Investigating the Function of CTGF in β-Cell Mass and Glucose Tolerance During Pregnancy

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KEYWORDS. Gestational diabetes, pancreas, β-cell

BRIEF. CTGF is necessary for glucose tolerance during pregnancy, but overall β-cell mass is unchanged at the measured time-point.

ABSTRACT. Gestational diabetes mellitus (GDM) affects between 3-20% of pregnancies and is characterized by abnormally high blood glucose (sugar) levels specifically during pregnancy. Blood glucose concentration is regulated by insulin, which is produced by the β-cells in the pancreatic islets of Langerhans. Normally during pregnancy, β-cell mass increases to maintain glucose homeostasis and adapt to the increased metabolic demands imposed by the developing fetus. The increase in β-cell mass is achieved through proliferation of existing β-cells and β-cell hypertrophy. Previous research indicates that the secreted protein, connective tissue growth factor (CTGF), is necessary for β-cell expansion during embryogenesis. While β-cell expansion also occurs during pregnancy, it is unknown if CTGF contributes to total β-cell mass in pregnant adult mice. Using a mutant CTGF mouse (CTGFLacZ/+) with body-wide loss of one CTGF allele, the role of the CTGF in β-cell mass expansion during pregnancy was determined. Immunohistochemistry was used to quantify β-cell mass in virgin and pregnant CTGFLacZ/+ mice, and this was compared to controls. While absence of CTGF impaired glucose tolerance during pregnancy, total β-cell mass was not different between pregnant CTGFLacZ/+ mice and pregnant controls. This indicates that CTGF is necessary for glucose tolerance during pregnancy, but does not affect β-cell mass at this time-point.

INTRODUCTION.

Diabetes is a significant health threat to individuals around the world, especially in American society. This disease is the 7th leading cause of death in the United States, and over 9.3% of the American population has some form of diabetes [1]. Furthermore, these affected individuals are more predisposed to adverse health conditions such as blindness, stroke, cardiovascular disease, and limb amputations [1]. Thus, researchers deem this chronic illness as an essential disease to cure. Diabetes is a result of insufficient insulin production or insufficient insulin signaling. There are three main types of diabetes: Type 1 diabetes (T1D), Type 2 diabetes (T2D), and gestational diabetes (GDM). T1D occurs when the immune system attacks and destroys the β-cells, cells located in the islets of the pancreas that secrete insulin in response to glucose. Loss of β-cells results in high blood sugar (hyperglycemia). The pancreas is located adjacent to the intestines. The endocrine pancreas consists of β-cells which produce insulin, α-cells which produce glucagon, δ-cells which produce somatostatin, and PP cells which produce pancreatic polypeptide. Insulin secreted by the β-cells lowers blood glucose after a meal.

Type 2 diabetes is also characterized by hyperglycemia, and it is caused by inadequate β-cell mass, insufficient insulin production, and improper insulin signaling in organs such as the liver, muscle and fat, usually due to obesity. Conversely, the cause of GDM remains more elusive. By definition, the disease only exists during pregnancy, making studying the biology of GDM in human patients difficult. Normally during pregnancy, β-cell mass and function increase to compensate for the increased metabolic demands imposed by the developing fetus [2]. Animal models indicate that GDM is caused by an insufficient compensatory response during pregnancy, but further research is needed to confirm this hypothesis [3]. For some forms of diabetes, treatment with exogenous insulin injection is a viable therapeutic option. However, this does not replace the function of insulin-producing β-cells. Moreover, giving exogenous insulin also increases the risk of hypoglycemia, which can increase patient morbidity and mortality and likely cause harm to the developing fetus [4]. As such, discovering mammalian genes that are necessary for proper β-cell mass and function will help advance our understanding of diabetes and promote the development of novel therapies to treat individuals with diabetes.

Connective Tissue Growth Factor (CTGF) is a secreted protein that has a wide tissue expression and is responsible for a diverse range of cellular functions. It is important in numerous developmental processes such as cell proliferation, adhesion, extracellular matrix deposition, bone formation, and vascularization. CTGF is expressed in the pancreatic blood vessels, ducts, and β-cells during embryogenesis and is necessary for proper levels of β-cell proliferation [4]. However, shortly after birth, the β-cells cease expression of CTGF. During pregnancy, β-cells re-express CTGF, suggesting that CTGF may be necessary for the increase in β-cell mass and function that normally occurs during this time [5]. However, the function of CTGF in the pancreas during pregnancy has yet to be reported.

Though β-cell expansion occurs during pregnancy, it is unknown if CTGF affects the total β-cell mass in pregnant adults. Due to the difficulty of studying the etiology of CTGF function and GDM in humans, mice were used as animal models in order to determine the role of CTGF in β-cell mass during pregnancy. The hypothesis tested was that CTGF would promote an increase in β-cell mass during pregnancy in female adult mice, and loss of CTGF would result in decreased beta cell mass during pregnancy. To test this hypothesis, we utilized a mouse model which carries one mutant CTGF allele ($CTGF^{LacZ/+}$) and thus expresses 50% of normal CTGF. Immunohistochemistry was employed to quantify β-cell mass in virgin and pregnant (gestational day 14.5) $CTGF^{LacZ/+}$ mice, and this was compared to wild-type controls. Additionally, glucose tolerance was assessed at this time.

MATERIALS AND METHODS.

Animals.

Genetically modified mouse models were used to examine CTGF in relation to GDM in adult pregnant mice. Mouse models were necessary for this project because β-cell mass cannot be measured in live humans, and sectioning of pancreata was essential in order to examine β-cells in the islet and thus allow for β-cell area and non-β-cell area to be calculated. Whole animal physiologic studies such as those examining β-cell mass expansion and glucose intolerance during pregnancy cannot be conducted using cell culture. For this research, two different forms of animals were used: wild-type mice and $CTGF^{LacZ/+}$ mice. These two genotypes of mice were divided into two additional cohorts, resulting in four experimental groups: wild-type virgin, wild-type pregnant, $CTGF^{LacZ/+}$ virgin, and $CTGF^{LacZ/+}$ pregnant.

The primary difference between the control group (wild-type) and experimental group ($CTGF^{LacZ/+}$) is their functioning copies of the CTGF allele. In wild-type mice, the CTGF allele contains five exons that encode the protein. Wild-type mice have two functioning copies of the CTGF allele and readily express CTGF. In $CTGF^{LacZ/+}$ mice, the mutant $CTGF^{LacZ}$ allele is a loss of function allele in which exons 3 and 4 have been completely replaced with the LacZ reporter cassette [5]. LacZ is a reporter gene that encodes the protein β-galactosidase. Thus, these mice have only one normal functioning copy of the CTGF allele while the other copy is a loss of function allele in which CTGF is not expressed due to inhibition by the LacZ gene. $CTGF^{LacZ/+}$ mice produce and express half as much CTGF compared to wild-type mice. $CTGF^{LacZ/+}$ mice constituted the experimental group and were categorized as $CTGF^{LacZ/+}$ mice while the con-

trol group was labeled as wild-type or CTGF$^{+/+}$ in order to indicate functioning CTGF alleles. Since mice with two copies of the CTGF^{LacZ} allele fail to live past birth due to unrelated defects in lung development [5], mice with one copy of the CTGF^{LacZ} allele and one wild-type CTGF allele (CTGF$^{LacZ/+}$) were utilized and compared to control mice (wild-type).

Tissue Processing.

The pancreata for virgin and pregnant CTGF$^{LacZ/+}$ and wild-type mice were processed for further immunohistochemistry experiments after harvesting. Pancreata were extracted from the mice, and the wet weight of each pancreas was assessed for later β-cell mass analyses. Pancreata were subsequently fixed in 4% paraformaldehyde. After dehydration through an increasing ethanol series and xylene washes, each pancreas was embedded in paraffin, a wax-like substance that preserved the pancreas tissue [6]. The samples were then serial sectioned at 5 μm, meaning the entire tissue was sectioned throughout on a microtome. Sections were placed on untreated glass slides.

Immunohistochemistry

β-cell mass quantification was used to determine if the loss of one CTGF allele in CTGF$^{LacZ/+}$ mice had an effect on the total average β-cell mass among the four experimental groups, especially the pregnant CTGF$^{LacZ/+}$ mice and wild-type mice. Immunohistochemistry for insulin protein was performed after the pancreas tissue was excised, weighed, and sectioned. Approximately every tenth slide that contained the pancreatic tissue was selected to immunolabel for insulin to quantify β-cell mass, allowing for a representative analysis of the whole pancreas. Primary antibodies detecting insulin (Dako, Carpinteria, CA) protein were followed by a conjugated species-specific secondary antibody (Jackson ImmunoResearch, West Grove, PA) that when reacted with DAB (3,3'-Diaminobenzidine, Vector Laboratories, Burlingame, CA) labels β-cells a dark brown color to differentiate them from surrounding non-β cell pancreatic tissue. Eosin stained the total pancreatic tissue. After staining was complete, slides were scanned using a bright field microscope (Aperio ScanScope CS), and images were captured digitally to analyze average β-cell and tissue area. A ratio of β-cell area to total pancreatic area was calculated, and this ratio was then applied to the total mass of the pancreas to determine approximate β-cell mass per animal [5].

In addition to quantifying β-cell mass, glucose tolerance was measured, and glucose homeostasis was determined in order to examine if the loss of one CTGF allele in CTGF$^{LacZ/+}$ mice influenced their ability to control blood glucose at appropriate levels. All intraperitoneal glucose tolerance tests (IPGTT) were conducted by a supervising scientist, did not cause more than momentary pain or distress, and were not done solely for the student's project. To measure β-cell function in response to glucose, mice were fasted overnight, and a baseline blood glucose reading was recorded. Next, 2 mg of glucose per gram body weight was administered, and blood glucose levels were recorded at 15, 30, 60, 90, and 120 minutes using an Accu-Chek glucometer (Roche, Indianapolis, IN) and glucose test strips. IPGTTs were conducted at 10 weeks of age for both virgin and pregnant mice. The IPGTT measured the clearance of blood glucose over the two-hour period. The glucose tolerance test was administered to pregnant mice at GD14.5, the period at which changes in β-cell proliferation is most dramatic [2]. The IPGTT was a necessary quantitative assessment to determine how each experimental mouse responded to high glucose levels in order to establish if there is a correlation between glucose tolerance and CTGF levels. These results would likely reflect the ability of beta-cells to secrete insulin in response to the glucose challenge.

Statistical Analysis

Statistical analyses was determined using GraphPad Prism 5.0 software. Statistical analysis of β-cell mass was determined with a one-way ANOVA followed by a Tukey's post-hoc test. Statistical analysis of the IPGTT was determined with a two-way ANOVA followed by a Bonferroni correction.

RESULTS.

There is no statistically significant difference in β-cell mass between wild-type and CTGF$^{LacZ/+}$ pregnant mice.

Because β-cell mass cannot be measured in live animals, pancreata from virgin and pregnant wild-type and CTGF$^{LacZ/+}$ mice were excised, sectioned, and processed for immunolabeling. The pancreata were not used solely for this project, nor were the animals sacrificed for this project. As the β-cells are the only cell type in the pancreas to produce insulin, immunolabeling was performed with an antibody specific to insulin. Eosin was used as a counterstain to help visualize non-β-cell tissue. After immunolabeling and counterstaining, slides were imaged with a bright field microscope. In the resulting images, β-cells are dark brown, while non-β-cell tissue appears pink. Using these images and the measured weight of the pancreas, the β-cell and non-β-cell area were measured, and β-cell mass for the individual specimen was calculated. No statistically significant differences were observed between any groups (Figure 1). Additionally, there were no statistically significant differences in β-cell mass between pregnant wild-type and CTGF$^{LacZ/+}$ mice. There was also no difference in β-cell mass between virgin and pregnant mice in the wild-type animals (the average of all groups was between 0.5 mg – 0.8 mg). The maximum increase in β-cell mass during pregnancy is observed at gestational day 16.5 (GD16.5) [5]. Thus it may be that the measurement at GD14.5 was too early to observe this difference between virgin and pregnant animals. Therefore, CTGF is not necessary for proper β-cell mass at GD14.5.

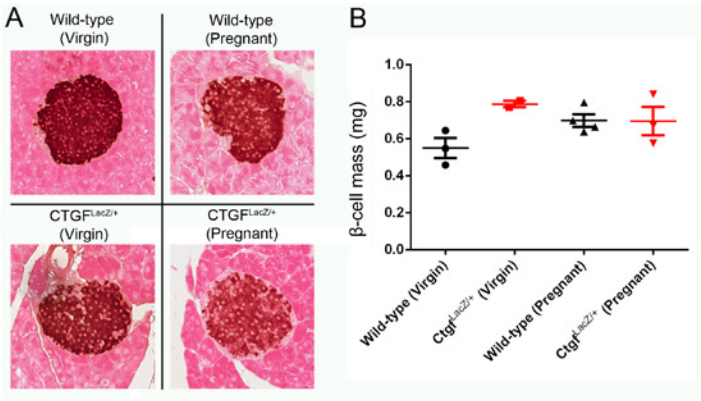

Figure 1. β-cell mass is similar between WT and CTGF$^{LacZ/+}$ virgin and pregnant mice. There was no statistically significant difference for β-cell mass among the mice. **A.** Representative images from immunolabeled sections of wild-type (virgin), wild-type (pregnant/GD14.5), CTGF$^{LacZ/+}$ (virgin), and CTGF$^{LacZ/+}$ pregnant/GD14.5) samples. β-cells are dark brown, and non-β-cell tissue is pink. **B.** Immunolabeled sections were used to quantify β-cell mass from 2-4 specimens per group. No statistically significant differences were observed between any groups.

Pregnant CTGF$^{LacZ/+}$ mice are glucose-intolerant.

In addition to measuring β-cell mass in wild-type and CTGF$^{LacZ/+}$ mice, glucose tolerance was also measured to determine if the loss of one functioning CTGF allele in CTGF$^{LacZ/+}$ mice affected their ability to regulate blood glucose levels. The glucose tolerance test was administered to pregnant mice at GD14.5, the period at which β-cell mass starts to increase, and the maximum increase in β-cell proliferation is observed [2]. Glucose tolerance was measured in 10 week old virgin and pregnant CTGF$^{LacZ/+}$ and wild-type control mice using intraperitoneal glucose tolerance tests (IPGTTs), which measured the clearance of blood glucose over a two-hour period time period. After a bolus of glucose was administered, blood glucose was measured at 15, 30, 60, 90, and 120 minutes. Glucose tolerance in 10 week old virgin CTGF$^{LacZ/+}$ mice was not significantly different from wild-type control mice. Conversely, glucose levels in 10 week old pregnant CTGF$^{LacZ/+}$ mice were higher at two early time points compared to pregnant wild-type controls, indicating glucose intolerance (Figure 2).

This result was surprising as no glucose intolerance was observed in virgin CTGF$^{LacZ/+}$ mice, nor was any deficiency in β-cell mass measured in pregnant CTGF$^{LacZ/+}$ mice compared to pregnant wild-type controls at this same time point (Figure 2). These data indicate that CTGF is essential for glucose tolerance during pregnancy independent of β-cell mass. These results emphasize the interesting fact that glucose tolerance can be adversely affected in pregnant animals even within the context of proper β-cell mass.

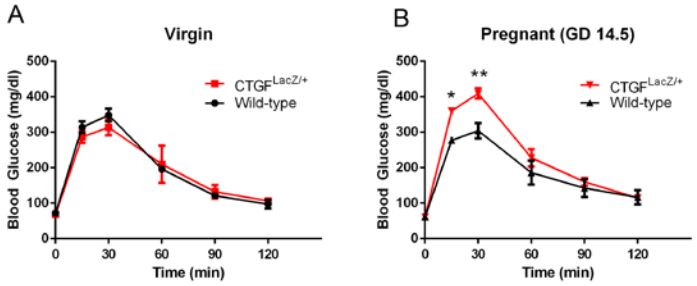

Figure 2. Pregnant, but not virgin, CTGF$^{LacZ/+}$ mice have impaired glucose tolerance. **A.** Glucose tolerance in 10 week old virgin CTGF$^{LacZ/+}$ mice was not significantly different from wild-type control mice. **B.** Glucose levels in 10 week old pregnant (GD: 14.5) CTGF$^{LacZ/+}$ mice were higher at two early time points, indicating glucose intolerance. n = 3-4 specimens per group (*: $p \leq 0.05$, **: $p \leq 0.01$).

DISCUSSION.

This research indicates that loss of one functional allele of CTGF does not impair β-cell mass in either virgin mice or at GD14.5. Surprisingly, we did not observe an increase in β-cell mass during pregnancy at GD14.5 even in the control animals. However, the increase in β-cell mass during pregnancy that has been reported by other labs does not peak until GD16.5, and thus our measurements may have been collected at a gestational time-point when the increase in mass have not yet been fully realized [2]. Thus, future research could measure β-cell mass at GD16.5 to more thoroughly explore if CTGF has a role in this adaptation during pregnancy. Notably, IPGTTs revealed no statistically significant difference in glucose tolerance between virgin wild-type and virgin CTGF$^{LacZ/+}$ mice. However, there was glucose intolerance during pregnancy in CTGF$^{LacZ/+}$ animals compared to wild-type pregnant animals. Unlike other forms of diabetes, GDM does not require an elevated fasting blood glucose levels for diagnosis [7, 8]. This finding suggests that pregnant CTGF$^{LacZ/+}$ mice have gestational diabetes, and that the defect in glucose homeostasis is not caused by a deficiency in β-cell mass. This surprising finding can be examined further by testing if CTGF affects β-cell function (i.e. insulin secretion) or insulin signaling specifically during pregnancy. For example, the β-cells present in the islets of pregnant CTGF$^{LacZ/+}$ mice may not release insulin properly. This potential phenotype could be tested by isolating islets from pregnant CTGF$^{LacZ/+}$ and pregnant wild-type mice and measuring how much insulin they secrete in response to a standard dose of glucose. Alternatively, pregnant CTGF$^{LacZ/+}$ mice may have defective insulin signaling, meaning that the cells that take up glucose in response to insulin are not functioning properly. This possibility could be assessed using hyperinsulinemic/euglycemic clamp technology. Overall, the findings demonstrate that CTGF has a previously unknown role in maintaining glucose homeostasis during pregnancy independent of β-cell mass, and future work is needed to determine how it affects β-cell function or insulin signaling.

From an overall medical and health standpoint, by examining CTGF's relationship to β-cell mass, glucose and insulin tolerance, it would be interesting to establish if defects or mutations in CTGF are related to GDM in human patients. In doing so, this could provide researchers, doctors, and those patients with GDM a better understanding of the etiology and development of this illness.

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