Differential regulation of embryonic and adult β cell replication

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Diabetes results from an inadequate functional β cell mass, either due to autoimmune destruction (Type 1 diabetes) or insulin resistance combined with β cell failure (Type 2 diabetes). Strategies to enhance β cell regeneration or increase cell proliferation could improve outcomes for patients with diabetes. Research conducted over the past several years has revealed that factors regulating embryonic β cell mass expansion differ from those regulating replication of β cells post-weaning. This article aims to compare and contrast factors known to control embryonic and postnatal β cell replication. In addition, we explore the possibility that connective tissue growth factor (CTGF) could increase adult β cell replication. We have already shown that CTGF is required for embryonic β cell proliferation and is sufficient to induce replication of embryonic β cells. Here we examine whether adult β cell replication and expansion of β cell mass can be enhanced by increased CTGF expression in mature β cells.

Introduction

Type 2 diabetes is a condition in which there is inadequate pancreatic β cell mass and/or insufficient insulin released by β cells, leading to hyperglycemia. β cell mass is dynamic, responding to stimuli such as pregnancy and weight gain, resulting in β cell mass expansion. Autopsy studies of obese, non-diabetic individuals reveal an expansion in β cell mass when compared with lean controls. However, this compensation is lost in individuals with Type 2 diabetes.[1] This inadequate β cell mass

expansion in individuals with diabetes has led to an increased focus of research into β cell proliferation and mechanisms of β cell mass expansion as a potential therapy for the disease. In the recent past, Type 2 diabetes was a disease that mainly affected adults as they aged due to decreasing physical activity and increased weight gain. However, in 2011, in the United States alone, 25.8 million children and adults had diabetes, representing 8% of the total population.[2] Strategies to increase β cell mass would thus have an impact on a significant number of people. Given the diverse nature of this population, it is unclear that strategies developed to augment β cell mass would show beneficial effects on both young patients and older adults.

Although it is becoming increasingly clear from studies in rodents that many differences exist between the mechanisms by which embryonic and adult β cell mass are increased and maintained, many of the transcription factors important in embryonic β cell development have distinct roles in maintaining adult β cell identity and functionality (**Table 1**). In the embryo, β cell differentiation from multipotent pancreatic progenitor cells is established by an extensive transcription factor regulatory network. This neogenesis continues through the first few weeks after birth, concomitant with proliferation, which begins at late gestation in the mouse (**Fig. 1**). In contrast, in the adult, there is a balance between β cell replication and apoptosis which dictates β cell mass; neogenesis is not thought to contribute to β cell mass expansion under normal circumstances in adult mice, although this is still unclear in humans.

Table 1. Factors regulating embryonic and postnatal β cell replication

Factor	Role during development	Role postnatally
Transcription factors		
Pdx1	Growth and branching of pancreatic epithelium,[6] required for endocrine cell differentiation,[20] contributes to β cell proliferation[21]	Required to maintain β cell mass and β cell function[27,69]
Ptf1a	Evagination of pancreatic buds[7]	Acinar cell gene expression[70]
Ngn3	Initiation of endocrine cell differentiation[8]	Deletion of NGN3 in mature β cells impairs β cell function[71]
Sox9	Regulates the number of Ngn3 positive cells[10]	Haploinsufficiency results in glucose intolerance[72]
Hnf6	Required for Ngn3 expression[9]	Expressed in acinar cells and ducts[73]
MafA	Critical to Pdx1 expression only in the absence of MafB[63]	Important for expression of genes involved in β cell function[24]
MafB	Critical to the development of mature β cells,[22] critical for Pdx1 expression[63]	Expressed in α cells[74]
FoxM1	No effect on β cell proliferation or β cell mass when inactivated[38]	Required for postnatal β cell proliferation[36-38] regulates p27 expression[38]
FoxO1	Regulates Pdx1 transcription[50]	Negatively regulates β cell proliferation[75]
NFAT	Unknown	Inactivating studies show decreased β cell replication[52]
Growth factors		
CTGF	Required for β cell proliferation. Can induce α and β cell replication[3,4]	No effect on β cell proliferation when overexpressed (this study)
PDGF	Unknown	Regulates Ezh2 expression[33]
Placental lactogen	Unknown	Stimulates β cell proliferation[44,56]
HGF	Unknown	Stimulates β cell proliferation by activating PI3 kinase[76]
Cell cycle activators		
Cyclin D2	No effect on β cell mass when inactivated[28]	Contributes to β cell replication[28]
Cdk4	No effect on neogenesis[29] but overexpression increases β cell proliferation[77]	Null mutants have decreased β cell proliferation at P10[29]
Cell cycle inhibitors		
P27[Kip1]	Negatively regulates β cell proliferation; proliferation increased when inactivated[18]	No effect on β cell proliferation when inactivated in young adults; inactivation in older adults improves β cell proliferation[18]
P16[Ink4a]	Unknown	Inhibits β cell proliferation with knockouts having increased β cell proliferation[30]
Cell cycle inhibitor regulators		
Bmi1	Unknown	Regulates p16[Ink4a][31]
Ezh2	Unknown	Regulates p16[Ink4a][32]
Regulators of the PI3K/Akt pathway		
mTORC2 (Rictor)	Regulates p27 and FoxO[149]	Critical for β cell replication[49]
PDK1	Essential for β cell expansion[78]	Unknown
Other		
Glucose	Ovine fetuses exposed to high glucose in utero showed increased β cell mass with no change in β cell proliferation[79]	Stimulates β cell proliferation[45,46]
PERK	Contributes to β cell proliferation[16]	Not required for β cell proliferation post-weaning[16]

Here we review what is currently known about regulation of embryonic and postnatal β cell mass. For focus and clarity, we have confined our analyses to in vivo studies using gene inactivation or transgenic overexpression studies. Within this context, we specifically explore the potential of the secreted protein, connective tissue growth factor (CTGF), to induce adult β cell mass expansion. We previously showed that CTGF is required for embryonic β cell proliferation[3,4] and that CTGF overexpression in embryonic cells increases β cell proliferation and β cell mass.[4] We now examine the effect of CTGF overexpression in adult cells.

Embryonic β Cell Neogenesis

The mouse pancreas develops from ventral and dorsal evaginations of the posterior foregut endoderm at embryonic day (e) 9.5, a process dependent on the transcription factors Pdx1 and Ptf1a.[6,7] Differentiation of all pancreatic endocrine cell types (α, β, Δ and PP) is dependent on the transcription factor, neurogenin 3 (Ngn3).[8] *Ngn3* expression is controlled by a variety of factors, including the Notch signaling pathway and the transcriptional regulators pancreatic and duodenal homeobox 1 (Pdx1), SRY-box 9 (Sox9) and hepatic nuclear factor 6 (Hnf6).[9-11] Although β cell neogenesis begins at e10.5, these early insulin-positive cells do not contribute to mature islets (**Fig. 1**). Instead, endocrine cells that will go on to contribute to the mature islets begin to differentiate at e13, a period known as the secondary transition (**Fig. 1**).[15] Some transcription factors critically involved in β cell differentiation include NK2 homeobox 2 (Nkx2.2), Nkx6.1, islet 1 (Isl-1), neuronal differentiation 1 (NeuroD1), motor neuron and pancreas homeobox 1 (Mnx1), paired box gene 4 (Pax4) and Pdx1.[12] The function of these factors in pancreatic endocrine differentiation has been extensively reviewed and will not be covered here.[13-15] cells formed during the secondary transition, which extends into the early postnatal period (**Fig. 1**), will serve as the source of cells for replication at late gestation, postnatally and in adults. Strategies to reactivate neogenic pathways in adult pancreas are under active research
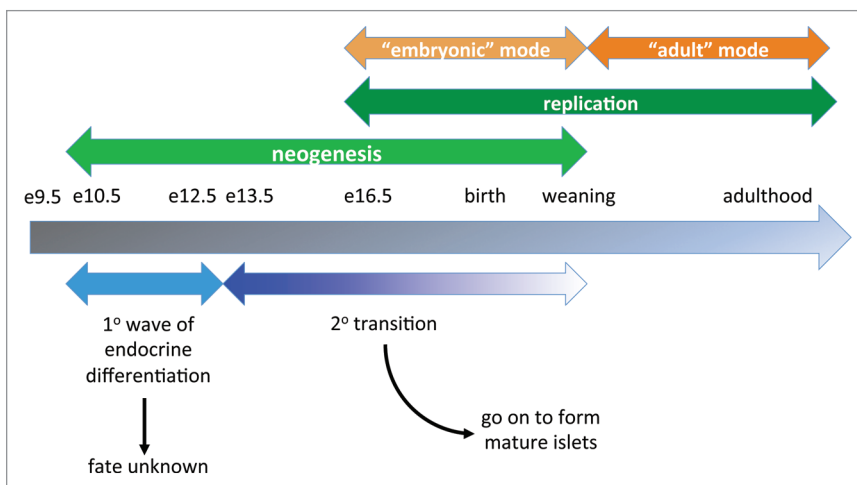

Figure 1. Schematic of β cell mass expansion throughout life. The timeline shows key events and time points in mouse pancreas development and β cell mass expansion under normal circumstances. Underlying the timeline (blue) are the approximate windows of primary and secondary endocrine differentiation. Overlying the timeline (green) are major mechanisms for β cell mass increase at those times: neogenesis early and replication later, although these overlap. The replicative phase is separated into stages (orange): "embryonic" mode and "adult" mode, indicating the switch from dependency on one set of regulatory factors to another around weaning.

and could aid in developing therapies to expand adult β cell mass.

Regulation of Embryonic and Neonatal β Cell Proliferation

Overall, less is known about regulation of embryonic β cell replication compared with adult β cell replication. Proliferation of existing cells can first be observed at e16.5 in the mouse (**Fig. 1**).[16] Studies in sheep have demonstrated that fetal overnutrition, in which pregnant ewes are fed a high fat diet, increases prenatal β cell proliferation.[17] Thus, as discussed below in adults, physiological stimuli can enhance β cell proliferation during development. Although several factors have been identified that play a role in the regulation of embryonic and neonatal β cell proliferation (**Table 1**), most of these have no obvious role in regulation of the cell cycle. One cell cycle regulator that does play a role in embryonic β cell proliferation is the cell cycle inhibitor, p27[Kip1]. Inactivation of *p27^{Kip1}* during embryogenesis results in an increase in β cell proliferation and subsequently β cell mass.[18] There was no change, however, in early postnatal β cell proliferation, suggesting that p27[Kip1] is not crucial to postnatal proliferation.

As mentioned above, Pdx1 is expressed in multipotent pancreatic progenitors in the early stages of pancreas development, but by e16.5, Pdx1 expression becomes enhanced in insulin-positive cells and is found at only low levels in exocrine cells.[19] This expression pattern is maintained into adulthood and Pdx1 plays a critical role in maintenance of the mature β cell phenotype.[20] Inactivation of *Pdx1* in embryonic insulin-expressing cells results in a dramatic decrease in β cell proliferation at late gestation, leading to decreased β cell mass at birth and early onset diabetes.[21] Two large Maf (musculoaponeurotic fibrosarcoma oncogene homolog) transcription factors that are closely related to one another, MafA and MafB, are critical for β cell differentiation and embryonic *Pdx1* expression[22] and therefore may have an indirect effect on embryonic β cell replication.

Inactivation of the eIF2α endoplasmic reticulum resident kinase, PERK (protein kinase RNA-like endoplasmic reticulum kinase), specifically in embryonic β cells (PERK[Δbeta]) results in a 2-fold decrease in β cell proliferation at e16.5, which persists through postnatal day (P) 8.[16] No difference in the number of insulin-expressing cells is evident in PERK[Δbeta] mice compared with control mice at e16.5,

suggesting that PERK deficiency does not affect β cell differentiation. Inactivation of *PERK* in adult β cells has no effect on β cell mass or function,[16] although its role in β cell mass expansion in response to stimuli such as pregnancy or obesity has not yet been examined.

CTGF, a member of the CCN (Cyr61, CTGF, Nov) family of secretory proteins, is involved in various cellular functions such as adhesion, migration, proliferation, extra-cellular matrix (ECM) remodeling and angiogenesis.[23] In the pancreas, CTGF is expressed in insulin-positive cells, blood vessel endothelium and ductal epithelium during embryogenesis in the mouse, but only in endothelium and ducts after P3.[3] We discovered that homozygous and heterozygous *Ctgf*-null mutant mice have a significant reduction in β cell proliferation at e18.5.[3] Hypothesizing that CTGF overexpression would increase β cell proliferation, we generated transgenic mice in which CTGF could be inducibly overexpressed.[4] Overexpression of CTGF in insulin-producing cells during embryogenesis induced proliferation of both α and β cells, resulting in increased α and β cell mass while maintaining the appropriate ratio of these two cell types. Interestingly, only MafA-negative insulin-positive cells were observed to proliferate in this model. Since MafA expression is associated with more mature cells,[24] these results suggest that only immature cells respond to CTGF. CTGF overexpression had no effect on the number of endocrine progenitors, indicating that CTGF influences endocrine cell proliferation and not neogenesis.

Regulation of Postnatal β Cell Proliferation

The perinatal period is characterized by heightened β cell proliferation and increased β cell mass.[25] At the time of weaning, there is considerable islet remodeling with significant β cell death but also a burst of β cell neogenesis; however, there is no significant contribution of neogenesis to β cell mass expansion post-weaning (**Fig. 1**).[25] Instead, adult β cell mass is mainly maintained and increased via proliferation.[26] In rats, by 30–40 d of age, β cell replication rates stabilize.[25] At this time, the α:β cell ratio is approximately 1:4 in mice.[27]

Postnatal β cell proliferation is heavily regulated by specific cell cycle activators and inhibitors that, surprisingly, play no role in embryonic β cell replication (**Table 1**). Although loss of the cell cycle activator Cyclin D2 during embryogenesis has no effect on β cell mass, postnatally, its absence causes decreased β cell replication and a 4-fold reduction in β cell mass compared with controls.[28] Similarly, the absence of the cell cycle activator cyclin-dependent kinase 4 (Cdk4) has no effect on embryonic β cell mass but *Cdk4*-null mutants have a 3.5-fold reduction in β cell proliferation at P10.[29] β cells have been shown to have less redundancy in cell cycle activators. For example, β cells lack Cdk6, a close homolog of Cdk4 that is co-expressed with Cdk4 in other cell types.[29] Thus, loss of a single activator results in a dramatic phenotype, although why the effects are often restricted to the postnatal period is not clear.

p16[Ink4a], a cell cycle inhibitor, increases in β cells with age and contributes to the normal age-related decline in β cell proliferation.[30] Germline deletion of *p16[Ink4a]* ameliorates this decline and β cells of aged mutant mice proliferate similarly to those of young control mice. Conversely, transgenic overexpression of *p16[Ink4a]* in β cells results in decreased β cell proliferation in young transgenic mice, similar to that seen in older control mice.[30] Bmi1 is an inhibitor of the *Ink4a/Arf* locus. At 2 weeks of age, *Bmi1* null mice show a significant increase in p16[Ink4a] with a subsequent reduction in β cell proliferation, demonstrating that Bmi-1 is necessary for p16[Ink4a] suppression.[31] However, the role of Bmi-1 during embryogenesis is yet to be elucidated. Conditional deletion of *Enhancer of zeste homolog 2* (*Ezh2*), another inhibitor of the *Ink4a/Arf* locus, resulted in a decrease in β cell proliferation and β cell mass by one month of age with a concomitant increase in p16[Ink4a] levels,[32] but the effect of *Ezh2* loss during embryogenesis has not been reported.

The effects of Ezh2 on β cell replication and the fact that platelet-derived growth factor (PDGF) is known to regulate Ezh2 signaling in fibroblasts led Chen and colleagues to analyze the role of PDGF signaling in β cell replication. They discovered that there is an age-related decline in expression of PDGF receptors (PDGFR) in β cells, which is coincident with the decrease in Ezh2 and increase in p16[Ink4a]. Inactivation of the *Pdgfr* gene results in a decrease in *Ezh2* mRNA levels and a subsequent decrease in β cell proliferation seen by 2–3 weeks of age. Conversely, transgenic overexpression of PDGF in β cells induces a 9-fold increase in β cell proliferation in 14-mo-old transgenic mice compared with control mice, suggesting that regulation of *Ezh2* by the PDGF signaling pathway can improve the age-related decline in β cell proliferation.[33]

A major regulator of genes involved in cell cycle progression in many cell types is the transcription factor forkhead box M1 (FoxM1). FoxM1 regulates genes involved in the G_1-S[34] and G_2-M[35] transitions of the cell cycle as well as karyokinesis and cytokinesis. Inactivation of *Foxm1* throughout the pancreatic epithelium using a Cre-lox mouse model demonstrated that FoxM1 is critical to preventing nuclear accumulation of the cell cycle inhibitor p27[Kip1]. In the absence of pancreatic *Foxm1*, there is decreased β cell proliferation and β cell mass expansion postnatally. In addition, *Foxm1* mutant β cells show a severe impairment in response to several proliferative stimuli, indicating that many different stimuli converge on FoxM1 to mediate increased proliferation.[36-38] Interestingly, the requirement for FoxM1 in β cell replication is first seen around weaning; *Foxm1* inactivation had no effect on β cell mass or proliferation during embryogenesis.[38] The reader is referred to other reviews of the role of cell cycle activators and inhibitors in β cell replication and aging.[39-42]

In addition to cell cycle activators and inhibitors regulating postnatal β cell replication, there are several known factors and signaling pathways that control β cell mass expansion in adults under normal conditions and in response to physiological stimuli (**Table 1**). For example, insulin resistance, which is often accompanied by increased insulin secretion, in the setting of either obesity or pregnancy is known to stimulate an increase in β cell mass via proliferation.[43,44] Likewise, elevated glucose is a stimulus for β cell replication. Adult rats given a 96-h glucose infusion

showed a transient increase in β cell replication accompanied by β cell hypertrophy, which led to an increase in β cell mass; replication declined within seven days after infusion.[45] Furthermore, with a six day glucose infusion, there is evidence of neogenesis in addition to an increase in β cell proliferation in adult rats.[46] The signaling mechanism(s) by which neogenesis or proliferation is stimulated by glucose has not yet been identified, although increased glucose metabolism increases secretion of insulin, which itself is a β cell growth factor.[47]

Insulin/insulin-like growth factor (IGF) signaling is one of several growth factor signaling pathways that plays a role in postnatal β cell mass expansion. Binding of insulin or IGF to their cognate receptors results in activation of a well-studied second messenger cascade culminating in the phosphorylation and activation of protein kinase B (PKB/AKT) by phosphoinositide-dependent protein kinase-1 (PDK1) and mammalian target of rapamycin complex 2 (mTORC2) kinases.[48] Deletion of either PDK1 or Rictor, a key component of mTORC2, results in a decrease in β cell replication in part through upregulation of the p27[Kip1] cell cycle inhibitor.[49] Activation of AKT leads to phosphorylation and inhibition of activity of the FoxO1 transcription factor, which then actively inhibits Pdx1 transcription by binding to the Pdx1 promoter.[50] Indeed, Pdx1 is essential for β cell mass expansion in response to insulin resistance.[51] These results highlight the importance of insulin/IGF induction of the AKT pathway to stimulate β cell proliferation through the inhibition of p27[Kip1] and FoxO1.

The transcription factor nuclear factor of activated T cells (NFAT) and its regulator, calcineurin, have recently been found to be critical in adult β cell replication. Loss of calcineurin b1 in a β cell-specific manner results in decreased β cell replication in mice at four weeks of age with a significant loss in β cell mass by 12 weeks of age.[52] Both calcineurin and NFAT are activated in response to increased glucose metabolism[53] and elevated intracellular calcium.[54]

Pregnancy is a time of metabolic stress in which β cell mass and function are enhanced to compensate for insulin resistance associated with weight gain, placental hormone production and fetal growth.[55] Circulating factors such as growth hormone, prolactin and placental lactogens (PL) are elevated during pregnancy and contribute to increased β cell mass, although PL is the major stimulus for maternal β cell proliferation during pregnancy.[44] PL-mediated β cell proliferation is FoxM1-dependent.[36] Transgenic overexpression of PL also stimulates β cell replication in non-pregnant mice.[56] Another secreted factor involved in maternal β cell replication during pregnancy is serotonin, although the activity of serotonin seems to be more of a local effect within the islet environment itself rather than systemic. The serotonin receptor subtype, Htr2b, is necessary for appropriate β cell proliferation and mass compensation during pregnancy.[57] It would be interesting to examine whether serotonin treatment in non-pregnant mice has an effect on β cell replication.

Other circulating factors that enhance adult β cell replication in vivo include hepatocyte growth factor (HGF),[58] Exendin-4/GLP-1[59] and IGF-1.[60] Our laboratory has been examining the potential role of CTGF in adult β cell mass expansion. CTGF increases proliferation in many cell types, including pancreatic endocrine cells during development,[4] but the mechanism by which this occurs in the endocrine pancreas is yet to be elucidated. Studies in immortalized mammary epithelial cells suggest that IGF1 regulates *Ctgf* expression via the PI3/Akt pathway.[61] Since this pathway is also involved in β cell replication, future studies should examine whether CTGF interacts with the insulin signaling pathway during times of β cell mass expansion. Although CTGF is not normally expressed in adult β cells in human or mouse, we have observed re-expression of CTGF in maternal islets during pregnancy in mice,[3] suggesting that it plays a role in adaptive changes that enhance β cell mass and/or function.

Overexpression of CTGF in Adult β Cells

CTGF is required for embryonic α and β cell proliferation, but whether it plays a role in adult β cell replication has yet to be determined. In order to assess whether CTGF treatment can improve β cell proliferation and/or function, we generated transgenic mice in which CTGF could be conditionally overexpressed under control of the Tetracycline operator (TetO).[4] Cell type-specific expression is thus controlled by the choice of promoter driving the TetO binding protein, reverse tetracycline transactivator (rtTA). Here we used the rat insulin promoter (RIP) to drive expression in insulin-producing cells.[5] The timing of CTGF expression in insulin-producing cells was controlled by doxycycline (DOX) administration. We first determined the level of *Ctgf* overexpression in our system using real time quantitative PCR. DOX was administered for one week and islets isolated for total RNA. We estimate that there is a 400-fold increase in total *Ctgf* in the CTGF-overexpressing islets (**Fig. S1**). While this may seem extremely high, it should be kept in mind that several different methods have shown that CTGF expression is normally undetectable in adult mouse or human islets.[3] Studies in which CTGF expression was induced in embryonic β cells had previously revealed a 10-fold increase in total CTGF in whole pancreata.[4]

Using this transgenic mouse model, we first examined whether induction of CTGF in β cells of ~7-week-old mice could increase β cell proliferation and β cell mass. Previous studies have shown that mouse β cells at this age are still highly responsive to several different proliferative stimuli.[62] *Ctgf* expression was induced for one, three and five weeks to determine whether longer exposure of β cells to CTGF had a more beneficial effect. cell function was assessed using intraperitoneal glucose tolerance tests. Given its re-expression in maternal islets during pregnancy, we hypothesized that CTGF might improve β cell function and thus glucose homeostasis. Animals expressing CTGF in their β cells showed no improvements in glucose homeostasis at any time point examined (**Fig. 2** and data not shown). We also examined glucose tolerance following longer periods of CTGF overexpression (up to 12 weeks), but no improvement was observed in glucose tolerance compared with littermate controls
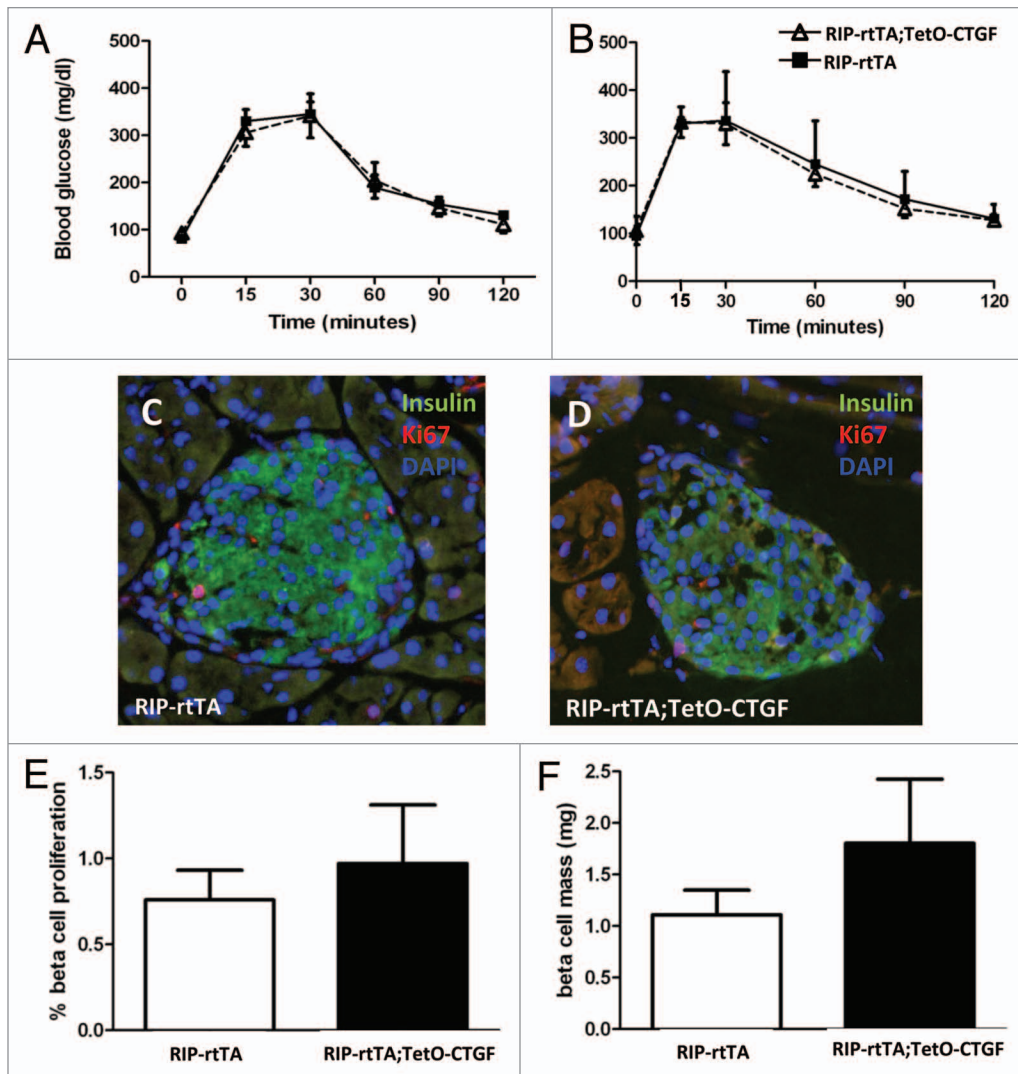
Figure 2. Effects of adult β cell CTGF overexpression on β cell function and mass. (A and B) Intraperitoneal glucose tolerance tests reveal no difference in glucose homeostasis between RIP-rtTA control mice (closed squares) and RIP-rtTA;TetO-CTGF mice (open triangles) treated with doxycycline for 1 week (A) or 5 weeks (B) beginning at ~7 weeks of age. (C and D) Immunolabeling of adult pancreata with antibodies against insulin (green) and Ki67 (red) to assess β cell proliferation (blue: DAPI, nuclei) (magnification 200). (E) There is no difference in the percent of β cell proliferation in CTGF-overexpressing mice compared with control mice after one week of doxycycline treatment (p = 0.61). (F) There is no statistically significant difference in β cell mass in CTGF-overexpressing mice compared control mice after one week of doxycycline treatment (p = 0.24). In (A), n = 8 for control, n = 4 for bigenic. In (B), n = 2 for control, n = 2 for bigenic. In (E), n = 3 for each genotype. In (F), n = 3 for each genotype.

(data not shown). Importantly, there were no observed deleterious effects of CTGF overexpression on glucose homeostasis at any time point examined.

Cell replication and cell mass were also examined after one week of DOX treatment and were found to be unchanged in CTGF overexpressing mice (**Fig. 2C–F**). Therefore, β cells of mice between 7–9 weeks of age do not seem to be responsive to CTGF, at least in this model. As noted previously, the ability of CTGF to induce β cell proliferation during embryogenesis was confined to MafA-negative, or immature, β cells. Thus, in retrospect, it may not be too surprising that adult β cells under non-stimulatory conditions do not respond to increased CTGF. However, at three weeks of age in the mouse, there remains a small population of immature MafA-negative β cells.[63] We therefore induced CTGF overexpression in animals right at weaning (three weeks of age) for one week and examined glucose homeostasis, β cell mass and β cell proliferation.

However, we observed no significant changes in any of these parameters (**Fig. 3**). Because pathological levels of CTGF are associated with fibrosis in models of pancreatitis[64] and pancreatic cancer,[65] pancreata from CTGF overexpressing and control mice were examined for collagen deposition using Direct Red 80/Fast Green staining. We were encouraged to observe no increase in peri-islet collagen was observed after one, three or five weeks of CTGF overexpression in β cells (**Fig. S2** and data not shown). In addition, there was no increase in periductal or peri-vascular collagen after one week of CTGF overexpression. However, increased collagen was apparent after 5

weeks of CTGF overexpression in peri-ductal and peri-vascular regions (**Fig. S2**). These results suggest caution when over-expressing CTGF, as there is a finite time period before fibrosis may develop.

Since CTGF is an inducer of angiogenesis in some settings,[66,67] we also examined whether CTGF overexpression in β cells would enhance islet vascularity. To this end, CTGF expression was induced in β cells for one or five weeks. There was no difference in islet vascular density as assessed by PECAM immunolabeling between control and CTGF overexpressing mice after one or five weeks of CTGF overexpression (**Fig. 4**).

Experimental Procedures

Animals. Generation of RIP-rtTA[5] and TetO-CTGF[4] transgenic mice has been described previously. Mice were administered 2 mg/ml of doxycycline (DOX) in a 2% Splenda solution in drinking water at the time of weaning (for 3-week-old mice) or at 6.5–7.5 weeks of age. Mice were treated with DOX for one, three and five weeks. All animal experiments were approved by the Institutional Animal Care and Use Committee of Vanderbilt University Medical Center.

PCR genotyping. Genotyping of RIP-rtTA;TetO-CTGF mice was performed using the following primers: forward 5'-GGA GGT CTA TAT AAG CAG AGC TCG-3' and reverse 5'-TTA AGT TAC GCC ATG TCT CCG TA-3' for TetO-CTGF; forward 5'-CGC TGT GGG GCA TTT TAC TTT AG-3' and reverse 5'-CAT GTC CAG ATC GAA ATC GTC for RIP-rtTa.

Real-time PCR for *Ctgf* expression. Entire pancreata were dissected and islets were isolated by the Vanderbilt University Islet Procurement and Analysis Core and placed immediately into RNAlater (Ambion). Total RNA was extracted using the RNAqueous kit (Ambion) according to the manufacturer's instructions. RNA concentration and integrity were assessed by the Vanderbilt University Genome Sciences Resource Core using the 2100 Electrophoresis Biolanalyzer (Agilent). cDNA was synthesized using the SuperScript III First-Strand synthesis system (Invitrogen). Reactions were performed in technical duplicate with iQ SYBR Green Supermix (Bio-Rad) according to the manufacturer's instructions at an annealing temperature of 61.1°C. 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 hypoxanthineguanine phosphoribosytransferase (HPRT). The following primers were used to detect total *Ctgf* expression: forward 5'-TTC TGC GAT TTC GGC TCC-3' and reverse 5'-ACC ATC TTT GGC AGT GCA CA-3'.

Tissue dissection, preparation and histology. At the end of the DOX treatment period, pancreata were dissected into cold PBS and fixed immediately in 4% paraformaldehyde at 4°C for 4 h. Tissues were incubated in a 30% sucrose solution overnight and were embedded in Surgipath© HSC 22 Frozen Section compound and sectioned at 7 μm and stored at -80°C.

Immunolabeling. Protein localization was assessed by incubation of tissue sections with various antibodies. Insulin and Ki67: labeling was performed on frozen sections and required antigen retrieval for Ki67 in 1 sodium citrate (10 mM) for 14 min at 40% power. Primary antibodies included guinea pig anti-insulin (1:1,000; Upstate) and mouse anti-Ki67 (1:500; BD PharMingen). Primary antibodies were detected by species-specific donkey secondary antibodies conjugated to either Cy2 or Cy3 flurophores (1:300; Jackson ImmunoResearch Laboratories, Inc.). PECAM: platelet endothelial β cell adhesion molecule (PECAM) labeling was performed on frozen sections after 3% hydrogen peroxide quenching using rat anti-PECAM (1:50; BD PharMingen) and the Vector M.O.M kit (Vector Labs). An anti-rat biotinylated secondary antibody (1:500; Vector Labs) applied with Vectastatin ABC kit (Vector Labs) was used for labeling followed by TSA amplification (Invitrogen).

All primary antibodies were incubated overnight in a humid chamber at 4°C. Fluorophores were excited and imaged using a ScanScope FL slide scanner (Aperio Technologies, Inc.). Images from each experiment were processed identically with ImageScope software (Aperio Technologies, Inc.).

Intraperitoneal glucose tolerance tests (IPGTT). IPGTTs were performed on control (RIP-rtTA) and bigenic (RIP-rtTA;TetO-CTGF) mice treated with DOX as described previously in reference 3, using an Accuchek Aviva glucometer and test strips (Roche Pharmaceuticals).

β cell proliferation. Entire pancreata were serially sectioned and slides were immunolabeled for insulin and Ki67. One section was analyzed every 250 μm (11–16 sections per animal). Every islet in that section was photographed and the total number of cells positive for insulin was counted, as were the number of proliferating cells (co-labeled for insulin and Ki67) using Metamorph 6.1 software (Molecular Devices). The percentage of proliferating cells was determined by dividing the number of Ki67/insulin double-positive cells by the total number of insulin-positive cells and multiplying by 100.

β cell mass. Entire pancreata were removed, weighed, fixed and serially sectioned. One section per slide was analyzed every 250 μm (11–16 sections per animal) throughout the pancreas as previously described in reference 3 and 41. Briefly, sections were prepared for insulin immunoperoxidase labeling and eosin counterstaining. Vector DAB Peroxidase Substrate kit (Vector Labs) was used for immunohistochemical labeling of insulin for β cell mass measurement. Images of anti-insulin-labeled sections were scanned using a ScanScope CS slide scanner (Aperio Technologies, Inc.). Images from each experiment were processed identically with the ImageScope software (Aperio Technologies, Inc.). β cell mass was measured by obtaining the fraction of cross-sectional area of pancreatic tissue positive for insulin as calculated by modified Genie algorithm within the Spectrum software (Aperio Technologies, Inc.,) and multiplying this by the pancreatic wet weight.

Collagen assessment. Entire pancreata were serially sectioned and three sections per animal, at least 250 μm apart, were randomly selected and analyzed. Sections were stained for collagen by incubating with Direct Red 80/Fast Green
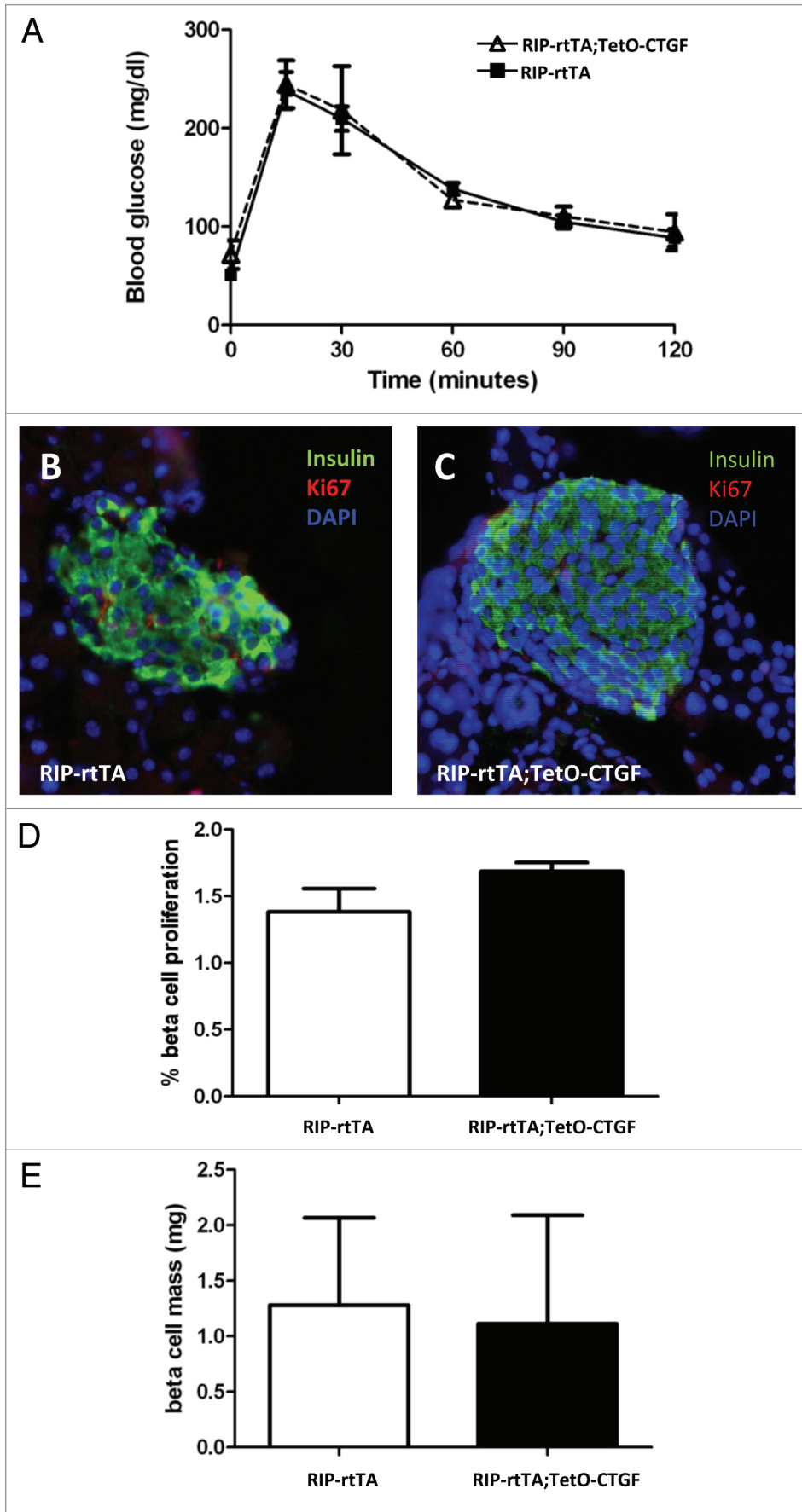
Figure 3 (See opposite page). Effects of β cell CTGF overexpression at weaning on β cell function and mass. (A) Intraperitoneal glucose tolerance tests reveal no difference in glucose homeostasis between RIP-rtTA control mice (closed squares) and RIP-rtTA;TetO-CTGF mice (open triangles) treated with doxycycline for one week, beginning at 3 weeks of age. (B and C) Immunolabeling of 4-week-old pancreata with antibodies against insulin (green) and Ki67 (red) to assess β cell proliferation after one week of CTGF overexpression (blue: DAPI, nuclei) (magnification 200). (D) There is no difference in the percent of β cell proliferation in CTGF overexpressing mice compared with control mice after one week of doxycycline treatment (p = 0.74). (E) β cell mass is unchanged after one week of CTGF overexpression (p = 0.91). In (A), n = 8 for control, n = 3 for bigenic. In (D), n = 3 for control, n = 2 for bigenic . In (E), n = 2 for each genotype.
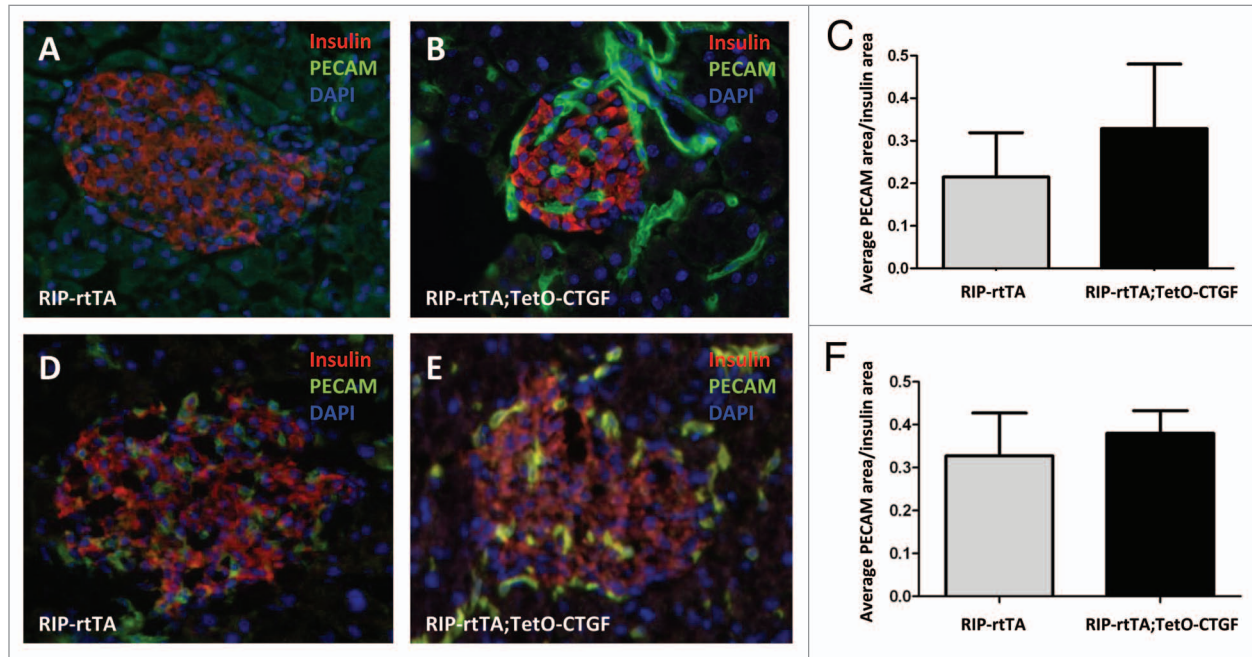
Figure 4. Effects of adult β cell CTGF overexpression on islet vascularity. Immunolabeling of control (A and D) and CTGF-overexpressing (B and E) pancreata after one (A and B) or five (D and E) weeks of CTGF induction (insulin, red; PECAM, green; DAPI, nuclei, blue) (magnification 200). Preliminary data suggests that islet density is not increased after 1 week (A–C) or 5 weeks (D–F) of CTGF overexpression in ~7-week-old mice. In (C), n = 1 for each genotype. In (F), n = 2 for control, n = 3 for bigenic for each genotype.

(Sigma-Aldrich Company) for one hour at room temperature in a humid chamber. The slides were washed in acidified water and dehydrated using an increasing ethanol series to xylene. Images of collagen-labeled sections were scanned using a ScanScope CS slide scanner (Aperio Technologies, Inc.).

Islet microvascular density. The blood vessel endothelium density per islet was determined by immunolabeling for insulin and PECAM. Entire pancreata were serially sectioned and slides immunolabeled for insulin and PECAM. One section per slide was analyzed every 250 μm (11–16 sections per animal) throughout the pancreas and the PECAM-positive area of every islet (designated as containing 10 insulin-positive cells or more) per section was quantified using Metamorph software. To determine the percentage of blood vessel area, the total PECAM area was divided by the total insulin-positive area and multiplied by 100.

Conclusions and Future Perspectives

The data presented here suggest that CTGF has no influence on β cell replication or mass in the post-weaning β cell under normal circumstances and that it can only induce replication of embryonic β cells.[4] Much work is still needed to elucidate the mechanism(s) by which CTGF promotes β cell proliferation, but from what is known in mammary cell lines, this regulation may occur via the PI3/Akt signaling pathway. Adult β cells are in general less receptive to proliferative stimuli than are embryonic or neonatal β cells, although the β cells at the time point we studied have been responsive in other studies.[62] It is possible that CTGF plays a role only in embryonic β cell replication or alternatively, would affect adult β cell replication in the presence of a strong proliferative stimulus such as obesity-induced insulin resistance, response to injury or pregnancy. These possibilities will be examined in the future.

Current efforts in the field are focused in large part on directed differentiation of human embryonic stem cells or induced pluripotent stem cells toward a β cell phenotype by mimicking normal pancreatic development. The best protocols allow for production of glucose-responsive β cells, although this process requires an in vivo maturation step that remains ill-defined.[68] CTGF is necessary for adequate β cell mass at birth and is sufficient to induce embryonic β cell mass expansion. Thus, it could be useful in stem cell directed differentiation protocols or in the expansion

of insulin-producing cells prior to their full maturation.

Clearly, expansion of immature insulin-positive cell populations is regulated by factors that differ from regulators of mature β cell mass expansion. In many cases, these changes in requirement from an "embryonic" form of regulation to an "adult" form of regulation occur around weaning (see model in **Fig. 1**). While the mechanism(s) underlying this switch have yet to be elucidated, it is possible that metabolic and physiological cues associated with switching from breast milk to chow could influence the mode of β cell replication. Alternatively, some sort of clock mechanism might come into play where a β cell undergoes a certain number of "embryonic" cell divisions and then switches to the postnatal form of replication. A better understanding of β cell development and the identification of factors important for β cell maturation and replication would be useful to further improving strategies for replacing β cell mass in individuals with diabetes. Changes in β cell mass are the result of a balance between the negative effects of apoptosis and the positive effects of proliferation or neogenesis. Successful treatments to increase β cell mass will attempt to tip the scales in the direction of proliferation and/or neogenesis and CTGF could be such a factor.

Acknowledgements

Supplemental Material

Supplemental materials may be found here: www.landesbioscience.com/journals/cc/article/20545

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