# MODULATION OF β-CELL INTRINSIC AND EXTRINSIC CHARACTERISTICS BY CTGF TO PROMOTE β-CELL MASS REGENERATION

By

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# CHAPTER I

# INTRODUCTION

## Significance

As of 2010, approximately 26 million Americans (8.3% of the population) have diabetes, a heterogeneous group of disorders characterized by a decrease in functional insulin-producing β-cells resulting in increased blood glucose. Diabetes results from absolute (type 1) or relative (type 2) loss of functional β-cell mass. Whereas type 1 diabetes is characterized by the selective autoimmune destruction of β-cells (1), type 2 diabetes occurs when the β-cell population fails to compensate for increased peripheral insulin resistance that is typically associated with obesity (2). Both forms of the disease would greatly benefit from treatment strategies that enhance β-cell regeneration, proliferation, and function. Although there were initial encouraging results from islet transplantation in achieving remission of type 1diabetes (3, 4), this was limited by the low amount of donor tissue obtainable. In addition, islet transplantation is short-lived and inefficient, requiring multiple islet donors per patient. Nearly all islet transplants fail with the first three years due to a combination of factors including ongoing autoimmunity and a less than ideal microenvironment in the liver (5).

The ability to induce the differentiation of β-cells or whole islets from resident adult pancreatic non-β-cells or other cell types *in vivo* or from embryonic stem (ES) cells

or induced pluripotent stem (iPS) cells *in vitro* would provide an alternative source to replenish β-cell mass in individuals with diabetes (6). Studies addressing the potential of adult pancreatic cell types to undergo proliferation, regeneration, transdifferentiation, and neogenesis to generate β-cells could lead to the restoration of β-cell mass in individuals with type 1 diabetes and enhanced β-cell compensation in patients with type 2 diabetes. Any approach to generate islet endocrine cells *in vivo* or *in vitro* will benefit greatly from a thorough understanding of the normal developmental processes that occur during pancreatic organogenesis (e.g., transcription factors, cell-signaling molecules, and cell–cell interactions that regulate endocrine differentiation and proliferation).

Much progress has been made in identifying factors involved in normal development and differentiation of the various pancreatic cell types. Interestingly, mutations in several developmentally important factors have been identified in individuals with diabetes. Knowledge of the pancreas development program is being used to generate insulin-producing cells or islets from stem cell sources (ES or iPS cells) *in vitro* or via transdifferentiation of embryonically related cells *in vivo* (7, 8). Of particular interest are the transcription factors, signaling molecules, and cell-cell interactions that regulate differentiation of progenitors into endocrine cells and their subsequent proliferation.

## Pancreas Development

### Summary of pancreatic development and islet genesis

The mature pancreas is composed of two distinct functional units: exocrine and endocrine. The exocrine component of the pancreas consists of clusters of acinar cells that produce and secrete digestive enzymes such as amylase and elastase, and the ductal network, which transports the acinar secretions into the rostral duodenum. The exocrine portion makes up ~98% of the adult organ. The endocrine compartment is composed of spherical clusters of at five hormone-producing cell types: insulin (β-cells), glucagon (α-cells), somatostatin (δ-cells), ghrelin (ε-cells), and pancreatic polypeptide (PP cells; Figures 1-1 and 1-2). These endocrine clusters comprise microorgans known as *islets of Langerhans.* Together, these islet hormones regulate glucose homeostasis by facilitating the uptake of ingested glucose into cells and stimulating glucose production by the liver during times of fasting. Acinar, ductal, and endocrine cells are all derived from the endoderm during embryonic development (9).

The epithelial component of the pancreas arises from dorsal and ventral outgrowths of the posterior foregut endoderm just caudal to the liver diverticulum in all vertebrates examined, including humans (see Figure 1-1; (10-14)). The dorsal and ventral buds later fuse to form a single organ (this occurs on embryonic day (e) 12.5 in the mouse). Pancreatic bud outgrowth can be observed as early as day 25 of gestation in humans (15), e9.5 in the mouse (16), and 24 hours postfertilization in zebrafish (see Figure 1-2; (12). In mammals, frogs, and chickens, both pancreatic buds consist of

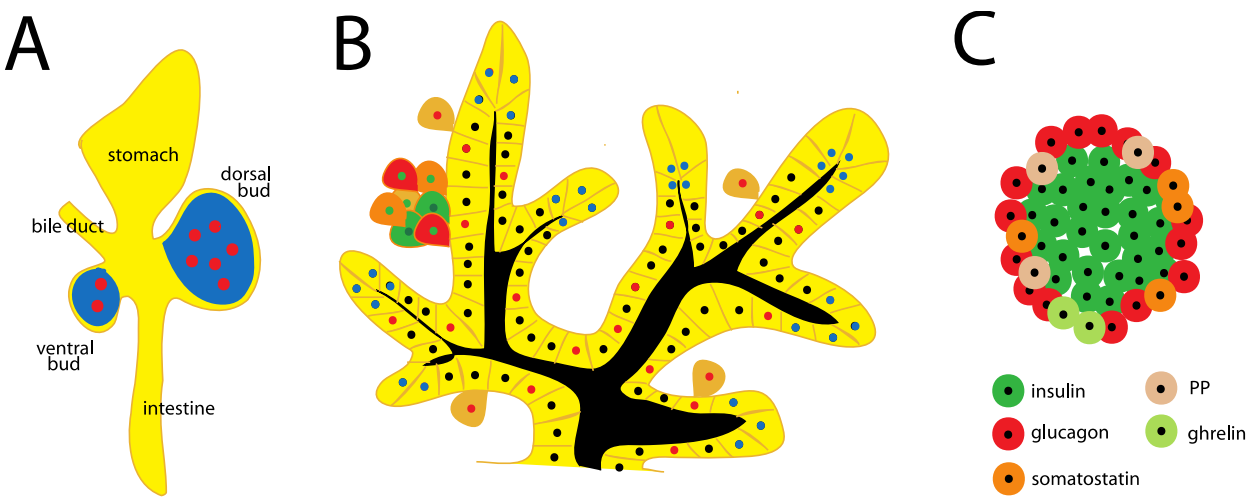


**Figure 1-1.** Schematic of pancreas and islet development. **A,** The pancreas arises as dorsal and ventral evaginations from the posterior foregut endoderm on embryonic day (e) 9.5, which is marked by the expression of the *Pdx1* transcription factor *(yellow)*. Markers of the early pancreatic buds include the transcription factors *Ptf1a* and *Hb9 (blue)*. Within the developing buds, a subset of cells expresses markers of the endocrine lineage, including *Ngn3, Isl-1,* and *Pax6 (red)*. **B,** As development proceeds, the pancreatic epithelium *(yellow)* becomes a highly branched ductal network. Endocrine cells *(green nuclei)* and exocrine cells *(blue nuclei)* arise from the ducts. Endocrine progenitors are scattered throughout the epithelium, and they express *Ngn3 (red nuclei)*. These cells maintain *Ngn3* expression as they delaminate from the epithelium *(tan-colored cells)*. *Ngn3* is downregulated as hormone expression begins *(green, red, and orange cells)*, and more general endocrine markers such as *Isl-1* and *Pax6* are expressed *(green nuclei)*. **C,** Mature islets begin to form during late gestation. In the mouse, insulin-producing β-cells *(green)* are found at the islet core, and all other hormone-producing cells are located at the periphery. From Pancreas Development and Regeneration. K. Riley and M. Gannon. *Principles of Developmental Genetics*, 2nd Edition, Copyright © [2014].

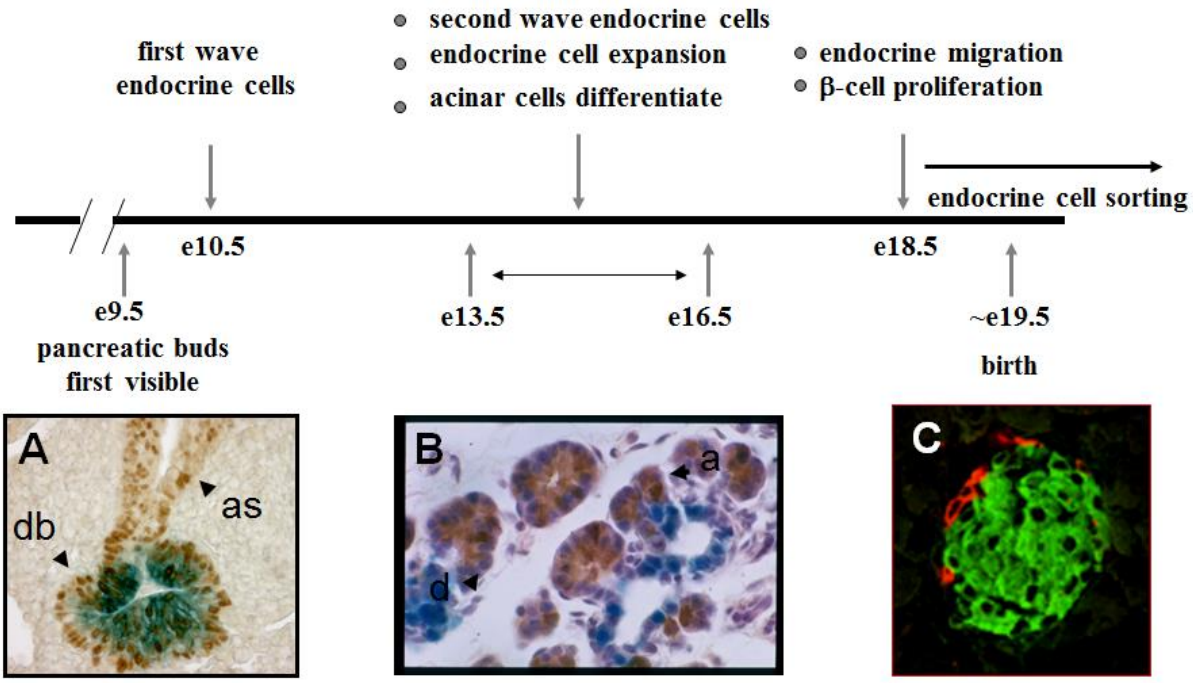


**Figure 1-2.** Timeline of pancreas development in the mouse. Key events in mouse pancreas development are shown. **A,** Pancreatic bud evagination can first be detected on e9.5. In this image, the *Pdx1* expression domain is marked by the brown nuclei, and it includes the antral stomach *(as)* and the dorsal pancreatic bud *(db)*, which is also marked by a pancreas-specific lacZ transgene *(blue)*. Endocrine differentiation occurs in two waves. The first begins on e10.5 and extends to e13.5. The second wave begins on e13.5 and continues until neonatal stages. **B,** Acinar gene expression *(a; amylase in brown)* begins on e14.5. Both acinar cells and endocrine cells bud off of the ductal epithelium *(d; blue)*. **C,** During late gestation, endocrine cells cluster, migrate away from the ductal epithelium, and organize into islets with β-cells at the core and other hormone cell types at the periphery. *Green,* Insulin, *red,* glucagon. From Pancreas Development and Regeneration. K. Riley and M. Gannon. *Principles of Developmental Genetics*, 2nd Edition, Copyright © [2014].

multipotent progenitor cells (MPCs) that generate exocrine and endocrine cells (It is currently not clear whether these MPCs represent a homogeneous or heterogeneous population.). MPCs of the early pancreatic buds are demarked by expression of *Pdx1, Ptf1a, Nkx6.1, Sox9, Hnf6, and Hnf1β*, each of which will be discussed later. By contrast, in zebrafish, the posterior (ventral) bud generates the endocrine tissue, which usually consists of a single islet, whereas the anterior (dorsal) bud gives rise primarily to the pancreatic duct and the acinar cells, although endocrine cells do arise from dorsal bud derivatives later during development (12).

Morphogenesis of the pancreatic epithelium yields a highly branched ductal network within which multipotent progenitors for both exocrine and endocrine cells reside (see Figures 1-1 and 1-3; (17). At approximately e12.5 in the mouse, the branching pancreatic epithelium becomes divided into 'tip' and 'trunk' domains via morphological and gene expression pattern changes. 'Tip' portions of the developing pancreas retain MPCs that express a unique combination of molecular markers compared with those found in the emerging buds earlier in development. The 'tip' MPCs express *Ptf1a, c-Myc,* and *Cpa1,* and continue to generate all pancreatic cell types (endocrine, duct or acinar). In addition, this MPC population expresses *Hnf6* and *Pdx1*. *Hnf6* and *Pdx1* are expressed at lower levels in tip cells than observed in the original pancreatic bud MPCs. 'Tip' cells lose their multipotency by e14.5, a period known as the "secondary transition" which is discussed in more detail below. At this time point, the 'tip' cell fate becomes restricted solely to the acinar lineage. The 'trunk' domain cells originate from the main duct and extend to the border of the 'tip' progenitor

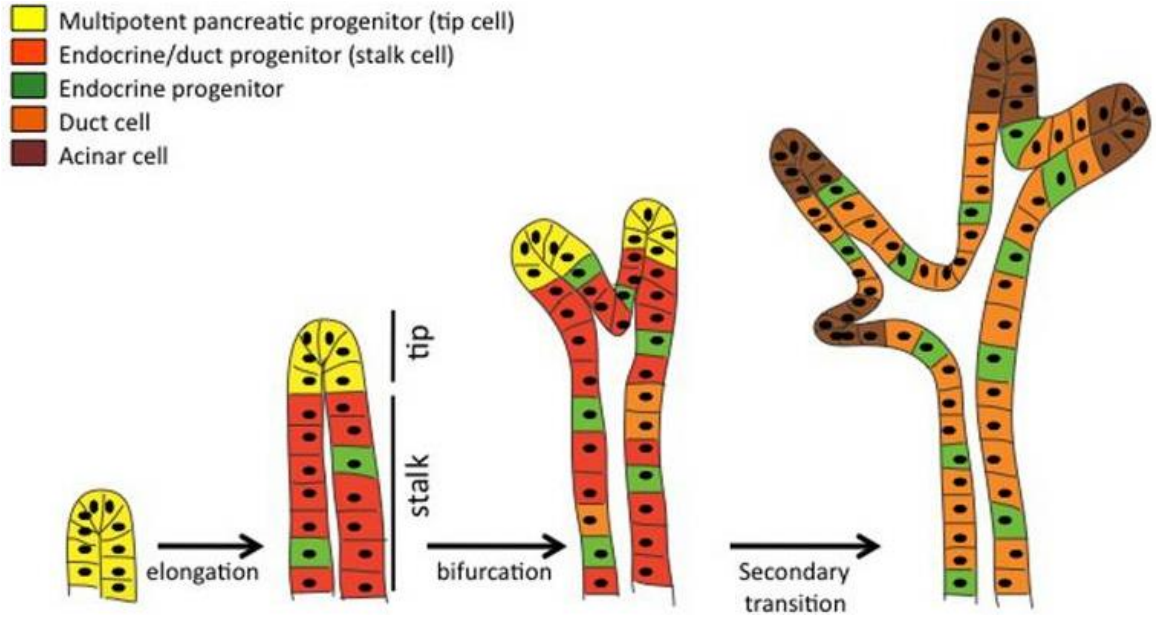


**Figure 1-3.** Location of pancreatic progenitors during branching morphogenesis. Multipotent pancreatic progenitors (yellow) are localized to tips of developing epithelial branches. As branches elongate at the tips, cells remaining in the stalks (red) lose the potential to differentiate as acinar cells. Cells that will give rise to definitive duct cells (orange) and endocrine progenitors (green) become specified in the stalks as development continues. Lastly, during the secondary transition, cells at the tips begin to differentiate as acinar cells (brown). Adapted from Pancreas Cell Fate. M. Guney and M. Gannon. *Birth Defects Research C Embryo Today*, 87(3), Copyright © [2009].

population. ‘Trunk’ cells express *Hnf6*, *Hnf1β, Sox9, Muc1,* and *Nkx6.1* and are capable of giving rise to either duct or endocrine cells.

Expression of endocrine hormones such as glucagon and insulin is detected even at early pancreatic bud stages (e10.5); exocrine-specific gene transcription does not commence until e14.5 (see Figure 1-2; (18). Pancreatic endocrine differentiation occurs in two waves during embryogenesis (see Figure 1-2; reviewed in (19). The first wave occurs between e9.5 and e13.5. Unlike second-wave endocrine cells, these early differentiating hormone-producing cells can develop in the absence of the pancreatic duodenal homeobox 1 (*Pdx1)* gene, a critical pancreatic transcription factor (20-22), and lack other genetic markers of mature islet endocrine cells (22-24), These early hormone+ cells have been shown by lineage-tracing analyses to not contribute to mature islets ((25, 26). During the second wave, or the “secondary transition,” of endocrine differentiation, which commences on e13.5 in the mouse, the numbers of endocrine cells greatly increase. These endocrine cells go on to populate the mature islets. The mechanism for the increase in endocrine differentiation at this stage is unknown. The formation of mature, optimally functional islets requires the generation of appropriate numbers of each endocrine cell type, and this process is likely regulated by positive and negative factors that influence cell proliferation and differentiation. As an example, recent studies indicate a role for two planar cell polarity (PCP) genes, Celsr2 and Celsr3, in β-cell differentiation during development; loss of these gene results in a decrease in β-cell number, while other endocrine cell types are not affected (27).

In a process reminiscent of neurogenesis in Drosophila and other organisms, islet organogenesis involves the delamination of specified endocrine cells from the ductal epithelium, migration away from the ducts, and the formation of adherent islet clusters (see Figure 1-1; reviewed in (28). As in Drosophila neurogenesis, the specification of endocrine progenitors within the ductal epithelium is dependent on cell–cell communication using the Notch-Delta signaling. Cells experiencing higher levels of Notch signaling remain within the epithelium and actively repress *neurogenin 3* (*Ngn3*; a transcription factor expressed in endocrine progenitors), whereas those cells in which Delta levels become elevated activate *Ngn3*, exit from the epithelium, and ultimately give rise to the endocrine population (see Figure 1-1; (29-34). Recent studies have shown that the Ptf1a transcription factor activates the Notch ligand, Dll1 in MPCs, thus providing an alternative, Ngn3-independent pathway to regulate Notch signaling and subsequent pancreas cell fate decisions (35).

After delamination, the endocrine cells begin to organize into clusters that are initially still located close to ducts (see Figure 1-1). Beginning around e18.5, these clusters lose their proximity to the ductal epithelium, and begin to form mature islets (see Figures 1-1 and 1-2). As islets form, the endocrine cells segregate such that, in mice, the insulin-producing β-cells are located at the core, and glucagon-, somatostatin-, ghrelin- and PP-producing cells are located at the periphery or mantle (see Figures 1-1 and 1-2). Little is known about how the different endocrine cell types and their precursors interact with one another to form functional islets. The processes of endocrine delamination and islet formation likely include changes in the expression of

lineage-specific transcription factors, cell adhesion molecules, and extracellular matrix components. For example, in the developing human pancreas, cell adhesion molecules and certain integrins mark endocrine progenitor cells within and delaminating from the ductal epithelium (36, 37).

**Inductive interactions during pancreas development**

Pancreas development is dependent on an interaction between epithelial (endodermal) and mesodermal components (reviewed in (19)). For example, signals from the notochord have been implicated in pancreas specification and early bud formation (10, 38-40), whereas pancreatic mesenchyme stimulates the overall growth of the endodermal epithelium (14, 41). In turn, the endoderm influences the character of the overlying mesoderm (16, 42).

Wessells and Cohen (1966) suggested that signals derived from dorsal axial tissue such as the notochord might be involved in inducing the outgrowth of the dorsal pancreatic bud. Experimental manipulations in chick embryos revealed that, in the absence of the notochord, the dorsal pancreatic bud undergoes only limited outgrowth and branching, and fails to activate the expression of pancreatic transcription factors (e.g., *Pdx1, Isl-1, Pax6*) and of markers of differentiated endocrine or exocrine cells (10). By contrast, the outgrowth and differentiation of the ventral pancreatic bud occurs normally in the absence of the notochord. Activin βB and fibroblast growth factor (FGF)-2 are likely to be the endogenous notochord-derived signals that induce dorsal pancreas bud outgrowth and differentiation (38). One of the main functions of

notochord-derived factors is repression of endodermal Sonic hedgehog (Shh) expression in the region that is destined to give rise to the pancreas (38). Transplantation of an ectopic notochord to nonpancreatic regions of the developing gut tube results in decreased Shh expression in the region that is adjacent to the transplant (38). Maintenance of Shh in presumptive pancreatic endoderm using a transgenic approach results in impaired development of the pancreatic epithelium and altered character of the overlying mesoderm such that it expresses markers consistent with small intestine smooth muscle (42).

The vasculature of the pancreas is derived from the mesodermal germ layer. Although islets represent only approximately 2% of the total mass of the pancreas in an adult, they receive up to 15% of the blood flow (43), facilitating secretion of hormones directly into the bloodstream. The morphology and architecture of endothelial cells differs among the different capillary beds (44). Capillaries in endocrine glands such as the islets are fenestrated (44, 45). Early differentiating pancreatic endocrine cells (both first- and second-wave cells) produce angiogenic factors including vascular endothelial growth factor (VEGF) and angiopoietin 1(46); the expression of these factors is maintained in adult islets suggesting that maintenance of a fenestrated endothelium is critical for mature islet function (47-49). In addition to islet endocrine cells communicating with vascular endothelial cells via secreted growth factors, endothelial cells also signal to the pancreatic epithelium, influencing the differentiation of endocrine cells (50-52).

Pancreatic bud outgrowth is initiated at sites in the posterior foregut endoderm, where it contacts the endothelium of major blood vessels. Endocrine differentiation initially occurs in cells that have direct contact with endothelial cells (49, 53). Endothelial cells also produce basement membrane components for developing islets (54). The importance of vascular endothelial cells in pancreatic endocrine differentiation has been demonstrated both in tissue recombination experiments and in genetically modified mice. For example, e8.5 endoderm cultured in the absence of endothelial cells failed to activate either Pdx1 or insulin protein expression; in undifferentiated endoderm cultured in combination with dorsal aorta, both Pdx1 and insulin were induced (49). Likewise, co-culture of mouse ES cells with human endothelial cells enhances the formation of insulin-producing cells (55). In VEGF receptor type 2 (VEGFR-2/flk-1) null mutant mice, which die before the second wave of endocrine differentiation, early insulin- and glucagon-positive cells fail to develop (49, 51). These mice express most pancreatic/endocrine transcription factors *(Pdx1, Hnf6, Ngn3, NeuroD, Prox1,* and *Hb9)*, with the exception of the early pancreatic bud marker, *Ptf1a* (51). Transgenic embryos expressing $VEGF_{164}$ throughout the entire pancreatic bud early during development show greatly increased vasculature in the pancreas and a corresponding threefold increase in islet number and islet area (50). The increase in endocrine area was at the expense of acinar tissue, suggesting endothelial factors promote the endocrine lineage (46). However, forced continuous over-expression of VEGF in embryonic β-cells results in impaired islet morphology and decreased β-cell proliferation and mass (56, 57), indicating the need for tight regulation of angiogenic factors.

These studies highlight the reciprocal communication between pancreatic endocrine cells and endothelial cells. Vascular endothelial cells produce several different secreted signaling molecules, including spingosine-1-phosphate, fibroblast growth factor (FGF), transforming growth factor (TGF)-β, Wnt, and hepatocyte growth factor (HGF; (58, 59). Pancreatic endothelial cells express HGF and HGF is mitogenic to β-cells (60-63). Thus, in the pancreas, endocrine-produced VEGF signaling through VEGF receptors on the endothelium may induce the expression of HGF, which in turn promotes endocrine proliferation.

**Endocrine specification and differentiation**

MPCs have the potential to give rise to all pancreatic cell types. Several lineage tracing studies have analyzed the cell fate potentials of these MPCs. Since these studies were performed on populations of cells and not clones derived from single cells, it remains unclear whether a single MPC has the potential to give rise to all the different pancreatic cell types. The MPC pool maintains itself through *Sox9*-mediated proliferation, and inactivation of *Sox9* in the developing pancreas results in severe pancreatic hypoplasia (64). Lineage tracing experiments revealed that *Sox9*+ MPCs give rise to exocrine, endocrine and ductal cells (65). *Pdx1* is also a marker of MPCs, as cells that at one time expressed *Pdx1* are able to give rise to all types of pancreatic cells (25, 32). Similarly, lineage tracing experiments showed that *Ptf1a* expressing cells have the capability of maturing into all pancreatic cell types, although not all endocrine cells derive from a *Ptf1a*+ cell (66). Another marker of MPCs in the early pancreatic bud is

hepatic nuclear factor 6 (Hnf6). Loss of *Hnf6* results in delayed *Pdx1* expression, pancreatic hypoplasia, and impaired endocrine and duct cell differentiation (67-69). The roles these and other transcription factors, play in pancreas differentiation are discussed in more detail in the following sections.

## A. Transcription Factors Involved in Endocrine Specification and Lineage Determination

Many lineage-restricted or lineage-specific transcription factors are expressed more broadly in the pancreatic epithelium or in the endocrine population early during development, gradually becoming restricted as development proceeds to refine the pattern of gene expression to what is observed in the adult islet. Gene-expression and mutational analyses in mice strongly correlate with gene function in humans; mutations in several genes involved in β-cell differentiation, including *Pdx1, Pax6,* and *NeuroD*, have been identified in individuals with type 2 diabetes.

*Prox1* is expressed in the posterior foregut endoderm in the presumptive pancreas region before bud outgrowth (70), and is essential for normal liver bud outgrowth (71). At e13.5, *Prox1* is expressed in most cells throughout the pancreatic epithelium. As the second wave of endocrine differentiation commences after e13.5, *Prox1* becomes more highly expressed in $Ngn3^+$ (an endocrine progenitor marker) and $Isl\text{-}1^+$ (a marker of all islet endocrine) cells, and is downregulated in differentiating acinar cells (72). After birth, *Prox1* expression is maintained at high levels in the ductal

epithelium and in peripheral islet cell types, with lower levels found in β-cells. At e15.5 (the time at which *Prox1*-deficient embryos die as a result of complications in other organ systems), the *Prox1* mutant pancreatic epithelium is less branched than that of wild type, and contains many fewer endocrine cells (72). By contrast, the number of differentiated acinar cells is relatively increased, and the pancreas has increased levels of *Ptf1a* with decreased levels of endocrine lineage markers (e.g., *Ngn3*). Thus, *Prox1* may be required within the bipotential endocrine/duct progenitors within the "trunk" population to repress the acinar fate and promote differentiation down the endocrine lineage.

Similar to *Pdx1* and *Ptf1a*, *Hnf6* is initially expressed throughout the early MPCs. Target genes for Hnf6 in the developing pancreatic region include *Foxa2, Pdx1,* and *Hnf4*, which are critical endodermal regulators: *Foxa2* is involved in the β-cell specific expression of *Pdx1,* and Hnf4 activates *Hnf1α.* In turn, Hnf1α activates *Pdx1,* and Hnf4 regulates *Hnf6* (see Figure 1-4; (73)). Thus, alterations in the expression of a single Hnf can affect the expression of multiple genes in this hierarchy.

As development proceeds, *Hnf6* is maintained in the ductal epithelium and in acinar cells, but becomes downregulated in endocrine cells as they begin to express hormones (69, 74, 75). This downregulation is critical for normal islet ontogeny and function: continued *Hnf6* expression in islets impairs the migration of endocrine cells from the ductal epithelium, disrupts the organization of endocrine cell types within the islet (core vs. mantle), and severely compromises β-cell maturation and function, leading to overt diabetes (76, 77).

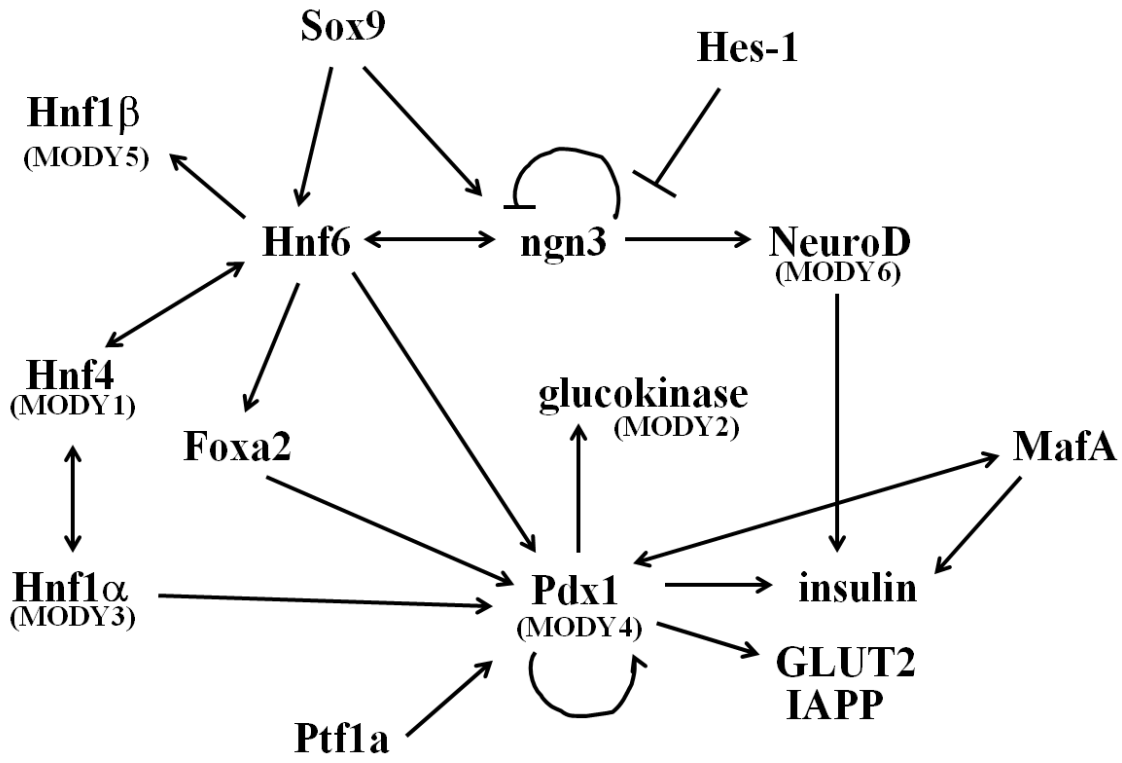


**Figure 1-4.** Simplified pancreas transcription factor network. Some of the factors that are important in the specification of the endocrine lineage and subsequent differentiated β-cells are shown. In particular, the interactions between different Mature onset diabetes of the young (MODY) genes, transcriptional targets of *Hnf6* and *Pdx1,* and transcription factors that transactivate the insulin promoter are highlighted. Arrows indicate direct transcriptional targets. From Pancreas Development and Regeneration. K. Riley and M. Gannon. *Principles of Developmental Genetics*, 2nd Edition, Copyright © [2014].

Hnf6 is an upstream activator of *Ngn3* (see Figure 1-4; (67, 78)), a transcription factor expressed in endocrine precursors. *Ngn3*$^{-/-}$ mice lack endocrine cells in both the pancreas and the small intestine (79). *Hnf6*$^{-/-}$ mice have decreased numbers of *Ngn3*$^{+}$ cells during embryogenesis, and lack islets at birth (67). Islets do develop later, but are abnormal, and the mice are diabetic. The presence of islets in the absence of *Hnf6* suggests that other factors can partially compensate for *Hnf6,* and, indeed, other closely related factors are also expressed in the developing pancreas (80). Conditional inactivation of *Hnf6* specifically in endocrine progenitors (cells that have activated *Ngn3* expression) results in fewer bipotential duct/endocrine progenitors committing to the endocrine lineage (69). *Hnf6* function is therefore required to generate endocrine progenitors in the appropriate numbers and its sustained function is required to irreversibly commit cells to the endocrine lineage.

*Neurogenin 3* (*Ngn3*) and *Beta2/NeuroD* (MODY6) are two closely related bHLH transcription factors involved in the early stages of pancreatic endocrine development. Early trunk progenitors express low levels of Notch and its ligand, Delta. Through as yet undetermined mechanisms, cells stochastically begin to express elevated levels of either Notch or Delta. Higher levels of Notch result in transcriptional repression of the proendocrine transcription factor, *Ngn3* (31). *Ngn3*-expressing cells are first detected scattered throughout the pancreatic epithelium on e9.5. Their numbers reach a peak on e15.5 decreasing to nearly undetectable levels by birth (79). Ngn3$^{+}$ cells are found within or adjacent to the ductal epithelium, and do not co-express islet endocrine hormones (see Figure 1-4). In the absence of *Ngn3,* endocrine markers within the

pancreas (including both broad and lineage-restricted transcription factors and all hormones) are missing (79). Thus, *Ngn3* represents the only known gene that specifically marks the endocrine progenitor population. Unfortunately, transgenic over-expression of Ngn3 in the pancreatic epithelium does not result in increased islet mass. Rather, expression of *Ngn3* throughout the pancreatic epithelium results in premature differentiation of glucagon-producing cells, with little to no β-cells being formed (29, 81, 82). Interestingly, the endocrine phenotype observed after *Ngn3* over-expression is identical to that seen in *Hes1*-deficient animals. In the absence of *Hes-1,* which represses *Ngn3* transcription in response to Notch signaling, *Ngn3* is over-expressed, thereby leading to an excess of glucagon-producing cells and the depletion of endocrine progenitors (30, 31).

*Ngn3* expression is biphasic, correlating with the first and second waves of endocrine cell differentiation (83). Competence of *Ngn3*-expressing progenitor cells changes during the secondary transition, with early differentiating endocrine cells favoring an α-cell fate and later cells preferentially differentiating as δ-cells or other endocrine cell types (84). In addition, through highly sensitive lineage tracing experiments, it was determined that *Ngn3*-expressing progenitor cells are unipotent and thus give rise to only one endocrine cell type (85). This suggests that the $Ngn3^{+}$ progenitor population is heterogeneous, although molecularly indistinguishable at this time.

*NeuroD* was isolated as a transactivator of the insulin gene (see Figure 1-4), but is expressed in all islet endocrine cell types during development and in the adult. It is a

direct transcriptional target of Ngn3 (see Figure 1-4). Loss of *NeuroD* results in a dramatic decrease in all islet endocrine cell types, suggesting that *NeuroD* functions in the expansion of the endocrine population or in endocrine cell survival (86). The remaining endocrine cells fail to organize into normal spherical islet structures, indicating that *NeuroD* also functions in islet morphogenesis (86). Removal of *NeuroD* in developing β-cells results in an immature β-cell phenotype and greatly impaired β-cell function (87). This agrees with the defects observed in humans with NeuroD mutations (MODY6) (88).

Members of the NKX class of homeodomain proteins also have roles in the pancreatic endocrine lineage. Both Nkx2.2 and Nkx6.1 are expressed in most pancreatic epithelial cells during early stages of development; however, by e15.5, Nkx2.2 becomes restricted to the endocrine cell population, and Nkx6.1 is found exclusively in insulin-producing cells and scattered cells within the ductal epithelium (89-91). During late gestation, Nkx2.2 can be detected in nearly all hormone$^+$ cells, except for the somatostatin-producing cells. After birth, both genes are expressed in the β-cell population.

*Nkx2.2* primarily functions as a transcriptional repressor in regulating endocrine differentiation (92). Mice lacking *Nkx2.2* have no detectable insulin$^+$ cells at any stage examined, and they also have a dramatic reduction in the number of glucagon-expressing cells and a more modest reduction in the number of PP$^+$ cells (89). Expression of Isl-1 and synaptophysin, general markers of islet endocrine cells, is normal in *Nkx2.2* mutants, suggesting that loss of *Nkx2.2* does not result in a dramatic

loss of endocrine cells in general. Subsequent analysis revealed that these "extra" endocrine cells in the *Nkx2.2* mutant pancreas were increased numbers of the ghrelin-producing ε-cell population (93). Thus, *Nkx2.2* is required to generate β-cells, maintain and expand α- and PP-cells, and repress ε-cell fate. Experiments in mice show that *Nkx2.2* and another transcription factor, *Arx*, work in concert to regulate the population of δ- and ε-cells in the developing pancreas (94).

*Nkx6.1* gene inactivation results in a highly specific profound loss of second-wave insulin$^+$ cells (after e13.5), with no alteration in the numbers of other islet endocrine cell types (90). Thus, in the absence of *Nkx6.1,* putative β-cells do not adopt an alternate islet endocrine cell fate. Genetic epistasis experiments have determined that *Nkx6.1* functions downstream of *Nkx2.2* in the expansion and terminal differentiation of the β-cell lineage (90). However, transgenic over-expression of *Nkx6.1* does not enhance β-cell mass or function (95). Upon deletion of both *Nkx6.1* and *Nkx2.2*, both α- and β-cells were decreased in number, indicating a role for both transcription factors in endocrine cell development (96, 97). Nkx6.1 actively represses the exocrine cell fate while promoting the endocrine cell fate. This is accomplished by Nkx6.1-mediated repression of *Ptf1a* expression (98).

Pax4 and Pax6, two members of the paired class of homeodomain-containing transcription factors, function in pancreatic endocrine differentiation. In the pancreas, Pax4 is specifically expressed in first- and second-wave insulin-producing cells, and is maintained at very low levels in adult β-cells (99). Pax4 is mainly thought to act as a transcriptional repressor to promote the β-cell fate (100-102). In the absence of Pax4,

first wave insulin-producing cells are observed, but mature β- and δ-cells fail to form, and there is an increase in glucagon- and ghrelin-producing cells (93, 99, 103). These data suggest that either Pax4 is separately required in the β- and δ-cell lineages or that these two islet cell types arise from a common progenitor that is dependent on Pax4. In addition, the increased numbers of α- and ε-cells suggests that cells that would have become β-or δ-cells have instead adopted one of these two cell fates or, alternatively, that β- and δ-cells normally produce something that inhibits the expansion of the α- and ε-cell populations. Despite Pax4 being dispensable for the formation of α- and PP-cell types, lineage tracing experiments have shown that *Pax4*-expressing cells give rise to α-, β-, and ε-cells, suggesting that Pax4 is expressed in a pluripotent endocrine progenitor (102). In addition, Pax4 promotes proper β-cell mass expansion in humans, with mutations in Pax4 resulting in diabetes (104).

In contrast to Pax4, Pax6 is expressed in all endocrine cell types within the pancreas both during embryonic development and in adults; however, global loss of Pax6 has a specific effect on the α-cell lineage. In the absence of Pax6, there is a complete loss of glucagon-producing cells; the other endocrine cell types are present in reduced numbers, and they fail to organize into normal islet structures (105-107). Mice that lack both Pax4 and Pax6 completely lack all pancreatic endocrine cell types (106). After birth, Pax6 functions to maintain islet function; gene inactivation in mature endocrine cells results in decreased expression of β-cell–specific genes and diabetes, with an increase in ghrelin-expressing cells (108, 109).

The Aristaless related homeobox (Arx) homeodomain–containing protein is expressed in scattered cells throughout the pancreatic buds between e10.5 and e12.5 and is later co-expressed with glucagon at e14.5 and also found in PP-cells. Inactivation of *Arx* causes a complete loss of second wave glucagon-producing α-cells, resulting in severe postnatal hypoglycemia and death (101, 110); glucagon stimulates the liver to release glucose into the bloodstream during times of fasting. The decrease in α-cell number is accompanied by an increase in both δ-cells and β-cells. Subsequent analyses strongly suggest that *Arx*$^{-/-}$ cells are diverted from an α-cell fate toward a β- or δ-cell fate instead. Indeed, the β-cell transcription factor Pax4 is upregulated in *Arx* mutants, whereas *Arx* is upregulated in Pax4 mutants (110, 111). Thus, these two genes have opposing actions within the endocrine lineage to establish β or δ-cells (*Pax4*) and α-cells (*Arx*).

The large Musculoaponeurotic fibrosarcoma (Maf) proteins are basic leucine zipper transcription factors first identified in an avian retrovirus. MafA was identified by several independent groups as an activator of insulin gene transcription (see Figure 1-4; (112-116)) and is specifically expressed in second-wave insulin$^{+}$ cells beginning on e13.5 and continuing into adulthood, thus making it a marker of more mature β-cells (117, 118). Despite its indication as a critical β-cell maturation factor, global inactivation of *MafA* had no effect on the number of insulin-producing cells generated during embryonic development. Instead, loss of MafA causes defects in β-cell gene expression and postnatal β-cell function, thus leading to diabetes (119). The lack of a developmental islet phenotype in *MafA* knockout animals may be the result of

compensation by another closely related Maf family member that is also expressed in developing endocrine cells, MafB (118, 120).

MafB is also capable of activating insulin reporter gene transcription in tissue culture cells, although, in adult islets, it is expressed only in α-cells, where it regulates the expression of the glucagon gene (120). During embryonic development, MafB is expressed in both first and second wave insulin- and glucagon-producing cells, becoming restricted to α-cells soon after birth. MafB promotes the activation of β-cell differentiation genes including *Pdx1, Nkx6.1, Glut2,* and *MafA* (118). Loss of *MafB* results in a reduction of insulin and glucagon positive cells (121).

## B. Signaling pathways involved in endocrine specification and lineage determination

### TGF-β Superfamily Signaling

The TGF-β superfamily of signaling molecules consists of TGF-β, Activin, Nodal, and BMP ligands. Through binding to type I and type II serine-threonine kinase receptors, these signaling molecules play a critical role in growth, differentiation, migration, adhesion, and apoptosis. Specifically, TGF-β, Activins, and Nodal activate Smads 2 and 3, while BMP family ligands activate Smads 1, 5, and 8 (122). This activation induces interaction with the co-Smad 4, and subsequent nuclear localization where it complexes further with co-factors to activate or repress transcription. TGF-β ligands (TGF-β 1, 2, 3) and receptors (ALK5, TGF-βRII) are expressed in the pancreatic

epithelium at e12.5. TGF-β1 is expressed in the acinar tissue by e15.5. In addition TGF-β2 and 3 are expressed in both the acinar and development cells. Respective receptor expression becomes localized to the ducts and blood vessels by e18.5 (123).

TGF-β superfamily components promote proper pancreatic development and specification (124). Pancreas-wide over-expression of Smad7, which inhibits TGF-β-specific Smads 2/3 and BMP-specific Smads 1/5/8, causes dramatic loss of β-cells and an increase in α-cells without alteration in α-cell proliferation or apoptosis (125). Inhibition of TGF-β signaling in the developing islet results in an immature ductal network (126). Yet, over-expression of TGF-β1 in insulin+ cells results in small, disorganized endocrine clusters in the mouse, suggesting that increased TGF-β signaling may negatively affect endocrine development and organization (127). However, in the adult islet, the role of TGF-β is less defined. Over-expression of Smad7 in adult β-cells results in loss of mature β-cell markers, such as MafA (125). Studies of TGF-β in β-cell function are conflicting; exogenous TGF-β added to cultured human islets *ex vivo* led to impaired insulin expression and glucose stimulated insulin secretion (128, 129), but promoted β-cell survival and differentiation (130). Yet, in cultured rat islets and β-cell lines, TGF-β improved insulin secretion (131, 132). Regardless, while complex, proper TGF-β signaling is critical for proper β-cell specification and mass expansion.

The BMP components of the TGF-β superfamily play a less understood role in the development pancreas. In the early pancreatic bud, BMP signaling is transcriptionally inhibited by GATA5 (133). In addition BMP signaling from the septum

transversum promotes liver fate over pancreas fate in the mouse (134). Ectopic expression of BMP in the specified pancreas results in conversion of pancreatic progenitors to hepatocytes (135). Later in pancreas development however, BMP ligands (BMP 4, 5, 7) and receptors (BMPR-1a, 1b, 2) are expressed in the pancreatic epithelium at e12.5. BMP4 and BMPR-1a are expressed in the developing endocrine cells at e15.5 (136). Inactivation of either does not alter islet development (137). Overall, there has been no role identified for BMP signaling in later stages of islet development.

**Wnt Signaling**

Activation of the highly conserved Wnt pathway induces axis patterning, cell proliferation, differentiation, gene transcription, and cell adhesion (138). Canonical Wnt signaling results in stabilization of β-catenin and eventual translocation to the nucleus and subsequent transcription of target genes. Wnt ligands bind to the Frizzled receptors and LdL-related protein receptor (LRP) 5/6 to activate the intracellular protein Disheveled (Dsh). Dsh disrupts the destruction complex, composed of glycogen synthase kinase (GSK) 3, APC, and Axin1, which typically phosphorylates β-catenin thus marking it for degradation. Once stabilized, β-catenin translocates to the nucleus and displaces the transcriptional repressor Groucho from the TCF/LEF1 complex of transcription factors.

Similar to BMP, Wnt expression is inhibited during pancreas specification (139). *In vivo* studies in which Wnt signaling is maintained in the developing pancreatic buds

induces endocrine precursor cells to switch to an intestinal cell fate, suggesting suppression of Wnt signaling is required for proper islet development (135). Stabilization of β-catenin in the developing pancreas resulted in an almost 5-fold increase in pancreas size, suggesting Wnt signaling may play a role in organ size regulation and post-natal growth (140). However, Wnt signaling does promote exocrine pancreas development at later stages. Wnt ligands (Wnt5a, 7b), receptors (Frizzled, LRP5/6) and activated β-catenin are observed in the acinar tissue at e12.5. Reduced Wnt signaling or ablation of β-catenin prior to or during the secondary transition results in a significant loss of exocrine tissue (141). Wnt signaling is also required for proper exocrine and endothelial cell development (142). In regards to the adult islet, proper Wnt signaling appears to be a requirement for proper glucose sensing and insulin secretion (143). In addition, Wnt signaling activation is typically seen in response to the proliferative stimuli in the adult islet (144).

**Integrin Signaling**

Integrins comprise a family of α/β heterodimeric receptors that mediate interactions with ECM components and elicit the activation of signaling from both inside and outside of cells. ‘Inside-out’ signaling occurs when intracellular signaling induce integrins to bind to matrix ligands (145, 146). ‘Outside-in’ signaling is inducted by the binding of an ECM component by integrin receptors, promoting integrin association with the actin cytoskeleton network and activation of intracellular second messenger signaling pathways (147, 148). The α subunit is required for proper recognition of ECM

ligands, while the β subunit function as the signal transduction molecule. This allows for α/β combination specific ligand specificity and signaling induction, resulting in integrins playing a role in various cellular processed such as cell adhesion, migration, proliferation, differentiation and cell survival (148-151).

Through the use of *in vitro* experiments involving pancreatic epithelium, integrins have been observed to play a critical role in proper pancreatic endocrine development (37, 152-157). Specifically, integrins αvβ3, αvβ5, and α6β4 regulate the migration of undifferentiated pancreatic epithelial cells from ductal networks (37, 155). Intriguingly, β1 integrin appears to regulate pancreatic cell proliferation and differentiation (36, 153, 154). Specifically in the endocrine compartment, removal of β1 integrins in the developing β-cells resulted impaired β-cell proliferation, β-cell mass expansion and islet morphogenesis (158).

## Connective Tissue Growth Factor (CTGF)

### CTGF structure and function

Connective tissue growth factor (CTGF), or CCN2, is a member of the CCN family of secreted molecules named for its found members, Cysteine rich 61 (CCN1), CTGF (CCN2), and Nephroblastoma over-expressed (Nov/CCN3). CCN proteins are vertebrate-specific extracellular matrix (ECM)-associated proteins (159). CTGF was discovered in 1991 as a mitogen secreted into the conditioned media of human umbilical vascular endothelial cells (HUVECs). The CTGF gene consists of 5 exons that

encode a 38 kD full-length cysteine-rich protein. CTGF has a modular structure; with an N-terminal secretory peptide followed by four conserved domains (See Figure 1-5;(160-162)). The first domain has sequence homology to insulin-like growth factor binding protein (IGFBP). While CTGF is capable of interacting with IGF, this occurs at a much lower affinity than classical IGF binding proteins (163). The functional significance of this domain remains unclear (164). The second module contains a von Willebrand type C domain (VWC), with similarities to the cysteine repeats in the BMP antagonist Chordin (165, 166) and interacts with TGF-β and BMP ligands. TGF-β promotes *Ctgf* gene expression. Extracellularly, CTGF enhances binding of TGF-β to its receptor, promoting TGF-β signaling (161). Via the VWC module, CTGF inhibits BMP signaling by preventing interaction of BMP with its receptors. Module 3, a thrombospondin type 1 repeat (TSP1), allows for ECM interactions, in particular, matrix glycoproteins. The TSP1 module is also a VEGF sink and interacts with integrins, limiting diffusibility. The final module, the carboxyl-terminal (CT), contains a cysteine knot motif. This motif binds the Wnt co-receptor LRP5/6, inhibiting Wnt signaling by blocking Wnt ligand binding. The CT module also allows interactions with integrins and extracellular heparin sulfate proteoglycans (160, 167, 168).

Though a specific CTGF receptor has not yet been identified, several studies have observed CTGF interacting with various integrins including $\alpha_v\beta_1$, $\alpha_v\beta_3$, $\alpha_5\beta_1$, and $\alpha_6\beta_1$. CTGF-elicited responses are dependent upon the integrin subtype (168-170). Through its modules and integrin interactions, CTGF modulates the activity of several

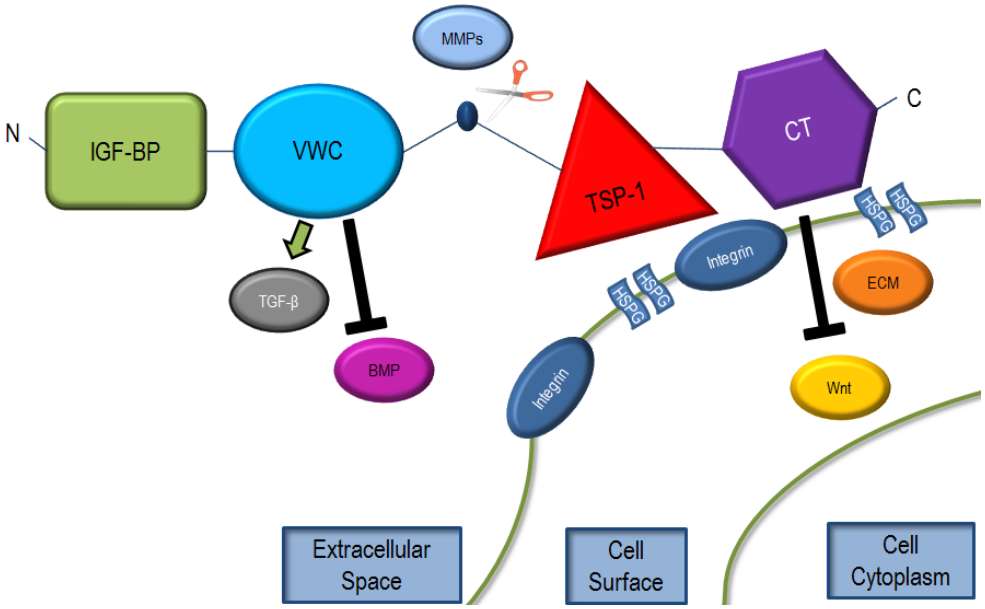


**Figure 1-5.** The modular structure of CTGF. The CTGF protein contains four domains: an insulin-like growth factor binding protein domain (IGF), a von Willebrand type C domain (VWC), a thrombospondin like-1 domain (TSP) and a cysteine rich C-terminal domain (CT). CTGF binds TGF-β and BMP ligands through its second module and interacts with the Wnt co-receptor LRP and αβ integrins through its C-terminal domain. Through the third domain, CTGF serves as a VegfA sink and interacts with several extracellular matrix components. CTGF is also proteolyticly cleaved between the second and third domains by MMPs.

growth factors and plays a role in many cellular processes such as proliferation, adhesion, ECM degradation, and angiogenesis.

**Genetic modulation of CTGF expression *in vivo***

The Lyons group generated a global null allele of *Ctgf* to determine the role of *Ctgf in vivo* (171). *Ctgf* null mice display gross skeletal defections including deformation of the craniofacial bones, kinked ribs, and bent long bones (171). Within the lung, impairment in proper proliferation and differentiation of lung epithelial cells was observed, resulting in lung hypoplasia (172). This paired with the aforementioned skeletal defects leads to respiratory failure. Thus, *Ctgf* null mice die soon after birth allowing only for embryonic studies into CTGF function (171). In addition, *Ctgf* null mice display decreased angiogenesis and *VegfA* expression within the growth plates, resulting in impaired bone ossification (171). These studies highlight the multi-faceted nature of CTGF in the requirement for proper proliferation, ECM remodeling and angiogenesis *in vivo*.

Through the employment of a conditional by inversion (COIN) strategy, conditional gene inactivation of *Ctgf* was achieved, thus allowing for analysis of CTGF in specific cell types in the adult mouse (173). Specific inactivation of *Ctgf* in adult bone resulted in impaired endochondral bone formation and a reduction in spongy bone trabeculae (173). Interestingly, mice with tissue-specific over-expression of CTGF in the developing bone display decreased bone mineral density and ossification (174, 175). The similarity between knock-out and over-expression phenotypes in the bone suggests

a critical and tightly regulated level of CTGF is required for proper bone development, and in particular, bone ossification. In corroboration, lung development depends on a specific level of CTGF. Either removal or over-expression of CTGF during lung development resulted in impaired alveolarization and vascularization of the lung (172, 176).

In addition to roles in lung and skeletal development, CTGF over-expression studies have elucidated a role in fibrosis. Fibroblast-specific over-expression of CTGF, through the use of the *collagen α2(1)* promoter, led to accelerated tissue fibrosis in the skin, vasculature, and kidney. This eventually results in death between 2 and 6 months of age (177). CTGF mediates its pro-fibrotic effects via enhanced fibroblast proliferation and upregulation of fibronectin, TIMPs, and α-smooth muscle actin, among other ECM components (177).

**CTGF and endocrine development**

CTGF was found to be downregulated in a transgenic mouse model of islet dysmorphogenesis and diabetes in which HNF6 was over-expressed in the endocrine population (178), suggesting CTGF plays a role in pancreas development. Using a lacZ knockin allele, it was observed that CTGF is expressed in insulin+ cells, ducts, and blood vessels during pancreas development as early as e12.5 (179) (See Figure 1-6). However, under normal conditions, CTGF expression is restricted to ducts and blood vessels by postnatal day 3. Global inactivation of CTGF results in impaired endocrine cell lineage allocations, with an observed increase in α-cells at the expense of β-cell

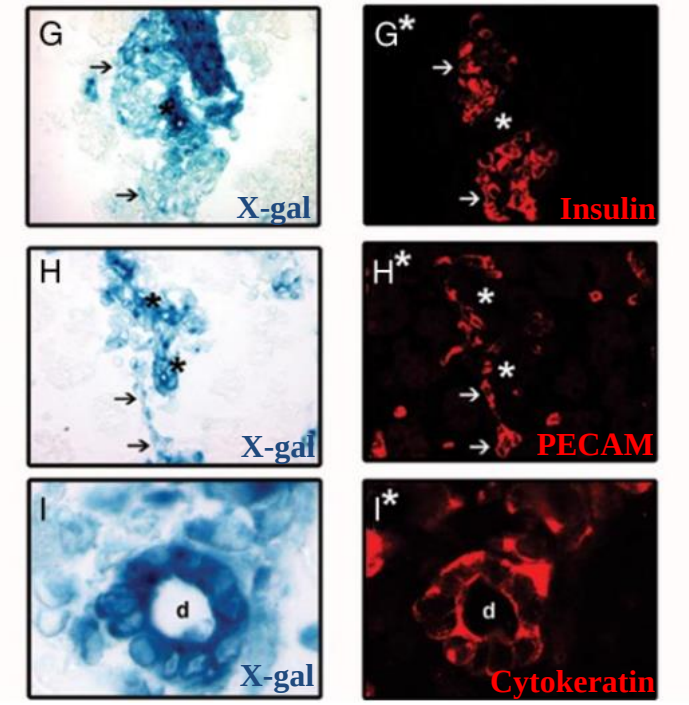


**Figure 1-6.** CTGF$^{lacZ}$ expression in the pancreas localizes with cell type-specific markers. X-gal staining (left column) along with co-labeling for cell type specific markers (right column) shows that CTGF$^{lacZ}$ is expressed in β-cells (insulin-positive) (G), endothelial cells (PECAM-positive) (H), and duct cells (cytokeratin-positive) (I) at e-18.5. Arrows indicate cells that co-express CTGF and the cell-type specific marker. Asterisks indicate cells which express CTGF but do not express the cell-type specific marker. d=duct. Adapted from Crawford *et al. Mol Endo,* 2009.

number by the beginning of the secondary transition (See Figure 1-7). In addition, these embryos displayed altered islet morphogenesis, with α-cells intermingled within the typically β-cell restricted core. However, no alterations to endocrine cell proliferation or apoptosis were observed in global CTGF mutants until e18.5, at which time there was a significant impairment in β-cell proliferation (179). These decreases in β-cell proliferation did not result in an overall decrease in total endocrine area or impaired β-cell function (under normal conditions) in part due to compensatory β-cell hypertrophy (179).

In order to probe the specific roles of CTGF in proper β-cell development further, tissue-specific models of CTGF knockout and over-expression were employed (52). Loss of CTGF from any one cell source in the embryonic pancreas resulted in significantly decreased β-cell proliferation, indicating that CTGF works in both a paracrine and autocrine manner to promote β-cell proliferation during development (52). In addition, via an inducible transgenic "Tet-On" system (described later), increased *Ctgf* expression in developing β-cells resulted in a 25% expansion in endocrine mass, due to enhanced β-cell and α-cell proliferation (See Figure 1-8)(52). These embryonic studies showed that CTGF plays a critical role in β-cell proliferation and mass expansion during development.

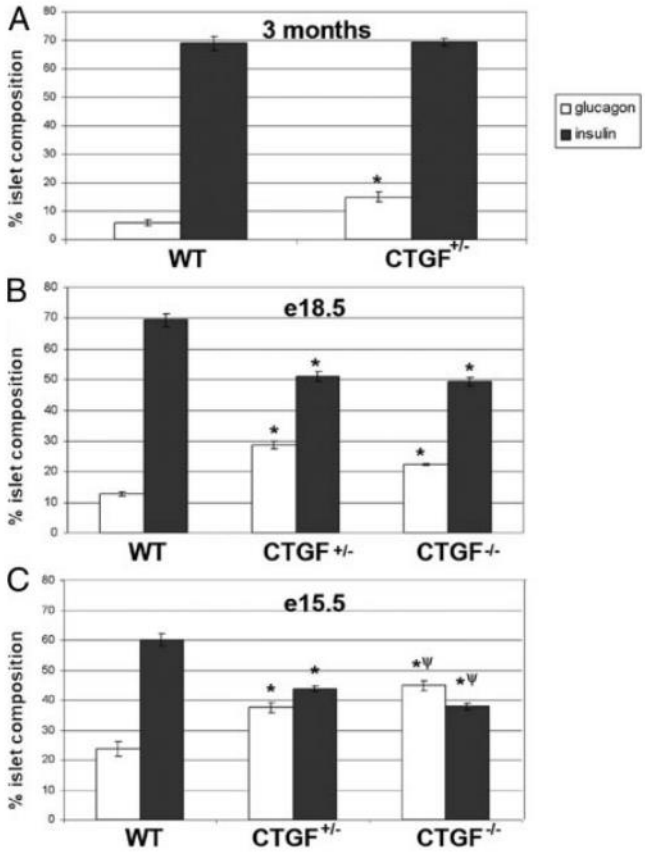


**Figure 1-7.** CTGF global mutants display altered islet composition. Morphometric analysis at (A) 3 months, (B) e18.5, and (C) e15.5 indicated alterations in the percentages of insulin-positive and glucagon-positive cells in CTGF heterozygous (CTGF $^{+/-}$) and null (CTGF$^{-/-}$) animals as compared to wildtype (WT). *p<0.01 compared to WT, ψp<0.05 compared to CTGF$^{+/-}$. Adapted from Crawford *et al. Mol Endo,* 2009.

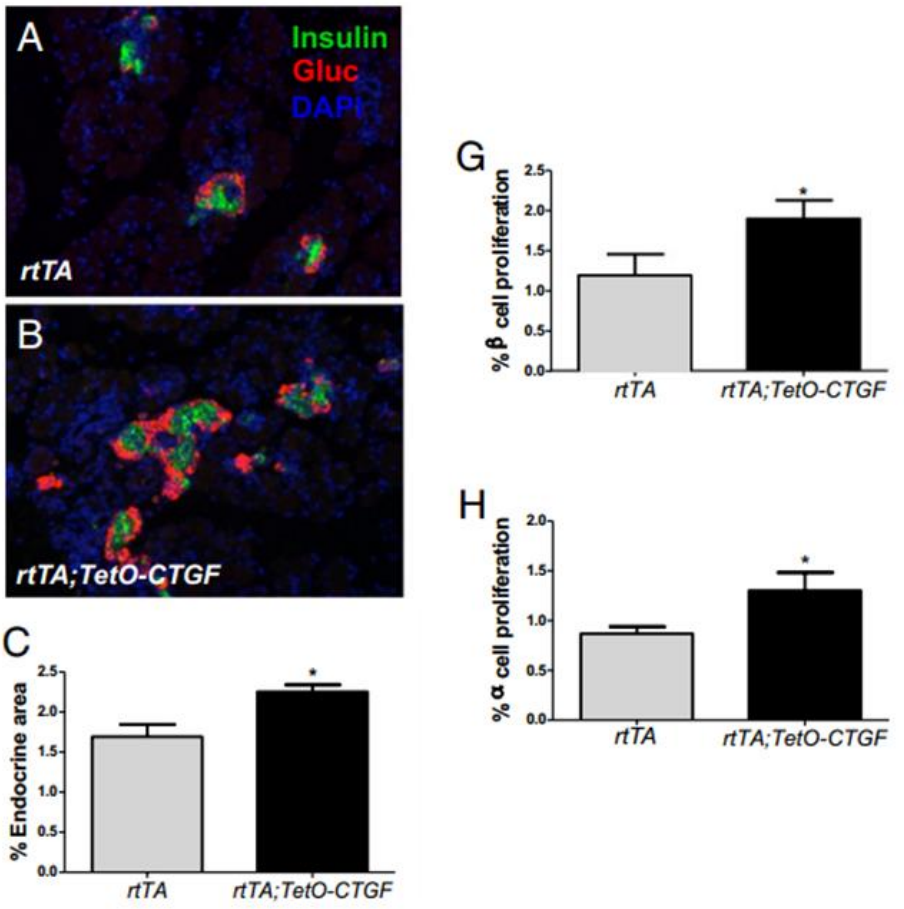


**Figure 1-8.** CTGF over-expression during development elicits increased endocrine mass. Immunofluorescence analysis of insulin (green) and glucagon (red) in control (A) and CTGF over-expression (B) pups at P1. (C) Quantification of percentage of pancreas area that is composed of endocrine tissue. This increase in endocrine area was due to an increase in both α- (H) and β-cell (G) proliferation in CTGF overexpression animals as compared to controls. *p<0.05 compared to rtTA. Adapted from Guney *et al. PNAS,* 2011.

## β-cell maturation and proliferation

### Post-natal β-cell maturation

β-cell maturation begins in late gestation, when key genes involved in adult function are first expressed. Many of these genes are critical for proper glucose responsiveness and subsequent insulin secretion. This ability to respond to changes in blood glucose is reliant on the expression of *Glut2* and the prohormone convertase PC1/3, which is required for cleavage of proinsulin to active insulin (180-182). β-cell maturation is also indicated by a transition from *MafB* to *MafA* expression (117, 118). Prior to the secondary transition *MafB* is expressed in both β- and α-cells, but is restricted to α-cells soon after birth. This expression in α-cells is maintained throughout adulthood (118, 120). MafA, however, is observed as early as e13.5 to be co-expressed with MafB in insulin-positive cells. Intriguingly, MafB is required for proper expression of several mature β-cell markers, such as MafA, Pdx1, Nkx6.1, and Glut2. Removal of MafB during pancreatic development results in loss of both α- and β-cells in the endocrine compartment of the pancreas (118, 183). MafA, while not required for expression of other key β-cell maturation genes, is critical for proper glucose tolerance and insulin secretion in the adult animal (119, 184). β-cell maturation completes around 3 weeks of age (weaning) in rodents, at this point the majority of the β-cell population (~80%) expresses *MafA* (121, 185).

Another hallmark of β-cell maturation is complete innervation of the pancreas, which initiates in the mouse at e10.5 via neural crest cells following endothelial cell

migration to the pancreas (186, 187). While inhibition of innervation via the deletion of *Foxd3* elicits an increase in endocrine area, these β-cells remain immature, with decreased *MafA* and *Glut2* expression (187). Overall, innervation is critical for proper maturation of the β-cell population.

## β-cell Proliferation

### A. β-Cell Replication Overview

The endocrine pancreas undergoes substantial remodeling during the neonatal period, including replication, apoptosis, and ongoing neogenesis (188-191). Between months 1 and 3 in the rat, the β-cell number doubles each month. After 3 months, the number of dying β-cells approaches the number of replicating cells (192). β-cell replication declines with age, and β-cell mass plateaus once the organism reaches mature body mass (193).

The majority of experimental evidence in mice indicates that most if not all new adult β-cells arise via proliferation of preexisting β-cells with little to no contribution from resident pancreatic stem cells or undifferentiated progenitors within the ductal epithelium (194-196). However, endocrine neogenesis may be ongoing in the adult human pancreas, based on biopsy and autopsy studies in which endocrine cells were observed within the pancreatic ducts of humans of different ages (197-199).

β-cell mass is dynamic, adapting to changing physiologic needs and increased functional demands. For example, β-cell mass increases during pregnancy and with the insulin resistance associated with obesity, whereas it decreases after parturition and after insulinoma implantation (200-202). In addition to increase β-cell proliferation, β-cells can also compensate for increased demand via improved function of individual cells. Functional adaptations include a reduced threshold for glucose-stimulated insulin secretion and increased glucokinase activity, both of which lead to enhanced insulin secretion (203, 204). Changes in β-cell mass are achieved by both hyperplasia (an increased number of cells) and hypertrophy (an increased individual cell size).

Glucose is one of the best stimuli for β-cell replication both *in vitro* and *in vivo* (205-207). Thus, in a normally functioning pancreas, sustained elevations in blood glucose levels should lead to increased β-cell mass, thereby providing compensation for the increased glucose load. Autopsies of human patients reveal a 40% increase in β-cell mass in obese non-diabetic individuals compared with lean subjects (208), suggesting that β-cell compensation does indeed occur with increasing insulin resistance. However, it is also possible those individuals who fail to progress to diabetes after becoming obese had a greater β-cell mass to begin with and that this is the reason they remain euglycemic. Currently there is no reliable method of measuring β-cell mass longitudinally or in living humans.

Overall, β-cells appear to be very long-lived with decreased replicative potential with age (209, 210). Gene products affecting the renewal, proliferation, or turnover of β-

cells are therefore candidate factors in diabetes susceptibility and therapeutics. In general, human β-cells are more recalcitrant to proliferative stimuli than rodent islets (190). However, introduction of specific cell cycle regulators does increase human β-cell proliferation in culture (211, 212).

**B. Secreted Factors that Affect β-Cell Replication**

Several secreted factors and their receptors play a role in β-cell mass dynamics. For example, the epidermal-growth-factor–related ligand, betacellulin, promotes differentiation and proliferation of β-cells *in vivo* and in pancreatic bud culture (213-217). The TGF-β family member, activin, plays a role in β-cell specification and/or proliferation *in vivo* as well; defects in activin-receptor signaling during embryogenesis result in islet hypoplasia (218, 219). Hepatocyte growth factor (HGF) is a mesenchyme-derived growth factor that stimulates the proliferation of both fetal and adult islets in culture (220, 221), acting through the c-Met receptor, which is highly expressed in β-cells (222). In transgenic mice, over-expression of HGF specifically in insulin-producing cells leads to increased β-cell proliferation and β-cell hypertrophy, and protects β-cells from apoptosis (61-63). Loss of HGF/c-Met signaling during pregnancy impairs β-cell mass expansion resulting in gestational diabetes (60). Placental lactogen is a circulating factor that directly stimulates β-cell replication and is required for maternal β-cell mass expansion during pregnancy (223, 224). More recently, betatrophin, produced by the liver in response to insulin resistance, was identified as a factor that when increased in

the circulation can promote β-cell proliferation and mass expansion *in vivo* (225). To date, there is no evidence that betatrophin can directly stimulate β-cell proliferation.

Secreted gut hormones such as glucagon-like peptide-1 (GLP-1) and gastrin also promote β-cell neogenesis and regeneration after injury. In animal models, the long-acting GLP-1 analog Exendin 4 stimulates both β-cell neogenesis from ductal progenitors and the proliferation of existing β-cells (226, 227). Compounds that mimic GLP-1 action or prevent its degradation in the circulation are currently being used for the treatment of type 2 diabetes. However, there remains controversy as to whether this treatment also stimulates proliferation of other epithelial cells in the pancreas leading to increased susceptibility to pancreatitis and pancreatic cancer (228).

**C. Cell Cycle Regulators and β-Cell Proliferation**

Progression through the cell cycle requires the activity of the heterodimeric cyclin/cyclin-dependent kinase (CDK) complexes. Progression from the G1 phase to S phase is mediated by the D class of cyclins and their partners, CDK4 and CDK6. Murine β-cells are particularly sensitive to loss of CDK4 and cyclin D2, resulting in decreased postnatal β-cell proliferation (229-232). CDK4 null mutant mice have a 50% reduction in body and organ size, are infertile, and develop diabetes at ~two months old (229, 230). Although CDK4 does not seem to be required for embryonic β-cell proliferation, it is essential for establishing the pool of NGN3+ endocrine progenitor cells from which β-cells arise (233). Likewise, cyclin D2 inactivation has no effect on embryonic β-cell

proliferation since mutants are born with normal β-cell mass; however postnatal β-cell expansion is impaired as a result of decreased proliferation, and mutants become diabetic by three months (231, 232).

**D. Transcription Factors that Affect β-Cell Proliferation**

The FoxM1 winged helix transcription factor regulates cell cycle progression genes including several cyclins, *Cdc25B*, and *Survivin* (234-236). FoxM1 is dispensable for embryonic β-cell proliferation and islet development, but is essential for postnatal β-cell replication and maintenance of β-cell mass (193, 237). Pancreas-specific inactivation of *Foxm1* results in diabetes by nine weeks of age as a result of greatly impaired post-weaning β-cell replication, while the absence of *Foxm1* in maternal islets during pregnancy results in gestational diabetes due to impaired β-cell mass expansion (238). Human islets transduced with a FoxM1-containing adenovirus show increased β-cell replication (211).

The Pdx1 transcription factor is required for embryonic β-cell replication and postnatal β-cell expansion in response to insulin resistance (239, 240). The winged helix transcription factor FoxO1 inhibits *Pdx1* expression and β-cell proliferation (241, 242). Thus, inhibition of *FoxO1* activity in β-cells is necessary for proliferation.

The transcription factor nuclear factor nuclear factor of activated T cells (NFAT*)* links increased glucose metabolism and a rise in intracellular calcium with β-cell replication. Inactivation of the NFAT pathway specifically in β-cells results in reduced β-

cell proliferation and subsequent decreases in β-cell mass in mice, while constitutive activation promotes β-cell proliferation, in part through FoxM1 induction (243, 244).

As discussed above, differential requirements exist for β-cell proliferative factors during embryonic development versus postnatal stages. Thus, redundant or parallel pathways are likely present during embryogenesis to ensure the generation of appropriate numbers of β-cells, whereas mature β-cells are highly susceptible to perturbations in cell cycle gene expression. For example, while many cell types express both CDK4 and CDK6, pancreatic β-cells express only one (CDK4 in mouse, CDK6 in human) (190). The ability to activate these cell cycle genes or to prevent the age-dependent decline in their expression may facilitate the expansion of β-cell mass *in vivo* or *in vitro*. Indeed, the expression of an activated form of CDK4 in islet β-cells using the rat insulin promoter results in β-cell hyperplasia and improved insulin secretion without hypoglycemia and without the formation of insulinomas (245, 246). Similarly, human islets transduced with a lentivirus expressing activated CDK4 show increased β-cell proliferation (246).

**CTGF in maturing and adult endocrine pancreas**

The secreted protein CTGF promotes proper β-cell development and mass expansion (52, 179). However, CTGF expression is restricted to only pancreatic ducts and endothelial cells soon after birth (179) (See Figure 1-6). Interestingly, when CTGF is over-expressed during development and through early postnatal stages, an increase

in β-cell proliferation is observed (52) (See Figure 1-8). However, this increase in β-cell proliferation is observed in the immature (MafA-) β-cells which typically make up 25% of the total β-cell number at this stage (52, 118) (See Figure 1-9). Thus, it may be that only immature β-cells are receptive to CTGF in the postnatal pancreas.

Interestingly, CTGF is re-expressed in adult β-cells during pregnancy and high fat diet feeding (179), suggesting it plays a role in β-cell mass expansion and/or improved β-cell function in response to physiological stimuli. However, when CTGF is over-expressed in adult β-cells under normal conditions, no increase in β-cell proliferation or mass expansion is observed (189) (See Figure 1-10). Overall, it appears that only in unique microenvironments of increased demand, such as development, pregnancy and high fat diet feeding are adult β-cells responsive to CTGF.

Additionally, CTGF has been widely studied in the context of pancreatic disease states. In pancreatic ductal adenocarcinomas (PDACs), CTGF is upregulated in both the tumor and surrounding stroma (247). In addition, CTGF promotes angiogenesis and thus metastasis of these tumors (247). Thus CTGF has been examined as a potential therapeutic target for PDAC. Administration of a neutralizing CTGF-specific monoclonal antibody to mice both resulted in reduced tumor growth, impaired angiogenesis, and metastasis (248, 249). In a pancreatic cancer cell line, it was observed that CTGF expression was dependent on ras/MEK/ERK signaling rather than TGF-β (250), a known player in pancreatic cancer progression(251), suggesting CTGF may be regulated by multiple signaling pathways in pancreatic cancer cells.

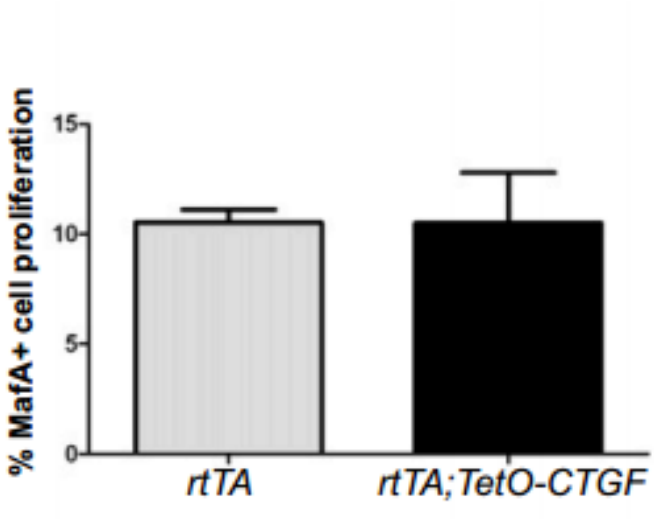


**FIGURE 1-9.** CTGF over-expression during development promotes proliferation of MafA- β-cells. No significant change was observed between groups. Adapted from Guney *et al. PNAS,* 2011.

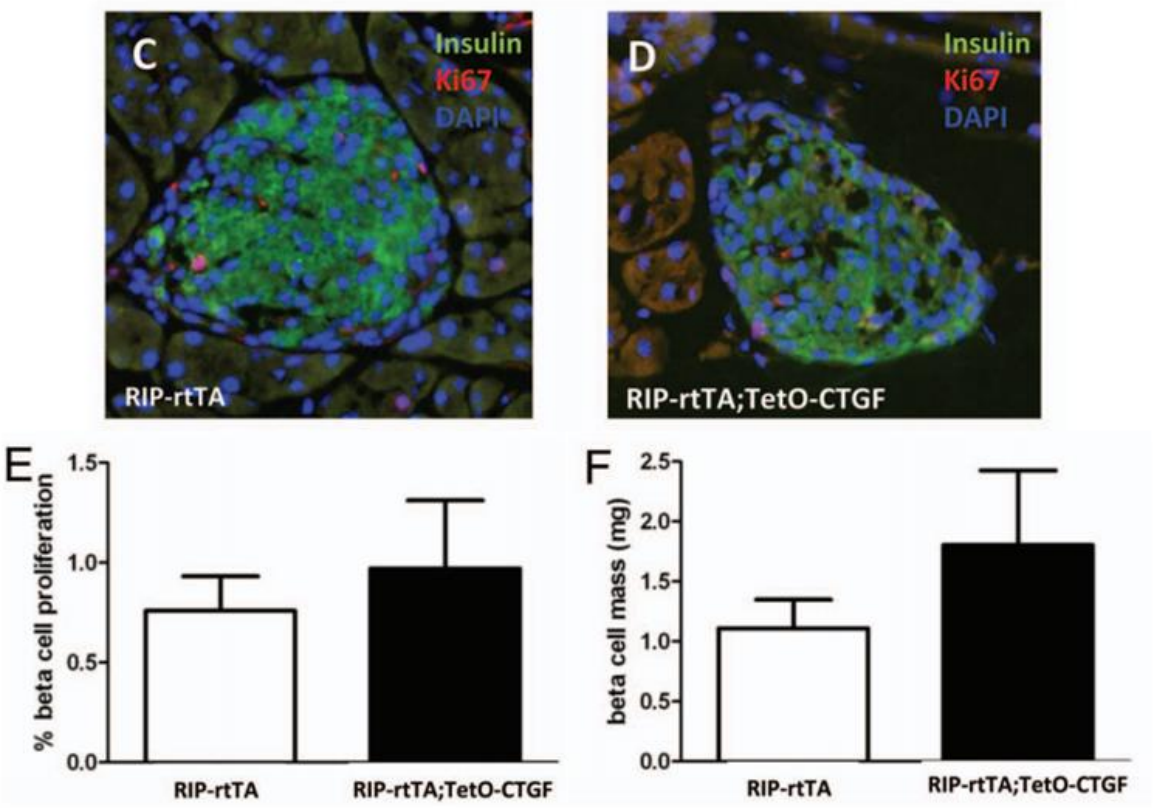


**Figure 1-10.** Over-expression of CTGF in adult β-cells does not yield increased β-cell proliferation or mass expansion. (C-D) Immunolabeling of adult pancreata for insulin (green) and Ki67 (red) to assess β-cell proliferation. (E) Quantification of the percentage of proliferating β-cells ($Insulin^{+};Ki67^{+}$). (F) Quantification of β-cell mass. Adapted from Gunasekaran *et al.*, *Cell Cycle*, 2012.

Expression of CTGF is also upregulated in pancreatitis, a disease characterized by a destruction of the pancreatic acinar cells and subsequent inflammation and fibrosis (252). The stellate cells in the pancreas produce the majority of CTGF in pancreatitis. Ethanol and TGF-β both stimulate CTGF expression in the stellate cells, resulting in integrin activation and subsequent migration, collagen synthesis and ultimately fibrosis (168, 253, 254). Expression of CTGF during pancreatitis also promotes chemotaxis of several immune cell populations, mainly macrophages and neutrophils, which may further enhance the inflammation phenotype of pancreatitis (255, 256).

## Pancreas dysfunction and diabetes

### Diabetes disease state within islet dysfunction

Autoimmune-mediated destruction of insulin-secreting β-cells leads to type 1 diabetes (T1D), while insulin resistance in peripheral tissues combined with β-cell failure leads to type 2 diabetes (T2D). The primary treatment for T1D is exogenous insulin therapy to maintain euglycemia; as cadaveric islet transplants eventually fail or succumb to autoimmunity (3, 4). Several alternative therapies have been proposed for β-cell mass recovery, including transdifferentiation, directed differentiation of embryonic or induced pluripotent stem cells, and induced proliferation of cadaveric islets (See Figure 1-11). Additionally, identification of novel factors that enhance β-cell proliferation and mass regeneration *in vivo* while retaining optimal function would serve as an ideal

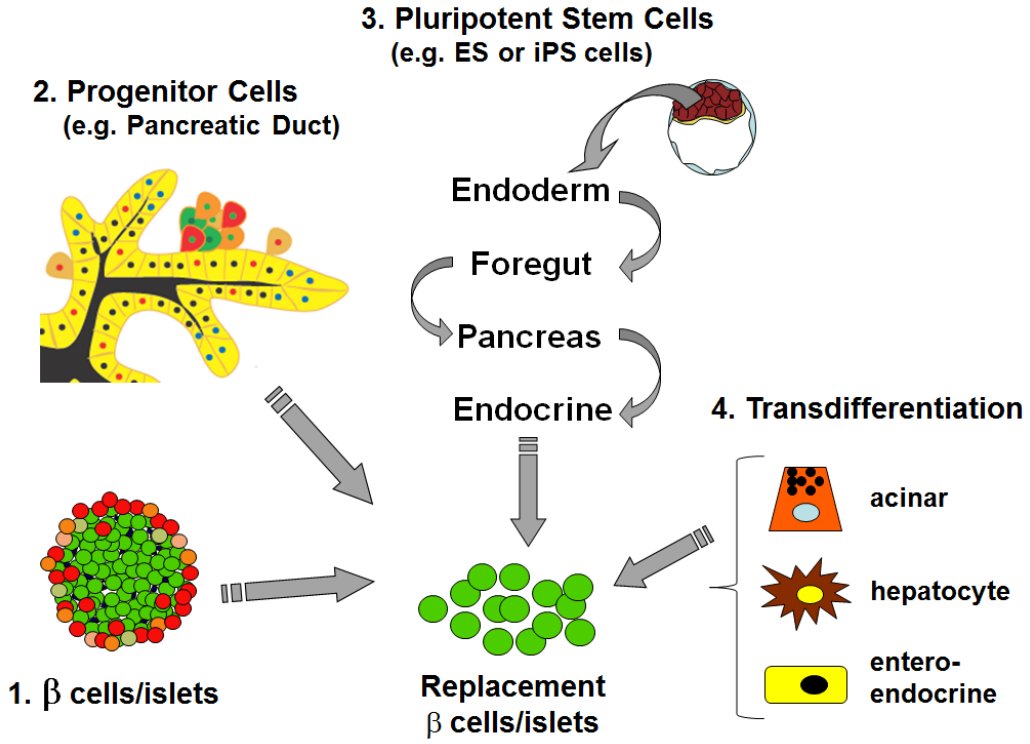


**Figure 1-11.** Potential sources of transplantable β-cells/islets. There are several potential avenues being explored to generate and expand mature β-cells or functional islets *in vitro* as a replenishable supply for use in transplantation: (1) the proliferation and expansion of existing β-cells; (2) the induction of β-cell differentiation from endogenous progenitors (embryonic ductal cells) or from adult ductal epithelium; (3) the induction of β-cell differentiation from embryonic or induced pluripotent stem cells; and (4) the transdifferentiation of other cell types (i.e. acinar or entero-endocrine cells) into β-cells. These strategies are more fully discussed in the text. Adapted from Pancreas Development and Regeneration. K. Riley and M. Gannon. *Principles of Developmental Genetics*, 2nd Edition, Copyright © [2014].

strategy for remediation of all forms of diabetes. However, these therapies, in order to be functional, would still require the suppression of the immune system in patients with T1D.

Adult β-cell mass adapts to changing physiological needs and demands, such as pregnancy and obesity (257). β-cell mass expansion and regeneration occur primarily by replication of existing β-cells (192, 258, 259). Additionally, the proportion of replicative β-cells declines dramatically with age (257). This age-dependent decline in basal proliferation, and reduced ability of β-cells to re-enter the cell cycle limits the innate regenerative potential of adult β-cells (258). Unfortunately, the processes that mediate the age-dependent decrease in proliferative and regenerative capacity remain poorly understood (192, 259, 260). Thus, further investigation into the underlying mechanisms or signaling pathways of β-cell replication and maturation is leading to the development of novel therapeutic strategies for diabetes.

**Therapeutic strategies for diabetes**

The mammalian pancreas (including that of humans) has the ability to partially regenerate after insult or injury, although not to the same extent as the liver (261, 262). Regeneration of duct and acinar tissue is undisputed, but controversy exists as to whether new β-cells can arise from a stem cell or facultative progenitor cell within the pancreas during regeneration. While β-cell neogenesis does not seem to occur under normal conditions in the adult pancreas (194), neogenesis was reported in several models of pancreas regeneration, including after β-cell destruction using chemical

toxins such as alloxan or streptozotocin (262-264), after the induction of pancreatitis (262, 265, 266), after cellophane wrapping (267), after partial pancreatectomy (193, 268), and after the targeting of inflammatory cytokines to the β-cell (269). However, recent lineage tracing studies have challenged the idea that β-cell neogenesis can occur after embryogenesis, even in the setting of injury or β-cell destruction (65, 270, 271).

β-cell mass expansion in the adult pancreas appears to occur through replication of existing β-cells and not from a resident stem or progenitor cell (194, 195, 231, 271). Genes and pathways involved in maintaining or altering β-cell mass are candidates for being affected in diabetic individuals. Functional analysis of these genes and pathways may lead to new *in vivo* therapeutic strategies for increasing existing β-cell mass in people with diabetes, and may facilitate the production of β-cells *in vitro* from embryonic stem cells (ESCs) or induced pluripotent stem cells (iPSCs) for cell-based therapies.

**A. Generating islet/β-cells from stem or progenitor cells**

There are several potential sources for the large number of insulin-producing cells that would be needed to make a cell-based therapy an option for individuals with diabetes (See Figure 1-11), including: (1) proliferation and expansion of existing β-cells *in vivo* or *ex vivo*; (2) induction of β-cell differentiation from endogenous progenitors; (3) induction of β-cell differentiation from ESCs or iPSCs; and (4) transdifferentiation of closely related cell types such as acinar, liver, and intestinal enteroendocrine cells. All of

these avenues are experimentally feasible, and are currently being examined in animal models. It may be that more than one of these methods will ultimately be used to derive a steady supply of insulin-producing cells. Regardless of the source, experimentally derived insulin-producing cells must be rigorously tested for their survival, engraftment, physiologic function *in vivo*, and their ability to reverse diabetes and maintain euglycemia, and their lack of tumorigenicity in a relevant animal model. The ability of *in vitro*-derived insulin-producing cells to function as mature β-cells in an animal model *in vivo* is critical to translating this eventually to human patients.

**Generation of Insulin-producing Cells from Adult Cell Sources**

Several models of pancreatic or β-cell injury suggest that the adult ductal epithelium retains some capacity for the production of new β-cells (neogenesis). The isolation and culture of ductal epithelium yields islet-like clusters that contain functional insulin-producing cells (272-275). These studies suggest that a facultative (if not a genuine) endocrine stem cell exists in the adult pancreatic ducts that, when properly activated, is capable of giving rise to new, functional β-cells. However, the presence of an adult pancreatic “stem cell” is highly controversial, as discussed prior in the section on regeneration. Some reports suggest that the adult mouse pancreas does contain multipotent precursor cells that represent only a very small fraction of the total cell population in the adult (276, 277) and that these may be centroacinar cells (278).

## Generation of Insulin-producing Cells from ES and iPS Cells

Several methods for the *in vitro* directed differentiation of insulin-expressing cells from murine and human ESCs have been described. These protocols are mainly based on information gained from processes occurring during *in vivo* pancreas development (8, 279). In addition, high-throughput screening of small molecules that promote β-cell differentiation has shown promise (280).

Although much is known about the factors involved in pancreatic endocrine differentiation during murine embryonic development, there are still serious limitations to differentiating human ES or iPS cells into β-cells. Current protocols generate insulin-producing cells at a low efficiency of ~10-15% at the final *in vitro* differentiation step (8, 281). In addition, the functionality of *in vitro* generated insulin-producing cells is limited, lacking the ability to secrete insulin in response to glucose (8). Gene expression analyses reveal that these insulin-producing cells are immature, lacking MafA and Nkx6.1 transcription factors (279, 281). However, transplantation of cells under the mouse kidney capsule at an earlier stage of differentiation, specifically the pancreatic progenitor stage, generates mature fully functional β-cells *in vivo* after about 5 months (281). Encouragingly, a recent study from the Melton group developed a protocol that efficiently generates a large amount of glucose-sensitive insulin+ cells from a human embryonic stem cell source (7). In addition, these insulin+ clusters were able to remediate diabetes in a diabetic mouse model. This promising protocol, paired with immune suppression tactics, could potentially serve as therapeutic for individuals with all forms of diabetes.

## B. β-Cell Regeneration in Adult Pancreas

In several models of pancreas regeneration and ductal metaplasia, upregulation of the Pdx1 transcription factor is associated with increased ductal proliferation and reported islet neogenesis (282, 283). It was thought that after insult, neogenesis may occur from ductal cells in a manner recapitulating embryonic pancreas development (284). Indeed, after partial pancreatectomy, there is a proliferation of ductal epithelium and formation of new ductal complexes (284). However, lineage tracing studies have generated contradictory results (285-287). One study suggested that β-cell neogenesis from a ductal origin still occurs in early postnatal mice, but not in adult mice (65). However, other lineage tracing studies show little to no contribution of ductal cells to neonatal islets (270, 288).

There is very limited information regarding pancreas regeneration in humans (285). Regeneration is thought to occur via neogenesis from ductal cells in the neonatal period, but new pancreas tissue formation after injury occurs to a limited degree in adults (261). Evidence suggests that β-cell mass does not regenerate after partial pancreatectomy in humans (289).

In addition, parts of this chapter were previously published under the title "Pancreas Development and Regeneration" in the 2nd edition of Sally Moody's *Principles in Developmental Genetics* (290).

## Thesis overview

Previous studies in our lab have examined the function of *Ctgf* in endocrine development, specifically β-cell differentiation and proliferation, using mouse models that display total or reduced *Ctgf* expression in the developing pancreas (52, 179). These studies demonstrated that CTGF is expressed in β-cells, ducts, and endothelial cells in the pancreas during development. CTGF expression is only maintained in the pancreatic ducts and endothelial cells after birth. The removal of CTGF during pancreas development yielded impaired β-cell differentiation and proliferation, and islet dysmorphogenesis. Intriguingly, over-expression of CTGF in insulin+ cells during development led to increased β-cell proliferation and endocrine area, while over-expression of CTGF in normal adult β-cells does not yield either of these two phenotypes. Yet, CTGF is re-expressed in adult β-cells during periods of β-cell mass expansion, such as pregnancy and high fat diet feeding. Overall, this suggests that CTGF is critical during periods of increased β-cell demand and β-cell mass expansion, potentially including β-cell regeneration.

As diabetes continues to affect millions of people within the United States, identification of novel factors that may enhance β-cell proliferation and mass regeneration *in vivo* would serve as an ideal therapeutic strategy to combat this epidemic. Thus, the goals of the studies presented in this thesis were to determine the ability of CTGF to promote β-cell mass regeneration in a setting of partial β-cell

destruction. We hypothesized that over-expression of CTGF after β-cell mass ablation would promote β-cell mass regeneration primarily via enhanced proliferation of the surviving β-cells. Chapter II of this thesis describes methods used, and Chapter III will introduce the role of CTGF in β-cell mass regeneration after 50% β-cell destruction. Chapter IV describes how CTGF promotes β-cell mass regeneration via modulation of β-cell maturity and proliferation characteristics. Finally, the potential involvement of the immune system in this model of β-cell regeneration will be addressed in Chapter V. Conclusions, implications and future directions for the studies described in this dissertation are presented in Chapter VI.

# CHAPTER II

## Materials and Methods

### Animals

Generation of RIP-rtTA (291), TetO-CTGF (52), and RIP-DTR (292) transgenic mice has been described previously. Mice were administered 2 mg/ml of doxycycline (DOX) in a 2% Splenda solution (to avoid taste aversion) in drinking water either prior to, during and/or after diphtheria toxin (DT) administration. Mice were treated with DOX for 2 days, 2 weeks, 4 weeks, or 8 weeks. DT (126 ng; Sigma) was given I.P. three times at 8 weeks of age. Animals treated with uridine analogs (5-Chloro-2'-deoxyuridine (CldU) or 5-Iodo-2'-deoxyuridine (IdU) both from Sigma) were administered analogs at 1 mg/mL in DOX-treated drinking water. PBS or clodrinate liposomes (250µL; clodrinate liposomes.com) was given I.P. once a day for 8 days. The Vanderbilt University Institutional Animal Care and Use Committee approved all of our mice studies.

### PCR and Genotyping

Genotyping of RIP-rtTA;TetO-CTGF;RIP-DTR mice was performed by PCR on DNA isolated from ear punches using the following primers: RIP-rtTA- forward 5'-CGC TGT GGG GCA TTT TAC TTT AG-3' and reverse 5'-CAT GTC CAG ATC GAA ATC GTC-3'. TetO-CTGF- forward 5'-GGA GGT CTA TAT AAG CAG AGC TCG-3' and

reverse 5’ TTA AGT TAC GCC ATG TCT CCG TA-3’. RIP-DTR- forward 5’-ACG GCT GCT TCA TCT ACA AGG-3’ and reverse 5’TTG GTG TCC ACG TAG TAG TAG-3’.

## Intraperitoneal glucose tolerance tests (IPGTT)

IPGTTs were performed on all cohorts (RIP-rtTA;RIP-DTR and RIP-rtTA;TetO-CTGF;RIP-DTR) after various DOX treatment periods as indicated. Mice were fasted for 16 h and given IP injections of filter-sterilized glucose in PBS (2.0 mg dextrose/g body weight). Glucose concentrations were measured via tail vein blood sampling at 0, 15, 30, 60, 90 and 120 minutes using an Accuchek Aviva glucometer and test strips (Roche Pharmaceuticals).

## Tissue dissection, preparation and histology

At the end of DOX treatment periods, mice were sacrificed and pancreata were dissected and fixed for 4 hours in 4% paraformaldehyde at 4°C, dehydrated in an ascending ethanol series, cleared in xylenes, and embedded in paraffin. Tissues were then serial sectioned at 5 µM. For the generation of frozen tissue sections, dissected pancreata were fixed for 1 hour in 4% paraformaldehyde at 4ºC, and placed in a 30% sucrose solution overnight at 4ºC. Pancreata were then embedded in O.C.T compound (Tissue-Tek). Tissues were then serial sectioned on a cryostat at 7µM.

## Immunolabeling

Paraffin-embedded tissues were deparaffinzed in xylenes, rehydrated in a descending ethanol series to distilled water. Frozen tissues were thawed and washed in 1X PBS solution. Indirect protein localization was obtained by incubation of tissue with primary antibodies. All primary antibodies were incubated overnight in a humid chamber at 4°C, unless otherwise stated. See Table 2-1 for immunolabeling details. Nuclei were visualized with DAPI (Molecular Probes) staining. Imaging was with a ScanScope FL scanner (Aperio Technologies, Inc.) and quantified using Metamorph 6.1 (Molecular Devices). Neutrophil immunohistochemistry (Neutrophil Marker, Santa Cruz) was completed on frozen tissue sections at Tissue Pathology Shared Resource Core.

## β-cell mass

Entire pancreata were serially sectioned and slides every ~250 μm apart (10-12 slides per animal, 1-2% of entire pancreas) were immunolabeled for insulin followed by a peroxidase-conjugated secondary antibody. The primary antibody was visualized via a DAB Peroxidase Substrate Kit (Vector Laboratories), and counterstained with Eosin. One pancreatic section per slide (1-2% of entire pancreas) was scanned using a ScanScope CS slide scanner (Aperio Technologies, Inc.). Images from each experiment were processed identically with the ImageScope Software (Aperio, Inc.) (293). β-cell mass was measured by obtaining the ratio of insulin-positive area to total pancreas area of all scanned sections per animal and multiplied by the pancreatic wet weight.

**Table 2-1. Antibody Information.**

| Antibody | Source | Dilution/Temp/Time | Antigen Retrieval | Special Note |
|---|---|---|---|---|
| **Guinea pig anti-insulin** | DAKO | 1:500/4°C/ON | None | - |
| **Rabbit anti-glucagon** | Sigma-Aldrich | 1:500/4°C/ON | None | - |
| **Rabbit anti-Ki67** | AbCam | 1:500/RT/ON | 1XNaCitrate (10mM; ph 6.0) | Boil 14' on High |
| **Rabbit anti-MafA** | Bethyl Laboratories | 1:500/RT/ON | 1XTEG buffer (pH 9.0) | 1' on High, 7.5' on 10% |
| **Rabbit anti-MafB** | Bethyl Laboratories | 1:500/RT/ON | 1XTEG buffer (pH 9.0) | 1' on High, 7.5' on 10% |
| **Rabbit anti-CD31** | AbCam | 1:100/4°C/ON | 1XNaCitrate (10mM; ph 6.0) | Boil 14' on High |
| **Mouse anti-E-Cadherin** | BD Parmigen | 1:500/RT/ON | 1XTEG buffer (pH 9.0) | 1' on High, 7.5' on 10% |
| **Rabbit anti-p-ERK1/2** | Cell Signaling | 1:100/4°C/ON | 1XNaCitrate (10mM; ph 6.0) | Pressure Cooker: 15' on high, 45' in heat. |
| **Rabbit anti-Tph1** | AbCam | 1:150/4°C/ON | 1XNaCitrate (10mM; ph 6.0) | Pressure Cooker: 15' on high, 45' in heat. |
| **Mouse anti-BrdU** | BD Parmigen | 1:100/4°C/ON | 1XTEG buffer (pH 9.0) | See (195) |
| **Rat anti-BrdU** | ACSC | 1:250/4°C/ON | 1XTEG buffer (pH 9.0) | See (195) |
| **Rat anti-CD45** | BD Parmigen | 1:100/4°/ON | None | Frozen Sections Only |
| **Rat anti-F4/80** | Invitrogen | 1:100/4°/ON | None | Frozen Sections Only |
| **Rat anti-B220** | BD Parmigen | 1:100/4°/ON | None | Frozen Sections Only |
| **Rat anti-CD3** | BD Parmigen | 1:100/4°/ON | None | Frozen Sections Only |

Abbreviations: ON, overnight; RT, room temperature; TEG, Tris-EGTA

## β-cell proliferation

Entire pancreata were serially sectioned and slides were immunolabeled for insulin and Ki67. Five slides (at least 250 µm apart) per animal were selected. One pancreatic section from each slide was imaged via a ScanScope FL slide scanner (Aperio Technologies, Inc.). A minimum of 4,000 cells were counted using Metamorph 6.1 software (Molecular Devices). The percentage of proliferating cells was determined by dividing the number of Ki67/insulin double-positive cells by the total number of insulin cells.

## Analysis of α-cell:β-cell ratio

Entire pancreata were serially sectioned and slides every ~250 µm apart (10-12 slides per animal) were immunolabeled for insulin and glucagon. One pancreatic section from each slide (1-2% of entire pancreas) was imaged via a ScanScope FL slide scanner (Aperio Technologies, Inc.). A minimum of 4,000 cells were counted using Metamorph 6.1 software (Molecular Devices). The ratio of α:β-cells was calculated by dividing the total number of glucagon+ cells by the total number of insulin+ cells per animal.

## β-cell size

Entire pancreata were serially sectioned and slides were immunolabeled for insulin. Five slides at least 250 μm apart per animal were selected. One pancreatic section from each slide was imaged via a ScanScope FL slide scanner (Aperio Technologies, Inc.). Using Metamorph 6.1 software (Molecular Devices), the total β-cell area for each islet was determined. The number of β-cells in each islet was quantified, and average β-cell size was determined by dividing the area of the islet by number of β-cells. β-cells from a minimum of 125 islets per animal were assessed.

## Islet number and size

Entire pancreata were serially sectioned and slides every ~250 μm apart (10-12 slides per animal) were immunolabeled for insulin and glucagon. One pancreatic section from each slide (1-2% of entire pancreas) was imaged via a ScanScope FL slide scanner (Aperio Technologies, Inc.). All islets were counted and binned according to size (<8 cells or ≥8 cells, ≥ 200 islets).

## Islet microvascular density

Entire pancreata were serially sectioned and slides were immunolabeled for insulin and CD31 to determine the microvascular density of islets. Five slides at least 250 μm apart per animal were selected and immunolabeled with insulin and platelet

endothelial cell adhesion molecule (PECAM/CD31). One pancreatic section from each slide was imaged via a ScanScope FL slide scanner (Aperio Technologies, Inc.). Through Metamorph 6.1 software (Molecular Devices) the area of PECAM-positive area of at least 100 islets was measured. To determine the percentage of blood vessel area, the total PECAM area was divided by the total insulin-positive area.

### E-cadherin expression in Proliferating β-cells

Entire pancreata were serially sectioned and slides were immunolabeled for insulin, E-cadherin, and Ki67. One pancreatic section from each slide was imaged via a ScanScope FL slide scanner (Aperio Technologies, Inc.). Through Metamorph 6.1 software (Molecular Devices) the number of Ki67/E-cadherin/Insulin triple-positive cells and Ki67/Insulin double-positive cells was quantified.

### Collagen Deposition

Entire pancreata were serially sectioned and five slides at least 250 µm apart per animal were selected. Sections were stained for one hour at room temperature in a humid chamber with Sirius Red with a Fast Green (Sigma-Aldrich) counterstain to denote pancreatic tissue. Slides were then washed in acidified water and dehydrated in an increasing ethanol series concluding in xylenes. One pancreatic section from each slide was imaged via a ScanScope CS slide scanner (Aperio Technologies, Inc.). Images were processed identically with the ImageScope software (Aperio Technologies,

Inc.). Collagen deposition was analyzed by obtaining the percentage of collagen per pancreatic section area via a modified Genie algorithm within the Spectrum software (Aperio Technologies, Inc.). This proportion was then multiplied to the pancreatic wet weight to determine total collagen mass per pancreas.

## β-cell death

Terminal deoxynucletidyl transferase dUTP nick end labeling (TUNEL) assay was performed to detect β-cell apoptosis and necrosis using the ApoAlert DNA fragmentation assay kit (CLONTECH Laboratories, Inc.) according to the manufacturer's instructions. Slides were co-labeled with insulin as described above. Five slides at least 250 µm apart per animal were assessed. One pancreatic section from each slide was imaged via a ScanScope FL slide scanner (Aperio Technologies, Inc.). A minimum of 4,000 cells were counted using Metamorph 6.1 software (Molecular Devices). The percentage of apoptotic or necrotic β-cells was determined by dividing the number of TUNEL/insulin double-positive cells by the total number of insulin cells.

## Analysis of β-cell maturity

Entire pancreata were serially sectioned and slides were immunolabeled for insulin, MafA, and Ki67. Due to species cross-reaction concerns, Ki67 was detected via direct conjugation of a Cy5 flurophore to the rabbit anti-Ki67 primary antibody. Five slides at least 250 µm apart per animal were selected. One pancreatic section from

each slide was imaged via a ScanScope FL slide scanner (Aperio Technologies, Inc.). A minimum of 4,000 cells were counted using Metamorph 6.1 software (Molecular Devices). The percentage of mature β-cells within the islet was determined by dividing the number of MafA/insulin double-positive cells by the total number of insulin+ cells. The percentage of immature β-cells within the islet was determined by dividing the number of MafB/insulin double-positive cells by the total number of insulin+ cells. To assess the maturity state of proliferating β-cells, the number of Ki67/MafA/Insulin triple-positive cells was divided by the total number of MafA/insulin double-positive cells. Subsequently, the percentage of immature proliferating β-cells was assessed by dividing the number of Ki67+/MafA-/insulin+ cells by the total number of MafA-/insulin+ cells.

### Analysis of β-cell refractory period

In order to assess changes in the length of the β-cell refractory period, a system of dual uridine analog labeling was employed. Mice were treated with CldU for 2 days after DT treatment, followed by a 1 or 3 week washout period and ended with a 5 day treatment with IdU. DOX was also administered from the completion of DT administration to the completion of these studies. Whole pancreata were then harvested, serially sectioned, and slides immunolabeled for insulin, CldU and IdU. CldU and IdU were visualized through the use of analog-specific BrdU primary antibodies (195). Five slides at least 250 μm apart per animal were selected. One pancreatic section from each slide was imaged via a ScanScope FL slide scanner (Aperio

Technologies, Inc.). A minimum of 4,000 cells were counted using Metamorph 6.1 software (Molecular Devices). The percentage of β-cells undergoing replication in the first two days, or last five days of DOX treatment was determined by dividing the number of CldU/Insulin double-positive cells or IdU/Insulin double-positive cells by the total number of insulin+ cells, respectively. To determine the percentage β-cells undergoing replication during both labeling periods, the number of CldU/IdU/Insulin triple-positive cells were divided against the total number of insulin+ cells. An increase in the number of triple-positive cells over controls is indicative of a shortened refractory period. Changes in the ratios between the three labeling groups (CldU+, IdU+, CldU+;IdU+) were assessed by determining ratios between subgroups (i.e. CldU+:IdU+, CldU: CldU+;IdU+, and IdU: CldU+; IdU+) and analyzing differences via Student's T-test.

### Analysis of Dual-Labeled β-cells' Maturity State

In order to assess the maturity state of dual-labeled β-cells, the previously described system of uridine analog labeling was employed. Pancreata were sectioned and five slides at least 250 μM apart per animal were selected. Slides were immunolabeled for MafA, CldU, and IdU. CldU and IdU were visualized as previously described (195). One pancreatic section from each slide was imaged via a ScanScope FL slide scanner (Aperio Technologies, Inc.). All dual-labeled (CldU+/IdU+ cells) were counted using Metamorph 6.1 software (Molecular Devices). To determine whether β-cells undergoing replication during both labeling periods were mature (MafA+) or

immature (MafA-), the number of MafA/CldU/IdU triple-positive cells or MafA-/CldU/IdU dual-positive cells were divided against the total number of dual-positive (Cldu+/IdU+) cells.

## Islet Isolation and RNA acquisition

Islets were isolated from 10 week old adult female mice via collagenase digestion through the main pancreatic duct at $37^0$C and hand-picking of islets from exocrine tissue (294). Picked islets were placed immediately in 1mL Trizol reagent and lysed by vortexing. RNA was isolated using the RNeasy Mini kits (Qiagen) according to manufacturer's instructions and eluted in 30 µL $dH_2O$. RNA concentration and integrity were assessed using a ND-1000 Spectrphotometer (NanoDrop) and the 2100 Electrophoresis Bioanlyzer (Agilent) at Vanderbilt Technologies for Advanced Genomics (VANTAGE) Core.

## *E-cadherin* and *p16* expression analysis

50 ng cDNA was prepared from islet RNA using the SuperScript III First Stand Synthesis System according to the manufacturer (Invitrogen). Real time reactions were with iQ SYBR Green supermix (Bio-Rad). E-cadherin: Forward, AGGCGGGAATCGTGGC; Reverse, AAGGATTCCGAGGATGGCA. p16: Forward, CCGTCGTACCCCGATTCAG; Reverse, GCACCGTAGTTGAGCAGAAGAG.

## Analysis of gene expression by TaqMan Low Density array (TLDA)

To assess changes in islet gene expression, islet RNA was isolated and >250 ng cDNA was prepared using the SuperScript III First Stand Synthesis System Kit according to the manufacturer's directions (Invitrogen). Genes were assessed using TaqMan Universal PCR Mastermix (with UNG, Applied Biosystems) and appropriate primer sets. TLDAs were conducted on a 7900HT Fast Real-Time PCR system. Acquired data was analyzed using SDS RQ Study software (Applied Biosystems, Life Technologies). All samples were run in duplicate.

## Western Blotting

Islets were isolated from 8 week old animals as described above. Following isolation, islets were immediately lysed in RIPA buffer and protein content was quantified using the Bio-Rad DC protein assay (Bio-Rad). 3.5 micrograms of protein per sample were electrophoresed on 4-12% Bis-Tris gels under denaturing conditions and blotted onto PVDF membrane using the NuPAGE Western blotting system (Invitrogen). Blots were blocked and probed with the following primary antibodies diluted in 5% nonfat milk in 1XTBST and incubated overnight at 4°C: rabbit anti-phospho-Smad3 (AbCam, 1:1,000), rabbit anti-Smad2/3 (Cell Signaling, 1:500), and rabbit anti-β-tubulin (Santa-Cruz, 1:5,000). HRP-conjugated rabbit secondary antibody (Jackson Immunoresearch, 1:5,000) was used for protein detections, and facilitated by an ECL

Prime detection system (Amersham) using Kodak X-Omat Blue film. Protein levels were quantified using ImageJ software.

## Quantification of Immune Cell Populations

To assess changes in immune cell infiltration to both exocrine and endocrine tissue, entire frozen pancreata were serially sectioned and at least three slides were immunolabeled for insulin and various immune cell markers; CD45 (pan-immune), B220 (B cells), CD3 (T cells), and F4/80 (Macrophages). One pancreatic section from each slide was imaged via a ScanScope FL slide scanner (Aperio Technologies, Inc.). Five random insulin+ areas ($4000^2$ pixels) were extracted per slide. Through Metamorph 6.1 software (Molecular Devices) immune cells were binned as either islet associated or within the exocrine compartment.

## Statistics

Results are expressed as mean ± SEM. Statistical significance was calculated by Student's T test, One-way, or Two-way ANOVA analysis where applicable. $p \leq 0.05$ was considered significant.

# CHAPTER III

## CTGF INDUCES β-CELL REGENERATION FOLLOWING 50% β-CELL ABLATION VIA INCREASED β-CELL PROLIFERATION

### Introduction

Our lab initially became interested in studying CTGF in the context of pancreas development and function when we observed it to be downregulated in a transgenic model of HNF6 islet cell over-expression (178). We have subsequently determined that CTGF is expressed in ductal epithelium, vascular endothelium and embryonic insulin-producing cells; expression in β-cells is silenced soon after birth (179). Our lab showed that CTGF is required for β-cell proliferation during embryogenesis and that transgenic over-expression of CTGF in embryonic insulin-producing cells increases β-cell proliferation and mass (52). Interestingly, CTGF over-expression elicited increased proliferation in immature (MafA-) β-cells. In contrast, induction of CTGF in adult β-cells, under normal conditions, does not increase β-cell proliferation or mass (189). However, CTGF is re-expressed in adult β-cells during pregnancy and in response to HFD feeding ((179), Mosser and Gannon, unpublished observations), suggesting that it plays a role in β-cell compensation during known periods of β-cell mass expansion, such as β-cell regeneration.

In order to test the hypothesis that CTGF could promote β-cell mass regeneration in the setting of β-cell destruction, we paired our transgenic model of CTGF induction (52) with a diphtheria toxin (DT)-mediated model of 50% β-cell ablation (292). Neither CTGF over-expression nor 50% β-cell destruction alters glucose homeostasis in the

mouse. We found that CTGF induction only after 50% β-cell destruction elicits an increase in adult β-cell proliferation, resulting in 50% β-cell recovery. We did not observe other mechanisms of β-cell mass regeneration in this particular model; such as β-cell neogenesis, loss of cell-cell contacts, or β-cell hypertrophy. Additionally, in this particular model of DT-mediated β-cell destruction, CTGF does not serve as a prophylactic in regards to enhancing β-cell survival. Together these data demonstrate for the first time that CTGF has the potential to promote β-cell mass regeneration after partial (50%) β-cell destruction.

## Results

### Mouse models of β-cell ablation and CTGF induction

CTGF is expressed by β-cells during known periods of physiological β-cell mass expansion, such as development (52), pregnancy (179), and HFD feeding (Mosser and Gannon, unpublished observations), we hypothesized that CTGF would promote β-cell mass regeneration following injury (i.e. β-cell loss). To assess this, a diphtheria toxin (DT)-mediated model of β-cell ablation was employed (292). In this model the rat insulin promoter (RIP) drives expression of the DT receptor (DTR) (See Figure 3-1A). This transgene was targeted to the *hprt* locus on the X chromosome, allowing for gender-specific differences in the proportion of β-cells in which DTR is expressed. In males and homozygous females, DT injection results in 99% β-cell ablation (292). We employed the less severe model of 50% β-cell ablation afforded by hemizygous RIP-DTR female

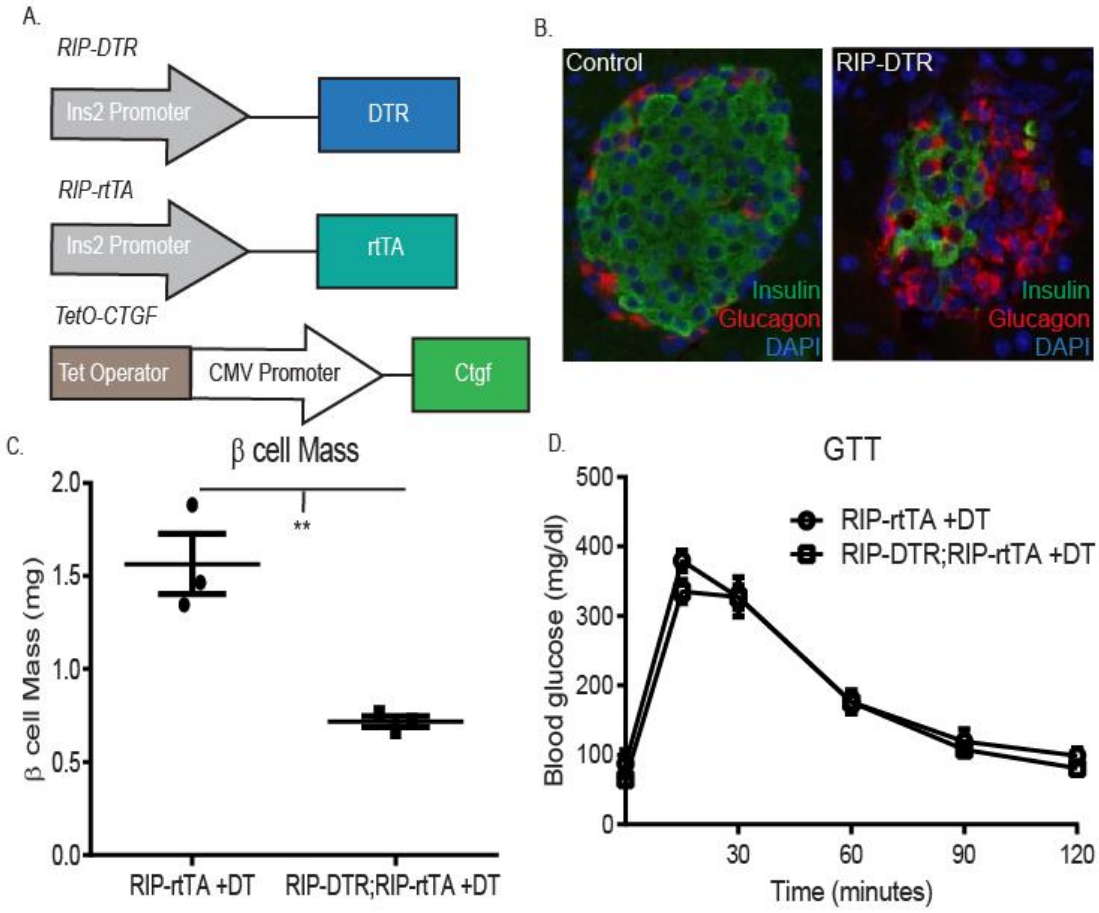


**Figure 3-1.** Validation of murine transgenic models. **A.** Transgenic models employed. Top: β-cell-specific diphtheria toxin receptor (DTR) is driven by the rat insulin (Ins2) promoter. Middle: β cell-specific reverse tetracycline transactivator (rtTA) is driven by the rat Ins2 promoter. Bottom: Tet-operator sequence binds rtTA to drive CTGF expression in the presence of Dox. **B.** Left- Islet from control DT-injected animal (RIP-rtTA or TetO-CTGF). Right- Islet from experimental DT-injected animal (hemizygous RIP-DTR). **C.** β cell mass (circles) and animals hemizygous for RIP-DTR (squares). **D.** 50% β cell ablation (squares) and controls (circles) blood glucose homeostasis as measured by intraperitoneal glucose tolerance tests. n=3, **p=0.0068. Adapted from Riley *et al.*, *Diabetes* (2015).

mice (which express DTR in 50% of β-cells on average due to random X-inactivation). We were able to consistently replicate the previously reported 50% β-cell ablation in hemizygous RIP-DTR female mice at 8 weeks of age (See Figure 1-3). β-cell mass was not affected in control animals (RIP-rtTA or TetO-CTGF) after DT administration (See Figure 3-1B, C) and α-cell mass was unaffected in all treatments. Additionally, no changes in blood glucose clearance were observed after 50% β-cell ablation, as assessed by intraperitoneal glucose tolerance test (IP-GTT) (See Figure 3-1D). This was anticipated as 50% β-cell mass is sufficient to maintain euglycemia and glucose tolerance under normal conditions.

To assess if CTGF promotes β-cell mass regeneration, an inducible CTGF bi-transgenic model was utilized in which insulin-producing cells express the reverse tetracycline transactivator (RIP-rtTA), which binds the Tet-Operator to drive CTGF (TetO-CTGF; See Figure 3-1A). This binding occurs solely in the presence of doxycycline (Dox), which is administered in the drinking water. Specificity of CTGF induction has been previously confirmed (189). Homozygous RIP-DTR females were interbred with RIP-rtTA;TetO-CTGF males. The resulting hemizygous RIP-DTR;RIP-rtTA;TetO-CTGF females and hemizygous RIP-DTR;RIP-rtTA female littermate controls were employed. Since CTGF up-regulation is associated with increased fibrosis in some situations (252, 295), collagen deposition was quantified. No increase in islet associated collagen was observed, as assessed by Sirius Red staining, after 8 weeks of continuous CTGF over-expression (See Figure 3-2). This is in agreement with our previously published results using this model (189), and demonstrates that CTGF can be over-expressed in islets for several weeks with no deleterious effects on fibrosis.

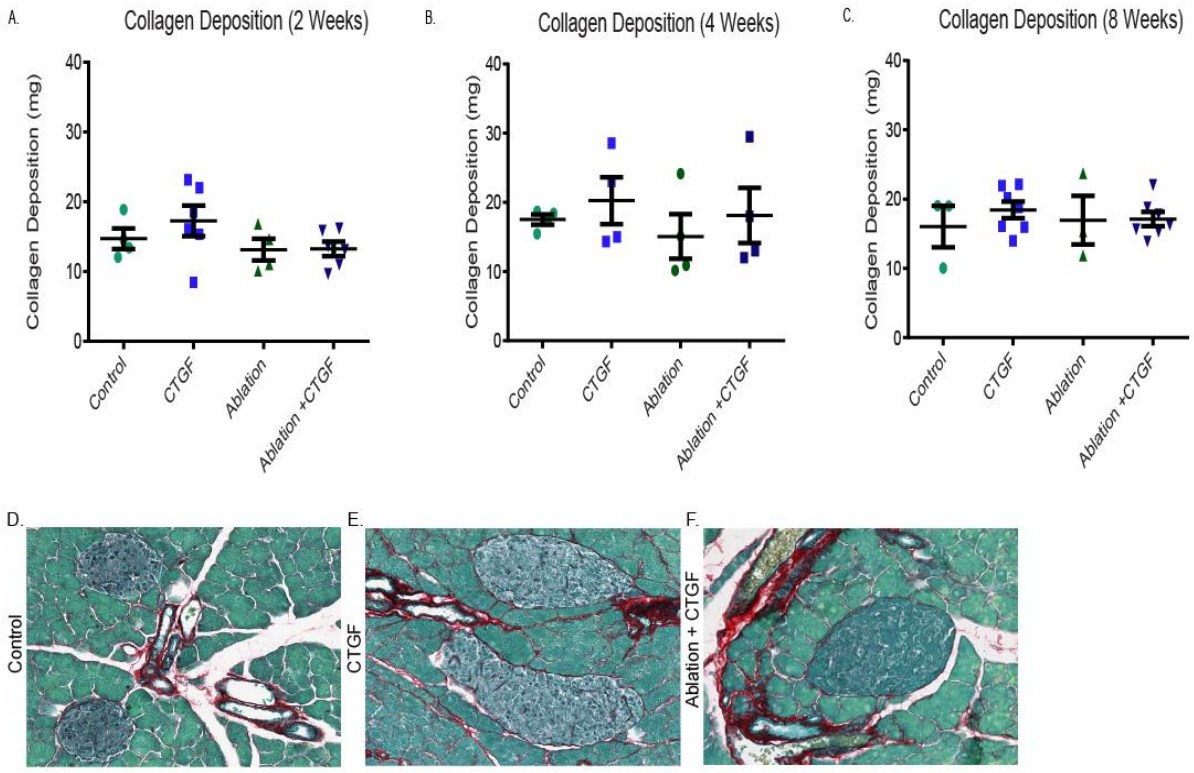


**Figure 3-2.** Continuous CTGF induction for up to 8 weeks does not promote excess collagen deposition or fibrosis. Quantification of collagen deposition per pancreas at 2 weeks **A.**, 4 weeks **B.** and 8 weeks **C.** of Dox administration. **D-F:** Representative images of collagen staining, with Sirius Red demarking collagen against a pancreatic tissue counterstain with Fast Green. n=3-7. Adapted from Riley *et al.*, *Diabetes* (2015).

**CTGF induces β-cell regeneration after 50% β-cell ablation**

DT was administered at 8 weeks of age to RIP-DTR;RIP-rtTA controls ("Ablation") and RIP-DTR;RIP-rtTA;TetO-CTGF experimental animals ("Ablation + CTGF") and CTGF induced by Dox treatment for 2 days, 2 weeks, or 4 weeks after DT injection. Non-DT injected animals included controls ("Control") and those in which CTGF was induced without ablation ("CTGF") (See Figure 3-3A). Prior to sacrifice, an IP-GTT was performed. At all treatment periods neither 50% β-cell ablation, nor CTGF induction alone had an effect on glucose homeostasis (See Figures 3-1D and 3-3). CTGF induction under normal conditions in adult islets elicited no increase in either β-cell mass expansion or β-cell proliferation (See Figure 3-4A,B; 2 - CTGF) in agreement with our previous observations (189). Likewise, 50% β-cell ablation alone did not induce β-cell regeneration or proliferation (See Figure 3-4A, B; 3 - Ablation). This finding was unsurprising, as these animals maintain euglycemia and thus have no physiological impetus to expand β-cell mass.

Over-expression of CTGF after ablation resulted in partial restoration of β-cell mass at 2 weeks, and reached 50% mass recovery at 4 weeks of CTGF treatment (See Figure 3-4A, C; 4 - Ablation+CTGF). The observed β-cell mass expansion was further corroborated by an improvement in the α-cell:β-cell ratio of the "Ablation+CTGF" cohort after both 2 and 4 weeks of CTGF induction, indicative of an increase in β-cell number (See Figure 3-5). α-cell mass was unaltered under any of our treatment conditions and 50% β-cell ablation did not stimulate α-cell transdifferentiation to a β-cell fate (Thorel and Hererra, manuscript submitted).

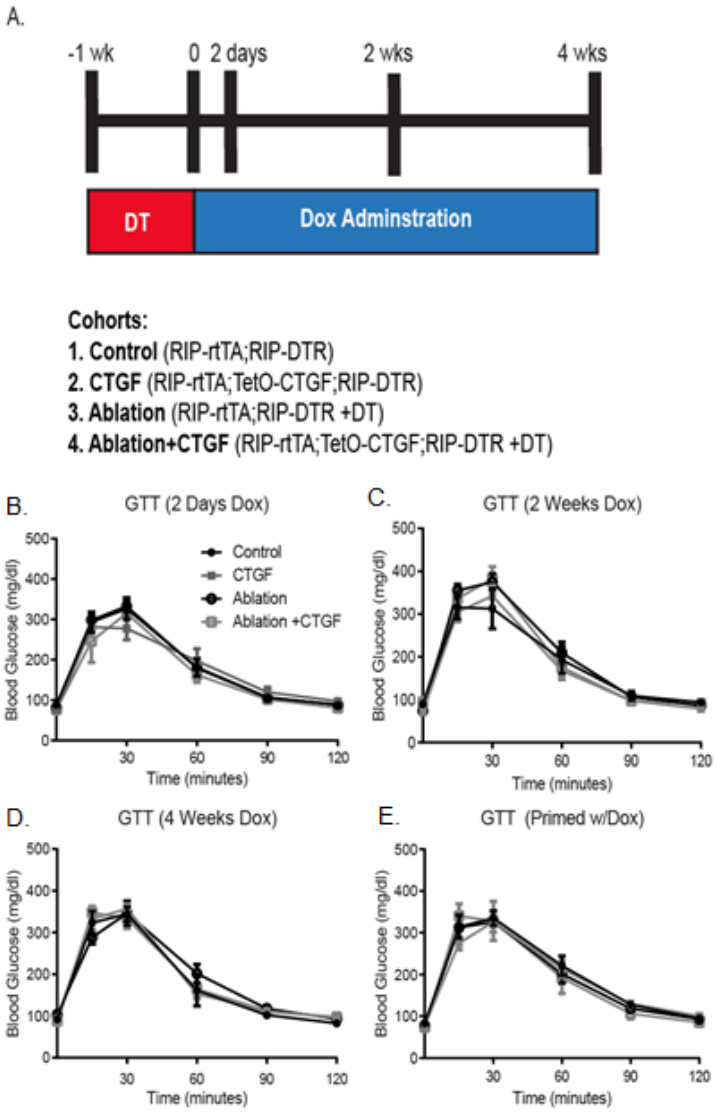


**Figure 3-3.** Experimental outline and glucose tolerance tests. **A.** Experimental outline. Mice were administered 2 mg/ml of doxycycline (Dox) in 2% Splenda in drinking water. Diphtheria toxin (DT; 126 ng; Sigma) was given IP 3 times at 8 weeks of age. **B-E.** Intraperitoneal glucose tolerance tests reveal no difference in glucose homeostasis between Control (filled circles), CTGF treated (filled squares), Ablation (open circles), or Ablation+CTGF (open squares) mice treated with Dox for 2 days **A.**, 2 weeks **B.**, 4 weeks **C.**, or for 1 week prior to, during and 2 days after DT injections **D.** beginning at 8 weeks of age. n=6 for 2 day and primed timepoints. n=8 for 2 and 4 week timepoints. Adapted from Riley *et al.*, *Diabetes* (2015).

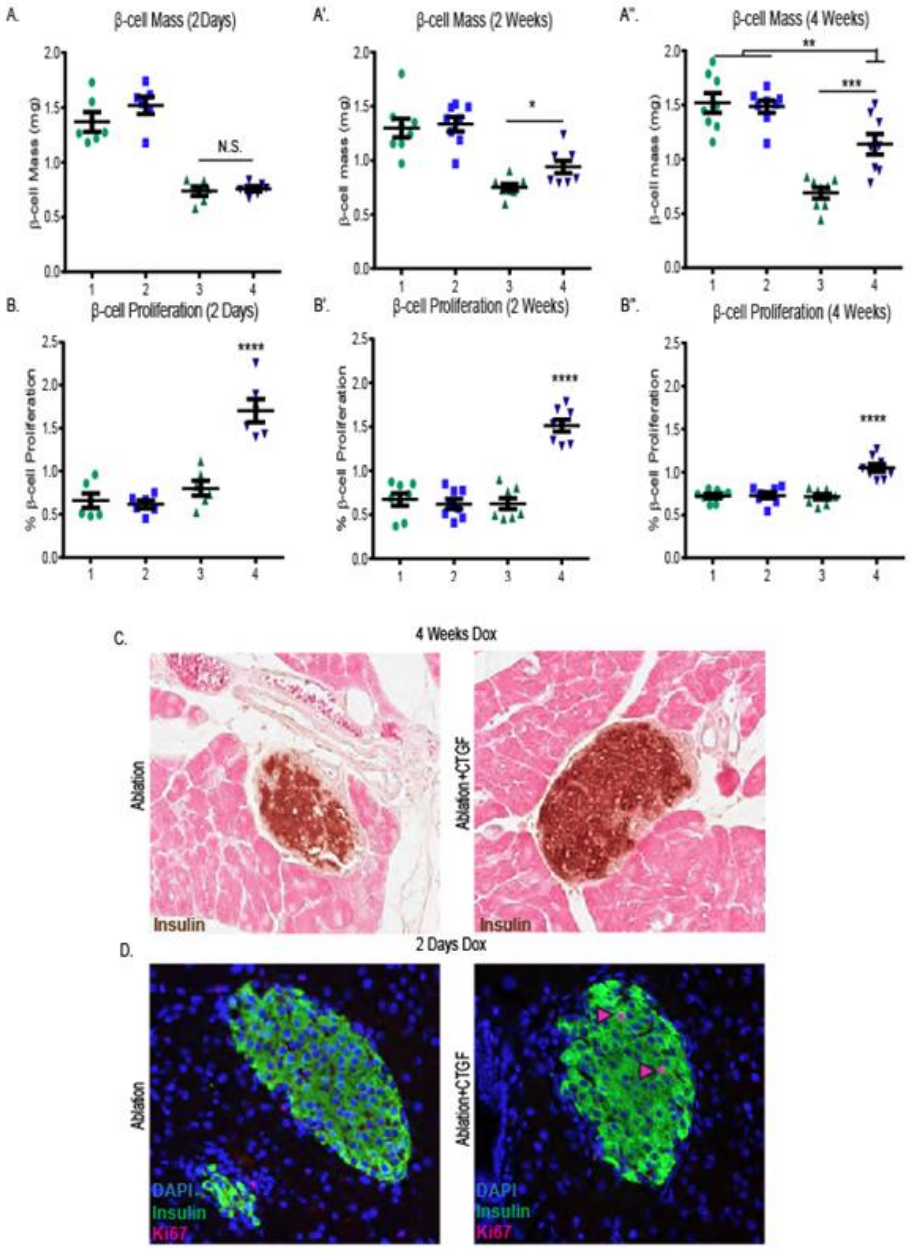


**Figure 3-4.** CTGF promotes β-cell mass regeneration and proliferation. (**A-A''**) β-cell mass and (**B-B''**) proliferation. **C.** Representative images of β-cell mass at 4 weeks. **D.** Representative images of β-cell proliferation 2 days after ablation with or without CTGF. Nuclei were visualized with DAPI (Molecular Probes). Primary antibodies were detected by species-specific donkey secondary antibodies conjugated to either Alexa 488 or Cy3 fluorophores. Pink arrows: proliferating β-cells. 2 day timepoint n=6, 2 and 4 week timepoints n=8. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001. Adapted from Riley *et al.*, *Diabetes* (2015).

A.

| | β-cells | α-cells | β-cell % |
|---|---|---|---|
| Control | 7672 | 2384 | 76.3% |
| Ablation | 3775 | 2487 | 60.3% |
| Ablation +CTGF | 5607 | 2443 | 69.6% |

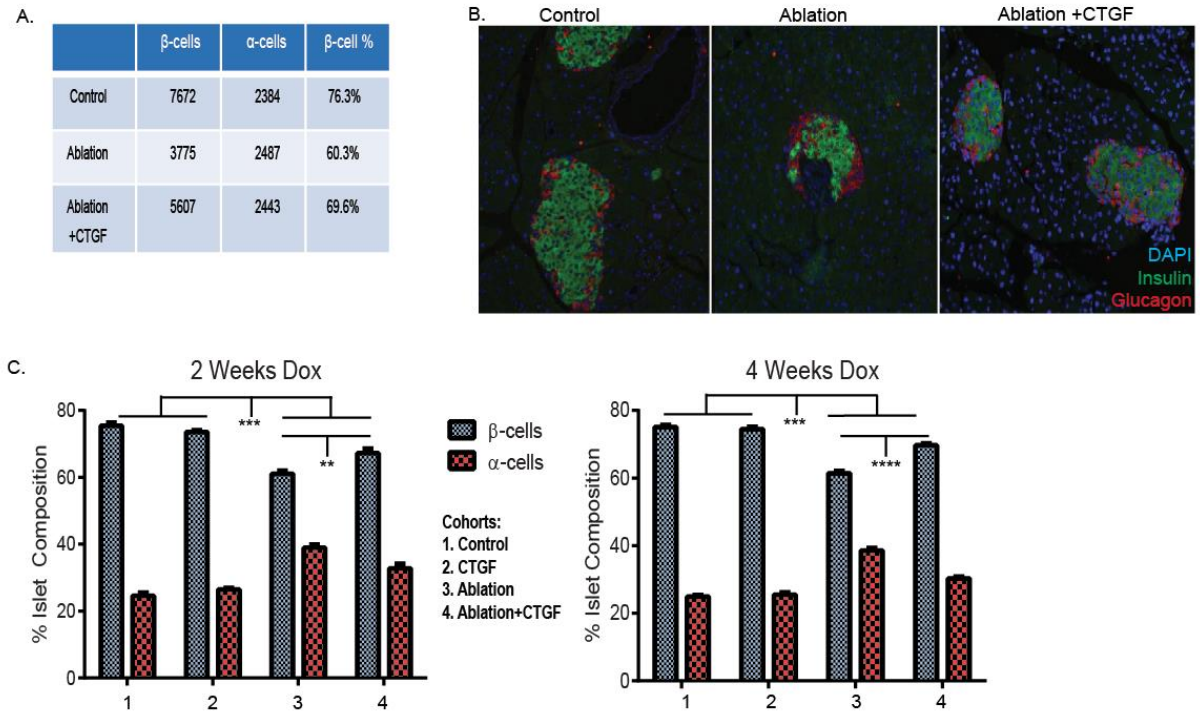


**Figure 3-5.** CTGF treatment after 50% β-cell destruction elicits improved α-cell:β-cell ratios. **A-B**. Example of actual raw data obtained: The number of insulin+ (green) and glucagon+ (red) cells was quantified. Under normal conditions, β-cells constitute ~75% of the total number of counted cells (Control). After DT injection, 50% of β-cells are ablated while α-cells remained unchanged (Ablation). This results in β-cells accounting for ~60% of counted cells. In Ablation+CTGF animals, β-cells account for ~70% of counted cells. **C**. Quantification of α-cell:β-cell ratios after 2 (left) and 4 (right) weeks of Dox administration. n=8, **p<0.01, ***p<0.001, ****p<0.0001. Adapted from Riley *et al.*, *Diabetes* (2015).

**CTGF elicits enhanced β-cell proliferation after 50% β-cell ablation**

In order to determine the underlying mechanism of the observed β-cell mass regeneration, β-cell proliferation was assessed. CTGF induction under normal conditions in adult islets elicited no increase in β-cell proliferation (See Figure 3-4C; 2 - CTGF) in agreement with our previous observations (189). Likewise, 50% β-cell ablation alone did not induce β-cell proliferation (See Figure 3-4C; 3 - Ablation).

As anticipated, the increase in β-cell mass was, at least in part, due to increased β-cell proliferation, which was enhanced after only 2 days of CTGF treatment and was maintained at all time-points, although the degree of enhanced β cell proliferation declines at 4 weeks of CTGF over-expression (See Figure 3-4C,E; 4 - Ablation+CTGF). Thus, the increase in β-cell proliferation precedes the observed β-cell mass recovery. The elevated β-cell proliferation at 4 weeks suggested that a further increase in β-cell mass recovery was possible. However, 8 weeks of continuous CTGF treatment after 50% β-cell ablation elicited no further improvement in β-cell mass recovery, suggesting a limit of restoration had been reached (data not shown). Thus, although adult β-cells are unresponsive to CTGF under non-stimulatory conditions, CTGF can induce replication and β-cell mass expansion in the unique microenvironment of β-cell destruction.

**No evidence of other modes of β-cell mass recovery**

We also examined whether other compensatory mechanisms also contributed to regeneration. Individual β-cell size remained constant regardless of treatment (See Figure 3-6A), indicating that β-cell mass expansion was not due to β-cell hypertrophy.

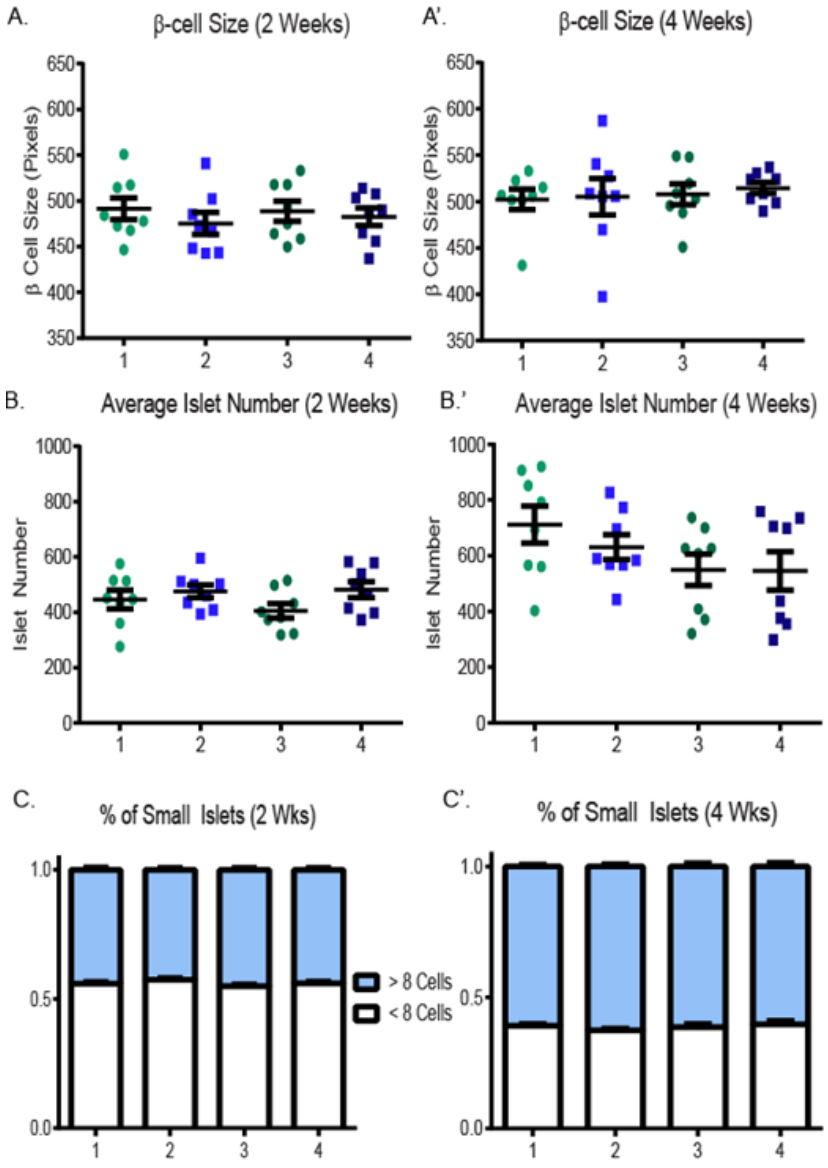


**Figure 3-6.** CTGF does not mediate β-cell regeneration via hypertrophy or neogenesis. 1. Control, 2. CTGF, 3. Ablation, 4. Ablation+CTGF. **A**. β-cell size after either 2 (**A**) or 4 (**A'**) weeks of CTGF treatment. **B**. Average number of islets per animal after 2 (**B**) or 4 (**B'**) weeks of CTGF induction. **C.** Number of small insulin+ clusters after CTGF induction for 2 (**C**) or 4 (**C'**) weeks. n=8. Adapted from Riley *et al.*, *Diabetes* (2015).

As surrogates for islet neogenesis, we evaluated total islet number and percentage of small insulin+ clusters. No change in either parameter was observed (See Figure 3-6B,C). Overall, CTGF does not appear to promote β-cell neogenesis in this model of β-cell regeneration, although in the absence of a specific marker for neogenesis, we cannot conclusively rule this out. However, this result was not surprising as neogenesis has been primarily described in more severe models of massive combined acinar and islet cell ablation (286).

Pancreatic islets are highly vascularized and their endothelium produces factors such as hepatocyte growth factor (HGF), which induce β-cell mass expansion (63, 296, 297). In addition, as CTGF is an angiogenic factor (162), it could promote β-cell regeneration indirectly via enhanced vascularization. We observed that CTGF did not elicit an increase in islet vascular density, as assessed by PECAM+ area (See Figure 3-7), in agreement with our previously published results (189), although it remains possible that CTGF induces production of endothelial-derived β-cell growth factors.

Finally, based on a recent study showing that *E-cadherin* knockdown enhances β-cell proliferation (298), we hypothesized that 50% β-cell ablation might result in decreased cell-cell contacts and decreased E-cadherin expression, thus contributing to increased β-cell proliferation. Therefore, we assessed *E-cadherin* expression via qRT-PCR after 2 days of CTGF induction (the peak of β-cell proliferation). *E-cadherin* levels (See Figure 3-8A) and the percentage of proliferating β-cells that were E-cadherin+ or E-cadherin- was the same across all cohorts (See Figure 3-8B). β-cell replication did not correlate with decreased E-cadherin protein membrane localization (See Figure 3-8B,C). Thus, CTGF promotes β-cell mass expansion primarily via enhanced β-cell

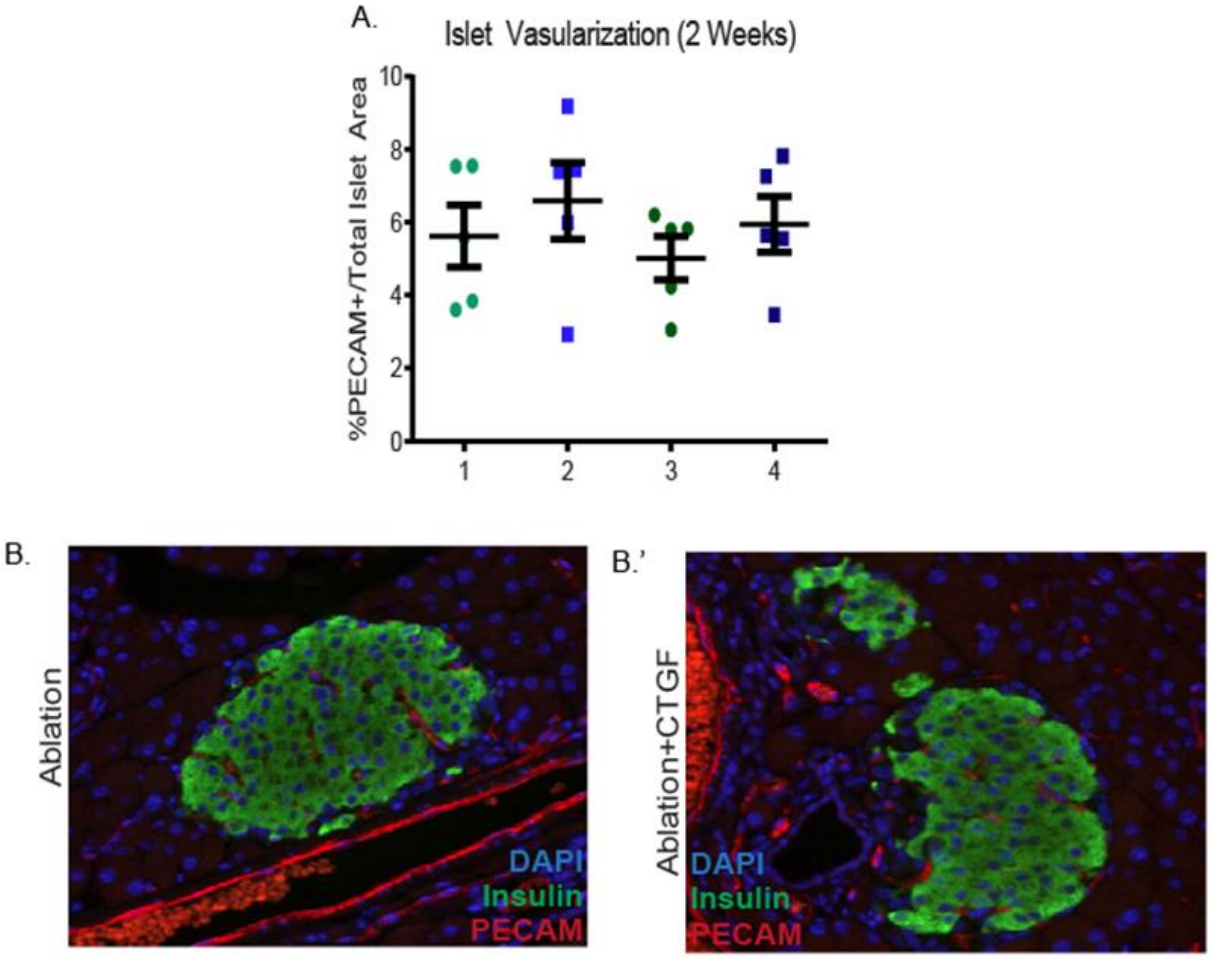


**Figure 3-7.** CTGF does not mediate β-cell regeneration via increased vascularization. 1. Control, 2. CTGF, 3. Ablation, 4. Ablation+CTGF. **A-B**. Islet vascularization quantification, as assessed by immunolabeling for blood vessels (PECAM; red) within islets (Insulin; green). n=8. Adapted from Riley *et al.*, *Diabetes* (2015).

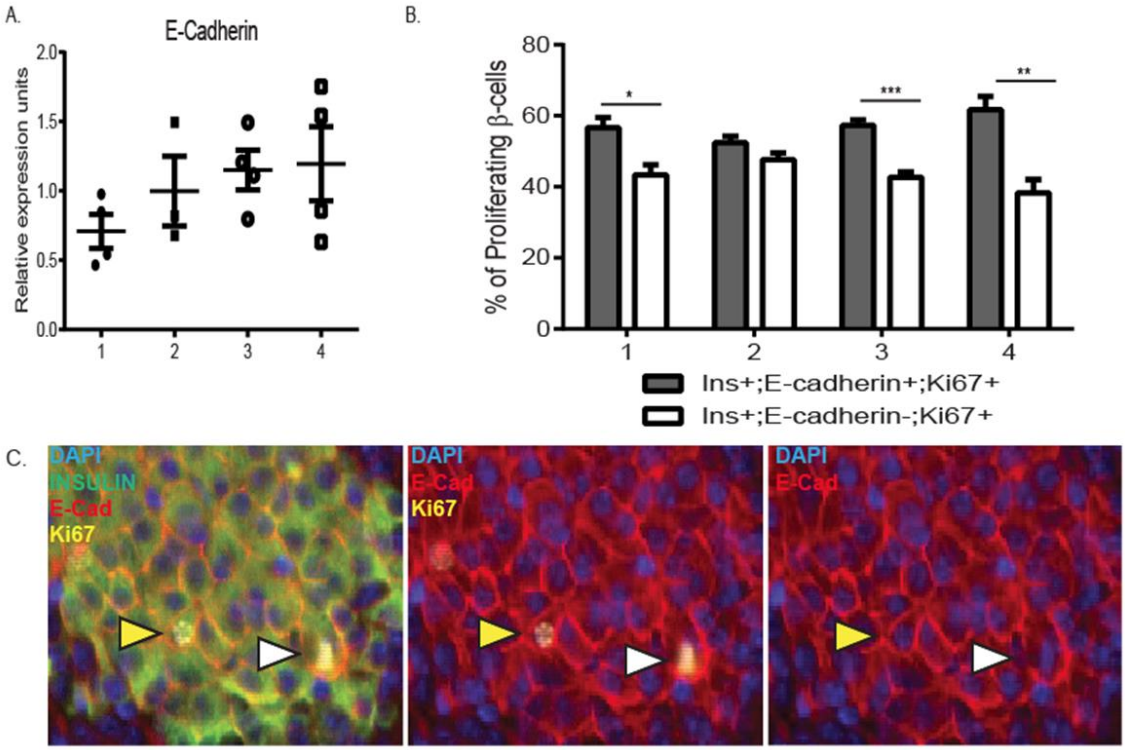


**Figure 3-8.** CTGF does not mediate β-cell regeneration via modulation of cell-cell contacts. 1. Control, 2. CTGF, 3. Ablation, 4. Ablation+CTGF. **A**. No change in E-cadherin mRNA expression as assessed by qRT-PCR. Real-time reactions were carried out in technical duplicates on a CFX Real-Time PCR Detection system (Bio-Rad). **B**. No alteration in the percentage of proliferating β-cells either completely (black bars) or incompletely surrounded (white bars) by E-cadherin. **C**. Representative images of Ablation+CTGF islets at 2 days Dox. Immunolabeling for insulin (green), E-cadherin (red), and Ki67 (yellow). Yellow arrows indicate a proliferating β-cell surrounded by E-cadherin localized to the membrane. White arrows indicate a proliferating β-cell (yellow arrow) with incomplete E-cadherin membrane localization. A cell was considered E-cadherin positive if more than 75% of the cell membrane displayed E-cadherin immunolabeling. For qRT-PCR n=3 for CTGF, and 4 for Control, Ablation, and Ablation+CTGF. For proliferation analysis, n=4. *p<0.05, **p<0.01, ***p<0.001. Adapted from Riley *et al.*, *Diabetes* (2015).

proliferation and not in combination with other compensatory mechanisms.

**CTGF does not enhance β-cell survival in the setting of DT-mediated β-cell destruction**

We next tested whether CTGF improves β-cell mass regeneration by enhancing cell survival. In order to assess this, CTGF expression was induced for 1 week prior to, during, and for 2 days following β-cell destruction (See Figure 3-9 A). Firstly, no impairment to blood glucose homeostasis was observed (See Figure 3-3E). Additionally, no difference in β-cell death (TUNEL) was observed across all cohorts, indicating that CTGF cannot protect against β-cell death in this particular model (See Figure 3-9B, C); although this does not exclude the possibility that CTGF may have prophylactic affects in other models of β-cell death.

Additionally, we assessed whether “priming” islets with CTGF prior to DT-mediated β-cell destruction would elicit further enhancement to the regeneration phenotype. However, “primed” animals displayed no improvement in either β-cell mass (See Figure 3-9D) or proliferation (See Figure 3-9E) compared to 2 days CTGF treatment. Thus, CTGF is not a prophylactic for DT-induced β-cell ablation.

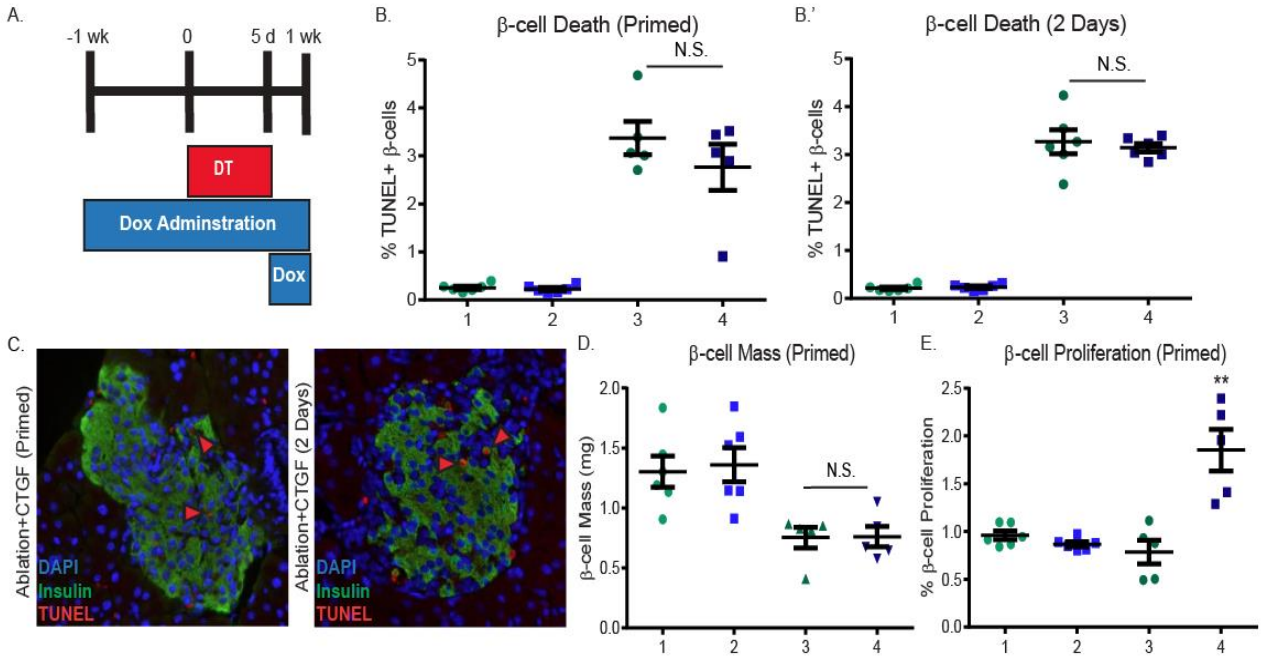


**Figure 3-9:** Priming islets with CTGF does not improve β-cell survival, or enhance β-cell proliferation and mass. **A**. Experimental outline. Mice were administered 2 mg/ml of Dox in 2% Splenda in drinking water. DT (126 ng) was given IP 3 times at 8 weeks of age. Cohorts are as follows: 1. Control 2. CTGF 3. Ablation 4. Ablation+CTGF. **B-B'**. β-cell survival as assessed by quantifying the percentage of TUNEL+ β-cells. The percentage of apoptotic or necrotic β-cells was determined by dividing the number of TUNEL/insulin double-positive cells by the total number of insulin cells. A minimum of 4,000 cells were counted. **C**. Representative images of β-cell death in prophylactic (left) and therapeutic (right) treated islets. (Green: Insulin, Red: TUNEL, Blue: DAPI) **D**. β-cell mass. **E**. β-cell proliferation. For Priming timepoint n=5. For 2 day timepoint n=6. **p=0.0027. Adapted from Riley *et al.*, *Diabetes* (2015).

## Discussion

Here we investigated the potential of CTGF to promote adult β-cell mass expansion in the face of reduced functional β-cell mass. Since both Type 1 and Type 2 diabetes result from insufficient β-cell mass, identification of signaling pathways that can be activated to promote adult β-cell mass expansion could translated into therapies for endogenous β-cell mass regeneration. Here we show that CTGF, a critical regulator of embryonic β-cell proliferation, can induce adult β-cell mass proliferation and regeneration, specifically under conditions of decreased β-cell mass. This study represents the first report examining the potential of CTGF to promote β-cell mass regeneration.

Importantly, we show that under normal islet conditions, β-cells are unresponsive to CTGF, consistent with previous findings (189), and upon restoration of 75% normal β-cell mass, the effects of CTGF begin to decline. Thus, responsiveness to CTGF seems to be restricted to periods of increased functional demand: late embryogenesis, pregnancy, HFD, and reduced β-cell mass, where the strain on individual β-cells has increased. Importantly, CTGF treatment of adult β-cells does not result in uncontrolled growth or hypoglycemia. In addition, the effects of CTGF on β-cell mass recovery do not appear to be due to factors other than increased β-cell proliferation. Although we did not directly assess β-cell neogenesis through lineage tracing studies, there was no change in several surrogate markers of neogenesis, leading us to conclude that β-cell neogenesis did not contribute to the β-cell mass recovery mediated by CTGF.

Although CTGF is angiogenic in some circumstances (162), no increased islet vascularization was observed in response to β-cell specific CTGF induction. Thus, it does not seem probable that CTGF mediates β-cell regeneration via increased vascularity; however, CTGF could increase production of endothelial-derived proliferative growth factors, such as HGF (297). This possibility, among others, will be pursued in Chapter IV. Overall, CTGF appears to mediate β-cell regeneration primarily by promoting the proliferation of pre-existing β-cells.

Taken together our findings suggest that adult β-cell ablation results in an islet microenvironment that facilitates CTGF-induced β-cell regeneration solely through increased replication of existing β-cells. Thus, following ablation, changes intrinsic to β-cells themselves or in the islet microenvironment allow for responsiveness to CTGF. β-cell intrinsic changes that we considered in the following chapter include alterations in the maturation state, replication refractory period, and expression of genes involved in key signaling pathways.

# CHAPTER IV

# CTGF PROMOTES β-CELL REGENERATION VIA MODULATION OF β-CELL MATURITY AND PROLIFERATION CHARACTERISTICS

## Introduction

Intriguingly, CTGF induction promotes β-cell mass expansion only in the setting of 50% β-cell mass destruction, and not under normal conditions in adult animals (See Figure 3-4, (189)). However, CTGF induction during development also elicits β-cell mass expansion via increased β-cell proliferation (52). Additionally, CTGF is re-expressed during periods of increased metabolic demand in the adult mouse, such as pregnancy and in response to high fat feeding ((179), Mosser and Gannon, unpublished observations). This suggests that during instance of β-cell mass expansion, the islet microenvironment changes in such a way to allow for β-cell responsiveness to CTGF. Here we began to address these changes in our model of β-cell regeneration.

Initially, we analyzed several characteristics intrinsic to the β-cells. During development, CTGF induction promotes proliferation of only MafA- β-cells (52), suggesting that immature β-cells may be more responsive to CTGF induction. We found that 50% β-cell ablation alone results in an increase in immature β-cells, and that this is further heightened by CTGF over-expression after 50% β-cell ablation. In addition, we observed that while CTGF induction enhanced proliferation of both mature (MafA+) and immature (MafA-) β-cells, a larger proportion of the proliferating β-cells were immature.

We also assessed whether CTGF over-expression after β-cell destruction altered the proliferative refractory period of the β-cells. Under normal conditions, β-cells have an extensive replicative refractory period (195). However, this period is labile and shortens in period of increased demand (195). In our model of β-cell regeneration, we observed that only CTGF induction after injury yields a shortened replicative refractory period. In addition, immature β-cells underwent multiple round of replication at a larger proportion as compared to mature β-cells, strengthening the hypothesis that immature β-cells are more responsive to CTGF.

In addition to these β-cell specific analyses, we also assessed whole islet gene expression changes. CTGF induction in the setting of 50% β-cell ablation increased expression of several cell cycle regulators, further corroborating our β-cell proliferation analysis. CTGF induction also promoted expression of several TGF-β signaling components along with several other known β-cell proliferative stimuli. Together these data suggest that CTGF induces β-cell mass regeneration via the alteration of several β-cell characteristics along with whole islet gene expression changes which promote a proliferative islet microenvironment.

## Results

### CTGF promotes proliferation in mature and immature β-cells after β-cell destruction

There is controversy about whether β-cell maturity is maintained during replication (299-301). The MafA transcription factor is associated with β-cell maturity

and optimal function (117, 118, 299). Previous studies from our lab demonstrated that CTGF over-expression in embryonic β-cells resulted in increased proliferation of MafA-/insulin+ cells; MafA+ cells did proliferate, but their proliferation was unaltered with CTGF (52). Based on our embryonic analysis (52), and that adult β-cells are unresponsive to increased CTGF under normal conditions, we hypothesized that CTGF may specifically enhance β-cell proliferation in immature (MafA-) β-cells following β-cell ablation. Approximately 15-20% of adult β-cells are normally MafA- ((118), See Figure 4-1A); this population of cells may be the most responsive to CTGF treatment.

To assess whether β-cell immaturity or partial de-differentiation is associated with enhanced permissiveness to CTGF, we examined MafA expression in insulin+ cells at 2 days (See Figure 4-1A). β-cell ablation alone resulted in a significantly higher percentage (~28%) of immature (MafA-) β-cells compared to controls (~20%). CTGF treatment enhanced the increase in MafA- β-cells after ablation (~35%), but had no effect on the number of MafA-/insulin+ cells under normal conditions. Additionally, MafB expression in insulin+ cells was assayed at 2 days. In mice, the MafB transcription factor is normally expressed in embryonic and early neonatal β-cells and is indicative of an immature β-cell (118). Correlating with the decrease in MafA+ cells (See Figure 4-1A), a significant increase (~4%) in immature (MafB+) β-cells was detected in Ablation alone or Ablation+CTGF cohorts as compared to controls (~1%) (See Figure. 4-1B).

To determine whether CTGF specifically promotes replication of immature β-cells in the setting of regeneration, mature (MafA+;Ki67+/MafA+;Ins+) and immature (MafA-;Ki67+/MafA-;Ins+) proliferating β-cells were quantified (See Figure 4-1C, D). 50% β-cell destruction alone did not provoke an increase in proliferation in immature β-cells.

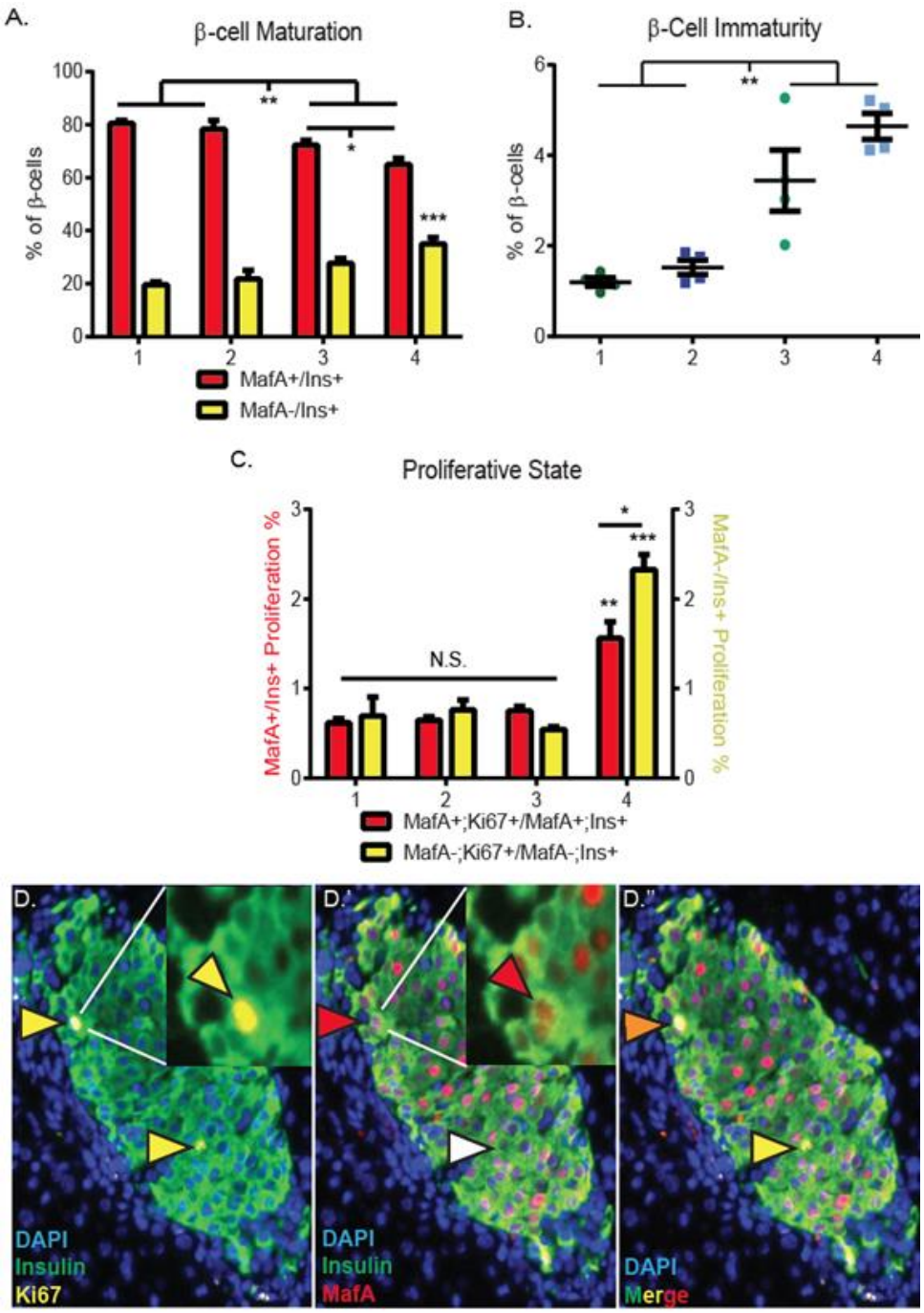


**Figure 4-1.** β-cell proliferation characteristics in response to ablation and CTGF-maturity state. 1. Control, 2. CTGF, 3. Ablation, 4. Ablation+CTGF. **A-B**. β-cell maturation and **C**. proliferative state. Mature β-cells (red bars), immature β-cells (yellow bars). **D.** Representative images of Ablation+CTGF islets at 2 days CTGF. Insulin (green), Ki67 (yellow), MafA (red). Mature β-cells (orange arrows), immature β-cells (yellow arrows). n=6. *p<0.05, **p<0.01, ***p<0.001. Adapted from Riley *et al.*, *Diabetes* (2015).

However, CTGF expression after ablation yielded an increase in proliferation of both mature and immature β-cells (See Figure 4-1C). While the increase in mature β-cell proliferation was significant, the percentage of immature β-cells proliferating was strikingly increased 3-fold as compared to all other cohorts (See Figure 4-1C). Overall, these data indicate that in the setting of β-cell destruction, CTGF stimulates proliferation in both mature and immature β-cells. The increase in immature β-cells after β-cell destruction is particularly exploited by CTGF as a mechanism for β-cell regeneration.

**CTGF induction shortens the replicative refractory period**

Between self-renewal cycles, β-cells undergo an extensive refractory period and are unable to reenter the cell cycle (192, 195). This refractory period is labile, shortening in periods of increased demand, such as pregnancy and 50% partial pancreatectomy (195). To determine if 50% β-cell ablation and/or CTGF treatment induces changes in the replicative refractory period, we used a dual labeling system involving two distinct uridine analogs (See Figure 4-2A, D; (195)). Following DT administration, CTGF was induced for 2 or 4 weeks. 5-Chloro-2'-deoxyuridine (CldU) was administered with Dox for the first 2 days. During the final 5 days of Dox treatment, the second uridine analog, 5-Iodo-2'-deoxyuridine (IdU) was administered. A Dox-only washout period occurred for either 1 or 3 weeks between uridine analogs (See Figure 4-2A). This process allows for labeling of newly synthesized DNA, marking actively proliferating β-cells, at two distinct time-points (See Figure 4-2D, D'). β-cell nuclei incorporating both labels are indicative of two rounds of replication during the labeling period (See Figure 4-2D'').

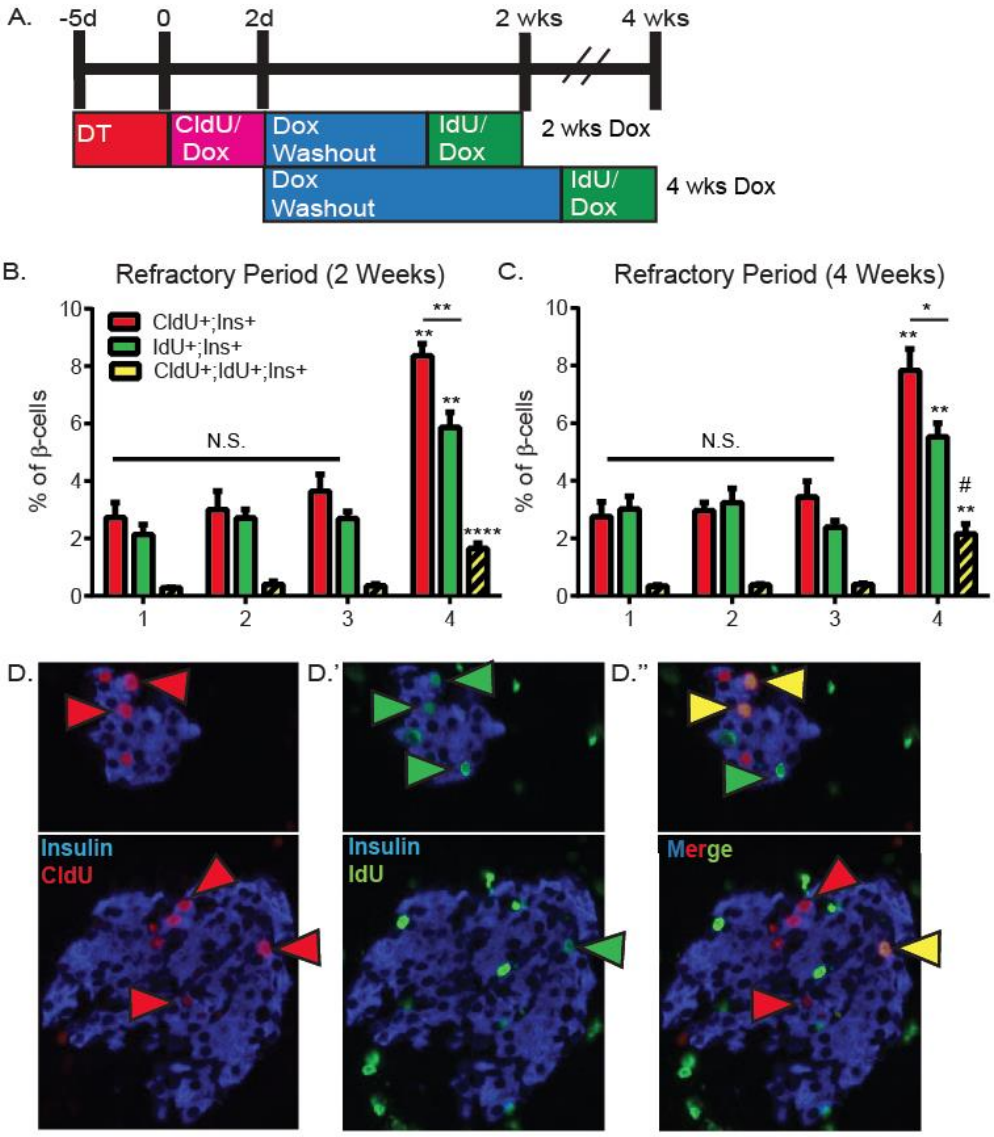


**Figure 4-2.** β-cell proliferation characteristics in response to ablation and CTGF-replicative refractory period length. Cohorts: 1. Control, 2. CTGF, 3. Ablation, 4. Ablation+CTGF. **A.** Experimental outline for double uridine analog labeling. Mice were administered Dox in drinking water. DT (126 ng) was given IP 3 times at 8 weeks of age. Uridine analogs (5-Chloro-2'-deoxyuridine (CldU) or 5-Iodo-2'-deoxyuridine (IdU)) were administered in Dox-treated drinking water. **B-C.** β-cell replication during the first 2 days (red bars), last five days (green bars), and both labeling periods (yellow bars) at 2 (**B**) and 4 (**C**) weeks was determined. The percentage of dual-labeled β-cells at 4 weeks was significantly higher than at 2 weeks (demarked by #). **D.** Representative images at 4 weeks. Replicating β-cells in the first 2 days incorporated CldU (red arrows), in the last 5 days incorporated IdU (green arrows). Replicating cells in both periods incorporated CldU and IdU (yellow arrows). n=6. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001, #=0.0414. Adapted from Riley *et al.*, *Diabetes* (2015).

We quantified the number of single+ (CldU+ or IdU+) and dual positive (CldU+;IdU+) β-cells after 2 or 4 weeks of CTGF treatment (See Figure 4-2B,C). At both time-points, islets from Ablation+CTGF animals displayed an increase in the percentage of single labeled β-cells. Additionally, in this cohort a greater number of β-cells underwent replication in the first 2 days (CldU+) compared to the last 5 days of labeling (IdU+) (red vs. green bars). A prominent increase in the number of dual-labeled β-cells was observed in the Ablation+CTGF cohort, indicative of a shortened β-cell replicative refractory period. An increase in dual-labeled β-cells was observed with 4 weeks of CTGF induction after β-cell ablation as compared to the 2 week time-point (See Figure 4-2 B,C; $p=0.0414$). This increase is not surprising given the longer washout period and thus additional time for re-entry into the cell cycle. We calculated the ratio of single-labeled cells vs. dual-labeled cells to ensure the increase in dual-labeled cells was not simply proportional to the overall increase in proliferation. In the Ablation+CTGF cohort, there was a statistically significant decrease in the average ratio of single-labeled β-cells to dual-labeled β-cells (4.2:1 for CldU; 2.9:1 for IdU) as compared to control animals (8.2:1 for CldU; and 8.4:1 for IdU).

As our previous studies indicated that immature β-cells proliferate at higher percentages than mature β-cells in our Ablation+CTGF cohort (See Figure 4-1), we assessed whether immature β-cells were more likely to be dual-labeled (and thus have undergone two cycles of replication). We quantified the number of mature (MafA+;CldU+;IdU+) and immature (MafA-;CldU+;IdU+) β-cells at the 2 week timepoint (See Figure 4-3). In all cohorts, dual-labeled β-cells were not specific to the immature (MafA-) phenotype, as both mature and immature β-cells underwent multiple rounds of

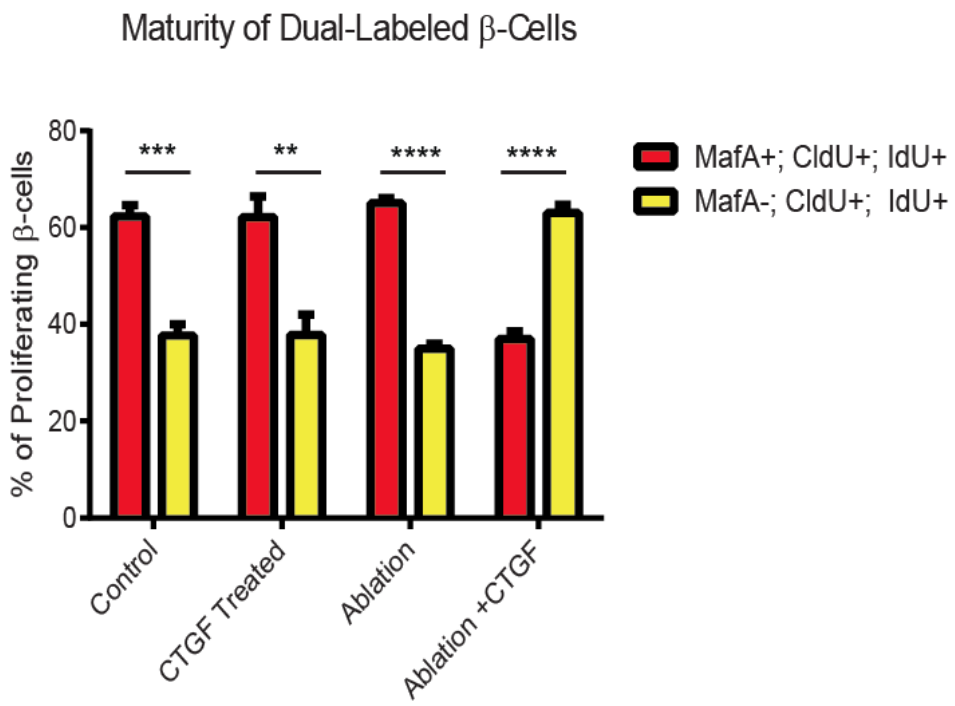


**Figure 4-3.** Maturity state of dual-labeled β-cells was determined at 2 weeks of CTGF treatment. All dual-labeled β-cells were quantified as either mature (MafA+; red bars) or immature (MafA-; yellow bars). n=4. **p<0.01, ***p<0.001, ****p<0.0001. Adapted from Riley *et al.*, *Diabetes* (2015).

replication in the 2 week timecourse. Most (~60%) of the dual-labeled β-cells in the Control, CTGF-treated, and Ablation cohorts were mature. However, in congruence with our findings in Figure 4-1C, the Ablation+CTGF cohort had an increase in the number of dual-labeled immature β-cells (~60%) with a shortened refractory period. Therefore, in the setting of β-cell destruction, CTGF elicits β-cell mass regeneration both by increasing the number of proliferating β-cells, and by reducing the period of time before these cells can re-enter the cell cycle.

**Gene expression changes at two day timepoint**

To gain insight into signaling pathways altered by CTGF with or without β-cell ablation, gene expression analysis was conducted on islets isolated from animals following 2 days of CTGF induction in vivo. We specifically probed changes in genes associated with β-cell functional maturity, proliferation, and growth factor signaling pathways (See Figure 4-4,5,6). Gene expression alterations specific to CTGF treatment, β-cell ablation, and CTGF treatment after β-cell ablation were determined.

There were several changes in key cell cycle regulators in response to ablation and/or CTGF treatment (See Figures 4-5). CTGF induction under normal conditions elicited an increase in *cyclinD3* expression, while ablation alone induced an increase in *cyclinB1*, *cyclinD1, cyclinD2*, and *Ki67*. β-cell ablation also up-regulated *Foxm1*, a key transcriptional regulator of β-cell proliferation (193, 237, 238, 302). These results are intriguing as no increase in β-cell proliferation is detected in the “Ablation” cohort (See Figure 3-4B). *CyclinD3, Ki67,* and *PCNA* mRNAs were strikingly up-regulated in the “

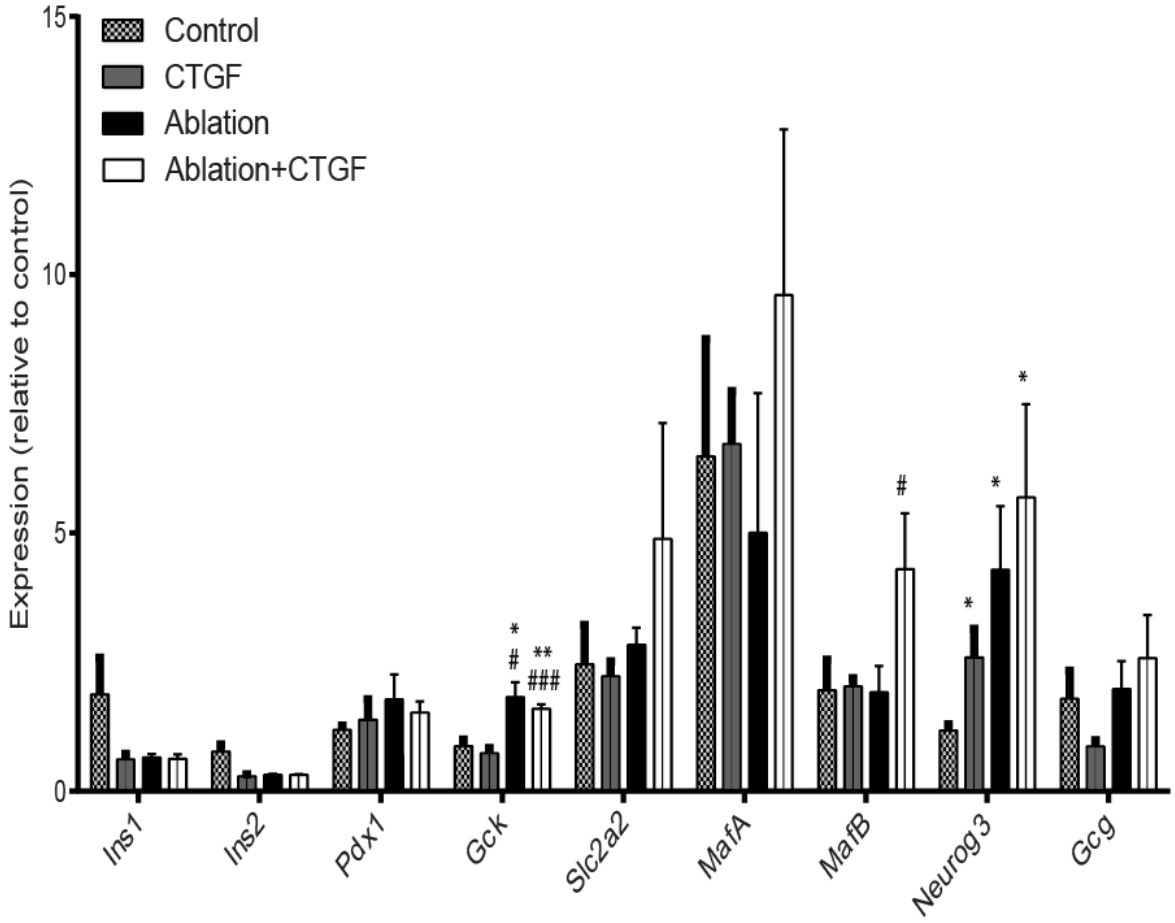


**Figure 4-4.** CTGF induces expression of islet cell markers. Gene expression analysis on whole islets using TaqMan Universal PCR Mastermix. Islets isolated from animals with/without β-cell ablation +/- CTGF treatment for 2 days. All samples were run in duplicate. n=4. *compared to Control, #compared to CTGF, ^compared to Ablation. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001. Adapted from Riley *et al.*, *Diabetes* (2015).

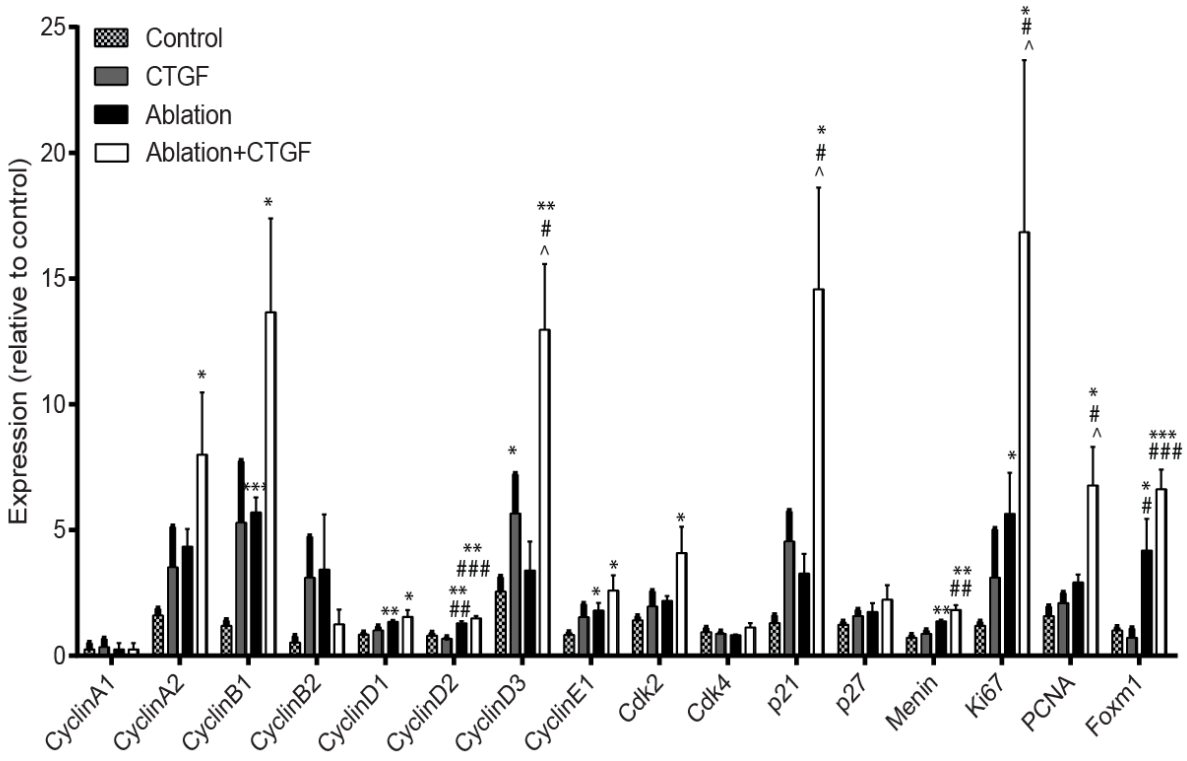


**Figure 4-5.** CTGF induces expression of cell cycle regulators. Gene expression analysis on whole islets using TaqMan Universal PCR Mastermix. Islets isolated from animals with/without β-cell ablation +/- CTGF treatment for 2 days. All samples were run in duplicate. n=4. *compared to Control, #compared to CTGF, ^compared to Ablation. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001. Adapted from Riley *et al.*, *Diabetes* (2015).

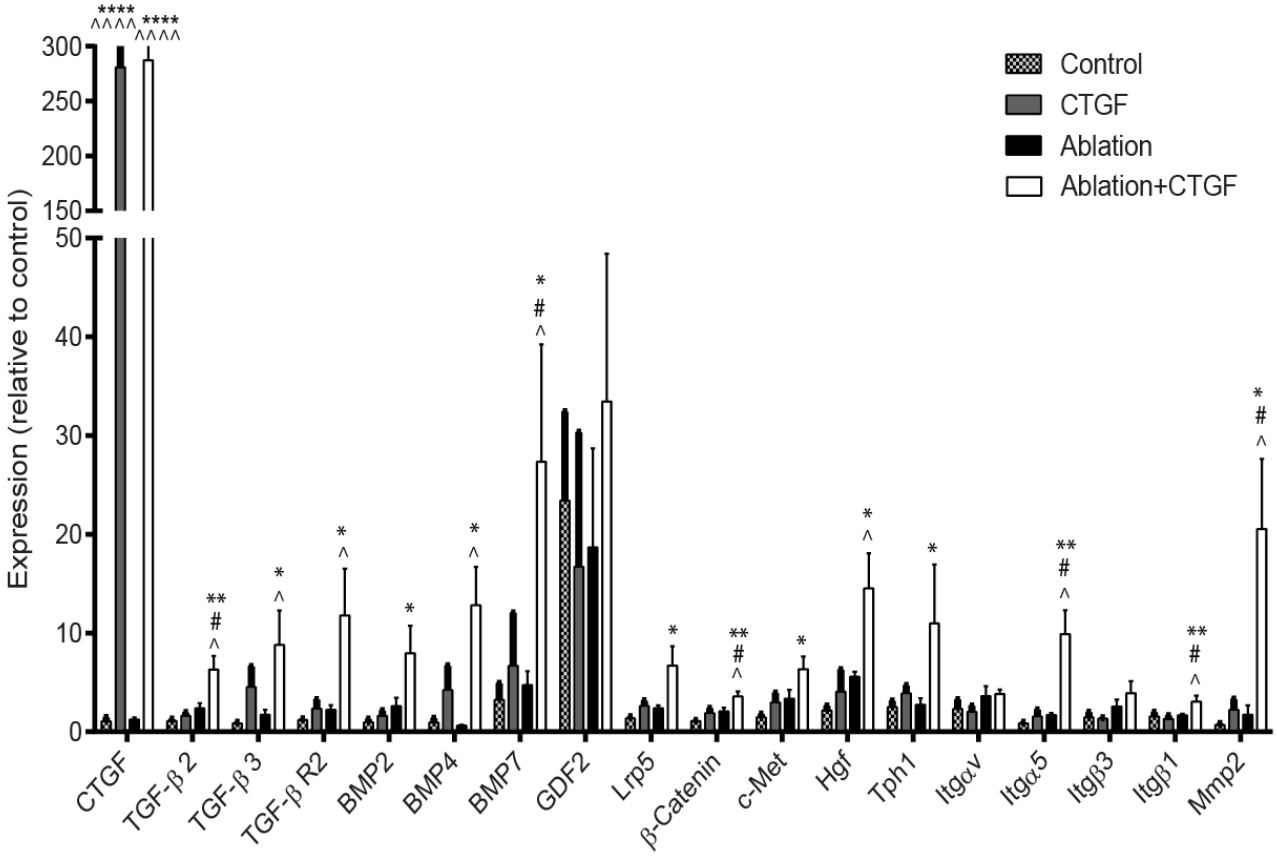


**Figure 4-6.** CTGF induces expression of genes involved in key signaling pathways. Gene expression analysis on whole islets using TaqMan Universal PCR Mastermix. Islets isolated from animals with/without β-cell ablation +/- CTGF treatment for 2 days. All samples were run in duplicate. n=4. *compared to Control, #compared to CTGF, ^compared to Ablation. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001. Adapted from Riley *et al.*, *Diabetes* (2015).

Ablation+CTGF" cohort, in agreement with the highly significant increase in β-cell proliferation. Up-regulation of the cell cycle inhibitor *Cdkn1a* (p21) was also observed only in the "Ablation+CTGF" cohort, suggesting the increased β-cell proliferation triggers an inhibitory feedback loop. However, other cell cycle inhibitors, *Cdkn1b* (p27) and *Cdkn2a* (p16), displayed no alteration in gene transcription (See Figure 4-5, data not shown).

With regard to growth factor signaling pathways, CTGF treatment after 50% ablation resulted in an increase in TGF-β (*TGF-β2, TGF-β3, TGF-βR2*), BMP (*BMP2, BMP4, BMP7*), and Wnt (*β-catenin, Lrp5)* associated genes (See Figure 4-6). CTGF promotes, and is promoted by, TGF-β signaling in other tissues, yet, no significant changes in p-SMAD3 protein levels were detected in any experimental cohort (See Figure 4-7) (161, 303). However, in other tissues CTGF expression is promoted by SMAD-independent TGF-β signaling via ERK (304-306). Thus, in this model of β-cell regeneration, CTGF may be promoted by TGF-β signaling in an ERK-dependent manner. Additionally, BMP is a known inhibitor of CTGF (307), indicating CTGF expression regulatory networks are activated in response to CTGF up-regulation.

In addition, an increase in expression of tryptophan hydroxylase (Tph1), the serotonin (5-HT) synthesizing enzyme, was observed in the Ablation+CTGF group (See Figure 4-6). Several other genes were up-regulated only in the presence of both 50% β-cell ablation and CTGF treatment, including *HGF*, its receptor *c-Met,* and integrins α5 (*Itgα5*) and β1 (*Itgβ1*) (See Figure 4-6). As 5-HT, integrin β1, and HGF each promote β-cell proliferation (158, 297), these findings indicate that CTGF-mediated β-cell regeneration involves recruitment of other pro-proliferative factors.

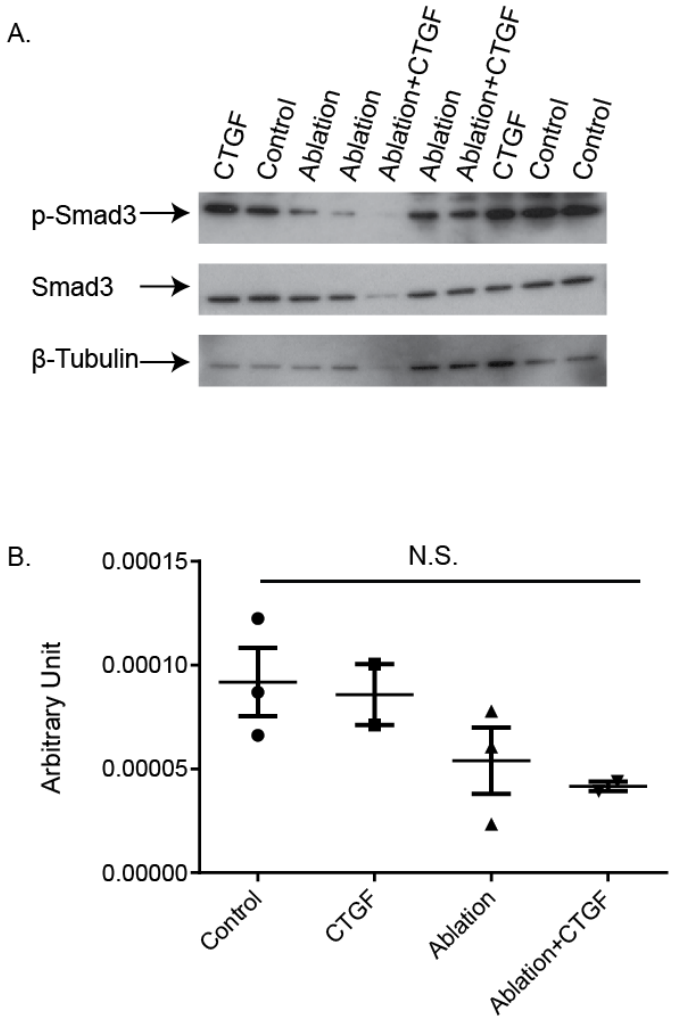


**Figure 4-7.** Protein levels of total and phospho-Smad3 in response to CTGF treatment and/or ablation. A: Isolated islet protein was probed with Smad3, p-Smad3, and β-tubulin antibodies for immunoblot analysis. B: Densitometric quantification of activated p-Smad3 relative to total Smad3 protein provided. n=3 for Control and Ablation, n=2 for CTGF and Ablation+CTGF. Adapted from Riley *et al.*, *Diabetes* (2015).

Gene expression analysis revealed no significant alteration to several key β-cell functional identity genes (See Figure 4-4). This was unsurprising as all cohorts remain euglycemic at all time-points and suggests that the surviving 50% of β-cells in the ablation cohorts up-regulate expression of these genes to wild-type levels. Intriguingly, upon 50% β-cell ablation, an increase in *glucokinase,* involved in both β-cell proliferation and function (308), is observed. *Ngn3* expression increased with CTGF treatment, and/or ablation, supporting the concept that these treatments promote a less differentiated state. Finally, up-regulation of *MafB,* a gene expressed in α-cells and immature β-cells, is observed only in the "Ablation+CTGF" cohort. This correlates with the observed increase in the percentage of MafB+/Ins+ cells in the "Ablation+CTGF" cohort (See Figure 4-1). No alteration in *MafA* expression was observed in any cohort. This is not unexpected as only an 8 or 15% decrease in MafA+ cells was observed in our "Ablation" or "Ablation+CTGF" cohorts, respectively (See Figure 4-1) and changes in MafA protein may be disconnected from changes in *MafA* gene expression (299).

**Verification of gene expression changes at two day timepoint**

We then sought to corroborate our gene expression analysis with assessment of protein expression of genes of interest. An increase in expression of tryptophan hydroxylase (Tph1), the serotonin (5-HT) synthesizing enzyme, was observed specifically in islets of the "Ablation+CTGF" cohort as compared to all other cohorts (See Figure 4-8A-D). This further underscores the potential of 5-HT to be involved in this mechanism of β-cell mass regeneration. Additionally, immunohistochemistry

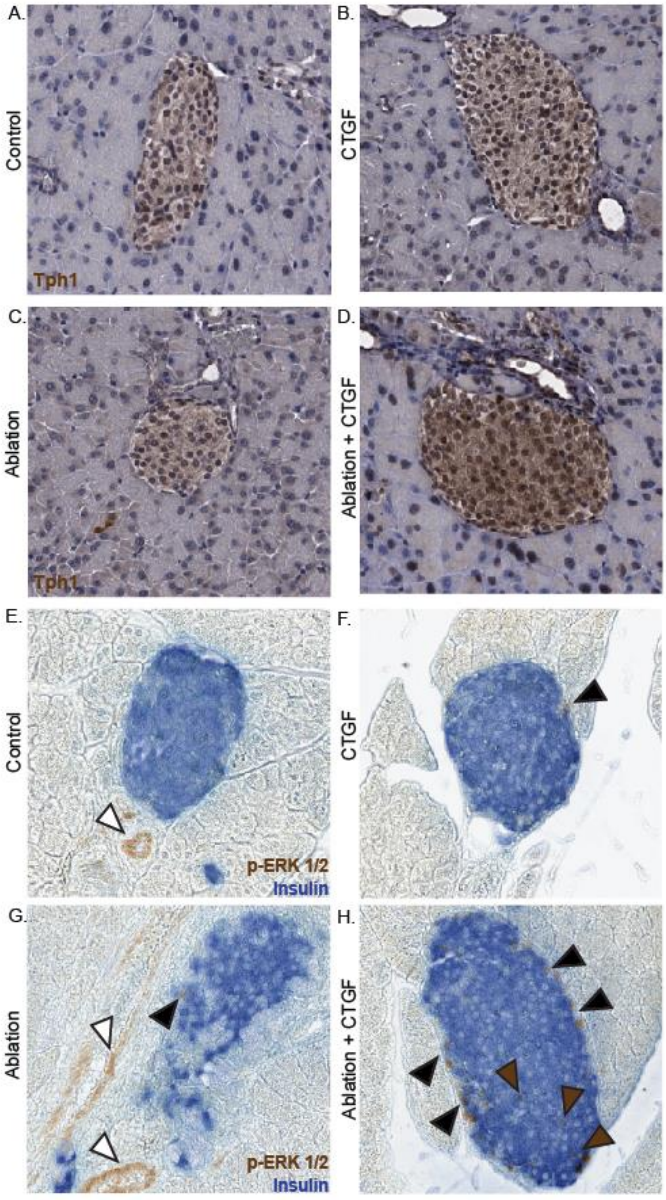


**Figure 4-8:** Alterations in Tph1expression and ERK1/2 signaling in response to CTGF and/or ablation. **A-D:** Representative images of islets at 2 days of CTGF**: A,E.** Control, **B,F.** CTGF, **C,G.** Ablation, **D,H.** Ablation+CTGF. Tph1: Primary antibodies were visualized via a DAB Peroxidase Substrate Kit, and counterstained with Hematoxylin. *p-ERK1/2*: Primary antibodies for p-ERK1/2 and Insulin were visualized via a DAB Peroxidase Substrate Kit, and by an alkaline phosphatase Vector Blue Substrate Kit, respectively. Brown and black arrows demark β-cells or other islet cells with activated Erk 1/2 signaling, respectively. White arrows demark endothelial cells with activated Erk 1/2 signaling. n=4 for Tph1, and n=3 for p-ERK1/2. Adapted from Riley *et al.*, *Diabetes* (2015).

revealed an increase in phosphorylated ERK1/2, a known downstream mediator of activated integrin β1 (158, 309), in islets of the “Ablation+CTGF” cohort as compared to all other cohorts (See Figure 4-8E-H). Interestingly, this strengthens the hypothesis that CTGF may be activating TGF-β signaling in an ERK-dependent fashion.

## Discussion

Here we investigated the mechanism by which promotes adult β-cell proliferation and mass expansion in the face of reduced functional β-cell mass. CTGF in the setting of β-cell destruction increased expression of several cell cycle regulators, TGF-β signaling components, and other known β-cell proliferative stimuli (e.g. HGF and serotonin synthesis). We also investigated the changing β-cell intrinsic characteristics in the setting of CTGF-mediated β-cell regeneration.

During embryonic development, both MafA-/ins+ and MafA+/ins+ cells proliferate; however, CTGF enhances proliferation of only MafA- β-cells (52). The lack of responsiveness of MafA+/ins+ cells to CTGF under normal conditions might explain the inability of CTGF to induce replication in adult β-cells in vivo under conditions of normal β-cell mass. In the setting of β-cell ablation, we find that CTGF induction enhanced proliferation of both mature and immature β-cells. However, CTGF consistently elicits a greater increase in proliferation of immature β-cells. Ablation alone also significantly increased the number of MafA- β-cells, although these show no increase in proliferation, despite increased expression of proliferative gene such as *FoxM1*. β-cells normally have a refractory period on the order of months but this period is labile, and was

shortened specifically with β-cell ablation plus CTGF. Thus, CTGF appears to mediate β-cell regeneration via enhanced β-cell proliferation, particularly of immature β-cells, and by shortening the β-cell replicative refractory period. It may be that cells that are slightly less mature are more responsive to proliferative stimuli, such as CTGF. Whether these effects are unique to CTGF, or are a property of other β-cell proliferative factors remains to be tested.

TLDA analysis served as a way to begin to dissect the pathways through which CTGF elicits β-cell regeneration. Increased expression of TGF-β, BMP, and Wnt genes was observed only upon induction of CTGF after β-cell ablation. CTGF and TGF-β are in a positive feedback loop (303). Increases in BMP and Wnt components points towards an attempt to negatively regulate the effects of CTGF induction (310). The roles these signaling pathways play in β-cell proliferation are unclear, although TGF-β signaling may be required for β-cell regeneration (311), and Wnt signaling is involved downstream of GLP-1-mediated β-cell proliferation (144).

A specific receptor for CTGF has not been identified, rather, CTGF interacts with integrins (167). Increased expression of integrins α5 and β1 was observed in "Ablation+CTGF" animals. It was recently shown that integrin β1 is absolutely critical for β-cell proliferation and mass expansion at all ages (158). Additionally, an increase in phosphorylated ERK1/2, a known downstream effector of integrin β1-mediated β-cell proliferation (158, 309), was observed solely in islets from the "Ablation+CTGF" cohort. Thus, it is highly likely that CTGF promotes β-cell proliferation through integrin signaling. Additionally, CTGF induction after β-cell ablation increases expression of *Hgf* and the

serotonin synthesizing enzyme *Tph1*, indicating that CTGF-mediated β-cell regeneration involves the recruitment of other pro-proliferative factors. (297, 312, 313).

β-cell ablation and CTGF induction are each necessary, but not sufficient, to induce in vivo β-cell proliferation in this model. Adult β-cell cell cycle re-entry may therefore require combinatorial stimuli to successfully initiate. All forms of diabetes are characterized by insufficient functional β-cell mass. Thus, strategies to promote the replication, and subsequent mass regeneration, of pre-existing β-cells are critical. The ability to elicit β-cell mass regeneration is a novel role for CTGF, and suggests that manipulation of CTGF signaling may serve as a therapeutic for diabetes. Additionally, our studies highlight the vital role the islet microenvironment plays in β-cell responsiveness to proliferative stimuli. In the next chapter we assess the potential additional role the immune system may play in CTGF-mediated β-cell regeneration.

# CHAPTER V

## CTGF PROMOTES β-CELL REGENERATION VIA IMMUNE SYSTEM MODULATION

### Introduction

CTGF treatment after 50% β-cell ablation promotes β-cell regeneration by modifying several β-cell intrinsic characteristics (See Chapter IV). However, it is probable that CTGF induction elicits β-cell mass regeneration by also mediating changes to extrinsic factors of the islet micro-environment. Although we found that CTGF induction does not promote heightened islet vascularization in this model of β-cell regeneration (See Figure 3-7); CTGF may promote the recruitment of particular immune cell populations, and their secreted products could prime β-cells to respond to CTGF.

The role of the immune system in regeneration is well established in multiple tissues (314, 315), including β-cell regeneration in other models of β-cell ablation (294, 316, 317). Specifically, macrophages are critical for proper β-cell mass regeneration after injury by partial duct ligation (317) and VEGF-mediated islet endothelial cell expansion (294). Additionally, several groups have proposed that T cells promote β-cell mass regeneration in the setting of diabetes mellitus (316, 318). However, it remains unclear how either macrophages or T cells promote β-cell proliferation and subsequent β-cell mass regeneration.

CTGF also promotes wound repair via immune system involvement in other organs (319). In the kidney, CTGF induction promoted infiltration of both macrophages

and T cells (320), suggesting that CTGF may serve as an immune cell chemoattractant. Further, in a model of pancreatic injury via ethanol injection, CTGF over-expression resulted in increased recruitment of neutrophils and T-cells to the pancreas (256). However, CTGF induction post injury can also yield deleterious effects. In a mouse fibrosis model, CTGF injections increased mast cell and macrophage number over time, resulting in fibrosis (321). Overall, there appears to be a critical window of CTGF expression post injury for maximal tissue repair and regeneration without deleterious effects of fibrosis.

In our model of β-cell regeneration, we initially observed an increase of leukocytes in pancreata of animals after 50% β-cell ablation. We analyzed several specific immune cell populations and observed an increase in macrophages in response to 50% β-cell ablation, which is further heightened by CTGF induction after β-cell destruction. In addition, we observed an increase in T cells in the pancreatic parenchyma only in pancreata from animals with CTGF induction after 50% β-cell ablation. In addition to these immunofluorescent analyses, we also assessed whole islet gene expression changes in immune cell populations and responses. CTGF induction after β-cell ablation increased the expression of several macrophage and T cell marker and chemoattractant genes. Thus it appears that CTGF treatment after 50% β-cell ablation promotes an increase in both T cells and macrophages.

In addition, we assessed the requirement for macrophages in CTGF-mediated β-cell regeneration via a macrophage depletion study. We observed that a reduction in macrophages in this model of β-cell destruction inhibits the proliferative effects elicited by CTGF treatment. Also, preliminary findings suggest that macrophage depletion may

also, via an unknown mechanism, promote a more mature β-cell phenotype. Together these data suggest a critical role for the recruited immune cell populations during CTGF-mediated β-cell regeneration.

## Results

### Evaluation of immune cell populations after two days of CTGF treatment, the peak of β-cell proliferation

In order to assess whether CTGF induction increased the population of immune cells in the pancreas, Hematoxylin and Eosin (H&E) staining was conducted after two days of CTGF induction (See Figure 5-1). This time was chosen since we had previously determined that this was the peak of β-cell proliferation in Ablation+CTGF pancreata. In both the Ablation and Ablation+CTGF cohorts, analysis of H&E staining indicated the presence of inflammation and increased immune cells from the myeloid lineage, in the absence of fibrosis (See Figure 5-1C, D). In order to confirm the increase in immune cells in the pancreas parenchyma, immunohistochemistry with the pan-leukocyte marker, CD45, was conducted at the two day timepoint. CTGF induction under normal conditions in adult islets elicited no general increase in leukocyte number (See Figure 5-2A, D; 2-CTGF). However, 50% β-cell ablation alone did promote an increase in the number of leukocytes in the pancreas (See Figure 5-2A; 3-Abation); although these cells were not targeted preferentially to the islets (See Figure 5-2B, E). The increase in immune cell number in response to ablation was further heightened

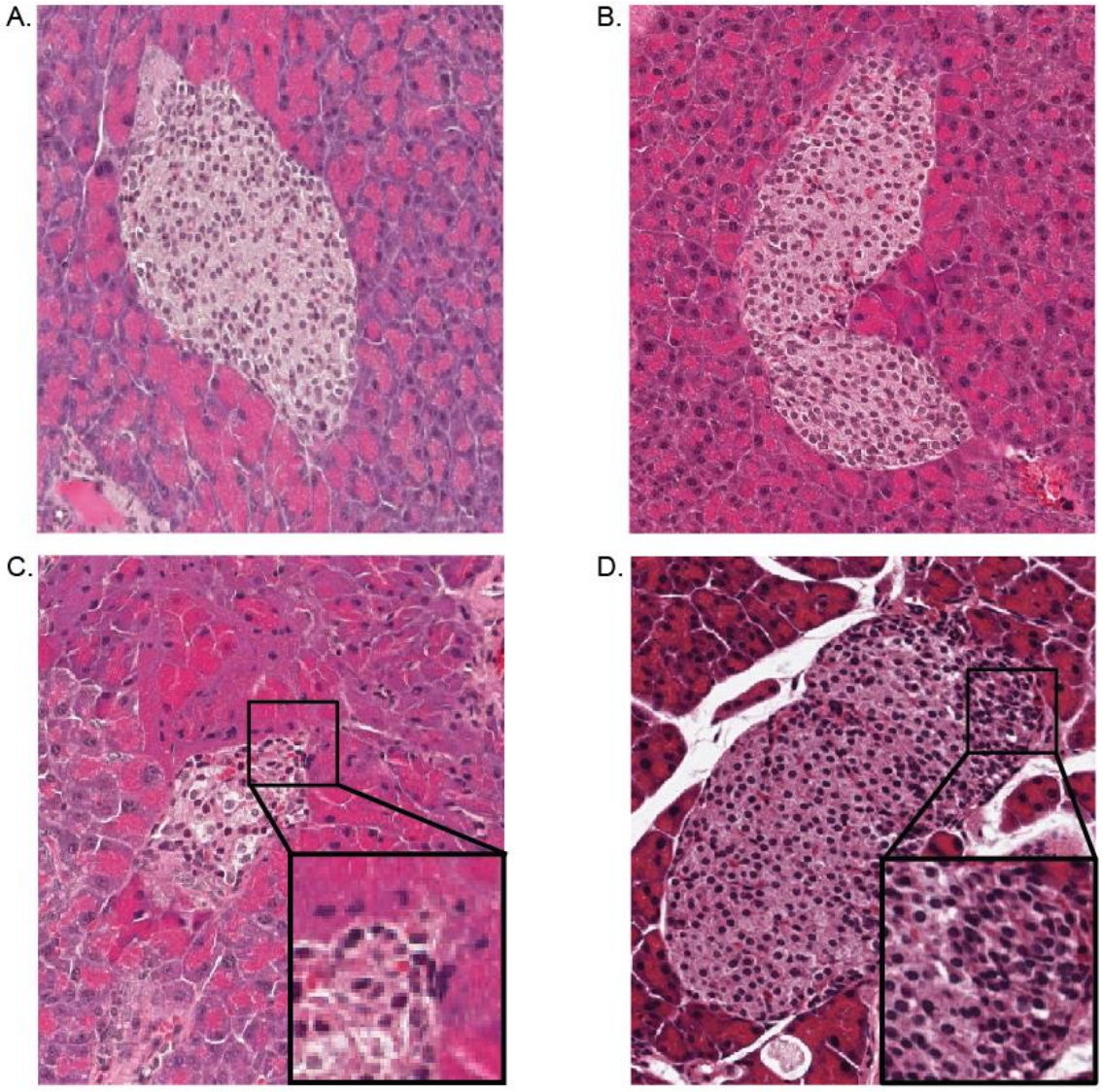


**Figure 5-1.** H&E staining for immune cell detection adjacent to islets. **A-D.** Representative images of Control (**A.**), CTGF treated (**B.**), Ablation (**C.**), and Ablation+CTGF (**D.**) islets after 2 days CTGF. Insets highlight small, dark and closely clustered nuclei that are indicative of myeloid cells.

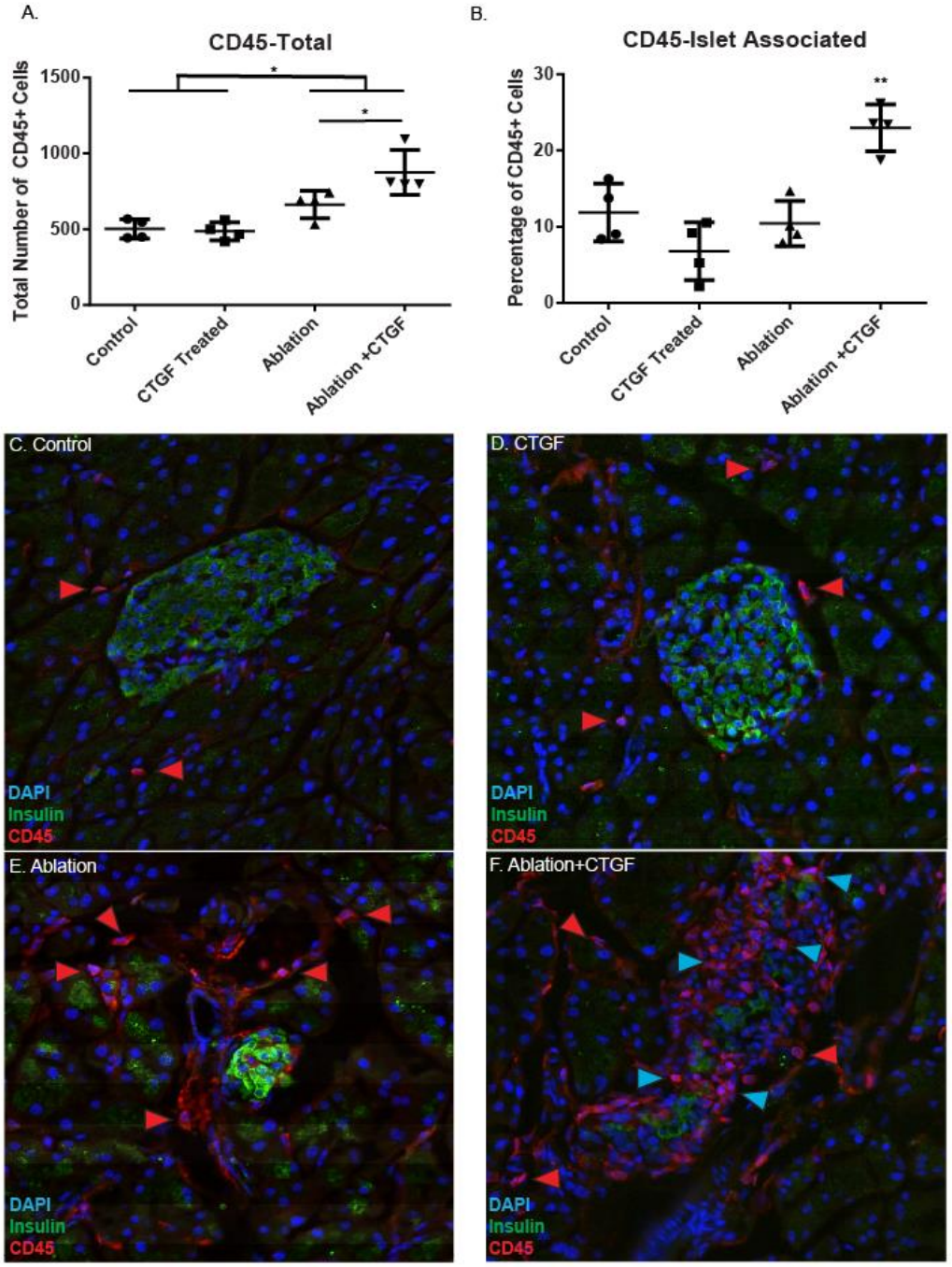


**Figure 5-2.** β-cell ablation and CTGF induction each promote increased pancreatic immune cells. **A**. Total number of pancreatic CD45+ cells and **B.** Proportion of islet-localized CD45+ cells. **C-F.** Representative images of Control (**C.**), CTGF treated (**D.**), Ablation (**E.**), and Ablation+CTGF (**F.**) islets after 2 days CTGF. Insulin (green), CD45 (red). CD45+ cells within the exocrine or endocrine compartments are demarked by red and blue arrows, respectively. n=4. *p<0.05, **p<0.01.

upon CTGF induction after injury (See Figure 5-2A; 4-Ablation+CTGF), and there was a greater proportion of these cells localized to islets (See Figure 5-2B, F).

To determine which specific immune populations were increasing following ablation and CTGF, immunohistochemistry for various immune cell populations was conducted. The largest proportion of immune cells in any of the cohorts was macrophages, as detected by immunolabeling against F4/80 (See Figure 5-3C-F). 50% β-cell ablation did elicit a general increase in macrophages (See Figure 5-3A; 3-Ablation), while CTGF treatment further enhanced this increase (See Figure 5-3A; 4-Ablation+CTGF). Additionally, a greater proportion of macrophages had were islet associated in the Ablation+CTGF cohort as compared to all other groups (See Figure 5-3B, F). Interestingly, only CTGF induction after β-cell ablation elicited a modest increase in T cells (See Figure 5-4A; 4-Ablation+CTGF), as assessed by CD3 immunolabeling (See Figure 5-4C-F). The increased T cells did not appear to be specifically targeted to the pancreatic islets (See Figure 5-4B). Very few B cells, as detected by B220 immunolabeling (See Figure 5-5C-F), were observed within the pancreatic parenchyma of any cohort, and were rarely observed close to islets (See Figure 5-5A, B). Finally, as CTGF has been shown to recruit neutrophils in other models of pancreatic injury (ref), we assessed this immune population via “neutrophil marker” immunohistochemistry (See Figure 5-6). However, in our model of β-cell ablation, no significant increase of neutrophils was observed in any cohort (See Figure 5-6A).

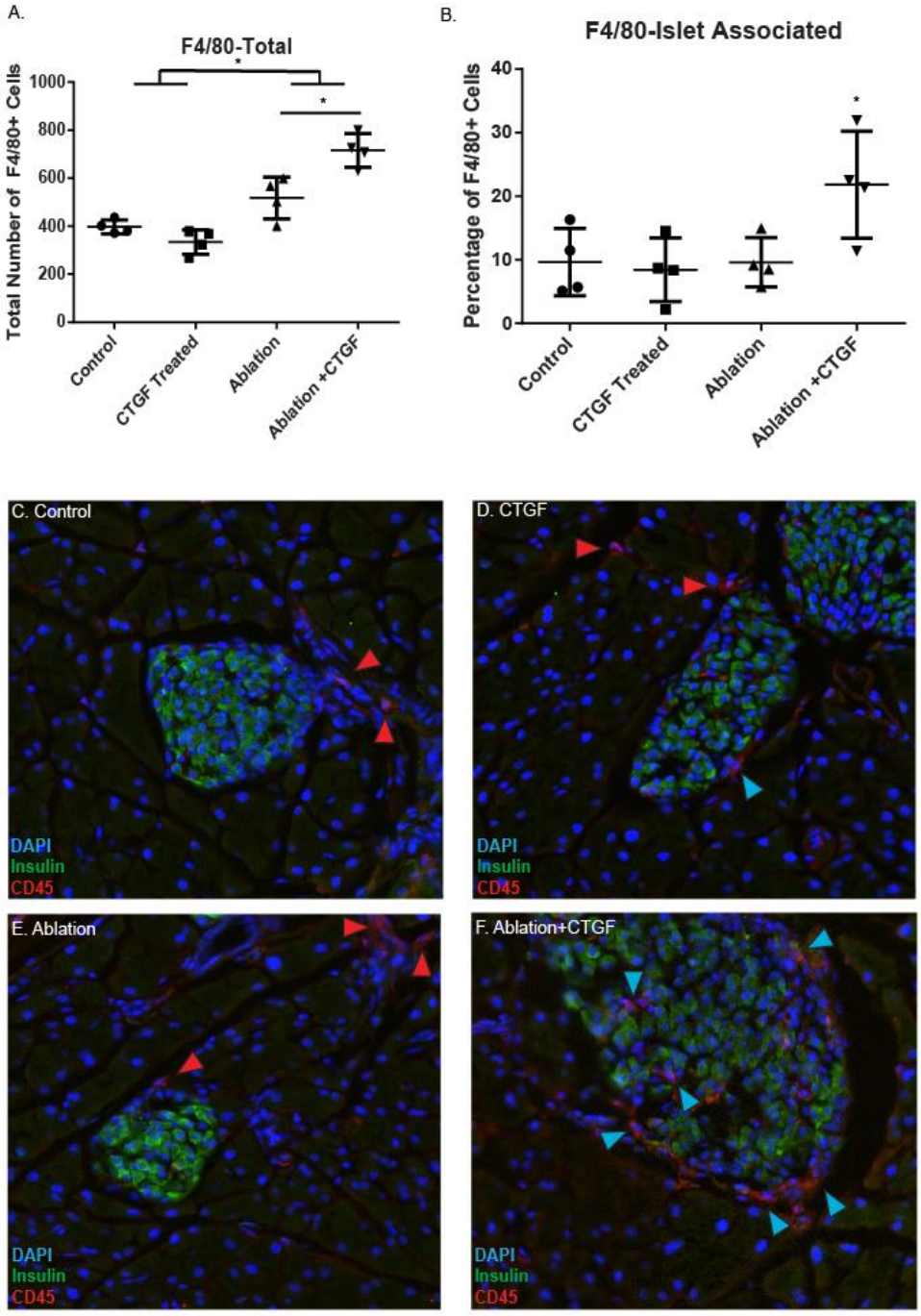


**Figure 5-3.** β-cell ablation and CTGF induction each promote increased pancreatic macrophages. **A**. Total number of F4/80 positive cells and **B.** Proportion of islet-localized macrophages. **C-F.** Representative images of Control (**C.**), CTGF treated (**D.**), Ablation (**E.**), and Ablation+CTGF (**F.**) islets after 2 days CTGF. Insulin (green), F4/80 (red). F4/80+ cells within the exocrine or endocrine compartments are demarked by red and blue arrows, respectively. n=4. *p<0.05.

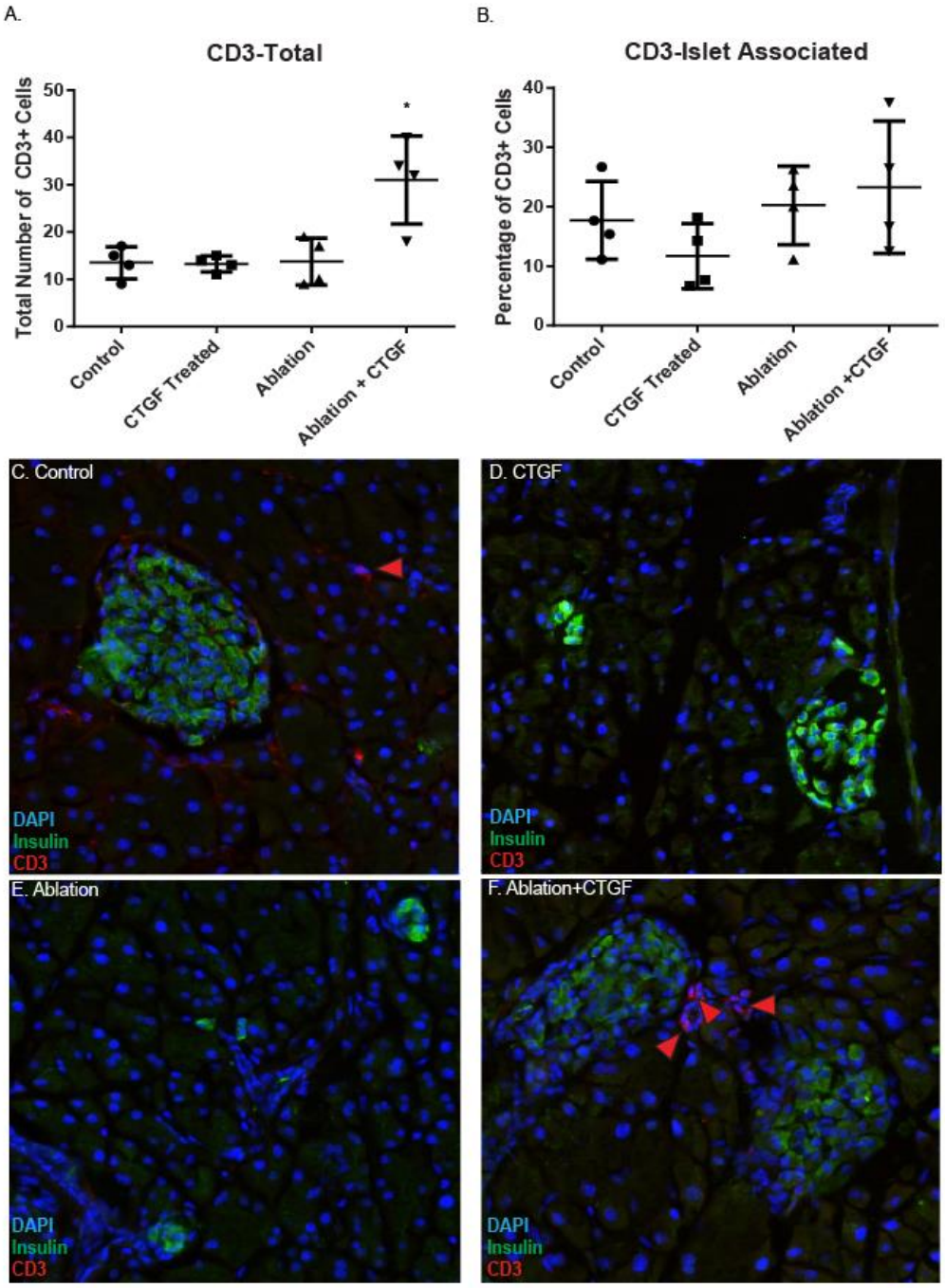


**Figure 5-4.** β-cell ablation with CTGF induction promotes increased pancreatic T cells. **A**. Total number of CD3 positive cells and **B.** Proportion of islet-localized T cells. **C-F.** Representative images of Control (**C.**), CTGF treated (**D.**), Ablation (**E.**), and Ablation+CTGF (**F.**) islets after 2 days CTGF. Insulin (green), CD3 (red). CD3+ cells are demarked by red arrows. n=4. *p<0.05.

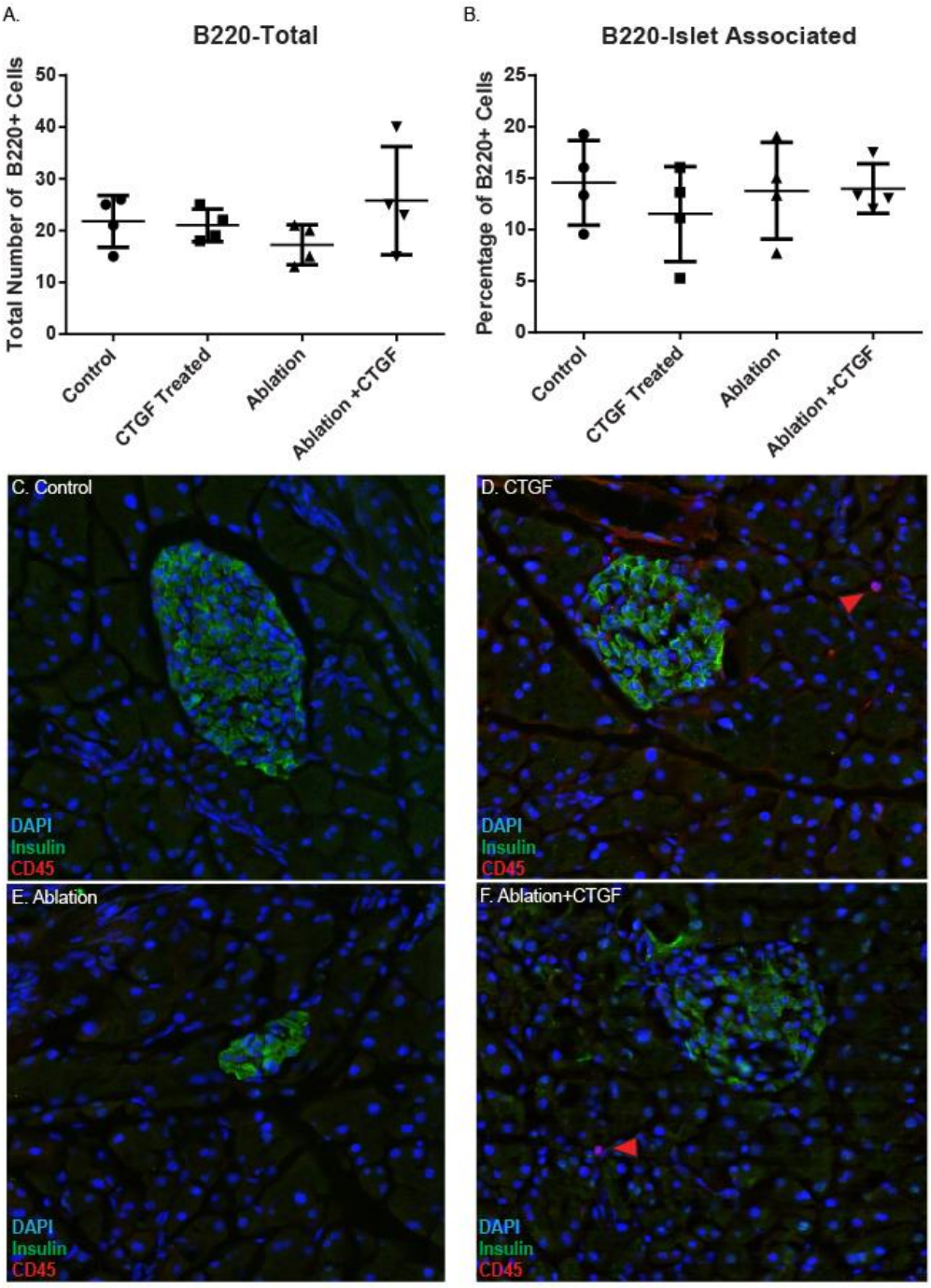


**Figure 5-5.** Neither β-cell ablation nor CTGF induction promotes increased pancreatic B cells. **A**. Total number of B220 positive cells and **B.** Proportion of islet-localized B cells. **C-F.** Representative images of Control (**C.**), CTGF treated (**D.**), Ablation (**E.**), and Ablation+CTGF (**F.**) islets after 2 days CTGF. Insulin (green), B220 (red). B220+ cells are demarked by red arrows. n=4.

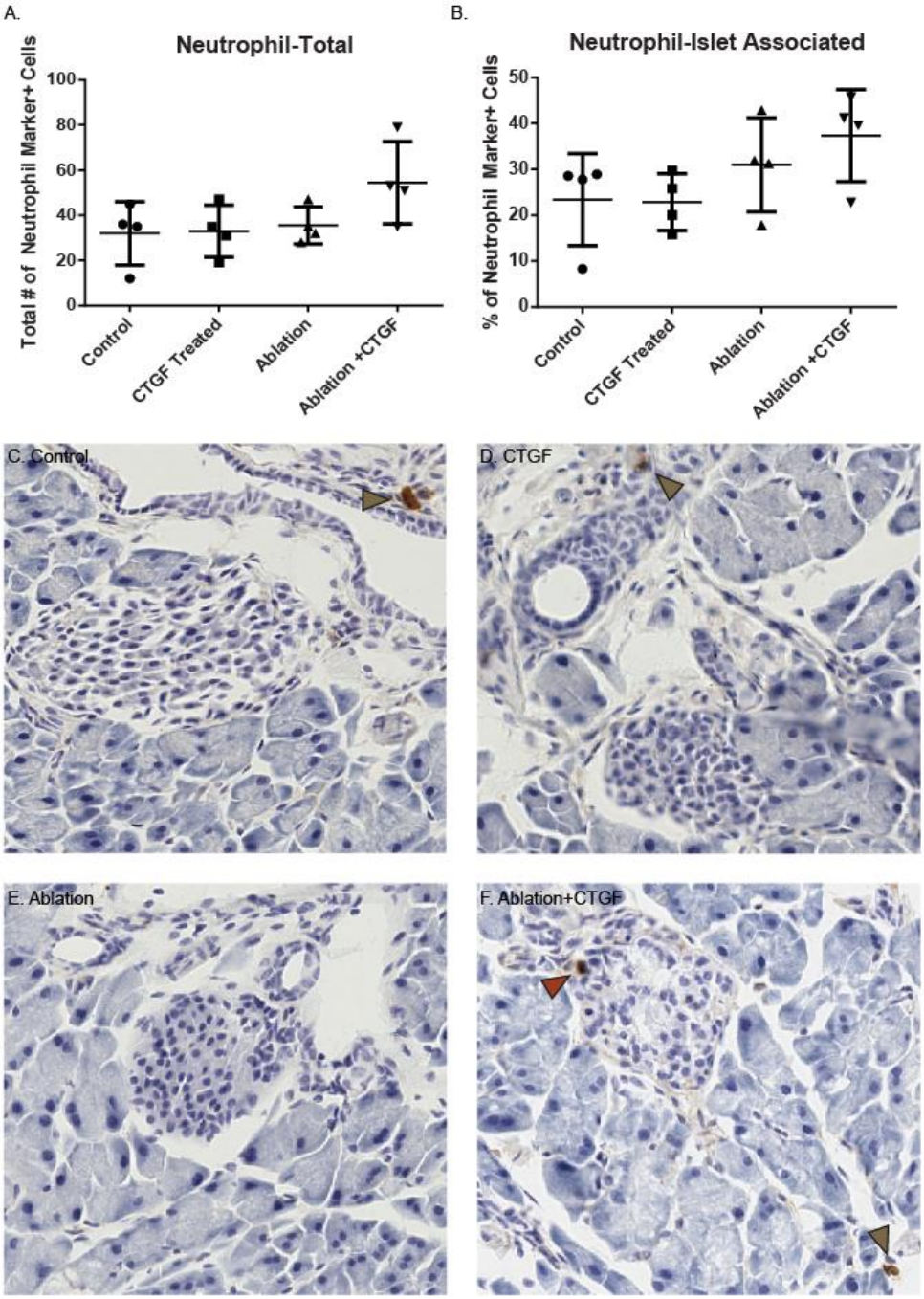


**Figure 5-6.** Neither β-cell ablation nor CTGF induction promotes increased pancreatic neutrophils. **A**. Total number of neutrophil marker-positive cells and **B.** Proportion of islet-localized neutrophils. **C-F.** Representative images of Control (**C.**), CTGF treated (**D.**), Ablation (**E.**), and Ablation+CTGF (**F.**) islets after 2 days CTGF. Neutrophil marker (brown), counterstained with Hematoxylin. Neutrophils within the exocrine or endocrine compartments are demarked by brown and red arrows, respectively. n=4.

**Gene expression changes at two day timepoint**

To gain further insight into which immune cell populations and associated signaling pathways are altered by CTGF with or without β-cell ablation, gene expression analysis was conducted on islets isolated from animals following 2 days of CTGF induction in vivo. We specifically assessed changes in expression of genes associated with the innate and adaptive immune cell response, including cytokine expression changes (See Figures 5-7-9). In addition, gene expression alterations in ECM components, vascular markers, and the stress response due to CTGF treatment, β-cell ablation, and CTGF treatment after β-cell ablation were determined (See Figures 5-10, 11).

There were several changes in key innate immune response genes following ablation and/or CTGF treatment (See Figure 5-7). CTGF induction under normal conditions or after β-cell ablation elicited an increase in *MCP1* (Macrophage Chemoattractant Protein 1), *RANTES (*Regulated on Activation, Normal T cell Expressed and Secreted/CCL5*)*, and *Ccr2* (C-C chemokine receptor type 2). MCP1 and its receptor, Ccr2, serve as chemoattractants for macrophages (322, 323), fitting with our immunolabeling data (See Figure 5-3). In addition, RANTES promotes macrophage activation along with T cell recruitment (324), further corroborating the observed increase in macrophages and T cells in our Ablation+CTGF cohort (See Figures 5-3, 4). β-cell ablation alone and in conjunction with CTGF induction increased expression of *CD45*, a pan-leukocyte marker, and *CD68*, a marker of macrophages, once again aligning with immunolabeling study findings (See Figures 5-2,3). This is highly suggestive of a critical role of macrophages in CTGF-mediated β-cell regeneration. The

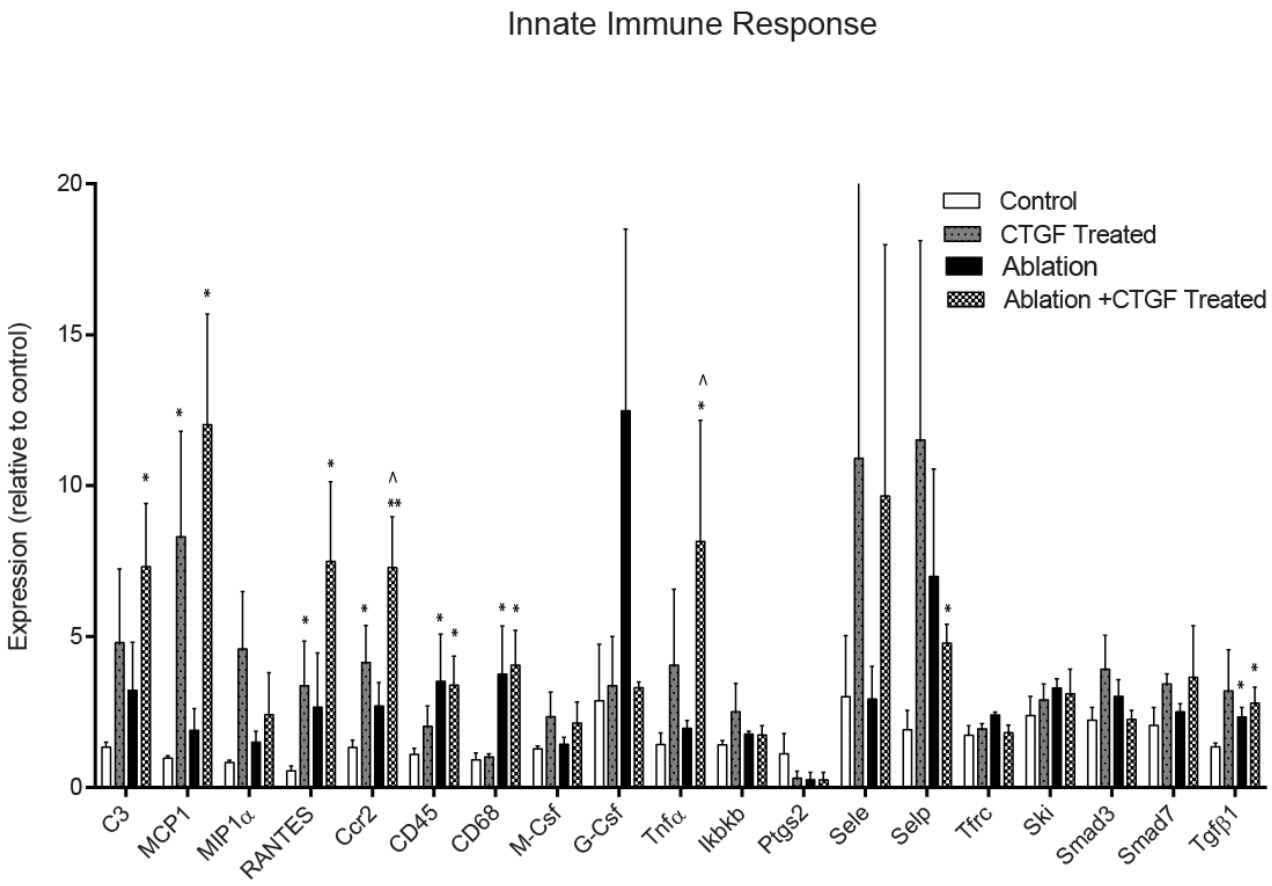


**Figure 5-7.** CTGF induces expression of genes involved in the innate immune response. Gene expression analysis on whole islets using TaqMan Universal PCR Mastermix. Islets isolated from animals with/without β-cell ablation +/- CTGF treatment for 2 days. All samples were run in duplicate. n=4. *compared to Control, ^compared to Ablation. *p<0.05, **p<0.01.

unique setting of CTGF induction after β-cell ablation resulted in the specific upregulation of *C3* (Complement Component 3), *TNFα* (Tissue Necrosis Factor α), and *Selp* (Selectin P). These genes are all associated with inflammation (325, 326), while *Selp* also serves as a leukocyte chemoattractant (327). Finally, in line with our hypothesis that CTGF may promote β-cell mass regeneration in a Smad-independent manner (See Figure 4-7), no increase in expression of *Smad3* or *Smad7* was observed in any cohort, while β-cell ablation alone or with CTGF treatment increased expression of the TGF-β response gene *Tgfβ1* (See Figure 5-7).

Alterations in expression of genes associated with the adaptive immune response focused primarily on T cells (See Figure 5-8). CTGF induction under normal conditions did not promote the expression of any genes associated with the adaptive immune response (See Figure 5-8). However, β-cell ablation alone or with CTGF treatment increased the expression of *CD3e*, a marker of T cells, and *Stat4*, a promoter of $Th_1$ development (See Figure 5-8; Ablation; (328)). Expression of several genes was increased only in the Ablation+CTGF cohort (See Figure 5-9; Ablation+CTGF). These included several additional markers of T cells, including; *CD4* (T helper cells), *CD28* (immature T cells), and *CD8a* (Cytotoxic T cells). Additionally, CTGF induction after β-cell ablation elicited the increased expression of macrophage expressed genes that promote T cell activation (*CD86*) and trafficking (*Ccl19*) (329, 330). Finally, CTGF treatment alone promoted the expression of *Ctla4* (Cytotoxic T Lymphocyte Associated protein 4), which is expressed on T helper and T regulatory cells to inhibit cytotoxic T cell activity (331), suggesting an attempt to remediate the inflammation occurring at this

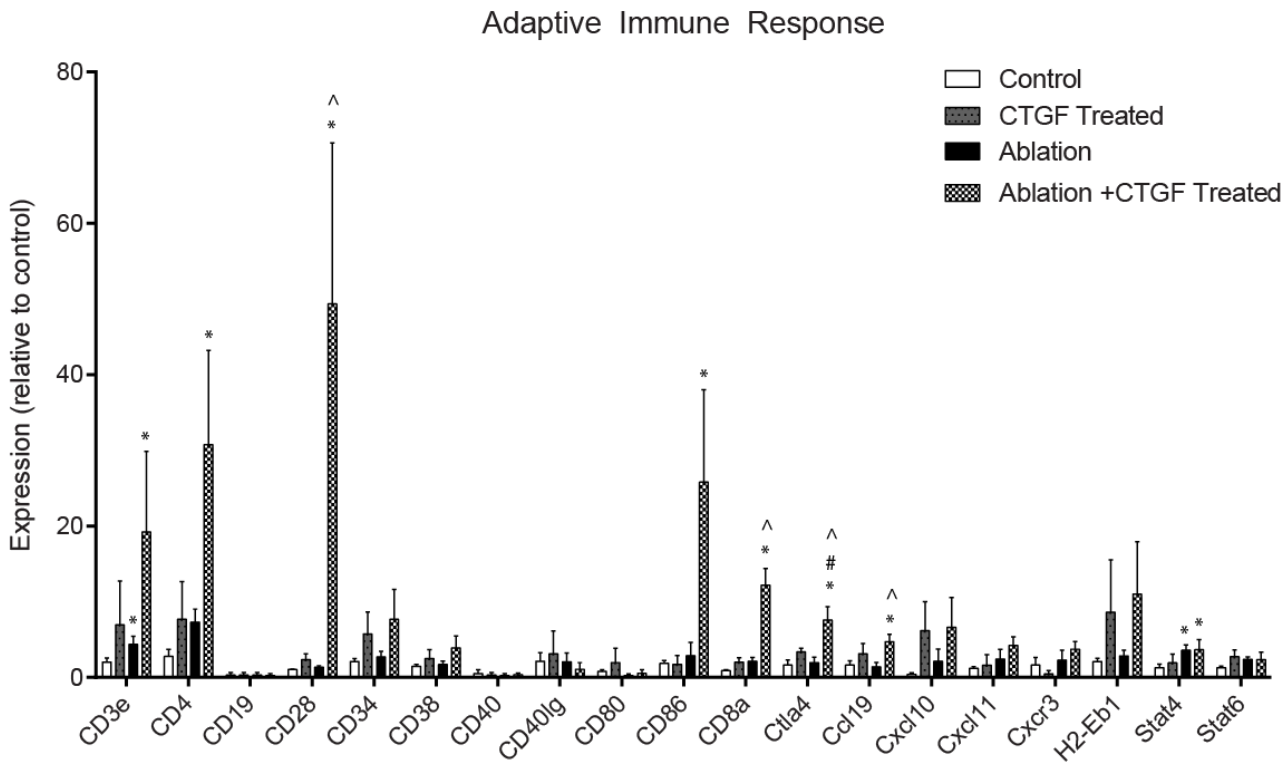


**Figure 5-8.** CTGF induces expression of genes involved in the adaptive immune response. Gene expression analysis on whole islets using TaqMan Universal PCR Mastermix. Islets isolated from animals with/without β-cell ablation +/- CTGF treatment for 2 days. All samples were run in duplicate. n=4. *compared to Control, #compared to CTGF Treated, ^compared to Ablation. * # ^p<0.05.

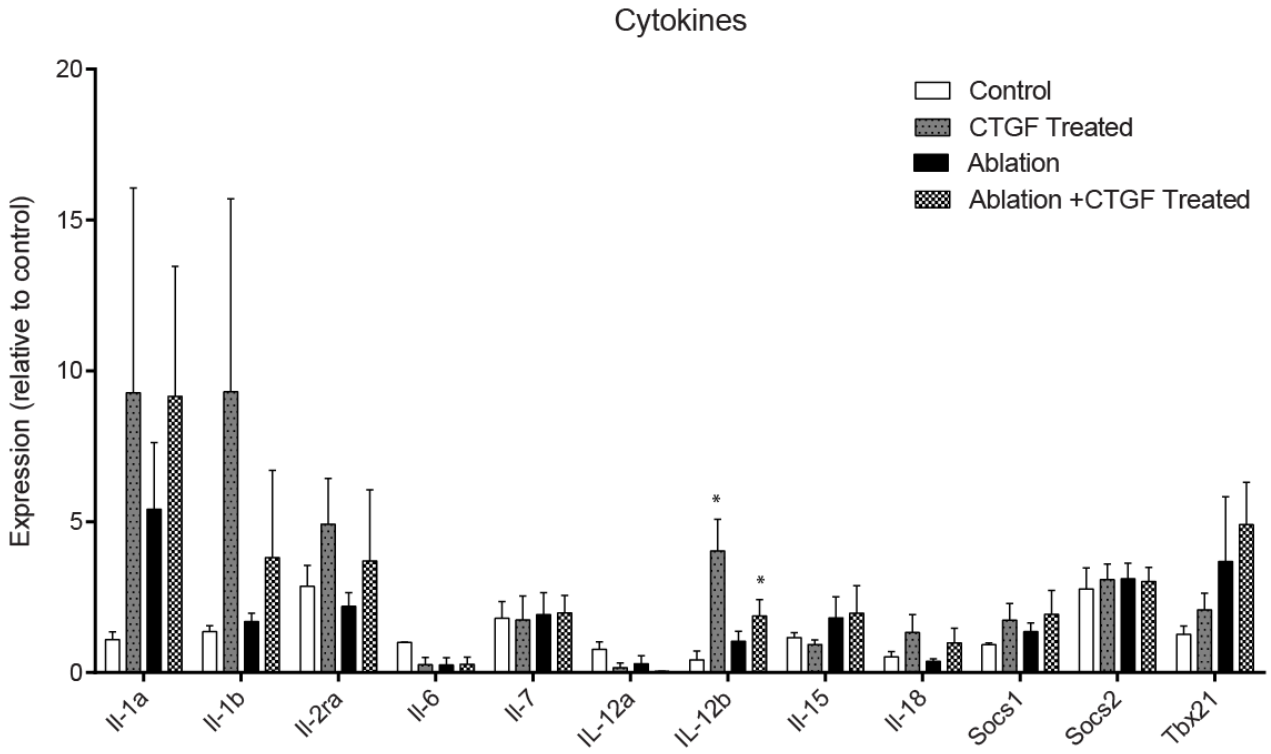


**Figure 5-9.** CTGF induces expression of the cytokine IL-12b. Gene expression analysis on whole islets using TaqMan Universal PCR Mastermix. Islets isolated from animals with/without β-cell ablation +/- CTGF treatment for 2 days. All samples were run in duplicate. n=4. *compared to Control. *$p<0.05$.

timepoint (See Figure 5-7). As predicted by our immunolabeling data, we did not observe changes in expression of genes associated with B cells (See Figure 5-8, *CD34,* CD38). We also assessed changes in the expression of several cytokines (See Figure 5-9). However, the only observed alteration was with *IL-12b* (Interluekin-12b), which was induced by CTGF expression after β-cell ablation and under normal settings (See Figure 5-9). *IL-12b* is expressed by macrophages and aids T helper cell development (332). Overall, these findings align well with our observed increase in T cells in the Ablation+CTGF cohort (See Figure 5-4), suggesting that CTGF induction promotes β-cell regeneration through macrophages and/or T cells.

Finally, we assessed alterations to genes associated with the ECM and vasculature, which play key roles in immune cell trafficking (See Figure 5-10). However, in our model of β-cell regeneration very few changes to the ECM or vasculature were observed. In fact, *Vcam1* (Vascular Cell Adhesion Molecule 1) was the sole gene significantly upregulated under any condition, that specifically being CTGF induction after β-cell ablation (See Figure 5-10; Ablation+CTGF). As Vcam1 is critical for adhesion of leukocytes to endothelial cells and subsequent signal transduction (333), this increase in expression suggested to us that the increase in macrophages and T cells was due to increased extravasation from the pancreatic vasculature. We also assessed whether our model of CTGF mediated β-cell regeneration involved induction or alterations to the cellular stress response (See Figure 5-11). However, no alteration to any gene assessed in any cohort was observed. Thus, it appears that in CTGF

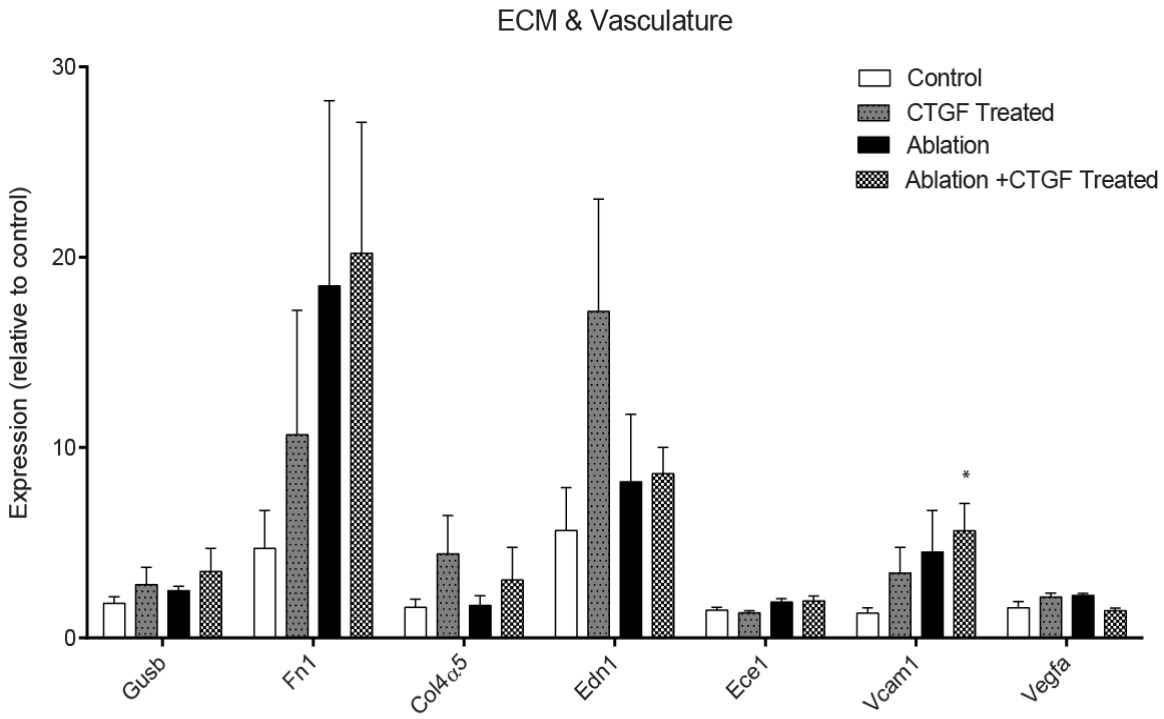


**Figure 5-10.** CTGF after β-cell ablation induces expression of Vcam1. Gene expression analysis on whole islets using TaqMan Universal PCR Mastermix. Islets isolated from animals with/without β-cell ablation +/- CTGF treatment for 2 days. All samples were run in duplicate. n=4. *compared to Control. *$p<0.05$.

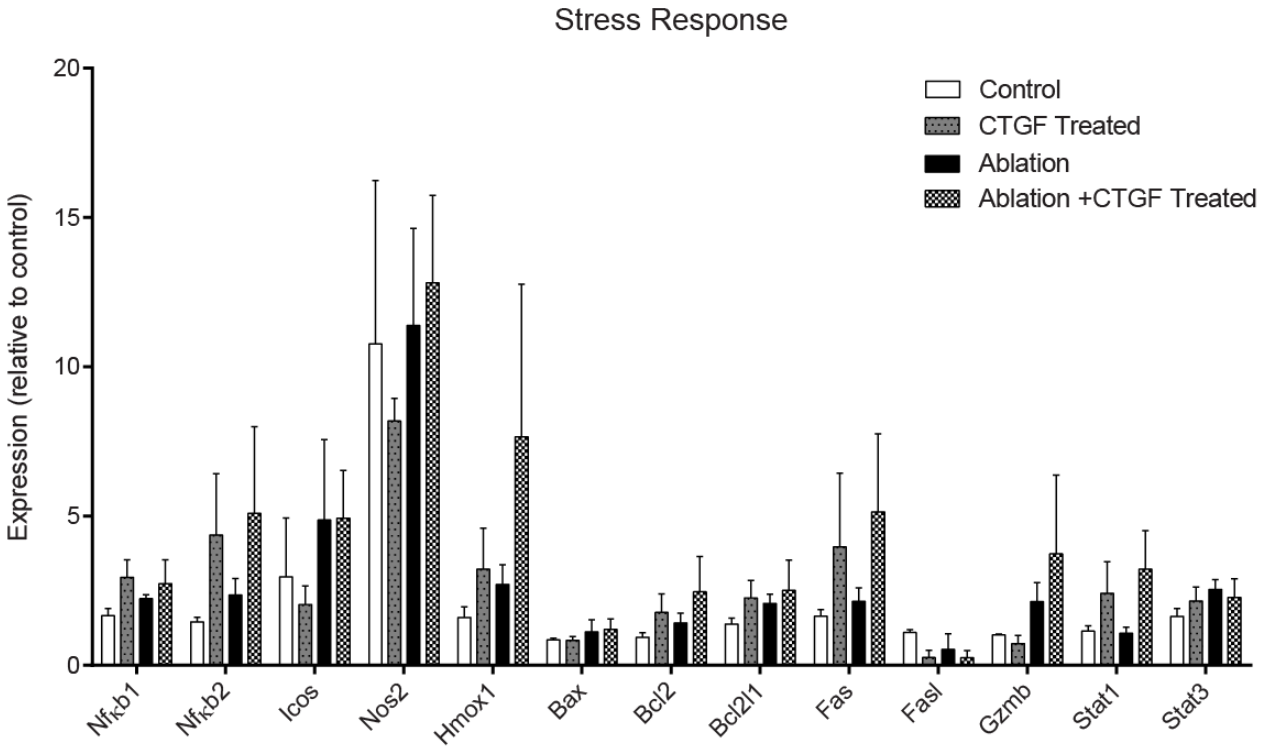


**Figure 5-11.** Neither β-cell ablation nor CTGF treatment induce expression of stress response genes. Gene expression analysis on whole islets using TaqMan Universal PCR Mastermix. Islets isolated from animals with/without β-cell ablation +/- CTGF treatment for 2 days. All samples were run in duplicate. n=4.

mediated β-cell mass expansion, CTGF induction promotes an increase in and activation of primarily macrophages and T cells.

**Macrophage depletion study**

In order to assess whether infiltrating macrophages were, at least in part, mediating CTGF-elicited β-cell regeneration, a macrophage depletion study was conducted. To ablate macrophages, liposomes containing clodrinate were employed. Liposomes are artificially prepared lipid vesicles, and can be used to encapsulate aqueous substrates, such as clodrinate, a non-toxic bisphosphonate. After injection, liposomes, acting as a Trojan horse, are ingested and digested by macrophages followed by intracellular release and accumulation of clodrinate. At high intracellular concentrations, clodrinate induces apoptosis (334). Clodrinate liposomes were administered once daily, one day prior, during and for 2 days following DT injections in 8 week old RIP-DTR;RIP-rtTA controls ("Ablation+Clodrinate") and RIP-DTR;RIP-rtTA;TetO-CTGF experimental animals ("Regeneration+Clodrinate") and CTGF induced by Dox treatment for 2 days after DT injection. Additional controls included RIP-DTR;RIP-rtTA animals injected with PBS-containing liposomes ("Ablation+PBS") and DTR;RIP-rtTA;TetO-CTGF animals injected with PBS-containing liposomes in which CTGF was induced ("Regeneration+PBS") (See Figure 5-12A). F4/80 immunolabeling confirmed a clodrinate treatment-specific depletion of macrophages (See Figure 5-12B). Additionally, as macrophages can serve as T cell chemoattractors (335), we assessed whether removal of macrophages also decreased the observed increase in T cells in our "Ablation+CTGF" cohort (See Figure 5-4). In our model, clodrinate liposome

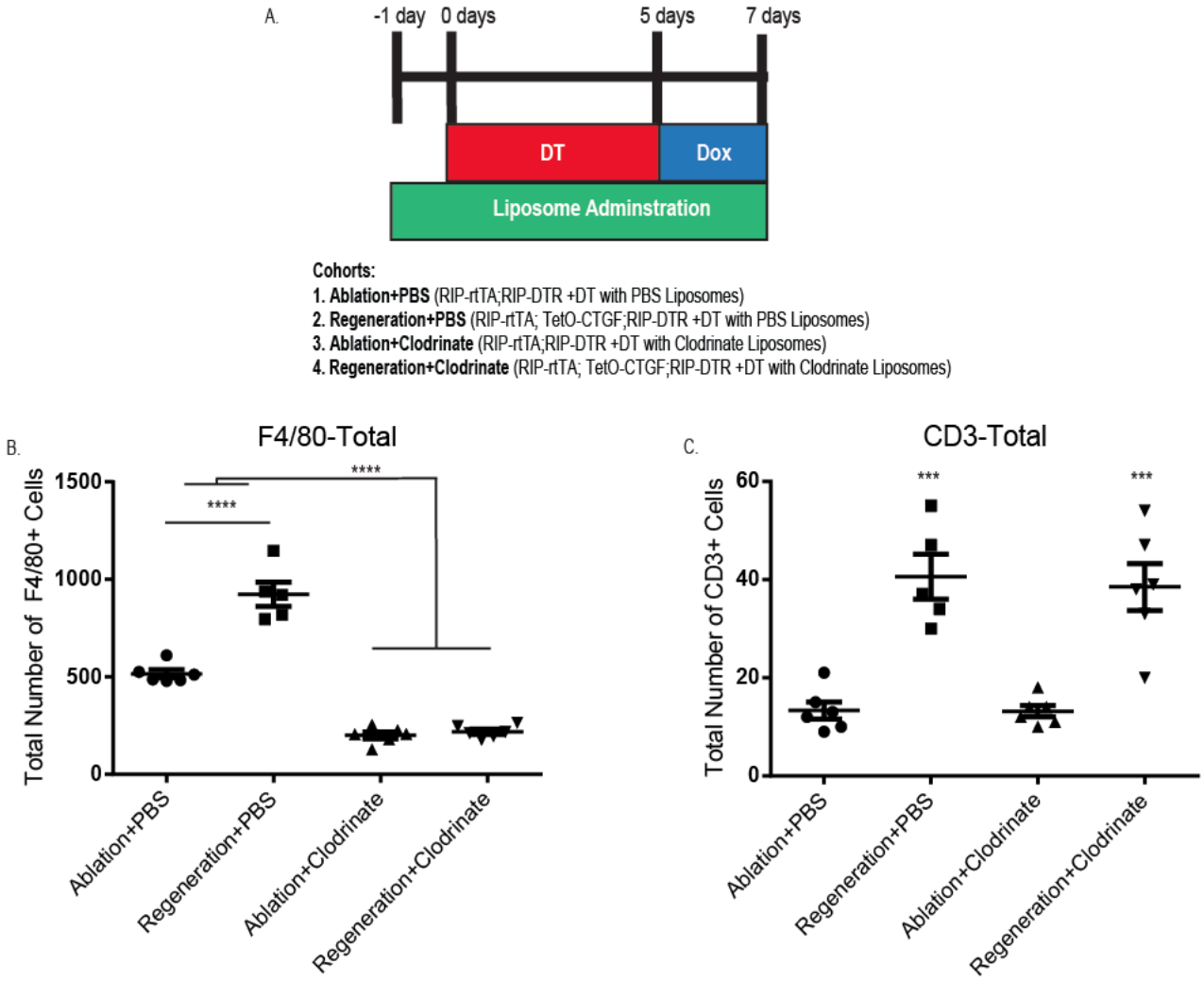


**Figure 5-12.** Experimental outline and macrophage depletion verification. **A.** Experimental outline. Mice were administered 250 µL of either clodrinate or PBS filled liposomes once daily. Diphtheria toxin (DT; 126 ng; Sigma) was given IP 3 times at 8 weeks of age. Mice were administered 2 mg/ml of doxycycline (Dox) in 2% Splenda in drinking water. **B.** Total number of F4/80 positive cells. **C.** Total number of CD3 positive cells. n=6 for Ablation+PBS, Ablation+Clodrinate and Regeneration+Clodrinate, n=5 for Regeneration+PBS. ***p<0.001, ****p<0.0001.

treatment does not result in a decrease in the T cell population within the pancreatic parenchyma (See Figure 5-12C).

CTGF treatment, elicits β-cell regeneration by increasing β-cell proliferation. Thus, we assessed whether the increase of macrophages after β-cell ablation was required for increased β-cell proliferation. Interestingly, depletion of macrophages during CTGF-mediated β-cell regeneration decreased the percentage of proliferating β-cells to control cohort levels (See Figure 5-13A; compare "Regeneration+Clodrinate" vs. "Ablation+PBS"). Thus, macrophages are essential for CTGF-mediated increases in β-cell proliferation.

Since 50% β-cell ablation promotes a more immature β-cell phenotype, which is further heightened upon CTGF treatment (See Figure 4-1A), we also assessed the requirement of macrophages for this effect. Intriguingly, macrophage depletion enhanced the percentage of mature (MafA+) β-cells as compared to the regeneration control cohort (See Figure 5-13B; "Regeneration+Clodrinate" vs. "Regeneration+PBS"). However, it must be noted that "Regeneration+Clodrinate" islets remain more phenotypically immature as compared to control, non-ablated islets (See Figures 5-13B and 4-1A). In addition, preliminary findings display no change in the percentage of immature (MafB+) β-cells across all cohorts (See Figure 5-13C). Regardless, it is possible that macrophages are required for both the β-cell proliferative and maturity state alterations mediated by CTGF after 50% β-cell ablation.

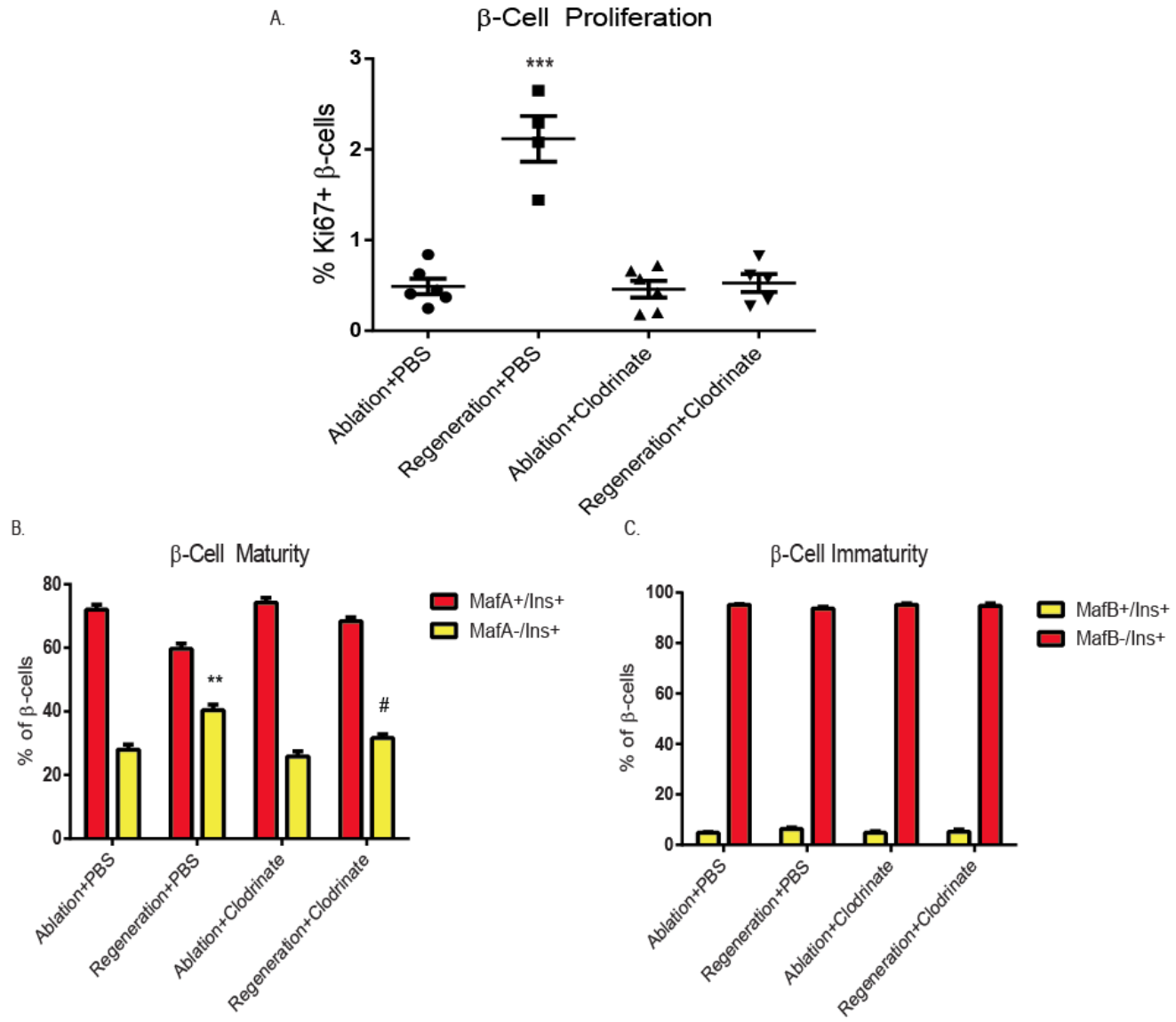


**Figure 5-13.** Examination of β-cell intrinsic characteristics in response to macrophage depletion. **A**. β-cell proliferation. **B**. Proportion of MafA+ (red bars) or MafA- (yellow bars) β-cells. **C.** Proportion of MafB+ (yellow bars) or MafB- (red bars) β-cells. **A.** n=6 for Ablation+PBS and Ablation+Clodrinate, n=5 for Regeneration+Clodrinate, n=4 for Regeneration+PBS. ***p<0.001. **B.** n=5 for Ablation+Clodrinate and Regeneration+ Clodrinate. N=4 for Ablation+PBS and Regeneration+PBS. **p<0.01 against all groups. #p<0.05 against Ablation+PBS and Ablation+Clodrinate. **C.** n=6 for Ablation+PBS, n=5 for Ablation+Clodrinate, n=4 for Regeneration+PBS, and n=3 for Regeneration+ Clodrinate.

## Discussion

From previous studies, we determined that CTGF treatment after 50% β-cell ablation promotes β-cell regeneration in part by modulation of several β-cell intrinsic characteristics (See Chapter IV). However, CTGF induction may also alter several extrinsic aspects to the islet-microenvironment during β-cell regeneration, outside of islet vascularization (See Figure 3-7). Indeed, in other pancreatic injury models, CTGF recruits several immune populations to the site of injury (256). Thus, here we investigated the potential role of the immune system in CTGF-mediated β-cell regeneration.

β-cell destruction and CTGF treatment in the setting of DT-mediated β-cell ablation increased the total number of macrophages in the pancreatic parenchyma. In fact, macrophages comprised the vast majority of immune cells within the pancreas. Our findings are supported by studies from other groups who have shown that macrophage involvement is critical for β-cell mass regeneration in multiple injury settings (294, 317). However, it is interesting that CTGF treatment after β-cell injury heightened the number of macrophages within the pancreata, and specifically within the endocrine compartment. Additionally, gene expression analysis showed CTGF induction-specific increases in expression of several macrophage markers and chemoattractant genes. Intriguingly, several genes (*CD86*, *Ccl19*, *IL-12b*) associated with the pro-inflammatory M1 macrophage polarization were observed to be increased in the setting of CTGF treatment after β-cell destruction. Future studies employing flow

cytometry will allow for verification of the macrophage polarization phenotype in this model of β-cell regeneration.

Additionally, we observed an increase in T cells specifically in the setting of CTGF treatment after β-cell destruction. This was not unexpected as studies have proposed that T cells promote β-cell mass regeneration in the setting of diabetes mellitus (316, 318). Also, CTGF has been observed to promote T cell recruitment in the kidney (320). Gene expression analysis on whole islets corroborated our immunofluorescence findings as upregulation of several T cell markers and chemoattractant genes was observed in the Ablation+CTGF cohort. While several of the T cell marker genes pointed towards a T helper cell population, future flow cytometry studies will allow us to determine the specific T cell sub-population(s) involved in CTGF-mediated β-cell mass regeneration.

However, in our model of β-cell mass regeneration we cannot determine whether CTGF treatment after β-cell ablation recruits macrophages and T cells from outside the pancreas to the pancreas/islets or if CTGF promotes proliferation of the pre-existing resident pancreatic macrophages and T cells. Further studies using bone marrow reconstitution with GFP-labeled bone marrow cells will allow us to determine if CTGF treatment is inducing recruitment or proliferation of macrophages.

However, it remained unclear whether either macrophages or T cells were involved in CTGF-mediated β-cell proliferation and subsequent β-cell mass regeneration. Through clodrinate liposome-based macrophage depletion techniques, we observed that macrophages are critical for CTGF-mediated increases in β-cell proliferation in the setting of β-cell destruction. Additionally, preliminary studies suggest

that macrophages may play also play a role in β-cells adapting a more immature phenotype after β-cell ablation, as removal of macrophages in the presence of Ablation+CTGF appears to prevent the decline in MafA+ β-cells we observed in Ablation+CTGF alone. However, these are only preliminary observations and further continuation and expansion of these studies are currently underway. Additionally, we plan to assess the potential role of T cells in CTGF-mediated β-cell mass regeneration via CD3 antibody-mediated T cell depletion studies.

CTGF mediated β-cell mass regeneration involves the modification of several β-cell intrinsic characteristics. However, it now appears that the immune system, specifically macrophages, is also required for effective β-cell mass expansion by CTGF. This is a novel interaction between CTGF and the immune system to promote β-cell mass expansion. However, it remains to be determined if CTGF promotes immune cell infiltration which in turn directly enhances β-cell proliferation, or if recruited immune cells promote a more CTGF-permissive islet microenvironment. Regardless, as all forms of diabetes are demarked by insufficient functional β-cell mass, further characterization of factors that can promote β-cell mass expansion is critical. Our studies highlight the significance of understanding the role of the immune system in promoting β-cell regeneration.

# CHAPTER VI

## SUMMARY AND FUTURE DIRECTIONS

The secreted protein CTGF modulates several biological processes including proliferation, migration, adhesion, ECM remodeling, and angiogenesis. In the pancreas, CTGF is critical for proper β-cell development and β-cell mass expansion during pregnancy, and has been observed to promote tumor migration and survival. Our lab initially became interested in CTGF in the context of normal pancreas development, as it is downregulated in the Hnf6 over-expressing transgenic mouse model of islet dysmorphogenesis and diabetes. We found that CTGF is expressed in insulin+ cells, ducts and blood vessels during pancreas development, but is restricted to duct and blood vessels in adult pancreata (179). CTGF is re-expressed in adult β-cells during pregnancy and high fat diet feeding (179), suggesting it plays a role in β-cell mass expansion and/or improved β-cell function in response to physiological stimuli.

Additionally, our laboratory determined that CTGF is crucial for islet development. Animals lacking CTGF display decreased β-cell proliferation and mass (52). Loss of CTGF from any one cell source in the embryonic pancreas resulted in significantly decreased β-cell proliferation, indicating that CTGF works in both a paracrine and autocrine manner to promote β-cell proliferation during development (52). In addition, increased *Ctgf* expression in developing β-cells resulted in a 25% expansion of the endocrine compartment, due to enhanced β-cell and α-cell proliferation (52). These embryonic studies revealed the critical role CTGF plays in β-cell proliferation and

mass expansion during development. However, CTGF induction in normal adult β-cells does not stimulate β-cell proliferation or β-cell mass expansion ((189); See Figure 3-4). Therefore, while CTGF expression is induced under certain physiological conditions to promote β-cell mass expansion, such as pregnancy or high fat diet, over-expression under normal conditions has no effect. Thus, we hypothesized that the islet microenvironment plays a critical role in regulating β-cell responsiveness to CTGF and potentially other proliferative signals.

We assessed the potential role of CTGF in β-cell mass regeneration through the use of a DT-mediated β-cell ablation model. This was paired with our conditional CTGF over-expression model in order to induce CTGF treatment after 50% β-cell ablation. We found that CTGF induction only after 50% β-cell ablation resulted in β-cell mass expansion, reaching a top threshold of 75% of the original β-cell mass restored by 4 weeks of treatment. 50% β-cell ablation or CTGF induction alone did not induce β-cell mass regeneration, leading us to hypothesize that adult β-cell mass expansion may therefore require combinatorial stimuli. As far as we have been able to assess, this observed β-cell mass regeneration occurred solely due to an increase in β-cell proliferation. This specific increase in β-cell proliferation was observed as early as two days of CTGF induction and was maintained, although to a lesser degree, out to our 4 week timepoint. However, while the CTGF-mediated 50% recovery of lost β-cell mass is significant, full β-cell mass recovery was unachievable in this model. This is not unsurprising as there is no physiological impetus for β-cell mass expansion at 50% β-cell loss. CTGF responsiveness may be restricted to periods of increased functional demand. Repeating this study in a more severe β-cell destruction model, such as the

~85% DT-mediated system (316), may result in a more robust CTGF-mediated recovery of β-cell mass. However, if CTGF cannot elicit greater than 50% β-cell mass regeneration. It may be that once euglycemia is achieved and individual β-cell strain is reduced that β-cell responsiveness to any proliferative factor is impeded.

Additionally, it would be of note to determine the functional window of CTGF treatment. It is currently unclear whether CTGF induction must occur directly after insult, as it does in the studies presented in this thesis, or if CTGF treatment onset can be delayed and still result in 50% β-cell mass expansion. Simple timecourse studies of delayed Dox induction would allow for determination of the functional window for CTGF-mediated β-cell regeneration. We hypothesize, due to the requirement of macrophages for CTGF-mediated β-cell proliferation enhancement that CTGF induction must occur soon after insult. As interplay between macrophages and CTGF appears to exist, it is likely that CTGF treatment must occur while macrophages are still present in the pancreatic parenchyma.

While CTGF promotes β-cell mass regeneration by increased β-cell proliferation, we sought to determine other potential mechanisms by which CTGF could elicit regenerative effects. In the current model of β-cell ablation, CTGF was unable to promote β-cell survival. Future experiments in other models of β-cell ablation, namely streptozotocin, could further explore the potential prophylactic role of CTGF. Additionally, through analysis of several surrogate markers; CTGF does not promote β-cell mass regeneration by stimulating β-cell neogenesis. However, it may be of interest to preform lineage tracing studies to fully confirm the lack of neogenesis in our model of β-cell regeneration. Finally, β-cell hypertrophy was not observed in our model. This was

of interest to us as animals heterozygous for CTGF display β-cell hypertrophy as a compensatory mechanism to maintain euglycemia (179).

In order to further understand how CTGF promoted β-cell mass expansion, we analyzed potential changes to intrinsic β-cell characteristics. We first assessed whether CTGF induction and/or β-cell destruction altered the maturity state of the β-cells. This was particularly intriguing to us because during development CTGF induction only enhances proliferation of immature (MafA-) β-cells (52). This suggests that permissiveness to CTGF may be restricted to specific β-cell sub-populations. Indeed, several groups have suggested that β-cells undergo partial de-differentiation prior to proliferation (reviewed in (336)). In our regeneration model, CTGF induction promoted proliferation of both mature and immature β-cells. However, a greater proportion of immature β-cells proliferated in response to CTGF, lending further credence to the hypothesis that immature or partially de-differentiated β-cells are more responsive to CTGF or other proliferative factors.

We determined whether CTGF induction after β-cell destruction affected the proliferative refractory period of β-cells. While β-cells typically have an extensive replicative refractory period, this period is labile and shortens in response to physiological stimuli of increased demand (195). Only the combination of CTGF induction and injury resulted in a shortened replicative refractory period. Intriguingly, while both mature and immature β-cells displayed a shorted refractory period, a greater percentage of immature β-cells underwent multiple rounds of replication. Overall, it appears that CTGF promotes β-cell regeneration via a multi-pronged approach; an increased proportion of β-cells proliferate and the replicative refractory periods of these,

primarily immature, β-cells are shortened. It would be of interest to assess whether this mechanism of β-cell regeneration is unique to CTGF or endemic to most proliferative stimuli.

As CTGF is associated with several signaling pathways, TLDA analysis was employed to begin to parse out potential pathways through which CTGF mediates β-cell regeneration. Of primary interest was the observed increase in several integrins, namely integrin β1, which is essential for adult β-cell proliferation and mass expansion (158). The potential role of integrin β1 was further corroborated by an observed increase in phosphorylated ERK1/2, an integrin β1 downstream effector (158, 309). Thus, it is highly likely that CTGF promotes β-cell mass regeneration through integrin signaling. Further studies could examine whether CTGF activation of β1 integrin signaling is required or responsible for the increased expression of other proliferative factors (i.e. *Hgf* and *Tph1*) observed in our model of β-cell mass regeneration. Unfortunately, genetic *in vivo* studies of conditional integrin β1 knockout in islets would be difficult to achieve in our already complicated mouse model. However, this could be circumvented by applying rCTGF to islets from a β-cell specific integrin β1 knockout animal. Additionally, integrin β1 signaling could be inhibited pharmacologically, for example using obustatin (337), or using endogenous antagonists, such as SHARPIN (338), in an *ex vivo* setting to analyze the role of β1 integrin signaling in CTGF-mediated β-cell mass regeneration.

Through various domains, CTGF either promotes (TGF-β, integrin) or inhibits (BMP, Wnt) different signaling pathways. While our gene expression data showed increased expression of response genes for all these pathways, it is incredibly difficult to

determine which, if any, are directly associated with the proliferative effects of CTGF on β-cells. However, reporter lines for various pathways (i.e. BRE-β-galactosidase (339) for BMP signaling, SMAD-TK-luciferase (340) for TGF-β signaling, and BAT-GAL (341) for Wnt signaling) could be interbred with our mouse model. This would allow us to assess which signaling pathways are being activated in the islet, or surrounding parenchyma, in response to CTGF-mediated β-cell regeneration. Additionally, our preliminary biochemical studies showed the observed increase in TGF-β signaling occurred in a SMAD-independent manner. Further biochemical or pharmacological studies would be difficult to execute *in vivo*. However, other studies from our lab have shown that recombinant CTGF promotes β-cell proliferation in an *ex vivo* setting. Thus, an *ex vivo* β-cell model could be employed to examine the direct effect of loss of TGF-β, BMP, or Wnt on CTGF-mediated β-cell proliferation.

CTGF is also proteolytically cleaved between its second and third domains by MMPs (342). CTGF cleavage is typically associated with wound healing, as it results in the release of VegfA from the thrombospondin domain located in the C-terminal half of CTGF (164). In addition, after cleavage, the C-terminal fragment may be tethered to the cell surface due to interactions with integrins and heparin sulfate proteoglycans while the N-terminus, which lacks these interaction domains, likely diffuses away from the cellular source (168, 343). Thus, it may be that CTGF induction in β-cells occurring after β-cell injury facilitates β-cell proliferation either in a cell autonomous or non-autonomous manner. It is currently unknown whether CTGF-mediated β-cell regeneration is dependent on full length CTGF or only on a specific cleavage fragment. Through studies in pancreatic and hepatic stellate cells, chondrocytes and murine fibroblasts, it

has been observed that the C-terminal fragment promotes cell adhesion, angiogenesis, migration, and survival primarily through integrin activation (167-169, 343, 344). Additionally, several integrins, namely integrin β1, have been observed to promote β-cell proliferation (158). The N-terminal fragment promotes TGF-β signaling, enhancing β-cell proliferation and survival in diabetic models (345), yet also enhances collagen deposition (303). In order to address if a specific fragment of CTGF is sufficient to drive β-cell regeneration, an ongoing study in our lab involves generating recombinant CTGF fragments (i.e. N-terminal- or C-terminal-specific domains). These peptides could be used *ex vivo* to determine the smallest functional unit of CTGF in regards to β-cell proliferation. This is an important step in establishing CTGF as a potential therapeutic for diabetes.

In addition to our various findings on how CTGF alters several β-cell intrinsic characteristics during β-cell mass regeneration, we also assessed changes to the islet microenvironment. We first assessed islet vascular density, as CTGF is an angiogenic factor (162). Vascular endothelial cells produce several different secreted signaling molecules, including spingosine-1-phosphate, fibroblast growth factor (FGF), and hepatocyte growth factor (HGF; (58, 59). HGF is expressed by pancreatic endothelial cells and is mitogenic to β-cells (60-63). Thus, in the pancreas, endocrine-produced VEGF signaling through VEGF receptors on the endothelium may induce the expression of HGF, which in turn promotes endocrine proliferation. Although CTGF did not promote enhanced islet vascularity, it remains possible that endothelial-derived proliferative factors, such as HGF, contribute to CTGF-mediated increases in β-cell proliferation and mass expansion.

We also examined the role of the immune system in our model of β-cell mass regeneration. It is probable that CTGF induction elicits β-cell mass regeneration by also mediating changes to extrinsic factors of the islet micro-environment. Although we found that CTGF induction does not promote heightened islet vascularization in this model of β-cell regeneration; CTGF may promote the recruitment of particular immune cell populations, and their secreted products could prime β-cells to respond to CTGF. The role of the immune system in regeneration is well established in multiple tissues (314, 315), including β-cell regeneration in other models of β-cell mass loss (294, 316, 317). We observed that CTGF induction after β-cell ablation results in an increased number of immune cells, primarily macrophages, in the pancreatic parenchyma. Through macrophage depletion studies, I determined that CTGF-mediated β-cell proliferation is dependent on macrophages. In addition, these macrophages also appear to modify the maturity state of β-cells, as removal of macrophages in the setting of Ablation+CTGF increased the number of MafA+; insulin+ cells compared with Ablation+CTGF alone. To our knowledge, no other group has observed an association between macrophages and β-cell maturity state.

While our preliminary studies on the role of the immune system in CTGF-mediated β-cell mass regeneration are exciting, they raise several new questions. It would be of great importance to attempt to determine the immune cell-derived factor that elicits the aforementioned effects on the islet microenvironment. This daunting task would be greatly aided via the determination of which specific immune cell types are critical for CTGF-mediated β-cell regeneration. In regards to macrophages, is it activated resident or infiltrating macrophages that are responsible for β-cell

proliferation? Future bone marrow reconstitution studies using GFP-tagged bone marrow will allow us to determine if CTGF promotes macrophage infiltration from the periphery and if these macrophages are essential for β-cell proliferation. It should also be noted that a less robust increase in T cells was also observed in the "Ablation+CTGF" cohort. The potential role of T cells in our model has yet to be determined. Pharmacological depletion studies, such as CD3 antibody-based depletion techniques, could be employed to assess which, if any, changes in the islet microenvironment during β-cell regeneration are due to T cells. If T cells are found to be critical for CTGF-mediated β-cell regeneration, future studies would focus on determining the specific T cell subpopulation involved in β-cell regeneration. Flow cytometry using specific T cell subpopulation makers (i.e. CD8 for cytotoxic T cells, CD4 for T helper cells) would allow for accurate quantification of the predominate T cell subpopulations in our model of β-cell regeneration. Additionally, time courses should be determined for the immune cell populations of interest. In order to determine the mechanism by which macrophages and potentially T-cells aid in CTGF-mediated β-cell regeneration, it would beneficial to understand when immune cell invasion/expansion begins in the pancreatic parenchyma and at what point do the immune cells disperse. Does this dispersal of immune cells correlate with the decrease in β-cell proliferation observed after the two week timepoint of CTGF induction?

Despite our extensive findings on the previously unknown role of CTGF in β-cell regeneration, a true mechanism has yet to be determined. We propose two major theories on how CTGF promotes β-cell mass expansions (See Figure 6-1). Generally, an increase in macrophages is observed following DT administration. However, these

macrophages alone are unable to promote β-cell regeneration. Thus we believe that CTGF induction elicits one of two major effects on macrophages; that of CTGF promoting macrophage recruitment over a regenerative threshold or CTGF altering the character of macrophages to a more proliferative phenotype. In regards to the first theory, CTGF induction elicits an increase in immune cells (resident or infiltrating) over a regenerative threshold, which in turn, via secretion of an unknown factor, alter the islet microenvironment to promote permissiveness to proliferative stimuli (See Figure 6-1; 1.). Secondly, CTGF induction following β-cell ablation results in an alteration to the character of the macrophages (see Figure 6-1; 2.). These altered macrophages subsequently secrete an unknown factor that promotes permissiveness to proliferative stimuli. Regardless of mechanism, the CTGF-mediated involvement of macrophages alters the islet microenvironment to become more responsive to proliferative stimuli. However, it is unknown at this time which proliferative factor is promoting β-cell proliferation and mass expansion. We propose three potential candidates; 1) CTGF itself (See Figure 6-1; A.), 2) other proliferative factors (i.e. HGF, 5-HT; See Figure 6-1; B.) or 3) the macrophage secreted factor alone promotes β-cell proliferation (See Figure 6-1; C).

It may be that β-cell injury alone triggers the observed increase in immune cells which promote β-cell permissiveness to CTGF (Not displayed). However, there are significant reservations with this theory as it must be noted that the increase in macrophages observed in the Ablation cohort was not enough to elicit β-cell proliferation. Thus giving credence to the theory that a threshold of macrophages

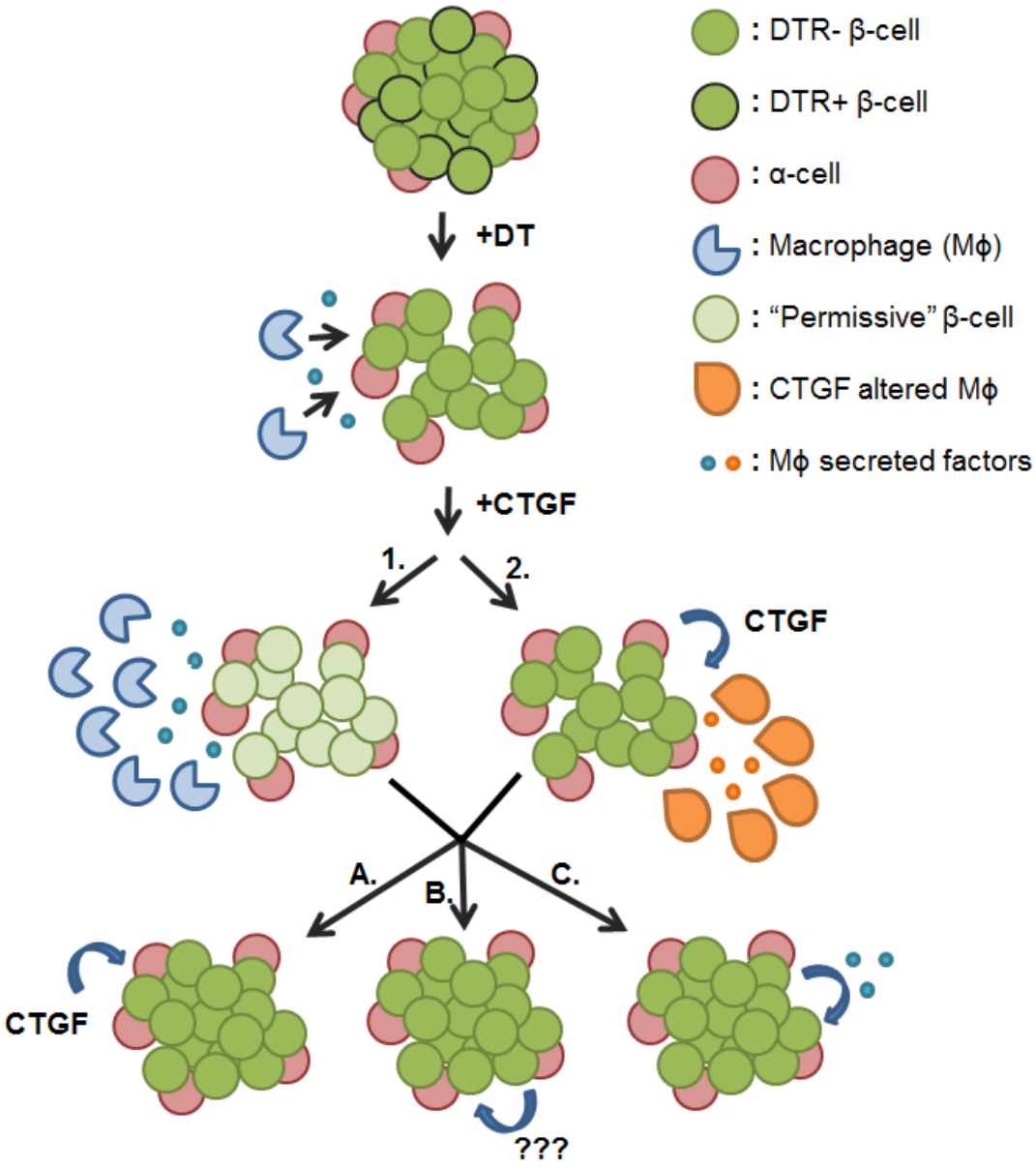


**Figure 6-1.** Potential mechanisms of CTGF-mediated β-cell mass regeneration. After 50% β-cell ablation, macrophages enter the islet to remove dead β-cells. **1.** Upon CTGF induction, more macrophages are recruited to the islet and secrete an unknown factor to promote β-cell permissiveness. **2.** Upon CTGF induction, recruited macrophages are altered by CTGF (i.e. polarization, chemokine profile etc.). These altered macrophages, in turn, secrete factors that promote β-cell permissiveness. Macrophage dependent β-cell proliferation is achieved either directly or indirectly through the following mechanisms **A.** CTGF itself, **B.** other proliferative factors, such as 5-HT, Integrin β1, or HGF, or **C.** directly by the macrophage secreted factors.

number must be reached in order to elicit β-cell proliferation and this can only occur in our model in the presence of CTGF induction after β-cell injury.

Regardless of mechanism, it would be of note to determine if any perturbation to the islet is impetus enough for immune cells to promote β-cell proliferation. We plan to use a Glucagon-DTR transgenic mouse model of α-cell ablation, paired with our CTGF over-expression model to determine if a non-β-cell injury to the islet can promote β-cell permissiveness to CTGF. α-cells have also been shown to be a potential pool of β-cells after near complete β-cell ablation (292). Following 99% β-cell ablation, α-cells transdifferentiated into β-cells to promote β-cell mass regeneration (292). As CTGF over-expression promotes α-cell proliferation during development, CTGF induction in a severe model of β-cell destruction may further enhance α-cell to β-cell transdifferentiation.

Future studies are planned in the lab to examine the ability of CTGF to improve the outcomes of islet transplantation. The process of islet transplantation involves the severing of the endogenous vasculature; however, some intraislet endothelial cells survive and become incorporated into the post-transplantation revascularization of the islet (346). Studies have shown that increased expression of angiogenic factors enhances the revascularization and survival of the islet grafts (346). Thus, while CTGF did not promote islet vascularization in our regeneration model, it may enhance islet transplant survival, by not only promoting β-cell proliferation, but by enhancing angiogenesis as well. We will be able to assess this possibility in a variety of methods. First, islets from our CTGF over-expression mice will be transplanted under the kidney capsule of wildtype donor mice. Doxycycline will be administered to induce CTGF over-

expression specifically in the transplanted islets. Additionally, wildtype donor islets could be transplanted with poly(lactic-co-glycolic acid) (PLGA) microspheres containing recombinant human CTGF (rhCTGF). PLGA microspheres offer a safe, biodegradable, and FDA approved method of therapeutic drug delivery (347, 348). The survival, proliferative state, and vascularization of the islet will be assessed and compared to controls. It would be of note to determine whether immune system involvement is required for any potential proliferative or angiogenic effects of CTGF in transplantation. Should CTGF promote enhanced murine islet graft survival, we would also assess translates to similar results with human islets. For this xenograft study, immunodeficient NOD-SCID mice would be used as the recipient mice. Human islets would once again be co-transplanted with rhCTGF containing PLGA microspheres. Encouragingly, preliminary findings have shown that rhCTGF can promote human β-cell proliferation *ex vivo* (R.C. Pasek, unpublished). However, this encouraging observation may be confounded due to several factors; a high glucose (11mM) culture condition which can stimulate β-cell proliferation (207), undetermined injury to islets during harvesting, and the potential favorable genetic makeup of the donor towards β-cell permissiveness to proliferative factors. Additionally, in my studies I observed the requirement of macrophages for CTGF-mediated β-cell proliferation. In cultured human islets, the immune cell population is extremely limited. Thus, it may be that in order for CTGF to promote β-cell mass expansion, as second “hit” is required. This secondary alteration to the islet micro-environment may be macrophages, high glucose, or some other unknown factor.

Through the studies presented in this thesis, we have demonstrated the novel role of CTGF in β-cell regeneration. However, we did not directly assess the therapeutic potential of CTGF in a diabetic setting. This deficiency could be addressed using a leptin receptor knockout mouse (*db/db*) of T2D. In this model β-cell mass declines due to glucolipotoxicity, which results in overt T2D at 6 weeks of age (349). CTGF over-expression would be induced in the β-cells either prior to or after disease onset in order to assess the prophylactic or therapeutic effects of CTGF, respectively. We would assess if CTGF delays, or prevents, T2D onset by assessing changes in body mass or blood glucose levels. Increased adiposity and elevated *ad lib* blood glucose levels are hallmarks of T2D in the *db/db* model (349). We predict that CTGF may promote improved blood glucose control by promotion of β-cell mass expansion, via β-cell proliferation, or survival in the pre-diabetic state. Alternatively, this question could be addressed through a HFD feeding model of T2D, where we have previously shown that CTGF is upregulated in β-cells. It would important in both of these models to determine the role of the immune system in mediating the effects of CTGF. Both of these models are characterized by increased inflammation (350), thus it may be that infiltrating and/or activated resident macrophages would promote β-cell permissiveness to CTGF in a T2D model as we suggest for the regeneration model.

Overall, this work has demonstrated the novel role for CTGF in promoting β-cell mass regeneration. The importance of CTGF as a potential diabetic therapeutic is highlighted by the fact that it is able to drive β-cell proliferation in an environment with no overt physiological impetus. Here we have shown that CTGF promotes β-cell regeneration by enhancing the number of β-cells undergoing proliferation and by also

reducing the length of time before the cell can re-enter the cell cycle. Our results also underscore the importance of the islet microenvironment in allowing permissiveness to proliferative stimuli. Neither CTGF induction, nor β-cell destruction alone allow for β-cell mass regeneration, thus β-cell ablation and CTGF induction are each necessary, but not sufficient, to induce in vivo β-cell proliferation in this model. β-cell destruction elicits an increase in macrophages, and their presence is required for CTGF to promote β-cell proliferation. Thus, adult β-cell cell cycle re-entry appears dependent upon multiple stimuli to successfully initiate. As all forms of diabetes are characterized by insufficient functional β-cell mass, effective strategies to promote replication and subsequent mass regeneration, of pre-existing β-cells are vital.

# APPENDIX

## Generation of Pdx1$^{PB}$-rtTA transgenic mouse

The goal of these studies was to generate transgenic mice in which the reverse tetracycline transactivator (rtTA) expression was driven specifically to pancreatic endocrine cells beginning at e11.5 using a 1 kb PstI to BstEII (PB) genomic fragment from the 5' region of *Pdx1* (76, 351). The transgenic line, termed Pdx1$^{PB}$-rtTA, was generated using the Pdx1$^{PB}$ expression vector which contains the 1 kb PB genomic fragment upstream of the hsp68 minimal promoter and intron 2 and polyadenylation sequences from the rabbit β globin gene. The rtTA cDNA was obtained from the pUHG 17-1 plasmid (352). The 1.1 kb rtTA fragment was excised by EcoR1 and BamH1 digestion of the plasmid, and gel purified using a Gel Extraction Kit (Qiagen), according to manufacturer's instructions. Concentration and purity of the fragment was determined by ND-1000 Spectrophotometer (NanoDrop, (VANTAGE)). The rtTA fragment was cloned into the BamH1 site following the hsp68 minimal promoter of the Pdx1$^{PB}$ vector, following BamH1 digestion and dephosphorylation of the Pdx1$^{PB}$ vector by Calf Intestinal Phosphatase (NEB). Pdx1$^{PB}$ and rtTA were incubated overnight at 15ºC with T3 DNA ligase according to manufacturer's instructions (NEB). Ligation products were transformed in DH5α bacteria by standard technique, allowed to recover for 1 hour in a shaking incubator at 37$^0$C, and 100 µL were plated on Luria broth (LB) plates. Resultant cultures were purified using a Miniprep Kit (Qiagen) according to manufacturer's instructions and eluted in 30 µL elution buffer. DNA (3ng/µL) was injected into pronuclei of one-cell embryos from B6D2 females, and embryos transplanted into

pseudopregnant ICR females (Transgenic/ESC Shared Resource facility at Vanderbilt University). Three founders were generated and used to establish transgenic lines. Pdx1$^{PB}$-rtTA transgenic mice were identified by PCR genotyping on DNA isolated from ear punches using the RIP-rtTA genotyping primers described in Chapter II. Pdx1$^{PB}$-rtTA transgenic mice were maintained on an inbred hybrid (B6D2) background. All mice were kept on a 12 hour light/dark cycle, fed Mouse diet 5015 (LabDiet), and given water ad lib.

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