

# The Impact of Connective Tissue Growth Factor on Glucose Homeostasis and Islet Physiology in Virgin and Pregnant Mice

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KEYWORDS. Connective Tissue Growth Factor (Ctgf), Islet, Physiology, Glucose, Diabetes

BRIEF. The loss of Connective tissue growth factor (Ctgf) does not affect islet physiology, indicating other existing problems in glucose homeostasis.

**ABSTRACT.** Approximately 9.2% of pregnancies are characterized by the inability to properly regulate blood glucose levels, a condition referred to as gestational diabetes mellitus (GDM) [1]. Blood glucose is regulated by the islets, which are clusters of endocrine cells primarily composed of  $\beta$ -cells and  $\alpha$ -cells. Glucose homeostasis is dependent on normal  $\beta$ -cell mass and islet architecture. Previous research shows that Connective tissue growth factor (Ctgf) is critical for proper islet morphology and mass during mouse development, but its impact in adults was unclear. To determine the impact of Ctgf on the islets in adult mice, this research utilized a conditional endocrine cell knockout (Ctgf <sup>$\Delta$ Endo</sup>) and a global Ctgf haploin-sufficiency (Ctgf<sup>LacZ/+</sup>) model. In a previous study [2], pregnant Ctgf <sup>$\Delta$ Endo</sup> mice developed GDM, and Ctgf<sup>LacZ/+</sup> mice displayed reduced pregnancy-induced  $\beta$ -cell proliferation. Analyzing the islets of Ctgf <sup>$\Delta$ Endo</sup> and Ctgf<sup>LacZ/+</sup> mice demonstrated that loss of Ctgf did not impair  $\beta/\alpha$ -cell ratios, islet vascularization, or  $\beta$ -cell mass. These results indicate that islet dysfunction can exist in the context of normal islet architecture, and that impaired  $\beta$ -cell proliferation does not always lead to a deficiency in  $\beta$ -cell mass. This work is significant as it clearly links the lack of Ctgf to GDM development in an animal model, which can potentially aid with its prevention and treatment in human patients.

## INTRODUCTION.

Gestational diabetes mellitus (GDM) is described as the onset of diabetes specifically during pregnancy [3]. The underlying causes of GDM are largely unknown. In healthy individuals, blood glucose is regulated by clusters of endocrine cells located in the pancreas, which are known as islets of Langerhans [4]. Two of the major cell types in the islets are the  $\beta$ -cells and  $\alpha$ -cells, and in a normal islet, the ratio of  $\beta/\alpha$ -cells is 3:1. The  $\beta$ -cells produce insulin in order to lower blood sugar levels while the  $\alpha$ -cells secrete glucagon to elevate blood sugar levels. During gestation, the maternal  $\beta$ -cells adapt to the metabolic demands of pregnancy by compensating with an increase in proliferation, cell size, and glucose-stimulated insulin secretion [5].

One protein that is necessary for the islets to develop properly is Connective tissue growth factor (Ctgf) [6]. Ctgf is not exclusive to the  $\beta$ -cells; it is also expressed in many different cell types. Ctgf regulates cell proliferation, cell migration, vasculature, and extra-cellular matrix remodeling in many tissues [7]. During embryonic development in mice, Ctgf expression in the pancreas is found in the  $\beta$ -cells, ductal cells, and also in the vasculature [6].

Previous research indicates that loss of Ctgf can impair pregnancy-induced  $\beta$ -cell proliferation and disrupt normal glucose-stimulated insulin secretion in the islets in pregnant mice, demonstrating that underlying  $\beta$ -cell dysfunction can contribute to GDM. However, it is unclear what impact loss of Ctgf has on  $\beta$ -cell mass and islet morphology in adult mice. This research project sought to determine if a disruption in the underlying morphology of the islets contributed to the impaired islet function during pregnancy and thus contributed to GDM.

Expression of Ctgf is necessary for  $\beta$ -cell proliferation and proper islet morphology during development. However, after the mouse is born, Ctgf expression in the  $\beta$ -cells and ductal cells is silenced, but expression in the vasculature continues [6]. Ctgf is also necessary for proper embryonic skeletal development, and mice homozygous for a loss-of-function allele of the Ctgf gene die shortly

after birth due to respiratory defects [8]. In contrast, mice with only one copy of the loss-of-function Ctgf allele survive and thrive. Mice with only one copy of the loss-of-function Ctgf allele have reduced  $\beta$ -cell proliferation during pregnancy, but it is unclear if this reduction causes a deficiency in  $\beta$ -cell mass [2]. As sufficient  $\beta$ -cell mass is necessary to produce normal levels of insulin during pregnancy, investigating models with impaired  $\beta$ -cell compensatory mechanisms during pregnancy will further our understanding of the development of GDM. In addition, previous research shows that mice with loss of endocrine-derived Ctgf (Ctgf <sup>$\Delta$ Endo</sup> mice) have impaired glucose tolerance as adult virgins and GDM during pregnancy [2]. Further analysis revealed this is accompanied by impairment in glucose-stimulated insulin secretion (GSIS) in the  $\beta$ -cells, despite the fact that Ctgf expression in  $\beta$ -cells is restricted to embryogenesis. Therefore, the different models were utilized to determine if there were changes in islet architecture.

To study the function of Ctgf in adult mice, two different genetic mouse models were used in this study. The first mouse model utilized a conditional allele of Ctgf; that is, a copy of the Ctgf gene that has normal function unless the cell also expresses the Cre recombinase protein which converts the conditional Ctgf allele to a loss-of-function Ctgf allele (Ctgf <sup>$\Delta$ Endo</sup> mice) [9]. The second mouse model used in this study is a global heterozygous model that only has one functional allele for Ctgf (Ctgf<sup>LacZ/+</sup>), causing a global reduction in Ctgf, including reduced Ctgf expression from the vasculature of the pancreas [6]. The use of pancreatic tissue from these two mouse models helped to determine the impact of endocrine-derived Ctgf versus vasculature-derived Ctgf in adult mice. Islets in Ctgf <sup>$\Delta$ Endo</sup> mice were analyzed to determine if disruptions in normal islet morphology contributed to the loss of glucose homeostasis and GDM phenotypes in adult mice. Islet morphology was assessed by measuring the  $\beta/\alpha$ -cell ratio and islet vascularization in control and Ctgf <sup>$\Delta$ Endo</sup> mice in both virgins and at gestational day 14.5 (GD14.5). Importantly, GD14.5 approximately corresponds to the relative time when GDM is diagnosed in human patients [3]. These two parameters were further investigated to see if these are the reasons that adult mice with endocrine-specific Ctgf ablation display reduced  $\beta$ -cell function.

Additionally, the  $\beta$ -cell mass of Ctgf<sup>LacZ/+</sup> mice was measured to determine if the decrease in  $\beta$ -cell proliferation during pregnancy observed in these mice caused a decrease in  $\beta$ -cell mass.

Together, these two mouse models allowed the examination of the relationship between islet morphology and  $\beta$ -cell function, as well as study how  $\beta$ -cell proliferation during pregnancy can impact overall  $\beta$ -cell mass.

## MATERIALS AND METHODS.

### Experimental Animal.

The experimental animals were generated for projects conducted by lab members. Tissue from the remaining samples, after the projects were completed, were utilized in this study.

Ctgf<sup>LacZ/+</sup> mice have been described previously [6]. Wild-type, age-matched, and sex-matched siblings were used as controls for experiments using Ctgf<sup>LacZ/+</sup> mice. Ctgf <sup>$\Delta$ Endo</sup> mice were bred by crossing Pax6-Cre mice (provided by Roland Stein, Vanderbilt University) (15) to mice homozygous for a previously described conditional by inversion (COIN) Ctgf allele [5, 8]. Control mice from these breedings were COIN/, COIN/COIN, or Pax6-Cre. Analyses were

performed when mice were 10 weeks old. All processes and procedures were approved by and conducted according to the Vanderbilt Institutional Animal Care and Use Committee under the supervision of the Division of Animal Care. Mice were housed in temperature-controlled environments with a 12-h night-day cycle and access to a high-energy diet and water, except when otherwise noted.

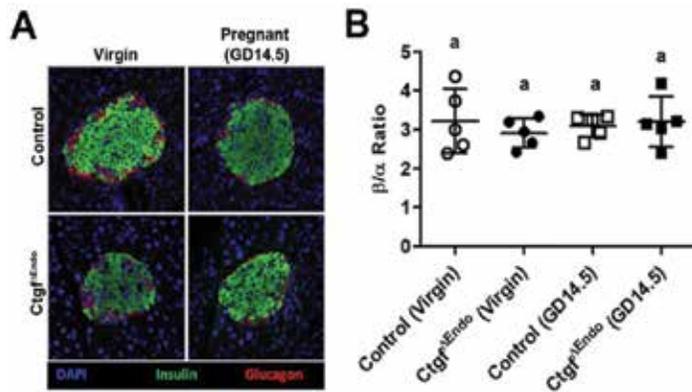
#### Processing Pancreas Tissue for Immunofluorescence Analysis.

Paraffin-embedded pancreata were sectioned holistically with a microtome, with a range of 90-130 slides per pancreata and 5 micrometer-wide sections, and then placed onto glass microscope slides. These slides were then dewaxed with xylene and rehydrated using a series of ethanol solutions and distilled water. For  $\beta/\alpha$ -cell ratio, dewaxed slides were heated in a sodium citrate solution to facilitate antibody-epitope binding.

For islet vascularization analysis, dewaxed slides were heated in Tris-EGTA solution to facilitate antibody-epitope binding. Afterwards, slides were incubated in a 5% normal donkey serum block solution to further prevent non-specific antibody binding. In this project, the following primary antibodies were used at a 1:500 dilution: guinea pig anti-insulin (to detect insulin), rabbit anti-glucagon (to detect glucagon), and rabbit anti-CD31 (to detect vasculature). The following secondary antibodies were used at a 1:400 dilution: Cy2-conjugated anti-guinea pig and Cy3-conjugated anti-rabbit. DAPI stain was added to visualize nuclei. Coverslips were added to the slides using AquaMount to preserve the tissue and the immunofluorescence staining.

#### Immunofluorescence for Determining $\beta/\alpha$ -cell Ratio.

After immunofluorescence labeling with insulin and glucagon, samples were visualized and images acquired using a ScanScope FL fluorescence microscope. Samples were analyzed by using MetaMorph 6.1 to quantify the number of  $\beta$ -cells and  $\alpha$ -cells. Each  $\beta$ - and  $\alpha$ -cell were counted manually in every islet in the randomly selected scanned images of slides gathered so as to form a representative sample. At least 5,000 cells were counted per sample.



**Figure 1.**  $\beta$ -cell-derived Ctgf is not necessary for normal  $\beta/\alpha$ -cell ratio in adult mice. (A) Pancreata from virgin and gestational day 14.5 (GD14.5) mice were sectioned and labeled for insulin and glucagon using immunofluorescence. Nuclei are labeled with DAPI (blue), insulin labels the  $\beta$ -cells (green), and glucagon labels the  $\alpha$ -cells (red). The  $\beta$  and  $\alpha$ -cells were manually counted and average ratios were determined. (B) The  $\beta/\alpha$ -cell ratios in the Ctgf <sup>$\Delta$ Endo</sup> mice are not significantly different from the control mice, cohorts labeled with the same letter are not statistically different. Significance was determined using a one-way ANOVA followed by a Tukey honest significance test.  $p \leq 0.05$ .

#### Immunofluorescence for Measuring Islet Vascularization.

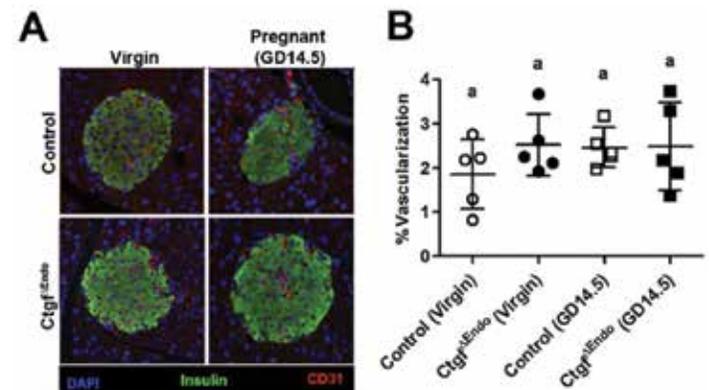
Slides were labeled for insulin and CD31 and images were acquired using a ScanScope FL fluorescence microscope. Briefly, total insulin positive area was measure relative to total CD31 positive area using MetaMorph 6.1 computer software to calculate the percentage of the islet composed of vasculature.

#### Immunohistochemistry for $\beta$ -cell Mass Analysis.

During experimentation, slides were washed in a 3% hydrogen peroxide solution to reduce background. Subsequently, the slides were incubated with the guinea pig anti-insulin primary antibody overnight at 4°C. The next day, the slides were incubated with the horseradish peroxidase anti-guinea pig antibody (HRP-conjugated anti-GP antibody). Then, the DAB (3,3'-diaminobenzidine) substrate was added to the slides, which reacts with the HRI-conjugated antibody to stain the islets a brown color. Eosin was used to counterstain the slides. Slides were then dehydrated with ethanol and xylene and coverslips were added with xylene-based mounting media. Slides were imaged using the ScanScope CS brightfield microscope system.  $\beta$ -cell mass was measured by using ImageScope computer software to analyze the ratio of insulin positive area to total pancreas area of tissue sections and multiplying by the wet weight of the entire pancreas.

#### Statistics to Determine Significance of Data.

For the  $\beta/\alpha$ -cell ratio, islet vascularization analysis, and  $\beta$ -cell mass analysis, statistical significance was determined using one-way ANOVA and Tukey post hoc test. Statistical analysis was conducted using GraphPad Prism 6 software. Statistical significance was set at  $p \leq 0.05$ .



**Figure 2.**  $\beta$ -cell-derived Ctgf is not necessary for normal islet vascularization in adult mice. (A) Pancreata from virgin and gestational day 14.5 (GD14.5) mice were sectioned and labeled for insulin (green) and CD3 (red) using immunofluorescence. Nuclei are labeled with DAPI (blue). (B) Islet vascularization as quantified using MetaMorph software, cohorts with the same letter are not statistically different. Statistical significance was determined using a one-way ANOVA followed by a Tukey honest significance test.  $p \leq 0.05$ . Results reported in [2].

## RESULTS.

### $\beta/\alpha$ -cell Ratio is Normal in Both Virgin and Pregnant Ctgf <sup>$\Delta$ Endo</sup> Mice.

As  $\beta$ -cells decrease blood glucose levels while  $\alpha$ -cells increase it, normal  $\beta/\alpha$ -cell ratios are essential for proper glucose homeostasis in the body. Extensive immunofluorescence analysis using digital microscopy was used to analyze  $\beta/\alpha$ -cell ratios in control and Ctgf <sup>$\Delta$ Endo</sup> mice in virgins and at gestational day 14.5 (GD14.5). In the immunofluorescent images of pancreas sections shown in Figure 1A, similar-sized islets are shown for easy comparison. In all samples, green shows the distribution of insulin, which labels the  $\beta$ -cells, and red labels the glucagon secreting  $\alpha$ -cells. Figure 1B is a quantified analysis of the  $\beta/\alpha$ -cell ratio, which is approximately 3:1 for all four cohorts. These results are consistent with the findings published by other laboratories that also describe a 3:1  $\beta/\alpha$ -cell ratio in wild-type mice. No statistically significant differences were observed between any groups.

### Islet Vascularization is Normal in Both Virgin and Pregnant Ctgf <sup>$\Delta$ Endo</sup> Mice.

Proper islet vascularization is critical in islet health and hormone secretion to peripheral tissues [10]. Islet vascularization was analyzed through immunofluorescence staining of insulin and the vasculature marker CD31. In this analysis, as seen in Figure 2, vascularization is generally distributed throughout the islet,

with no obvious differences between virgin and pregnant samples in either the control or  $Ctgf^{Endo}$  mice. No statistically significant difference between islet vascularization was observed between the different groups. This suggests that the lack of endocrine-derived  $Ctgf$  is not necessary for proper vascularization in islets of adult mice, and a lack of vascularization does not contribute to the GDM developed in  $Ctgf^{Endo}$  mice, which was portrayed in a previous study [2].

#### $\beta$ -cell Mass is Normal in Both Virgin and Pregnant $Ctgf^{LacZ/+}$ Mice.

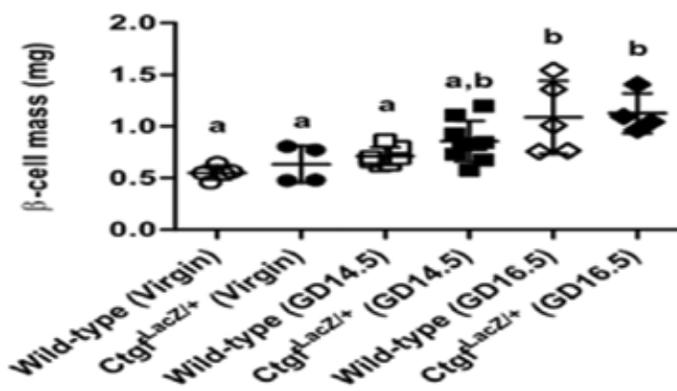
In the  $Ctgf^{LacZ/+}$  mouse model, an insufficient increase in  $\beta$ -cell proliferation during pregnancy was previously reported [2]. As diabetes is often caused by insufficient  $\beta$ -cell mass, this portion of the project sought to determine if the decrease in  $\beta$ -cell proliferation correlated with decreased  $\beta$ -cell mass expansion during pregnancy in the  $Ctgf^{LacZ/+}$  mouse model, which was observed in a previous study [2].

Immunohistochemistry was used for  $\beta$ -cell mass analysis in virgin, GD14.5, and GD16.5 mice. These time-points were analyzed as the maximum increase in  $\beta$ -cell proliferation was previously observed to be at GD14.5, while the maximum increase in  $\beta$ -cell mass occurs at GD16.5 [11]. The samples were analyzed using IHC and the results organized into the graph in Figure 3. Statistically significant differences between groups are shown through the use of letters; groups with different letters are statistically different. This led to the conclusion that proliferation during pregnancy can cause an increase in  $\beta$ -cell mass by GD16.5, and that this increase is not impaired in  $Ctgf^{LacZ/+}$  mice, despite the deficiency in  $\beta$ -cell proliferation shown in a previous study [2].

#### DISCUSSION.

As shown in Figure 1, the  $\beta/\alpha$ -cell ratios were not affected by the endocrine-specific  $Ctgf$  inactivation. This shows that the glucose intolerance phenotype is not caused by a disruption in the proper  $\beta/\alpha$ -cell ratio. After thorough analyses, the  $\beta/\alpha$ -cell ratios were shown to be similar throughout all four cohorts. This ultimately suggests that  $\beta/\alpha$ -cell ratios were not impaired by the lack of endocrine-derived  $Ctgf$ .

Shown in Figure 2, the vascularization was also not altered by the lack of endocrine-derived  $Ctgf$ . Similarly, after extensive immunofluorescent analyses, the percentages of vascularization were shown to be similar in all cohorts, demonstrating that neither pregnancy nor the absence of endocrine-derived  $Ctgf$  affects islet vascularization.



**Figure 3.** Proliferation during pregnancy does cause an increase in  $\beta$ -cell mass at GD16.5.  $\beta$ -cell mass was shown to be increased during GD16.5 for both wild-type and  $Ctgf^{LacZ/+}$  mice. Letters (a and b) represent statistical significance in regards to each cohort. Significance was determined using a one-way ANOVA followed by a Tukey honest significance test.  $p \leq 0.05$ .

Figure 3 shows that  $\beta$ -cell mass was not affected in a  $Ctgf$  global haploinsufficient mouse model ( $Ctgf^{LacZ/+}$ ). A proper amount of  $\beta$ -cell mass is necessary to produce and secrete a proper amount of insulin. After various trials of immunohistochemistry, the percentages of  $\beta$ -cell mass were shown to be similar in all four cohorts by GD16.5. Immunohistochemistry demonstrates that both wild-type and  $Ctgf^{LacZ/+}$  mice have increased  $\beta$ -cell mass by GD16.5, indicating that even with a deficiency in  $\beta$ -cell proliferation,  $\beta$ -cell mass can still be normal. Potentially, the deficiency in  $\beta$ -cell proliferation was not severe enough to impair overall  $\beta$ -cell mass, but further research is needed to clarify this possibility.

Loss of  $Ctgf$  expression can cause a disruption in glucose homeostasis and the development of GDM. Various components of islet physiology, including  $\beta/\alpha$ -cell ratio, islet vascularization, and  $\beta$ -cell mass were analyzed to see if the loss of  $Ctgf$  had caused any significant changes in this phenotype, thus potentially accounting for the impairment in glucose homeostasis. It was determined that these components of physiology were not affected by the lack of  $Ctgf$ , demonstrating that normal islet architecture can coexist with impaired islet function.

In the future, further investigations regarding the mechanisms behind the loss of glucose homeostasis in  $Ctgf^{Endo}$  mice will be conducted by analyzing the expression levels of genes necessary for  $\beta$ -cell function, such as  $MafA$ ,  $Glut2$ , and  $FoxM1$ ; this is essential to gather a more complete understanding of GDM.

Extensive analyses showed that islet physiology was normal. These results imply that pre-existing disruptions in glucose homeostasis can lead to the development of GDM. Additionally, it was discerned that glucose homeostasis can be disrupted even when the overall islet structure is normal. This work is significant as it has linked the loss of  $Ctgf$  to the development of gestational diabetes, and demonstrates that impairment of  $\beta$ -cell compensatory actions does not always lead to insufficient  $\beta$ -cell mass. This can aid further research on the prevention and treatment of this prevalent disease.

#### ACKNOWLEDGMENTS.

I would like to thank my mentor Dr. Raymond Pasek for teaching me all of the facts and skills necessary for completing this project, always indulging my curiosity, and for his unwavering support. I would also like to acknowledge the Gannon lab for providing me with all of the resources necessary to conduct my research. In addition, I would like to thank the School for Science and Math at Vanderbilt for giving me the opportunity to do research at Vanderbilt during the summer of 2016. Last but not least, I would like to thank Dr. Eeds for all of her help and support in submitting my research to the different scientific competitions.

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