# THE ROLE OF CONNECTIVE TISSUE GROWTH FACTOR IN ISLET MORPHOGENESIS AND β CELL PROLIFERATION

By

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# CHAPTER I

# GENERAL INTRODUCTION

## Significance

The mature pancreas is comprised of two functionally distinct tissue types. The exocrine pancreas, consisting of acinar cells that secrete digestive enzymes into a complex ductal network, makes up approximately 98 percent of the adult organ. Interspersed within the acinar parenchyma are the islets of Langerhans containing hormone-producing endocrine cells, which are responsible for maintaining glucose homeostasis within the organism. Each islet is a microorgan containing five different hormone-producing cell types including β (insulin), α (glucagon), δ (somatostatin), ε (ghrelin), and PP (pancreatic polypeptide) cells. Insulin is the main hormone produced by the islet which stimulates the uptake of glucose into peripheral tissues such as the liver and muscle. Diabetes mellitus results from insulin insufficiency caused by either a selective autoimmune destruction of the β cells (type 1) or a failure of β cells to compensate for peripheral insulin resistance, usually associated with obesity (type 2). There has been some therapeutic success with transplanted cadaveric islets into patients with type 1 diabetes; several patients achieved insulin independence for a limited period of time. However, insufficient amounts of donor tissue and ongoing autoimmunity have prevented islet transplantation from becoming a widely available treatment option. Consequently, researchers are currently trying to develop ways to generate replacement sources of β cells by expanding existing β cells *in vivo* or generating them de novo *in vitro* (Figure 1-1). One current avenue of study involves the

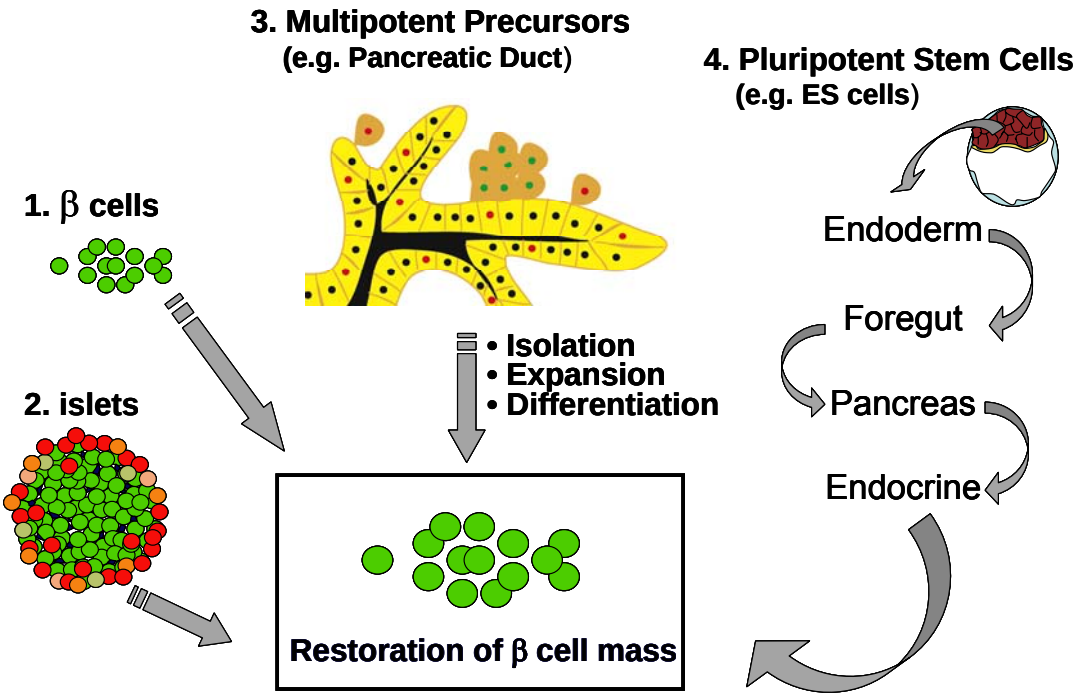


**Figure 1-1**. Possible sources of cells for restoration of β cell mass include: (1) the isolation and expansion of preexisting β cells, (2) the expansion of islets, (3) the isolation, expansion, and differentiation of multipotent pancreatic precursors into β cells, and (4) differentiation of pluripotent stem cells down the normal path of development into β cells.

directed differentiation of human embryonic stem (ES) cells or induced pluripotent stem (iPS) cells down the normal path of pancreas development into glucose-responsive β cells. A thorough understanding of how pancreas organogenesis occurs in the embryo should provide insight into how β cells can be generated more efficiently and effectively using these differentiation protocols. 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 and islet morphogenesis

## Pancreas development

Pancreas organogenesis can be thought of as a sequential continuum of morphological and molecular events including: regionalization of the endoderm and early bud formation, bud outgrowth and cell specification, endocrine lineage allocation, endocrine proliferation and maturation, and islet morphogenesis (Figure 1-2).

### Regionalization of the endoderm and early bud formation

Pancreas development begins at embryonic day (e) 9.5 in the mouse (gestational day 25 in humans) as two evaginations from the posterior foregut that ultimately fuse to form the mature organ. The pancreas forms from a region of the foregut endoderm located posterior to the developing liver and anterior to the duodenum. Overlapping expression domains of transcription factors initially broadly pattern the endoderm along the anterior to posterior axis (Figure 1-3). For example, Sox2 is expressed in the anterior domain of endoderm that will give rise to the esophagus and stomach [1]; Pdx-1 expression is found in the antral stomach, presumptive pancreas, common bile duct, and

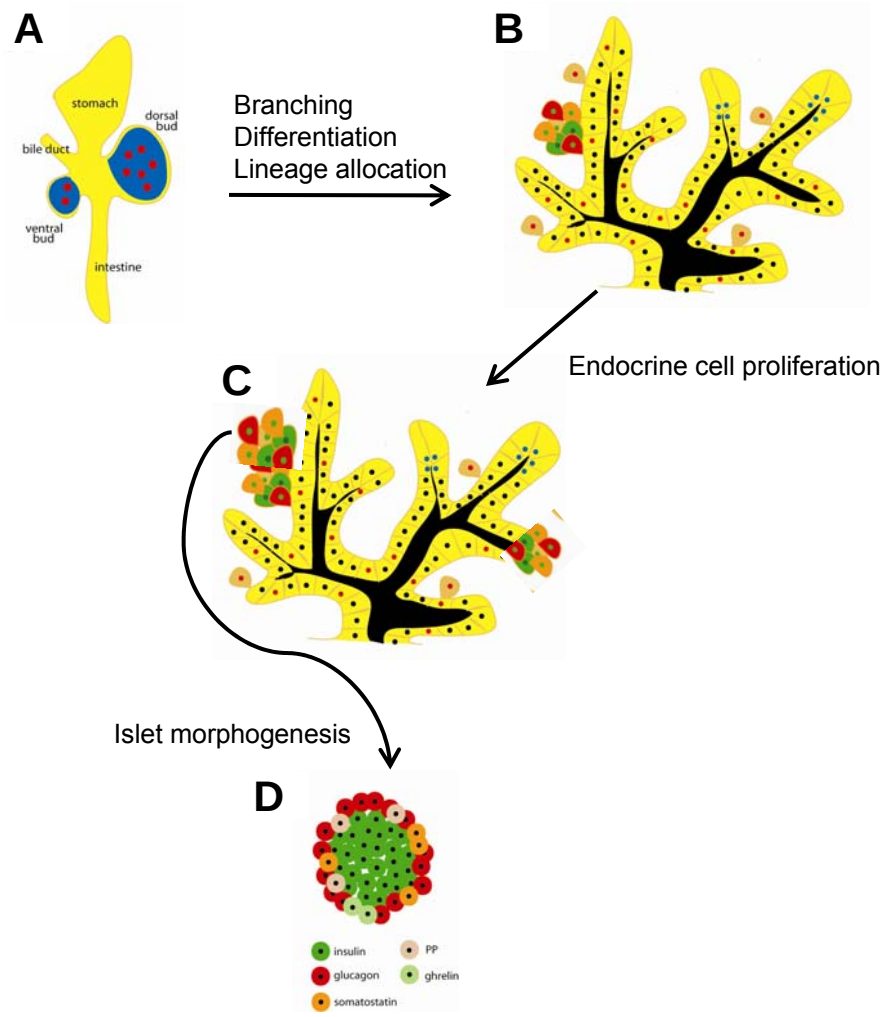


**Figure 1-2**. A schematic of some the morphological and molecular events required for pancreas development. (A) The endoderm becomes regionalized and the dorsal and ventral pancreatic buds evaginate at e9.5 (red circles are pancreatic progenitors). (B) The pancreas undergoes growth and branching morphogenesis to form a ductal epithelium. Endocrine progenitors within the ductal epithelium delaminate and differentiate into the various hormone-expressing cell types. (C) At late gestation endocrine cells proliferate and (D) organize into islets within the acinar tissue. In the mouse, islets have a characteristic architecture with the β cells in the center and the other cell types at the periphery.

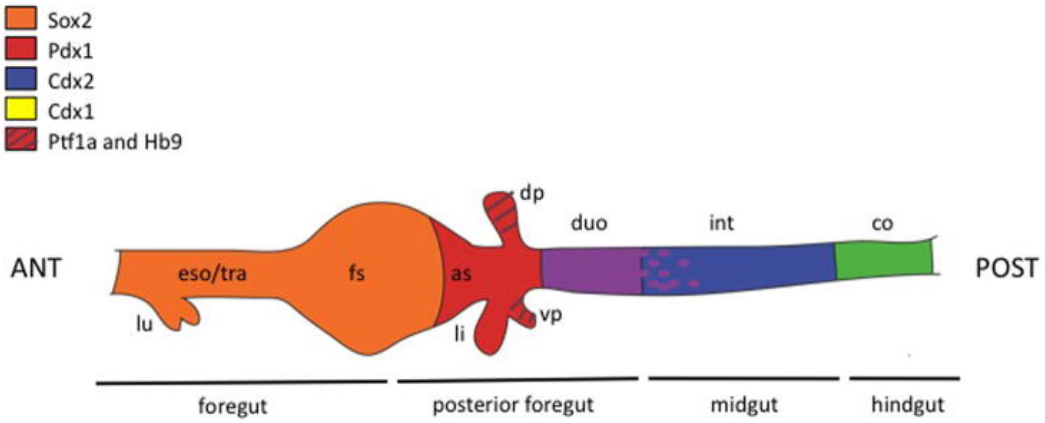


**Figure 1-3.** Anterior/posterior patterning of the digestive tract. Expression of various transcription factors is regionalized along the anterior-posterior axis of the developing gut tube endoderm. Regions of common expression are shown in blended colors: purple, overlapping expression of Pdx1 and Cdx2; green, overlapping expression of Cdx1 and Cdx2 in the hindgut. The posterior boundary of Pdx1 expression is diffuse (purple dots). Abbreviations: ANT, anterior; as, antral stomach; co, colon; dp, dorsal pancreas; duo, duodenum; eso, esophagus; fs, forestomach; int, intestine; li, liver; lu, lung; POST, posterior; tra, trachea; vp, ventral pancreas. From Pancreas Cell Fate. M. Guney and M. Gannon. *Birth Defects Research C Embryo Today*, 87(3), Copyright © [2009].

rostral duodenum [2]; and Cdx2 is expressed in the entire post gastric epithelium in the regions which will form intestine [3].

Initially, the presumptive pancreatic domain is marked by overlapping expression of Pdx-1, Ptf1a, and homeobox gene 9 (Hb9) [2,4,5,6]. In the absence of Hb9, the dorsal pancreatic bud fails to develop although the adjacent endoderm and surrounding mesenchyme are patterned normally, indicating Hb9 is absolutely required for epithelial bud specification and outgrowth [5,6]. At e9.5, a subset of cells within the Pdx-1 expression domain begins to express the bHLH transcription factor, Ptf1a/p48 [7]. It is the Ptf1a/Pdx-1 double-positive cells that will give rise to the pancreatic anlagen, while Pdx-1+/Ptf1a- cells contribute instead to the antral stomach and rostral duodenum (Figure 1-3). Ptf1a is the tissue-specific component of a heterotrimeric transcription factor complex known as PTF1 that also includes the proteins p65 and p75 [8,9]. Although it was originally thought to be required solely for acinar cell development, Ptf1a is now known to be indispensable for specification of the pancreas as well as for the development of the endocrine and exocrine cell types [4,10]. Lineage tracing analysis showed that Ptf1a-expressing progenitors give rise to all of the cell types in the pancreas [4]. Mice lacking *Ptf1a* are apancreatic except for a severely hypoplastic dorsal bud [4,10]. Interestingly, in the absence of Ptf1a, cells that would normally become ventral pancreas survive and proliferate, are found within the duodenum, and express intestinal cell markers, suggesting a role for Ptf1a in directing bipotential endodermal progenitor cells towards the pancreatic fate and away from the intestinal lineage [4]. The fact that *Ptf1a* mutant cells adopt an intestinal fate rather than undergo apoptosis indicates the highly plastic nature of the endoderm during gut formation. Along these lines, studies have shown that ectopic expression of Ptf1a in the Pdx-1 domain can convert the duodenum and stomach to pancreas [11,12].

Suppression of Wnt signaling in the anterior endoderm is required for both liver and pancreas development [13]. As development proceeds, the mesodermal tissues surrounding the posterior foregut provide secreted signals that promote either liver or pancreas development from a common region of endoderm. In the ventral foregut, bone morphogenetic proteins (BMP2 in zebrafish and chick, and BMP4 in mouse) secreted by the septum transversum mesenchyme and FGF1 and 2 produced by the cardiac mesoderm promote liver development while concomitantly suppressing the pancreatic differentiation program [14,15,16,17,18]. When cultured in the absence of cardiac mesoderm, ventral foregut endoderm normally fated to become liver instead expresses Pdx-1, suggesting that in vivo, FGFs direct bipotent cells away from the "default" pancreatic identity towards the liver fate [14]. Recent work in *Xenopus* has identified the co-repressor, TGF-β induced factor 2 (TGIF2), as a factor that acts to limit BMP signaling within the endoderm and promote the expression of pro-pancreas genes [19].

Retinoic acid (RA) produced by the foregut mesoderm may be involved in setting up the anterior and posterior boundaries of the posterior foregut within the endoderm. Treatment of both zebrafish and *Xenopus* embryos with exogenous RA expands the pancreatic field anteriorly, although a conserved role for RA in endoderm patterning has not yet been shown in mammals [20]. The ability of RA to act as a posteriorizing agent has also been shown in organs derived from the other germ layers including the chick heart (mesoderm) and neural tube (ectoderm) [21,22].

Dorsally, signals from the notochord also induce pancreas formation. From the time it is formed, the notochord is in contact with the prepancreatic endoderm until e8 in the mouse at which time the dorsal aortae fuse between the notochord and endoderm. In experiments using chick embryos, removal of the notochord at a time when it normally contacts the presumptive pancreatic endoderm results in a reduction in epithelial branching as well as a loss of expression of pancreas/endocrine transcription factors

such as Pdx-1, Islet 1 (Isl1), and Pax6, as well as insulin [23,24]. Interestingly, while recombination of the notochord with prepancreatic endoderm induced pancreatic gene expression, placing the notochord in contact with endoderm isolated from a more posterior location failed to induce pancreas gene expression, suggesting that the endoderm is already patterned at this stage and only a particular domain is competent to form pancreas in response to notochord signals. The notochord promotes pancreas formation by repressing the expression of the secreted morphogen, sonic hedgehog (Shh), in the underlying endoderm [24]. Although Shh is highly expressed in the endoderm rostral and caudal to the developing pancreas, it is markedly absent from the presumptive pancreas epithelium. Studies from the Edlund lab suggest that exclusion of Shh from the pancreatic endoderm is required to inhibit intestinal fates. Ectopic expression of Shh in the Pdx-1 expression domain is incompatible with normal pancreas development; pancreatic mesoderm in Shh over-expressing transgenic mice expressed markers of intestinal mesoderm including smooth muscle $\alpha$-actin [25]. FGF2 and Activin-$\beta$ are likely to be endogenous signals secreted from the notochord which mediate its suppressive effects on Shh in the pancreatic region [24]. In contrast, the ventral pancreas develops in the absence of any contact with the notochord. This is just one example of the divergent developmental programs resulting in dorsal versus ventral pancreatic bud formation.

At e9 the two dorsal aortae fuse, disrupting the contact of the notochord with the prepancreatic endoderm. Signals from blood vessel endothelial cells are also important for pancreas development *in vitro* and *in vivo*, although the relevant endothelial-derived molecule(s) await identification. Co-culture experiments demonstrated that signals from the endothelium are required for maintenance of Pdx-1 expression, dorsal bud outgrowth, and initiation of *Ptf1a* and *insulin* gene expression [26,27]. Conversely,

transgenic mice over-expressing vascular endothelial growth factor A (VEGFA) under control of the *Pdx-1* promoter had an increase in pancreatic blood vessels with a concomitant increase in pancreatic islets and ectopic insulin positive cells within the posterior stomach [26]. Since Pdx-1 is also expressed in this region of the stomach early in development, these data suggest that endothelial cells are able to induce β cell fate in competent regions of endoderm. Consistent with other data suggesting the ventral pancreas develops quite differently from the dorsal pancreas, ventral bud evagination was not affected in the absence of endothelium.

In zebrafish, in addition to its role in anterior/posterior patterning, RA signals to the underlying endoderm to induce pancreas differentiation [28]. Interfering with RA signaling using an RA-receptor antagonist or antisense morpholinos oligonucleotides directed against the RA synthesis enzyme RALDH2, leads to a loss of Pdx-1 expression, the early endocrine marker Isl1, and ultimately insulin [28,29]. Studies in quail and *Xenopus* provide support for these data by showing that blocking RA receptor signaling results in a failure of dorsal, but not ventral, pancreas bud formation [20,30]. An important role for RA in pancreas bud formation is also conserved in mammals. Mice lacking *Raldh2* display dorsal pancreas agenesis [31,32], while mice expressing a dominant-negative form of the retinoic acid receptor α driven by the *Pdx-1* promoter show loss of both dorsal and ventral pancreatic buds [33], suggesting that another RA synthesizing enzyme (perhaps Raldh1) acts within the ventral pancreas. The expression patterns of other factors involved in early steps of pancreas development such as Hb9 and Shh, however, are normal suggesting that RA acts prior to the initiation of Pdx-1 expression but after pancreas specification [32].

**Pancreas outgrowth and cell specification**

The pancreatic buds undergo elongation and branching within the pancreatic mesenchyme to yield a highly branched ductal network. During this process, the pool of pancreatic progenitors is marked by Pdx-1 expression and lineage tracing indicates that all of the epithelial lineages in the pancreas come from a Pdx-1-expressing cell [2,34]. However, it is unclear whether any or all of these progenitor cells are truly multipotent, with the capacity to differentiate into any or all of the different pancreatic cell types, or whether these progenitors are already specified to a particular pancreatic cell lineage(s) at an early stage within the undifferentiated epithelium. Pdx-1 is critical for pancreas outgrowth; loss of Pdx-1 expression leads to pancreas agenesis in mice and humans [2,35]. Interestingly, however, Pdx-1 is not required for the specification of pancreatic endoderm or for the formation of early endocrine cells, since *Pdx-1* null embryos have a minimally branched dorsal ductule and low numbers of insulin and glucagon-expressing cells [2,36].

FGFs produced by the mesenchyme surrounding the pancreatic bud are also important for outgrowth of the epithelium. Global inactivation of FGF10 in mice leads to pancreatic hypoplasia due to decreased proliferation of Pdx-1-positive progenitor cells [37]. Conversely, over-expression of FGF10 throughout the pancreatic epithelium beginning early in development significantly increased overall pancreas size, enhanced proliferation, and impaired endocrine differentiation [38,39]. *In vitro* culture experiments using FGF ligands 1,7, and 10 demonstrated similar effects on epithelial proliferation and progenitor expansion, further supporting the idea that FGF stimulates growth of the epithelium and inhibits differentiation [40].

Wnts are another family of extrinsic factors which play a role in pancreas growth and differentiation. Wnt ligands bind to Frizzled (Frz) receptors and LRP5/6 co-receptors on the cell surface [41]. Activation of Wnt signaling leads to inhibition of the

Axin/APC/GSK3-β complex, which in the absence of Wnt, phosphorylates and targets β-catenin for proteosomal degradation. Stabilization of β-catenin causes it to localize to the nucleus where it interacts with TCF/LEF transcription factors and activates transcription. Multiple Wnt ligands, Frizzleds, Wnt inhibitors, and LRP5/6 are expressed in the pancreas during development and several studies have examined the effects of modulating Wnt activity on pancreas development [42]. Data indicate that Wnt signaling may have multiple spatiotemporal-specific roles in the pancreas with one of them being to regulate epithelial progenitor proliferation [43]. For example, over-expressing a soluble dominant-negative form of Frz8 in the pancreas results in a reduction in pancreas size which was shown to be due to a reduction in epithelial cell proliferation during mid-gestation [44].

During branching morphogenesis, the pancreatic epithelium repeatedly evaginates to form new branches with a lumen that remains contiguous with the main pancreatic duct. Molecular marker analyses and lineage tracing studies suggest that the branching pancreatic epithelium consists of molecularly and functionally distinct microdomains termed “tip cells” and “stalk cells” [45]. In other branching tissues such as the lung and the kidney, multipotent progenitors are physically separated within the developing branch from the more differentiated populations of cells [46,47]. Similarly, recent lineage tracing evidence in the pancreas suggests that much of branch growth and elongation occurs at the tips of the developing ductal tree, while cells left behind in the wake of the growing tip become incorporated into the “stalk” of the branch [45]. The tips of the branching epithelium are marked by expression of Carboxypeptidase A1 (Cpa1), Pdx-1, Ptf1a, and c-Myc. Lineage tracing of tip cells revealed that early in pancreas development progeny of these cells give rise to endocrine cells, ducts, and acinar cells, and can thus be considered multipotent progenitors (Figure 1-4). The first

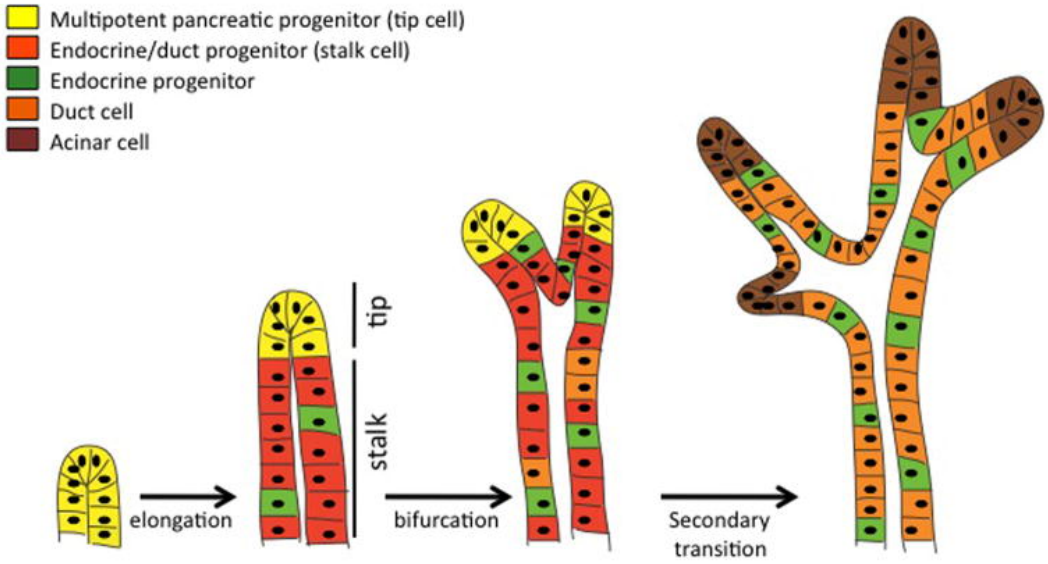


**Figure 1-4.** Location of pancreatic progenitors during branching morphogenesis. Multipotent pancreatic progenitors (yellow) are localized to tips of developing epithelial branches. As branches elongate at the tips, cells remaining in the stalks (red) lose the potential to differentiate as acinar cells. Cells that will give rise to definitive duct cells (orange) and endocrine progenitors (green) become specified in the stalks as development proceeds. Finally, during the secondary transition, cells at the tips begin to differentiate as acinar cells (brown). From Pancreas Cell Fate. M. Guney and M. Gannon. *Birth Defects Research C Embryo Today*, 87(3), Copyright © [2009].

cells to be deposited in the stalk or trunk region give rise to endocrine and ductal cells. Tip cell progenitors become restricted as development proceeds such that by e14.5 the majority differentiate into acinar cells. Thus, in addition to being spatially regulated, the decision between endocrine and exocrine differentiation is also temporally regulated with the endocrine progenitors being specified earlier and the exocrine cells later.

Within the trunk ductal epithelium, juxtacrine Notch-Delta signaling regulates the differentiation of endocrine progenitor cells [48,49]. It is hypothesized that this occurs in a manner similar to what occurs within equivalence groups during *Drosophila* neurogenesis [50]. Cells initially express low levels of both the Notch receptor and its ligand, Delta. Interactions between adjacent cells lead to stochastic up-regulation of either Notch or Delta. Stabilization of Notch signaling leads to activation of the target gene, Hes1, which represses expression of the pro-endocrine bHLH transcription factor, neurogenin 3 (Ngn3) [51]. Cells that fail to activate Ngn3 remain in an undifferentiated state while cells in which Ngn3 becomes activated initiate the endocrine differentiation program and delaminate from the ductal epithelium [52,53].

All endocrine cell types arise from a Ngn3-expressing progenitor and currently Ngn3 is the earliest known marker of an endocrine progenitor [52,53]. *In vivo* studies have also demonstrated an important role for Ngn3 in regulating the endocrine differentiation program within the pancreatic epithelium. Ngn3 expression is biphasic, correlating with the “waves” of endocrine cell differentiation [54]. The first wave of endocrine cells appear in the pancreas at approximately e10.5; however these cells do not express markers of mature endocrine cells and it is thought that they do not contribute to mature islets; the ultimate fate of these cells is still unclear [55,56,57,58,59]. At approximately e13–16 in the mouse, during a period known as the ‘‘secondary transition,’’ there is a dramatic increase in the number of endocrine cells budding from the ductal epithelium. These endocrine cells contribute to the mature islet.

The expression of Ngn3 begins around e9.5 and peaks during the secondary transition but by birth Ngn3 expression is nearly undetectable [52]. Consistent with the hypothesis that Ngn3 marks endocrine progenitors, $Ngn3^{high}$-positive cells do not co-express pancreatic hormones and are found within or adjacent to the ductal epithelium. Lineage tracing analysis has recently been used to show that Ngn3 cells are unipotent, that is, a Ngn3 positive cell will only give rise to one endocrine cell type at birth [60]. *Ngn3* null embryos lack all endocrine cell types and die two to three days after birth due to diabetes [52]. The role of Ngn3 in promoting endocrine differentiation is further evidenced by over-expressing Ngn3 throughout the pancreas using the *Pdx-1* promoter. *Pdx-1*-Ngn3 transgenic embryos have a hypoplastic pancreas, with a dramatic decrease in carboxypeptidase-positive acinar cells and an increase in differentiated (mainly glucagon-expressing) endocrine cells [48,61]. Ectopic expression of Ngn3 throughout the pancreatic epithelium thus leads to a loss of multipotent pancreatic progenitors and specifically favors the development of $\alpha$ cells with very few insulin-positive cells [48,61,62].

Expression of a proper level of Hepatic nuclear factor 6 (Hnf6/OC-1) is also required for endocrine development. HNF6 is a member of the ONECUT family of transcription factors and regulates genes involved in liver and pancreas development. Hnf6 expression co-localizes with Pdx-1 in the pancreatic epithelium at e10.5 but is not expressed in hormone-positive cells; however, expression is maintained throughout adulthood in the ducts and at low levels in exocrine tissue [63],[64],[65]. Hnf6 mutant mice have a hypoplastic pancreas, a dramatic decrease in Ngn3 expression, and a marked reduction in insulin and glucagon expression [65,66]. After birth, these mice have impaired glucose homeostasis. Consistent with these results, Hnf6 binds and activates the *Ngn3* and *Pdx-1* promoters [66,67]. Studies from our laboratory using a conditional Hnf6 allele demonstrate that irreversible commitment to the endocrine fate

requires a threshold of Hnf6-dependent Ngn3 expression. *Ngn3-Cre*-mediated inactivation of Hnf6 in putative endocrine progenitors results in a significant decrease in total endocrine area at birth [65]. Lineage tracing analyses indicate that in the absence of sustained Hnf6 expression, a subset of cells (14%) that had activated Ngn3 became diverted to the exocrine lineage and express markers of terminally differentiated acinar and ductal cells, while in normal development, ~1% of cells that activate the *Ngn3* promoter become incorporated into exocrine tissue [68]. Thus, *Ngn3* gene activation does not necessarily commit a cell to the endocrine fate. These data also suggest that continued Hnf6 activity at the *Ngn3* promoter is required in order for Ngn3 protein to reach a required level within the cell and commit multipotent pancreatic progenitors to an endocrine fate (Figure 1-5). Consistent with this idea, mice carrying a hypomorphic allele of Ngn3 have a significant reduction in overall endocrine cell number and an increase in acinar and ductal cells [69]. It is likely that a high level of Ngn3 is required to activate downstream genes involved in endocrine cell differentiation and maintenance such as the transcription factors Neurod1, Myt1, and Pax4.

Ptf1a also regulates the exocrine vs. endocrine cell fate decision. However, in contrast to Ngn3, Ptf1a expression directs progenitor cells away from the endocrine lineage and promotes acinar cell fate. Mice hemizygous for a hypomorphic allele of Ptf1a and a null allele display decreased pancreatic size and impaired acinar cell differentiation [70]. While Pdx-1 is normally only found at low levels in acinar tissue postnatally, persistent exocrine Pdx-1 expression is detected in Ptf1a hypomorphic pancreata, suggesting these cells may be activating the β cell differentiation program. Ptf1a gene dosage may play a role in determining whether pancreatic progenitors adopt an exocrine or endocrine fate in zebrafish as well [71]. In fish carrying a hypomorphic Ptf1a allele, cells normally fated to become exocrine cells co-express the endocrine cell marker, Isl1. These data suggest that high levels of Ptf1a are necessary to fully commit

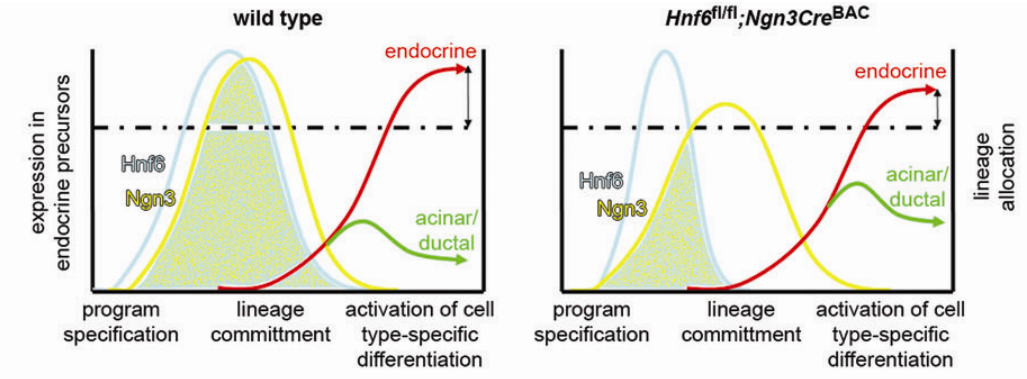


**Figure 1-5**. A threshold of Ngn3 is required to generate fully committed endocrine cells. Inactivation of Hnf6 subsequent to Ngn3 gene activation (*Hnf6*$^{fl/fl}$;*Ngn3Cre*$^{BAC}$) results in a reduced number of differentiated endocrine cells. In wild type mice (left graph), Hnf6 expression (light blue) precedes Ngn3 expression (yellow). Hnf6 expression must be maintained for a certain period of time to allow Ngn3 expression to exceed a critical threshold (dotted line), allowing for adequate endocrine differentiation (red). When Hnf6 expression is prematurely extinguished (right graph), Ngn3 levels reach this threshold in fewer cells, resulting in reduced endocrine mass. From Pancreas Cell Fate. M. Guney and M. Gannon. *Birth Defects Research C Embryo Today*, 87(3), Copyright © [2009].

pancreatic progenitor cells towards the exocrine lineage while concomitantly suppressing the endocrine lineage. After e13.5, Ptf1a becomes enriched in acinar cells and has been shown to bind the promoters of exocrine genes, such as elastase and trypsin [8,72]. Accordingly, Ptf1a is absolutely required for exocrine development and no acinar tissue is found in Ptf1a null mice. The switch between the early and late roles of Ptf1a in pancreas development is mediated by its interaction with two different isoforms of the vertebrate suppressor of hairless protein, RBPJ [73]. The Notch-dependent, RBPJκ form of the protein is found in the PTF1 complex early in pancreas development and is swapped for the Notch-independent, RBPJL form at the onset of acinar cell development [73]. It is exclusively the RBPJL form that is found bound to the promoters of acinar-specific genes [73,74].

A recent study from the Sander lab demonstrates that Ptf1a activity is antagonized by the transcriptional repressors Nkx6.1 and Nkx6.2. Nkx6 factors are expressed in multipotent pancreatic progenitors and Nkx6.1/Nkx6.2 compound null mutants have a reduction in both $\alpha$ and $\beta$ cell numbers [75]. This reduction in endocrine cells is accompanied by an increase Ptf1a expression and in the number of acinar cells [76]. Furthermore, over-expression of Nkx6.1 throughout the Pdx-1 domain promoted endocrine differentiation and inhibited acinar differentiation; this was shown to be due to direct binding of Nkx6.1 to the *Ptf1a* promoter. Conversely, expression of Ptf1a in Pdx-1 expressing cells inhibits expression of Nkx6.1 [76]. However, since Ptf1a is thought to be a transcriptional activator, the repression of Nkx6.1 by Ptf1a is likely not due to direct transcriptional inhibition.

Loss of the *prospero*-related transcription factor, Prox1 results in a decrease in secondary transition endocrine cells, with a concomitant increase in differentiated acinar cells [77]. Prox1 is expressed at higher levels in endocrine progenitors with lower levels of expression detected in differentiating exocrine cells [78]. Although it is unclear how

Prox1 acts to promote endocrine cell fate, Prox1 is required for normal branching morphogenesis and it is possible that Prox1-deficient embryos lack sufficient numbers of stalk cells required for endocrine progenitor specification, and thus more of the epithelium differentiates as acinar cells.

**Endocrine cell lineage allocation and differentiation**

*Transcription factors*

All endocrine cells are derived from Ngn3-expressing progenitors, but the mechanisms by which these endocrine progenitors are specified to the individual hormone-positive lineages are not well characterized. Genetic analyses have revealed that α and β cells develop from independent lineages, while β and PP cells may arise from a common endocrine precursor [58]. Hormone-expressing cells differentiate in the pancreas in a cell type-specific pattern with α cells appearing earliest followed by β, δ, and PP cells. The sequential pattern of hormone expression suggests that endocrine differentiation is temporally regulated and that competence of the Ngn3-positive progenitors changes as development proceeds. To test this hypothesis, the Grapin-Botton laboratory used an “add back” strategy, expressing a tamoxifen-inducible Ngn3-ER fusion protein throughout the pancreatic epithelium using the *Pdx-1* promoter at different developmental time points in the *Ngn3* null background (Figure 1-6). These studies identified specific windows of competence for the differentiation of the individual endocrine cell types [79]. The pancreatic epithelium becomes competent to form glucagon-positive cells at the earliest stages of pancreas development, consistent with previous findings that over-expression of Ngn3 using the *Pdx-1* promoter induces the differentiation of mostly α cells [48,62,79]. Competency to form insulin-producing cells appears to occur between e10.5 and 14.5, with the greatest number of β cells formed

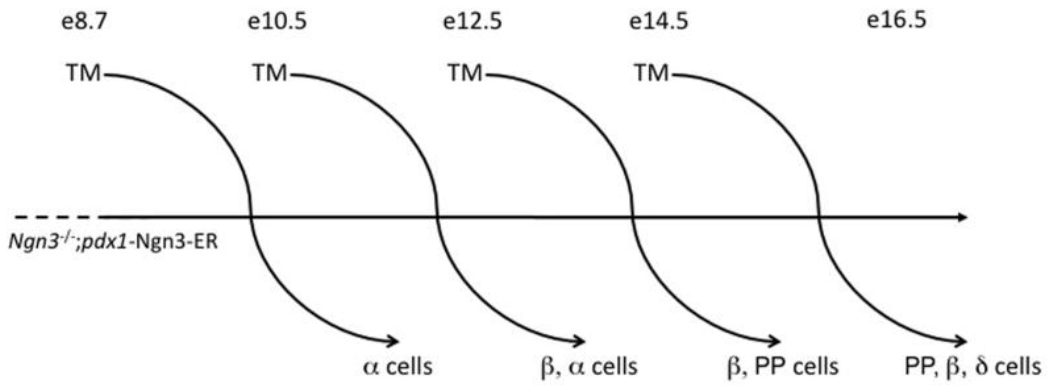


**Figure 1-6.** Temporally-dependent specification of Ngn3-expressing endocrine progenitors. Using the *Pdx1* promoter to drive expression of a tamoxifen (TM)-inducible Ngn3-estrogen receptor (ER) fusion protein, *Ngn3* expression was restored to *Ngn3* null mutant embryos at different developmental time points with the addition of TM. Examination of endocrine differentiation two days after a single TM injection revealed that endocrine progenitors preferentially differentiate as a particular hormone-producing cell depending on when during development they were generated. α cells are formed from the earliest Ngn3-producing cells, while β, PP, and δ cells subsequently form, in that order. From Pancreas Cell Fate. M. Guney and M. Gannon. *Birth Defects Research C Embryo Today*, 87(3), Copyright © [2009].

when Ngn3 is induced at e12.5. Later in development, the epithelium loses the ability to differentiate into glucagon-positive cells and acquires the competence to form PP and δ cells. Interestingly, these shifts in developmental competence appear to be mediated by intrinsic changes within epithelium rather than in the character of the surrounding mesenchyme [79].

The formation of β and α cells is thought to be regulated by the opposing actions of the transcription factors Pax4 and Arx. Pax4 is a paired homeodomain factor and its mRNA can be detected within cells in the pancreas beginning at e9.5 [80]. Its expression is restricted to first and second wave insulin cells, but becomes down-regulated soon after birth and is not detected in adult islets [81]. Pancreata from *Pax4* null embryos express Pdx-1 and Hb9 and contain first wave insulin-producing cells, but lack mature β cells indicating that Pax4 is required during the secondary transition for β cell differentiation [80,82]. Additionally, *Pax4* null mutant mice have a decrease in δ cells and an increase in the numbers of glucagon- and ghrelin-expressing cells [80,83]. Since no changes in endocrine cell proliferation or apoptosis were observed in *Pax4* mutants, it is likely that a common progenitor of both β and δ cells is directed toward alternate endocrine lineages in the absence of Pax4 [82]. Many of the glucagon-expressing cells in *Pax4* null pancreata co-expressed ghrelin [83,84]. Small numbers of glucagon/ghrelin co-expressing cells are normally found in wild type embryos; however, the number of these cells is significantly increased in *Pax4* mutants.

Pax4 is thought mainly to function as a transcriptional repressor. It directly binds and represses both the *glucagon* and *ghrelin* promoters, thus providing a mechanism for the increased expression of these two hormones observed in *Pax4* mutants [85,86,87]. In addition, Pax4 inhibits the expression of Arx, a transcription factor that promotes α cell differentiation. Although Pax4 is 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 pluripotent endocrine progenitors [87]. Furthermore, ectopic expression of Pax4 in pancreatic progenitors or in α cells induces their respecification towards a β cell fate [88]. Glucagon deficiency in Pax4-expressing mice leads to increased α cell neogenesis; however once α cells differentiate they are converted to β cells, resulting in oversized islets [88].

In contrast to the role of Pax4, Arx expression within the endocrine progenitor population promotes the development of α cells. Arx acts downstream of Ngn3 and *Arx* null embryos show a complete loss of α cells with a concomitant increase in β cells and δ cells [86]. The ability of Arx to direct cells towards the α cell lineage is further evidenced by studies in which Arx was over-expressed under control of the *Pdx-1* promoter [89]. These mice have an increased number of α and PP cells at the expense of the β and δ lineages; the total number of endocrine cells was unchanged. Additionally, the authors used an inducible system to drive Arx expression in mature β cells and found that insulin-positive cells were converted to the α and PP lineages [89]. *Arx* mutants have an increase in Pax4 expression, and Arx expression is up-regulated in *Pax4* mutants, indicating that Arx and Pax mutually inhibit each other's expression [86,90]. Indeed, binding sites for Arx were found in the *Pax4* promoter and vice versa [90]. However, Arx is not is not sufficient to repress *Pax4* within the α cell linage since *Pax4* levels are not reduced in transgenic mice over-expressing Arx. These results underscore the importance of the balance of Pax4 and Arx in establishing the β and α cell lineages. Inactivation of both *Pax4* and *Arx* leads to a loss of both β and α cell lineages along with a dramatic increase in somatostatin-producing cells, suggesting that glucagon-producing cells may normally inhibit the development of δ cells.

Pax6, another paired homeodomain transcription factor, is expressed at e9.5 and e10.5 in a subset of cells in the pancreatic epithelium, and is later expressed in cells committed to the endocrine lineage [49,91,92]. Although Pax6 is expressed in both insulin-positive and glucagon-positive cells, it only appears to be essential only for the formation of α cells. *Pax6* mutant mice have a dramatic loss of glucagon-expressing cells and a lesser reduction in the other islet cell types [92,93]. Thus, Pax6 may be important not only for allocation to the α cell lineage, but for expansion of the endocrine population as a whole [84,94]. In *Pax6* mutants there is an increase in ghrelin-expressing cells without an increase in proliferation of this cell population, suggesting that a reduction in Pax6 levels may direct endocrine progenitors towards the ε cell fate [84].

The NK homeodomain factor, Nkx2.2, regulates β cell differentiation in a pathway parallel to Pax4 [82]. Nkx2.2 is expressed throughout the whole pancreatic bud at early developmental stages, as well as in Ngn3-expressing endocrine progenitors [61,95]. Although Nkx2.2 expression is detected in all hormone-positive cells except δ cells during late gestation, it is only essential for β cell differentiation. *Nkx2.2* null embryos completely lack β cells but have reduced numbers of α and PP cells [95]. The function of *Nkx2.2* appears to be conserved; morpholino knockdown in zebrafish leads to a similar phenotype as seen in mice [96]. The fact that the expression of early endocrine markers such as Isl1 and synaptophysin were normal suggests that cells lacking *Nkx2.2* are specified correctly as endocrine cells. Similar to embryos lacking *Pax4*, *Nkx2.2* mutants also have an increase in ghrelin-expressing cells [83]. Using an Nkx2.2 repressor-fusion construct, the Sussel lab demonstrated that the repressor function of Nkx2.2 can partially rescue the defects in β and α cell development as well as the increase in ghrelin expression found in null mutant mice [97]. Thus, Nkx2.2 acts as a repressor during

endocrine specification however its activator function appears to be required for later steps of β cell maturation. The rescued β cells in these transgenic mice did not express MafA (a marker of mature β cells and a transactivator of the insulin promoter) and, consistent with these results, Nkx2.2 was found in other studies to bind and activate the MafA promoter [97,98].

MafB is a member of the large Maf family of basic leucine zipper transcription factors that also includes MafA. MafB is expressed in some Ngn3-positive cells, as well as in first and second wave insulin- and glucagon-expressing cells. In the adult pancreas, MafB is only detected in α cells [99]. MafB is not required for endocrine specification; embryos lacking *MafB* still express markers of the endocrine lineage such as Isl1 and Pax6. However, first wave endocrine cells are absent in *MafB* mutant pancreata and MafB is required for the differentiation of second wave α and β cells [100]. *MafB* mutant embryos have a 50% decrease in insulin- and glucagon-expressing cells compared to wild type littermates [100]. Consistent with the reduction of α and β cells, MafB was shown to bind to the *insulin*, *glucagon* and *MafA* promoters [100].

Pdx-1 expression becomes elevated in β cells at late gestation during islet formation. The *Pdx-1* promoter contains a conserved *cis*-regulatory region, termed “Areas I-II-III”. Deletion of this region from the endogenous *Pdx-1* locus generates a hypomorphic allele [101]. When placed in *trans* to a *Pdx-1* null allele, the hypomorphic allele is incapable of promoting normal pancreas development, with defects similar to those observed in *Pdx-1* null mice. Mice heterozygous for the hypomorphic allele displayed altered islet architecture, an increase in α and PP cells, and impaired glucose tolerance. The reduction of β cells found in these mice suggests that, similar to Ngn3, a threshold of Pdx-1 is required to activate the full β cell differentiation program; reduced Pdx-1 levels may favor the differentiation of α and PP cells.

*Signaling molecules*

The TGF-β superfamily of signaling molecules includes TGF-β, Activin, Nodal, and BMP ligands. These ligands bind to type I and type II serine-threonine kinase receptors, resulting in phosphorylation of downstream receptor Smad (R-Smad) proteins. TGF-β, Activins, and Nodal activate Smads 2 and 3, while ligands in the BMP family have been shown to activate Smads 1, 5, and 8 [102]. R-Smad phosphorylation leads to interaction with Smad 4, and subsequent nuclear localization where the complex interacts with co-factors to activate and repress transcription. There is conflicting *in vitro* evidence that TGF-β signaling regulates the ratio of exocrine and endocrine cell types during pancreas development [103,104]. The number of ligands and receptors make isolating specific effects of a given ligand difficult. Thus, despite several studies inactivating specific ligands or making use of dominant negative receptors, a clear requirement for this family of growth factors in endocrine lineage allocation has yet to be revealed *in vivo* [105,106,107].

Pancreas-wide over-expression of Smad7, which inhibits signaling mediated by Smads 2/3 and 1/5/8, causes a dramatic loss of β cells and an increase in the number of α cells without any changes in proliferation or apoptosis, thereby supporting a role for the TGF-β signaling pathway in lineage allocation [108]. It must be remembered, however, that Smad7 inhibits signaling by multiple ligands. Inactivation of the TGF-β ligand, growth differentiation factor 11 (GDF11), leads to an increase in undifferentiated Ngn3-expressing progenitors and a decrease in the α to β cell ratio, similar to what was seen in Smad7 transgenic mice [109]. GDF11 acts through Smad2/3 and, consistent with these data, the pancreatic defects in Smad2 heterozygous mice phenocopy those found in GDF11 mutants [109,110]. In contrast, an alternative study of GDF11 function in pancreas development found an increase in Ngn3-positive cells, but no alterations in

endocrine cell mass or ratios [111]. The reason for the conflicting results in these studies is not clear, but it may be due to differences in genetic background of the mouse strains used. Although GDF11 interacts with the Activin A type IIB (ActRIIB) receptor, inactivation of this receptor (in combination with heterozygous deletion of the Activin A type IIA receptor) indicates that Activin receptors function to regulate pancreas and islet size, but not endocrine lineage specification, since *ActR* mutant mice show pancreatic and islet hypoplasia, with no change in endocrine cell ratio [110,112,113].

**Endocrine cell proliferation**

Although there is little proliferation of endocrine cells during early and mid-gestation, the percentage of proliferating endocrine cells substantially increases in late gestation and in the early neonatal period. Adequate proliferation is clearly required for adequate numbers of endocrine cells in the adult; however, few factors have been identified to affect embryonic endocrine proliferation *in vivo*.

The eIF2$\alpha$ kinase PERK is required for both endocrine and exocrine function [114]. Mice with global PERK inactivation display diabetes due to insufficient $\beta$ cell mass and a progressive loss of exocrine tissue after four weeks of age. These mice also display skeletal abnormalities and together with the pancreas dysfunction this model bears similarity to human Wolcott-Rallison syndrome [114]. PERK localizes to the membrane of the endoplasmic reticulum (ER) and is hyperactivated in response to ER stress caused by increasing concentrations of unfolded proteins or inadequate levels of protein chaperones. Hyperactivated PERK phosphorylates eIF2$\alpha$, repressing global protein synthesis and ultimately leading to apoptosis. However, inactivation of PERK does not cause significant apoptosis in $\beta$ cells [115]. In fact, $\beta$ cell proliferation in adult $\beta$ cell-specific PERK knockout animals is not significantly altered; the decrease in $\beta$ cell

mass in the adult can be attributed to significant defects in embryonic and neonatal β cell proliferation. The decrease in embryonic proliferation may reveal a role for PERK in pathways other than the unfolded protein response. Gene expression profiling of postnatal day (P)2 control and knockout islets indicated that the failure of PERK neonatal β cells to expand may be due to decreased expression of genes important for progression of the G2 and M phases of the cell cycle such as *cyclinA* and *CDK1* [115]. PERK may also have roles in regulating β cell maturation or function. Global or pancreas-wide inactivation of PERK leads to decreased *MafA*, *Pdx-1*, and *insulin* expression as well as impaired glucose stimulated insulin secretion [115].

β cells may also provide signals regulating the numbers of other cell types, in particular the α cell population. Removal of *Pdx-1* specifically in embryonic β cells, for example, leads to a significant decrease in β cell proliferation and an increase in α cell proliferation at late gestation [116]. Mice lacking *Pdx-1* in β cells have elevated blood sugar at birth and progress to overt diabetes in the adult, possibly also due to the misregulation of genes that are required for mature β cell function [117,118]. The fact that β and α cell proliferation are reciprocally altered when *Pdx-1* is inactivated suggests that embryonic β cells normally provide a signal to the α cell population, inhibiting its expansion to generate proper numbers of each different cell type within the islets.

Glucagon-positive cells are found in the pancreas as early as e10.5, but no metabolic function is known for these cells at this time. Therefore, some have postulated that these early hormone-expressing cells provide a signal that regulates formation of other cell types within the developing pancreas. Global inactivation of the glucagon receptor or deletion of pro-hormone convertase-2 (PC2), the enzyme responsible for converting pro-glucagon to glucagon, leads to an increase in proliferation of the pro-glucagon-expressing cell population and in the percentage of glucagon- and

somatostatin-expressing cells in embryonic islets [119,120]. In addition to negatively regulating its own cell population, glucagon-positive cells may also send signals to regulate β cell proliferation since both models of glucagon inactivation show increased postnatal β cell proliferation [119,120]. Loss of glucagon expression leads to a decrease in first wave β cells and in mature β cell markers, suggesting glucagon also has non-cell autonomous roles in β cell differentiation [121].

**Islet morphogenesis**

At e17.5 newly differentiated endocrine cells lie close to the ductal epithelium but beginning at e18.5 and continuing after birth, the endocrine cells organize into islets within the acinar parenchyma [122]. In the mouse, islets have a characteristic architecture with the β cells in the core of the islet and the other cell types such as the α and δ cells forming a mantle around the β cells. This organization is thought to promote proper cell-cell communication and enhance islet function [123,124]. Although many of the cellular events required for islet morphogenesis have not been identified, they likely include changes in expression of cell adhesion molecules, modifications in extracellular matrix proteins, and paracrine and juxtacrine cell–cell communication. Real-time imaging of GFP-labeled β cells in pancreatic explants revealed that β cells migrate during islet development [125]. Similar to other migrating cell types, migrating β cells extend cytoplasmic filapodia, suggesting that migration is an active process on the part of the β cell. However, it is not clear whether islets are formed by individual cells which lose connections to neighboring cells, migrate to form clusters, and reestablish cell adhesions or whether groups of cells migrate in clusters while maintaining cell-cell contact. A recent study by the Hara lab suggests that during the neonatal period, increased β cell proliferation generates extensive cord-like structures of endocrine cells which undergo

fission to form single islets [126]. Although this report favors a model of islet morphogenesis that does not involve β cell migration, it does not rule out the possibility that active β cell migration is required for earlier steps or that islet morphogenesis is a heterogeneous process.

Cell migration requires dynamic regulation of cell-cell and cell-extracellular matrix (ECM) adhesion. Islets in mice lacking the epidermal growth factor receptor (EGFR) are elongated and closely opposed to the ductal epithelium [127]. EGFR may act, in part, by modulating activity of Rac1, a Rho-GTPase involved in migration and adhesion in other cell types. Expression of a dominant form of Rac1 in β cells also leads to failure of endocrine tissue to separate away from ducts due to increased E-cadherin at cell-cell contacts [128]. Furthermore, islets expressing dominant negative Rac1 fail to spread on ECM when treated with betacellulin, an EGFR ligand, suggesting that Rac1 may function downstream of EGFR [128]. Integrin signaling is also involved in modulating interactions between endocrine cells and ECM or ductal cells during islet morphogenesis. Inhibiting $\alpha_v$ integrins using cyclic RGD peptides in fetal pancreas explants blocks the emergence of endocrine cells from the ductal epithelium [129]. EGFR and integrin signaling both regulate the activity of matrix metalloproteinases (MMPs) and *in vitro* experiments using a chemical inhibitor indicate that MMP2 activity is required for proper budding of endocrine tissue [130]. These data suggest that ECM remodeling is required for proper islet morphogenesis. However, islet formation is not altered in a mouse model of combined MMP2 and 9 inactivation, making it likely that the relevant molecules which mediate ECM modeling during islet morphogenesis have not yet been identified [131].

While a reduction in adhesion is required for separation of endocrine tissue away from the ductal epithelium, cell-cell contacts are required for the formation of islet architecture and for cell sorting. Functional E-cadherin is necessary for endocrine cell

clustering—β cells expressing a dominant negative form remain dispersed throughout the pancreas as individual cells and small aggregates containing only a few cells [132]. Neural cell adhesion molecule (N-CAM), which is expressed in aggregating endocrine tissue, is not required for islet clustering, but rather is required for cell type segregation; loss of N-CAM causes α and β cells to be randomly distributed throughout the islet [133]. It has been hypothesized that the segregation of α and β cells may be due to differential expression of adhesion molecules making β cells more cohesive than α cells [133,134]. These differences in cohesiveness are thought to generate variations in surface tension between cell populations and therefore the tissue with the lower surface tension (i.e. α cells) will envelop the tissue of higher surface tension (i.e. β cells). In fact, β cell lines are substantially more cohesive than α cell lines and when mixed will form islet-like aggregates in culture with β cells in the center and α cells towards the outside [134]. Furthermore, increasing cohesiveness in α cells by over-expressing the adhesion molecule P-cadherin disturbs this architecture [134].

### The HNF6 transgenic model of islet dysmorphogenesis and diabetes

Expression of the transcription factor HNF6 in the multipotent progenitor population is required for regulating genes involved in endocrine differentiation [66]. However, HNF6 expression is downregulated in progenitors specified to the endocrine lineage and is not expressed in hormone positive cells [65]. In order to determine whether downregulation of HNF6 is required for pancreas development or function, transgenic mice were generated which express HNF6 from an islet-specific fragment of the *Pdx-1* promoter [135]. HNF6 transgenic mice develop diabetes due to impaired insulin secretion and have alterations in islet composition and architecture [135,136].

Islets of HNF6 transgenic pups are closely apposed to the ductal epithelium, indicating that downregulation of HNF6 in islets is required for the islets to move away from the ducts (Figure 1-7) [135]. Furthermore, islet morphology was altered in transgenic mice with islets appearing larger than control islets and displaying defects in cell sorting with $\alpha$ cells dispersed throughout the $\beta$ cell core [135]. During development, the number of $\alpha$ cells is also increased in HNF6 transgenic embryos due to increased $\alpha$ cell proliferation [137]. Together, these data indicate that proper temporal regulation of HNF6 is important for islet development and function.

The defects in islet morphogenesis in the HNF6 transgenic mouse line suggested that it may be of interest to use this model to identify factors that are important for normal islet formation. To determine which genes may be altered in HNF6 transgenic islets, microarray analysis was performed at e18.5 and P1 on control and HNF6 transgenic pancreata [137]. It is not surprising that the list of altered genes include those involved in insulin biosynthesis, secretion, proliferation, adhesion, and migration. One gene which was particularly interesting to the Gannon lab was connective tissue growth factor (CTGF). CTGF is downregulated approximately 2-fold in HNF6 transgenic pancreata at e18.5 and has been shown in other systems to be important in processes involved in islet morphogenesis such as cell migration, adhesion and ECM remodeling suggesting that it may play a role in pancreatic islet development [137].

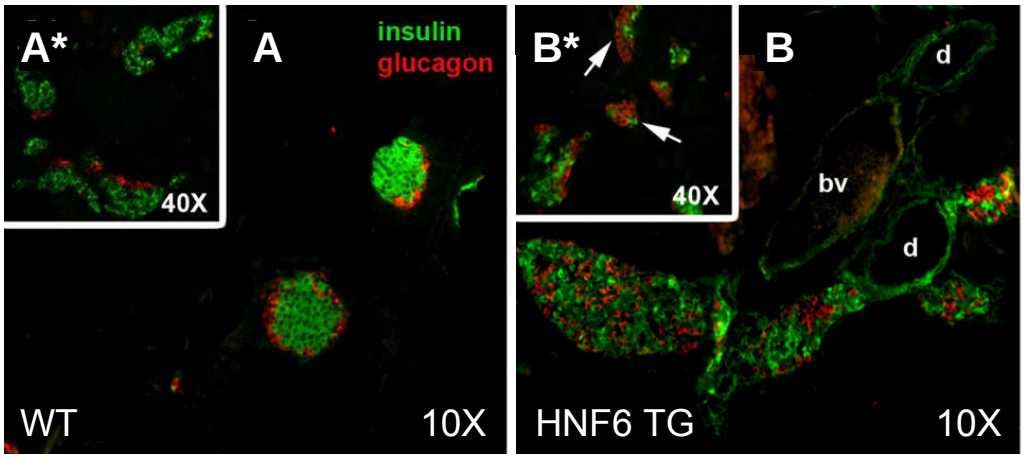


**Figure 1-7**. Transgenic islets over-expressing HNF6 have disrupted islet morphology. (A) Four week-old wild type (WT) islets have a characteristic architecture with β cells in the core and α cells at the periphery. (B) At the same age, HNF6 transgenic (HNF6 TG) islets have increase in α cells and islets which are larger and more closely apposed to ducts. α cells are also mixed within the core of the islet. (A*) Islets begin to form at e18.5 in wild type pancreata. (B*) At e18.5 HNF6 TG pancreata have increased numbers of α cells and clusters of α cells with only a few, if any, β cells (arrows). Abbreviations: bv, blood vessel, d, duct. Modified from Wilding Crawford et al., *PloS One*, 2008.

## Connective Tissue Growth Factor (CTGF)

### CTGF structure and function

CTGF, also known as CCN2 and Fisp2, is a member of the CCN family of secreted proteins named for the originally identified members cysteine rich 61 (CCN1), CTGF, and nephroblastoma over-expressed (Nov/CCN3) (reviewed in Moussad, 2000). CTGF was discovered in 1991 as a mitogen secreted into the conditioned media of human umbilical vascular endothelial cells (HUVECs). The CTGF gene has 5 exons which encode a 38 kD full length cysteine-rich protein which consists of a signal sequence followed by 4 functional domains [138] (Figure 1-8). Module 1 is an insulin-like growth factor binding module through which CTGF interacts with IGF, albeit at a lower affinity than classical IGF binding proteins [139]. The second module is a von Willebrand factor type C repeat that is thought to participate in oligomerization and interacts with BMP and TGF-$\beta$ ligands [140]. Module 3 is a thrombospondin homology domain which is found in other secreted proteins and may bind to matrix glycoproteins. It is thought that this module mediates interactions with ECM through binding specific integrin subtypes as well as through associations with the low-density lipoprotein receptor-associated protein (LRP) [141,142]. The fourth module contains a cysteine knot which is also found in other growth factors such as TGF-$\beta$, nerve growth factor (NGF), and platelet-derived growth factor (PGDF). Module 4 also binds to integrins as well as heparin sulfate proteoglycans [143,144].

Unlike classical growth factors, a specific receptor for CTGF has not been identified. Instead, CTGF exerts its biological effects by interacting with integrins including $\alpha_V\beta_1$, $\alpha_V\beta_3$, $\alpha_6\beta_1$, and $\alpha_5\beta_1$, and elicits specific responses depending on the integrin subtypes expressed on the cell [145,146],[147]. CTGF also acts by modulating

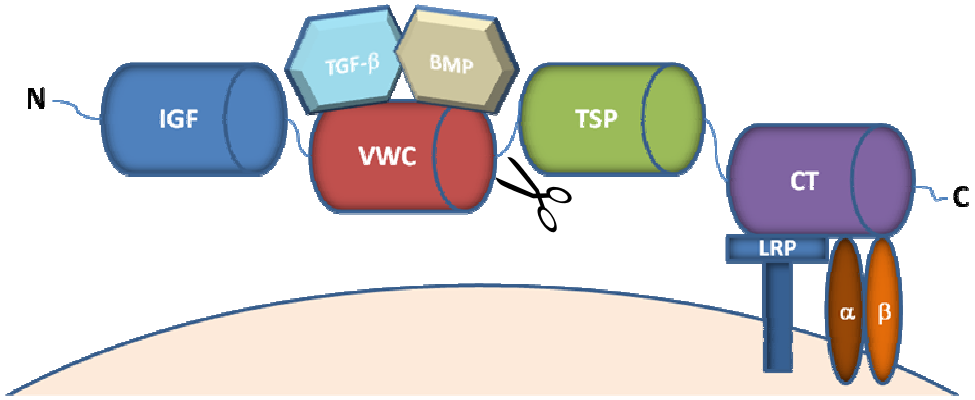


**Figure 1-8**. The modular structure of CTGF. The CTGF protein contains four domains: an insulin-like growth factor binding protein domain (IGF), a von Willebrand type C domain (VWC), a thrombospondin like-1 domain (TSP) and a cysteine rich C-terminal domain (CT). CTGF binds TGF-β and BMP ligands through its second module and interacts with the Wnt co-receptor LRP and αβ integrins though its C-terminal domain. CTGF is also proteolyticly cleaved between the second and third domains.

signaling pathways; it enhances TGF-β and inhibits BMP signaling by binding directly to TGF-β and BMP ligands [140]. CTGF also inhibits Wnt signaling through binding to the Wnt co-receptor LRP [142]. Overall, CTGF is thought to modify the interactions between the cell and the extracellular matrix and affect important cellular functions as proliferation, differentiation, adhesion, and extracellular matrix remodeling. Many of these downstream effects are not mediated by CTGF alone, but in combination with other growth factors or ECM components.

MMPs, chymotrypsin, and plasmin proteolytically cleave CTGF between the second and third modules to yield two fragments which retain biological activity and display different biological activities [148,149]. Treatment of fibroblasts with the purified N-terminal fragment of CTGF promotes ECM deposition and myofibroblast differentiation, whereas treatment with the C-terminal fragment enhances proliferation [150]. Furthermore, modules 3 and 4 interact with heparan sulfate proteoglycans and fibronectin localizing them to the ECM suggesting that the C-terminus may participate in more local signaling while the N-terminus may participate in long-range signaling [144,151]. It is not known how far CTGF can diffuse once secreted; however, CTGF and its cleavage products can be detected in serum, cerebrospinal, and peritoneal fluids [148,152].

**Biological activities of CTGF**

The role of CTGF in promoting proliferation has been widely studied in a number of different cell types including smooth muscle cells, fibroblasts, mesangial cells, and pancreatic stellate cells. CTGF has traditionally been thought to be a downstream mediator of TGF-β mitogenic activity. However, the mechanism by which CTGF affects proliferation is cell-type specific. In fibroblasts, CTGF is required for TGF-β mediated cell

cycle progression [153]. Treatment of cells with CTGF induces S-phase by upregulating cyclin A levels. A reduction in the level of the cell cycle inhibitor p27 was also detected in CTGF treated cells; however, it is unclear how CTGF might affect p27 expression or stability. CTGF treatment increases [$^{3}$H]thymidine incorporation into cellular DNA in isolated hepatic stellate cells (HSCs) [144]. Studies performed by the Brigstock laboratory indicate that CTGF-stimulated HSC proliferation is dependent on ERK1/2 phosphorylation and expression of the immediate early gene c-fos [144]. Although extracellular events were not extensively examined in this study, proliferation was shown to involve binding to heparan sulfate proteoglycans, In mesangial cells, CTGF acts via interactions with heparan sulfate proteoglycans and the tyrosine kinase receptor TrkA to promote ERK1/2 phosphorylation, suggesting that proliferation in HSC may be mediated by TrkA as well [154]. Interestingly, although integrin signaling is required for the effects of CTGF on HSC adhesion, it does not appear to be involved in proliferation in this system suggesting that CTGF may exert diverse downstream effects in a cell by interacting with multiple cell surface receptors [144].

Conversely, CTGF induces apoptosis and reduces cell survival in human aortic smooth muscle cells, COS-7 cells, and retinal pericytes [155]. Over-expression of CTGF in retinal perictytes leads to anoikis, a form of apoptosis caused by decreased ECM attachment [156]. CTGF-mediated apoptosis in retinal pericytes and lung cancer cells has been shown to involve increased expression of p53 which activates cell cycle inhibitors in other cell types [157]. Interestingly, the addition of exogenous CTGF to COS-7 cells stimulates growth, while over-expression of a form of CTGF that is sequestered in the cytoplasm induces apoptosis [158]. These data suggest that the effects of CTGF may depend on whether CTGF interacts with extracellular or intracellular factors. Alternatively, the level of CTGF over-expression may be greater

than the amount of exogenously added CTGF in these assays and point to an important role for CTGF levels in regulating the balance between proliferation vs. apoptosis.

CTGF also modulates cellular adhesion and migration. Pancreatic stellate cells, skin fibroblasts, and human vascular endothelial cells (HUVECs) display increased adhesion when plated on CTGF coated plates [145,146,159]. CTGF stimulates both directional and non-directional migration and it is thought that the pro-migratory effect of CTGF is due in part to ECM remodeling; over-expression of CTGF in vascular smooth muscle and chondrosarcoma cells increases migration and MMP activity [160,161]. Much of the effects of CTGF on adhesion vs. migration are attributed to its interactions with specific integrins and heparin sulfate proteoglycans. For example, adhesion of skin fibroblasts to CTGF involves formation of $\alpha_6\beta_1$-containing focal adhesion complexes and activation of focal adhesion kinase (FAK) and Rac [162]. On the other hand, CTGF-$\beta_3$ integrin interactions have been shown to stimulate dephosphorylation of FAK, disassembly of the cytoskeleton, and remodeling of focal adhesions to promote mesangial cell migration [163].

Angiogenesis is another complex physiological process that involves CTGF activity. CTGF is angiogenic *in vivo*; adding CTGF to chick chorioallantoic assays and injecting CTGF into the backs of mice stimulates blood vessel formation [164]. Additionally, adding CTGF to a monolayer of bovine aortic endothelial cells (BAECs) stimulates the formation of a capillary like network of tube structures [164]. In cultured endothelial cells, CTGF can also promote many of the biological processes required for angiogenesis. Exposure of human dermal microvascular endothelial cells (HMVECs) to CTGF promotes migration and adhesion in a dose dependant fashion [145]. CTGF also stimulates proliferation of BAECs and HMVECs [145,164]. CTGF may mediate endothelial cell proliferation by enhancing the activity of other growth factors such as

basic fibroblast growth factor (bFGF), one of the first angiogenic factors characterized and a well known endothelial cell mitogen [165]. CTGF is also upregulated by VEGF-A, a factor required for vascular formation, in endothelial cells. Interestingly however, CTGF complexes with VEGF-A and interferes with the binding of VEGF-A to the VEGF-R2 receptor suggesting that in some circumstances, CTGF may inhibit angiogenesis [166]. Indeed, transgenic over-expression of CTGF in the lung decreased capillary formation [167,168]. MMPs and ADAM28 (a disintegrin and metalloproteinase 28) have been shown to cleave the CTGF-VEGF complex restoring the angiogenic activity of VEGF-A [149,169]. Together, these data suggest that CTGF and VEGF may participate in a feedback loop to fine tune angiogenesis.

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

Few studies have examined effects of CTGF loss-of-function *in vivo*. A global null allele of CTGF was generated by the Lyons lab and first studied in the context of skeletogenesis [170]. CTGF null mice have skeletal defects including deformation of the craniofacial bones, bent long bones, and kinked ribs. Proliferation and differentiation in the lung were later found to be impaired in CTGF null mice as well [171]. The defects in rib formation combined with lung hypoplasia leads to respiratory failure and thus CTGF null mice die at birth, limiting analysis of the CTGF global loss-of-function phenotype to embryonic stages [170]. The defects in skeletal formation are caused by decrease in proliferation of pre-hypertrophic chondrocytes and altered expression of cartilage ECM components which together impair ossification. VEGF expression and angiogenesis within the growth plates was also decreased in CTGF null mutants [170]. Together, the phenotype of CTGF global null mice highlights the requirement of CTGF in promoting proliferation, ECM remodeling and angiogenesis *in vivo*.

Conditional inactivation of CTGF using a conditional by inversion (COIN) strategy was used to bypass the requirement for CTGF in embryonic skeletal development and examine the role of CTGF in the perinatal and postnatal skeleton [172]. Inactivation of CTGF in the limb bud or in adult bone revealed defects in endochondral bone formation and a reduction in spongy bone trabeculae in adult mice further supporting the role of CTGF in bone development.

Recently, an allele of CTGF was generated with which CTGF levels can be modulated in a tissue-specific manner [173]. In this system, the endogenous CTGF 3'UTR was replaced by the mouse *FBJ osteosarcoma* oncogene (*c-Fos*) 3'UTR flanked by loxP sites. A bovine growth hormone (BGH) 3'UTR was also placed downstream of the floxed *c-Fos* 3'UTR [173]. The *c-Fos* 3'UTR destabilizes the CTGF message, creating a hypomorphic allele by decreasing CTGF expression. In the presence of Cre, the *c-Fos* 3'UTR is excised, placing the CTGF message under the control of the BGH 3'UTR. The BGH 3'UTR renders the CTGF message more stable than the endogenous CTGF 3'UTR and thus increases CTGF expression. CTGF over-expression using a globally-expressed Cre led to embryonic lethality before e13.5 and abnormal vascular, hindbrain, eye, and pharyngeal arch development [173]. Thus, both global loss and over-expression of CTGF is incompatible with normal development. However, 4-fold over-expression of CTGF in the adult using a global tamoxifen-inducible Cre did not cause any gross abnormalities 3 months after tamoxifen administration indicating that this level of CTGF over-expression is not lethal after development [173].

Transgenic mice with tissue-specific over-expression of CTGF have also been generated. Mice over-expressing CTGF in developing bone under the direction of either the type XI collagen or the osteocalcin promoter have decreased bone mineral density and ossification [174,175]. Interestingly, the phenotype of over-expressing CTGF in the bone is similar to the defects observed when CTGF is removed in a tissue-specific

manner [172]. These data suggest that a certain level of CTGF is very important for regulating bone formation and either too much or too little impairs ossification. Similarly, while CTGF is required for proper lung development, increased expression of CTGF in the neonatal lung also has detrimental effects [168,171]. Lungs over-expressing CTGF displayed disrupted alveolarization and vascular development. Furthermore, the lung pathology of CTGF over-expressing mice resembled those in human bronchopulmonary dysplasia, a disease found in premature infants.

Over-expression of CTGF specifically in fibroblasts in mice using a fragment of the *collagen $\alpha$2(I)* promoter leads to accelerated tissue fibrosis in the skin, kidney, and vasculature; these mice die between 2 and 6 months of age [176]. The fibrogenic effects of CTGF are attributed to increased fibroblast proliferation and upreguation of key matrix genes such as fibronectin, TIMPs, and $\alpha$-smooth muscle actin [176]. In contrast, the over-expression of CTGF in hepatocytes and kidney podocytes does not to lead to any alterations in normal development or function; however, these mice have an increased susceptibility to fibrosis under injury or disease conditions [177,178]. Animals with hepatocyte-specific CTGF over-expression display an increased propensity to develop liver fibrosis after bile duct ligation or treatment with a heptatotoxin [179]. In human diabetic nephropathy, CTGF expression is upregulated in podocytes and mesangial cells [178]. Twelve weeks after streptozotocin induced diabetes, podocyte-specific CTGF-transgenic mice showed a more severe diabetic nephropathy than control mice, suggesting that upregulation of CTGF contributes to the development of diabetic complications [178].

Finally, cardiac tissues of mice and rats over-expressing CTGF in cardiomyocytes do not show defects in basal function or increased fibrosis [180]. In fact, CTGF over-expression has a protective effect on cardiomyocyte function in a cardiac

injury model [180]. However, over time CTGF over-expressing mice developed severe age dependent cardiac dysfunction, suggesting that a certain level of CTGF may be beneficial but long term over-expression may activate pathological pathways.

**CTGF and the pancreas**

In the pancreas, CTGF has mainly been studied in the context of pancreatic disease states. CTGF is upregulated in pancreatic ductal adenocarcinoma where it is expressed in both the tumor and the surrounding stroma [181]. CTGF expression promotes angiogenesis and metastasis of tumors [181]. Knock-down of CTGF levels in pancreatic tumor cell lines reduces tumor growth both *in vitro* and when transplanted subcutaneously into nude mice [181]. Furthermore, administration of a neutralizing CTGF-specific monoclonal antibody to mice with established tumors inhibited angiogenesis, metastasis and tumor growth [182,183]. TGF-β signaling is also thought to play an important role in pancreatic cancer, and CTGF may interact with TGF-β signaling to promote cancer progression [184]. Examination of the *cis*-acting elements and signaling cascades required for CTGF expression in PANC-1 cells, a pancreatic cancer cell line, revealed that CTGF expression is dependent on ras/MEK/ERK signaling rather than TGF-β signaling suggesting that multiple signaling pathways may regulate CTGF in pancreatic cancer cells [185].

Expression of CTGF is also upregulated in pancreatitis, a disease characterized by a destruction of the pancreatic acinar cells and subsequent inflammation and fibrosis [186]. Stellate cells, resident fibroblast-like cells in the pancreas, are thought to be the main CTGF-producing cell type in pancreatitis. TGF-β and ethanol stimulate CTGF expression in stellate cells which in turn acts through integrins to promote migration, adhesion, collagen synthesis, and ultimately fibrosis [146,187].

CTGF was found to be downregulated in the HNF6 transgenic model of islet dysmorphogenesis and diabetes [137]. This data suggested that CTGF may also be involved in pancreas development and function. To determine whether CTGF is normally expressed in the pancreas during development, antibody labeling and an allele with lacZ replacing part of the CTGF coding sequence were used to examine CTGF expression [188]. X-gal staining to detect lacZ expression and thus CTGF expression revealed that CTGF expression can be detected in the pancreas as early as e12.5 in the developing ductal epithelium and in the surrounding mesenchyme (Figure 1-9). As development proceeds, CTGF expression is localized to the developing endocrine cords and vasculature, but expression cannot be detected in acinar tissue. Cell type-specific markers were used along with X-gal staining to determine which cell types in the pancreas express CTGF at late gestation [188]. CTGF can be detected in $\beta$ cells, endothelial cells, and ducts but not in $\alpha$ cells or in acinar tissue during development (Figure 1-10) [188]. Co-immunofluorescence for $\beta$-galactosidase and cell-type specific markers was used to confirm the expression pattern of CTGF. In the adult pancreas, CTGF can no longer be detected in $\beta$ cells, but expression is maintained in endothelial cells and ducts. Although few studies have examined CTGF expression in normal pancreatic tissue in humans, CTGF has also been shown to be expressed in adult human pancreatic ducts and endothelial cells [189]. The expression pattern of CTGF in the pancreas suggests that CTGF may be involved in the regulation of pancreas development.

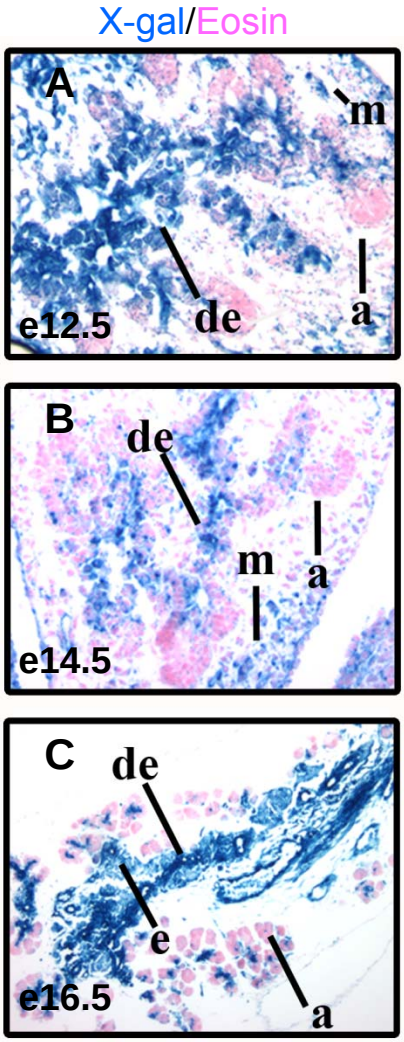


**Figure 1-9**. X-gal staining was used to examine CTGF$^{LacZ}$ expression throughout development. (A) At e12.5 CTGF$^{LacZ}$ is expressed in the pancreatic ductal epithelium (de) and in the surrounding mesenchyme (m). (B) At e14.5 CTGF$^{LacZ}$ is expressed in the ductal epithelium, mesenchyme, but not in surrounding acinar (a) tissue. At e16.5 it is evident that CTGF$^{LacZ}$ is expressed in the developing endocrine clusters and ductal epithelium but not in acinar tissue. Modified from Crawford et al. *Mol Endo*, 2009.

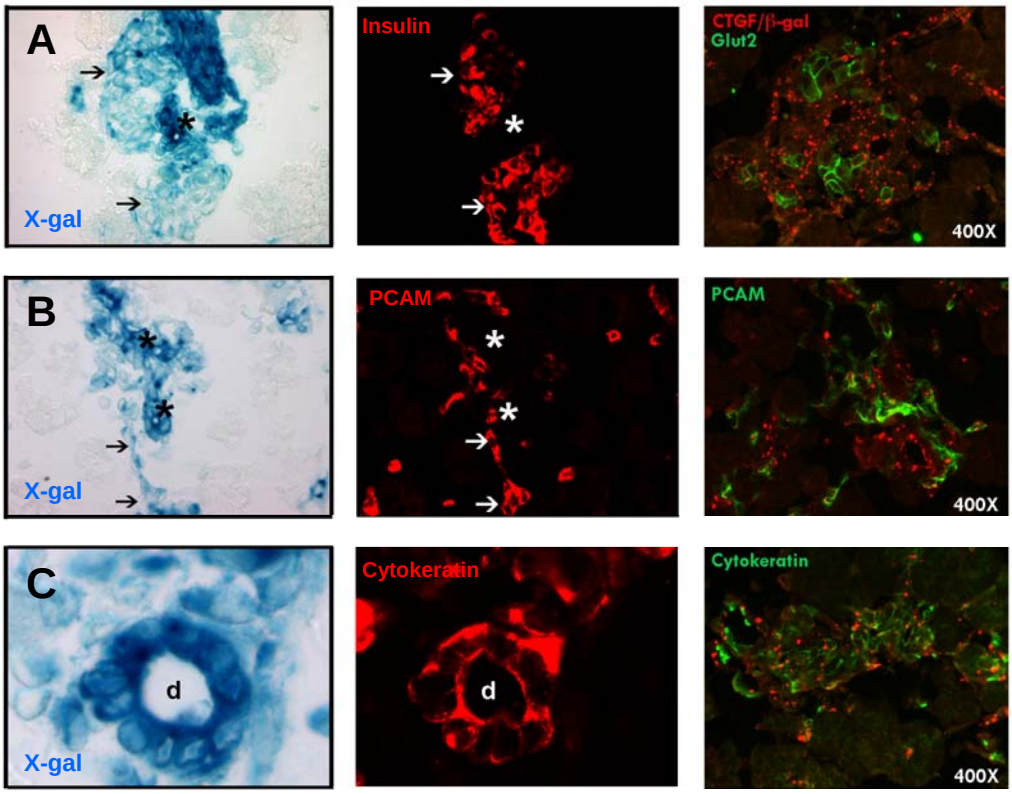


**Figure 1-10.** CTGF$^{LacZ}$ expression in the pancreas localizes with cell type-specific markers. X-gal staining (left column) along with co-labeling for cell type specific markers (middle column) revealed that CTGF$^{LacZ}$ is expressed in insulin-positive cells (β cells) (A), PCAM-positive cells (endothelial cells) (B), and cytokeratin-positive cells (ducts) (C) at e18.5. Immunolabeling for β-galactosidase (β -gal) and cell-type specific markers (right column) confirmed CTGF$^{LacZ}$ localization. CTGF$^{LacZ}$ contains a transmembrane domain and therefore the β-gal staining appears as membrane-localized puncta. Arrows indicate cells which co-express CTGF and the cell-type specific marker. Asterisks indicate cells which express CTGF but do not express the cell-type marker. d=duct. Modified from Crawford et al. *Mol Endo*, 2009.

## Thesis overview

Factors which can promote β cell proliferation, differentiation or islet morphogenesis in the pancreas may ultimately aid in generating replacement β cells to treat diabetes either *in vivo* or *in vitro*. Connective tissue growth factor (CTGF) is a modular secreted protein which stimulates proliferation, differentiation, adhesion, and migration in multiple cell types. The Gannon lab has demonstrated that CTGF is expressed in β cells, ducts, and endothelial cells in the pancreas during development. Studies using a global null allele of CTGF indicate that CTGF is required for proper endocrine cell lineage allocation, β cell proliferation, and islet morphogenesis during development. Chapter II of this thesis describes the methods used, and Chapter III summarizes the pancreatic phenotype of global CTGF inactivation. Because CTGF has been shown to interact with integrin, TGF-β, BMP, and Wnt signaling in other systems, it is unclear from these initial studies with which signaling pathways CTGF acts to promote pancreas development. Chapter IV describes efforts to examine whether CTGF interacts with TGF-β during endocrine development and to explore the mechanism of CTGF action in the embryonic pancreas at the cellular level.

The fact that CTGF is expressed in multiple pancreatic cell types raises the question of whether CTGF acts in an autocrine or paracrine manner to regulate β cell proliferation, endocrine cell lineage allocation, and islet morphogenesis. To determine which pancreatic cell type(s) are producing the required CTGF for each aspect of pancreas development, mice carrying a conditional allele of CTGF were generated and interbred to tissue-specific Cre lines to inactivate CTGF in each of the tissues in which it is expressed in the pancreas. Chapter V describes our results indicating that CTGF acts in both an autocrine and paracrine manner during pancreas development. Because loss

of CTGF leads to a reduction in insulin-positive area, we hypothesized that increased CTGF levels during embryogenesis may increase β cell mass. Chapter IV describes the use of an inducible transgenic system to specifically over-express CTGF in β cells and our data which indicates that over-expression of CTGF in β cells is sufficient to increase endocrine cell mass and β cell proliferation. Conclusions, implications, and future directions for the studies described in this dissertation are presented in Chapter VII.

# CHAPTER II

# MATERIALS AND METHODS

## Mice

### *CTGF$^{lacZ}$*

The generation of the CTGF$^{lacZ}$ allele has been previously described [188]. CTGF$^{lacZ}$ mice were maintained on a mixed C57BL/6/129SvJ/B6D2 background. Genotyping for the presence of the null allele was performed by PCR amplification of a portion of CTGF exon 4 which is missing in the null allele. Amplification of a 193-bp fragment from the endogenous allele was performed using the following primers: for 5'aag aca cat ttg gcc cag ac 3' and rev 5' ttt tcc tcc agg tca gct tc rev 3'. CTGF$^{lacZ/+}$ mice were distinguished from wild type by either X-gal staining or PCR amplification of the lacZ gene using the following primers: for 5' gcc gtc tga att tga cct ga 3' and rev 5' tct gct tca atc agc gtc cc 3'.

### *MIP-GFP*

Mice expressing green fluorescent protein (GFP) from the mouse *insulin* promoter (*MIP-GFP*) were provided by Dr. David Piston (Vanderbilt University). The generation of *MIP-GFP* mice has been previously described [190]. PCR genotyping was performed using the following primers: for 5' act ggg ctt aca tgg cga tact c 3' and rev 5'gaa gac aat agc agg cat gct g 3'.

***CTGF$^{e2COIN}$***

The generation of the *CTGF$^{e2COIN}$* allele has been previously described [172]. Targeted *CTGF$^{e2COIN}$* ES cells (Regeneron Pharmaceuticals) were used to generate chimeric male mice at the Transgenic/ESC Shared Resource facility at Vanderbilt University. Chimeras were bred to mice expressing the FLPE recombinase from the *Protamine 1* promoter (provided by Dr. Chin Chiang, Vanderbilt University) for the removal of the *Hyg∆TK* selection cassette. The removal of the selection cassette was confirmed by PCR using the following primers: for 5' acg agc ggg ttc ggc cca tt 3' and rev tgc ggc cat tgt ccg tca gg 3'. Mice exhibiting germline transmission of the *CTGF$^{e2COIN}$* allele were bred to create homozygous *CTGF$^{e2COIN/e2COIN}$* mice. Genotyping of *CTGF$^{e2COIN/e2COIN}$* mice was performed by PCR on DNA isolated from ear punches (adults) or pieces of tails (embryos) using the following primers: for 5' cac ttt cta ctc tgt gac 3'; and rev 5' cct tac atg ttt tac tag 3'. PCR amplification of the CTGF wild type exon 2 was used to distinguish *CTGF$^{e2COIN/e2COIN}$* mice from *CTGF$^{e2COIN/+}$* mice using the following primers: for 5' cct gct atg ggc cag gac tg 3' and rev 5' cca aaa ggt gag gcc tct gc 3'. Tissue-specific inactivation of *CTGF* was achieved by breeding *CTGF$^{e2COIN/e2COIN}$* to the following Cre deleter lines: *Tie-1-Cre* (provided by Dr. Scott Baldwin, Vanderbilt University), *Pdx-1-Cre* (provided by Dr. Guoqiang Gu, Vanderbilt University) and *Ngn3-Cre$^{BAC}$* (provided by Dr. Christopher Wright, Vanderbilt University) [53,68,191] Genotyping for the Cre transgene was performed using the following primers: for 5' tgc cac gac caa gtg aca gc 3' and rev cca ggt tac gga tat agt tca tg 3'. Inversion of the *CTGF$^{e2COIN}$* allele was confirmed by PCR using DNA isolated from pancreatic tissue sections using the following primers for 5' cct tac atg ttt tac tag 3' and rev 5' ctc aga gta ttt tat cct cat ctc 3'.

***RIP-rtTA* and *TetO-CTGF***

To over-express CTGF in β cells, transgenic mice were generated in which expression of the CTGF cDNA (Open Biosystems, Huntsville, AL) is driven by the tetracycline operator (TetO, plasmid was a gift from Dr. Tim Blackwell, Vanderbilt University) rendering transgene expression DOX-dependent. *TetO-CTGF* mice were interbred to homozygous mice expressing the reverse tetracycline transactivator (*rtTA*) from a fragment of the *rat insulin 2* promoter (*RIP-rtTA*), which were generously provided by Dr. Alvin Powers (Vanderbilt University) [192]. The following primers were used to genotype animals for the presence of the *TetO-CTGF* transgene: for 5' gga ggt cta tat aag cag act tcg 3' and rev 5' tta agt tac gcc atg tct ccg ta 3'. To genotype for the presence of the *rtTA* transgene, the following PCR primers were used: for 5' gcg tgt ggg gca ttt tac ttt ag 3' and rev 5' cat gtc cag atc gaa atc gtc rev 5'. Pregnant mothers were given 2 mg/ml of doxycycline (Sigma) in a 2% Splenda solution in their drinking water beginning on day 9.5 of gestation to expose the embryos to doxycycline prior to *RIP* activation, which normally occurs at e11.5. Doxycycline was administered in a colored water bottle to protect from light exposure and was replaced every 48 hours.

All animal experiments were approved by the Institutional Animal Care and Use Committee of Vanderbilt University Medial Center.

**DNA extraction**

Ear punches or tails were digested overnight at 55°C in 80 µl per sample of tissue buffer [35.2 mM Tris, 2.5 mM, EDTA, 2.5 mM sodium citrate, 8.8 mM ammonium sulfate, 5% (v/v) Tween20] supplemented with fresh 0.3 mg/ml Proteinase K and 0.3 mg/ml RNase A. Tissues were the digested at 37°C for 15 minutes and heat-inactivated at 95°C for 10 minutes followed by centrifugation at 13,000 rpm for 10 minutes. The

supernatant was stored at 4°C. For DNA extraction from paraffinized tissue sections, sections were deparaffinized in xylene and washed with 100% ethanol prior to the extraction procedure as described above.

### Pancreatic explant cultures

Pancreata were dissected at e12.5 and cultured in 3D collagen gels. To make the collagen gel, 10x RPMI, 1N NaOH, and complete medium [1xRPMI (Invitrogen), 10% fetal bovine serum (FBS) and 1% Penicillin/Streptomycin] were mixed with type I rat tail collagen (BD Biosciences). 50 µl mounds of collagen were placed in a 24-well plate and allowed to solidify at 37°C. Pancreata were placed on top of the collagen and 20 µl of collagen was added to cover the pancreata. The collagen "sandwiches" were then allowed to solidify, and complete media was added to each well. For the TGF-β experiments, 5 ng/ml recombinant human TGF-β1 (R&D systems) was added to both the collagen and the media. The media was changed every 48 hours. After 7 days, the cultures were fixed overnight at 4°C in 4:1 methanol/DMSO. The collagen gels were then washed twice in methanol and immunolabeled in whole mount for insulin and glucagon. For confocal imaging, the cultures were cleared in BABB (1:2 benzyl alcohol: benzyl benzoate) and placed on slides with footed coverslips. Confocal Z-stacks were taken of each bud and Metamorph 6.1 software was used to quantify the percentage of the bud epithelium that was composed of insulin-positive and glucagon-positive area.

## Pancreatic cell migration assays

Transwell inserts (24 well, 8 μm pores, Costar), were coated on the underside with either 50 mg/ml ultrapure BSA as a control (Calbiochem) or 10 μg/ml collagen IV (Sigma) overnight at 4°C. Wild type pancreata were dissected at e18.5 and placed in dissociation buffer [9 ml Hanks buffered saline solution (HBSS), 72 μl of 25 mg/ml Liberase RI (Roche), 90 μl of 10 mg/ml DNase I, and 225 μl of 1M $CaCl_2$]. The pancreata were incubated at 37°C for 2 minutes while triturating then washed in HBSS. Trypsin (0.025% in EDTA) was added and the cells were incubated at 37°C for 5 minutes or until a single-cell suspension was obtained. The cells were then washed in complete medium and resuspended in migration buffer (1xRPMI 1640, 0.5% ultrapure BSA, and 0.4 mM $MnCl_2$). Cells ($5x10^4$ cells/well in 100 μl migration buffer) were added to the pre-coated transwells. To determine the effect of exogenous CTGF on cell migration, migration buffer containing either control high-performance liquid chromatography (HPLC) buffer or increasing concentrations of recombinant human CTGF (a gift from Dr. David Brigstock, Ohio State University) was added to each well. Each concentration of CTGF was performed in triplicate. For migration assays using cells isolated from wild type and CTGF null pancreata, $CTGF^{lacZ/+}$ heterozygotes were interbred to generate litters with wild type $CTGF^{lacZ/+}$, and $CTGF^{lacZ/lacZ}$ embryos. Pancreatic cells were isolated and the embryos were genotyped as described above. The isolated cells were stored in complete medium at 4°C for 2-3 hours until genotyping was completed.

After incubation for 24 hours at 37°C, the cells on the underside of the membrane were fixed in 4% PFA for 20 minutes at room temperature and the cells on the top of the membrane were removed with a cotton swab. The remaining cells were stained with toluidine blue (1% toluidine blue O in 1% sodium tetraborate) for 20 minutes at room temperature. The membranes were removed from the inserts using a scalpel and

mounted on slides. Five random fields of view at 100x magnification were photographed of each membrane and all of the cells in each field of view were counted. The average number of migrating cells/field of view was determined.

### Adhesion assays

To determine whether CTGF stimulates adhesion of pancreatic cells, adhesion assays were performed. 96-well medium binding plates (Costar) were coated with either 50 mg/ml ultrapure BSA or increasing concentrations of CTGF (in triplicate) diluted in sterile PBS overnight at 4°C. Non-specific adhesion was blocked by incubating wells with ultrapure BSA for 1 hour at room temperature. Dissociated cells from e18.5 pancreata (see above) were plated at a concentration of $1x10^5$ cells/well in 100 µl of migration buffer (see above) and the plate was incubated at 37°C for 1.5 hours. The adherent cells were fixed with 4% PFA for 20 minutes at room temperature and the wells were then washed with PFA to remove non-adherent cells. The wells were then stained with toluidine blue for 20 minutes at room temperature and washed in $dH_2O$.

### Real-time PCR

Pancreata were dissected and placed immediately into RNAlater (Ambion). Total RNA was extracted using the RNAqueous kit (Ambion) according to the manufacturer's instructions and eluted in 50 µl of elution buffer. RNA samples were treated to remove DNA contamination using the Turbo-DNase kit (Ambion). RNA concentration and integrity were assessed using the ND-1000 Spectrophotometer (NanoDrop) and the 2100 Electrophoresis Biolanalyzer (Aligent) at the Vanderbilt Functional Genomics Shared Resource. cDNA was synthesized using the Superscript III First-Strand

synthesis system (Invitrogen) using 500-1000 ng of RNA. Real-time reactions were carried out in technical duplicate with iQ SYBR Green supermix (Bio-Rad) according to the manufacturer's instructions at an annealing temperature of 58°C using the following primers to detect total CTGF: for 5' ttc tgc gat ttc ggc tcc 3' and rev 5' acc atc ttt ggc agt gca ca 3'. Primers used to detect endogenous CTGF were designed against the 3'UTR which is absent from the CTGF cDNA: for 5' ctg ggg aca atg aca tct 3' and rev 5'gtt cgt gtc cct tac ttc ct 3'. Data were collected using an iCycler iQ Real-time PCR Detection System (Bio-Rad) and software (Bio-Rad). Primers were optimized by melting curve and standard curve assays first before application. Expression levels were normalized against the levels of *hypoxanthine-guanine phosphoribosytransferase* (*HPRT*) using the following primers: for 5' agt caa cgg ggg aca taa aa 3' and rev 5' tgc att gtt tta cca gtg tca a 3'.

**Sequencing of CTGF exon 2**

The e2COIN intron is placed within exon 2 of the CTGF locus, splitting it into two parts. In order to determine if, in the absence of Cre, the splicing of the e2COIN allele alters the CTGF message, exon 2 was sequenced using cDNA from $CTGF^{e2COIN/e2COIN}$ pancreata. RNA was isolated from whole pancreas from $CTGF^{e2COIN/e2COIN}$ e18.5 embryos and cDNA was generated as described above. Exon 2 was PCR amplified using the high-fidelity DNA polymerase PfuUltra (Agilent) and the following primers: for 5' tgc tat ggg cca gga ct 3' and rev 5' cga aat cgc aga aga gg 3'. The 250 bp product was gel purified and sequenced by the Vanderbilt University DNA sequencing facility with the primers used for PCR amplification. The sequence of $CTGF^{e2COIN/e2COIN}$ exon 2 was compared to wild type mouse CTGF exon 2 as listed on the UCSC genome browser (www.genome.ucsc.edu)

### *In situ* hybridization

CTGF anti-sense and sense RNA probes were generated to detect CTGF expression in a cell type-specific manner. The pCRII vector (Invitrogen) containing a CTGF full length cDNA placed with the multiple cloning site was obtained from Dr. David Brigstock at Ohio State University. The vector was linearized with restriction enzymes SgrAI (anti-sense probe) or EcoRV (sense probe). Digoxigenin-UTP-labeled probes were generated by an *in vitro* transcription reaction using SP6 and T7 RNA polymerases (DIG RNA Labeling Kit, Roche). The probes were treated with DNase I and precipitated with 4 M LiCl and 100% EtOH at -80°C overnight, washed in 80% EtOH, and resuspended in RNase-free water.

Paraffin slides were dewaxed and rehydrated using a decreasing ethanol series and washed in RNase-free 1x PBS. Slides were treated with 4% PFA for 10 minutes at room temperature and treated with proteinase K (400 ng/ml) for 4 minutes at room temperature. The slides were incubated in TEA (3.7 g triethanolamine, 448 µl 10 N NaOH, and 250 µl acetic anhydride in 200 ml RNase-free water) for 10 minutes and then washed in 1x PBS. Slides were then pre-hybridized for 2-6 hours in hybridization buffer [5 ml 100% formamide, 2.5 ml 20x SSC, 1 ml 50x Denhardt's Solution (Invitrogen), 250 µl 10 mg/ml yeast tRNA, 500 µl 10mg/ml herring sperm DNA, and 750 µl RNase-free water] at room temperature. Probes were hybridized at a concentration of 0.3 ng/µl overnight at 68°C. The next day, sides were washed in 0.2x SSC for 1 hour at 55°C and then in maleic acid buffer, pH 7.5 (2.9 g maleic acid and 2.175 g NaCl in 250 ml distilled deionized water). The slides were then blocked with commercial blocking powder (Roche) and incubated with alkaline phosphatase-conjugated anti-digoxigenin Fab fragments (Roche) at a dilution of 1:1500 for 2 hours at room temperature. A 1:1 ratio of

nitroblue tetrazolium chloride (NBT, Roche) and 5-bromo-4-chloro-3-indoxyl phosphate (BCIP, Roche) was used for detection of the labeled probes.

### Tissue dissection, preparation, and histology

For embryonic studies, the morning of the vaginal plug was considered to be e0.5. Digestive organs were dissected in cold PBS and fixed immediately in 4% paraformaldehyde at 4 °C for 1-1.5 hours. Tissues were dehydrated, embedded in paraffin and sectioned at 5 μm. Serial sections were deparaffinized and rehydrated using a decreasing ethanol series to distilled water. Indirect protein localization was obtained by incubation of tissue with the following primary antibodies: guinea pig anti-insulin (1:1000; Upstate), rabbit anti-glucagon (1:1000, Millipore), rabbit anti-phosphorylated histone H3 (pH3, 1:200; Millipore), and mouse anti-Neurogenin3 (Ngn3, 1:1000; Developmental Studies Hybridoma Bank, The University of Iowa). Ducts were labeled with biotinylated *Dolichos biflorus* agglutinin (DBA, 1:1000; Vector Laboratories) followed by detection with Cy3-conjugated streptavidin (1:500; Vector Laboratories). Detection of pH3 and Ngn3 required antigen retrieval in 10 mM sodium citrate buffer, pH 6.0. Slides were microwaved at full power until boiling and then boiled for 6 minutes. All primary antibodies were incubated overnight in a humid chamber at 4 °C. Primary antibodies were detected by species-specific donkey secondary antibodies conjugated to either Cy2 or Cy3 fluorophores (1:200; Jackson ImmunoResearch Laboratories, Inc). Ngn3 detection required tyramide signal amplification (Invitrogen). Fluorophores were excited using an Olympus BX41 research microscope (Tokyo, Japan) and digital images were captured using MagnaFire software (Optronics Engineering, Goleta, CA). TIFF images from each experiment were processed identically with Adobe Photoshop.

## Morphometric analyses

### α and β cell proliferation

Entire pancreata were serially sectioned and slides were immunolabeled for pH3 and insulin or glucagon. One section was photographed every 250 μm (7-10 sections per animal) throughout the pancreas. Using Metamorph 6.1 software (Molecular Devices), the total number of cells that were positive for insulin or glucagon was counted as were the number of proliferating cells (co-labeled for hormone and pH3). The percentage of proliferating α or β cells was determined by dividing the number of proliferating cells by the total number of hormone-positive cells.

### Insulin and glucagon area/percent endocrine area

Entire pancreata were serially sectioned and slides were immunolabeled for insulin and glucagon. One section was photographed every 250 μm throughout the pancreas. Using Metamorph 6.1 software, the insulin+ and glucagon+ area of each section was determined by thresholding. The percentage of the total endocrine area that was composed of insulin or glucagon was calculated. For the CTGF over-expression studies, the percentage of the pancreas area composed of endocrine tissue was calculated by combining the insulin positive and glucagon positive area on each section and dividing it by the total pancreas area of each section.

### β cell size

Every thirtieth slide of pancreas tissue was immunolabeled for insulin and every islet from one section on each slide was photographed. Metamorph 6.1 software was used to determine the total β cell area for each islet. The number of β cells in each islet

was counted, and average β cell size for each islet was determined by dividing the total β cell area by the number of β cell nuclei. β cells from at least 125 islets from three animals per genotype were used to determine the average β cell size. As an alternative method to measure β cell size, rabbit anti-mouse Glut2 (from Dr. Bernard Thorens, University of Lausanne, 1:500) was used to visualize the plasma membrane of β cells. Metamorph software was used to determine the area of 100 individual β cells in at least 5 different islets per mouse. Three mice per genotype were analyzed to determine the average β cell size.

**Proximity of endocrine tissue to ducts**

In order to determine whether tissue-specific inactivation of CTGF leads to changes in the proximity of the endocrine tissue to the ducts, the distance between the endocrine tissue and the ducts was quantified at P1. Every twentieth slide of pancreas was immunolabeled with anti-insulin and anti-glucagon antibodies raised in the same species. DBA was used to label ducts. One section from each slide was photographed and Metamorph software was used to quantify the distance between each endocrine cluster and the nearest duct. The average distance between the endocrine tissue and the ducts was determined for each animal. At least 3 animals of each genotype were examined.

**α and β cell number**

Entire pancreata were serially sectioned and slides were immunolabeled for insulin and glucagon. One section was photographed every 250 μm throughout the pancreas. The number of the insulin-positive and glucagon-positive cells were counted and divided by the total area of the section in $\mu m^2$.

### Quantification of Ngn3-positive cells

Entire pancreata were sectioned and slides were immunolabeled for Ngn3 and DAPI. One section every 150 μm was photographed. The number of Ngn3 positive nuclei were counted and divided by the total pancreatic epithelial area in $\mu m^2$.

## Statistical analysis

Results are expressed as mean ± SEM. For two group comparison, Student's t test was used. $p<0.05$ was considered statistically significant.

# CHAPTER III

## GLOBAL CTGF INACTIVATION LEADS TO DEFECTS IN ISLET CELL LINEAGE ALLOCATION, β CELL PROLIFERATION AND ISLET MORPHOGENESIS DURING DEVELOPMENT

### Introduction

Our lab initially became interested in studying CTGF in the context of pancreas development and function because it was found to be downregulated in a transgenic mouse model of HNF6 islet cell over-expression [137]. HNF6 transgenic mice display islet dysmorphogenesis, insulin secretion defects, and diabetes [135,136]. CTGF has also been shown in other systems to regulate proliferation, extracellular matrix remodeling, and migration—processes which are all thought to be important for pancreas development [122,129,130]. In the developing mouse pancreas, CTGF is expressed as early as e12.5 in the mesenchyme, ductal epithelium, endothelial cells, and insulin-producing cells [188]. The phenotype of the HNF6 transgenic model combined with the embryonic expression pattern of CTGF prompted us to investigate whether CTGF is required for proper pancreas development and function. Analysis of the CTGF global knockout phenotype was initially performed by a former graduate student in the lab, Dr. Laura Crawford. While rotating in the lab I performed some of these analyses, and took over the project once Dr. Crawford left the lab.

CTGF null mice die at birth due to skeletal and lung defects; therefore analysis of the CTGF null phenotype was limited to embryonic stages [170]. However, we found that CTGF null embryos display defects in islet morphogenesis, alterations in the ratio of endocrine cells with an increase in glucagon-positive area and a decrease in insulin-positive area, and a decrease in β cell proliferation specifically at e18.5 [188]. The

phenotype of adult CTGF heterozygous animals was also examined. These mice have alterations in islet morphology and an increase in α cells, but β cell area is normalized due to an increase in individual β cell size. Together these data demonstrate for the first time that CTGF is required for normal pancreas development and may act downstream of HNF6 to promote proper lineage allocation, β cell proliferation, and islet morphogenesis.

## Results

### CTGF is required for the proper number of the different endocrine cell types

Mice carrying a null allele of CTGF which contains the *lacZ* gene knocked into the CTGF locus (*CTGF*$^{lacZ}$) were used to examine the role of CTGF in pancreas development [188]. In order to determine whether CTGF inactivation led to defects in endocrine development, morphometric analysis was used to examine islet endocrine composition at e18.5. The proportion of the islets which were composed of insulin- and glucagon-positive area was significantly altered in CTGF null embryos (*CTGF*$^{lacZ/lacZ}$) compared to wild type embryos [188] (Figure 3-1). Islets from wild type embryos had approximately 12% of their endocrine area occupied by glucagon-producing cells compared to 25% in *CTGF*$^{lacZ/lacZ}$ embryos (Figure 3-2A). The increase in glucagon-positive cells in islets from null embryos was accompanied by a decrease in insulin area compared with wild type (70% in wild type vs. 49% in *CTGF*$^{lacZ/lacZ}$). This corresponds to an α to β cell ratio of 1:4 in wild type and 1:2 in mutant embryos. CTGF heterozygous embryos (*CTGF*$^{lacZ/+}$) also displayed a similar increase in α cells and a decrease in β cells, suggesting one functional copy of CTGF is not sufficient to promote proper proportions of the endocrine cell types. There was no difference in average islet size or

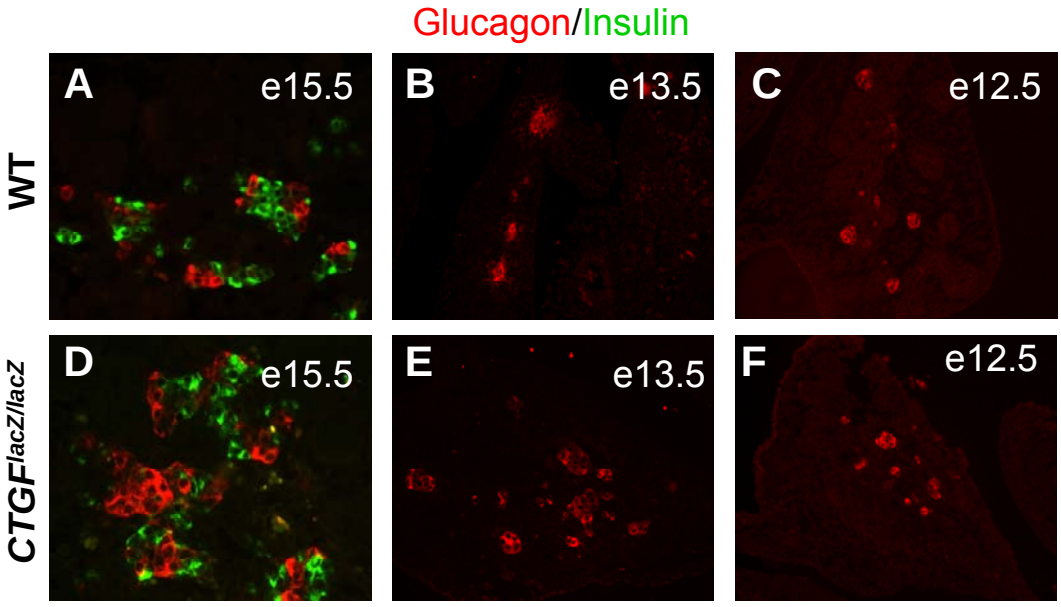


**Figure 3-1**. CTGF is required for proper endocrine cell ratios. Compared to controls (A), CTGF null embryos displayed an increase in glucagon positive area and a decrease insulin positive area at e15.5 (D). The increase in glucagon positive area is detected at e13.5 in mutant embryos (E), but not at e12.5 (C and F). Modified from Crawford et al. *Mol Endo*, 2009.

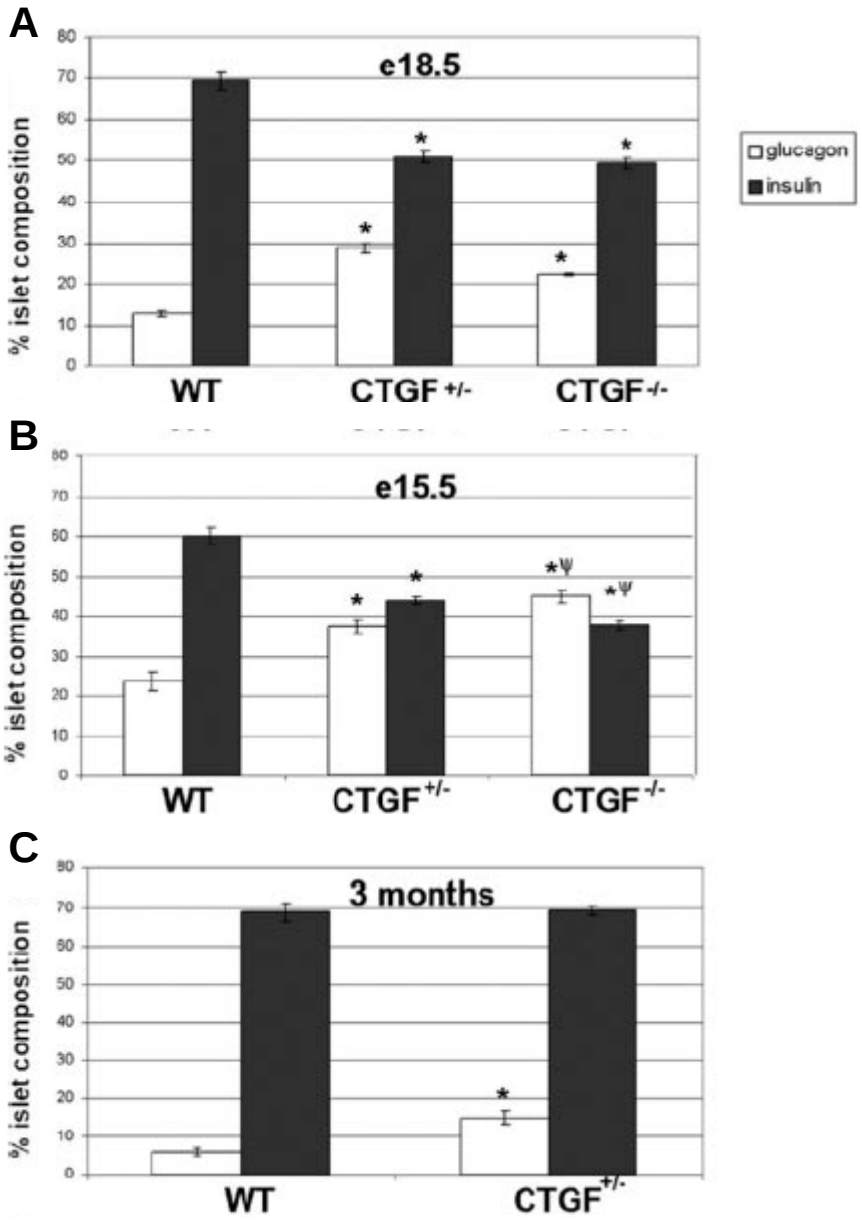


**Figure 3-2**. CTGF mutant animals have an altered islet composition. Morphometric analysis at (A) e18.5, (B) e15.5, and (C) adult stages indicated alterations in the proportions of insulin-positive and glucagon-positive cells in CTGF heterozygous ($CTGF^{+/-}$) and null ($CTGF^{-/-}$) animals compared to wild type (WT). n=5 for e18.5, n=3 for 15.5 and adult. *$p<0.01$ compared with WT; Ψ, $p<0.05$ compared with $CTGF^{+/-}$. Modified from Crawford et al. *Mol Endo*, 2009.

in the distribution of different size islets in CTGF heterozygous or null embryos compared to controls (data not shown).

In order to determine how early the changes in endocrine cell ratio occur, islet composition was examined at e15.5 (Figure 3-1). The change in islet cell types seen at e18.5 were also present at e15.5—in *CTGF*$^{lacZ/lacZ}$ embryos nearly 45% of endocrine area was occupied by glucagon positive cells, compared with 23% in wild type embryos (Figure 3-2B). Insulin-positive cells occupied 37% of endocrine area in *CTGF*$^{lacZ/lacZ}$ at e15.5 compared with 60% in wild type controls. Even in control embryos, there are not a significant number of insulin-expressing cells in the pancreas at e13.5; therefore we only examined the number of $\alpha$ cells in control and CTGF null embryos at this stage. Compared to control embryos, there was an increase in the number of glucagon-positive cells in *CTGF*$^{lacZ/lacZ}$ pancreata as well. However, one day earlier at e12.5, the number of glucagon-positive cells in CTGF null embryos was not significantly different from controls suggesting that CTGF plays a role in regulating endocrine cell ratios beginning at the secondary transition.

**CTGF is required for $\beta$ cell proliferation and for lineage allocation**

In order to determine the mechanism leading to altered endocrine cell ratios, endocrine cell proliferation and apoptosis was quantified at various stages throughout development. TUNEL staining was used to quantify the number of apoptotic cells; however, there were no changes in $\alpha$ or $\beta$ cell apoptosis detected at any stage examined (data not shown). The percentage of $\alpha$ and $\beta$ cells undergoing proliferation at e13.5, e14.5, e15.5, e16.5, e17.5 and e18.5 in wild type and CTGF mutant embryos was quantified using double immuofluorescence for phosphorylated histone H3 (pH3) and insulin or glucagon. Consistent with previous results, we detected few proliferating cells

at e13.5, e14.5, e15.5, and e16.5. There were also no significant changes in the percentage of pH3-positive α cells between wild type and *CTGF*$^{lacZ/lacZ}$ or *CTGF*$^{lacZ/+}$ pancreata at any stage examined (data not shown), indicating that the increase in glucagon-positive area is not due to an increase in α cell proliferation. We also detected no differences in β cell proliferation between wild type and CTGF mutants at e13.5, e14.5, e15.5, e16.5 or e17.5; however, there was a significant decrease in β cell proliferation in both *CTGF*$^{lacZ/lacZ}$ and *CTGF*$^{lacZ/+}$ embryos at e18.5 compared with wild type (Figure 3-3A) [188]. Whereas nearly 1.0% of β cells were proliferating at e18.5 in wild type embryos, *CTGF*$^{lacZ/+}$ and *CTGF*$^{lacZ/lacZ}$ had less than 0.02% and less than 0.01% proliferating β cells, respectively. β cell proliferation increases at late gestation and continues at relatively high rates during the neonatal stages; therefore we determined whether loss of CTGF affects β cell proliferation after birth. Because CTGF null pups die at birth, β cell proliferation was examined at P2 in CTGF heterozygotes (Figure 3B). The percentage of β cells proliferating in *CTGF*$^{lacZ/+}$ tended to be less than in wild type pups but did not reach significance, likely due to the variation in the percentage of proliferating β cells between individual pancreata. The variability may be due to the fact that the mice are on a mixed genetic background or due to differences in individual nutritional intake after birth. Together, these data indicate that CTGF is required for β cell proliferation specifically at e18.5. Because there were no changes in α or β cell proliferation or apoptosis before e18.5, it is likely that the changes in the ratio of endocrine cells observed earlier than e18.5 are due to alterations in endocrine cell lineage allocation. Thus, in the absence of CTGF a multipotent endocrine progenitor may preferentially differentiate into an α cell rather than a β cell.

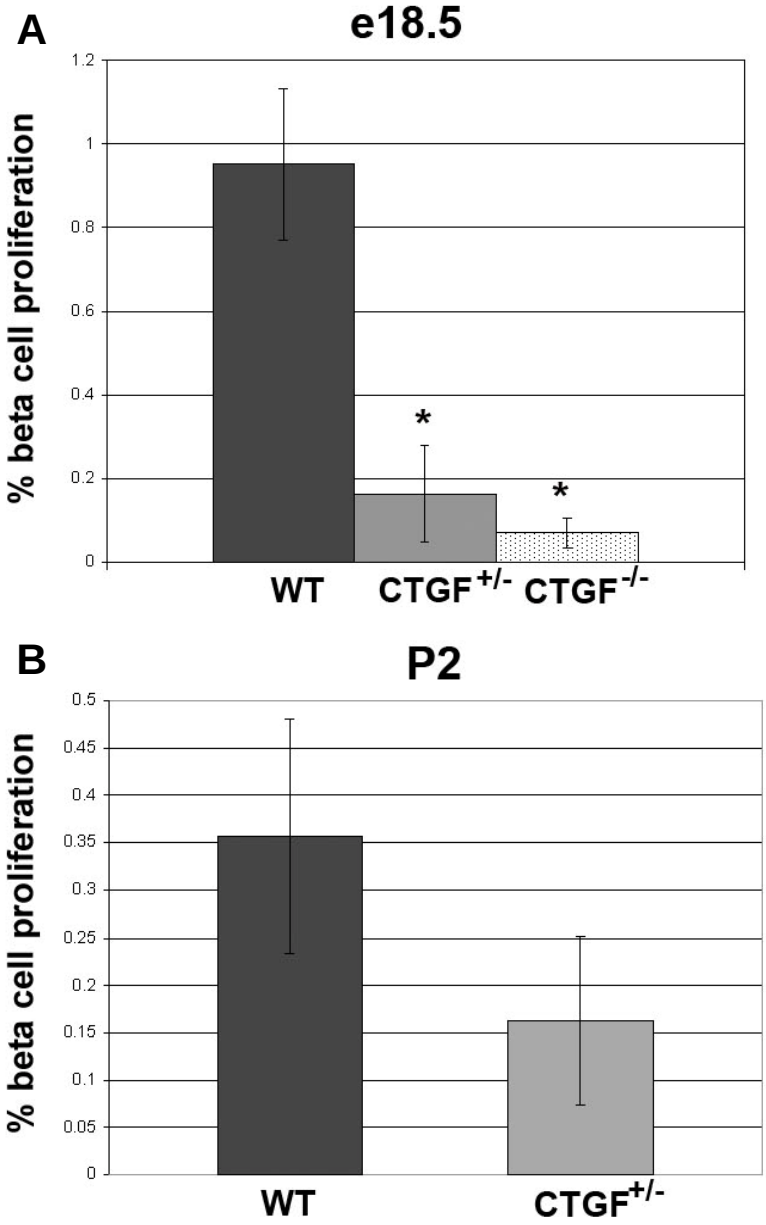


**Figure 3-3**. CTGF mutant animals have decreased β cell proliferation. (A) β cell proliferation is significantly decreased compared to wild type (WT) in $CTGF^{lacZ/+}$ ($CTGF^{+/-}$), and $CTGF^{lacZ/lacZ}$ ($CTGF^{-/-}$) pancreata at e18.5. (B) CTGF heterozygous pancreata ($CTGF^{+/-}$) trend toward a decrease in β cell proliferation at P2, but this does not reach statistical significance. n=3. *p<0.05 compared with WT. Figure reproduced from: Connective tissue growth factor (CTGF) inactivation leads to defects in islet cell lineage allocation and β cell proliferation during embryogenesis, Crawford et al. *Mol Endo* 23(3)324-336, *Copyright 2009, The Endocrine Society*.

**CTGF mutant embryos have disrupted islet morphogenesis**

During development, endocrine progenitor cells initially reside within the ductal epithelium but delaminate and differentiate into endocrine cells. At e17.5, newly differentiated endocrine cells are located close to the ductal epithelium, but beginning at e18.5 and continuing after birth they organize into islets within the acinar parenchyma [122,193]. HNF6 transgenic islets have alterations in islet morphogenesis; islets appear disorganized and remain closely apposed to the ductal epithelium instead of separating away from the ducts [135,136]. Because CTGF is downregulated in HNF6 transgenic islets, duct and islet morphology were examined in *CTGF*$^{lacZ/lacZ}$ embryos (Figure 3-4). Cytokeratin (a pan-keratin marker of ductal structures) and synaptophysin (a general marker of endocrine tissues) co-labeling of wild type and mutant pancreata revealed that endocrine clusters in *CTGF*$^{lacZ/lacZ}$ were very closely apposed to the ductal epithelium at e18.5. Thus, similar to the HNF6 transgenic embryos, CTGF null embryos have defects in islet formation.

**CTGF heterozygous adults display compensatory β cell hypertrophy**

Studies of adult pancreas structure and function were limited to CTGF heterozygous animals. The size and gross morphology of CTGF heterozygous pancreata were normal at all ages examined; however analysis of pancreatic tissue from adult *CTGF*$^{lacZ/+}$ animals revealed alterations in islet architecture. Some islets exhibited morphology indistinguishable from wild type islets; while some islets within *CTGF*$^{lacZ+}$ pancreata had a mixed-islet phenotype, with α cells found within the β cell core [188]. Morphometric analysis of pancreata from 3 month old wild type and CTGF heterozygous mice revealed a disrupted endocrine cell composition in *CTGF*$^{lacZ+}$ animals. In wild type animals, only 5% of the islet area was occupied by α cells, whereas in *CTGF*$^{lacZ+}$ animals

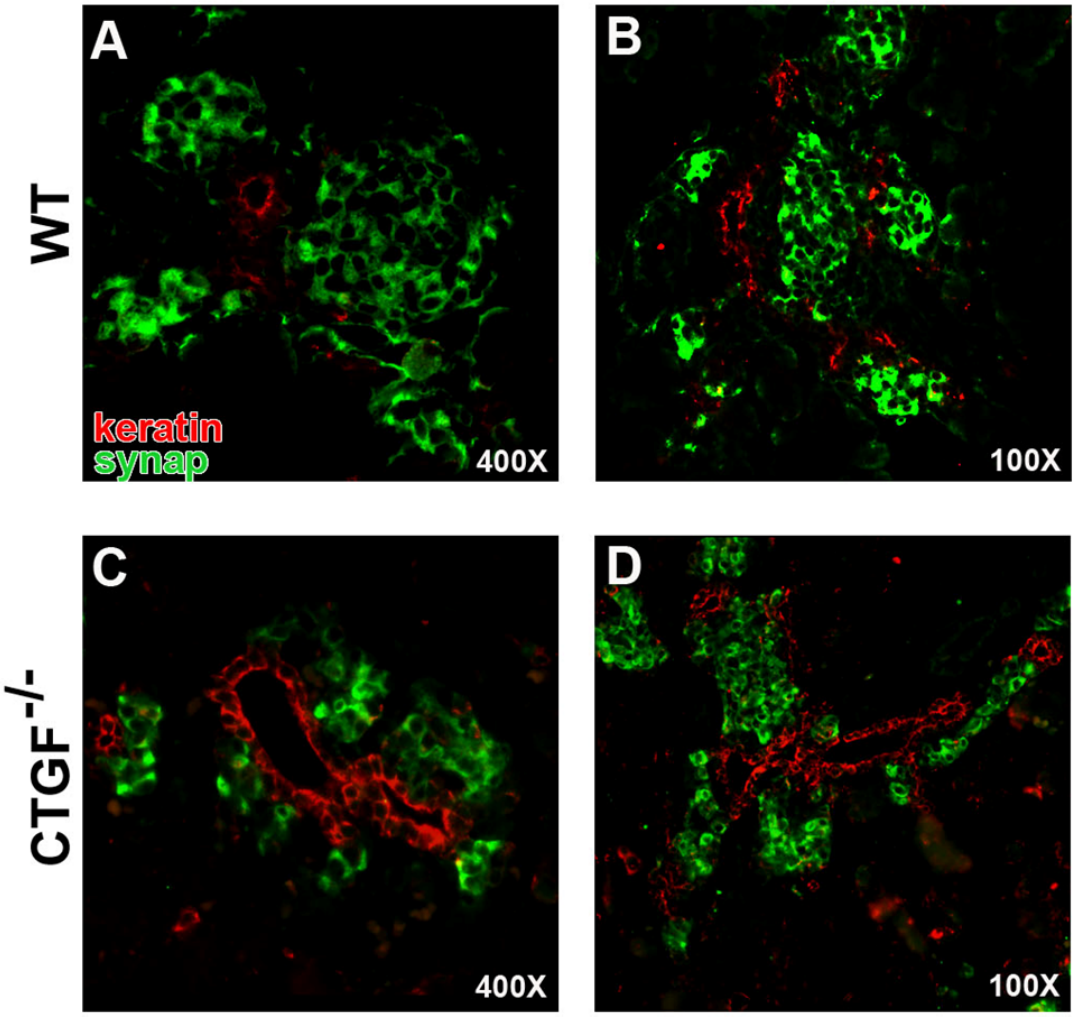


**Figure 3-4**. CTGF null embryos display islet dysmorphogenesis. Cytokeratin (keratin, red) and synaptophysin (synap, green) labeling of e18.5 WT (A and B) and CTGF null (C and D) pancreata revealed that endocrine tissue in CTGF null embryos is more closely apposed to the ductal epithelium than in WT pancreata. Figure reproduced from: Connective tissue growth factor (CTGF) inactivation leads to defects in islet cell lineage allocation and β cell proliferation during embryogenesis, Crawford et al. *Mol Endo* 23(3)324-336, *Copyright 2009, The Endocrine Society*.

α cells make up nearly 15% of islet area (Figure 3-2C). Despite the increase in α cell area, the area occupied by β cells was not different between wild type and *CTGF*$^{lacZ+}$ adult animals; thus *CTGF*$^{lacZ+}$ animals showed an overall larger average islet size compared with wild type [188]. Analysis of the other endocrine cell types revealed no detectable difference in the proportion of δ cells and a slight increase in the number of PP cells in *CTGF*$^{lacZ/+}$ animals compared with wild type (data not shown).

In order to understand why insulin positive area was decreased at e18.5 but normal at 3 months of age, β cell proliferation was quantified at 3 months of age to determine if proliferation in *CTGF*$^{lacZ+}$ animals was increased compared to controls. There was no significant difference in the percentage of proliferating β cells between control and CTGF heterozygous animals at this stage (data not shown). Therefore we hypothesized that the normalization of insulin positive area might be due to an increase in individual cell size. Two independent methods were utilized to quantify β cell size. The first involved measuring the total insulin positive area of the islet and dividing it by the total number of β cell nuclei in the islet. The second involved co-immunolabeling for insulin and the membrane-localized glucose transporter (Glut2) and determining the area of individual β cells. Both methods yielded similar results; the β cells of CTGF heterozygotes were significantly larger than β cells in wild type animals, suggesting that β cell hypertrophy contributes to the increase in β cell area that occurs between e18.5 and 3 months of age (Figure 3-5). Cell hypertrophy in response to an absolute decrease in β cell number has been observed in other models of decreased β cell proliferation [194]. However, these results do not exclude the possibility that β cell proliferation also contributes to the restoration of β cell mass postnatally in CTGF heterozygous animals.

The ability of CTGF heterozygous animals to respond to a glucose challenge was also examined. *CTGF*$^{lacZ/+}$ adults had normal fasting blood glucose levels and glucose

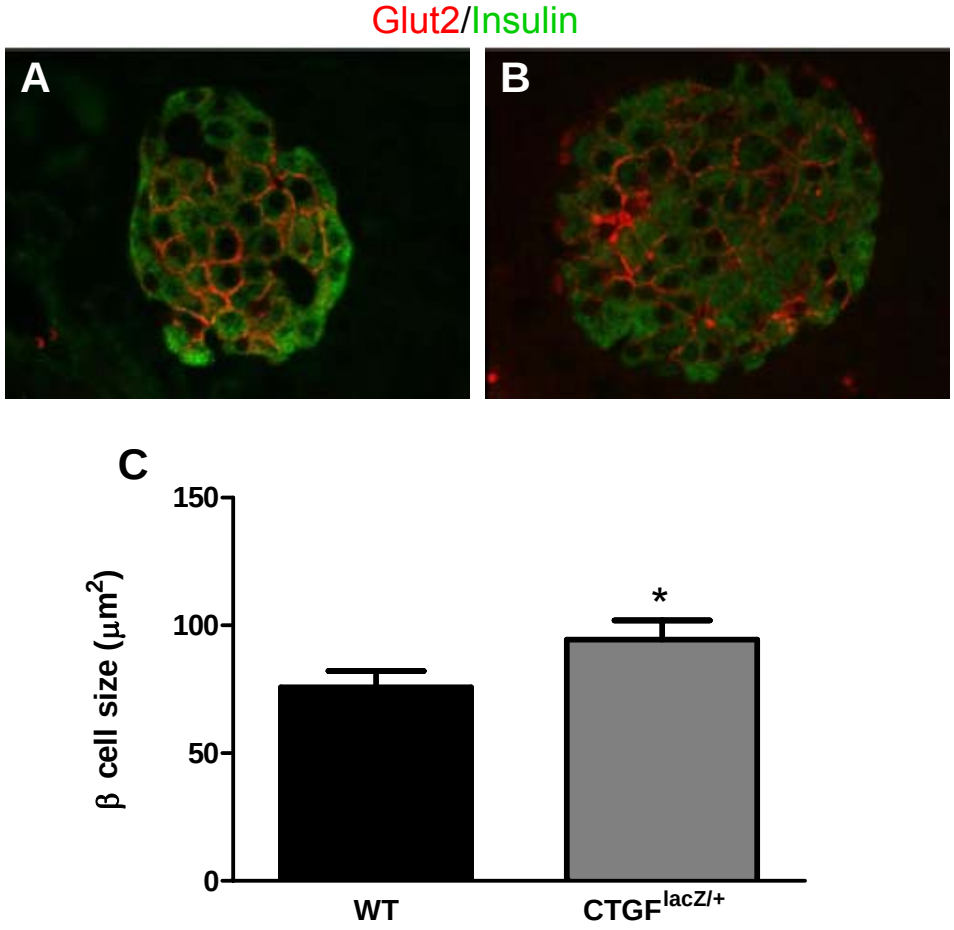


**Figure 3-5**. β cell hypertrophy in *CTGF*$^{lacz/+}$ islets. β cell size was measured by labeling for Glut2 (red) and insulin (green) in control (A) and CTGF heterozygous adults (B). The average size of β cells in both genotypes was quantified (C). *p<0.05. n=3 animals of each genotype. Modified from Crawford et al. *Mol Endo*, 2009.

clearance (data not shown). Wild type and *CTGF*$^{lacZ/+}$ adults were also placed on a high-fat diet for 12 weeks to determine if they are more susceptible to developing defects in glucose homeostasis under conditions of metabolic stress. Similar to mice that are on a chow diet, *CTGF*$^{lacZ/+}$ animals did not display any alterations in glucose tolerance, suggesting that a 50% reduction in CTGF levels does not affect glucose homeostasis [188]. However, these results do not rule out the possibility that CTGF null mice may display defects in adult β cell proliferation or glucose homeostasis that was not revealed by these assays.

## Discussion

This study represents the first report examining the function of CTGF in the developing pancreas. CTGF expression is elevated in pancreatic diseases such as cancer and pancreatitis and contributes to pancreatic tumor growth and metastasis, but prior to this study, nothing was known about its potential role in pancreas development and function. Analysis of the CTGF null phenotype demonstrated that CTGF is required for proper islet cell composition, β cell proliferation at e18.5, and islet morphogenesis. The fact that endocrine composition is changed beginning at the secondary transition without concomitant changes in total endocrine area, islet size, α or β cell proliferation, or apoptosis at that stage, suggests that CTGF is also required for the proper allocation of endocrine progenitors to the α and β cell fates.

Although there have been several factors which have been identified to be required for postnatal β cell proliferation, there are few genes known to affect the proliferation of embryonic β cells. Thus, CTGF can be added to the small list of genes, which include the transcription factor *Pdx-1* and the eIF2α kinase PERK, that have been

shown to be required for embryonic β cell proliferation *in vivo* [115,195]. The fact that CTGF null embryos have a defect in β cell proliferation specifically at e18.5 suggests that CTGF may have a unique time window of action which correlates with the increase in endocrine cell proliferation that normally occurs at late gestation.

The fact that CTGF mutants have defects in the separation of the endocrine tissue from the ductal epithelium suggests that CTGF may also be involved in promoting the migration of pancreatic epithelial cells during islet morphogenesis. CTGF could affect cell migration directly or could be required for extracellular matrix remodeling or for changes in cell adhesion, although these possibilities are not mutually exclusive.

During development, CTGF is expressed in pancreatic ducts, vasculature, and β cells. Therefore, it is unclear from the analysis of CTGF null embryos which cell type(s) is responsible for the defects in pancreas development. It is possible that the endocrine phenotype is due to paracrine effects of loss of CTGF from the ducts or vasculature. Early pancreatic endocrine cells delaminate from the ductal epithelium and previous studies have shown that blood vessel endothelial cells are a source of developmental signals promoting endocrine differentiation [26,27,196,197]. Cell-type specific inactivation of CTGF using a conditional allele will reveal which cellular source of CTGF functions to promote proper lineage allocation, β cell proliferation, and islet morphogenesis. Furthermore, conditional inactivation of CTGF specifically from β cells in the adult will be useful to determine whether CTGF is required for adult β cell proliferation and function without the confounding effects of embryonic lethality or potential changes in peripheral insulin sensitivity that could occur in the global knockout.

Microarray analysis revealed a 2-fold reduction in CTGF expression in the HNF6 transgenic model of islet dysmorphogenesis and diabetes [137]. Recently, the *CTGF* promoter was shown to be directly repressed by the pro-endocrine transcription factor,

Ngn3 [198]. Ngn3 expression is increased in the HNF6 model, which may partially account for the decrease in CTGF expression in these transgenic mice. Interestingly, analysis of CTGF mutant animals showed an islet phenotype strikingly similar to the HNF6 transgenic phenotype. Both animal models have an increase in $\alpha$ cells at e18.5 and endocrine tissue which is closely apposed to ducts. These similarities suggest that the downregulation of CTGF may be at least partially responsible for the phenotype of the HNF6 transgenic mice. While HNF6 and Ngn3 may lie upstream of CTGF, it remains unknown which factors and signaling pathways are downstream of CTGF during pancreas development. CTGF has been shown to interact with integrins, TGF-$\beta$, BMPs and Wnts in other systems [199]. The phenotypes of inactivating integrin or TGF-$\beta$ signaling are similar to the phenotype of CTGF inactivation suggesting that CTGF may act by enhancing integrin or TGF-$\beta$ signaling. Blocking integrin activity in human fetal pancreas explants inhibits the separation of endocrine cells from ducts and leads to an increase in glucagon producing cells [129]. Additionally, inducible over-expression of Smad7, a TGF-$\beta$ and BMP inhibitor, during pancreas development alters the ratio of endocrine cells with a decrease in insulin-positive cells and an increase in glucagon-positive cells [108]. On the other hand, data from our lab indicates that $\beta$-catenin, a downstream effector of activated Wnt signaling, is mislocalized in CTGF mutants. In control pancreata, $\beta$-catenin is localized to the plasma membrane; however, in CTGF mutants, there is a decrease in $\beta$-catenin at the cell membrane although the total level of $\beta$-catenin is not changed. We hypothesize that this is due to increased $\beta$ catenin in the nucleus and that Wnt signaling may be activated in CTGF mutants. [188]. Thus, the identification of the specific signaling pathways CTGF modulates during pancreas development requires further investigation.

# CHAPTER IV

## EXAMINING THE ROLE OF CTGF IN CELL PROLIFERATION, DIFFERENTIATION, AND MIGRATION USING *EX VIVO* CULTURE METHODS

### Introduction

Analysis of the CTGF global knockout phenotype demonstrated that CTGF is required for proper lineage allocation, β cell proliferation, and islet morphogenesis during pancreas development. It is unclear from these previous studies how CTGF acts to promote these processes at the cellular and molecular level. Therefore, we sought to identify the signaling pathways with which CTGF interacts to regulate lineage allocation and β cell proliferation and to determine the cellular mechanism underlying the defects in islet morphogenesis in CTGF null embryos. We employed *ex vivo* culture assays to address each of these questions.

### CTGF participates in a positive feedback loop with TGF-β

Although a CTGF-specific receptor has not been identified, CTGF exerts its downstream effects by interacting with integrins and modulating signaling pathways such as TGF-β, BMP, and Wnt. However, it is not known which of these interactions play a role during pancreas development. CTGF modulates signaling through several integrins including $\alpha_V\beta_3$. Interestingly, treatment of human fetal pancreas explants with cyclic RGD peptide analogues to inhibit $\alpha_V$ integrins prevents the separation of endocrine cells from ducts and leads to an increase in glucagon-producing cells [129]; a phenotype similar to what is observed in the CTGF loss-of-function model. However, the interactions between

CTGF and the TGF-β pathway are the most well characterized in other cell types; therefore we chose to focus our initial studies on the TGF-β pathway.

TGF-β mediates its effects by binding to two distinct cell surface type II and type I serine/threonine kinase receptors and initiating intracellular signaling cascades (Figure 4-1). Type II receptors are constitutively active and phosphorylate and activate type I receptors upon ligand binding. The activated receptor complex phosphorylates TGF-β receptor Smads (R-Smads) 2 and 3 which interact with the Co-Smad, Smad4. The phosphorylated R-Smad-Smad4 complex is localized to the nucleus where it binds to GC rich sequences and acts with other co-factors to either activate or repress gene expression. Smad7 is an inhibitory Smad that negatively regulates TGF-β signaling by interfering with Smad-receptor or Smad-Smad interactions [102]. CTGF enhances TGF-β signaling by physically binding TGF-β ligands extracellularly through its cystine rich domain and CTGF may also suppress the transcription of Smad7 [200,201]. Additionally, TGF-β regulates the expression of CTGF, as numerous studies have shown that CTGF is upregulated in response to the addition of TGF-β in a variety of cell types. A TGF-β/Smad response element has been identified in the CTGF promoter, further supporting the idea that CTGF is an immediate early gene in response to TGF-β signaling [202].

Multiple studies have indicated that members the TGF-β signaling family are important for pancreas development. In the pancreas, TGF-β isoforms are expressed in β cells as well as the ductal epithelium. The Type II TGF-β receptor localizes to ducts in the adult mouse suggesting that TGF-β may play a role in the normal regulation of the growth and differentiation of the ducts [203]. Several studies suggest that TGF-β signaling in the pancreas promotes the development of the endocrine compartment and limits the development of exocrine tissue. Over-expression of TGF-β under the direction of the insulin promoter resulted in pancreatic hypoplasia and a relative decrease in

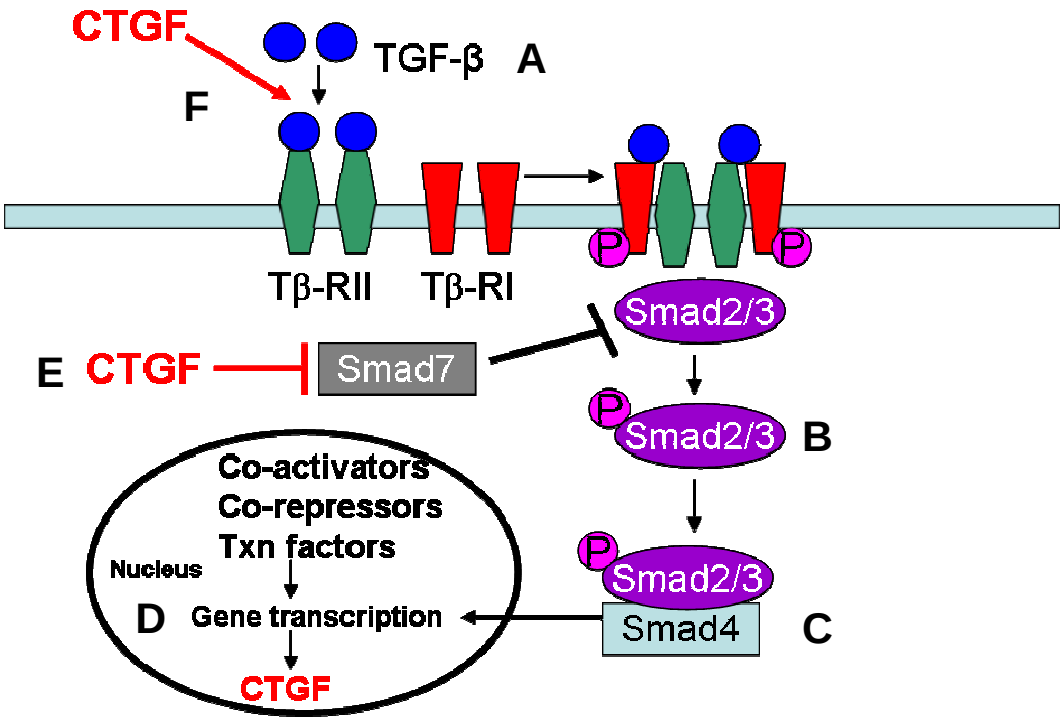


**Figure 4-1**. CTGF participates in a positive feed back loop with TGF-β signaling. (A) TGF-β binds its type II receptor which recruits a type I receptor. (B) The activated receptor complex phosphorylates Smad2/3. (C) Smad2/3 dimerizes with Smad4 and the complex translocates to the nucleus affecting transcription. (D) CTGF expression is increased in response to TGF-β. (E) CTGF reinforces TGF-β signaling by inhibiting the expression of Smad7, an inhibitory Smad. (F) CTGF also enhances the binding of TGF-β to its receptor.

acinar tissue [204]. Interestingly, the islet phenotype observed when certain TGF-β signaling components are mutated bears striking similarity to the defects in the islet phenotype of CTGF mutants. Mice deficient in GDF11, a TGF-β ligand, have reduced numbers of β cells and increased numbers of α cells [109] and pancreas-wide over-expression of Smad7 also leads to an altered α to β cell ratio [108]. Additionally, Smad7 transgenic mice have clusters of glucagon positive cells that are not associated with islets. The evidence of the link between CTGF and TGF-β signaling combined with similarities in their islet phenotypes suggests that CTGF may modulate TGF-β during pancreas development and that the defects in CTGF global knockouts may be due to impaired TGF-β signaling. However, this hypothesis has not been previously been tested.

Pancreatic bud culture systems have been used to investigate the role of TGF-β in pancreas development. Embryonic pancreata grown in 3-D collagen gels are able to differentiate into exocrine tissue and endocrine tissue organized into islet-like structures. Treatment of pancreatic bud cultures with exogenous TGF-β leads to an increase in the proportion of endocrine tissue and the addition of a TGF-β neutralizing antibody to the cultures disrupts the morphogenesis of the explants [104,130]. We utilized this system to investigate whether CTGF interacts with TGF-β during pancreas development. We hypothesized that if CTGF both enhances TGF-β signaling and is a downstream effector of TGF-β, it may be required for the pro-endocrine effects of TGF-β on the epithelium (Figure 4-2). Therefore, wild type and CTGF global null pancreatic buds were cultured in the presence of TGF-β and endocrine development was examined. In contrast to previous results, treatment of wild type pancreatic bud explants with TGF-β did not increase the proportion of endocrine tissue in the buds in our experiments. Thus, we were unable to determine whether CTGF interacts with TGF-β using this system.

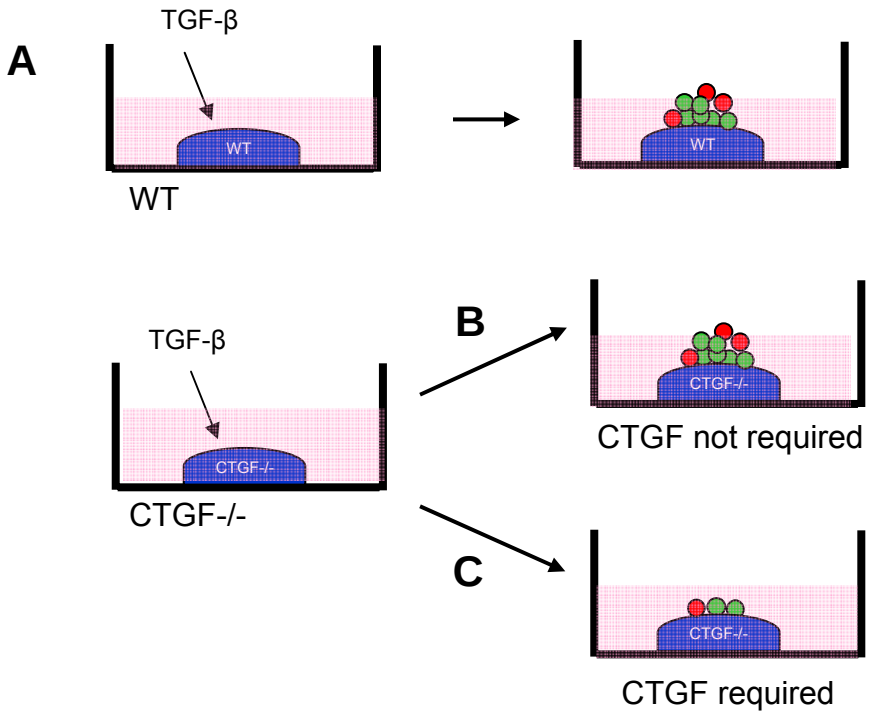


**Figure 4-2**. Schematic of bud culture experiments. (A) TGF-β has been shown to increase endocrine mass when added to wild type (WT) buds. (B) If TGF-β has the same effect on WT and CTGF null ($CTGF^{-/-}$) buds, CTGF is not acting downstream of TGF-β. (C) If TGF-β has less of a pro-endocrine effect on CTGF null buds, CTGF may be acting downstream of TGF-β.

**CTGF regulates cell migration and adhesion**

CTGF has been shown to affect the processes of cell migration, adhesion, and extra cellular matrix remodeling in other tissue types. For example, pancreatic stellate cells, skin fibroblasts, and human vascular endothelial cells (HUVECs) display increased adhesion when plated on CTGF [145,146,159]. CTGF also stimulates both directional and non-directional migration of multiple cell types. Over-expression of CTGF in vascular smooth muscle and chondrosarcoma cells increases migration and MMP activity, suggesting that the degradation of extracellular matrix in response to CTGF may contribute to changes in cell motility [161,205]. A recent study demonstrated that CTGF expression induces migration and metastasis of gastric carcinoma cells in nude mice [206]. The effects of CTGF on adhesion and migration are attributed, in part, to its interactions with specific integrins and heparin sulfate proteoglycans. Adhesion of skin fibroblasts to CTGF involves formation of $\alpha 6\beta 1$-containing focal adhesion complexes and activation of focal adhesion kinase (FAK) and Rac [162]. On the other hand, CTGF-$\beta 3$ integrin interactions have been shown to stimulate dephosphorylation of FAK and focal adhesion and cytoskeleton disassembly leading to mesangial cell migration [163]. These data combined with the defects in islet morphogenesis in CTGF null mutants led us to ask whether CTGF affects migration or adhesion of cells in the developing pancreas. We performed migration and adhesion assays on cells isolated from e18.5 pancreata in the presence or absence of exogenously added CTGF. Our data indicate that CTGF does not promote migration in our system; however, preliminary data suggests that CTGF may promote cellular adhesion. Additionally, CTGF null cells did not appear to have defects in migration.

## Results

### Examining the role of CTGF in TGF-β-mediated endocrine differentiation and proliferation

We hypothesized that if CTGF acts downstream of the TGF-β pathway during pancreas development, it may be required for the effects of TGF-β on endocrine differentiation and proliferation (Figure 4-2). To determine whether CTGF null pancreas responds similarly to wild type pancreas to treatment with exogenous TGF-β, pancreatic buds were dissected at e12.5 from litters containing wild type, CTGF heterozygous, and null embryos. Pancreata were cultured in 3D collagen I gels with or without the addition of 5 ng/ml TGF-β. In culture, development of the buds resembles pancreas development *in vivo*; buds grow, undergo branching, and develop exocrine and endocrine tissue (Figure 4-3). After 7 days in culture, the explants were fixed and immunolabeled in whole mount for insulin and glucagon. Confocal microscopy was used to optically section and image the buds. The proportion of the total epithelial area which was composed of insulin and glucagon positive tissue was then quantified using Metamorph software.

Previous studies indicated that the addition of TGF-β to cultured pancreatic buds led to an increase in the proportion of the bud that was composed of endocrine tissue [104]. In our cultures, although the mesenchyme exhibited morphological changes with the addition of 5 ng/ml of TGF-β, we did not detect a significant increase the amount of endocrine tissue or any changes in the percentages of insulin or glucagon-positive area in wild type buds (Figure 4-4). The fact that the pancreatic mesenchyme displayed morphological changes suggested that the TGF-β was biologically active; however it is possible that the TGF-β was unable to diffuse into the center of the culture. In order to monitor endocrine development in real-time throughout the culture period without having to first fix and immunolabel the buds, e12.5 pancreas buds were cultured from mice

**Insulin/Glucagon**

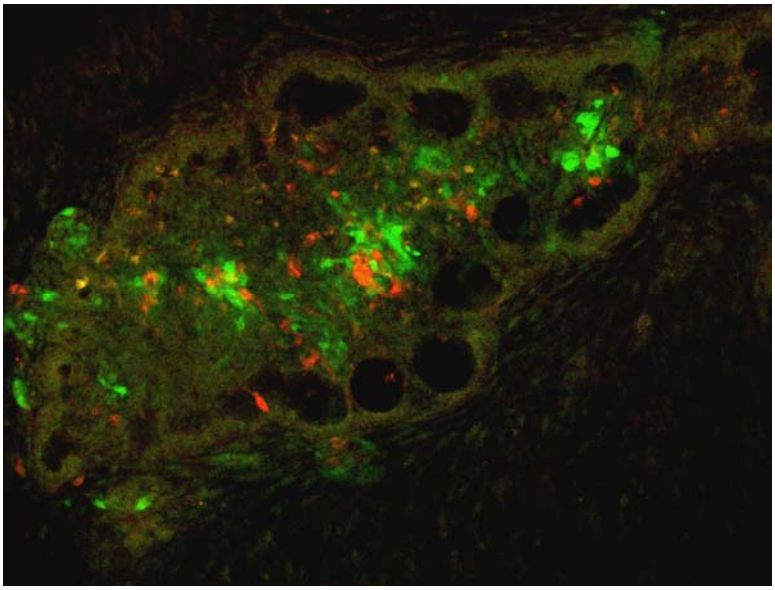

**Figure 4-3**. Pancreatic explants develop normally in culture. An optical section taken through a pancreatic bud that was dissected at e12.5, cultured for 7 days, and stained in whole mount for insulin (green) and glucagon (red).

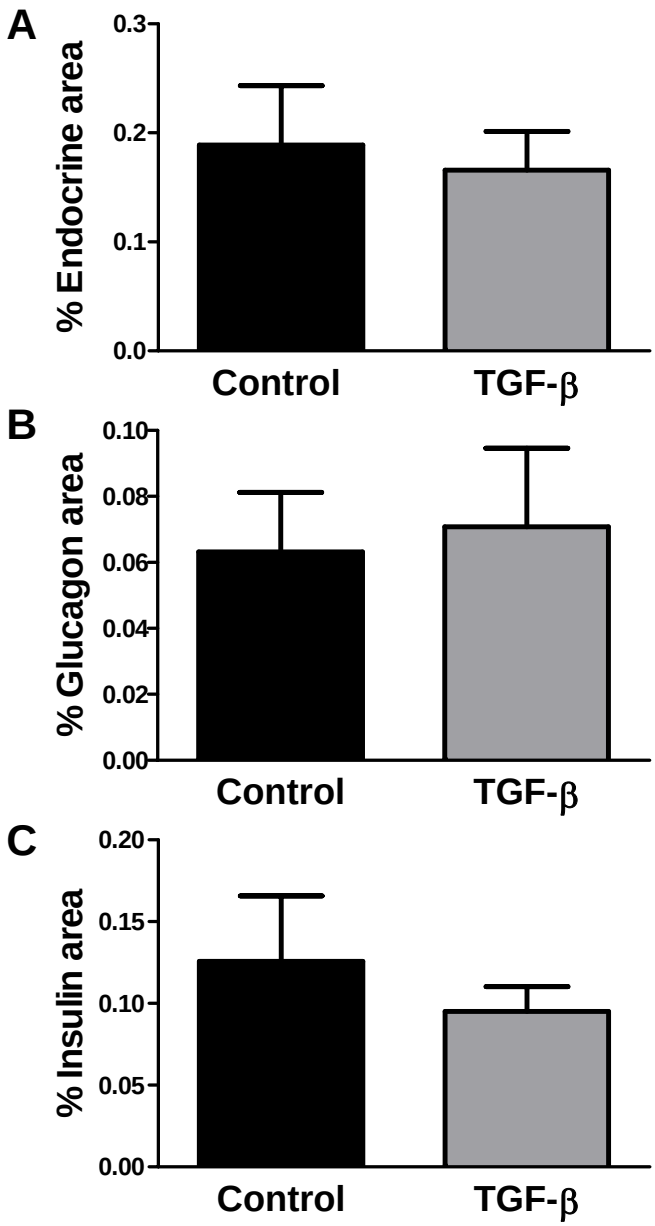


**Figure 4-4**. TGF-β does not have a pro-endocrine effect on pancreatic bud cultures. Wild type pancreatic explants treated with TGF-β show no difference compared to untreated buds (control) in the percentage of the total bud area composed of endocrine tissue (A), insulin-positive area (B) or glucagon positive-area (C). Buds were dissected at e12.5 and cultured in the presence or absence of 5 ng/ml TGF-β for 7 days. n=4 control and 5 TGF-β treated.

expressing green fluorescent protein (GFP) from the *mouse insulin I* promoter (*MIP-GFP*) [190]. The *MIP-GFP* buds developed normally during the 7 day culture period, and it was possible to repeatedly visualize GFP expression in the same bud after 2 days in culture (Figure 4-5A). The β cells (GFP-positive cells) in explants treated with TGF-β appeared more scattered compared to untreated *MIP-GFP* pancreas buds (Figure 4-5B), suggesting that the TGF-β was indeed active within the epithelium. However, it did not appear that the number of β cells was increased compared to untreated buds, indicating that similar to our previous experiments, TGF-β did not have a pro-endocrine effect on pancreatic explants in our hands. Because TGF-β did not increase the amount of endocrine tissue in wild type buds, we were unable to use this assay to further examine the effect of TGF-β on CTGF null buds.

**CTGF does not promote migration of e18.5 pancreatic cells**

In order to determine whether CTGF affects the migration of embryonic pancreatic cells, migration assays were performed using dissociated pancreata from embryos at e18.5, the stage at which islet morphogenesis occurs. Cells were plated on transwell filter inserts that were coated on the underside with BSA as a control or collagen IV, which has been previously shown to stimulate pancreatic cell migration (Dr. Vincenzo Cirulli, University of Washington, personal communication). Increasing concentrations of recombinant human CTGF were added to the media below the transwells. After 24 hours, the cells that migrated to the underside of the transwells were fixed and the cells on the upperside of the transwells were removed. The inserts were stained with toluidine blue and the number of cells that had migrated to the underside of the transwell was quantified. Compared to BSA, 10 μg/ml collagen IV significantly stimulated pancreatic cell migration (Figure 4-6A). This data is consistent with previous

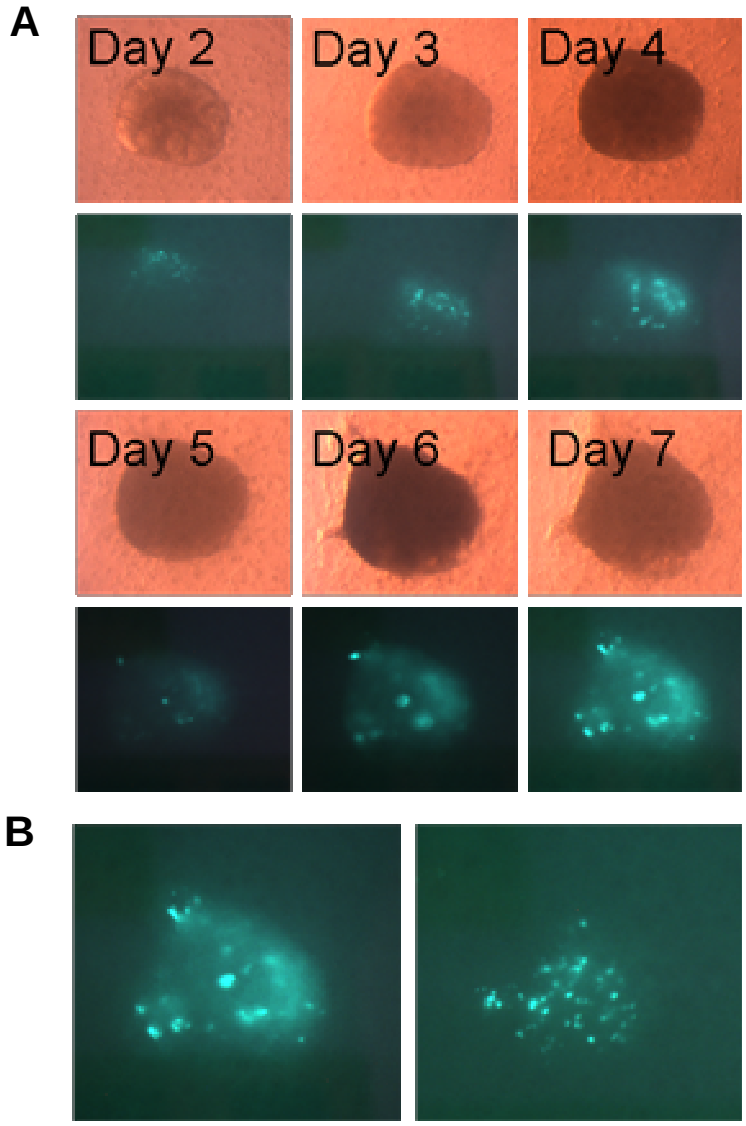


**Figure 4-5**. (A) Time course of *MIP-GFP* buds cultured for 7 days in collagen gels. The GFP image is below the corresponding brightfield image for each day. (B) *MIP-GFP* buds cultured for 7 days with 5 ng/ml TGF-β (right) showed scattered GFP positive cells compared to untreated buds (left), but no difference in the total number of GFP positive cells.

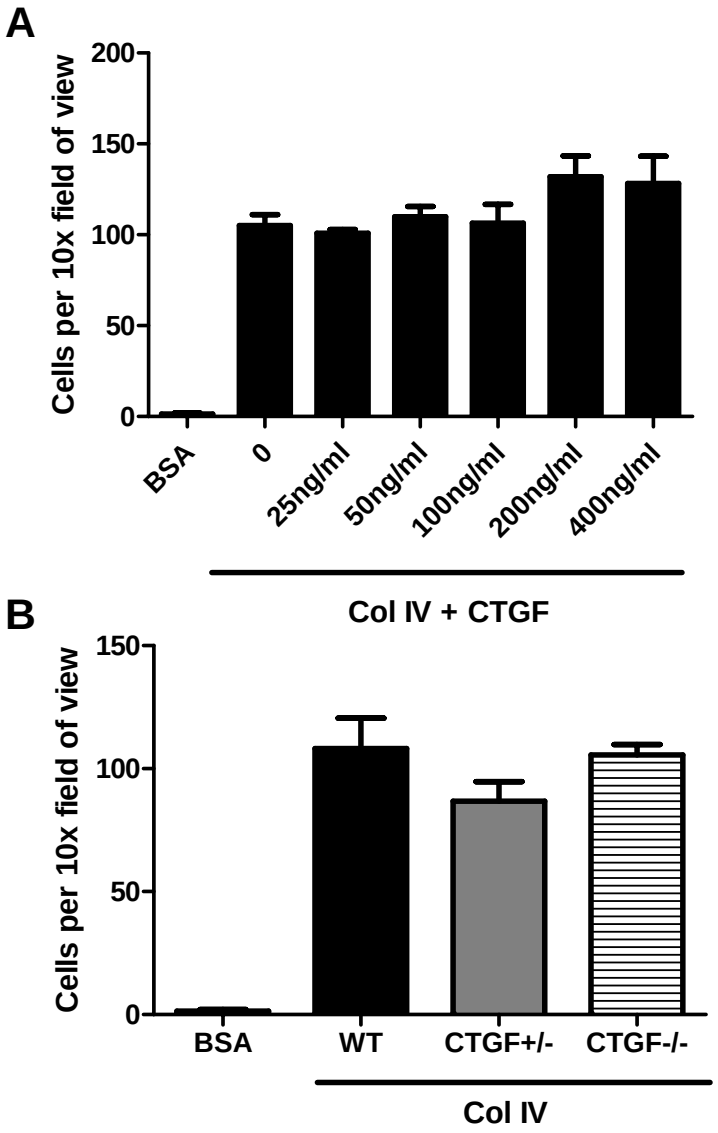


**Figure 4-6.** Migration assays using cells from e18.5 pancreata. (A) CTGF did not stimulate migration. Wild type cells were plated either on transwells coated with BSA or 10 µg/ml collagen IV (Col IV). Increasing concentrations of CTGF were added to the media below the transwells. The dose response curve was repeated twice. (B) Wild type (WT), CTGF heterozygous (CTGF+/-) or CTGF null (CTGF-/-) cells were plated on col IV coated transwells. No significant difference in migration was seen between the WT and CTGF-/- cells. At least 3 wells per genotype were used.

reports that collagen IV stimulates migration of embryonic pancreatic cells and suggests that the cells in our system were capable of migration. However, the addition of increasing concentrations of CTGF to the culture media did not significantly stimulate migration (Figure 4-6A). It may be that migration is maximally stimulated by collagen IV and CTGF is unable to induce any further migration. To address this possibility, cells were plated on uncoated transwells and increasing concentrations of CTGF were added to the media below the filter. Very few cells migrated on uncoated wells, and the addition of CTGF did not enhance migration (data not shown). These results suggest that in this system, CTGF does not appear to act as a chemoattractant for pancreatic cells. It should be kept in mind, however, that the cells used for these assays are a heterogeneous mixture of all cell types found in the pancreas and it is unclear exactly which cell types are migrating in this assay.

While the addition of exogenous CTGF may not be sufficient to stimulate migration in vitro, endogenous CTGF may still be required for pancreatic cell migration. To determine if cells lacking CTGF have defects in migration, dissociated pancreatic cells from wild type, CTGF heterozygous, and CTGF global null embryos were plated on transwells coated with collagen IV. No significant difference in migration was seen between the different genotypes, suggesting that CTGF is not required for pancreatic cells to migrate on a collagen IV matrix (Figure 4-6B).

Finally, we asked whether exogenous CTGF affects the adhesion of pancreatic cells. Dissociated cells from e18.5 pancreata were plated in well plates coated with BSA or increasing concentrations of CTGF and incubated for 1 hour. The non-adherent cells were removed and those that remained attached to the plate were quantified. Preliminary data suggest that CTGF may stimulate adhesion of pancreatic cells, which is consistent with data indicating that CTGF stimulates adhesion of other cell types (Figure 4-7).

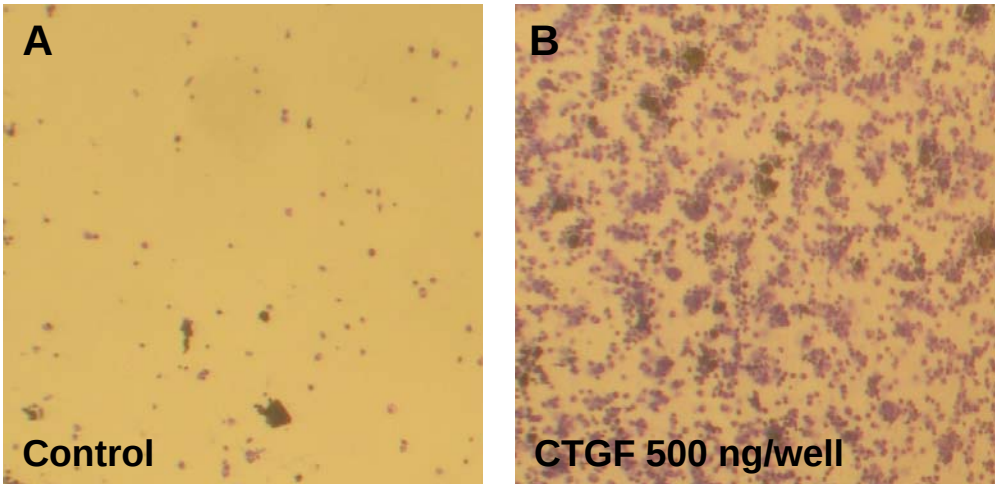


**Figure 4-7**. Preliminary data indicates that CTGF may promote adhesion of dissociated pancreatic cells. Cells from e18.5 wild type pancreata were plated in wells that were coated with buffer (A) or 500 ng/well CTGF (B). After 1 hour, cells that were non-adherent were washed away, and the wells were stained with toluidine blue to stain all remaining cells. n=3 wells per treatment, the experiment was performed once.

## Discussion

### Exogenous TGF-β did not promote endocrine differentiation or proliferation *in vitro*

In our assays, exogenous TGF-β did not expand the endocrine population in cultured pancreata. The reason for the differing results between these studies and those of Sanvito et al. is not known. There may be slight differences in culturing conditions between the two studies which are not discernable by examining the published methods described in the previous study. Alternatively, since there are not many studies published reporting a pro-endocrine role for TGF-β *in vitro*, perhaps this study is not reproducible. The reports in the literature describing the role of TGF-β signaling components in pancreas development are seemingly contradictory. Although there is data which supports the idea that TGF-β signaling promotes endocrine and β cell development [108,109], other data indicates that TGF-β signaling has no effect on the endocrine compartment and may be more important for duct and acinar development [107,204,207]. Furthermore, some studies demonstrate that TGF-β signaling actually may act to limit the amount of endocrine tissue [107]. Thus, it is likely that the role of TGF-β in pancreas development is determined by timing, level of activation, and the specific ligands and receptors involved.

Future studies will be aimed at understanding the signaling pathways with which CTGF interacts to regulate pancreas development. To determine whether CTGF enhances TGF-β signaling during pancreas development *in vitro*, pancreatic bud cultures could be treated with recombinant human CTGF and the activation of TGF-β could be assessed by examining the expression of phosphorylated Smad2/3 or known TGF-β target genes. Alternatively, the relationship between CTGF and TGF-β could be

examined *in vivo*. The global null allele of CTGF could be interbred to mice carrying a transgenic reporter allele containing a Smad binding element to assay for TGF-β activation in CTGF null embryos. If CTGF normally acts to enhance TGF-β activity, we would expect to see reduced reporter activation in the CTGF null embryos. We have also generated a transgenic mouse model of inducible CTGF over-expression specifically in β cells (see Chapter VI). Over-expression of CTGF during development leads to increased endocrine proliferation and endocrine mass. The mechanisms leading to the increase in endocrine proliferation could also be examined by interbreeding CTGF over-expressing mice to the TGF-β reporter mice to determine whether increased levels of CTGF enhances TGF-β activity [208]. Preliminary data from our lab indicates that β-catenin, an effector of Wnt signaling, may be mislocalized in CTGF null pancreata, suggesting that loss of CTGF may affect Wnt signaling. To determine if CTGF inhibits Wnt signaling in the pancreas, CTGF over-expressing mice could be interbred with mice carrying a reporter allele containing a multimerized LEF/TCF binding site driving lacZ expression [209]. Similarly, to examine whether BMP signaling is altered in CTGF over-expressing pancreata, a BMP reporter allele could be used [210,211].

**CTGF does not act as a chemotactic factor during pancreas development**

Analysis of CTGF global null embryos indicated that CTGF is required for separation of endocrine tissue from the ductal epithelium. We hypothesized that the defects in islet morphology may be due to defects in pancreatic cell migration. Therefore, migration assays were performed using cells isolated from wild type, CTGF heterozygous, and CTGF null pancreata. Our results suggest that CTGF null cells do not have defects in migration on transwells coated with collagen IV. Although CTGF null cells are able to migrate similarly to wild type cells on collagen IV, CTGF null cells may

have defects in signaling though integrins which do not interact with collagen IV. Therefore defects in migration may be observed if CTGF null cells are plated on transwells coated with a different ECM or a combination of matrices that more closely resembles the ECM which is present in the developing pancreas. Alternatively, a scratch assay could be used to determine if cells lacking CTGF have defects in migration independent of interactions with ECM; however this assay requires a confluent layer of cells, which do not form from dissociated pancreatic cells. Another explanation for these data is that loss of CTGF may only affect the migration of endocrine cells, which are a small proportion of the total cells in the pancreas. To determine if endocrine cells fail to migrate, the underside of the transwells could be immunolabeled with markers of endocrine cells and the number of migrating wild type and CTGF mutant endocrine cells could be quantified. Alternatively, mice carrying the CTGF global null allele could be interbred with *MIP-GFP* mice to generate CTGF null β cells which are GFP-positive. Live cell imaging of control and CTGF null explants could reveal whether lack of CTGF impairs β cell migration. Finally, the loss of CTGF may not affect the migration of pancreatic cells directly; in the absence of CTGF adhesion or extracellular matrix remodeling may be altered instead.

These studies also demonstrated that exogenous CTGF does not stimulate migration of pancreatic cells isolated from e18.5 embryos that are plated on uncoated transwells or transwells coated with collagen IV. One possible explanation for these results is that CTGF may not stimulate the migration of dissociated pancreatic cells in solution. Cell-cell or cell-ECM interactions may be necessary for CTGF to stimulate migration and they are likely disrupted during the enzymatic digestions required to dissociate the pancreas into a single cell suspension. The interactions between CTGF and MMPs also may be disrupted by the dissociation of these cells. It has previously

been shown that MMPs cleave CTGF into different fragments which retain biological activity in culture. Furthermore, these fragments have different biological activities. The full length CTGF added in our migration assays may not stimulate migration of pancreatic cells; CTGF may need to be cleaved into a form which promotes migration. Purified CTGF cleavage fragments could be added to the cultures both separately and in combination to determine if they can stimulate migration. These results may also indicate that CTGF does not act by stimulating the migration of endocrine cells. Alternatively, CTGF may promote the degradation of the extracellular matrix surrounding the endocrine cells, allowing them to migrate in a manner that is independent of CTGF.

Preliminary data indicate that CTGF may promote the adhesion of embryonic pancreatic cells. These data are surprising considering that CTGF null embryos have endocrine tissue which is more closely apposed to ducts than in wild type embryos. One might therefore hypothesize that CTGF promotes migration by inhibiting the adhesion of pancreatic cells, thus allowing them to migrate. The fact that CTGF appeared to increase the adhesive property of cells in this assay may indicate that CTGF may promote the adhesion of specific cell types, for example, ductal cells, thereby allowing endocrine cells to migrate. Immunostaining the wells with cell-type specific markers after performing an adhesion assay would indicate whether CTGF increases the adhesion of a certain cell type. We have data that demonstrates that the level of CTGF in the pancreas is important for regulating pancreas development. In our assays, the amount of CTGF added to the cultures could shift the biological activity of CTGF from decreasing adhesion to stimulating adhesion. Performing a more precise dose response curve examining CTGF amount vs. adhesion would address this question. Finally, inactivating CTGF in a cell type-specific manner using a conditional allele of CTGF (see Chapter V) along with tissue-specific Cre recombinases may provide insight to the role of CTGF in pancreatic cell adhesion. Performing adhesion assays using dissociated pancreas from

embryos with CTGF inactivated from specific cell types may reveal whether CTGF produced by a certain cell type is required for adhesion.

# CHAPTER V

## DISSECTING THE ROLE OF CTGF IN ISLET DEVELOPMENT USING CONDITIONAL GENE INACTIVATON

### Introduction

We previously determined that the secreted factor connective tissue growth factor (CTGF) is also required for embryonic β cell proliferation. *CTGF* null embryos display a significant decrease in β cell proliferation specifically at e18.5 and also have defects in pancreatic endocrine lineage allocation and islet morphogenesis [188]. In the mouse, CTGF is expressed in the developing pancreas as early as e12.5 and is localized to the developing blood vessels, β cells, ducts, and mesenchyme [188]. As development proceeds, CTGF remains highly expressed in ducts and endothelial cells but its expression decreases in β cells. By postnatal day (P) 3, CTGF is no longer detected in β cells but is still expressed in ducts and endothelial cells in adult mice and humans. CTGF null embryos die at birth due to skeletal defects, limiting analysis of the pancreatic phenotype to embryonic stages [170]. At early stages, embryos lacking CTGF show normal pancreas development but have an altered endocrine cell ratio beginning at e13.5; α cells are increased without change in total endocrine area, proliferation or apoptosis [188]. These data suggest a role for CTGF in regulating endocrine cell fate decisions specifically beginning at the secondary transition, a stage between e13 and e16 when it is thought that the majority of endocrine cells that will contribute to the mature islet are generated.

CTGF is a modular protein that acts in other systems as both an autocrine and paracrine factor via its interactions with integrins, TGF-β, BMPs, and Wnts [199].

Because CTGF is expressed by multiple cell types in the pancreas, it is not clear from our previous studies whether CTGF acts in an autocrine or paracrine manner to promote proper endocrine lineage allocation, β cell proliferation, and islet morphogenesis. We hypothesized that the various sources of CTGF may act differently to promote the cellular processes necessary for pancreas development. To determine how the various CTGF-producing cells within the pancreas contribute to its role in endocrine differentiation and proliferation, a conditional null allele of CTGF was used along with tissue-specific Cre recombinases to inactivate CTGF from the pancreatic epithelium, vasculature, or endocrine progenitors. These data indicate that CTGF acts as paracrine factor produced by endothelial cells that promotes embryonic β cell proliferation. Additionally, CTGF produced by the β cells themselves is required for β cell proliferation, making it the first autocrine regulator of embryonic β cell proliferation identified. In contrast, different CTGF sources function redundantly to promote lineage allocation and islet morphogenesis. Inactivating CTGF from the endothelial cells, epithelium, or β cells did not lead to significant alterations in the endocrine cell ratio or in islet morphogenesis, suggesting that the remaining sources of CTGF may compensate for the loss of CTGF from one source with respect to these processes.

## Results

### Characterization of a novel conditional-by-inversion (COIN) allele of CTGF

To address how CTGF-mediated autocrine and paracrine communication between the different pancreatic cell types regulates the differentiation of progenitors into endocrine cells and their subsequent proliferation and islet morphogenesis, *CTGF* was conditionally inactivated in a cell type-specific manner using a conditional-by-

inversion (*CTGF*$^{e2COIN}$) allele generated by Regeneron Pharmaceuticals (Figure 5-1) [172]. The *CTGF*$^{e2COIN}$ allele contains a GFP cassette placed within exon 2 of the CTGF locus, dividing it into two parts (Figure 5-2). In the absence of Cre recombinase, this cassette is spliced out due to the presence of splice donor and splice acceptor sites and a normal CTGF message is produced. Cre mediates the inversion of the *CTGF*$^{e2COIN}$ allele and places the GFP cassette in the correct orientation to be expressed and a polyA sequence such that the transcription of the CTGF message is terminated prematurely. The resulting protein is a fusion protein that consists of the CTGF signal sequence, a small portion of exon 2, and a transmembrane GFP. Real-time PCR was used to determine that the presence of the e2COIN intron within exon 2 did not affect CTGF message levels (Figure 5-3A). Furthermore, to determine whether splicing of the *CTGF*$^{e2COIN}$ allele introduces or alters nucleotides that may ultimately disrupt the function of the CTGF protein, the region of exon 2 separated by the *CTGF*$^{e2COIN}$ allele was PCR-amplified using cDNA from *CTGF*$^{e2COIN/e2COIN}$ pancreata. The fragment was sequenced and the nucleotide sequence was compared to the sequence of wild type CTGF. The results showed that the sequence of exon 2 in the *CTGF*$^{e2COIN}$ allele is identical to the wild type allele (data not shown). This data is consistent with previous results from Dr. Aris Economides at Regeneron Pharmaceuticals indicating that the splicing of other COIN alleles also does not introduce or alter any nucleotides within the mRNA sequence. *CTGF*$^{e2COIN/e2COIN}$ mice develop normally and do not display any overt skeletal defects, suggesting that the *CTGF*$^{e2COIN}$ allele does not act as a hypomorphic allele in the absence of Cre.

In order to confirm the Cre-dependent inversion of the *CTGF*$^{e2COIN}$ allele, *CTGF*$^{e2COIN/e2COIN}$ mice were interbred to mice expressing Cre from the *Sox2* promoter [212] (from Dr. Mark Magnuson, Vanderbilt University) to generate mice carrying a globally inverted form of the COIN allele (*CTGF*$^{e2COIN\text{-}INV}$). *CTGF*$^{e2COIN\text{-}INV}$ mice were

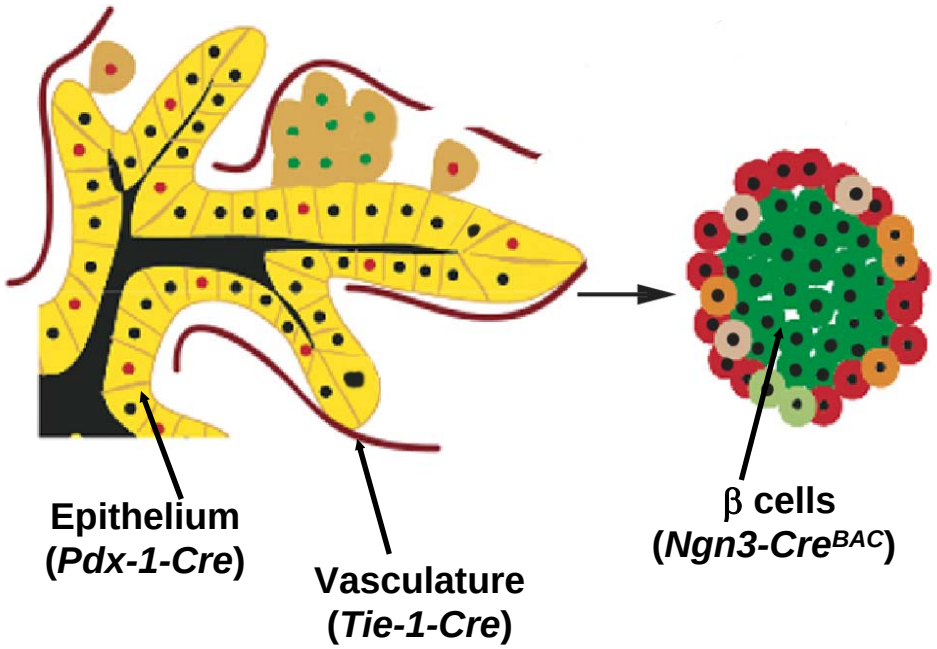


**Figure 5-1**. Schematic of tissue-specific CTGF inactivation. In order to determine which cellular source(s) of CTGF are required for lineage allocation, β cell proliferation and islet morphogenesis, a conditional allele of CTGF was bred to tissue specific Cre lines to inactivate CTGF throughout the pancreatic epithelium, vasculature, or early in the endocrine lineage.

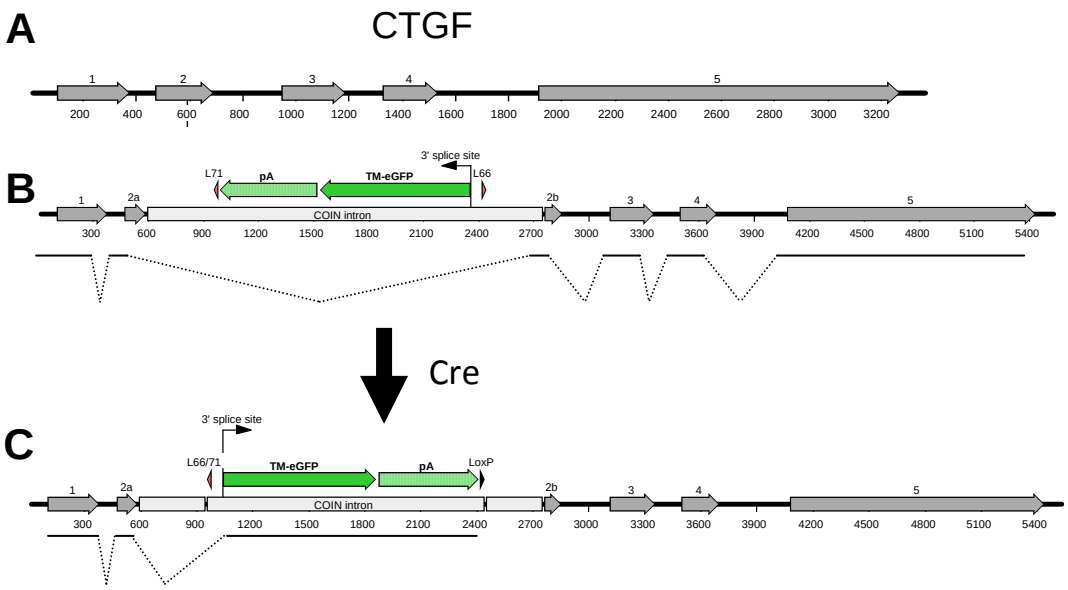


**Figure 5-2**. A schematic of the *CTGF*$^{e2COIN}$ allele. (A) The CTGF locus contains five exons. (B) The COIN intron is placed within exon2 dividing it into two parts (2a and 2b). In the absence of Cre the COIN intron is spliced out. The solid lines below each diagram indicate regions that are included in the CTGF message after splicing, while the dotted lines indicate regions which are spliced out. (C) In the presence of Cre, the COIN intron is inverted, placing the GFP (TM-eGFP) in the correct orientation to be expressed and a polyA sequenence (pA) such that it terminates the transcription of the CTGF message. The arrowheads indicate loxp sites.

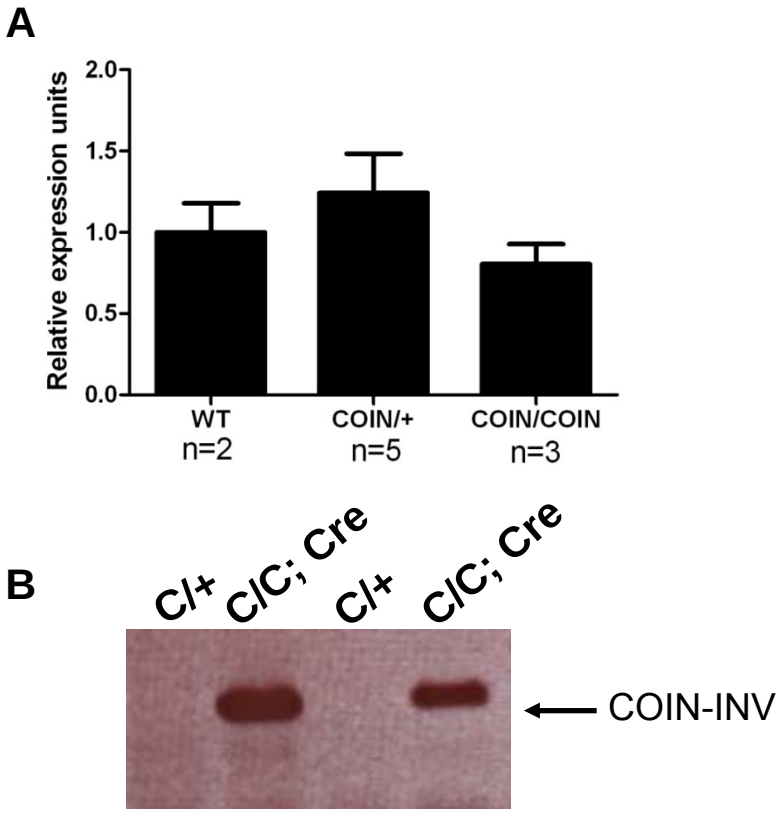


**Figure 5-3**. The COIN allele does not affect CTGF expression in the absence of Cre. (A) Real-time PCR for *Ctgf* expression in wild type (WT), *CTGF*$^{e2COIN/+}$ (COIN/+), and *CTGF*$^{e2COIN/e2COIN}$ (COIN/COIN) pancreata. (B) PCR to specifically detect the inverted form of the *CTGF*$^{e2COIN}$ allele (COIN-INV) using DNA isolated from pancreatic sections from *CTGF*$^{e2COIN/+}$ (C/+) and *CTGF*$^{e2COIN/e2COIN}$;*Pdx-1*-Cre (C/C;Cre) pancreata.

interbred with CTGF global null mice (*CTGF*$^{LacZ}$) to generate *CTGF*$^{e2COIN\text{-}INV/LacZ}$ mice. Similar to global CTGF null mice, *CTGF*$^{e2COIN\text{-}INV/LacZ}$ pups died at birth and displayed skeletal defects. Furthermore, northern blotting analysis of *CTGF*$^{e2COIN\text{-}INV/LacZ}$ pancreata revealed that no CTGF message is detected (data not shown). Together, these data suggest that the *CTGF*$^{e2COIN}$ acts as a null allele in its inverted form. The Cre-dependent inversion of the *CTGF*$^{e2COIN}$ allele was confirmed in individual pancreata using PCR on DNA isolated from paraffin sections of embryonic pancreata (Figure 5-3B). In theory, an advantage of using the *CTGF*$^{e2COIN}$ allele over a traditional conditional allele is that inactivation of CTGF should coincide with the expression of GFP and allow for assessment of recombination efficiency at the cellular level. However, we were unable to detect GFP fluorescence or detect GFP expression by indirect protein localization in tissues from mice which carried the *CTGF*$^{e2COIN}$ and expressed Cre. This was later determined to be due to the fact that the GFP is linked to a transmembrane domain which causes misfolding and degradation of the protein (Dr. Aris Economides, personal communication).

To determine the efficiency of CTGF inactivation in a cell-type specific manner, immunohistochemistry was performed using several commercial anti-CTGF antibodies as well as with antibodies generated by our collaborator Dr. David Brigstock at Ohio State University. In our hands, we were unable to detect a specific CTGF signal in pancreatic samples using any of these antibodies. As an alternative, a riboprobe was generated in order detect CTGF mRNA by *in situ* hybridization. Although CTGF mRNA could be detected by *in situ* hybridization using this probe on sections of intestine and kidney (Figure 5-4), no specific signal could be detected in pancreatic tissue. This is likely due to the abundance of RNAses in pancreatic exocrine tissue which quickly degrade RNA during the tissue dissection and RNA isolation process. Thus, at this time

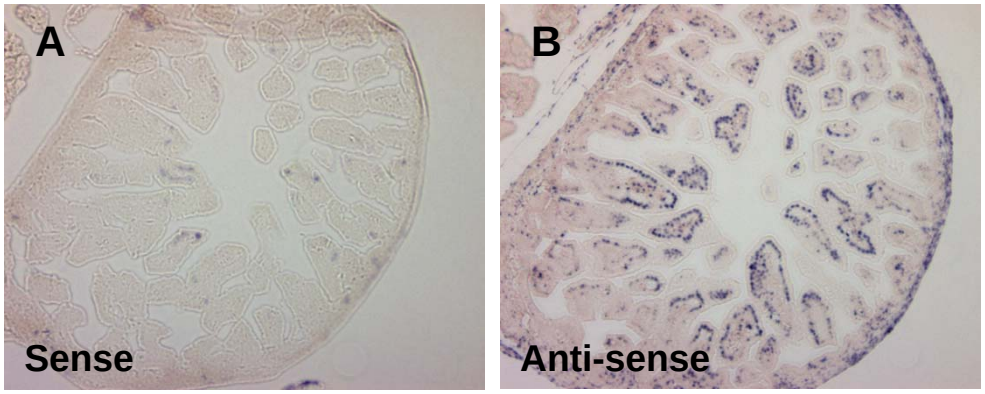


**Figure 5-4.** CTGF anti-sense probes detect CTGF expression. *In situ* hybridization of e18.5 intestine using CTGF sense (A) and anti-sense (B) probes.

we are unable to identify recombined cells or quantify the efficiency of recombination at the cellular level.

**CTGF acts in both a paracrine and autocrine manner to promote proper levels of β cell proliferation during embryogenesis**

Previous work demonstrated a role for endothelially-derived factors in regulating islet development. To determine whether CTGF produced by the endothelium promotes β cell proliferation during embryogenesis, *CTGF*$^{e2COIN}$ mice were interbred to transgenic mice expressing Cre recombinase from the *Tie-1* promoter to ultimately generate litters containing embryos in which *CTGF* is inactivated specifically in the vasculature during development (*CTGF*$^{e2COIN/e2COIN}$;*Tie-1-Cre*) [191]. Because the presence of the e2COIN intron does not affect expression of CTGF and *CTGF*$^{e2COIN/+}$ and *CTGF*$^{e2COIN/e2COIN}$ mice develop normally, *CTGF*$^{e2COIN/+}$ and *CTGF*$^{e2COIN/e2COIN}$ and littermates were used interchangeably as controls in all subsequent experiments. β cell replication was analyzed in control and *CTGF*$^{e2COIN/e2COIN}$;*Tie-1-Cre* embryos at e18.5 using immunohistochemistry to examine expression of phosphorylated histone H3 (pH3) and insulin. Compared to control embryos, embryos in which *CTGF* was inactivated in endothelial cells had a significant decrease in β cell proliferation (Figure 5-5A). At this stage, 10.2 ± 0.49% of β cells were proliferating in control embryos, while *CTGF* mutants had nearly a 60% reduction in β cell proliferation (4.4 ± 0.56%, p=0.002), indicating that CTGF secreted by endothelial cells is required for embryonic β cell proliferation at late gestation. Since CTGF is still produced by the pancreatic epithelium (ducts and β cells) in *CTGF*$^{e2COIN/e2COIN}$;*Tie-1-Cre* mutant embryos, we conclude that the level of CTGF produced by the epithelium is not sufficient to promote normal β cell proliferation.

To address whether CTGF produced by an epithelial source in the pancreas, either the ducts or the β cells, also affects β cell proliferation, *CTGF* was inactivated

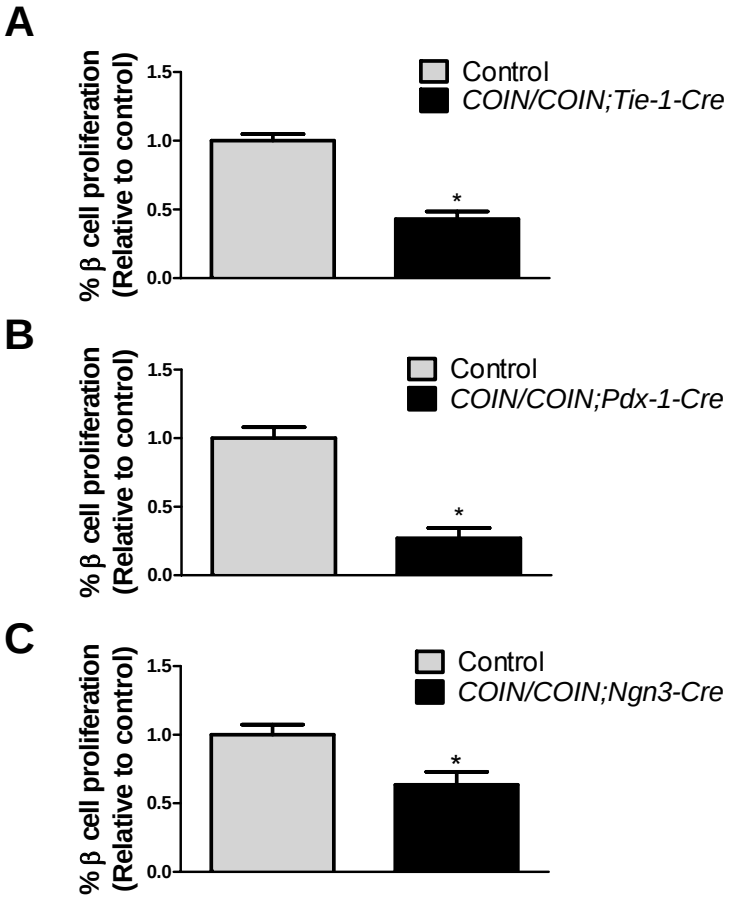


**Figure 5-5**. CTGF from multiple sources is required for β cell proliferation during embryogenesis. Quantification of β cell proliferation normalized to control littermate embryos in *CTGF$^{e2COIN/e2COIN}$;Tie-1-Cre* (A), *CTGF$^{e2COIN/e2COIN}$;Pdx-Cre* (B), and *CTGF$^{e2COIN/e2COIN}$;Ngn3-Cre$^{BAC}$* (C) embryos at e18.5 (A and B) and P1 (C). n=3-5 animals per genotype. *p<0.05 compared with Control.

throughout the pancreatic epithelium beginning at e9.5 by interbreeding mice carrying the *CTGF*$^{e2COIN}$ allele to *Pdx-1-Cre* mice [53]. Analysis of β cell proliferation at e18.5 indicated that the apparent percentage of replicating β cells in control mice was approximately 10-fold lower than in control mice from *Tie-1-Cre* litters. This difference was determined to be due to a change in pH3 antibody lot availability and an apparent change in overall sensitivity of detection between the two lots (Figure 5-6). Nonetheless, *CTGF*$^{e2COIN/e2COIN}$;*Pdx-1-Cre* embryos displayed a significant reduction in the percentage of replicating β cells compared to control littermates (Figure 5-5B). Control embryos displayed 0.5 ± 0.04% β cell proliferation, while *Pdx-1-Cre* mutants had 0.14 ± 0.34% (p=0.001). Thus, CTGF produced by an epithelial source is also required for β cell proliferation.

Because *Pdx-1-Cre* removed CTGF function from both the ductal epithelium and β cells, *CTGF*$^{e2COIN}$ mice were also bred to *Ngn3-Cre*$^{BAC}$ mice [68] to inactivate *CTGF* early from the endocrine lineage. CTGF expression is not localized to any other hormone-expressing cell type in the mouse pancreas; therefore using *Ngn3-Cre*$^{BAC}$ assayed for the role of CTGF in autocrine β cell signaling. *CTGF*$^{e2COIN/e2COIN}$;*Ngn3-Cre*$^{BAC}$ embryos had a significant (36 percent) reduction in β cell proliferation at P1 compared to controls (Figure 5-5C). While an average of 0.87 ± 0.06% of β cells were proliferating in controls, *CTGF*$^{e2COIN/e2COIN}$;*Ngn3-Cre*$^{BAC}$ mutants had an average of 0.56 ± 0.08% (p=0.02) proliferating β cells. These data indicate that autocrine CTGF-mediated signaling is also required to promote proper levels of embryonic β cell proliferation. Currently a Cre driver line with which *CTGF* can specifically and efficiently be inactivated from embryonic ducts is not available. Therefore, we were unable to directly investigate the role of CTGF produced by the ducts in β cell proliferation at this time. Inactivating *CTGF* in endothelial cells, the pancreatic epithelium, or β cells each lead to a defect in β

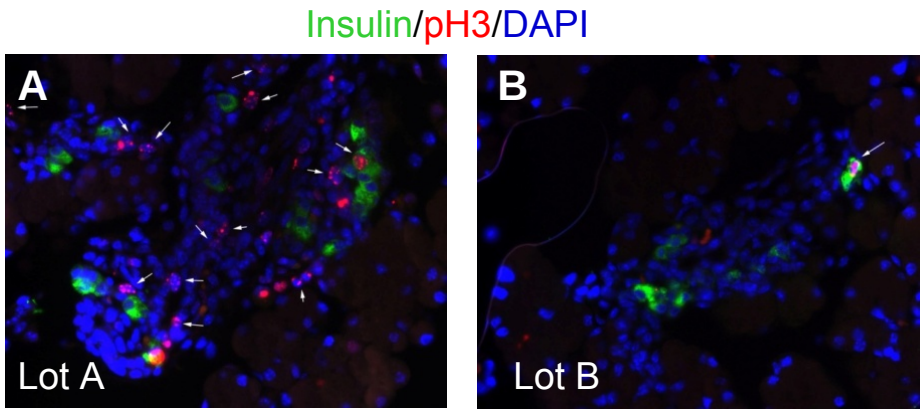


**Figure 5-6.** The sensitivity of the pH3 antibody to detect proliferating cells varied depending on the lot. Adjacent slides from a *CTGF*$^{e2COIN/e2COIN}$ pancreas were stained with two different lots of pH3 antibody. Lot A (A) appeared to be more sensitive at detecting proliferating cells than Lot B (B). Arrows indicate proliferating cells.

cell proliferation, suggesting that the function of CTGF produced by each one of these sources is non-redundant with regards to β cell proliferation.

**Different CTGF sources function redundantly to promote lineage allocation and islet morphogenesis**

Global inactivation of *CTGF* leads to defects in endocrine lineage allocation and islet morphology. CTGF null mutant islets have an increase in glucagon-positive area and a concomitant decrease in insulin-positive area, islets fail to separate from the ductal epithelium, and islet architecture is perturbed such that peripheral cell types are found scattered throughout the islet [188]. Therefore, we investigated which source(s) of CTGF are required for generating the correct proportions of endocrine cell types and for islet morphogenesis. The proportion of endocrine tissue composed of insulin and glucagon-positive area was quantified at P1 in $CTGF^{e2COIN/e2COIN}$;*Tie-1-Cre*, $CTGF^{e2COIN/e2COIN}$;*Pdx-1-Cre*, and $CTGF^{e2COIN/e2COIN}$;$Ngn3\text{-}Cre^{BAC}$ conditional-mutant embryos as well as in littermate controls. Endocrine composition was not significantly different from controls in any of the tissue-specific mutants (Figure 5-7A-C). The proximity of islets to ducts and islet architecture was also examined in each of the tissue-specific mutants and was found to be similar to controls (Figure 5-7D and E), suggesting that the remaining sources of CTGF compensate for the loss of CTGF from one source with respect to both lineage allocation and islet morphogenesis. Alternatively, the reduction in CTGF levels when CTGF is inactivated from a single source may not be significant enough to lead to alterations in lineage allocation.

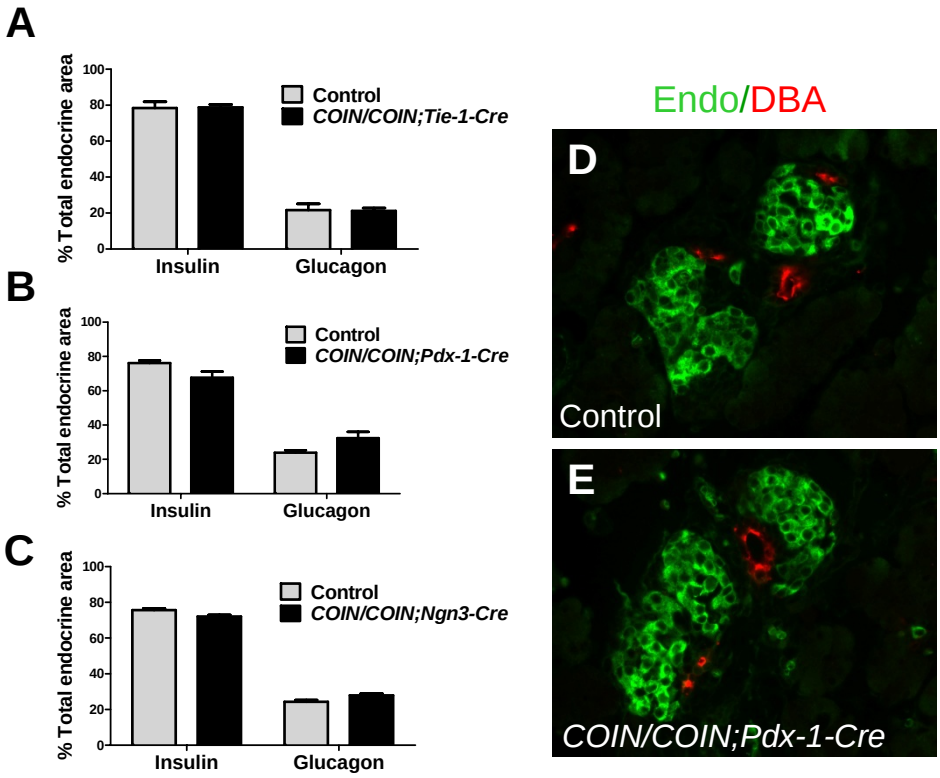


**Figure 5-7**. Different sources of CTGF function redundantly to promote lineage allocation and islet morphogenesis. The proportion of the endocrine tissue composed of insulin-and glucagon-positive area was not significantly altered in *CTGF*$^{e2COIN/e2COIN}$*;Tie-1-Cre* (A), *CTGF*$^{e2COIN/e2COIN}$*;Pdx-1-Cre* (B) and *CTGF*$^{e2COIN/e2COIN}$*;Ngn3-Cre*$^{BAC}$ (C) mutants at P1. n=3 animals of each genotype. (D-E) Islet morphogenesis was also unaltered in conditional mutants. Sections from P1 pancreata were immunolabeled with insulin and glucagon (Endo, green) and DBA (red). The distance between the endocrine tissue and ducts was measured in control (D) and mutant (E) pancreata and found not to be significantly different. Although only *Pdx-1-Cre* mutants are shown, mutants from other Cre lines were analyzed and found not to be significantly different from controls.

## Discussion

In this study, we investigated the tissue interactions by which CTGF promotes normal pancreatic islet development. We used a conditional gene inactivation strategy to inactivate CTGF from endothelial cells and the entire pancreatic epithelium and found that β cell proliferation was significantly impaired when CTGF is lost from either source. Furthermore, we showed that the β cells themselves are a required source of CTGF within the epithelium, making CTGF the first secreted β cell-derived factor that has been demonstrated to be required for embryonic β cell proliferation. Although we were unable to directly assess the role of CTGF produced by pancreatic ducts in this study, it is possible that CTGF produced by the ductal epithelium also contributes to β cell proliferation.

It is noteworthy that although CTGF is a secreted factor, the remaining sources of CTGF were unable to completely compensate for the defect in β cell proliferation that results when CTGF was lost from a single source. We therefore conclude that the process of β cell proliferation in the embryo is particularly sensitive to the total level of CTGF to which the β cells are exposed, and that the overall level of CTGF in the pancreas is more important for promoting β cell proliferation than a specific cellular source. These data are consistent with our previous finding that β cell proliferation is significantly impaired in CTGF heterozygous mutant embryos, which have a 50 percent reduction in overall CTGF expression in the pancreas [188]. In contrast, the processes of endocrine cell lineage allocation and islet morphology may be less sensitive to the overall levels of CTGF than β cell proliferation, as they were unaffected when CTGF was removed from only one pancreatic source.

To address the hypothesis that the overall levels of CTGF are more important than one particular cellular source, we planned to examine the amount of CTGF protein remaining in each of tissue-specific mutant lines using western blotting and determine whether the reduction in CTGF in each of the Cre lines correlates with the reduction in β cell proliferation. Pancreata from e18.5 control and mutant embryos from each Cre line were dissected and sent to our collaborator for analysis. Unfortunately, our collaborators were unable to detect any CTGF in our control samples, so we were unable to compare control and mutant levels of CTGF. This is likely due to the fact that the assay used was not sensitive enough to detect the amount of CTGF in pancreas tissue at that time. As an alternative, we are currently generating embryos to perform real-time PCR on control and mutant RNA samples to examine CTGF message levels. Although we do not yet have data regarding the percentage of reduction in CTGF levels in each of the tissue-specific mutant lines, we hypothesize that the reduction in CTGF is less than 50 percent of controls. $CTGF^{LacZ/+}$ embryos have approximately a 50 percent reduction in CTGF protein and display defects in β cell proliferation, lineage allocation, and islet morphogenesis. The fact that the tissue-specific mutants have normal lineage allocation and islet morphogenesis suggest that the remaining level of CTGF in the pancreas is sufficient to promote these processes.

Alternatively, we may not detect a difference in the overall level of CTGF in pancreata from each of the conditional mutants compared to control embryos. CTGF is proteolytically cleaved into N and C-terminal fragments; the N-terminal fragment can diffuse away from the cellular source while the C-terminal fragment is tethered to the cell membrane due to interactions with integrins and heparan sulfate proteoglycans. Therefore, it is possible that the local concentration of CTGF seen by the β cells is

decreased in the tissue-specific mutants and impairs β cell proliferation, but that this change cannot be detected by gene expression profiling of whole pancreata.

There has been a longstanding appreciation of the intricately connected relationship between the pancreatic vasculature and the endocrine tissue; however the relevant molecules which mediate the interaction between the endothelium and the pancreatic epithelium have not yet been identified. Elegant studies have demonstrated that endothelial cells have the ability to induce pancreas outgrowth and endocrine differentiation during development [26,27,196]. Furthermore, in adult islets, the vascular basement membrane has been shown to produce laminins which promote insulin expression and β cell proliferation [213]. Our studies indicate that CTGF mediates the interaction between the vasculature and the pancreatic epithelium. Furthermore, CTGF is the first factor identified to be secreted by the endothelium that is required for β cell proliferation in the embryo, indicating that the importance of the vasculature-endocrine interaction can be extended to include a role for endothelial cells in embryonic β cell proliferation. CTGF produced by the vasculature may also promote proper endocrine lineage allocation; however, we were unable to determine the role of endothelial-derived CTGF in endocrine differentiation because the other sources of CTGF appear to be able to compensate for the loss of CTGF from one source.

During pregnancy in mice and humans, β cell mass expands to meet the increased metabolic demand for insulin and in rodents this expansion is due to a three-fold enhancement of β cell proliferation [214]. An increase in vascular growth has been shown to precede β cell proliferation in pregnant rats, suggesting a role for endothelial cells in stimulating β cell proliferation in response to pregnancy [215]. The fact that CTGF is also highly expressed in adult islet vasculature suggests that CTGF may also promote the proliferation of adult β cells in response to metabolic stress or pregnancy.

Interestingly, we previously found that although CTGF is not normally expressed in adult β cells, it is re-expressed in β cells during pregnancy; therefore CTGF may act as both an autocrine and paracrine signal in regulating β cell proliferation in the adult as well as the embryo [188]. Studies are underway to determine the role of CTGF in normal adult β cell proliferation and under conditions of metabolic stress such as pregnancy.

One question remaining from our studies is whether the decrease in β cell proliferation observed when CTGF is removed from endothelial cells is due to a direct role of endothelial cell-derived CTGF on β cell proliferation, or whether loss of CTGF affects blood vessel density and thus β cell proliferation indirectly. Although the role of CTGF in neovascularization and angiogenesis has not been examined in the pancreas, CTGF promotes endothelial cell migration and angiogenesis *in vitro* and *in vivo* in other systems [216]. Therefore, it is possible that in *CTGF*$^{e2COIN/e2COIN}$;*Tie-1-Cre* mutant embryos, blood vessel density may be decreased and result in the decrease of an unidentified blood vessel-derived signal which is required for proper β cell proliferation. Studies to address this question are currently underway.

We are also currently investigating whether the decrease in β proliferation observed during embryogenesis in each of the conditional mutants will translate to a decrease in β cell mass in the adult. In some mouse models, a decrease in embryonic or neonatal beta cell proliferation leads to a permanent reduction in β cell mass [194]. However, *CTGF*$^{LacZ/+}$ animals have decreased β cell proliferation and β cell area at e18.5 but normal β cell area in adulthood. This is due, in part, to individual β cell hypertrophy which compensates for the defect in proliferation. Therefore, if adult CTGF conditional mutants do not display decreased β cell mass, it may be that the defect in proliferation during embryogenesis is not severe enough to significantly impact adult β cell mass, or due to an increase in individual β cell size.

# CHAPTER VI

## INDUCIBLE OVER-EXPRESSION OF CTGF DURING EMBRYOGENESIS INCREASES ISLET MASS BY ENHANCING ENDOCRINE CELL PROLIFERATION

### Introduction

CTGF is required for proper lineage allocation, β cell proliferation, and islet morphogenesis during pancreas organogenesis. Since inactivation of CTGF leads to a decrease in β cell proliferation and β cell mass, we hypothesized that increased levels of CTGF may increase β cell mass. Increased levels of CTGF have been shown to promote proliferation and differentiation *in vitro*; however, few studies have examined the effects of over-expressing CTGF *in vivo*. In the postnatal lung, conditional over-expression of CTGF in respiratory epithelial cells from P1 to P14 using an inducible system led to increased myofibroblast differentiation, fibrosis and proliferation [167]. A recent study demonstrated that 10-fold global over-expression of CTGF throughout development led to embryonic lethality [173]. Although the cause of lethality was not clear, these embryos displayed defects in craniofacial, brain, and vascular development. In contrast, global over-expression of CTGF in adult mice did not affect viability [173].

To study the effects of enhanced CTGF signaling on pancreas development, CTGF was over-expressed specifically in β cells during development using a transgenic inducible system and islet mass, endocrine cell proliferation, and differentiation were examined. We found that an increased level of CTGF in β cells was sufficient to increase islet mass and endocrine cell proliferation, while endocrine differentiation was not significantly altered. These data suggest that CTGF may be an attractive candidate for

inclusion in directed differentiation protocols where CTGF may be able to enhance the proliferation of newly differentiated β cells.

## Results

In order to determine whether increased levels of CTGF can enhance β cell mass, CTGF was specifically over-expressed in β cells during development using a tetracycline-inducible system (Figure 6-1). Mice expressing the reverse tetracycline transactivator from the rat insulin promoter (*RIP-rtTA*) [192] were inter-bred to a CTGF responder line (*TetO-CTGF*) and doxycycline (dox) was administered continuously in the drinking water of pregnant dams beginning at day 9.5 of gestation in order to expose the embryos to dox before the activation of *RIP* which normally occurs at e11.5. Over-expression of CTGF was confirmed by real-time PCR of pancreata at e16.5 (Figure 6-2A) and indicated that *Ctgf* expression in bigenic (*rtTA;TetO-CTGF*) embryos was increased by approximately 10-fold compared to control embryos (*rtTA*). Primers which did not detect *Ctgf* expressed from the *TetO-CTGF* transgene were also used to quantify endogenous *Ctgf* levels in control and bigenic embryos. In contrast to total *Ctgf* levels, endogenous *Ctgf* levels were not significantly changed in bigenic embryos indicating that the transgenic over-expression of *Ctgf* does not affect *Ctgf* expression in general (Figure 6-2B). Although it would also be useful to know the fold over-expression and localization of the CTGF protein, these analyses were not performed in this study due to the lack of available antibodies that can be successfully used for immunohistochemistry or western blotting. Alternatively, we could have over-expressed a tagged version of the CTGF cDNA and used antibodies designed against the tag to detect the CTGF protein. When tagging proteins it is critical to place the tag such that it does not disrupt the function of

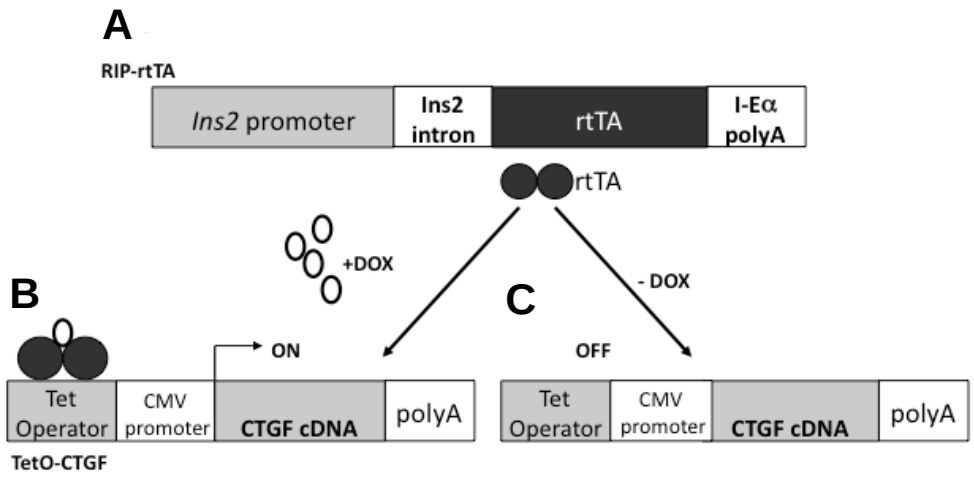


**Figure 6-1.** A schematic of the transgenic system used to conditionally over-express CTGF specifically in β cells. (A) Expression of *rtTA* is driven by the *Ins2* promoter (*RIP-rtTA*). (B) In the presence of doxycycline (DOX), the *rtTA* activates expression of a CTGF cDNA placed dowstream of the Tet Operator (*TetO-CTGF*). (C) *rtTA* does not activate *TetO-CTGF* in the absence of DOX.

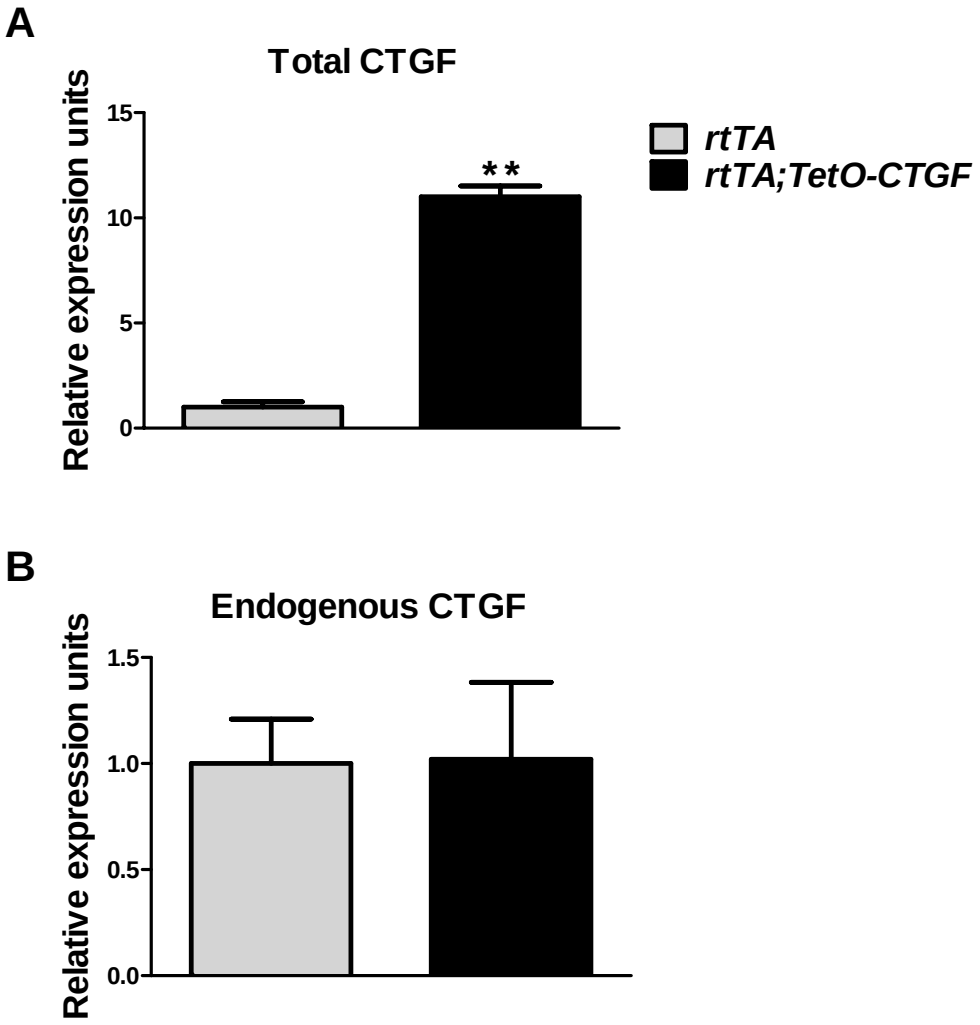


**Figure 6-2.** CTGF is over-expressed in pancreata from *RIP-rtTA;TetO-CTGF* embryos. (A) The level of *Ctgf* in bigenic pancreata was increased approximately 10-fold compared to control embryos at e16.5. Doxycycline was initiated at e9.5 in pregnant mothers' drinking water. (B) Primers specifically amplifying endogenous *Ctgf* demonstrated that endogenous *Ctgf* levels are not significantly different between control (gray bars) and CTGF over-expressing embryos (black bars). *p<0.0001. n=3 animals of each genotype.

the protein. Because functional studies using a tagged version of CTGF have not been performed, we did not know where to place the tag on CTGF so that it did not alter CTGF function. Furthermore, the fact that CTGF is cleaved would make interpreting the results of immunohistochemistry or western blotting against the tagged CTGF difficult. For these reasons, we chose not to express a tagged version of CTGF in this study.

Pancreata from bigenic pups and littermate controls were analyzed for endocrine mass at P1. There was no significant difference in the total pancreatic area between control and bigenic pancreata (Figure 6-3); however, the proportion of the pancreatic area composed of endocrine tissue was significantly increased by approximately 25 percent in bigenic neonates (Figure 6-4). These studies indicate that increased levels of CTGF are sufficient to increase islet mass. Relative to the total pancreatic area, the number of both $\alpha$ and $\beta$ cells were significantly increased in bigenic pancreata (Figure 6-4D).

To determine whether the increase in endocrine cells was due to changes in neogenesis or proliferation, the number of Ngn3-expressing cells was quantified. There was no significant difference in the number of Ngn3-positive cells in the CTGF over-expressing pancreata compared to control pancreata (Figure 6-5A), indicating that the increased number of endocrine cells is not due to an overall increase in the endocrine progenitor population. The ratio of insulin to glucagon positive cells was also similar to control pups (Figure 6-5B). Thus, over-expression of CTGF in $\beta$ cells does not affect allocation to the different endocrine lineages. However, the proportion of proliferating $\beta$ and $\alpha$ cells was significantly increased in bigenic neonates compared to control embryos at P1 (Figure 6-5C and D). CTGF over-expression in the pancreas appears to increase the number of endocrine cells by enhancing both $\beta$ and $\alpha$ cell proliferation, rather than promoting neogenesis.

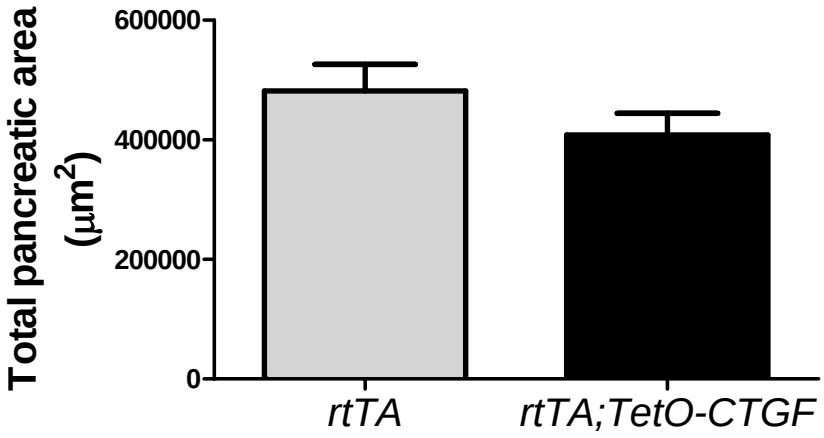


**Figure 6-3.** Total pancreatic area in CTGF over-expressing pancreata. Total pancreatic area was not significantly changed between control (*rtTA*) and bigenic (*rtTA;TetO-CTGF*) neonates at P1. n=3 animals of each genotype.

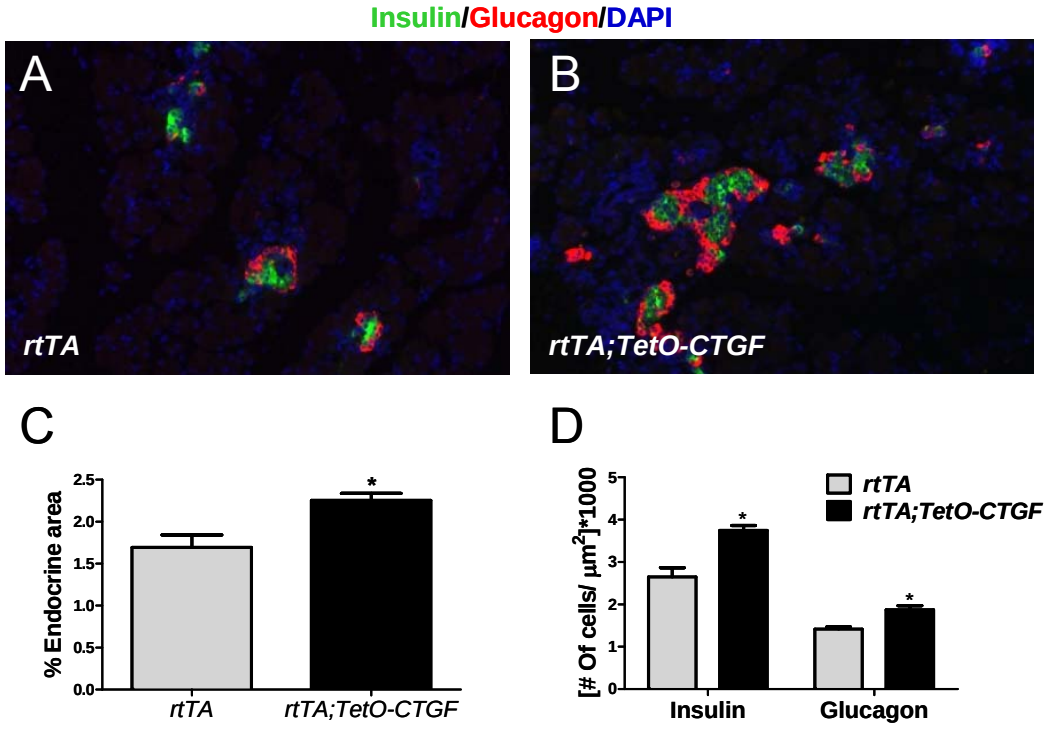


**Figure 6-4.** CTGF over-expression during development led to increased endocrine mass. Immunohistochemical analysis of insulin (green) and glucagon (red) in control (A) and bigenic (B) pups at P1. (C) Quantification of the proportion of pancreas that is composed of endocrine area at P1 in control (*rtTA*) and bigenic (*rtTA;TetO-CTGF*) pups. (D) The number of insulin and glucagon positive cells in control and bigenic pups normalized to total pancreatic area. n=3 of each genotype. *p<0.05 compared to *rtTA*.

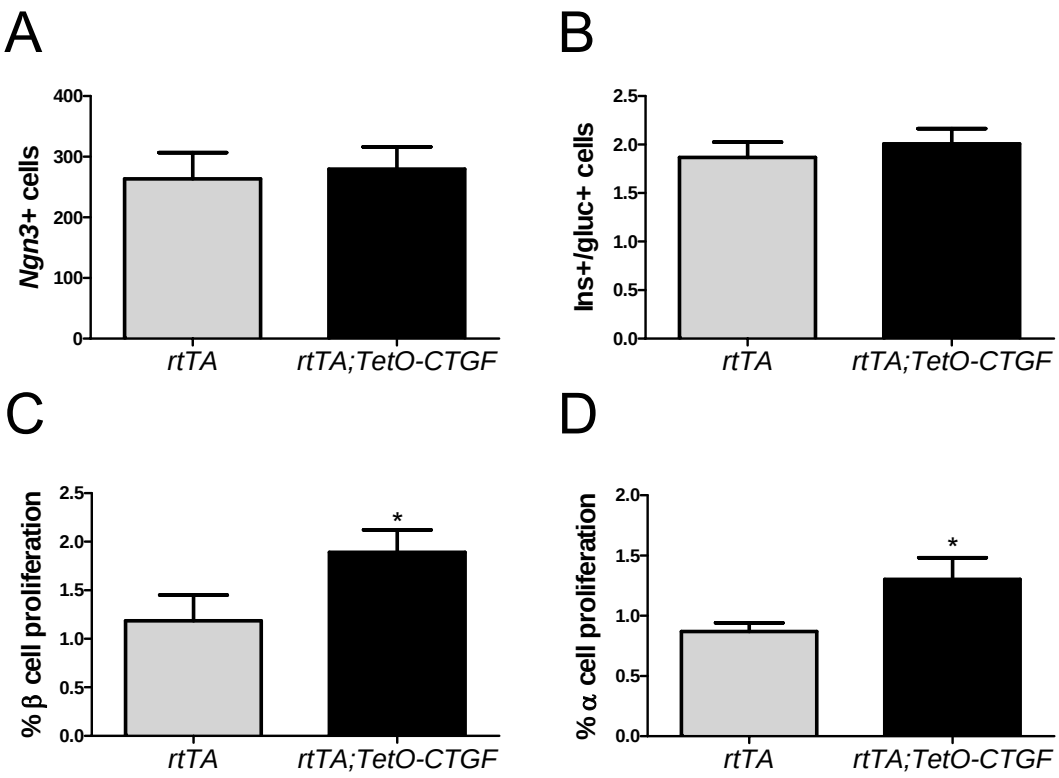


**Figure 6-5.** CTGF over-expression led to an increase in insulin and glucagon-positive cell proliferation but not neogenesis. (A) Quantification of the number of *Ngn3*-positive cells in control and CTGF over-expressing pancreata at e14.5. (B) The ratio of insulin-positive to glucagon-positive cells was not altered in CTGF over-expressing neonates. (C) The percent of proliferating β cells was increased in CTGF over-expressing pups at P1 using pH3 immunofluorescence. (D) α cell proliferation also was significantly increased in CTGF over-expressing pups. n=3 of each genotype. *p<0.05 compared to *rtTA*.

## Discussion

In this study, we found that embryos in which CTGF is over-expressed specifically in β cells display increased endocrine cell proliferation and endocrine mass. Thus, an increase in CTGF levels was sufficient to stimulate β and α cell proliferation *in vivo*. CTGF is not expressed in α cells, and α cell proliferation is not altered in the CTGF global inactivation model [188]. Therefore, although CTGF is not required for α cell proliferation, it is possible that increased paracrine CTGF signaling enhances α cell proliferation. Alternatively, the increased α cell proliferation may be an indirect result of communication between the different endocrine cell types, whereby an increase in the number of β cells concomitantly stimulates α cell proliferation. Our results indicate that CTGF over-expression in insulin-expressing cells did not affect endocrine cell neogenesis or the allocation of endocrine progenitors into the α and β lineages. The temporal or spatial pattern of CTGF over-expression in this study may have limited the effects of CTGF to cell proliferation; earlier or broader expression of CTGF in endocrine or pancreatic progenitors may reveal the ability of CTGF to promote neogenesis. Mice with the *tetracycline-regulated transactivator* (*tTA*) gene replacing part of the *Pdx-1* coding sequence have been previously generated and could be used in future experiments to express CTGF throughout the entire pancreatic epithelium as early as e9.5 [217].

The 10-fold increase in *Ctgf* expression in bigenic pups in this system is most likely due to the activity of the *rat insulin 2* promoter as the *insulin* gene is highly expressed in β cells. This data is consistent with reports from other groups indicating a high level of transgene expression using the *RIP-rtTA* driver line (personal communication, Qing Cai, graduate student in the laboratory of Dr. Alvin Powers).

Furthermore, co-transfecting the *RIP-rtTA* transgene construct and a *TetO-luciferase* reporter plasmid into βTC3 cells, a clonal pancreatic β cell line, led to a 15-fold induction of luciferase expression upon doxycycline treatment [218]. It is likely that the over-expression of CTGF in this system is beyond physiological levels and in future studies, the *Pdx-1-tTA* driver line could be used to examine the effect of more physiological levels of CTGF expression on pancreas development. Nevertheless, we did not observe any obvious off target effects of CTGF expression—bigenic pups developed normally and there did not appear to be excess stroma or excess ECM around the epithelium suggesting that CTGF over-expression did not lead to overt fibrosis in the pancreas. However, future studies will need to be performed to directly examine whether fibrosis occurs in these animals. The 10-fold increase in *Ctgf* expression led to approximately a 1.6-fold increase in β cell proliferation. This data suggests that at this level of *Ctgf* over-expression, there is not a linear correlation between *Ctgf* message levels and β cell proliferation. β cell proliferation is a tightly controlled process which is regulated at many levels [219]. Although CTGF is sufficient to increase β cell proliferation, there are likely cellular "breaks" in place to prevent uncontrollable β cell replication during embryogenesis which could lead to tissue overgrowth or cancer. Therefore, there may be a physiological limit to the amount β cell proliferation can increase *in vivo*. Additionally, CTGF acts to modulate the interactions between cells and their extracellular environment by regulating signaling pathways such as TGF-β, BMP, and Wnt. The indirect correlation between the level of *Ctgf* and the fold-increase in proliferation could also be due to the fact that the concentration of the ligands with which CTGF interacts are limiting during embryogenesis. Finally, *Ctgf* message levels may not necessarily correlate with protein levels. It will be interesting to quantify the fold increase in CTGF

protein in bigenic embryos in order to understand more about how CTGF levels affect β cell proliferation.

One question remaining from our studies is whether the increase in β cell proliferation observed when CTGF is over-expressed in β cells is due to a direct role of β cell-derived CTGF on β cell proliferation, or whether an increase in CTGF affects blood vessel formation and thus β cell proliferation indirectly. Although the role of CTGF in neovascularization and angiogenesis has not been examined in the pancreas, CTGF promotes endothelial cell migration and angiogenesis *in vitro* and *in vivo* in other systems [216]. Therefore, it is possible that in CTGF over-expressing embryos, blood vessel density may be increased and result in an increase in blood vessel-derived signals which promote β cell proliferation. Studies are currently underway to quantify pancreatic endothelial cell density in control and CTGF over-expressing pups.

Another question currently under investigation is whether over-expression of CTGF during embryogenesis is sufficient to promote increased β cell mass or improve glucose homeostasis in the adult. We are currently aging mice in which CTGF was over-expressed during development and will perform glucose tolerance tests as well as analyze β cell mass in these animals. In addition to promoting embryonic β cell proliferation, CTGF may also enhance adult β cell proliferation. Future studies will address whether over-expression of CTGF in adult β cells is sufficient to increase β proliferation and mass.

# CHAPTER VII

## SUMMARY AND FUTURE DIRECTIONS

CTGF is a secreted molecule which modulates diverse biological processes such as proliferation, migration, adhesion and differentiation in a number of cell types. In the pancreas, CTGF has previously been shown to promote pancreatic tumor migration and survival and mediate fibrosis during pancreatitis. Our lab initially became interested in studying CTGF in the context of normal pancreas development because it is downregulated in the HNF6 over-expressing transgenic mouse model of islet dysmorphogenesis and diabetes. CTGF is also expressed in the pancreas during development; it is expressed as early as e12.5 and can be localized to β cells, ducts, and endothelial cells, but is downregulated in β cells soon after birth [188]. The expression pattern of CTGF suggested that it may play a role in pancreas development. Indeed, global CTGF inactivation leads to alterations in the endocrine cell ratio beginning at the secondary transition, decreased β cell proliferation at e18.5, and impaired separation of endocrine tissue from the ductal epithelium [188]. Thus, these data suggest that as in other tissues, CTGF is also required for proper differentiation, proliferation, and tissue remodeling during pancreas development.

Because CTGF is produced by β cells, ducts, and endothelial cells during pancreas development, we sought to examine the tissue interactions by which CTGF promotes normal lineage allocation, β cell proliferation, and islet morphogenesis. We generated mice carrying a conditional allele of CTGF and used tissue-specific Cre lines to inactivate CTGF from the pancreatic epithelium, β cells, or endothelial cells and examined lineage allocation, β cell proliferation, and islet morphogenesis. We found that

removal of CTGF from each one of these tissue types led to a reduction in embryonic β cell proliferation. In contrast, tissue-specific inactivation from any one cell type did not significantly impair lineage allocation or islet morphogenesis. Overall, conditional inactivation of CTGF revealed that the processes of β cell proliferation, lineage allocation, and islet morphogenesis during development may require different levels of CTGF to occur properly (Figure 7-1). We hypothesize that when CTGF is removed from one pancreatic source, the CTGF produced by the remaining sources is sufficient to allow proper endocrine lineage allocation and islet morphogenesis. However, embryonic β cell proliferation appears to be exquisitely sensitive to the level of CTGF, such that inactivating CTGF from just one source leads to a decrease in proliferation. Studies are currently underway to determine the percent reduction in CTGF levels in each of the tissue-specific mutants. Interestingly, previous studies have indicated that a 50 percent reduction in CTGF levels leads to a defect in β cell proliferation as well as defects in lineage allocation and islet morphogenesis [188]. These studies suggest that even a more modest reduction in CTGF levels is insufficient to support proper β cell proliferation.

CTGF is proteolytically cleaved between its second and third domains by MMPs, chymotrypsin, and plasmin [148,149]. After being cleaved, the C-terminal fragment may be tethered to the cell surface due to interactions with integrins and heparan sulfate proteoglycans while the N-terminus, which lacks these interaction domains, may be able to diffuse away from the cellular source [144,151]. Thus, it is possible that CTGF secreted by specific cell types could participate in either local or long range signaling depending on the proteolytic enzymes expressed by the cell. Therefore, the role of CTGF in regulating the different aspects of pancreas development may be governed by not only the level of full length CTGF, but also by the levels of the individual cleavage

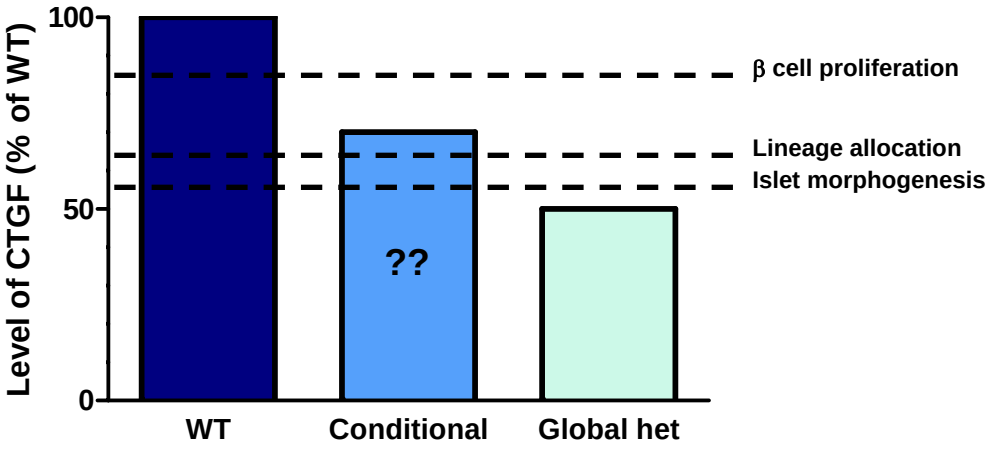


**Figure 7-1**. Model of the requirement of CTGF in regulating the different processes of pancreas development. We hypothesize that β cell proliferation, lineage allocation, and islet morphogenesis require different thresholds of CTGF levels (dotted lines). When the level of CTGF expression is below the threshold, defects in that process are observed. Wild type (WT), CTGF tissue-specific mutant (Conditional) and $CTGF^{lacZ/+}$ heterozygous (Global het) embryos each express different amounts of CTGF, although the amount of CTGF in the tissue-specific mutants is not currently known (denoted by question marks).

fragments. Antibodies have been generated which specifically recognize the N-terminal or C-terminal fragments of CTGF. Western blotting with these antibodies could be performed on pancreata isolated from CTGF tissue-specific mutants to determine whether the different sources of CTGF produce different CTGF cleavage products. The functional role of each of the domains of CTGF in pancreas development could be assessed by generating mice that lack a specific module of CTGF and analyzing the pancreatic phenotype of each mutant.

Tissue-specific inactivation of CTGF also revealed, for the first time, that a secreted factor produced by either β cells or endothelial cells is required for embryonic β cell proliferation. Few genes have been identified which regulate embryonic β cell proliferation and this study indicates that β cells themselves produce signals which are necessary for proper β cell proliferation. Previous to this study, it was known that endothelial cells produce signals which promote endocrine cell neogenesis, however the relevant molecules had not been identified [26]. We hypothesized that CTGF may act as an endothelial-derived molecule which promotes the differentiation of β cells. Due to the fact that lineage allocation was not significantly altered in any of the tissue-specific mutants, we were unable to assess the function of CTGF produced by endothelial cells in β cell differentiation; it is still possible that endothelial cell-produced CTGF normally plays a role in β cell differentiation. This study did, however, reveal a previously unappreciated role for endothelial cell-derived factors in the proliferation of embryonic β cells. Thus, one function of CTGF is to mediate the interactions regulating the reciprocal development of blood vessels and endocrine cells by promoting β cell proliferation. Studies are currently underway to determine whether the decrease in β cell proliferation observed when CTGF is inactivated from endothelial cells is an indirect consequence of

decreased blood vessel formation or directly due to a requirement of endothelial-produced CTGF on β cells.

The fact that embryonic β cell proliferation is particularly sensitive to the levels of CTGF raises the question of whether CTGF is also required for adult β cell proliferation under normal or stimulatory conditions. Future studies in the lab will examine whether inactivation of CTGF in adult β cells using the $CTGF^{e2COIN}$ allele and an islet-specific tamoxifen-inducible Cre ($Pdx\text{-}1^{PB}$-CreER) impairs β cell proliferation and glucose homeostasis. CTGF expression is not normally detected in adult β cells, and CTGF heterozygous adults do not have defects in β cell proliferation [188]. Therefore, we may find that CTGF is not required for normal adult β cell proliferation. Interestingly, however, CTGF is upregulated in β cells during pregnancy at all stages that have been examined [188], suggesting that CTGF may be required for the increase in β cell proliferation and expansion that occurs during pregnancy. We will therefore explore whether inducible islet-specific inactivation of CTGF impairs β cell proliferation or glucose homeostasis during pregnancy. It may also be interesting to use this inducible model of CTGF inactivation in adult β cells to examine whether CTGF is required for the compensatory β cell proliferation which occurs in response to the insulin resistance caused by high-fat diet feeding. If CTGF is required for expansion of β cell mass under stimulatory conditions, would also be worthwhile to determine if CTGF expression is altered in humans with diabetes.

In this study, we also asked whether increased levels of CTGF could increase β cell mass. Our results indicate that over-expression of CTGF specifically in β cells during development using an inducible transgenic system is sufficient to increase endocrine cell mass at birth. This increase in endocrine cell mass was due to an increase in both α and β cell proliferation, with no apparent change in endocrine cell differentiation. These

results indicate that in addition to being required for β cell proliferation, CTGF is also able to promote β cell proliferation. The fact that loss of CTGF does not impair α cell proliferation but over-expression increases α cell proliferation suggests that while CTGF is not required for α cell replication, it may be sufficient to promote it. Alternatively, communication between the endocrine cell types is thought to regulate α and β cell numbers within the islet. For example, removal of Pdx-1 from embryonic β cells leads to a decrease in β cell proliferation and a concomitant increase in α cell proliferation [195]. In the CTGF over-expression model, a signal may be produced by the increased number of β cells which concomitantly increases α cell proliferation. Studies are also underway to examine the endothelial density in CTGF over-expressing pancreata because it is possible that over-expression of CTGF could also indirectly increase endocrine cell number by enhancing blood vessel formation in the pancreas. Thus, the mechanism by which CTGF enhances endocrine mass may involve complex interactions between the different pancreatic cell types.

Current studies are underway in the lab to examine whether over-expression of CTGF in β cells can enhance β cell proliferation in the adult pancreas. Future studies will also address whether increased CTGF levels can promote pancreas regeneration in injury models by enhancing β cell proliferation or differentiation. Additionally, the ability of CTGF to improve the outcomes of islet transplantation is being investigated. The process of islet transplantation involves the severing of endogenous vasculature; however, some endothelial cells do survive and contribute to the post-transplantation revascularization of the islet [197]. Studies have shown that increased expression of angiogenic factors enhances the revascularization and survival of the islet grafts [197]. Thus, expression of CTGF in islets during the post-transplantation period may enhance islet survival, not only by promoting β cell proliferation, but by enhancing angiogenesis

as well. For these studies, islets from *RIP-rtTA;TetO-CTGF* adult mice will be transplanted into donor mice, and doxycycline will be administered to the recipient mouse in order to induce CTGF expression in the islet grafts. The survival and revascularization of CTGF over-expressing grafts will then be compared to control transplants. Alternatively, to avoid the use of genetic modifications, mouse or human islets could be cultured with soluble CTGF protein prior to transplantation.

Another question which still remains after these studies is how CTGF fits into the pancreas development regulatory network; that is, how CTGF is dynamically regulated and with which signaling pathways and factors CTGF interacts to promote proper pancreas development. CTGF has been shown to be regulated in other tissue types by several factors which play a role in pancreas development. The most well characterized of these signaling pathways is TGF-β. We sought to determine whether CTGF acts downstream of TGF-β to promote pancreas development using an *ex vivo* bud culture system. Although we were unable to use this method to determine whether CTGF functionally acts downstream of TGF-β signaling, in the future, we could determine whether treatment of pancreatic buds with TGF-β increases CTGF expression.

Alternatively, the Lyons lab has recently demonstrated that CTGF expression in chondrocytes is dynamically regulated by β catenin and Sox9, a gene which is also required for the maintenance of pancreatic progenitors [220,221,222]. Binding of Sox9 and β catenin to the CTGF proximal promoter is mutually exclusive [220]. During development, Sox9 represses CTGF in early immature chondrocytes, but is later replaced by β catenin to activate CTGF and the chondrocyte differentiation program [220]. CTGF is also upregulated by VEGF-A in vascular cells, suggesting that CTGF expression in pancreatic endothelial cells may be mediated by VEGF-A [223]. In a recent study, Ngn3 was inducibly expressed in mouse ES cells which had been

differentiated into pancreatic endoderm in order to promote differentiation to the endocrine lineage [198]. Interestingly, expression of Ngn3 was found to correlate with the repression of CTGF, suggesting that Ngn3 may inhibit CTGF expression. Together, these studies suggest that Sox9, Wnt, VEGF-A, and Ngn3 may lie upstream of CTGF; however understanding whether any or all of these factors regulate CTGF expression in the pancreas requires further investigation. It would be particularly interesting to examine CTGF expression in pancreata from Sox9, Wnt, VEGF-A, and Ngn3 loss or gain-of-function mutants to determine if CTGF lies downstream of any of these genes.

The similarity between the pancreatic phenotypes of CTGF global inactivation and some models of inhibition of integrin and TGF-β signaling suggests that the activity of these pathways may be altered in the absence of CTGF [108,109,110,129,188]. We therefore hypothesize that CTGF may normally modulate integrin or TGF-β signaling in the pancreas; however CTGF has also been shown to exert downstream effects by inhibiting BMP and Wnt signaling in other tissue types. Future studies will be directed towards understanding which signaling pathways CTGF interacts with to promote proper pancreas development. We plan to interbreed *RIP-rtTA;TetO-CTGF* mice to mice carrying TGF-β, BMP and Wnt reporter alleles to assess whether increased CTGF expression in β cells affects activation of these pathways *in vivo* [208,209,210,211]. Doxycycline will be administered in the drinking water of pregnant dams and antibodies will be used to detect expression of the appropriate reporter in embryonic pancreata. This analysis will reveal which pathways are normally active in the pancreas, and whether over-expression of CTGF leads to further activation of TGF-β or inhibition of BMP and Wnt signaling. Furthermore, co-labeling with cell type-specific markers of β cells, endothelial cells, or ducts will reveal whether increased levels of CTGF in β cells has cell autonomous and non-cell autonomous effects on TGF-β, BMP, and Wnt

signaling. Although there is unfortunately not a specific read-out of integrin activity available, it would also be interesting to examine the level of phosphorylated focal adhesion kinase in mice that have increased CTGF expression.

The fact that CTGF is both necessary and sufficient for embryonic β cell proliferation raises the question of whether CTGF can promote the generation of β cells *in vitro*. The current approaches to generating transplantation-quality β cells for the treatment of diabetes include the directed differentiation of ES cells or induced pluripotent stem cells (iPS) cells down the normal differentiation path from endoderm to insulin-producing cells by adding exogenous factors to the culture medium. These protocols have been successful in generating insulin-producing cells which can restore glucose homeostasis in diabetic models; however, in general, the percentage of β cells in these cultures is relatively low [224,225]. Recent studies have sought to identify small molecules or growth factors that are able to increase the yield of β cells, for example, by enriching early stage cultures for *Pdx-1* expressing pancreatic progenitors [226]. However, another viable approach could be to enhance the proliferation of the newly differentiated β cells. Our studies suggest that CTGF is an attractive candidate for inclusion in these directed differentiation protocols. Furthermore, because CTGF is a secreted factor, it could be directly added to the culture medium. We hypothesize that CTGF has the potential to increase the efficiency of the differentiation of stem cells at multiple steps of the protocol—either by increasing the number of endocrine cells that differentiate from endocrine progenitors by mimicking a blood vessel-derived signal or by stimulating the proliferation of immature β cells at later stages of the differentiation protocol (Figure 7-2).

Overall, this work has demonstrated an essential role for CTGF in promoting proper embryonic β cell proliferation. The importance of CTGF is highlighted by the fact

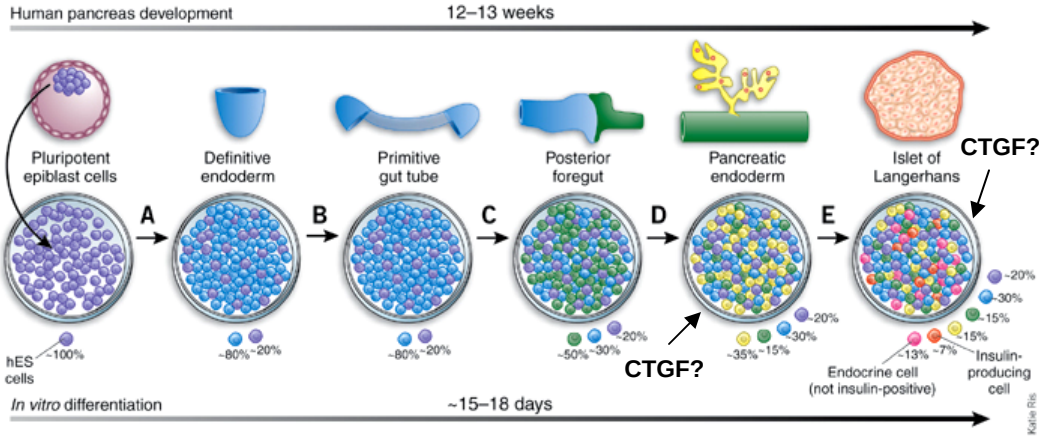


**Figure 7-2**. Directed differentiation of human ES cells into insulin-producing cells by mimicking embryonic development. Upper panel, key stages of pancreatic endocrine development. Below are the corresponding stages of the ES cell cultures. As the cells are cultured in five successive conditions (steps A to E), individual cells within the culture are differentiated toward pancreatic islet cells. Step A converts 80% of the hES cells to definitive endoderm. Step B converts all definitive endoderm cells to primitive gut tube (plate 3 is identical in color and pattern to plate 2). Step C converts 50% of cells to posterior foregut (green). Step D converts 35% to pancreatic precursors, and step E generates an average of 7% and up to 12% insulin-producing-cells (of a total of 20% endocrine cells). The fixed pattern of the cells in the successive dishes illustrates that red cells arise from yellow cells, which arise from green cells, which arise from blue cells. The two large arrows indicate both the directional flow and the contrast of the time scales: human fetal pancreatic development occurs over weeks, whereas directed differentiation *in vitro* is measured in days. We hypothesize that CTGF could enhance the differentiation of endocrine cells from progenitors or stimulate proliferation of newly differentiated β cells. Adapted by permission from Macmillan Publishers: *Nature Biotechnology*, Madsen, OD, Serup, P, Towards Cell Therapy for Diabetes, copyright 2006.

that inactivation of CTGF from any cellular source in the pancreas leads to defects in embryonic β cell proliferation. We believe that CTGF may have the potential to enhance β cell proliferation *in vitro* and *in vivo*. Here we have shown that CTGF is not only required for β cell proliferation, but that increased CTGF expression is sufficient to enhance β cell proliferation and β cell mass during development. CTGF is also a highly unique molecule in that it can modulate several signaling pathways and therefore could potentially have pleiotropic beneficial effects on β cell mass expansion, regeneration, and islet vascularization.

Our results also underscore the importance of autocrine and paracrine communication in regulating pancreas development. We have demonstrated that both endothelial-endocrine interactions and β cell-derived signals are required for proper embryonic β cell proliferation. Researchers attempting to derive β cells in vitro often discuss manipulating the differentiation protocol to produce greater proportions of definitive endoderm or pancreatic endoderm with improved efficiency. Yet during normal development, mesodermally-derived cell types, including endothelial cells, influence β cell differentiation. In the absence of other cell types in the culture, strategies to generate sufficient amounts of functional insulin-producing cells for transplantation should perhaps incorporate additional factors that mediate the heterotypic and homotypic interactions that normally occur between pancreatic cell types during development. To our knowledge, CTGF is the first reported secreted β cell or endothelial cell-derived factor that promotes normal embryonic β cell proliferation; however, our studies raise the possibility that other such factors may be produced by these cell types.

## REFERENCES


1. Ishii Y, Rex M, Scotting PJ, Yasugi S (1998) Region-specific expression of chicken Sox2 in the developing gut and lung epithelium: regulation by epithelial-mesenchymal interactions. Dev Dyn 213: 464-475.

2. Offield MF, Jetton TL, Labosky PA, Ray M, Stein RW, et al. (1996) PDX-1 is required for pancreatic outgrowth and differentiation of the rostral duodenum. Development 122: 983-995.

3. Silberg DG, Swain GP, Suh ER, Traber PG (2000) Cdx1 and cdx2 expression during intestinal development. Gastroenterology 119: 961-971.

4. Kawaguchi Y, Cooper B, Gannon M, Ray M, MacDonald RJ, et al. (2002) The role of the transcriptional regulator Ptf1a in converting intestinal to pancreatic progenitors. Nat Genet 32: 128-134.

5. Li H, Arber S, Jessell TM, Edlund H (1999) Selective agenesis of the dorsal pancreas in mice lacking homeobox gene Hlxb9. Nat Genet 23: 67-70.

6. Harrison KA, Thaler J, Pfaff SL, Gu H, Kehrl JH (1999) Pancreas dorsal lobe agenesis and abnormal islets of Langerhans in Hlxb9-deficient mice. Nat Genet 23: 71-75.

7. Burlison JS, Long Q, Fujitani Y, Wright CV, Magnuson MA (2008) Pdx-1 and Ptf1a concurrently determine fate specification of pancreatic multipotent progenitor cells. Dev Biol 316: 74-86.

8. Krapp A, Knofler M, Frutiger S, Hughes GJ, Hagenbuchle O, et al. (1996) The p48 DNA-binding subunit of transcription factor PTF1 is a new exocrine pancreas-specific basic helix-loop-helix protein. Embo J 15: 4317-4329.

9. Rose SD, Swift GH, Peyton MJ, Hammer RE, MacDonald RJ (2001) The role of PTF1-P48 in pancreatic acinar gene expression. J Biol Chem 276: 44018-44026.

10. Krapp A, Knofler M, Ledermann B, Burki K, Berney C, et al. (1998) The bHLH protein PTF1-p48 is essential for the formation of the exocrine and the correct spatial organization of the endocrine pancreas. Genes Dev 12: 3752-3763.

11. Afelik S, Chen Y, Pieler T (2006) Combined ectopic expression of Pdx1 and Ptf1a/p48 results in the stable conversion of posterior endoderm into endocrine and exocrine pancreatic tissue. Genes Dev 20: 1441-1446.

12. Jarikji ZH, Vanamala S, Beck CW, Wright CV, Leach SD, et al. (2007) Differential ability of Ptf1a and Ptf1a-VP16 to convert stomach, duodenum and liver to pancreas. Dev Biol 304: 786-799.

13. McLin VA, Rankin SA, Zorn AM (2007) Repression of Wnt/beta-catenin signaling in the anterior endoderm is essential for liver and pancreas development. Development 134: 2207-2217.

14. Deutsch G, Jung J, Zheng M, Lora J, Zaret KS (2001) A bipotential precursor population for pancreas and liver within the embryonic endoderm. Development 128: 871-881.

15. Rossi JM, Dunn NR, Hogan BL, Zaret KS (2001) Distinct mesodermal signals, including BMPs from the septum transversum mesenchyme, are required in combination for hepatogenesis from the endoderm. Genes Dev 15: 1998-2009.

16. Zhang W, Yatskievych TA, Baker RK, Antin PB (2004) Regulation of Hex gene expression and initial stages of avian hepatogenesis by Bmp and Fgf signaling. Dev Biol 268: 312-326.

17. Shin D, Shin CH, Tucker J, Ober EA, Rentzsch F, et al. (2007) Bmp and Fgf signaling are essential for liver specification in zebrafish. Development 134: 2041-2050.

18. Chung WS, Shin CH, Stainier DY (2008) Bmp2 signaling regulates the hepatic versus pancreatic fate decision. Dev Cell 15: 738-748.

19. Spagnoli FM, Brivanlou AH (2008) The Gata5 target, TGIF2, defines the pancreatic region by modulating BMP signals within the endoderm. Development 135: 451-461.

20. Stafford D, Hornbruch A, Mueller PR, Prince VE (2004) A conserved role for retinoid signaling in vertebrate pancreas development. Dev Genes Evol 214: 432-441.

21. Yutzey KE, Rhee JT, Bader D (1994) Expression of the atrial-specific myosin heavy chain AMHC1 and the establishment of anteroposterior polarity in the developing chicken heart. Development 120: 871-883.

22. Blumberg B (1997) An essential role for retinoid signaling in anteroposterior neural specification and neuronal differentiation. Semin Cell Dev Biol 8: 417-428.

23. Kim SK, Hebrok M, Melton DA (1997) Notochord to endoderm signaling is required for pancreas development. Development 124: 4243-4252.

24. Hebrok M, Kim SK, Melton DA (1998) Notochord repression of endodermal Sonic hedgehog permits pancreas development. Genes Dev 12: 1705-1713.

25. Apelqvist A, Ahlgren U, Edlund H (1997) Sonic hedgehog directs specialised mesoderm differentiation in the intestine and pancreas [published erratum appears in Curr Biol 1997 Dec 1;7(12):R809]. Curr Biol 7: 801-804.

26. Lammert E, Cleaver O, Melton D (2001) Induction of pancreatic differentiation by signals from blood vessels. Science 294: 564-567.

27. Yoshitomi H, Zaret KS (2004) Endothelial cell interactions initiate dorsal pancreas development by selectively inducing the transcription factor Ptf1a. Development 21: 21.

28. Stafford D, White RJ, Kinkel MD, Linville A, Schilling TF, et al. (2006) Retinoids signal directly to zebrafish endoderm to specify insulin-expressing beta-cells. Development 133: 949-956.

29. Stafford D, Prince VE (2002) Retinoic acid signaling is required for a critical early step in zebrafish pancreatic development. Curr Biol 12: 1215-1220.

30. Chen Y, Pan FC, Brandes N, Afelik S, Solter M, et al. (2004) Retinoic acid signaling is essential for pancreas development and promotes endocrine at the expense of exocrine cell differentiation in Xenopus. Dev Biol 271: 144-160.

31. Molotkov A, Molotkova N, Duester G (2005) Retinoic acid generated by Raldh2 in mesoderm is required for mouse dorsal endodermal pancreas development. Dev Dyn 28: 28.

32. Martin M, Gallego-Llamas J, Ribes V, Kedinger M, Niederreither K, et al. (2005) Dorsal pancreas agenesis in retinoic acid-deficient Raldh2 mutant mice. Dev Biol 284: 399-411.

33. Ostrom M, Loffler KA, Edfalk S, Selander L, Dahl U, et al. (2008) Retinoic acid promotes the generation of pancreatic endocrine progenitor cells and their further differentiation into beta-cells. PLoS ONE 3: e2841.

34. Gu G, Brown JR, Melton DA (2003) Direct lineage tracing reveals the ontogeny of pancreatic cell fates during mouse embryogenesis. Mech Dev 120: 35-43.

35. Jonsson J, Carlsson L, Edlund T, Edlund H (1994) Insulin-promoter-factor 1 is required for pancreas development in mice. Nature 371: 606-609.

36. Ahlgren U, Jonsson J, Edlund H (1996) The morphogenesis of the pancreatic mesenchyme is uncoupled from that of the pancreatic epithelium in IPF1/PDX1-deficient mice. Development 122: 1409-1416.

37. Bhushan A, Itoh N, Kato S, Thiery JP, Czernichow P, et al. (2001) Fgf10 is essential for maintaining the proliferative capacity of epithelial progenitor cells during early pancreatic organogenesis. Development 128: 5109-5117.

38. Hart A, Papadopoulou S, Edlund H (2003) Fgf10 maintains notch activation, stimulates proliferation, and blocks differentiation of pancreatic epithelial cells. Dev Dyn 228: 185-193.

39. Norgaard GA, Jensen JN, Jensen J (2003) FGF10 signaling maintains the pancreatic progenitor cell state revealing a novel role of Notch in organ development. Dev Biol 264: 323-338.

40. Miralles F, Czernichow P, Ozaki K, Itoh N, Scharfmann R (1999) Signaling through fibroblast growth factor receptor 2b plays a key role in the development of the exocrine pancreas. Proc Natl Acad Sci U S A 96: 6267-6272.

41. Liu Z, Habener JF Wnt signaling in pancreatic islets. Adv Exp Med Biol 654: 391-419.

42. Heller RS, Dichmann DS, Jensen J, Miller C, Wong G, et al. (2002) Expression patterns of Wnts, Frizzleds, sFRPs, and misexpression in transgenic mice suggesting a role for Wnts in pancreas and foregut pattern formation. Dev Dyn 225: 260-270.

43. Heiser PW, Lau J, Taketo MM, Herrera PL, Hebrok M (2006) Stabilization of beta-catenin impacts pancreas growth. Development 133: 2023-2032.

44. Papadopoulou S, Edlund H (2005) Attenuated Wnt signaling perturbs pancreatic growth but not pancreatic function. Diabetes 54: 2844-2851.

45. Zhou Q, Law AC, Rajagopal J, Anderson WJ, Gray PA, et al. (2007) A multipotent progenitor domain guides pancreatic organogenesis. Developmental Cell 13: 103-114.

46. Hogan BL, Grindley J, Bellusci S, Dunn NR, Emoto H, et al. (1997) Branching morphogenesis of the lung: new models for a classical problem. Cold Spring Harb Symp Quant Biol 62: 249-256.

47. Costantini F (2006) Renal branching morphogenesis: concepts, questions, and recent advances. Differentiation 74: 402-421.

48. Apelqvist A, Li H, Sommer L, Beatus P, Anderson DJ, et al. (1999) Notch signalling controls pancreatic cell differentiation. Nature 400: 877-881.

49. Jensen J, Heller RS, Funder-Nielsen T, Pedersen EE, Lindsell C, et al. (2000) Independent development of pancreatic alpha- and beta-cells from neurogenin3-expressing precursors: a role for the notch pathway in repression of premature differentiation. Diabetes 49: 163-176.

50. Fisher A, Caudy M (1998) The function of hairy-related bHLH repressor proteins in cell fate decisions. Bioessays 20: 298-306.

51. Jensen J, Pedersen EE, Galante P, Hald J, Heller RS, et al. (2000) Control of endodermal endocrine development by Hes-1. Nat Genet 24: 36-44.

52. Gradwohl G, Dierich A, LeMeur M, Guillemot F (2000) neurogenin3 is required for the development of the four endocrine cell lineages of the pancreas. Proc Natl Acad Sci U S A 97: 1607-1611.

53. Gu G, Dubauskaite J, Melton DA (2002) Direct evidence for the pancreatic lineage: NGN3+ cells are islet progenitors and are distinct from duct progenitors. Development 129: 2447-2457.

54. Villasenor A, Chong DC, Cleaver O (2008) Biphasic Ngn3 expression in the developing pancreas. Dev Dyn 237: 3270-3279.

55. Herrera PL, Huarte J, Zufferey R, Nichols A, Mermillod B, et al. (1994) Ablation of islet endocrine cells by targeted expression of hormone-promoter-driven toxigenes. Proc Natl Acad Sci U S A 91: 12999-13003.

56. Pang K, Mukonoweshuro C, Wong GG (1994) Beta cells arise from glucose transporter type 2 (Glut2)-expressing epithelial cells of the developing rat pancreas. Proc Natl Acad Sci U S A 91: 9559-9563.

57. Lee YC, Damholt AB, Billestrup N, Kisbye T, Galante P, et al. (1999) Developmental expression of proprotein convertase 1/3 in the rat. Mol Cell Endocrinol 155: 27-35.

58. Herrera PL (2000) Adult insulin- and glucagon-producing cells differentiate from two independent cell lineages. Development 127: 2317-2322.

59. Wilson ME, Kalamaras JA, German MS (2002) Expression pattern of IAPP and prohormone convertase 1/3 reveals a distinctive set of endocrine cells in the embryonic pancreas. Mech Dev 115: 171-176.

60. Desgraz R, Herrera PL (2009) Pancreatic neurogenin 3-expressing cells are unipotent islet precursors. Development 136: 3567-3574.

61. Schwitzgebel VM, Scheel DW, Conners JR, Kalamaras J, Lee JE, et al. (2000) Expression of neurogenin3 reveals an islet cell precursor population in the pancreas. Development 127: 3533-3542.

62. Grapin-Botton A, Majithia AR, Melton DA (2001) Key events of pancreas formation are triggered in gut endoderm by ectopic expression of pancreatic regulatory genes. Genes Dev 15: 444-454.

63. Landry C, Clotman F, Hioki T, Oda H, Picard JJ, et al. (1997) HNF-6 is expressed in endoderm derivatives and nervous system of the mouse embryo and participates to the cross-regulatory network of liver- enriched transcription factors. Dev Biol 192: 247-257.

64. Rausa F, Samadani U, Ye H, Lim L, Fletcher CF, et al. (1997) The cut-homeodomain transcriptional activator HNF-6 is coexpressed with its target gene HNF-3 beta in the developing murine liver and pancreas. Dev Biol 192: 228-246.

65. Zhang H, Ables ET, Pope CF, Washington MK, Hipkens S, et al. (2009) Multiple, temporal-specific roles for HNF6 in pancreatic endocrine and ductal differentiation. Mech Dev 126: 958-973.

66. Jacquemin P, Durviaux SM, Jensen J, Godfraind C, Gradwohl G, et al. (2000) Transcription factor hepatocyte nuclear factor 6 regulates pancreatic endocrine cell differentiation and controls expression of the proendocrine gene ngn3. Mol Cell Biol 20: 4445-4454.

67. Jacquemin P, Lemaigre FP, Rousseau GG (2003) The Onecut transcription factor HNF-6 (OC-1) is required for timely specification of the pancreas and acts upstream of Pdx-1 in the specification cascade. Dev Biol 258: 105-116.

68. Schonhoff SE, Giel-Moloney M, Leiter AB (2004) Neurogenin 3-expressing progenitor cells in the gastrointestinal tract differentiate into both endocrine and non-endocrine cell types. Dev Biol 270: 443-454.

69. Wang S, Yan J, Anderson DA, Xu Y, Kanal MC, et al. Neurog3 gene dosage regulates allocation of endocrine and exocrine cell fates in the developing mouse pancreas. Dev Biol 339: 26-37.

70. Fukuda A, Kawaguchi Y, Furuyama K, Kodama S, Horiguchi M, et al. (2008) Reduction of Ptf1a gene dosage causes pancreatic hypoplasia and diabetes in mice. Diabetes 57: 2421-2431.

71. Dong PD, Provost E, Leach SD, Stainier DY (2008) Graded levels of Ptf1a differentially regulate endocrine and exocrine fates in the developing pancreas. Genes Dev 22: 1445-1450.

72. Cockell M, Stevenson BJ, Strubin M, Hagenbuchle O, Wellauer PK (1989) Identification of a cell-specific DNA-binding activity that interacts with a transcriptional activator of genes expressed in the acinar pancreas. Mol Cell Biol 9: 2464-2476.

73. Masui T, Long Q, Beres TM, Magnuson MA, MacDonald RJ (2007) Early pancreatic development requires the vertebrate Suppressor of Hairless (RBPJ) in the PTF1 bHLH complex. Genes Dev 21: 2629-2643.

74. Beres TM, Masui T, Swift GH, Shi L, Henke RM, et al. (2006) PTF1 is an organ-specific and Notch-independent basic helix-loop-helix complex containing the mammalian Suppressor of Hairless (RBP-J) or its paralogue, RBP-L. Mol Cell Biol 26: 117-130.

75. Henseleit KD, Nelson SB, Kuhlbrodt K, Hennings JC, Ericson J, et al. (2005) NKX6 transcription factor activity is required for alpha- and beta-cell development in the pancreas. Development 132: 3139-3149.

76. Schaffer AE, Freude KK, Nelson SB, Sander M (2010) Nkx6 transcription factors and Ptf1a function as antagonistic lineage determinants in multipotent pancreatic progenitors. Dev Cell 18: 1022-1029.

77. Wang J, Kilic G, Aydin M, Burke Z, Oliver G, et al. (2005) Prox1 activity controls pancreas morphogenesis and participates in the production of "secondary transition" pancreatic endocrine cells. Dev Biol 286: 182-194.

78. Burke Z, Oliver G (2002) Prox1 is an early specific marker for the developing liver and pancreas in the mammalian foregut endoderm. Mech Dev 118: 147-155.

79. Johansson KA, Dursun U, Jordan N, Gu G, Beermann F, et al. (2007) Temporal control of neurogenin3 activity in pancreas progenitors reveals competence windows for the generation of different endocrine cell types. Dev Cell 12: 457-465.

80. Sosa-Pineda B, Chowdhury K, Torres M, Oliver G, Gruss P (1997) The Pax4 gene is essential for differentiation of insulin-producing beta cells in the mammalian pancreas. Nature 386: 399-402.

81. Smith SB, Ee HC, Conners JR, German MS (1999) Paired-homeodomain transcription factor PAX4 acts as a transcriptional repressor in early pancreatic development. Mol Cell Biol 19: 8272-8280.

82. Wang J, Elghazi L, Parker SE, Kizilocak H, Asano M, et al. (2004) The concerted activities of Pax4 and Nkx2.2 are essential to initiate pancreatic beta-cell differentiation. Dev Biol 266: 178-189.

83. Prado CL, Pugh-Bernard AE, Elghazi L, Sosa-Pineda B, Sussel L (2004) Ghrelin cells replace insulin-producing {beta} cells in two mouse models of pancreas development. Proc Natl Acad Sci U S A 101: 2924-2929. Epub 2004 Feb 2917.

84. Heller RS, Jenny M, Collombat P, Mansouri A, Tomasetto C, et al. (2005) Genetic determinants of pancreatic epsilon-cell development. Dev Biol.

85. Ritz-Laser B, Estreicher A, Gauthier BR, Mamin A, Edlund H, et al. (2002) The pancreatic beta-cell-specific transcription factor Pax-4 inhibits glucagon gene expression through Pax-6. Diabetologia 45: 97-107.

86. Collombat P, Mansouri A, Hecksher-Sorensen J, Serup P, Krull J, et al. (2003) Opposing actions of Arx and Pax4 in endocrine pancreas development. Genes Dev 17: 2591-2603.

87. Wang Q, Elghazi L, Martin S, Martins I, Srinivasan RS, et al. (2008) Ghrelin is a novel target of Pax4 in endocrine progenitors of the pancreas and duodenum. Dev Dyn 237: 51-61.

88. Collombat P, Xu X, Ravassard P, Sosa-Pineda B, Dussaud S, et al. (2009) The ectopic expression of Pax4 in the mouse pancreas converts progenitor cells into alpha and subsequently beta cells. Cell 138: 449-462.

89. Collombat P, Hecksher-Sorensen J, Krull J, Berger J, Riedel D, et al. (2007) Embryonic endocrine pancreas and mature beta cells acquire alpha and PP cell phenotypes upon Arx misexpression. J Clin Invest 117: 961-970.

90. Collombat P, Hecksher-Sorensen J, Broccoli V, Krull J, Ponte I, et al. (2005) The simultaneous loss of Arx and Pax4 genes promotes a somatostatin-producing cell fate specification at the expense of the alpha- and beta-cell lineages in the mouse endocrine pancreas. Development 132: 2969-2980.

91. Turque N, Plaza S, Radvanyi F, Carriere C, Saule S (1994) Pax-QNR/Pax-6, a paired box- and homeobox-containing gene expressed in neurons, is also expressed in pancreatic endocrine cells. Mol Endocrinol 8: 929-938.

92. St-Onge L, Sosa-Pineda B, Chowdhury K, Mansouri A, Gruss P (1997) Pax6 is required for differentiation of glucagon-producing alpha-cells in mouse pancreas. Nature 387: 406-409.

93. Sander M, Neubuser A, Kalamaras J, Ee HC, Martin GR, et al. (1997) Genetic analysis reveals that PAX6 is required for normal transcription of pancreatic hormone genes and islet development. Genes Dev 11: 1662-1673.

94. Heller RS, Stoffers DA, Liu A, Schedl A, Crenshaw EB, 3rd, et al. (2004) The role of Brn4/Pou3f4 and Pax6 in forming the pancreatic glucagon cell identity. Dev Biol 268: 123-134.

95. Sussel L, Kalamaras J, Hartigan-O'Connor DJ, Meneses JJ, Pedersen RA, et al. (1998) Mice lacking the homeodomain transcription factor Nkx2.2 have diabetes due to arrested differentiation of pancreatic beta cells. Development 125: 2213-2221.

96. Pauls S, Zecchin E, Tiso N, Bortolussi M, Argenton F (2007) Function and regulation of zebrafish nkx2.2a during development of pancreatic islet and ducts. Dev Biol 304: 875-890.

97. Doyle MJ, Loomis ZL, Sussel L (2007) Nkx2.2-repressor activity is sufficient to specify alpha-cells and a small number of beta-cells in the pancreatic islet. Development 134: 515-523.

98. Raum JC, Gerrish K, Artner I, Henderson E, Guo M, et al. (2006) FoxA2, Nkx2.2, and PDX-1 regulate islet beta-cell-specific mafA expression through conserved sequences located between base pairs -8118 and -7750 upstream from the transcription start site. Mol Cell Biol 26: 5735-5743.

99. Artner I, Le Lay J, Hang Y, Elghazi L, Schisler JC, et al. (2006) MafB: An Activator of the Glucagon Gene Expressed in Developing Islet {alpha}- and {beta}-Cells. Diabetes 55: 297-304.

100. Artner I, Blanchi B, Raum JC, Guo M, Kaneko T, et al. (2007) MafB is required for islet beta cell maturation. Proc Natl Acad Sci U S A 104: 3853-3858.

101. Fujitani Y, Fujitani S, Boyer DF, Gannon M, Kawaguchi Y, et al. (2006) Targeted deletion of a cis-regulatory region reveals differential gene dosage requirements for Pdx1 in foregut organ differentiation and pancreas formation. Genes Dev 20: 253-266.

102. Massague J, Gomis RR (2006) The logic of TGFbeta signaling. FEBS Lett 580: 2811-2820.

103. Crisera CA, Maldonado TS, Kadison AS, Li M, Alkasab SL, et al. (2000) Transforming growth factor-beta 1 in the developing mouse pancreas: a potential regulator of exocrine differentiation. Differentiation 65: 255-259.

104. Sanvito F, Herrera P, Huarte J, Nichols A, Montesano R, et al. (1994) TGF-beta 1 influences the relative development of the exocrine and endocrine pancreas in vitro. Development 120: 3451-3462.

105. Bottinger EP, Jakubczak JL, Roberts IS, Mumy M, Hemmati P, et al. (1997) Expression of a dominant-negative mutant TGF-beta type II receptor in transgenic mice reveals essential roles for TGF-beta in regulation of growth and differentiation in the exocrine pancreas. Embo J 16: 2621-2633.

106. Goulley J, Dahl U, Baeza N, Mishina Y, Edlund H (2007) BMP4-BMPR1A signaling in beta cells is required for and augments glucose-stimulated insulin secretion. Cell Metab 5: 207-219.

107. Tulachan SS, Tei E, Hembree M, Crisera C, Prasadan K, et al. (2007) TGF-beta isoform signaling regulates secondary transition and mesenchymal-induced endocrine development in the embryonic mouse pancreas. Dev Biol 305: 508-521.

108. Smart NG, Apelqvist AA, Gu X, Harmon EB, Topper JN, et al. (2006) Conditional expression of Smad7 in pancreatic beta cells disrupts TGF-beta signaling and induces reversible diabetes mellitus. PLoS Biol 4: e39.

109. Harmon EB, Apelqvist AA, Smart NG, Gu X, Osborne DH, et al. (2004) GDF11 modulates NGN3+ islet progenitor cell number and promotes {beta}-cell differentiation in pancreas development. Development 131: 6163-6174. Epub 2004 Nov 6117.

110. Oh SP, Yeo CY, Lee Y, Schrewe H, Whitman M, et al. (2002) Activin type IIA and IIB receptors mediate Gdf11 signaling in axial vertebral patterning. Genes Dev 16: 2749-2754.

111. Dichmann DS, Yassin H, Serup P (2006) Analysis of pancreatic endocrine development in GDF11-deficient mice. Dev Dyn 235: 3016-3025.

112. Andersson O, Reissmann E, Ibanez CF (2006) Growth differentiation factor 11 signals through the transforming growth factor-beta receptor ALK5 to regionalize the anterior-posterior axis. EMBO Rep 7: 831-837.

113. Kim SK, Hebrok M, Li E, Oh SP, Schrewe H, et al. (2000) Activin receptor patterning of foregut organogenesis. Genes Dev 14: 1866-1871.

114. Zhang P, McGrath B, Li S, Frank A, Zambito F, et al. (2002) The PERK eukaryotic initiation factor 2 alpha kinase is required for the development of the skeletal system, postnatal growth, and the function and viability of the pancreas. Mol Cell Biol 22: 3864-3874.

115. Zhang FD, Li Y, Iida K, McGrath B, Cavener DR (2006) PERK EIF2AK3 control of pancreatic beta cell differentiation and proliferation is required for postnatal glucose homeostasis. Cell Metabolism 4: 491-497.

116. Gannon M, Ables ET, Crawford L, Lowe D, Offield MF, et al. (2008) pdx-1 function is specifically required in embryonic beta cells to generate appropriate numbers of endocrine cell types and maintain glucose homeostasis. Dev Biol 314: 406-417.

117. Ahlgren U, Jonsson J, Jonsson L, Simu K, Edlund H (1998) beta-cell-specific inactivation of the mouse Ipf1/Pdx1 gene results in loss of the beta-cell phenotype and maturity onset diabetes. Genes Dev 12: 1763-1768.

118. Brissova M, Shiota M, Nicholson WE, Gannon M, Knobel SM, et al. (2002) Reduction in pancreatic transcription factor PDX-1 impairs glucose-stimulated insulin secretion. J Biol Chem 277: 11225-11232.

119. Vincent M, Guz Y, Rozenberg M, Webb G, Furuta M, et al. (2003) Abrogation of protein convertase 2 activity results in delayed islet cell differentiation and maturation, increased alpha-cell proliferation, and islet neogenesis. Endocrinology 144: 4061-4069.

120. Vuguin PM, Kedees MH, Cui L, Guz Y, Gelling RW, et al. (2006) Ablation of the glucagon receptor gene increases fetal lethality and produces alterations in islet development and maturation. Endocrinology 147: 3995-4006.

121. Prasadan K, Daume E, Preuett B, Spilde T, Bhatia A, et al. (2002) Glucagon is required for early insulin-positive differentiation in the developing mouse pancreas. Diabetes 51: 3229-3236.

122. Pictet RL, Clark WR, Williams RH, Rutter WJ (1972) An ultrastructural analysis of the developing embryonic pancreas. Dev Biol 29: 436-467.

123. Bosco D, Orci L, Meda P (1989) Homologous but not heterologous contact increases the insulin secretion of individual pancreatic B-cells. Exp Cell Res 184: 72-80.

124. Samols E, Stagner JI, Ewart RB, Marks V (1988) The order of islet microvascular cellular perfusion is B----A----D in the perfused rat pancreas. J Clin Invest 82: 350-353.

125. Puri S, Hebrok M (2007) Dynamics of embryonic pancreas development using real-time imaging. Dev Biol 306: 82-93.

126. Miller K, Kim A, Kilimnik G, Jo J, Moka U, et al. (2009) Islet formation during the neonatal development in mice. PLoS One 4: e7739.

127. Miettinen PJ, Huotari M, Koivisto T, Ustinov J, Palgi J, et al. (2000) Impaired migration and delayed differentiation of pancreatic islet cells in mice lacking EGF-receptors. Development 127: 2617-2627.

128. Greiner TU, Kesavan G, Stahlberg A, Semb H (2009) Rac1 regulates pancreatic islet morphogenesis. BMC Dev Biol 9: 2.

129. Cirulli V, Beattie GM, Klier G, Ellisman M, Ricordi C, et al. (2000) Expression and function of alpha(v)beta(3) and alpha(v)beta(5) integrins in the developing pancreas: roles in the adhesion and migration of putative endocrine progenitor cells. J Cell Biol 150: 1445-1460.

130. Miralles F, Battelino T, Czernichow P, Scharfmann R (1998) TGF-beta plays a key role in morphogenesis of the pancreatic islets of Langerhans by controlling the activity of the matrix metalloproteinase MMP-2. J Cell Biol 143: 827-836.

131. Perez SE, Cano DA, Dao-Pick T, Rougier JP, Werb Z, et al. (2005) Matrix metalloproteinases 2 and 9 are dispensable for pancreatic islet formation and function in vivo. Diabetes 54: 694-701.

132. Dahl U, Sjodin A, Semb H (1996) Cadherins regulate aggregation of pancreatic beta-cells in vivo. Development 122: 2895-2902.

133. Esni F, Taljedal IB, Perl AK, Cremer H, Christofori G, et al. (1999) Neural cell adhesion molecule (N-CAM) is required for cell type segregation and normal ultrastructure in pancreatic islets. J Cell Biol 144: 325-337.

134. Jia D, Dajusta D, Foty RA (2007) Tissue surface tensions guide in vitro self-assembly of rodent pancreatic islet cells. Dev Dyn 236: 2039-2049.

135. Gannon M, Ray MK, Van Zee K, Rausa F, Costa RH, et al. (2000) Persistent expression of HNF6 in islet endocrine cells causes disrupted islet architecture and loss of beta cell function. Development 127: 2883-2895.

136. Tweedie E, Artner I, Crawford L, Poffenberger G, Thorens B, et al. (2006) Maintenance of hepatic nuclear factor 6 in postnatal islets impairs terminal differentiation and function of beta-cells. Diabetes 55: 3264-3270.

137. Wilding Crawford L, Tweedie Ables E, Oh YA, Boone B, Levy S, et al. (2008) Gene expression profiling of a mouse model of pancreatic islet dysmorphogenesis. PLoS ONE 3: e1611.

138. Moussad EE, Brigstock DR (2000) Connective tissue growth factor: what's in a name? Mol Genet Metab 71: 276-292.

139. Kim HS, Nagalla SR, Oh Y, Wilson E, Roberts CT, Jr., et al. (1997) Identification of a family of low-affinity insulin-like growth factor binding proteins (IGFBPs): characterization of connective tissue growth factor as a member of the IGFBP superfamily. Proc Natl Acad Sci U S A 94: 12981-12986.

140. Abreu JG, Ketpura NI, Reversade B, De Robertis EM (2002) Connective-tissue growth factor (CTGF) modulates cell signalling by BMP and TGF-beta. Nat Cell Biol 4: 599-604.

141. Bardeesy N, Cheng KH, Berger JH, Chu GC, Pahler J, et al. (2006) Smad4 is dispensable for normal pancreas development yet critical in progression and tumor biology of pancreas cancer. Genes Dev 20: 3130-3146.

142. Mercurio S, Latinkic B, Itasaki N, Krumlauf R, Smith JC (2004) Connective-tissue growth factor modulates WNT signalling and interacts with the WNT receptor complex. Development 131: 2137-2147.

143. Segarini PR, Nesbitt JE, Li D, Hays LG, Yates JR, 3rd, et al. (2001) The low density lipoprotein receptor-related protein/alpha2-macroglobulin receptor is a receptor for connective tissue growth factor. J Biol Chem 276: 40659-40667.

144. Gao R, Ball DK, Perbal B, Brigstock DR (2004) Connective tissue growth factor induces c-fos gene activation and cell proliferation through p44/42 MAP kinase in primary rat hepatic stellate cells. J Hepatol 40: 431-438.

145. Babic AM, Chen CC, Lau LF (1999) Fisp12/mouse connective tissue growth factor mediates endothelial cell adhesion and migration through integrin alphavbeta3, promotes endothelial cell survival, and induces angiogenesis in vivo. Mol Cell Biol 19: 2958-2966.

146. Gao R, Brigstock DR (2006) A novel integrin alpha5beta1 binding domain in module 4 of connective tissue growth factor (CCN2/CTGF) promotes adhesion and migration of activated pancreatic stellate cells. Gut 55: 856-862.

147. Schober JM, Chen N, Grzeszkiewicz TM, Jovanovic I, Emeson EE, et al. (2002) Identification of integrin alpha(M)beta(2) as an adhesion receptor on peripheral blood monocytes for Cyr61 (CCN1) and connective tissue growth factor (CCN2): immediate-early gene products expressed in atherosclerotic lesions. Blood 99: 4457-4465.

148. Brigstock DR, Steffen CL, Kim GY, Vegunta RK, Diehl JR, et al. (1997) Purification and characterization of novel heparin-binding growth factors in uterine secretory fluids. Identification as heparin-regulated Mr 10,000 forms of connective tissue growth factor. J Biol Chem 272: 20275-20282.

149. Hashimoto G, Inoki I, Fujii Y, Aoki T, Ikeda E, et al. (2002) Matrix metalloproteinases cleave connective tissue growth factor and reactivate angiogenic activity of vascular endothelial growth factor 165. J Biol Chem 277: 36288-36295. Epub 32002 Jul 36211.

150. Grotendorst GR, Duncan MR (2005) Individual domains of connective tissue growth factor regulate fibroblast proliferation and myofibroblast differentiation. Faseb J 19: 729-738.

151. Hoshijima M, Hattori T, Inoue M, Araki D, Hanagata H, et al. (2006) CT domain of CCN2/CTGF directly interacts with fibronectin and enhances cell adhesion of chondrocytes through integrin alpha5beta1. FEBS Lett 580: 1376-1382.

152. Brigstock DR, Kim GY, Steffen CL, Liu A, Vegunta RK, et al. (1996) High molecular mass forms of epidermal growth factor in pig uterine secretions. J Reprod Fertil 108: 313-320.

153. Kothapalli D, Grotendorst GR (2000) CTGF modulates cell cycle progression in cAMP-arrested NRK fibroblasts. J Cell Physiol 182: 119-126.

154. Wu SH, Lu C, Dong L, Chen ZQ (2008) Signal transduction involved in CTGF-induced production of chemokines in mesangial cells. Growth Factors 26: 192-200.

155. Abraham D (2008) Connective tissue growth factor: growth factor, matricellular organizer, fibrotic biomarker or molecular target for anti-fibrotic therapy in SSc? Rheumatology (Oxford) 47 Suppl 5: v8-9.

156. Liu H, Yang R, Tinner B, Choudhry A, Schutze N, et al. (2008) Cysteine-rich protein 61 and connective tissue growth factor induce deadhesion and anoikis of retinal pericytes. Endocrinology 149: 1666-1677.

157. Chien W, Yin D, Gui D, Mori A, Frank JM, et al. (2006) Suppression of cell proliferation and signaling transduction by connective tissue growth factor in non-small cell lung cancer cells. Mol Cancer Res 4: 591-598.

158. Kubota S, Hattori T, Shimo T, Nakanishi T, Takigawa M (2000) Novel intracellular effects of human connective tissue growth factor expressed in Cos-7 cells. FEBS Lett 474: 58-62.

159. Kireeva ML, Latinkic BV, Kolesnikova TV, Chen CC, Yang GP, et al. (1997) Cyr61 and Fisp12 are both ECM-associated signaling molecules: activities, metabolism, and localization during development. Exp Cell Res 233: 63-77.

160. Docherty K (2001) Growth and development of the islets of Langerhans: implications for the treatment of diabetes mellitus. Curr Opin Pharmacol 1: 641-650.

161. Tan TW, Lai CH, Huang CY, Yang WH, Chen HT, et al. (2009) CTGF enhances migration and MMP-13 up-regulation via alphavbeta3 integrin, FAK, ERK, and NF-kappaB-dependent pathway in human chondrosarcoma cells. J Cell Biochem 107: 345-356.

162. Chen CC, Chen N, Lau LF (2001) The angiogenic factors Cyr61 and connective tissue growth factor induce adhesive signaling in primary human skin fibroblasts. J Biol Chem 276: 10443-10452.

163. Crean JK, Furlong F, Finlay D, Mitchell D, Murphy M, et al. (2004) Connective tissue growth factor [CTGF]/CCN2 stimulates mesangial cell migration through integrated dissolution of focal adhesion complexes and activation of cell polarization. FASEB J 18: 1541-1543.

164. Shimo T, Nakanishi T, Nishida T, Asano M, Kanyama M, et al. (1999) Connective tissue growth factor induces the proliferation, migration, and tube formation of vascular endothelial cells in vitro, and angiogenesis in vivo. J Biochem 126: 137-145.

165. Kolesnikova TV, Lau LF (1998) Human CYR61-mediated enhancement of bFGF-induced DNA synthesis in human umbilical vein endothelial cells. Oncogene 16: 747-754.

166. Inoki I, Shiomi T, Hashimoto G, Enomoto H, Nakamura H, et al. (2002) Connective tissue growth factor binds vascular endothelial growth factor (VEGF) and inhibits VEGF-induced angiogenesis. Faseb J 16: 219-221. Epub 2001 Dec 2014.

167. Wu S, Platteau A, Chen S, McNamara G, Whitsett J, et al. Conditional overexpression of connective tissue growth factor disrupts postnatal lung development. Am J Respir Cell Mol Biol 42: 552-563.

168. Chen S, Ron M, Platteau A, Hehre D, Smith H, et al. CTGF Disrupts Alveolarization and Induces Pulmonary Hypertension in Neonatal Mice: Implication in the Pathogenesis of Severe Bronchopulmonary Dysplasia. Am J Physiol Lung Cell Mol Physiol.

169. Mochizuki S, Tanaka R, Shimoda M, Onuma J, Fujii Y, et al. Connective tissue growth factor is a substrate of ADAM28. Biochem Biophys Res Commun 402: 651-657.

170. Ivkovic S, Yoon BS, Popoff SN, Safadi FF, Libuda DE, et al. (2003) Connective tissue growth factor coordinates chondrogenesis and angiogenesis during skeletal development. Development 130: 2779-2791.

171. Baguma-Nibasheka M, Kablar B (2008) Pulmonary hypoplasia in the connective tissue growth factor (Ctgf) null mouse. Dev Dyn 237: 485-493.

172. Canalis E, Zanotti S, Beamer WG, Economides AN, Smerdel-Ramoya A (2010) Connective tissue growth factor is required for skeletal development and postnatal skeletal homeostasis in male mice. Endocrinology 151: 3490-3501.

173. Doherty HE, Kim HS, Hiller S, Sulik KK, Maeda N A mouse strain where basal connective tissue growth factor gene expression can be switched from low to high. PLoS One 5: e12909.

174. Smerdel-Ramoya A, Zanotti S, Stadmeyer L, Durant D, Canalis E (2008) Skeletal overexpression of connective tissue growth factor impairs bone formation and causes osteopenia. Endocrinology 149: 4374-4381.

175. Nakanishi T, Yamaai T, Asano M, Nawachi K, Suzuki M, et al. (2001) Overexpression of connective tissue growth factor/hypertrophic chondrocyte-specific gene product 24 decreases bone density in adult mice and induces dwarfism. Biochem Biophys Res Commun 281: 678-681.

176. Sonnylal S, Shi-Wen X, Leoni P, Naff K, Van Pelt CS, et al. Selective expression of connective tissue growth factor in fibroblasts in vivo promotes systemic tissue fibrosis. Arthritis Rheum 62: 1523-1532.

177. Remedi MS, Kurata HT, Scott A, Wunderlich FT, Rother E, et al. (2009) Secondary consequences of beta cell inexcitability: identification and prevention in a murine model of K(ATP)-induced neonatal diabetes mellitus. Cell Metab 9: 140-151.

178. Yokoi H, Mukoyama M, Mori K, Kasahara M, Suganami T, et al. (2008) Overexpression of connective tissue growth factor in podocytes worsens diabetic nephropathy in mice. Kidney Int 73: 446-455.

179. Tong Z, Chen R, Alt DS, Kemper S, Perbal B, et al. (2009) Susceptibility to liver fibrosis in mice expressing a connective tissue growth factor transgene in hepatocytes. Hepatology 50: 939-947.

180. Panek AN, Posch MG, Alenina N, Ghadge SK, Erdmann B, et al. (2009) Connective tissue growth factor overexpression in cardiomyocytes promotes

cardiac hypertrophy and protection against pressure overload. PLoS One 4: e6743.

181. Bennewith KL, Huang X, Ham CM, Graves EE, Erler JT, et al. (2009) The role of tumor cell-derived connective tissue growth factor (CTGF/CCN2) in pancreatic tumor growth. Cancer Res 69: 775-784.

182. Dornhofer N, Spong S, Bennewith K, Salim A, Klaus S, et al. (2006) Connective tissue growth factor-specific monoclonal antibody therapy inhibits pancreatic tumor growth and metastasis. Cancer Res 66: 5816-5827.

183. Aikawa T, Gunn J, Spong SM, Klaus SJ, Korc M (2006) Connective tissue growth factor-specific antibody attenuates tumor growth, metastasis, and angiogenesis in an orthotopic mouse model of pancreatic cancer. Mol Cancer Ther 5: 1108-1116.

184. Ijichi H, Chytil A, Gorska AE, Aakre ME, Fujitani Y, et al. (2006) Aggressive pancreatic ductal adenocarcinoma in mice caused by pancreas-specific blockade of transforming growth factor-beta signaling in cooperation with active Kras expression. Genes Dev 20: 3147-3160.

185. Pickles M, Leask A (2007) Analysis of CCN2 promoter activity in PANC-1 cells: regulation by ras/MEK/ERK. J Cell Commun Signal 1: 85-90.

186. di Mola FF, Friess H, Martignoni ME, Di Sebastiano P, Zimmermann A, et al. (1999) Connective tissue growth factor is a regulator for fibrosis in human chronic pancreatitis. Ann Surg 230: 63-71.

187. Gao R, Brigstock DR (2005) Connective tissue growth factor (CCN2) in rat pancreatic stellate cell function: integrin alpha5beta1 as a novel CCN2 receptor. Gastroenterology 129: 1019-1030.

188. Crawford LA, Guney MA, Oh YA, Deyoung RA, Valenzuela DM, et al. (2009) Connective Tissue Growth Factor (CTGF) Inactivation Leads to Defects in Islet Cell Lineage Allocation and {beta}-Cell Proliferation during Embryogenesis. Mol Endocrinol 23: 324-336.

189. di Mola FF, Friess H, Riesle E, Koliopanos A, Buchler P, et al. (2002) Connective tissue growth factor is involved in pancreatic repair and tissue remodeling in human and rat acute necrotizing pancreatitis. Ann Surg 235: 60-67.

190. Hara M, Wang X, Kawamura T, Bindokas VP, Dizon RF, et al. (2003) Transgenic mice with green fluorescent protein-labeled pancreatic beta -cells. Am J Physiol Endocrinol Metab 284: E177-183.

191. Gustafsson E, Brakebusch C, Hietanen K, Fassler R (2001) Tie-1-directed expression of Cre recombinase in endothelial cells of embryoid bodies and transgenic mice. J Cell Sci 114: 671-676.

192. Milo-Landesman D, Surana M, Berkovich I, Compagni A, Christofori G, et al. (2001) Correction of hyperglycemia in diabetic mice transplanted with reversibly

immortalized pancreatic beta cells controlled by the tet-on regulatory system. Cell Transplant 10: 645-650.

193. Teitelman G, Lee J, Reis DJ (1987) Differentiation of prospective mouse pancreatic islet cells during development in vitro and during regeneration. Dev Biol 120: 425-433.

194. Zhang H, Ackermann AM, Gusarova GA, Lowe D, Feng X, et al. (2006) The Foxm1 Transcription Factor is Required to Maintain Pancreatic Beta Cell Mass. Mol Endocrinol 20: 1853-1866.

195. Ackermann Misfeldt A, Costa RH, Gannon M (2008) β-Cell Proliferation, but not Neogenesis, Following 60% Partial Pancreatectomy is Impaired in the Absence of FoxM1. Diabetes 57: 3069-3077.

196. Jacquemin P, Yoshitomi H, Kashima Y, Rousseau GG, Lemaigre FP, et al. (2006) An endothelial-mesenchymal relay pathway regulates early phases of pancreas development. Dev Biol 290: 189-199.

197. Brissova M, Shostak A, Shiota M, Wiebe PO, Poffenberger G, et al. (2006) Pancreatic islet production of vascular endothelial growth factor--a is essential for islet vascularization, revascularization, and function. Diabetes 55: 2974-2985.

198. Kapasa M, Serafimidis I, Gavalas A, Kossida S (2008) Identification of phylogenetically conserved enhancer elements implicated in pancreas development in the WISP1 and CTGF orthologs. Genomics.

199. Gressner OA, Gressner AM (2008) Connective tissue growth factor: a fibrogenic master switch in fibrotic liver diseases. Liver Int 28: 1065-1079.

200. Abreu JG, Ketpura NI, Reversade B, De Robertis EM (2002) Connective-tissue growth factor (CTGF) modulates cell signalling by BMP and TGF-beta. Nat Cell Biol 4: 599-604.

201. Wahab NA, Weston BS, Mason RM (2005) Modulation of the TGFbeta/Smad signaling pathway in mesangial cells by CTGF/CCN2. Exp Cell Res 307: 305-314.

202. Grotendorst GR, Okochi H, Hayashi N (1996) A novel transforming growth factor beta response element controls the expression of the connective tissue growth factor gene. Cell Growth and Differentiation 7: 469-480.

203. Rane SG, Lee JH, Lin HM (2006) Transforming growth factor-beta pathway: role in pancreas development and pancreatic disease. Cytokine Growth Factor Rev 17: 107-119.

204. Lee MS, Gu D, Feng L, Curriden S, Arnush M, et al. (1995) Accumulation of extracellular matrix and developmental dysregulation in the pancreas by transgenic production of transforming growth factor-beta 1. Am J Pathol 147: 42-52.

205. Fan WH, Karnovsky MJ (2002) Increased MMP-2 expression in connective tissue growth factor over-expression vascular smooth muscle cells. J Biol Chem 277: 9800-9805.

206. Mao Z, Ma X, Rong Y, Cui L, Wang X, et al. Connective tissue growth factor enhances the migration of gastric cancer through downregulation of E-cadherin via the NF-kappaB pathway. Cancer Sci 102: 104-110.

207. Sanvito F, Nichols A, Herrera PL, Huarte J, Wohlwend A, et al. (1995) TGF-beta 1 overexpression in murine pancreas induces chronic pancreatitis and, together with TNF-alpha, triggers insulin-dependent diabetes. Biochem Biophys Res Commun 217: 1279-1286.

208. Lin AH, Luo J, Mondshein LH, ten Dijke P, Vivien D, et al. (2005) Global analysis of Smad2/3-dependent TGF-beta signaling in living mice reveals prominent tissue-specific responses to injury. J Immunol 175: 547-554.

209. Maretto S, Cordenonsi M, Dupont S, Braghetta P, Broccoli V, et al. (2003) Mapping Wnt/beta-catenin signaling during mouse development and in colorectal tumors. Proc Natl Acad Sci U S A 100: 3299-3304.

210. Korchynskyi O, ten Dijke P (2002) Identification and functional characterization of distinct critically important bone morphogenetic protein-specific response elements in the Id1 promoter. J Biol Chem 277: 4883-4891.

211. Monteiro RM, de Sousa Lopes SM, Korchynskyi O, ten Dijke P, Mummery CL (2004) Spatio-temporal activation of Smad1 and Smad5 in vivo: monitoring transcriptional activity of Smad proteins. J Cell Sci 117: 4653-4663.

212. Hayashi S, Lewis P, Pevny L, McMahon AP (2002) Efficient gene modulation in mouse epiblast using a Sox2Cre transgenic mouse strain. Mechanisms of Development 119S: S97-S101.

213. Nikolova G, Jabs N, Konstantinova I, Domogatskaya A, Tryggvason K, et al. (2006) The vascular basement membrane: a niche for insulin gene expression and Beta cell proliferation. Dev Cell 10: 397-405.

214. Parsons JA, Brelje TC, Sorenson RL (1992) Adaptation of islets of Langerhans to pregnancy: increased islet cell proliferation and insulin secretion correlates with the onset of placental lactogen secretion. Endocrinology 130: 1459-1466.

215. Johansson M, Mattsson G, Andersson A, Jansson L, Carlsson PO (2006) Islet endothelial cells and pancreatic beta-cell proliferation: studies in vitro and during pregnancy in adult rats. Endocrinology 147: 2315-2324.

216. Brigstock DR (2002) Regulation of angiogenesis and endothelial cell function by connective tissue growth factor (CTGF) and cysteine-rich 61 (CYR61). Angiogenesis 5: 153-165.

217. Holland AM, Hale MA, Kagami H, Hammer RE, MacDonald RJ (2002) Experimental control of pancreatic development and maintenance. Proc Natl Acad Sci U S A 99: 12236-12241. Epub 12002 Sep 12239.

218. Thomas MK, Devon ON, Lee JH, Peter A, Schlosser DA, et al. (2001) Development of diabetes mellitus in aging transgenic mice following suppression of pancreatic homeoprotein IDX-1. J Clin Invest 108: 319-329.

219. Ackermann AM, Gannon M (2007) Molecular regulation of pancreatic beta-cell mass development, maintenance, and expansion. J Mol Endocrinol 38: 193-206.

220. Huang BL, Brugger SM, Lyons KM Stage-specific control of connective tissue growth factor (CTGF/CCN2) expression in chondrocytes by Sox9 and beta-catenin. J Biol Chem 285: 27702-27712.

221. Seymour PA, Freude KK, Dubois CL, Shih HP, Patel NA, et al. (2008) A dosage-dependent requirement for Sox9 in pancreatic endocrine cell formation. Dev Biol 323: 19-30.

222. Seymour P, Freude K, Tran M, Mayes E, Jensen J, et al. (2007) SOX9 is required for maintenance of the pancreatic progenitor cell pool. Proc Natl Acad Sci U S A 104: 1865-1870.

223. Suzuma K, Naruse K, Suzuma I, Takahara N, Ueki K, et al. (2000) Vascular endothelial growth factor induces expression of connective tissue growth factor via KDR, Flt1, and phosphatidylinositol 3-kinase-akt-dependent pathways in retinal vascular cells. J Biol Chem 275: 40725-40731.

224. D'Amour KA, Bang AG, Eliazer S, Kelly OG, Agulnick AD, et al. (2006) Production of pancreatic hormone-expressing endocrine cells from human embryonic stem cells. Nat Biotechnol 24: 1392-1401.

225. Kroon E, Martinson LA, Kadoya K, Bang AG, Kelly OG, et al. (2008) Pancreatic endoderm derived from human embryonic stem cells generates glucose-responsive insulin-secreting cells in vivo. Nat Biotechnol 26: 443-452.

226. Borowiak M, Maehr R, Chen S, Chen AE, Tang W, et al. (2009) Small molecules efficiently direct endodermal differentiation of mouse and human embryonic stem cells. Cell Stem Cell 4: 348-358.