adult mouse β cells under unstressed, euglycemic conditions [87]. β-cell-specific CCN2 is able to induce β-cell proliferation during embryogenesis, after 50% β-cell ablation, and rhCCN2 can induce β-cell proliferation in islets *ex vivo* [133,392]. Although animals in which 50% of β cells were ablated remained euglycemic, we hypothesize that the remaining β cells must increase production and secretion of insulin, and that this leads to increased β-cell stress. Furthermore, the process of islet isolation imposes stress on islets. Thus, β-cell stress is a common factor in situations where CCN2 induces β-cell proliferation. The mechanism by which CCN2 promotes β-cell proliferation and the general effect of CCN2 on the β cell is unclear – one possibility is that it functions to modulate β-cell stress to induce β-cell proliferation. Thus, the studies detailed in this chapter aim to elucidate whether CCN2 modulates β-cell stress *ex vivo*.

To test the hypothesis that CCN2 modulates β-cell stress, *ex vivo* islet stress assays were conducted. Male and female RIP-rtTA;TetO-CTGF and monogenic control (RIP-rtTA and TetO-CTGF alone) mice received DOX two days prior to islet isolation to induce β-cell-specific expression of CCN2 *in vivo*. Islets were then isolated and pooled from these animals and cultured in media containing DOX to prolong CCN2 expression for the duration of the assays. To induce acute ER stress, isolated adult mouse islets were treated for 24 hours with 10 nM thapsigargin (TG) which acts to deplete ER

calcium, leading to activation of ER stress pathways and the unfolded protein response [410]. ER stress is closely linked to oxidative stress [411]. Thus, qRT-PCR was used to examine expression of both ER (*Grp78, Ddit3* (CHOP)*, sXbp1, tXbp1, Atf6, Eif2s1*) and oxidative (*Nfe2l2* (Nrf2)*, Sod1, Txn1*) [412,413] stress genes. The selection of ER stress markers included genes from the three ER unfolded protein response (UPR) pathways. The oxidative stress genes *Sod1* and *Txn1* were selected based on previous studies demonstrating the importance of these genes in preserving β-cell function and aiding β-cell survival during diabetogenic conditions [414-416]. After treatment, islets were harvested, and qRT-PCR was conducted to examine expression of the ER and oxidative stress markers listed above.

While CCN2 induction during thapsigargin treatment did not attenuate upregulation of some ER stress markers, there was upregulation of the antioxidant transcription factor *Nfe2l2*, suggesting that CCN2 in the presence of cell stress may function to alleviate oxidative stress. Since *Nfe2l2* expression was increased, expression of the Nrf2 target genes *Gpx2, Hmox1,* and *Nqo1* was assessed. However, there was no difference in expression of these target genes across all groups suggesting that while an increase in *Nfe2l2* occurred, it did not translate to direct activation of these genes.

## Results

### Thapsigargin efficiently induces expression of several ER and oxidative stress markers.

Before conducting *ex vivo* assays with islets with CCN2 induction, pilot studies

were conducted to optimize a protocol for TG treatment. In these studies, islets were isolated from wild-type C57BL/6J mice of both sexes aged eight-to-ten weeks old. Islets were treated with TG at varying concentrations (ranging from 10 nM to 0.5 μm) for varying time points (24h to 96h – not shown). After treatment, islets were harvested, RNA isolation was conducted, and cDNA was synthesized. qRT-PCR was utilized to examine the expression of ER and oxidative stress markers. For these studies, the aim was to induce mild cellular stress to avoid inducing apoptosis. It was found that treating islets with 10 nM TG for 24h was sufficient to induce an at least 1.5-fold increase in expression of all stress markers assessed [Figure 4-1A-G]. As a means of assessing the health of islets after treatment with TG, expression of various markers of β-cell identity was analyzed. There was no difference in expression of *Pdx1, Glut2, Ins1* and *Nkx6.1* [Figure 4-1H-K] after treatment with TG compared to islets treated with DMSO vehicle. Thus, the established protocol for future TG *ex vivo* assays was 24h treatment with 10 nM TG.

**β-cell-specific CCN2 induction does not attenuate upregulation of ER stress markers, but induces expression of oxidative stress markers after TG treatment.**

Islets were isolated from RIP-rtTA;TetO-CTGF and monogenic control (RIP-rtTA or TetO-CTGF alone) mice of both sexes aged eight-to-ten weeks old. *CCN2* expression was maintained in culture, with RIP-rtTA;TetO-CTGF islets having a 100-fold increase in expression compared to monogenic control islets. After overnight culture, islets were treated with either DMSO vehicle or 10 nM TG for 24h. After 24h, islets were harvested, RNA was isolated, and cDNA was synthesized. qRT-PCR was utilized to assess expression of previously mentioned ER and oxidative stress markers. It was

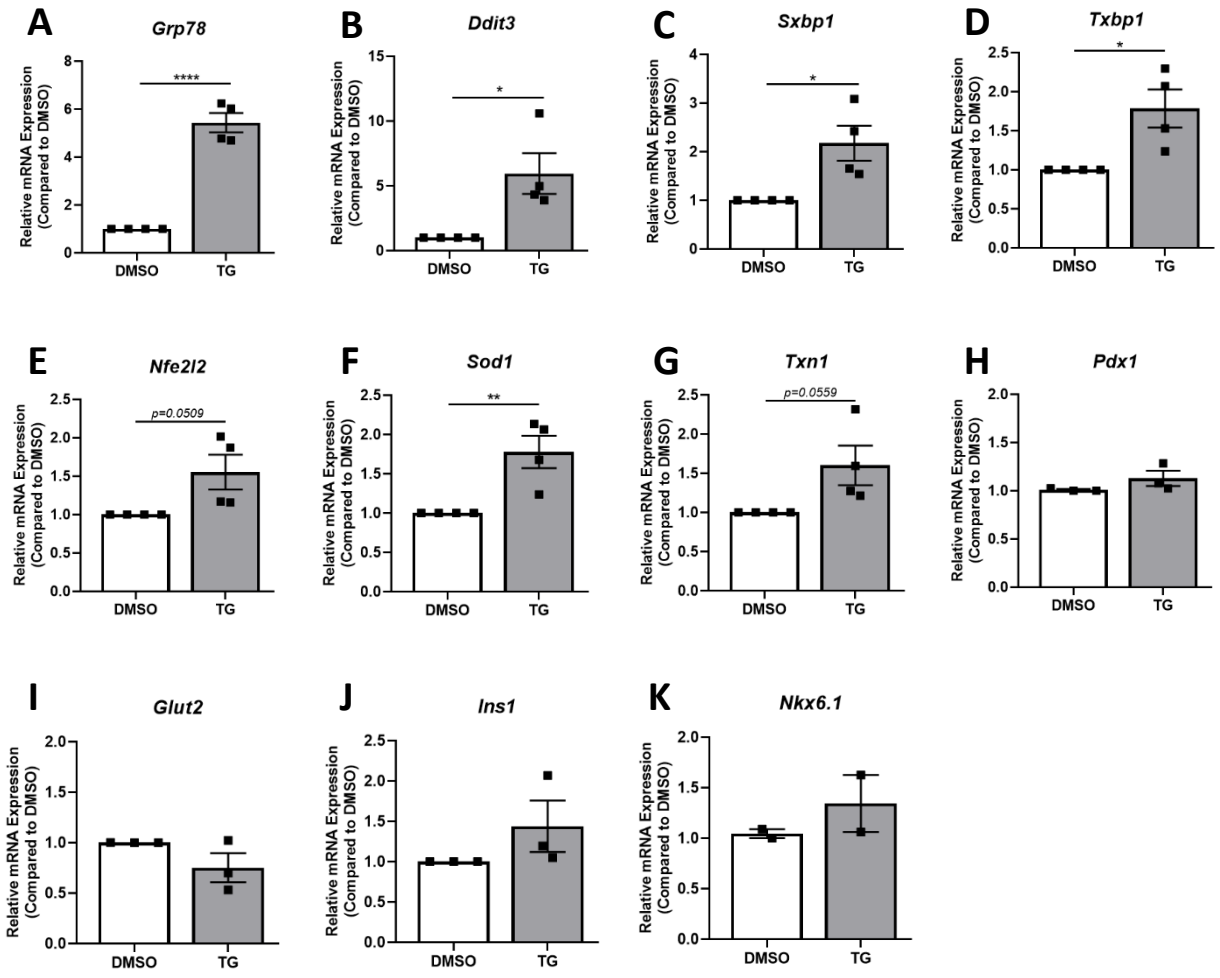



**Figure 4-1. 10 nM thapsigargin (TG) treatment for 24h efficiently induces expression of several ER and oxidative stress markers without affecting expression of key β-cell genes**. **A-G)** Wild-type C57BL/6J mouse islets were treated with 10 nM TG for 24h. 10 nM TG treatment induced significant upregulation of various ER and oxidative stress markers. **H-J)** 10 nM TG treatment for 24h does not affect expression of key β-cell genes. *n*=2-4 biological replicates. All samples were run in duplicate for these assays. Islets from both male and female mice were pooled for these assays. Data were analyzed using two-tailed Student's t-test. **p*<0.05 ***p*<0.01 *****p*<0.0001

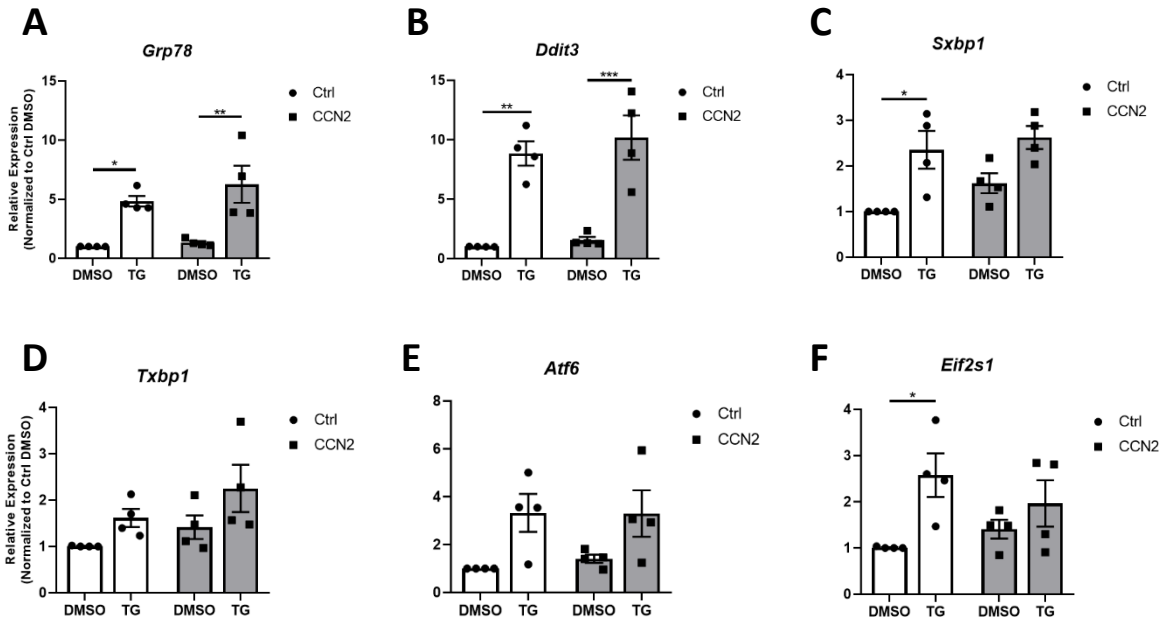



**Figure 4-2**. **CCN2 induction during thapsigargin treatment attenuates upregulation of some ER stress genes. A)** CCN2 induction has no effect on *Grp78* expression during TG treatment. Genotype: F=1.158, *p*=0.3031. Treatment: F=29.03, *p*=.0002. Interaction: F=0.4667, *p*=0.5075. **B)** CCN2 induction has no effect on *Ddit3* expression during TG treatment. Genotype: F=0.7865, *p*=0.3926. Treatment: F=58.64, *p*<0.0001. Interaction: F=0.1325, *p*=0.7222. **C)** CCN2 induction during TG treatment attenuates upregulation of *Sxbp1* following TG treatment. Genotype: F=2.786, *p*=0.1209. Treatment: F=19.54, *p*=0.0008. Interaction: F=0.4289, *p*=0.5249. **D)** Neither TG or CCN2 induction affect expression of *Txbp1*. Genotype: F=3.006, *p*=0.1086. Treatment: F=5.736, *p*=0.0338. Interaction: F=0.1400, *p*=0.7148. **E)** Neither TG or CCN2 induction affect expression of *Atf6.* Genotype: F=0.08973, *p*=0.7696. Treatment: F=11.01, *p*=0.0061. Interaction: F=0.1198, *p*=0.7352. **F)** CCN2 induction during TG treatment attenuates upregulation of *Eif2s1*. Genotype: F=0.08732, *p*=0.7727. Treatment: F=8.712, *p*=0.0121. Interaction: F=1.977, *p*=0.1850. Islets from both male and female mice were pooled for these assays. Data were analyzed using two-way ANOVA and Tukey *post hoc* analysis. **p*<0.05, ***p*<0.01, ****p*<0.001.

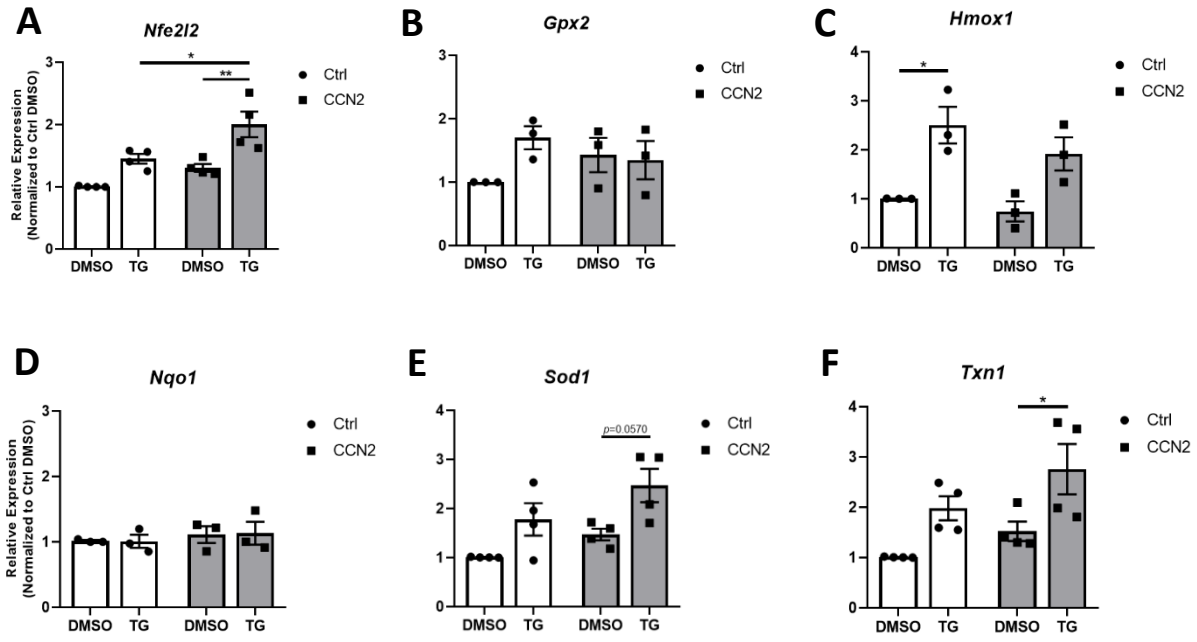



**Figure 4-3**. **CCN2 induction during thapsigargin treatment causes upregulation of antioxidant genes. A)** CCN2 induction in the presence of TG upregulates expression of *Nfe2l2.* Genotype: F=13.85, *p*=0.0029. Treatment: F=25.10, *p*=.0003. Interaction: F=1.225, *p*=0.2900. **B)** CCN2 induction has no effect on *Gpx2* expression during TG treatment. Genotype: F=0.02764, *p*=0.8721. Treatment: F=1.956, *p*=0.1944. Interaction: F=3.139, *p*=0.1144. **C)** CCN2 induction during TG treatment has no effect on *Hmox1* expression. Genotype: F=2.426, *p*=0.1579. Treatment: F=24.2, *p*=0.0012. Interaction: F=0.3620, *p*=0.5640. **D)** Neither TG or CCN2 induction affect expression of *Nqo1*. Genotype: F=0.8314, *p*=0.3885. Treatment: F=0.004366, *p*=0.9489. Interaction: F=0.01104, *p*=0.9189. **E)** There is a trend toward upregulation of *Sod1* expression following CCN2 induction during TG treatment. Genotype: F=5.568, *p*=0.0361. Treatment: F=13.15, *p*=0.0035. Interaction: F=0.2157, *p*=0.6507. **F)** CCN2 induction during TG treatment causes upregulation of *Txn1*. Genotype: F=1.669, *p*=0.0478. Treatment: F=14.20, *p*=0.0027. Interaction: F=0.2072, *p*=0.6571. Islets from both male and female mice were pooled for these assays. Data were analyzed using two-way ANOVA and Tukey *post hoc* analysis. **p*<0.05, ***p*<0.01.

found that CCN2 induction did not attenuate upregulation of the ER stress markers *Grp78* and *Ddit3* [Figure 4-2A and B]. However, CCN2 induction during TG treatment attenuated upregulation of *Sxbp1* and *Eif2s1* [Figure 4-2C and F]. Expression of *Txbp1* and *Atf6* unchanged by TG treatment or CCN2 induction [Figure 4-2D and E]. It was discovered that CCN2 induction during TG treatment resulted in increased expression of the antioxidant transcription factor *Nfe2l2* (Nrf2) [Figure 4-3A]. Since Nrf2 is a master regulator of the antioxidant response, mRNA expression of the Nrf2 target genes *Gpx2, Hmox1,* and *Nqo1* was assessed. There was no difference in expression of all three genes across the four groups [Figure 4-3B-D]. CCN2 induction during TG treatment caused significant upregulation of the antioxidant enzymes *Sod1* and *Txn1* [Figure 4-3E and F]. Despite no change in expression of Nrf2 target genes, the results of this study suggest that CCN2 might function to alleviate oxidative stress in the setting of cell stress.

**Discussion**

In this study, we examined the potential of CCN2 to modulate β-cell stress by assessing expression of several stress markers after TG treatment. After treatment of isolated islets *ex vivo* with 10 nM TG for 24h, it was discovered that CCN2 did not attenuate upregulation of the ER stress markers *Grp78* and *Ddit3* (CHOP). However, CCN2 induction during TG treatment attenuated upregulation of *Sxbp1* and *Eif2s1*, demonstrating that CCN2 is capable of attenuating ER stress. When analyzing gene expression of various oxidative stress markers, it was revealed that CCN2 induction in the presence of TG resulted in upregulation of *Nfe2l2* suggesting that CCN2 may act to relieve oxidative stress in islets. Assessment of the downstream target genes of Nrf2

(*Gpx2, Hmox1,* and *Nqo1*) did not show differences in expression between all groups. However, assessment of expression of the antioxidant enzymes *Sod1* and *Txn1* demonstrated that CCN2 induction in the presence of TG upregulates genes involved in the resolution of oxidative stress.

There are few studies detailing the relationship between ER stress and CCN2 expression and activity. In fact, CCN2 has been demonstrated to be both beneficial and detrimental in situations of ER stress. One study examining neovascularization in tumor growth and progression found that glucose deprivation, an experimental situation that induces ER stress, caused upregulation of *Ccn2*. This suggested that UPR activation induces CCN2 expression [417]. In chondrocytes, CCN2 was demonstrated to be an ER stress-responsive gene and loss of CCN2 led to elevated levels of BiP and CHOP indicative of an increase in ER stress. Conversely, overexpression of CCN2 in chondrocytes decreased expression of BiP suggesting that CCN2 may reduce ER stress [418]. In another study, overexpression of CCN2 via adenoviral infection in hepatocytes led to an increase in ER stress, which was demonstrated by an increase in mRNA and protein expression of sXbp1, p-Eif2α, BiP, CHOP and cleaved caspase-3 [419]. The authors noted that CCN2 overexpression was supraphysiological, so it is possible that overexpression of the protein itself induced ER stress, but that was not further explored.

In this current study, CCN2 did not attenuate upregulation of the ER stress markers examined indicating that in islets, CCN2 does not act to reduce ER stress. Furthermore, CCN2 does not act to increase ER stress in my model as upregulation of stress markers after TG treatment is comparable between islets with CCN2 induction

and control islets. In the previous study utilizing adenoviral-mediated CCN2 overexpression, the level of overexpression was considered detrimental and induced ER stress [419]. In my study, when comparing the control group and group with CCN2 induction in the absence of stressors, there is no difference between expression of the stress markers assessed suggesting that CCN2 induction alone does not promote ER stress in islets. This is interesting because CCN2 induction with the RIP-rtTA;TetO-CTGF system results in a 300-fold increase in CCN2 mRNA when assessed in islets isolated two days after *in vivo* CCN2 induction. CCN2 induction still occurs in isolated islets *ex vivo*, with *CCN2* mRNA expressed 100-fold more in RIP-rtTA;TetO-CTGF islets compared to control. However, it is known that baseline CCN2 expression in adult islets is virtually zero; a 300-fold increase in mRNA may not translate to an equivalent increase in protein, and it is also possible that since baseline expression is so low, a 300-fold increase might still be minimal. Overall, though, it can be said that CCN2 induction does not cause an increase in islet ER stress in my study nor does it attenuate upregulation of ER stress markers after TG treatment.

The biological activity of CCN2 as it relates to oxidative stress has been well-studied in many models of disease. Oxidative stress has been demonstrated to induce CCN2 both *in vitro* and *in vivo*. Treatment of retinal pigment epithelium cells (ARPE19) with the oxidative stress-inducing corticosteroid suspension triamcinolone acetonide (TA) led to upregulation of both CCN2 mRNA and protein [420]. Another study by the same group used hypoxia/reoxygenation and paraquat exposure to induce oxidative stress in ARPE19 cells and found the same result when examining CCN2 mRNA both *in vitro* and *in vivo* [421]. Finally, treatment of human lens epithelial cells with hydrogen

peroxide directly induced CCN2 mRNA [422]. ER stress and oxidative stress are linked. Despite these findings, CCN2 was not induced in my TG treatment model, demonstrating that CCN2 in islets may not be an oxidative stress-responsive gene.

While *Nfe2l2* expression was increased, there was no change in expression of the target genes assessed. Studies have demonstrated that localization of Nrf2 is a more direct readout of Nrf2 activation with activated Nrf2 being localized to the nucleus. However, we did not assess sub-cellular localization in *ex vivo* assays. Furthermore, although the mRNA expression of target genes was unchanged, that does not directly translate to protein expression, so it is possible that Nrf2 activation led to increased protein expression and thus activity of Gpx2, Hmox1 and Nqo1. Altogether, the findings of this study demonstrate that CCN2 does not attenuate ER stress but does upregulate *Nrf2*, a gene associated with the antioxidant response, suggesting that CCN2 can act to mediate oxidative stress.

## CHAPTER V: CCN2 induction does not attenuate chronic β-cell stress *in vivo*.

### Introduction

My studies demonstrated that CCN2 does not attenuate upregulation of stress markers *ex vivo* after acute treatment with the ER stressor thapsigargin (TG). However, I wondered if CCN2 induction in a chronic model of β-cell stress could modulate β-cell stress. To this end, I utilized two mouse models: diet-induced obesity and mice heterozygous for the *Lepr*$^{db}$ mutation (*db/+).* Diet-induced obesity promotes β-cell stress by inducing glucolipotoxicity. In a study examining long-term HFD feeding in wild-type C57BL/6J mice, it was discovered that after 16 weeks of HFD there was significant glucose intolerance despite significantly increased plasma insulin, suggesting that insulin resistance was present [423]. Furthermore, immunohistochemical staining of pancreatic sections demonstrated that there was increased Atf6 and p-eIF2α expression in islets of HFD-fed mice compared to control, suggesting induction of ER stress. *Ex vivo* studies using the INS-1 β-cell line confirmed that treating cells with varying concentrations of palmitic acid did indeed cause ER stress as measured by ATF6 expression, suggesting that lipotoxicity promotes ER stress in the setting of diet-induced obesity [423]. Taken together, these data demonstrate that prolonged HFD is sufficient to promote ER stress in β cells.

Mice homozygous for the *Lepr*$^{db}$ mutation (db/db) are commonly used as a T2D mouse model in the field. db/db mice are hyperphagic, which contributes to increased body mass and eventual overt diabetes. In this model, prolonged glucolipotoxicity induces significant β-cell ER and oxidative stress, which causes eventual β-cell dysfunction and loss due to apoptosis and dedifferentiation. In my studies, I wanted to

utilize *in vivo* models where ER stress is present without significant loss of β-cell mass. Thus, I utilized mice heterozygous for the *Lepr*$^{db}$ mutation (db/+). While the field has regarded db/+ mice as euglycemic and comparable to wild-type mice, studies have not been conducted to characterize and compare the metabolic parameters in db/+ mice to wild-type mice. One focus of our group is to examine the effects of prostaglandin signaling in diabetes progression and we have used the db/db model for these studies. Our group has demonstrated that the islets of db/+ mice exhibit oxidative stress similar to that of db/db mice as measured by the nuclear presence of the oxidative stress marker 8-hydroxy-2'-deoxyguanosine (8-OHdG) [409]. In my study, ER and oxidative stress marker expression was compared between db/+ and wild-type mice. Examination of β-cell-specific nuclear 8-OHdG and BiP expression demonstrated that islets from db/+ mice express more of both markers, making db/+ mice a sufficient model of chronic β-cell stress. With these results known, I chose to utilize db/+ mice as an *in vivo* model of β-cell stress that occurs without hyperglycemia and β-cell dysfunction.

Markers of ER and oxidative stress in the presence of CCN2 induction were analyzed in the diet-induced obesity and db/+ mouse models. Expression of BiP, percent of β cells with nuclear expression of 8-OHdG, and percent of β cells with nuclear expression of phospho-Nrf2 (p-Nrf2) was assessed by immunofluorescence in islets of all groups of mice used in these experiments. BiP is an ER chaperone protein that assists with proper protein folding in the ER and 8-OHdG is a marker of oxidative DNA and RNA damage. Increased expression of BiP and 8-OHdG is associated with increased ER stress and oxidative stress, respectively. Analysis of pancreatic sections in both the diet-induced obesity and db/+ models demonstrated that CCN2 induction

does not alter expression of either BiP or 8-OHdG in the presence of chronic *in vivo* stress. The *ex vivo* stress assays demonstrated that CCN2 induction during TG treatment led to upregulation of *Nfe2l2* – thus, protein localization of p-Nrf2 (the activated form of the protein) was analyzed. No difference in nuclear localization of phospho-Nrf2 was found among all groups. Taken together with the data from *ex vivo* CCN2 induction assays, it appears that CCN2 does not modulate β-cell ER and oxidative stress in the setting of chronic *in vivo* stress.

## Results

### β-cell-specific induction of CCN2 does not alter cell stress markers in *in vivo* models of chronic β-cell stress.

In this study, male RIP-rtTA;TetO-CTGF and control (RIP-rtTA and TetO-CTGF alone) mice were weighed and assigned to one of the following groups: control diet (CD), HFD (HFD Ctrl), or HFD with CCN2 induction (HFD CCN2). All groups had access to their respective diet *ad libitum*. All groups were administered DOX for the duration of the diet to maintain CCN2 expression. Two timepoints were assessed in this model: one week and ten weeks. Previous studies from our group examining the effect of HFD on β-cell mass dynamics demonstrated that increased β-cell proliferation occurs in both the absence (one week) and presence (ten weeks) of insulin resistance [425]. Thus, I chose these two timepoints for my studies. After both one week and ten weeks of HFD, animals were euthanized and pancreata were dissected, fixed, embedded and sectioned. Pancreatic sections were immunolabeled for BiP/Ins, 8-OHdG/Ins, and p-Nrf2/Ins, imaged, and analyzed for BiP levels, percentage of β cells with nuclear 8-OHdG, and percentage of β cells with nuclear phospho-Nrf2. At both one and ten

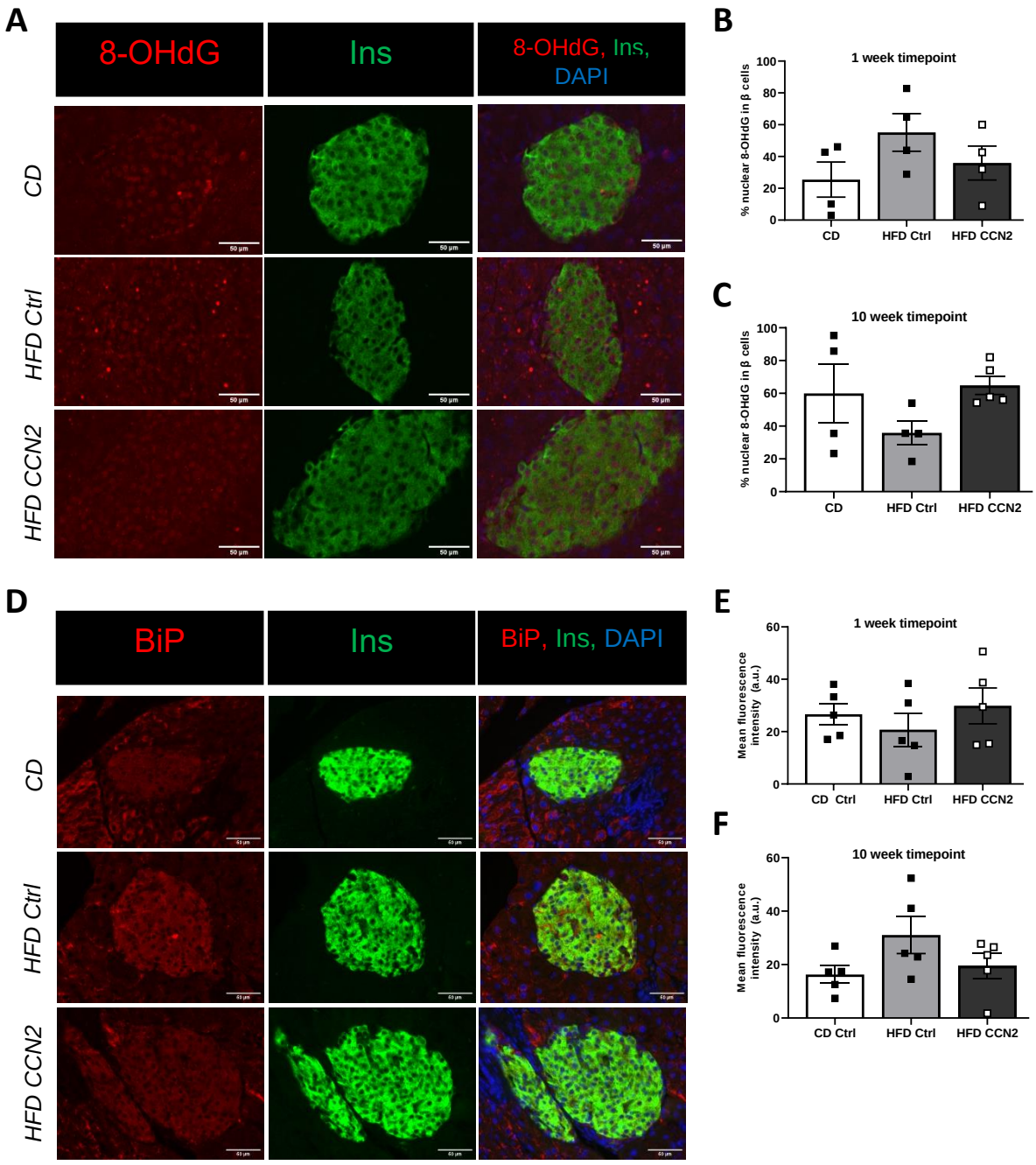



**Figure 5-1. CCN2 induction during HFD does not alter β-cell oxidative or ER stress. A)** Representative images of 8-OHdG immunolabeling in pancreata from male mice after CCN2 induction during one week of HFD. 8-OHdG – red, Ins – green, DAPI – blue. **B)** Quantification of percent of β cells expressing 8-OHdG in the nucleus after CCN2 induction during one week of HFD and **C)** 10 weeks of HFD. **D)** Representative images of BiP immunolabeling in paraffin-embedded pancreata after CCN2 induction during 10 weeks of HFD. BiP – red, Ins – green, DAPI - blue. **E)** BiP quantification in β cells after one week high-fat diet and CCN2 induction and **F)** 10 weeks high-fat diet and CCN2 induction. *n*=4-5 animals per group.

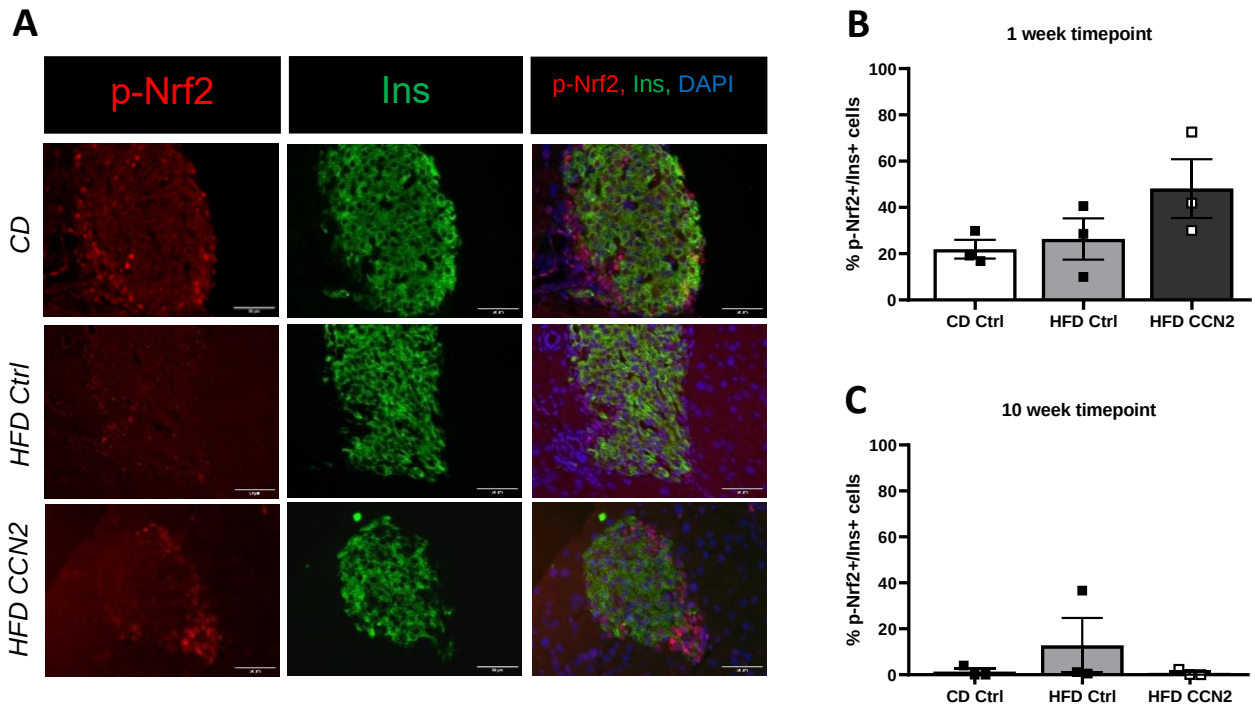



**Figure 5-2. CCN2 induction during HFD does not stimulate nuclear translocation of phospho-Nrf2. A)** Representative images of p-Nrf2 immunolabeling in pancreata from male mice after CCN2 induction during one week of HFD. p-Nrf2 – red, Ins – green, DAPI – blue. **B)** Quantification of percent of β cells expressing p-Nrf2 in the nucleus after CCN2 induction during one week of HFD and **C)** 10 weeks of HFD. *n*=3 animals per group.

weeks, there was no difference in either BiP levels or percentage of β cells with nuclear 8-OHdG between the three groups [Figure 5-1B, C, E, F]. Since *Nfe2l2* was upregulated following TG treatment in *ex vivo* islet TG assays, localization of phospho-Nrf2 was assessed. Increased nuclear expression of p-Nrf2 is indicative of an active form of the transcription factor. At both the one and ten-week timepoints, no difference in phospho-Nrf2 expression was noted [Figure 5-2A - C]. Altogether these findings suggest that CCN2 does not modulate cell stress as measured by BiP, 8-OHdG, and p-Nrf2 in a chronic *in vivo* model of β-cell stress.

For an additional model of chronic β-cell stress, RIP-rtTA;TetO-CTGF and db/+ mice were interbred to generate db/+;RIP-rtTA;TetO-CTGF experimental mice and monogenic db/+;RIP-rtTA and db/+;TetO-CTGF control mice. Male animals were administered DOX for two weeks at two timepoints: four to six weeks of age, and six to eight weeks of age. These timepoints were chosen because previous studies characterizing various metabolic parameters of db/db mice found that β-cell proliferation is significantly increased compared to controls at four to six weeks of age, but after six weeks of age β-cell proliferation wanes [211]. Since one hypothesis of these studies is that CCN2 stimulates β-cell proliferation in part by modulating β-cell stress, expression of stress markers was analyzed after two weeks of DOX administration at six and eight weeks of age. Analysis of expression of BiP and quantification of nuclear 8-OhDG found that CCN2 induction did not alter either parameter at either timepoint, demonstrating that CCN2 induction in this model does not function to reduce β-cell stress [Figure 5-4B, C, E, F].

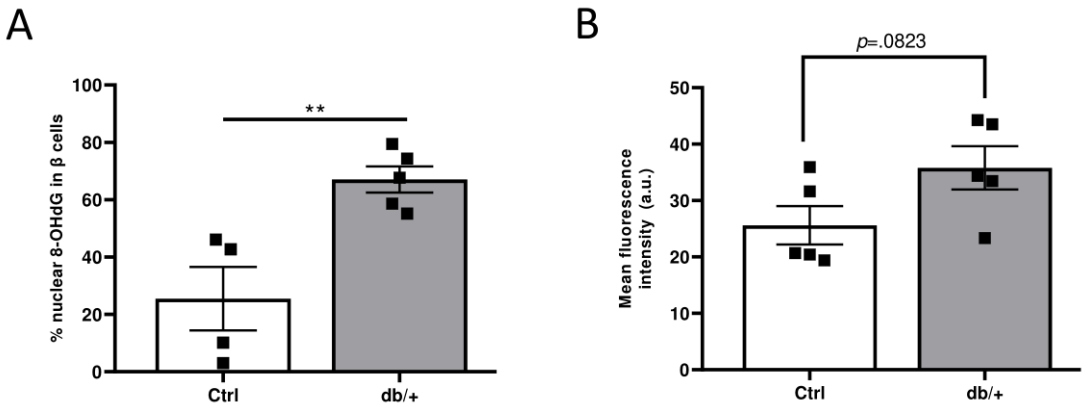



**Figure 5-3**. **db/+ mice exhibit more islet ER and oxidative stress than wild-type controls. A)** Nuclear expression of 8-OHdG in β cells is significantly higher in male db/+ mice compared to controls. **B)** Expression of BiP in β cells is higher in db/+ animals compared to controls. Data were analyzed using a two-tailed Student's *t test*. *n*=4-5 animals per group. ***p*<0.01.

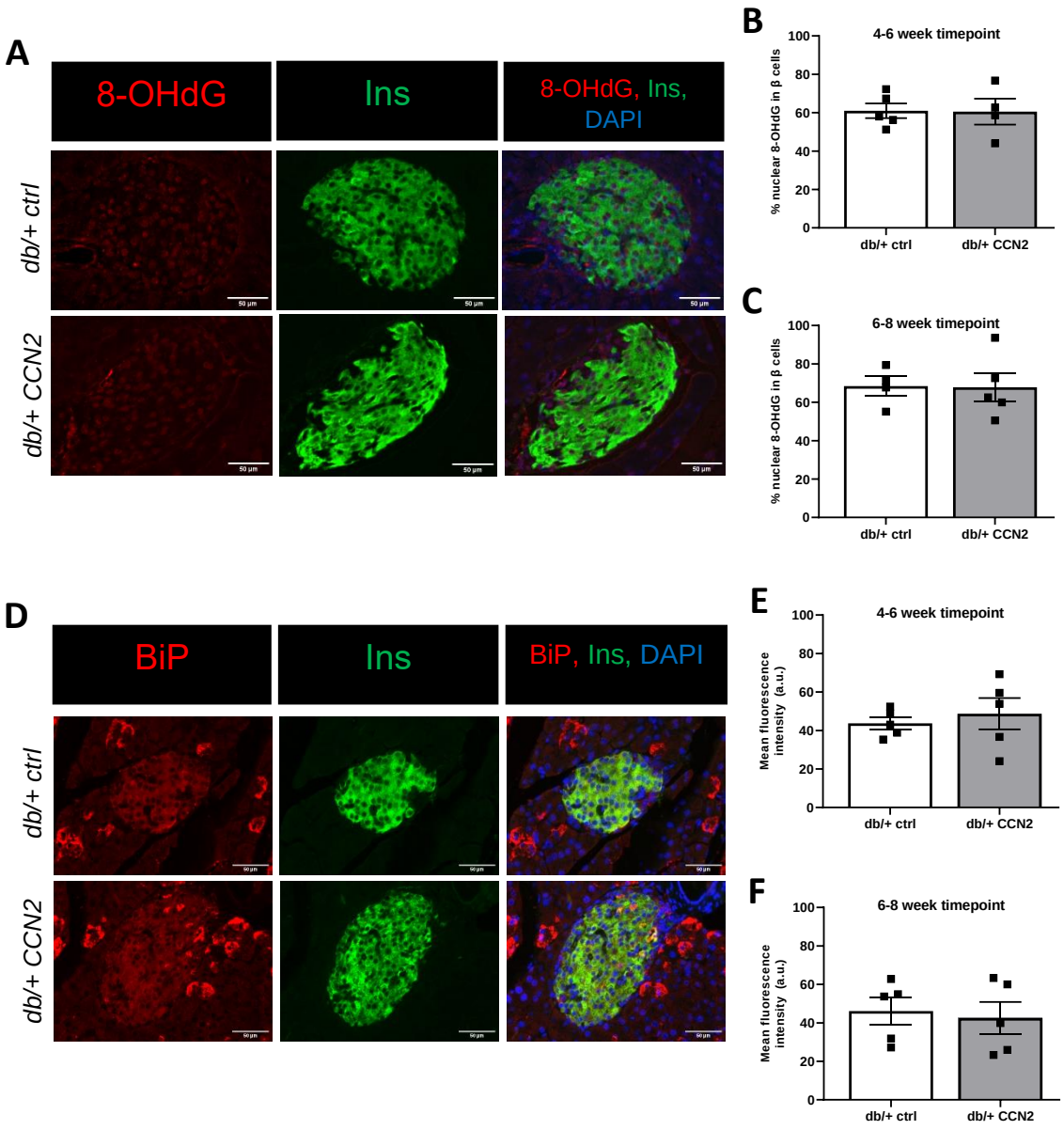



**Figure 5-4**. **CCN2 induction does not alter β-cell oxidative or ER stress in db/+ animals**. **A)** Representative images of 8-OHdG immunolabeling in pancreata from male db/+ animals with CCN2 induction from four to six weeks of age. 8-OHdG – red, Ins – green, DAPI – blue. **B)** Quantification of percent of β cells expressing 8-OHdG in the nucleus after CCN2 induction from four to six weeks of age and **C)** from six to eight weeks of age. **D)** Representative images of BiP immunolabeling in paraffin-embedded pancreata from db/+ animals with CCN2 induction from six to eight weeks of age. BiP – red, Ins – green, DAPI – blue. **E)** BiP quantification in β cells after CCN2 induction from four to six weeks of age and **F)** from six to eight weeks of age. *n*=5-6 animals per group. Data were analyzed using a two-tailed Student's *t test*. (Scale bar, 50μm)

## Discussion

The capability of CCN2 to modulate β-cell stress has not been previously explored. In this study, the action of CCN2 on ER and oxidative stress in chronic *in vivo* models of cell stress was assessed. Diet-induced obesity and db/+ heterozygosity were used as models of chronic *in vivo* cell stress. It was found that CCN2 induction in both models did not alter expression of the ER stress marker BiP nor nuclear expression of 8-OHdG demonstrating that CCN2 does not have the capability of altering cellular stress, at least as assessed by these markers. Furthermore, CCN2 induction during HFD did not stimulate nuclear translocation of p-Nrf2 after both one and ten weeks of HFD.

In the literature, CCN2 is primarily associated with detrimental outcomes when examining different pathophysiological disease models. For example, in a study examining renal ischemia and reperfusion, a situation of oxidative stress, it was found that CCN2 aggravates DNA damage following renal ischemia-reperfusion injury. Furthermore, CCN2 inhibition in the renal ischemia-reperfusion model utilized in that study alleviated DNA damage and lipid peroxidation observed after renal injury [425], implicating CCN2 in the detrimental outcomes in this model. A different study utilized a preclinical mouse model of non-alcoholic hepatosteatosis (NASH) by combining prolonged HFD feeding with low-dose streptozotocin injections in order to mimic the glucolipotoxic conditions observed in NASH. These glucolipotoxic conditions promote both ER and oxidative stress. NASH is a form of non-alcoholic fatty liver disease (NAFLD) that causes liver damage and can lead to fibrosis of the liver. Animals in the NASH group were administered a neutralizing CCN2 antibody to examine the role

CCN2 plays in disease pathogenesis in this model. Animals in the NASH group that received CCN2-neutralizing antibody had significant downregulation of collagen I and collagen III mRNA expression along with attenuation of phosphorylation of pro-fibrotic ERK signaling pathway in the liver [426]. This suggests that CCN2 expression in this separate model of disease is also detrimental.

CCN2 is pro-fibrotic and expression of CCN2 aggravates a multitude of diseases associated with fibrosis [427]. The potential of CCN2 to induce fibrosis in islets has been previously studied by our group. In the studies of CCN2 induction after 50% β-cell ablation, it was found that CCN2 induction did not promote fibrosis in islets with CCN2 induction [133]. Thus, it appears that CCN2 induction does not play a deleterious role in islets as it relates to ER and oxidative stress. While *in vivo* mRNA expression of CCN2 is 300-fold increased in RIP-rtTA;TetO-CTGF islets compared to control islets, it is possible that the amount of protein secreted is not to a level that would promote pathophysiological conditions. Nonetheless, while CCN2 does not attenuate cell stress as measured by BiP and 8-OHdG in two chronic *in vivo* models of β-cell stress, it is still of importance that CCN2 induction in this system did not aggravate β-cell stress. While the RIP is highly active and CCN2 mRNA is expressed 300-fold, this does not seem to induce β-cell stress, in agreement with my *ex vivo* studies. Taken together, using our RIP-rtTA;TetO-CTGF system it has been demonstrated that CCN2 does not have the ability to modulate ER and oxidative stress in the models utilized in these studies. Furthermore, unlike other pathophysiological models, CCN2 does not aggravate the existing β-cell stress in the models utilized in these studies.

## CHAPTER VI. Induction of CCN2 during prolonged HFD stimulates β-cell mass expansion.

### Introduction

Studies from our lab have demonstrated that CCN2 is a β-cell proliferative factor and can induce proliferation in multiple settings. Overexpression of CCN2 during embryogenesis stimulates proliferation of glucagon-producing and insulin-producing cells, leading to an increase in overall endocrine area compared to controls [392]. Induction of CCN2 during adulthood following 50% β-cell ablation promotes β-cell regeneration and partial restoration of β-cell mass [133]. Although animals with 50% β-cell ablation remained euglycemic, to meet the demand for insulin, existing β cells likely had to increase insulin processing and output which in turn increases β-cell stress. However, β-cell-specific induction of CCN2 during euglycemic, unstressed conditions fails to stimulate β-cell proliferation [87]. Thus, since β cells only respond to CCN2 when they are under stress, we hypothesized that β-cell stress is sufficient for CCN2 to induce β-cell proliferation.

To test this hypothesis, we utilized the diet-induced obesity model described in the last chapter as a model to promote chronic β-cell stress. While there was no alleviation of stress following CCN2 induction in this model [results detailed in Chapter V], it is known that diet-induced obesity promotes β-cell stress by inducing glucolipotoxicity. Diet-induced obesity also increases ER stress as measured by expression of Atf6 and p-eIF2α in pancreatic sections from animals administered a 45% HFD for 16 weeks [423]. In the studies in this dissertation, animals with or without CCN2 induction were administered chow diet (CD) or 60% HFD for one or ten weeks. Previous

studies by our group examining the relationship between HFD and β-cell proliferation and mass found that β-cell proliferation was increased as early as three days after HFD administration compared to CD, and β-cell mass was significantly increased after 11 weeks of HFD compared to CD [424]. It was also demonstrated that HFD-induced β-cell proliferation occurs in both the presence and absence of insulin resistance. Thus, in this model, diet was administered for either one (in the absence of insulin resistance) or ten weeks (in the presence of insulin resistance). We found that there were no changes in β-cell proliferation or mass after one week of HFD with concurrent CCN2 induction, but there was a significant increase in β-cell mass after ten weeks of HFD with concurrent CCN2 induction. I did not observe an increase in β-cell proliferation at either one week or ten weeks of HFD. β-cell size was also not different between the three groups at ten weeks. Expression of the β-cell identity marker Pdx1 was assessed at both time points. No difference in expression was found after one week of HFD and CCN2 induction. However, there was a trend toward reduced Pdx1 expression after ten weeks of HFD, while expression was unchanged in the HFD group with CCN2 induction compared to control. These data demonstrate that CCN2 can stimulate β-cell mass expansion in the setting of prolonged, but not short-term HFD.

## Results

### One week of HFD with concurrent CCN2 induction does not alter β-cell proliferation or mass compared to controls.

In these studies, male RIP-rtTA;TetO-CTGF and monogenic control (RIP-rtTA or TetO-CTGF alone) mice were weighed and assigned randomly to one of the following

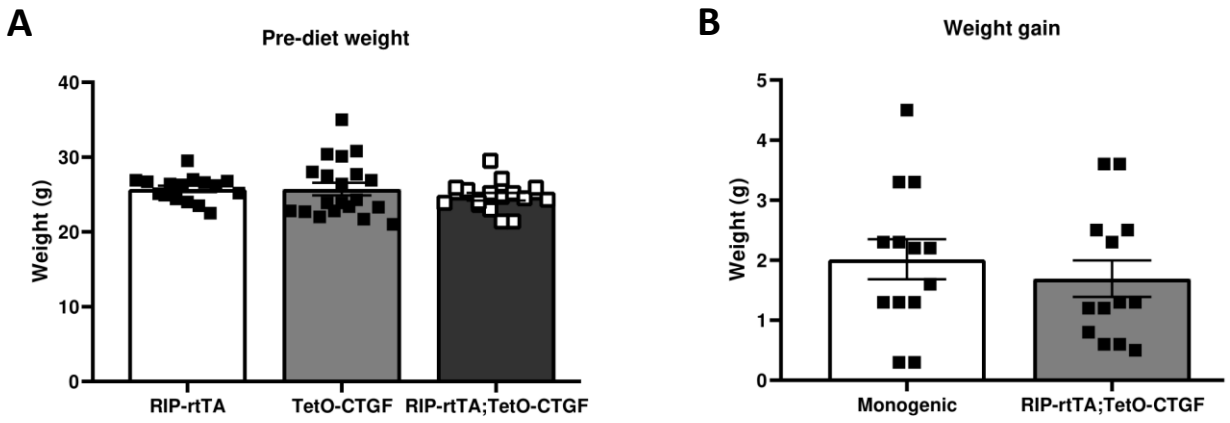



**Figure 6-1**. **The RIP-rtTA and TetO-CTGF transgenes do not alter pre-diet weight or weight gained after 1 week of HFD. A)** Pre-diet weight of RIP-rtTA, TetO-CTGF and RIP-rtTA;TetO-CTGF male mice. n=16,20,16 for RIP-rtTA, TetO-CTGF and RIP-rtTA;TetO-CTGF, respectively. **B)** Weight gained after one week of HFD in monogenic (RIP-rtTA and TetO-CTGF alone) and RIP-rtTA;TetO-CTGF mice. *n*=13 for each group.

groups: 1) CD (nutrient-matched) 2) 60% HFD alone (HFD Ctrl) and 3) 60% HFD with concurrent CCN2 induction (HFD CCN2). Before diet was administered, animals were weighed to determine if there were phenotypic differences between groups due to presence of the transgenes. It was found that pre-diet weight between RIP-rtTA, TetO-CTGF and RIP-rtTA;TetO-CTGF mice was similar [Figure 6-1A] . DOX was administered two days before the start of diet to induce β-cell-specific CCN2 expression and continued throughout the duration of diet. Originally, the first timepoint to be measured was three days post-HFD as previous studies in the lab demonstrated that β-cell proliferation is significantly increased after only three days of HFD [424]. However, when examining β-cell proliferation at this timepoint, there was no significant difference between the three groups. Previous studies examining the effect of HFD on β-cell proliferation and mass were conducted with wild-type C57BL/6J mice. However, the RIP-rtTA;TetO-CTGF mice are of a mixed genetic background which may affect the β-cell proliferative response to HFD. Furthermore, it cannot be ruled out that the presence of DOX may play a role in the lack of a proliferative response to HFD. This possibility has not been explored.

Since there was no change in β-cell proliferation after three days of HFD, the next timepoint assessed was one-week post-HFD. Male RIP-rtTA;TetO-CTGF and monogenic control mice were administered HFD or nutrient-matched CD concurrently with DOX for one week. Animals were weighed after the duration of diet to assess whether presence of the transgenes affected weight gain on HFD. There was no difference in weight gained between monogenic control mice and RIP-rtTA;TetO-CTGF mice [Figure 6-1B]. At the beginning of these studies, it was unknown whether HFD

altered expression of CCN2 since CCN2 is not normally expressed in adult islets. In a pilot study, animals were administered HFD for one week with DOX to induce CCN2. Islets were then isolated from CD, HFD Ctrl and HFD animals with CCN2 induction at the end of one week and expression of *CCN2* was assessed. As expected, there was no *CCN2* expression in the CD group [Figure 6-2A]. Furthermore, HFD did not induce expression of *CCN2* [Figure 6-2A]. In the HFD group with CCN2 induction, *CCN2* expression was approximately 300-fold more than both the CD and HFD control groups [Figure 6-2A]. This increase in expression is in agreement with previous studies utilizing the RIP-rtTA;TetO-CTGF induction model *in vivo* [133].

The effect of CCN2 induction on glucose tolerance has not been explored in a diet-induced obesity model. Perifusion studies utilizing islets with and without CCN2 induction demonstrated that CCN2 expression in adult β cells does not alter the insulin secretory response to multiple secretagogues [Figure 6-2B and C]. Furthermore, there is no difference in insulin content between groups [Figure 6-2D]. However, we wanted to explore if CCN2 induction would affect glucose tolerance during HFD feeding. After one week of HFD, glucose tolerance was assessed. As expected, HFD altered glucose tolerance compared to CD, but there was no difference in glucose tolerance between the HFD groups [Figure 6-2E and F], demonstrating that CCN2 induction does not alter glucose tolerance.

After IP-GTT, animals were sacrificed and pancreata were excised to assess α- and β-cell proliferation and β-cell mass. Previous studies examining the effect of β-cell-specific CCN2 overexpression during embryogenesis found an increase in glucagon-expressing cells due to increased proliferation at P1 [392]. In my studies, there was no

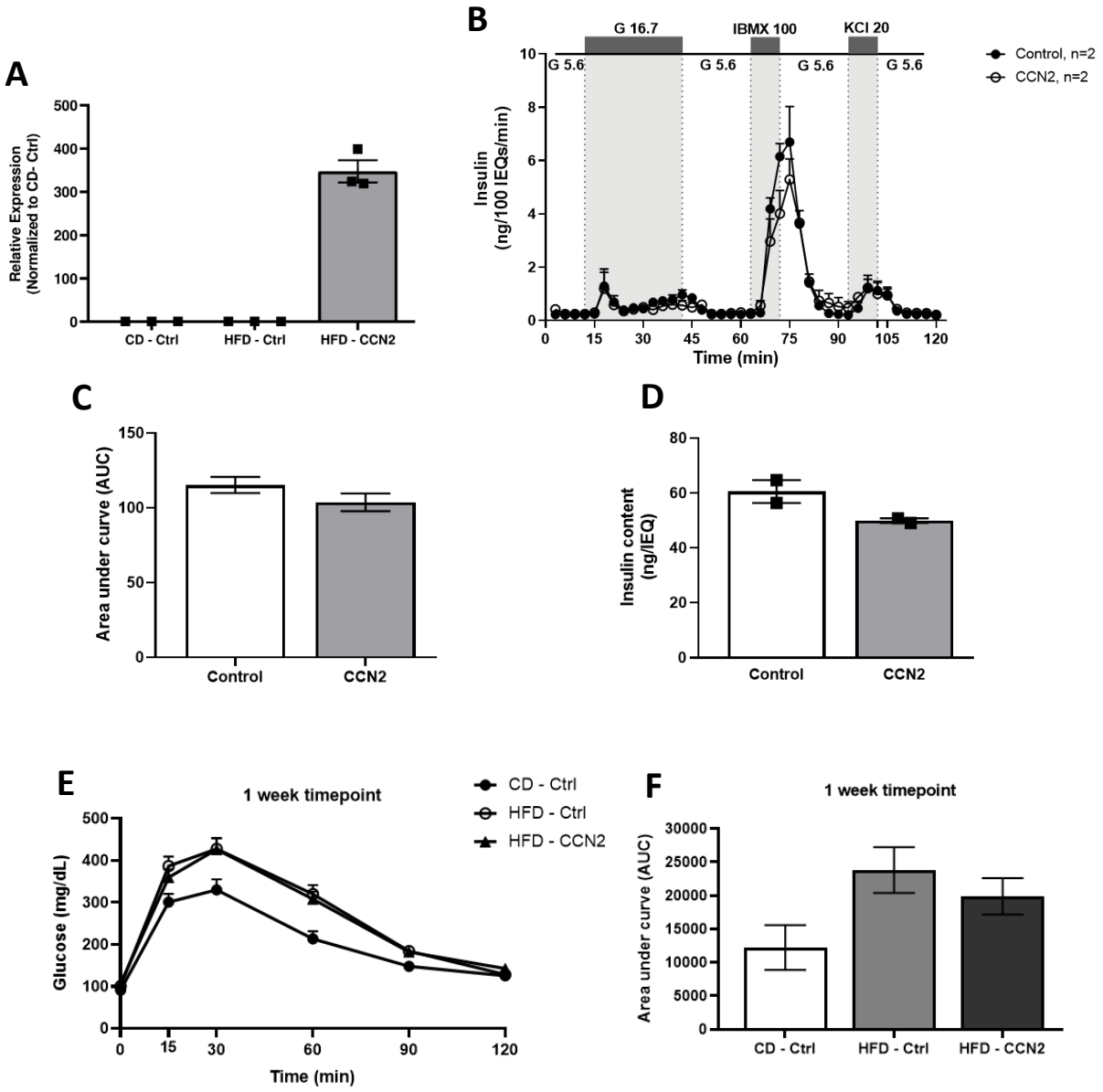



**Figure 6-2. CCN2 induction does not affect insulin secretion**. **A)** *CCN2* is expressed 300-fold in islets from male RIP-rtTA;TetO-CTGF mice after one week of DOX. HFD does not induce *CCN2.* **B)** Graph of perifusion assay showing insulin secretion responses to secretagogues in control (RIP-rtTA and TetO-CTGF alone) and RIP-rtTA;TetO-CTGF islets. **C)** Area under the curve quantification for perifusion assay in B. **D)** Insulin content in islets utilized for the perifusion assay in B. **E)** IP-GTT data from male mice after the one-week diet timepoint. **F)** Area under the curve quantification for IP-GTT in E. *n*=2-3 per group in A-D. *n*=5-10 per group in E and F. One-way ANOVA was conducted on data in **F.** F=3.119, *p*=0.0997.

difference in either α- and β-cell proliferation between the groups after one week of HFD [Figure 6-3A and B]. Furthermore, there was no difference in β-cell mass between all groups [Figure 6-3C]. Pdx1 expression was assessed, and no difference was observed between groups at this timepoint [Figure 6-3D]. Taken together, induction of CCN2 during one week of HFD does not alter glucose tolerance compared to HFD control nor stimulate β-cell proliferation and β-cell mass expansion.

**Ten weeks of HFD and concurrent CCN2 significantly increases β-cell mass.**

The end point for these studies was after ten weeks of diet. At the end point, animals from all groups were weighed, and a similar amount of weight was gained between the HFD groups [Figure 6-4A]. Glucose tolerance was assessed prior to sacrifice, and similarly to the one-week time point, CCN2 induction during ten weeks of HFD did not alter glucose tolerance compared to HFD control [Figure 6-4B and C]. After IP-GTT, animals were sacrificed and pancreata were excised to be analyzed for β-cell proliferation and mass. There was no difference in β-cell proliferation between any groups [Figure 6-5A]. However, β-cell mass was significantly increased in the HFD group with CCN2 induction, suggesting that CCN2 induction stimulated β-cell mass expansion [Figure 6-5B]. Since an increase in β-cell mass can occur via both proliferation and hypertrophy, β-cell size was examined, but no difference was found between the three groups [Figure 6-5C]. While previous studies reported a significant increase in β-cell mass after long-term HFD administration, that result was not seen in this study as there was no significant increase in β-cell mass after ten weeks of HFD alone. Pdx1 expression was analyzed at this timepoint, and there was a downward

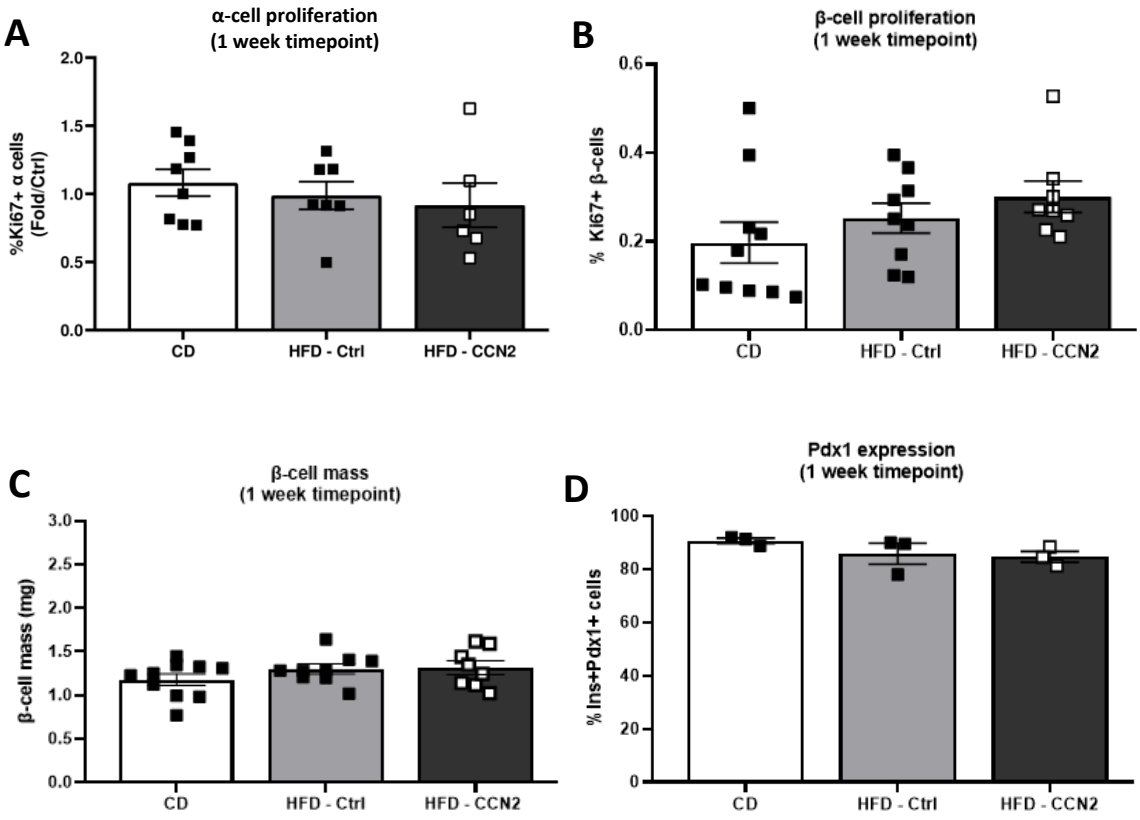



**Figure 6-3**. **CCN2 induction during one week of HFD does not induce α- and β-cell proliferation, or lead to an increase in β-cell mass after one week. A)** CCN2 induction for one week in male mice does not induce α-cell proliferation. *n*=8,7,6 for CD, HFD – Ctrl and HFD – CCN2 respectively. **B)** CCN2 induction for one week does not stimulate β-cell proliferation. *n*=10,9,8 for CD, HFD – Ctrl and HFD – CCN2 respectively. **C)** CCN2 induction for one week does not increase β-cell mass. *n*=10,9,8 for CD, HFD – Ctrl and HFD – CCN2. **D)** Pdx1 expression is similar between groups after one week of CCN2 induction. *n*=3 per group.

trend in Pdx1 expression after ten weeks of HFD alone. However, that expression was preserved in the HFD group with CCN2 induction [Figure 6-5D]. Assessment of β-cell death by TUNEL was also attempted, but it was found that the TUNEL immunolabeling was largely non-specific, so those results were inconclusive.

**Discussion**

In the studies described in this chapter, the capability of CCN2 to induce β-cell mass expansion during a period of chronic *in vivo* β-cell stress was assessed. β-cell specific CCN2 induction during the setting of diet-induced obesity did not increase β-cell proliferation or mass after one week of HFD, but lead to significantly higher β-cell mass after ten weeks of HFD. Other metabolic parameters assessed in this study were weight gained after HFD and glucose tolerance. Induction of CCN2 for both one and ten weeks did not alter weight gained on HFD, which suggests that induction of CCN2 in β cells does not cause any unexpected systemic metabolic effects. Finally, CCN2 induction during HFD did not alter glucose tolerance when comparing the HFD groups. Studies investigating the effect of CCN2 on β-cell function found that CCN2 induction did not alter the insulin secretory response to various secretagogues which may explain why CCN2 induction during HFD did not worsen or improve glucose tolerance.

When examining β-cell proliferation in response to HFD, no difference was found between groups at either the one- or ten-week timepoints. This was unexpected as previous studies demonstrated that only three days of HFD was sufficient to induce significant β-cell proliferation [424]. However, those studies were conducted with wild-type C57BL/6J mice on a pure genetic background while the animals utilized in these studies are maintained on a mixed genetic background. Furthermore, our model utilizes

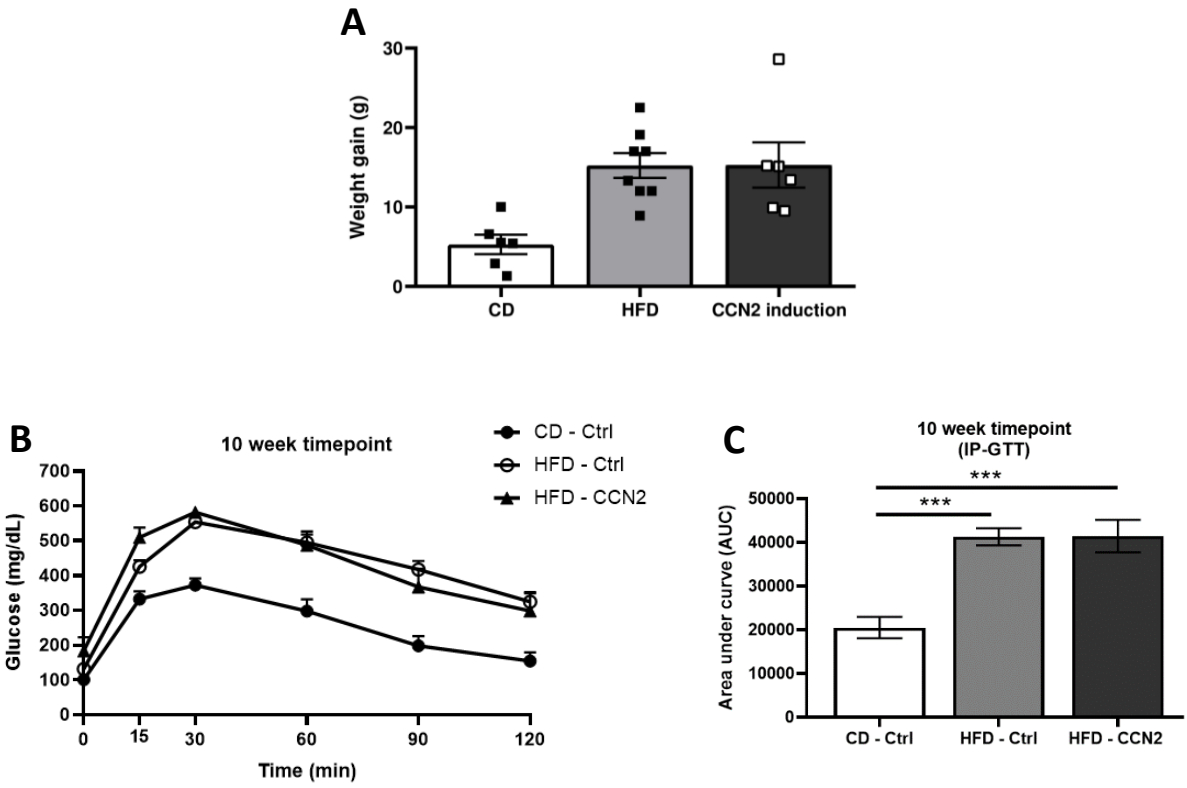



**Figure 6-4**. **CCN2 induction during HFD for ten weeks does not alter weight gain on HFD or glucose tolerance**. **A)** Weight gain is similar between the HFD groups. *n*=6,8,6 male mice per group respectively. **B)** CCN2 induction for 10 weeks during HFD does not alter glucose tolerance compared to HFD controls. *n*=5-10 per group. **C)** Area under the curve quantification corresponding to IP-GTT in B. *n*=5-10 per group. One-way ANOVA with Tukey *post hoc* analysis was conducted for data in **C**. F=18.45, *p*=0.0002. ****p*<0.0001.

DOX to induce CCN2. Previous studies have been conducted to assess the effect of DOX on β cells during pro-inflammatory settings such as in the db/db mouse model and diet-induced obesity. It was found that db/db animals receiving DOX had better glycemic control, less insulin resistance, and increased β-cell proliferation compared to controls, although this did not translate to an increase in β-cell mass [428]. In the study assessing the effect of DOX during HFD, it was discovered that the group receiving DOX had a significant increase in β-cell mass, although the mechanism for this increase was not explored [429]. In those studies, however, the dose of DOX that contributed to the beneficial effects observed was ten-fold less than the dose utilized in my studies [428,429]. Furthermore, the duration of DOX treatment in those studies differed from my studies. Thus, it is still unknown what the potential effect of DOX on β-cell proliferation in our model is. Previous studies from our lab assessing the effects of HFD feeding on β-cell mass found that one week of HFD was not sufficient to induce β-cell mass expansion [424]. In those studies, β-cell mass expansion was only evident after 11 weeks of HFD. Thus, it was not expected that one week of HFD with or without concurrent CCN2 induction would increase β-cell mass.

In my study, CCN2 induction during ten weeks of HFD stimulated β-cell mass expansion. This occurs only one week prior to when β-cell mass was significantly increased in the previously published study by our group examining the effect of HFD on β-cell proliferation and mass [424]. In my study, ten weeks of HFD alone did not stimulate β-cell mass expansion. In the literature, diet-induced obesity is used extensively for comparison of metabolic parameters after various treatments or genetic manipulations. However, due to different experimental questions being investigated by

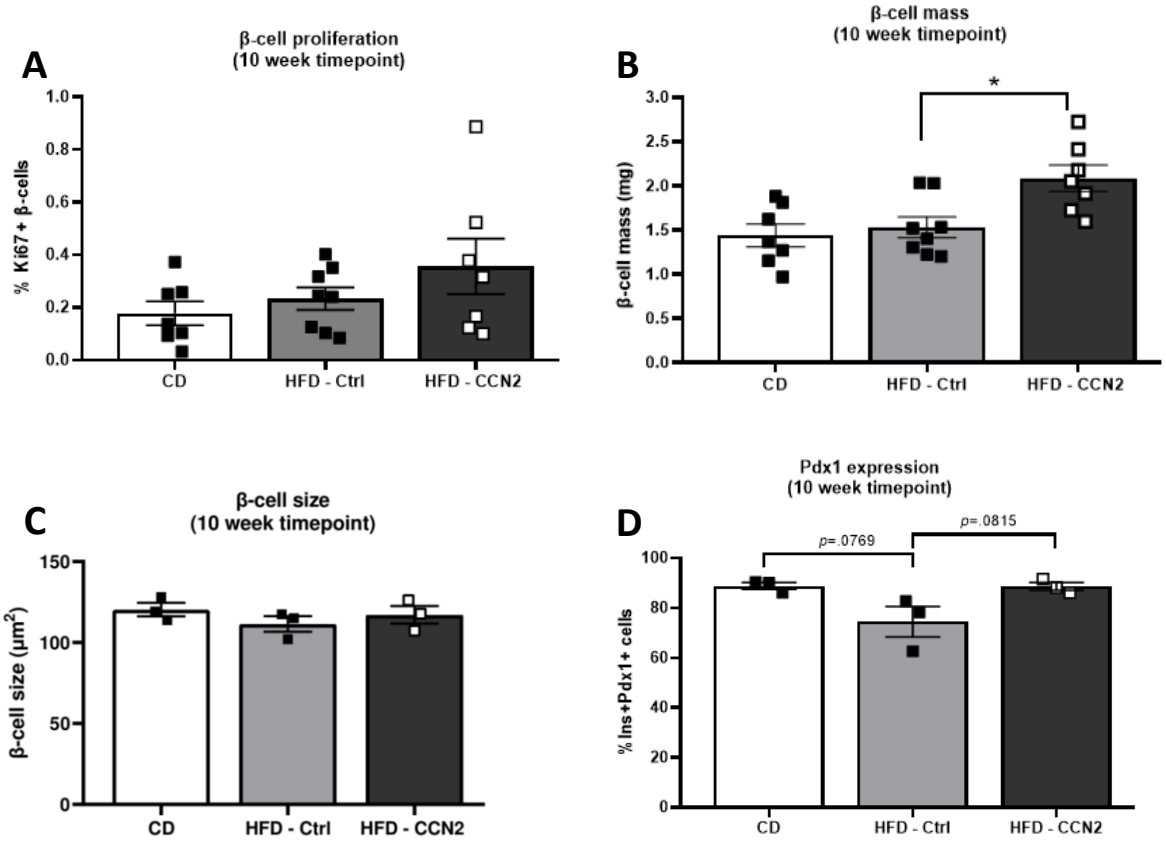



**Figure 6-5. CCN2 induction during HFD for ten weeks significantly increases β-cell mass**. **A)** CCN2 induction during 10 weeks of HFD does not stimulate β-cell proliferation. **B)** CCN2 induction for 10 weeks during HFD significantly increases β-cell mass. **C)** β-cell size is similar between the three groups. **D)** CCN2 induction during 10 weeks of HFD preserves Pdx1 expression. *n*=7,8,7 male mice per group in A and B. *n*=3 male mice per group in C and D. Data were analyzed using one-way ANOVA and Tukey *post hoc* analysis. **B**: F=6.852, *p*=0.0057. **p*<0.05.

multiple groups, the duration of HFD feeding used in studies greatly varies. The earliest timepoint found in the literature in which HFD feeding has induced significant β-cell mass expansion is eight weeks [430]. As mentioned previously, the mixed background of the mice in my study, combined with DOX administration could have affected HFD-induced β-cell mass expansion. However, it is possible that if assessed later, a significant increase in β-cell mass compared to CD would be apparent.

In my study, the expression of Pdx1 was assessed after both one and ten weeks of HFD. At the one-week timepoint, there was no difference in Pdx1 expression. However, at the ten-week timepoint, Pdx1 expression had a trend toward a reduction in animals in the HFD group. Expression of Pdx1 was unchanged in the HFD group with CCN2 induction compared to controls which suggests that CCN2 may aid in maintaining β-cell identity during prolonged HFD. Analysis of β-cell death by TUNEL immunolabeling was not successful. Finally, at the ten-week timepoint, there was no increase in β-cell proliferation observed. This led us to examine if hypertrophy was the mechanism by which β-cell mass expansion occurred. After calculating β-cell size, no differences were found between the three groups. Since an increase in β-cell proliferation was not observed at either the one- and ten-week timepoints, this suggests that the window of β-cell proliferation that led to increased β-cell mass at ten weeks was missed. Examination of intermediate timepoints may have captured the increase in β-cell proliferation. Altogether, these studies demonstrate that in the setting of HFD, prolonged β-cell stress is required for CCN2 to induce β-cell mass expansion, and likely β-cell proliferation.

## CHAPTER VII: Induction of CCN2 in db/+ mice results in a significant decrease in β-cell mass.

### Introduction.

Studies described in this dissertation demonstrate that db/+ mice exhibit more ER and oxidative stress compared to mice on a wild-type background [Figure 5-3 in Chapter V]. Thus, db/+ mice were used as another model of mild chronic β-cell stress. In these studies, db/+;RIP-rtTA;TetO-CTGF male mice were generated to allow for β-cell-specific CCN2 induction in the presence of DOX in the setting of db/db haploinsufficiency. CCN2 was induced in β cells at four-to-six weeks or six-to-eight weeks of age. It was found that CCN2 induction did not alter β-cell proliferation at either time point. CCN2 induction in β cells did not alter β-cell mass from four to six weeks of age, but significantly reduced β-cell mass from six to eight weeks of age. Further analysis to assess the method of β-cell loss demonstrated that there was no difference in expression of several apoptosis genes when comparing control db/+ islets with CCN2-expressing db/+ islets. Thus, unlike in the diet-induced obesity model, CCN2 induction in this model of chronic β-cell stress is detrimental and leads to a significant loss of β-cell mass.

### Results

#### CCN2 induction from six to eight weeks of age in db/+ mice leads to a significant reduction in β-cell mass.

db/+ mice were bred with RIP-rtTA;TetO-CTGF mice to generate db/+;RIP-rtTA;TetO-CTGF male mice, allowing for β-cell-specific CCN2 induction in the setting of db haploinsufficiency. db/+ animals with one transgene (RIP-rtTA or TetO-CTGF alone)

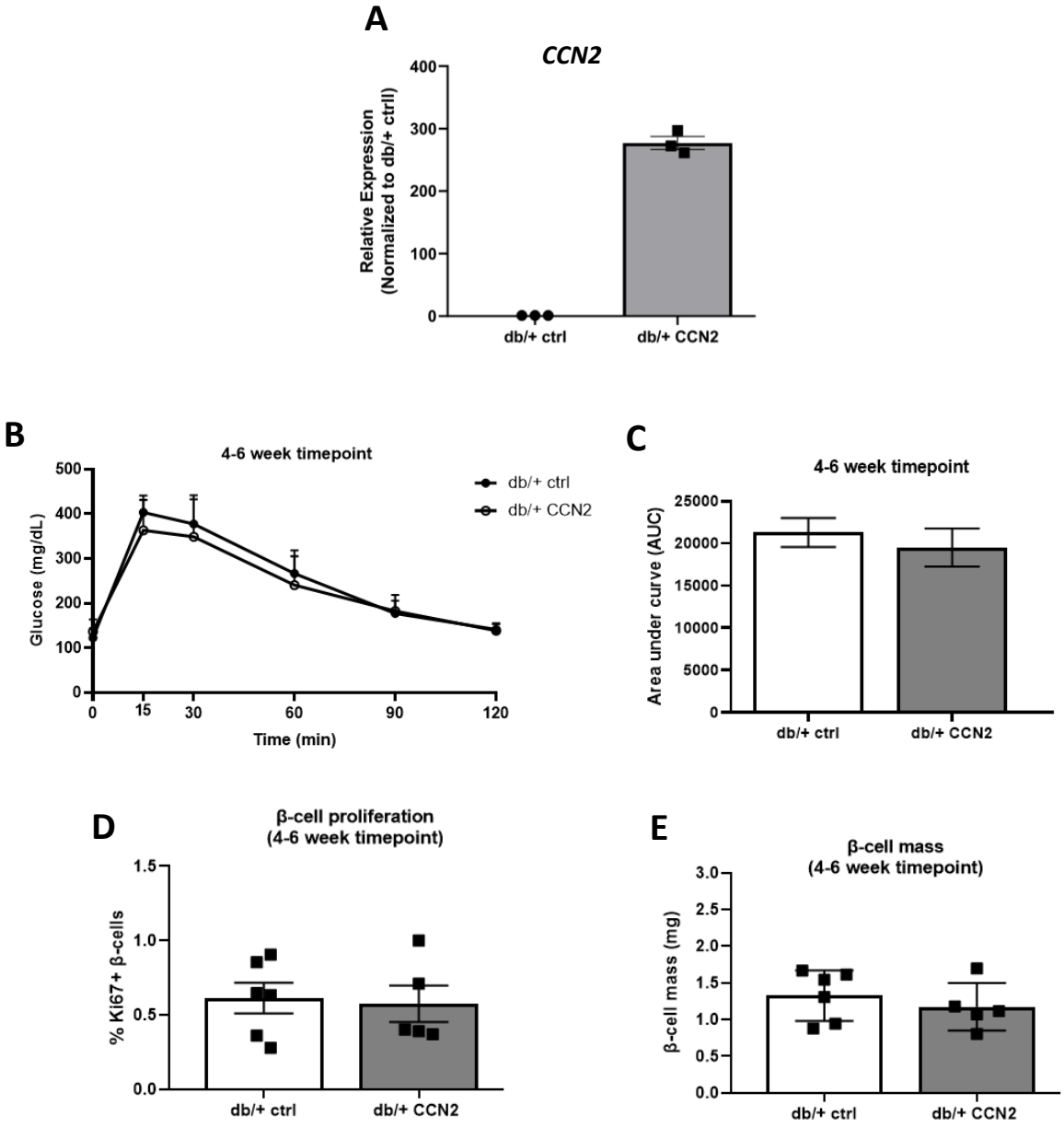



**Figure 7-1**. **CCN2 induction in db/+ mice from four to six weeks of age does not alter glucose tolerance or stimulate β-cell mass expansion**. **A)** DOX administration *in vivo* in db/+;RIP-rtTA;TetO-CTGF male mice results in a 300-fold increase in *CCN2.* **B)** IP-GTT was conducted on db/+ mice after CCN2 induction from four to six weeks of age. **C)** Area under the curve quantification of IP-GTT in A. **D)** CCN2 induction from four to six weeks of age in db/+ mice does not stimulate β-cell proliferation. **E)** CCN2 induction from four to six weeks of age in db/+ mice does not affect β-cell mass. *n*=3-4 per group in A - C. *n*=5-6 per group in C and D.

were utilized as controls. CCN2 was induced at two time points: between four to six and six to eight weeks of age. Expression of CCN2 in db/+;RIP-rtTA;TetO-CTGF was increased 300-fold compared to controls, which is in agreement with previously published studies [Figure 7-1A] [133].

At the four- to six-week timepoint, glucose tolerance was assessed before sacrifice, and it was found that CCN2 induction did not alter glucose tolerance compared to controls at either age [Figure 7-1B and C]. When examining β-cell proliferation at this timepoint, there was no difference between the control and CCN2 induction group [Figure 7-1D]. CCN2 induction from four to six weeks of age in db/+ mice did not change β-cell mass compared to control [Figure 7-1E]. CCN2 induction from six to eight weeks of age in db/+ mice did not alter glucose tolerance [Figure 7-2A and B] or β-cell proliferation [Figure 7-2C], but significantly reduced β-cell mass [Figure 7-2D]. To assess whether apoptosis led to loss of β-cell mass, qRT-PCR was conducted on islets from db/+ mice with/without CCN2 induction from six to eight weeks of age to examine expression of the apoptosis markers *Bad, Bax, Bcl-XL* and *Casp3*, and no difference in expression was found [Figure 7-3A]. β-cell size was assessed in this model, and there was no difference in cell size between the two groups [Figure 7-3B]. Finally, analysis of Pdx1 demonstrated no changes in expression in db/+ mice with CCN2 induction compared to control [Figure 7-3C].

**Discussion**

β-cell-specific CCN2 induction in db/+ animals did not alter β-cell proliferation at either age assessed. In contrast to the HFD studies, β-cell-specific CCN2 induction in

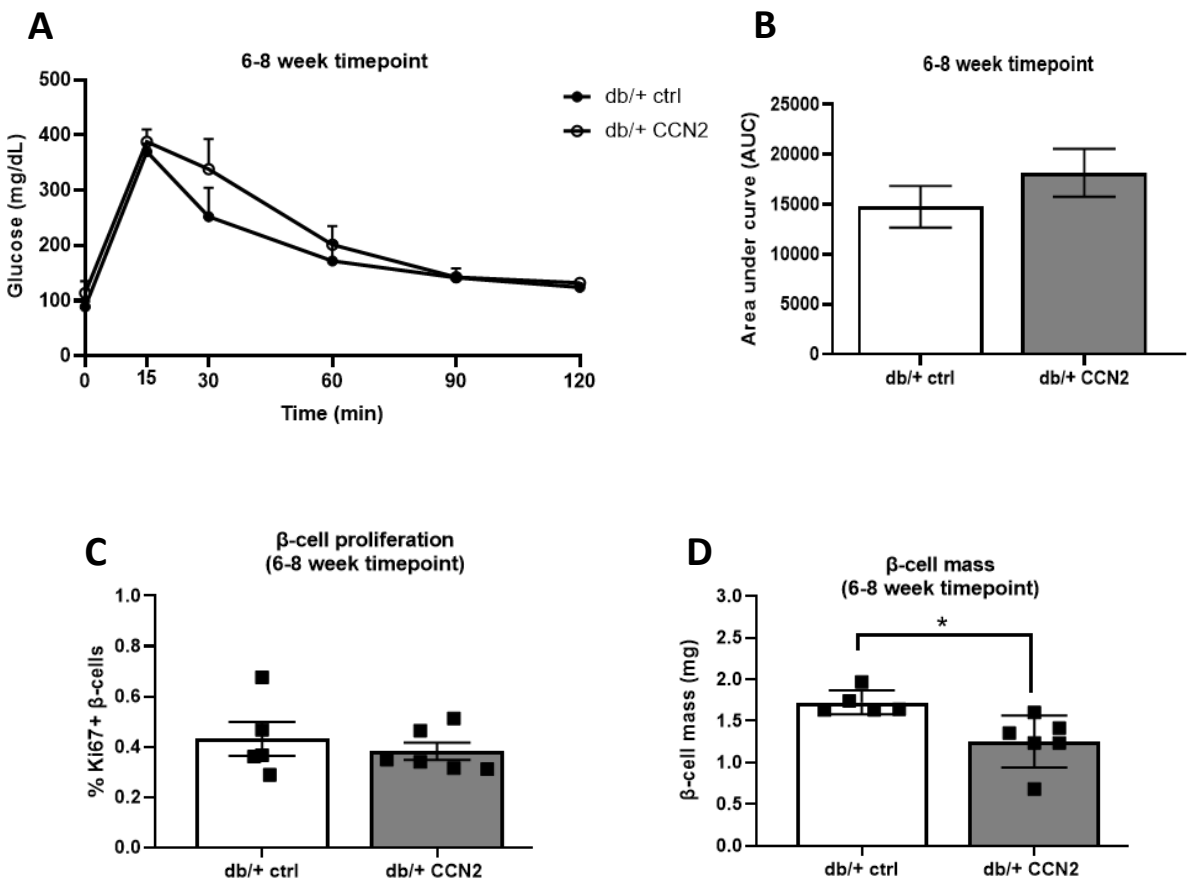



**Figure 7-2**. **CCN2 induction in db/+ mice from six to eight weeks of age does not alter glucose tolerance but significantly reduces β-cell mass**. **A)** IP-GTT was conducted on db/+ male mice after CCN2 induction from six to eight weeks of age. **B)** Area under the curve quantification of IP-GTT in A. **C)** CCN2 induction from six to eight weeks of age in db/+ mice does not stimulate β-cell proliferation. **D)** CCN2 induction from six to eight weeks of age in db/+ mice reduces β-cell mass. *n*=3-4 per group in A and B. *n*=5-6 per group in C and D. Two-tailed Student's t-test was used to analyze data. *$p$<0.05

db/+ animals significantly reduced β-cell mass. These findings suggest that cell stress is necessary but not sufficient for CCN2 to stimulate β-cell mass expansion.

The main potential mechanisms for loss of β-cell mass include apoptosis and dedifferentiation. TUNEL and caspase-3 immunolabeling were attempted to detect apoptosis, but the results were inconclusive. TUNEL immunolabeling was shown to be largely non-specific, while caspase-3 immunolabeling was positive in nearly all α cells, a phenomenon our lab has encountered before with these antibodies. As an alternative to immunolabeling, qRT-PCR was utilized to assess expression of the apoptosis genes *Bax, Bad, Bcl-xl* and *Casp3* in islets isolated from db/+ mice with or without β-cell-specific CCN2 induction. However, no differences were found in the expression of these genes between the two groups. While hypertrophy is a mechanism by which β-cell mass can increase, β-cell atrophy is less well characterized as a potential mechanism for decreased β-cell mass. Studies utilizing different mouse models from our group and others have demonstrated that β-cell atrophy does occur after loss of various signaling molecules, and was usually the mechanism by which total β-cell mass decreased in those models [431-434]. However, β-cell size was similar between the groups in my study. Other studies examining pancreatic samples from humans with T2D found that, while there was a slight decrease in β-cell size, a reduction in number of β cells was the main mechanism by which β-cell loss occurs during the course of the disease [435]. Thus, it is possible that db/+ mice with β-cell-specific CCN2 induction had a decrease in the number of β cells, but that was not determined in my study. Furthermore, it cannot be completely ruled out that apoptosis is the main cause of loss of β-cell mass because while mRNA expression is unchanged, expression of proteins associated with apoptosis

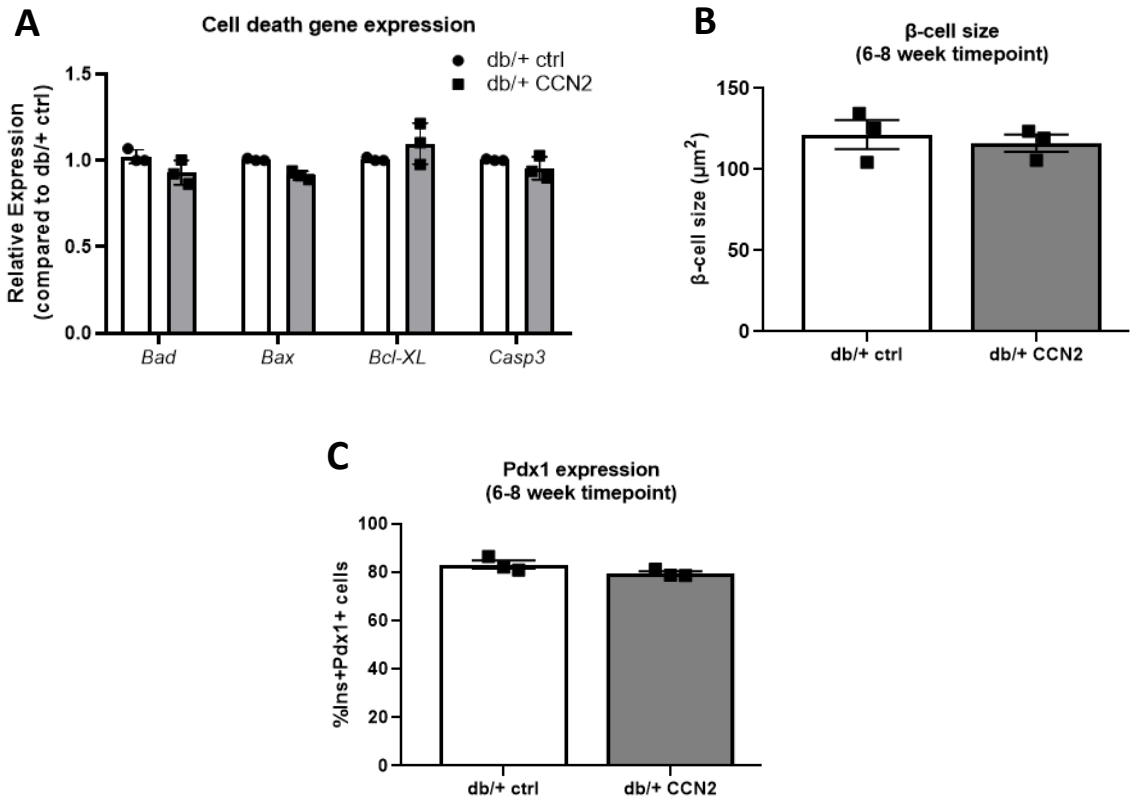



**Figure 7-3**. **There are no differences in cell death marker expression, β-cell size, or Pdx1 expression in db/+ mice with/without CCN2 induction from six to eight weeks of age**. **A)** Gene expression of various cell death markers was analyzed between control and db/+ male mice with CCN2 induction from six to eight weeks of age. **B)** β-cell size is not different between the two groups. **C)** CCN2 induction from six to eight weeks of age in db/+ mice does not affect Pdx1 expression. *n*=3 per group.

may still be increased.

It is surprising that CCN2 induction in one model of chronic *in vivo* β-cell stress stimulated β-cell mass expansion while promoting β-cell mass loss in the other model utilized. This suggests that the cellular microenvironment between the two models is distinct enough that CCN2 induction would be beneficial in one and detrimental in another. It was demonstrated by previous studies from our group and in this current study that db/+ animals are under more ER and oxidative stress than wild-type animals [409, Figure 5-3 in Chapter V]. When examining BiP and 8-OHdG between transgenic animals on and off the db/+ background, it was found that both markers of stress were expressed more highly in db/+ animals. The pre-existing stress in islets from mice on the db/+ background combined with the potential stress of β-cell-specific CCN2 induction could be detrimental to the health of β cells, leading to apoptosis. This may explain why β-cell-specific CCN2 induction leads to a loss of β-cell mass in db/+ animals. While the HFD model does induce β-cell stress, there was no pre-existing β-cell stress before HFD feeding and CCN2 induction which may explain why CCN2 induction in that model did not lead to a decrease in β-cell mass. It is also possible that the high fat content in the diet used in my studies acted as an additional stimulant for β-cell proliferation.

Although these results are contrary to my original hypothesis, they highlight that CCN2 is not capable of inducing β-cell mass expansion in every situation of β-cell stress. This suggests that there is a threshold of stress that, when crossed, promotes detrimental effects of CCN2 induction on β-cell health. This has greater implications for use of CCN2 as a potential therapeutic and reveals that stress alone may be sufficient

for CCN2 to stimulate β-cell mass expansion only below a certain threshold. Once that threshold is exceeded, induction of CCN2 may prove to be detrimental which may have implications for the different effect that CCN2 induction had on β-cell mass between the diet-induced obesity and db/+ models.

## CHAPTER VIII: Summary and future directions

CCN2 is a matricellular protein that is part of the CCN family of proteins. CCN2 has four distinct domains – IGF-BP, VWC, TSP-1 and CT – all of which are capable of binding to a variety of growth factors, cell surface proteins and extracellular matrix molecules [378]. Because of its capability to interact with various molecules, CCN2 is implicated in many biological processes such as adhesion, migration, proliferation and angiogenesis [376-379]. Our lab became interested in studying CCN2 and its role in pancreas development because it was discovered to be downregulated in a mouse model of defective β-cell differentiation and islet morphogenesis [375]. Since then, our lab has extensively studied CCN2 during both embryogenesis and the postnatal period. During embryogenesis, CCN2 is expressed in the ductal epithelium, insulin-positive cells, and the endothelium. Shortly after birth, CCN2 expression is downregulated in β cells, becoming restricted to the duct and blood vessels in adult pancreata [391]. Our prior studies have revealed that CCN2 is required for β-cell proliferation and specification during embryogenesis and causes an increase in total endocrine area following embryonic β-cell-specific overexpression [391,392]. During adulthood, CCN2 is required for maternal β-cell mass expansion during pregnancy [366] and can stimulate β-cell regeneration in a 50% β-cell ablation model [133]. However, induction of CCN2 *in vivo* during euglycemic, unstressed conditions does not promote β-cell proliferation [87]. Finally, treatment of isolated wild-type C57BL/6J mouse islets with CCN2 *ex vivo* stimulates β-cell proliferation [133].

While our lab has elucidated a role for CCN2 in both the embryonic and adult pancreas, there is much still to learn about the protein. First, CCN2 induces β-cell

proliferation in some cellular settings (as those described above) and not others. In the setting of embryogenesis, β cells are rapidly proliferating to reach a sufficient mass to maintain euglycemia in the postnatal period. During pregnancy, when more insulin resistance is present, maternal β-cell mass expansion occurs to meet the increased demand for insulin [436]. In the situation of 50% β-cell ablation, while euglycemia is maintained, there is more demand on the remaining β cells to supply sufficient insulin to maintain glucose homeostasis. Finally, islet isolation is a process which imposes stress on the islets due to the process of isolation along with loss of internal blood supply and nerve input. In the situations described, there is either insufficient β-cell mass or increased β-cell stress present. Based on these findings, we have been working under the hypothesis that cell stress is necessary for CCN2 to induce β-cell proliferation. This hypothesis is further strengthened by the finding that CCN2 induction during euglycemic, unstressed conditions does not promote β-cell proliferation [87]. Thus, the main hypothesis of my thesis was that β-cell stress would be necessary for CCN2 to induce β-cell proliferation, and that it relieves stress resulting in increased β-cell proliferation.

Many biological questions remain about CCN2. The overarching goal of studying CCN2 is to understand if and how this molecule can be utilized as a therapeutic. While CCN2 is not a very large protein at a molecular weight of 38 kDa, it would be beneficial to understand what portion of the protein induces β-cell proliferation. Discovering the smallest form of CCN2 that has biological activity would have implications for future therapeutic uses. CCN2 is readily cleaved between the second and third domains, VWC and TSP-1 respectively, to generate N- and C-terminal portions of the protein. In the

literature, the role of the C-terminal domain of CCN2 has been more thoroughly investigated. In vascular smooth muscle cells, the CT domain of C-terminal CCN2 upregulated expression of TGF-β receptor 2 and activated the Smad pathway [437]. In rat hepatic stellate cells, it was discovered that binding of the CT domain of C-terminal CCN2 to αvβ3 integrins and heparan sulfate proteoglycan stimulated adhesion [387]. Finally, it was demonstrated that C-terminal CCN2 activated fibroblast migration, stimulated assembly of focal adhesion complexes and promoted osteoclast differentiation [379]. CCN2 is involved in integrin-mediated proliferation of various cell types through the action of the CT domain which can interact with integrins [376,378,379]. The N-terminus of CCN2 has been demonstrated to bind growth factors such as TGF-β and VEGF and has been implicated in fibroblast differentiation [438]. Interestingly, one study provided evidence that N-terminal CCN2 acts to autoinhibit full-length CCN2 and CCN2 only becomes biologically active when cleavage by MMPs occurs to separate the protein into an inactive N- and active C-terminal domain [379]. No studies have reported a role for N-terminal CCN2 in cell proliferation.

To determine the domain of CCN2 that induces β-cell proliferation, our lab contracted GenScript to generate recombinant human N- and C-terminal CCN2. I utilized these recombinant proteins in *ex vivo* proliferation assays with wild-type C57BL/6J mouse islets and found that recombinant N- and C-terminal CCN2 alone did not induce β-cell proliferation. Rather, only when treating isolated islets *ex vivo* with both recombinant N- and C-terminal CCN2 together was β-cell proliferation stimulated. Previous studies utilizing full-length rhCCN2 in *ex vivo* proliferation assays found that a concentration of 250 ng/mL of rhCCN2 was required to stimulate β-cell proliferation.

Treatment with the combination of N- and C-terminal domain also induced β-cell proliferation at a concentration of 250 ng/mL, similar to the studies with full-length rhCCN2 [133].

Based on the knowledge of the action of CCN2 in other cell types, it was surprising that the C-terminal of the protein did not promote β-cell proliferation, as many studies have demonstrated that CCN2 induces cell proliferation through integrin-mediated signaling [406,407]. Since the N-terminal domain has not been as characterized as the C-terminal domain, it wasn't clear what the action of this domain would be on β-cell proliferation. Based on my studies, the importance of N-terminal CCN2 in promoting β-cell proliferation was revealed. I hypothesize that N-terminal CCN2 induces β-cell proliferation through a VEGF-mediated interaction. It has been demonstrated that full-length CCN2 increases gene expression of *Vegfc, Vegfd, Vegfr2 and Vegfr3* in primary cultured lymphatic endothelial cells [407]. The ability for N-terminal CCN2 to interact with VEGF has been previously elucidated [376,378,379]. While the role of VEGF-C and VEGF-D in β-cell biology has not been elucidated, it has been established that VEGF-A indirectly promotes β-cell proliferation.

VEGF-A binds to VEGFR2 on endothelial cells, subsequently resulting in the release of HGF. HGF is a classic β-cell mitogen and has been shown to play a role in β-cell regeneration and maternal β-cell compensation [153,154]. Furthermore, overexpression of HGF increases β-cell proliferation, islet number, and β-cell mass [152]. I hypothesize that CCN2 induces β-cell proliferation indirectly by stimulating HGF secretion from endothelial cells. Thus, it is possible that CCN2 in this situation indirectly promotes β-cell proliferation by upregulating VEGFR2 and enhancing VEGF-A binding

to its receptor on endothelial cells, leading to release of HGF. Indeed, studies from our lab show upregulation of HGF expression in whole islets in response to β-cell-specific CCN2 induction [133]. While I have not determined that N-terminal CCN2 promotes the upregulation of VEGFR2, it is still possible that this domain plays a role in this action. Despite a loosely defined role for N-terminal CCN2 in the literature, my study demonstrates that N-terminal CCN2 is required for stimulation of β-cell proliferation. More studies need to be conducted to fully define the role that N-terminal CCN2 plays in biology. However, the results of my study contribute to the understanding of the biological activity of the N-terminal domain of CCN2 as it contributes to β-cell biology.

The signaling mechanisms by which CCN2 induces β-cell proliferation are largely unknown. Because of this, a Kinexus phosphoarray was utilized to assess changes in protein expression and phosphorylation following treatment with rhCCN2. Preliminary data utilizing isolated islets *ex vivo*, revealed that treatment for 12h with rhCCN2 lead to an increase in total expression of RSK1, and an increase in activating phosphorylation events of both RSK1 and PLC-γ1. While there are no studies directly linking CCN2 to RSK1 phosphorylation, RSK1 is a downstream effector of the ERK/MAPK pathway which could indirectly link CCN2 to activation of RSK1. CCN2 has been established to activate ERK, which provides evidence that CCN2 signaling could lead to indirect activation of RSK1. This is further supported by the KiNetscape map [Figure 3-2 in Chapter III] which illustrates that both ERK1 and ERK2 phosphorylate RSK1 at the activating residues S363 and T359 after 12h of CCN2 treatment. Phosphorylation at these residues is indicative of activation of RSK1.

PLCγ1 is a protein that is involved in cell migration, growth, apoptosis, calcium signaling, brain development and proliferation [409]. Studies from our group examining prostaglandin signaling as it relates to β-cell proliferation demonstrated that activity of PLCγ1 was required for stimulation of β-cell proliferation in that experimental model [409]. In endothelial cells, it was shown that downstream activation of PLCγ1 by VEGF signaling was important for cell proliferation [410]. When examining the KiNetscape map [Figure 3-2 in Chapter III], there are multiple signaling pathways affected by CCN2 treatment that could activate PLCγ1. For example, EGFR, VEGFR2, and Src are all targets that were identified by the phosphoarray. This suggests that CCN2 activates signaling through the RTKs EGFR or VEGFR2 to cause activation of PLCγ1. Src is another molecule that can be activated by signaling through RTKs, which further suggests that CCN2 can act to activate RTK signaling. As mentioned previously, it is also known that CCN2 induction/treatment increases gene expression of *Vegfr2*, so it is possible that CCN2 induces more VEGFR2 expression while also activating signaling through VEGFR2 to cause activating phosphorylation events on PLCγ1 [407]. The results gained from the Kinexus phosphoarray provide more information about CCN2 signaling and identify targets that may be manipulated to stimulate β-cell mass expansion.

To further explore the role of RSK1 and PLCγ1 in CCN2-mediated β-cell proliferation, *ex vivo* assays using isolated islets could be conducted with purified rhCCN2. Treatment of isolated islets with purified CCN2 concurrently with a RSK1 or PLCγ1 pharmacological inhibitor would provide information about whether either of these molecules are required for CCN2 to stimulate β-cell proliferation. Previous studies

in the lab have already demonstrated that PLCγ1 is required for β-cell proliferation in the context of prostaglandin signaling utilizing pharmacological inhibitor treatment against PLCγ1 [409]. Although a role for RSK1 in β-cell proliferation has not been defined, utilizing pharmacological inhibitors for the molecule could potentially uncover that information. Loss of β-cell proliferation after treatment with CCN2 and pharmacological inhibitors for RSK1 and PLCγ1 would demonstrate that RSK1 and/or PLCγ1 act downstream of CCN2 and are required for CCN2 to induce β-cell proliferation. As mentioned previously, I hypothesize that the N-terminus of CCN2 functions to induce β-cell proliferation through a VEGF-mediated interaction. Thus, further experiments utilizing inhibitors of VEGFR2 during treatment with CCN2 would provide information about whether VEGF signaling is involved in CCN2-mediated β-cell proliferation. Results of these studies would provide more insight into how pathways activated by CCN2 could be manipulated to increase functional β-cell mass.

Previous studies have demonstrated that CCN2 is capable of inducing β-cell proliferation in a variety of settings but fails to induce β-cell proliferation during euglycemic, unstressed conditions [87,133,392,393]. Thus, I hypothesized that cellular stress would be sufficient for β cells to respond to CCN2, leading to increased proliferation and mass. Furthermore, I hypothesized that CCN2 acted to alleviate cellular stress in order to promote β-cell proliferation. However, it is also possible that CCN2, during situations of β-cell stress, stimulates β-cell proliferation to relieve stress. The studies described in this dissertation were conducted to investigate these possibilities. Using the RIP-rtTA;TetO-CTGF β-cell-specific CCN2 induction mouse model, I found that induction of CCN2 in islets treated *ex vivo* with the ER stressor

thapsigargin (TG) did not fully attenuate upregulation of expression of ER stress markers compared with TG treatment alone. CCN2 induction in the presence of TG led to increased expression of the antioxidant transcription factor *Nfe2l2*. Thus, it is possible that CCN2 utilizes antioxidant mechanisms to promote β-cell proliferation.

*Nfe2l2* (Nrf2) is a master regulator of the antioxidant response [280]. Activation of Nrf2 results in transcription of various molecules responsible for mediating oxidative stress, one of which is *Txn1* [281]. Further analysis of gene expression of known downstream targets of Nrf2 such as *Gpx2, Hmox1* and *Nqo1* found no difference in gene expression between groups. This would suggest that while Nrf2 expression was increased, this may not translate to activation of downstream effectors. However, mRNA expression does not always correlate with protein expression or activity, making it possible that CCN2 induction in the presence of TG does lead to activation of downstream effectors of Nrf2. It is possible that CCN2 may upregulate only Nrf2 mRNA and not protein; conducting western blots using islets with CCN2 induction after TG treatment would directly assess whether there are changes in protein expression of Nrf2 or any downstream effectors. Another direct readout of Nrf2 activation would be examining intracellular ROS levels in islets with CCN2 induction after TG treatment. There are many methods of detecting ROS in *ex vivo* systems utilizing fluorescence, luminescence, spectroscopy, and antibodies [439,440]. If CCN2 does act to alleviate oxidative stress in the presence of TG, assays to measure ROS could be utilized to compare islets with and without CCN2 induction in the presence of TG.

The *ex vivo* assays utilizing TG described in this dissertation were conducted for only 24 hours. While this duration was sufficient to capture changes in gene expression

of ER and oxidative stress markers, it is possible that more information could be gained about the action of CCN2 on ER and oxidative stress if the treatment period were longer. The decision to conduct these assays with 10 nM TG was made based on pilot studies demonstrating that that concentration is sufficient to induce mild cell stress without inducing apoptosis. While the gene encoding CHOP was significantly upregulated after 24h of TG treatment at that concentration, it is still possible that a longer treatment period could be utilized. Longer treatment with TG concurrently with CCN2 induction could potentially reveal Nrf2-mediated changes in gene expression or provide information about whether CCN2 could function to alleviate cell stress in the setting of more chronic stress compared to the conditions I tested.

Chronic *in vivo* models were utilized to assess if CCN2 can alleviate cell stress *in vivo*. The two models utilized were diet-induced obesity and db/+ heterozygosity. In the diet-induced obesity model, animals were placed on HFD for either one week or ten weeks concurrently with CCN2 induction. At both the one- and ten-week timepoints, it was found that CCN2 did not alleviate cell stress as measured by 8-OHdG (oxidative stress) and BiP (ER stress). There are other markers that could be assessed as a readout of oxidative and ER stress. Thus, it is possible that CCN2 could function to alleviate oxidative and ER stress but that was not captured in my model due to the markers chosen to assess these parameters. In the db/+ model, CCN2 induction did not alter the percentage of cells with nuclear localization of 8-OHdG or expression of BiP, similar to the results observed in the HFD model. However, CCN2 induction was detrimental in this model which will be discussed later.

While protein expression was not assessed on tissue sections from animals in the HFD studies, *ex vivo* TG assays demonstrated that *Nfe2l2* was increased. Phosphorylation of Nrf2 protein causes its activation and nuclear translocation. In the HFD model, localization of phospho-Nrf2 was assessed at both timepoints. At the one-week timepoint, the CCN2 induction group trended toward an increase in nuclear localized p-Nrf2; however, it was not significant. Addition of more samples may lead to a significant result suggesting that CCN2 functions to alleviate oxidative stress *in vivo*. At the ten-week timepoint, nuclear p-Nrf2 expression was detected at very low levels. It is possible that if assessed at other timepoints between one and ten weeks of HFD, there would be a noticeable difference in p-Nrf2 localization. While we can control the levels of ER and oxidative stress induced *ex vivo* by treating with TG at varying concentrations, the control over induction of ER and oxidative stress by HFD is not possible. Thus, it would be difficult to pinpoint what duration of HFD would be needed to significantly induce ER and oxidative stress, and that may affect the results obtained. A more thorough examination of cell stress throughout the ten weeks of HFD would provide more information about the capability of CCN2 to alleviate cell stress.

Using *in vivo* models of chronic β-cell stress, it was revealed that CCN2 induction in the setting of long-term diet-induced obesity resulted in significantly increased β-cell mass. This suggests, that in this model, CCN2 induction stimulates β-cell mass expansion. The timepoints used in the HFD studies were one week and ten weeks. At both timepoints, there was no increase in β-cell proliferation evident. However, when examining β-cell size after ten weeks of HFD, there was no difference in size between the three groups demonstrating that hypertrophy was not the mechanism by which

β-cell mass increased. This suggests that CCN2 induced β-cell proliferation at a timepoint that was not examined in these studies. In previous studies utilizing wild-type C57BL/6J mice to examine β-cell mass dynamics during HFD feeding, it was found that β-cell proliferation was significantly increased as early as three days after the start of HFD, and increased proliferation was evident up to seven weeks after HFD [424]. It is possible that if β-cell proliferation was assessed at different timepoints throughout the ten weeks of HFD, the window of proliferation that led to the increased β-cell mass would have been observed. In the future, intermediate timepoints could be added between one and ten weeks of HFD to examine β-cell proliferation and capture the window in which CCN2 stimulates β-cell proliferation to result in increased β-cell mass.

When examining the db/+ model, it was discovered that CCN2 induction was detrimental and led to a significant reduction in β-cell mass. These findings suggest that cell stress is necessary, but not sufficient for CCN2 to induce β-cell proliferation. It was surprising that CCN2 induction during one chronic setting of β-cell stress would stimulate β-cell mass expansion, while having detrimental effects in another setting. Multiple methods were attempted to investigate the significant decrease in β-cell mass observed in db/+ animals. TUNEL and caspase-3 immunolabeling was conducted on pancreatic tissue sections to determine if apoptosis was the cause of reduced β-cell mass. However, TUNEL labeling was largely non-specific and caspase-3 immunolabeling co-localized with primarily α cells, a phenomenon we have observed before. Another method of assessing whether apoptosis was increased in db/+ animals with CCN2 induction was gene expression analysis of multiple cell death markers such as *Bad, Bax, Bcl-xL* and *Casp3.* There was no difference in expression of any of these

markers between groups. As mentioned previously, mRNA expression does not always directly correlate with protein expression. Thus, immunolabeling for other cell death markers would be a more direct way of assessing if apoptosis was increased in db/+ animals with CCN2 induction. Finally, while β-cell atrophy is not a common mechanism by which β-cell mass is lost, β-cell size was analyzed and no difference between groups was observed.

Previous studies in the lab demonstrated that db/+ mice exhibit oxidative stress in β cells as measured by percentage of β cells with 8-OHdG. In that study, db/+ mice had comparable levels of 8-OHdG in β cells as db/db mice [409]. Studies conducted in this dissertation examined percentage of 8-OHdG in db/+ mice compared to mice on a wild-type background and a similar result was obtained [Figure 5-3 in Chapter V]. db/+ mice had significantly more nuclear 8-OHdG compared to wild-type mice, and there was a trend toward higher expression of BiP compared to wild-type mice. One reason why CCN2 induction is detrimental in db/+ mice could be the presence of pre-existing β-cell stress before induction of CCN2. It is possible that the synthesis and secretion of CCN2 by β cells adds additional stress to db/+ animals which could result in eventual β-cell death leading to a loss of β-cell mass. Further investigation needs to occur to understand how CCN2 induction resulted in a significant reduction in β-cell mass in this *in vivo* model of chronic stress.

Altogether, the results of the studies described in this dissertation demonstrate that cell stress is necessary, but may not be sufficient for CCN2 to induce β-cell proliferation past a certain threshold. Furthermore, contrary to my hypothesis, CCN2 did not function to alleviate β-cell stress as measured by 8-OHdG, BiP and p-Nrf2. Future

studies utilizing other models of β-cell stress could be conducted to determine if CCN2 can induce β-cell mass expansion in those settings. One model is partial pancreatectomy which involves loss of a significant portion of the pancreas leading to more stress on the remaining β cells to maintain glucose homeostasis, similar to what we hypothesize about the CCN2 induction during β-cell ablation model. Another model is pregnancy where there is gradual weight gain and increased insulin resistance, causing maternal β-cell mass expansion to occur to meet the demand for insulin [436]. Induction of CCN2 in these models would provide more details about in which settings CCN2 can induce β-cell mass expansion and further understanding about the protein.

While the long-term goal of studying CCN2 is to potentially provide a therapeutic for T2D, it is also possible that CCN2 could provide beneficial effects during pre-diabetic conditions. While it is known that prolonged β-cell stress due to glucolipotoxic conditions is present during diabetes, it has also been demonstrated that pre-diabetic conditions, characterized by insulin resistance and elevated plasma glucose (but not to the degree of overt diabetes), are also a source of β-cell stress. As mentioned previously, it may be that CCN2 can only stimulate β-cell mass expansion up to a certain threshold of β-cell stress. Thus, while CCN2 does not induce β-cell proliferation under euglycemic, unstressed conditions, the oxidative stress present in pre-diabetic hyperglycemic conditions could be sufficient for CCN2 to stimulate β-cell mass expansion. Usage of animal models of pre-diabetes could provide information about whether CCN2 can induce β-cell mass expansion during these milder stress conditions. The results of those studies could provide further information about whether CCN2 can be used for both pre-diabetes as a potential therapeutic or deterrent against the eventual development of

overt diabetes, and overt diabetes as a method of increasing insulin output to promote better glucose homeostasis.

## APPENDIX I: Investigating the role of c-Met signaling in CCN2-induced β-cell proliferation.

### Hepatocyte growth factor (HGF) and c-Met signaling

c-Met is a cell surface receptor tyrosine kinase that exists as a single-pass transmembrane disulfide bond-linked α/β heterodimer. This cell surface receptor is expressed on epithelial cells of many organs including the liver, pancreas, prostate, kidney, muscle and bone marrow during both embryogenesis and adulthood [441]. The high-affinity ligand for c-Met is hepatocyte growth factor (HGF), which was originally identified as a circulating factor in liver regeneration called scatter factor. When assessing its function, HGF was also identified independently as both a motility factor and scatter factor for hepatocytes. HGF acts as a pleiotropic factor and cytokine to promote proliferation, survival, motility, scattering, differentiation and morphogenesis. HGF is secreted by mesenchymal cells as a single-chain biologically inert precursor and gains biological activity when it is cleaved by extracellular proteases into its mature form which consists of an α and β chain held together by a disulfide bond [442].

HGF/c-Met signaling is crucial for development – global c-Met inactivation is embryonically lethal. Animals with c-Met global deletion have marked decrease in liver size, damage of liver parenchyma, complete loss of myotubes in limbs, shoulders and diaphragm, and defects in placental development. The phenotype of global c-Met deletion is very similar to global loss of HGF during embryogenesis, providing further evidence of the importance of HGF/c-Met signaling during the developmental process [443,444]. Briefly, HGF binding to c-Met results in receptor homodimerization and phosphorylation of two key tyrosine residues (Y1234 and Y1235) which are in the

catalytic loop of the tyrosine kinase domain. Two other tyrosine residues of importance are Y1349 and Y1356 which, when phosphorylated, cause the recruitment of multiple adaptor molecules such as GRB2 and SH2. The adaptor molecules then interact and activate effector molecules such as PI3K, PLCγ, and Src. Pathways activated include the MAPK pathway which results in cell proliferation, motility, and cell cycle progression. Furthermore, PI3K and subsequent Akt activation governs the cell survival phenotype of c-Met [445].

**HGF/c-Met signaling and the endocrine pancreas**

Expression of c-Met is dynamic throughout life. The level of c-Met mRNA is more than ten-fold higher in the fetal pancreas than in the adult pancreas. During adulthood, c-Met expression is concentrated in the islets, with isolated islets containing almost tenfold more c-Met expression than that of the total pancreas. In the adult islet, c-Met is primarily expressed on the surface of β cells. HGF is expressed during pancreas development, but is primarily expressed in islets in the intra-islet vascular endothelium during adulthood. The intra-islet vascular endothelium produces and secretes HGF, which can then bind to c-Met on the surface of β cells. It has been known for over two decades that HGF, through its interaction with c-Met on β cells, is a potent β-cell mitogen. In 1996, Otonkoski et al. demonstrated that conditioned medium from human fetal pancreatic fibroblasts stimulated β-cell proliferation in human fetal islet-like cell clusters. This proliferative effect was blocked by the addition of an anti-HGF antibody, providing early evidence that HGF acts as a mitogen for fetal β cells. To further supplement this result, the authors of this same study treated human fetal islet-like cell clusters with recombinant HGF and observed the same proliferative effect as seen

when treating with conditioned medium [446]. While the study described previously was performed with fetal islet-like cell clusters, later studies demonstrated that addition of recombinant HGF to adult human β cells in culture could stimulate β-cell proliferation [447]. The findings presented in both of these studies established that HGF is a potent β-cell mitogen in vitro.

There have been many studies using in vivo models of both HGF/c-Met overexpression and HGF/c-Met inactivation. A transgenic mouse model of HGF overexpression was generated using the RIP to overexpress HGF solely in β cells (RIP-HGF) [152]. Because the RIP is expressed during embryogenesis, mice with the RIP-HGF transgene experienced HGF overexpression beginning during embryogenesis and continuing into adulthood. HGF overexpression in β cells led to an increase in islet number and mass, with islet volume being two- to three-fold higher per unit of pancreatic volume, primarily due to an increase in β-cell proliferation by a factor of 2.5 compared to control [152]. Of note, this increase in islet volume was not accompanied by a decrease in exocrine volume, and thus was not due to a change in cell fate from multipotent pancreatic progenitors during development. Islets from RIP-HGF mice secreted significantly more insulin than normal islets when assessing glucose-stimulated insulin secretion (GSIS) by static incubation, leading to mild hypoglycemia and inappropriate hyperinsulinemia in vivo. It was postulated that this was due to the noted overexpression of insulin per β cell in the mice with HGF overexpression compared to controls [152]. This finding suggests that HGF may play a role in regulating β-cell function. In addition to promoting β-cell proliferation, HGF also enhanced β-cell survival in the setting of streptozotocin-induced diabetes; mice with HGF

overexpression became only mildly hyperglycemic compared with controls, which developed overt diabetes [132].

Multiple studies have been conducted to investigate how loss of HGF alters β-cell mass. It was found in two separate studies that disruption of HGF/c-Met signaling does not affect β-cell mass during basal conditions [153,154]. However, when presented with a challenge such as pregnancy or β-cell injury, it appears that HGF/c-Met signaling is required for β-cell compensation [153,154]. During pregnancy, maternal β-cell mass expansion is required to meet the demands for insulin. Furthermore, it has been established that wild-type pregnant mice display significant HGF upregulation at gestational day 15 (GD15), which is the peak of maternal β-cell proliferation [153]. To investigate the effect of embryonic loss of c-Met, mice with the Pdx1-Cre transgene were interbred with mice homozygous for the c-Met floxed allele. The loxP sites on the c-Met floxed allele flank exon 16 which encodes a critical ATP-binding site in the intracellular tyrosine kinase domain which is essential for activation of the receptor. Mice with the genotype c-Metfl/fl;Pdx-Cre (PancMetKO) thus have β-cell-specific c-Met inactivation beginning during embryogenesis and persisting into adulthood.

Adult PancMet KO mice displayed normal glucose homeostasis, β-cell mass, and β-cell proliferation under basal conditions, which has been reported previously. During pregnancy, body weight was similar between PancMet KO and wild-type pregnant dams. β-cell mass was also similar at GD11 and GD15 as well as during nonpregnant conditions. At GD19, which is the time when maximal β-cell mass expansion occurs in wild-type pregnant mice, it was noted that PancMet KO pregnant dams had significantly decreased β-cell mass which was attributed to significantly impaired β-cell proliferation

and significantly increased β-cell apoptosis [153]. Due to this lack of proper maternal β-cell adaptation as well as a concurrent decrease in insulin secretion, pregnant PancMet KO mice developed gestational diabetes [153]. Taken together, the results of this study concluded that HGF/c-Met signaling is required for sufficient β-cell mass expansion during pregnancy in order to maintain proper glucose homeostasis [153].

The role of HGF/c-Met signaling was also investigated in PancMet KO mice using two models of β-cell mass reduction: partial pancreatectomy (Ppx) removing ~50-60% of the pancreas, and multiple low-dose streptozotocin administration (MLDS) [154]. It was already well-established that HGF plays a role in liver regeneration, however it was unknown at the time whether HGF played a role in β-cell regeneration. Seven days post-Ppx, β-cell proliferation was significantly decreased in PancMet KO mice compared to controls [131]. Similar results were found when assessing the MLDS model 20 days post-MLDS treatment, indicating that HGF/c-Met signaling is required for β-cell regeneration in these models. In converse studies, wild-type mice were administered exogenous HGF seven days post-Ppx. Daily HGF administration significantly increased β-cell proliferation subsequently leading to significantly increased β-cell mass at twelve days post-Ppx [154]. This further underscores the importance of HGF/c-Met signaling in β-cell regeneration.

The role of CCN2 in the endocrine pancreas has been heavily discussed in this dissertation. More information that remains to be learned about CCN2 is through what pathway it signals. In the 50% β-cell ablation and CCN2 induction model, a TaqMan low density array (TLDA) was utilized to analyze gene expression in isolated islets from animals with 50% ablation and CCN2 induction for two days. It was found that there was

upregulation of various molecules associated with cell proliferation, including molecules that have been implicated in stimulating β-cell proliferation such as *Tph1, Hgf,* and *Met* [133]. As mentioned previously, HGF and c-Met have been discovered to be molecules that are crucial for β-cell proliferation and regeneration. In the islet, HGF is created and secreted by vascular endothelial cells, while c-Met is expressed on the surface of β cells [134]. One hypothesis by which CCN2 induces β-cell proliferation is through its action on the vascular endothelium. I hypothesized that CCN2 interacts with endothelial cells which then synthesize and secrete HGF. HGF then binds to c-Met on the surface of β cells to stimulate proliferation.

**Generation of mouse model with CCN2 overexpression in the setting of c-Met inactivation**

With this hypothesis in mind, I generated an embryonic mouse model to investigate if CCN2 induction in the setting of c-Met inactivation would attenuate CCN2-induced β-cell proliferation. This mouse model was generated by combining the RIP-rtTA, TetO-CTGF, c-Met$^{floxed}$, and RIP-CreER alleles. The c-Met floxed allele has loxP sites flanking exon 16 which encodes the cytoplasmic activation domain of the receptor [448]. Cre recombination would prevent signaling through the c-Met receptor as autophosphorylation and subsequent activation would not be able to occur upon HGF binding to the receptor. An animal harboring all four alleles would have β-cell-specific CCN2 induction in the presence of DOX, and inactivation of c-Met after tamoxifen administration. To test my hypothesis, animals harboring all four alleles were generated and four groups were created:

1. c-Met$^{fl/fl}$;RIP-CreER;RIP-rtTA;TetO-CTGF (no manipulation)

2. c-Met$^{fl/fl}$;RIP-CreER;RIP-rtTA;TetO-CTGF with DOX (CCN2 OX)
3. c-Met$^{fl/fl}$;RIP-CreER;RIP-rtTA;TetO-CTGF with tamoxifen (Met KO)
4. c-Met$^{fl/fl}$;RIP-CreER;RIP-rtTA;TetO-CTGF with DOX and tamoxifen (CCN2 overexpression in the setting of Met inactivation)

In this model, pregnant dams receive DOX at e9.5 (two days prior to RIP activation) to induce CCN2 in quadruple transgenic pups. Pregnant dams receive two injections of 7.5 mg tamoxifen at e13.5 and e15.5 (for a total of 15 mg) to promote β-cell-specific c-Met inactivation in quadruple transgenic pups. At e18.5, embryonic pancreata are dissected from the pups to be analyzed. These pancreata are fixed, paraffin-embedded, sectioned, and immunolabeled for insulin, glucagon and Ki67 to analyze α- and β-cell proliferation (analysis ongoing). If c-Met is required for CCN2 to induce β-cell proliferation, CCN2 overexpression in the setting of c-Met inactivation should not result in increased β-cell proliferation compared to the no manipulation condition. Furthermore, based on previous studies, the CCN2 overexpression alone group should have significantly more α- and β-cell proliferation than the other three groups. Total endocrine area will be assessed due to the finding that embryonic CCN2 overexpression caused an increase in total endocrine area [392].

Another method of examining if c-Met is required for CCN2-induced β-cell proliferation is assessment of downstream molecules activated by c-Met signaling. Examples of this are PLCγ1 and PKCζ, both molecules that have been demonstrated to be important for β-cell proliferation in other studies [153,449]. By using this embryonic experimental model, a potential signaling mechanism by which CCN2 stimulates β-cell

proliferation may be revealed. In the future, manipulation of this pathway could have therapeutic potential for the treatment of T2D.

## APPENDIX II: Investigating the role of β1 integrin in CCN2-induced β-cell proliferation.

Integrins are transmembrane heterodimeric cell surface receptors that bind to extracellular components and are expressed on virtually all cell types. Integrins are composed of one α and one β subunit. These cell surface receptors affect a variety of cell processes including adhesion, migration, differentiation, and cell growth. In mammals, 18 α and eight β subunits have been identified, which combine to create 24 unique heterodimers. Both the α and β subunits are involved in determining ligand specificity; however, the β subunit facilitates adhesion and activates intracellular second messenger cascades [450]. Common ligands of integrins are extracellular matrix components, such as laminin, fibronectin, and collagen.

Integrins can also engage in crosstalk with growth factor receptors. The signals that are activated by the integrin-focal adhesion kinase (FAK)-Src complex can be integrated with signals from growth factor receptor binding to subsequently activate the Ras-MEK-MAPK pathway, which is canonically associated with cell proliferation and survival [451]. Some examples of growth factor receptors with which integrins act synergistically are VEGFR, c-Met, and EGFR, all of which are involved in β-cell proliferation [102,146, 452]. Furthermore, integrins can regulate growth factor receptor availability by altering their rate of internalization and degradation, thus directly affecting signaling through these receptors [451].

As mentioned previously, there are 18 α and 8 β subunits of integrins which combine to create 24 distinct heterodimers, most of which contain the β1 subunit. The β1 integrin subunit has been shown to pair with multiple α subchains, forming twelve

different known integrins [145]. It has been established that β1 integrin is necessary for development, as global deletion of β1 is embryonically lethal [146]. Due to its ability to dimerize with multiple α subunits, this integrin subunit is also capable of binding multiple ECM components such as laminin, collagen, and fibronectin [141]. Common signaling molecules that are activated by stimulation of β1 integrin are FAK, ERK, Akt and the Src family of kinases, all of which are involved in either cell survival or proliferation.

In the pancreatic islet, the specific α and β subunits expressed on endocrine cells differ among species. Integrin signaling is critical for pancreatic endocrine specification during development. In the developing pancreas, differentiation of endocrine cells from bipotent duct/endocrine progenitors within the pancreatic trunk epithelium requires decreased interaction between $\alpha_5\beta_1$ integrin and the ECM substrate [453]. Similarly, inhibition of FAK or Src promotes endocrine differentiation from human embryonic stem cells [454]. On the β cell, β1 integrin is the most highly expressed integrin subunit. β1 integrin promotes β-cell proliferation and survival, and facilitates the migration of endocrine progenitors during pancreas development [102].

*Ex vivo* studies using INS1 and MIN6 cells demonstrated that blockade of β1 integrin signaling using specific function-neutralizing antibodies attenuates the beneficial effects that ECM components, such as collagen and laminin, have on β-cell proliferation and survival. Blockade of β1 integrin also decreases mRNA expression of *insulin*, elucidating another method by which β1 integrin can promote β-cell survival [137,139]. Similar findings have been demonstrated in primary rat β cells and fetal human islets plated on laminin where blockade of β1 integrin increased apoptosis and attenuated phosphorylation of both FAK and ERK, two kinases involved in promoting cell survival in

general. Furthermore, *insulin* and *Pdx1* mRNA were reduced under this condition [142,452]. These findings demonstrate that β1 integrin interaction with ECM components promotes β-cell proliferation and survival.

β1 integrin is highly expressed in developing duct, acinar, and insulin-producing cells of the pancreas. Inactivation of β1 integrin has been demonstrated to affect proliferation and survival of β cells *in vivo*. The Cirulli group investigated the role of β1 integrin in β-cell expansion by generating a β-cell-specific β1 integrin inactivation mouse model. Transgenic mice in which Cre recombinase is driven by the Rat Insulin Promoter (RIP) were used to inactivate the β1 integrin gene (*Itgb1*), thus removing β1 integrin from the surface of β cells (RIP-Cre/β1KO). To generate RIP-Cre/β1KO mice, mice with the RIP-Cre allele were interbred with mice harboring the β1 integrin-floxed allele. The loxP sites on the β1 integrin gene are flanking exon 3, which encodes for an extracellular portion of β1 integrin. Therefore, deletion of this exon inactivates β1 integrin signaling. RIP-Cre mediated recombination resulted in deletion of *Itgb1* in insulin-expressing during embryogenesis but only a slight reduction in expression of β1 integrin protein was observed on β cells. RIP-Cre/β1KO mice also had reduced β cell numbers, and β-cell area was reduced by ~80% in these mice compared to control in adulthood. RIP-Cre/β1KO mice also had significantly less larger islets (>100 cells) compared to control mice, which was due to a defect in proliferation at all stages examined (E17.5, P4, 8 weeks). The defect in proliferation was attributed to a downregulation of cell cycle genes including *Ccnd1, Ccnd2* and *Ccne1*, and upregulation of positive regulators of cell cycle arrest [147].

β-cell-specific β1 integrin inactivation postnatally also generates similar results. The Wang group used a tamoxifen-inducible β-cell-specific β1 integrin inactivation mouse line to inactivate β1 integrin on β cells during adulthood [148]. Tamoxifen was administered to four-week-old male and female mice to inactivate β1 integrin specifically in β cells. Eight weeks after tamoxifen injection, mice were characterized. Male mice with β-cell-specific β1 integrin inactivation (MIPβKO) were glucose intolerant and had significantly lower levels of plasma insulin during IPGTTs [148]. GSIS was assessed *ex vivo* using the static incubation assay, and it was observed that MIPβKO islets had significantly decreased insulin secretion under basal and high glucose conditions. It was observed that there was a significant reduction in proteins involved in exocytosis and insulin secretion such as *Snap25* and *Vamp2*, which is a potential mechanism for decreased insulin secretion in MIPβ1KO islets [148]. *Pdx1* mRNA and protein expression were significantly decreased in male MIPβKO mice, and β-cell mass was significantly reduced as well. MIPβKO pancreata had a significantly lower number of large islets compared to control. The decrease of β-cell mass was observed concurrently with decreases in β-cell proliferation and increases in the apoptotic marker cleaved-Poly (ADP-ribose) polymerase (c-PARP). Female MIPβKO mice exhibited the same phenotypes; however, significant differences were not observed until 16 weeks post-tamoxifen. It was also noted that these phenotypes were maintained as the mice aged [148]. In conclusion, the results of this study illustrate that β1 integrin plays a key role in β-cell mass expansion and β-cell mass maintenance from embryogenesis to the postnatal period.

There is a potential link between CCN2-induced β-cell proliferation and integrins, specifically β1 integrin. There is currently no known receptor for CCN2; however, it has been discovered that CCN2 can bind to integrins through its CT domain [review of ccn2], and that CCN2 signals through β1 integrin to affect adhesion, migration and differentiation of multiple cell types [387; 455]. Previous studies in the lab utilizing the CCN2 induction and β-cell ablation model discovered that expression of *Itgb1*, the gene for β1 integrin, was upregulated two days following CCN2 induction and β-cell ablation [ref]. Furthermore, studies previously described demonstrated that β1 integrin is involved in both embryonic and postnatal β-cell proliferation. Taking all this together, these data suggest that β1 integrin may play a role in CCN2-mediated β-cell proliferation. Thus, I conducted studies utilizing a β1 integrin-neutralizing antibody in the presence of recombinant human CCN2 (rhCCN2) to elucidate if CCN2 can induce β-cell proliferation in the setting of diminished β1 integrin signaling.

**Methods**

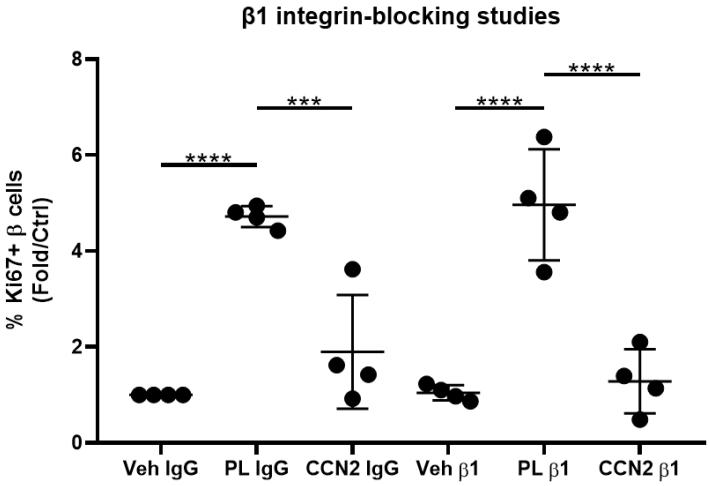
*Islet isolation*

Islets were isolated from male and female mice by the Islet and Pancreas Analysis Core. Briefly, after euthanasia, pancreata were perfused with 0.5 mg/mL type IV collagenase that was dissolved in Hanks Balanced Salt Solution as described previously [395]. Wild-type C57BL/6J islets from male and female mice were pooled, handpicked and cultured overnight in 11 mM glucose RPMI-1640 with 10% horse serum and 1% penicillin/streptomycin (P/S) in preparation for *ex vivo* assays.

*Proliferation assays with β1-integrin neutralizing antibody*

Forty islets per well were cultured overnight following islet isolation. After overnight recovery, islets were incubated in control non-specific IgG or β1-integrin neutralizing antibody for 24h in culture media (11 mM glucose RPMI-1640 with 10% horse serum and 1% penicillin/streptomycin (P/S)). Following the 24h incubation, islets were treated with either vehicle (PBS) or 250 ng/mL rhCCN2 for four days according to standard proliferation assay protocol. After four days, islets were harvested, cytospun onto charged slides and immunolabeled for insulin and Ki67 (antibody information listed in Methods section). Islets were imaged using a ScanScope FL scanner and a CytoNuclearFL algorithm in eSlideManager was utilized to analyze β-cell proliferation as measured by double-positive insulin/Ki67 cells (Aperio Technologies, Inc.).

**Results and Discussion**




**β1 integrin blockade does not alter basal β-cell proliferation nor supperss placental lactogen-induced β-cell proliferation.** The results of this study are hard to interpret because rhCCN2 alone did not induce β-cell proliferation. However, it is apparent that blockade of β1 integrin does not affect basal or PL-induced β-cell proliferation. n=4 biological replicates. One-way ANOVA with Tukey *post hoc* analysis was utilized to analyze this data. F=25.31, $p<0.0001$. ***$p<0.001$, ****$p<0.0001$

The goal of this study was to assess whether blockade of β1 integrin signaling would attenuate CCN2-induced β-cell proliferation. My hypothesis was that it would –

however, the experiment could not be carried out successfully because rhCCN2 alone did not induce β-cell proliferation which has been reported in the past. In the future, obtaining rhCCN2 with activity would allow for these studies to be repeated, and for the experimental question to be answered. However, at this time, it is still unknown whether β1 integrin signaling is required in part for CCN2-stimulated β-cell proliferation.

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