Enhanced Insulin Sensitivity Mediated by Adipose Tissue Browning Perturbs Islet Morphology and Hormone Secretion in Response to Autonomic Nervous Activation in Female Mice

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Running Title: Autonomic mediated islet adaptation to insulin sensitivity
ABSTRACT

Insulin resistance results in compensatory increase in insulin secretion to maintain normoglycemia. Conversely, high insulin sensitivity results in reduced insulin secretion to prevent hypoglycemia. The mechanisms for this inverse adaptation are not well understood. We utilized highly insulin-sensitive mice, due to adipocyte-specific overexpression of the FOXC2 transcription factor, to study mechanisms of the reversed islet adaptation to increased insulin sensitivity. We found that Foxc2TG mice responded to mild hyperglycemia with insulin secretion significantly lower than that of wild type mice, however when severe hyperglycemia was induced, Foxc2TG mice demonstrated insulin secretion equal to or greater than that of wild type mice. In response to autonomic nervous activation by 2-deoxyglucose, the acute suppression of insulin seen in wild-type mice was absent in Foxc2TG mice suggesting impaired sympathetic signaling to the islet. Basal glucagon was increased in Foxc2TG mice but they displayed severely impaired glucagon responses to cholinergic and autonomic nervous stimuli. These data suggest that the autonomic nerves contribute to the islet adaptation to high insulin sensitivity which is compatible with a neuro-adipo regulation of islet function being instrumental for maintaining glucose regulation.

Keywords: Insulin, Glucagon, White adipose tissue, Sympathetic nervous system, Parasympathetic nervous system

INTRODUCTION

Blood glucose homeostasis is one of the most tightly regulated processes in mammalian physiology. In healthy humans, glucose levels vary by only a few percent throughout a 24 hour period (40). During feeding this tight regulation is mainly mediated by interplay between insulin levels and the induction of glucose uptake in insulin-sensitive tissues (9). In contrast, during fasting, glucose regulation is mediated primarily by glucagon induced release of stored
glucose from the liver (21). In overweight and obese individuals the sensitivity of the liver, muscle and adipose tissue to insulin decreases, however the pancreatic beta cells adapt by secreting more insulin to maintain normal glucose uptake in these tissues and maintain normal blood glucose (41). Consequently, the loss of the ability of the beta cells to adapt to systemic insulin resistance is a primary defect causing type 2 diabetes (22). As tight glucose regulation is such a fundamental factor for normal energy homeostasis, it would stand to reason that an adaptation to high degrees of insulin sensitivity is required to continue to maintain normal blood glucose levels. This would also imply a consequent inverse relationship between insulin sensitivity and insulin secretion such that higher insulin sensitivity would be expected to result in lower insulin secretion (3, 11, 22). This was indeed found in a study of elite athletes with high degrees of insulin sensitivity showing that basal and stimulated levels of insulin were decreased compared to sedentary individuals with lower insulin sensitivity when blood glucose levels were equal (6). Similarly, the improved insulin sensitivity induced by weight reduction after bariatric surgery is associated with reduced insulin secretion (18). The mechanistic basis for this “reverse adaptation” to insulin sensitivity has not been characterized.

In recent years there has been an interest in the enhancement of insulin sensitivity by transitioning insulin-resistant white adipose tissue into energy-dissipating brown adipose tissue, so called “browning” of white adipose tissue (8). In rodents and humans, the transcription factor FOXC2 has been shown to be one of the key mediators of browning of white adipose tissue (15, 30). Mice with an adipose tissue-specific overexpression of the Foxc2 transcription factor show browning of white adipose tissue, with increased expression of brown fat associated genes UCP1, B3AR and others as well as increased oxygen consumption in mitochondria and multilocular lipid droplets in white adipocytes (15). The adipose tissue-specific Foxc2 transgenic mouse is lean and resistant to high fat diet-induced
obesity (28). However, whether and by which mechanism the enhanced insulin sensitivity affects islet function has not been defined in this model. We have therefore undertaken an in depth study to understand the cellular and physiological mechanisms that drive adaptation to increased insulin sensitivity to maintain normal glucose regulation, using multiple metabolic challenges in this unique model. We found that the reverse adaptation to insulin sensitivity is primarily mediated by altered neural signaling to the pancreatic islet such that enhanced systemic insulin sensitivity causes increased sympathetic nervous input to the islet to suppress insulin, enhance basal glucagon secretion and thus maintain normal glucose levels and avoid hypoglycemia.

METHODS

Animals. The generation of transgenic mice overexpressing the Foxc2 gene in adipose tissue under the direction of the AP2 promoter has been described previously (15). The AP2 gene promoter is specific for mature adipocytes and the transgene is active from the time of adipocyte maturity. Transgenic mice heterozygous for the Foxc2 transgene, on a C57BL6/J background, were backcrossed for multiple generations onto the C57BL6/JBomTac background. 4-5 month old female mice were used for all experiments. We have previously shown that the glucagon response to 2-D-deoxyglucose (2-DG) is more pronounced in female than in male mice (27). Ethical approval for all animal experiments was received from the regional ethical committee in Lund Sweden.

Euglycemic-hyperinsulinemic clamp studies. For euglycemic-hyperinsulinemic clamp experiments, mice were anesthetized with a single i.p injection of fentanyl (0.78 mg/kg) – fluanisone (25 mg/kg) [Hypnorm Vetpharma] and midazolam (12.5 mg/kg) and rested for 30 minutes prior to receiving a primed continuous infusion of insulin (30mU/kg/mouse; Actrapid Novo Nordisk A/S, Bagsvaerd, Denmark) and variable glucose (40% D-glucose in saline) for
90 minutes. Blood glucose was monitored every five minutes with a hand-held glucose
monitor (HemoCue, Ängelholm Sweden). Plasma samples were taken at time 0, 60 and 90
minutes for the measurement of insulin.

**Hyperglycemic clamp studies.** Anesthetized mice were given a single intravenous injection of
D-glucose in the tail vein to elevate blood glucose to the target level and then infused with
variable glucose (30-40% D-glucose in saline) to maintain blood glucose at the target level for
90 minutes. Plasma samples were taken at 0, 1, 5, 20, 50, 70 and 90 minutes for measurement
of insulin.

**Intravenous glucose, 2-deoxyglucose, arginine and carbachol challenges.** Anesthetized
mice were fasted for five hours and then given a single intravenous injection of D-glucose
(0.35 mg/kg or 1.0 mg/kg), 2-D-deoxyglucose (50 mg/ml), L-arginine (5 mg/mouse) or the
muscarinic receptor agonist carbachol (30µg/kg) in the tail vein. Blood was sampled from the
intraorbital retrobulbar sinus plexus at 0, 1, 5, 10, 20, 30 and 50 minutes for glucose; 0, 2, 10
and 30 minutes for 2-deoxyglucose and 0, 1, 5, 10, 20 and 50 minutes for arginine and
carbachol injections. Plasma was separated by centrifugation and glucose, insulin and
glucagon were measured.

**Ex vivo insulin and glucagon secretion from isolated islets.** Pancreata were perfused in situ
with Hank’s Balanced Salt Solution (HBSS) containing 2.7U Collagenase P (Roche,
Mannheim, Germany), removed and incubated at 37° C for 19 minutes. Digested pancreata
were washed with HBSS and islets were hand-picked in an inverted microscope. Freshly
picked islets were pre-incubated in HEPES buffer containing 125 mmol/l MgCl2, 25 mmol/l
HEPES (pH 7.4), 5.6 mmol/l glucose and 0.1% fatty acid free BSA (Boehringer Mannheim,
GmbH, Germany). Islets were then incubated in batches of three per well in eight replicates
and incubated with varying glucose concentrations with or without parasympathetic
(carbachol) or sympathetic (clonidine) neurotransmitter analogues (Sigma-Aldrich, St. Louis, MO, USA) for 60 minutes. Media samples are taken at 60 minutes for measurement of insulin and glucagon.

**Immunohistochemistry.** Pancreata were extracted and fixed in 4% paraformaldehyde in 0.1 mmol/L PBS containing 0.1% picric acid, prior to cryopreservation in 20% (w/v) sucrose in PBS. Pancreata were frozen in OCT compound and 10 μm sections were cut and mounted. Sections were immunostained using antiserum against rat proinsulin (1:1280 dilution), and rabbit glicentin for glucagon detection (1:10560 dilution) [both from Eurodiagnostica, Malmö, Sweden]. Glucose transporter 2 (GLUT2) was detected using rabbit anti-rat GLUT2 antiserum (1:200 dilution) [Chemicon International, Inc., Temecula, CA, USA]. Islet areas were quantified as described previously (36, 37). Briefly, Alexa-fluor coupled fluorescent secondary antibodies (goat anti-guinea pig Alexa546 for insulin and goat anti-rabbit Alexa488 for glucagon) were used in double staining experiments. Fluorescent images were captured in an Olympus BX 51 fluorescent microscope with an Olympus DP-50 digital camera. Islet size was determined manually by interactively defining outline of each individual islet and the area covered was determined in the program NIH image 1.06 after calibration. A total of five to seven sections from each pancreas were analyzed.

**Biochemical analyses.** Plasma glucose was determined by the glucose oxidase method with ABTS as the substrate. Plasma insulin was determined by radioimmunoassay (Linco, St. Louis, MO, USA). Plasma glucagon was determined by radioimmunoassay (Linco).

**Statistical Analyses.** All data are presented as mean ± SEM. The acute insulin and glucagon responses are calculated as the mean of the 1 and 5 minute values, after baseline subtraction, during an IVGTT or hyperinsulinemic clamp, whereas ∆AIR is the baseline subtracted 1 minute value only. For 2-deoxyglucose challenges the acute insulin and glucagon responses
were calculated as the baseline-subtracted mean 2 and 10 minute values, whereas ΔAIR was the baseline subtracted 2 minute value only. Area under the curve was calculated using the trapezoidal rule. Statistical significance was determined by the non-parametric Mann Whitney U-test, unless otherwise stated. Statistical calculations were made using the GraphPad Prism 6.0 software package.

RESULTS

Foxc2 transgenic mice have lower basal glucose and insulin and are highly insulin sensitive. Body weight and basal plasma parameters were obtained to assess the basal metabolic state of Foxc2 transgenic mice. Foxc2 transgenic mice had a slightly higher body weight compared to wild type mice (Figure 1A). Four hour and 16 hour fasting plasma glucose were significantly lower in Foxc2 transgenic mice (Figure 1B) as was four hour fasting insulin (Figure 1C) suggesting an increased insulin sensitivity. There was, however, no significant difference in plasma insulin after an overnight fast. To assess whole-body insulin sensitivity, euglycemic-hyperinsulinemic clamps were performed. Blood glucose was clamped at 6.5 mmol/l under a 30 minute steady state period (Figure 1D). During the steady state period, the glucose infusion rate needed to maintain euglycemia, a direct measure of insulin sensitivity, was 3-fold higher in Foxc2 transgenic mice compared to wild type mice (Figure 1E, F).

Foxc2 transgenic mice respond to high insulin sensitivity with a glycemia dependent reduction in insulin secretion in vivo. In order to determine the effect of the notable increase in insulin sensitivity on insulin secretion, Foxc2 transgenic mice were subjected to hyperglycemic clamp experiments at three different levels of hyperglycemia. The acute insulin response to glucose during the hyperglycemic clamps showed a distinct pattern that was dependent on the degree of hyperglycemia. When clamped at 11.1 mmol/l glucose, the
acute insulin response was significantly lower in Foxc2 transgenic mice and the second phase of insulin secretion was nearly absent, suggesting adaptation to increased insulin sensitivity with decreased insulin secretion (Figure 2A, D, G). When clamped at 16.7 mmol/l glucose, approximately 2-fold higher than normal nonfasted blood glucose, there was no difference in the acute insulin response or second phase insulin secretion between the wild type and Foxc2 transgenic mice (Figure 2B, E, H). When clamped at 33 mmol/l glucose, to establish maximal insulin secretory responses to glucose in vivo, Foxc2 transgenic mice exhibited significantly greater acute insulin responses than wild type controls, with no differences in second phase insulin apparent (Figure 2C, F, I).

**Foxc2 transgenic mice have normal insulin responses to arginine and cholinergic agonists but impaired response to autonomic nervous activation by 2-deoxyglucose.** In addition to glucose we also determined the insulin secretory responses to non-glucose secretagogues with different mechanisms of action. It was noted that both intravenous arginine and carbachol had demonstrable and opposing effects on plasma glucose in Foxc2 transgenic mice and wild type mice (Figure 3A, B). Acute intravenous arginine was associated with significantly greater increases in the plasma glucose excursion in Foxc2 transgenic mice compared to wild-type (Figure 3A). In contrast, stimulation with carbachol or 2-dexoyglucose resulted in significantly lower glucose excursions in Foxc2 transgenic mice than in wild type mice (Figure 3B, C). Insulin secretion in response to acute intravenous injection of arginine was similar between Foxc2 transgenic and wild type mice (Figure 3D, G). We next investigated the effect of the cholinergic muscarine receptor agonist carbachol on insulin secretion in Foxc2 transgenic mice. There were no differences in insulin secretion between Foxc2 transgenic and wild type mice in response to intravenous injection of carbachol (Figure 3E, H).
2-Deoxyglucose (2-DG) causes activation of the autonomic nervous system and signaling through sympathetic and parasympathetic nerve terminals of the endocrine pancreas as well as secretion of epinephrine and norepinephrine from the adrenal glands (13). Immediately after intravenous injection of 2-DG, there was a significant suppression of insulin in wild type mice (Figure 3F), which has been previously attributed to activation of the adrenergic receptors on the beta cell through the sympathetic nervous system (24). After this reduction of insulin, there is a clear increase in insulin secretion, which has been shown to be due to parasympathetic nerve activation (Karlsson and Ahrén, 1991). It was found that the acute suppression of insulin by 2-DG was completely absent in Foxc2 transgenic mice (Figure 3F, I). In contrast, the subsequent insulin secretion, from 2 minutes to 30 minutes, was not different between wild type and Foxc2 transgenic mice (Figure 3F).

Foxc2 transgenic mice have enhanced basal glucagon but impaired glucagon secretion in response to cholinergic and autonomic stimuli. As carbachol is a mimetic of acetylcholine and 2-deoxyglucose stimulates both cholinergic and adrenergic nervous activity, the diminished glucose excursions in response to these stimuli suggested impaired glucagon secretion and/or action in response to autonomic nervous activation in Foxc2 transgenic mice. We next investigated glucagon secretion in the basal and stimulated states in Foxc2 transgenic mice. Foxc2 transgenic mice had two-fold greater basal plasma glucagon than wild type mice after a five hour fast (Figure 4A). Interestingly, there was no increase in basal plasma glucagon in response to a prolonged fast in Foxc2 transgenic mice (Figure 4B).

We then proceeded to investigate glucagon secretion in response to the non-glucose secretagogues. Despite the higher basal plasma glucagon in Foxc2 transgenic mice (Figure 4C), the acute glucagon response to arginine was not significantly different between wild type and Foxc2 transgenic mice (Figure 4F).
While adrenergic receptor activation in the islet is inhibitory for insulin secretion and stimulatory for glucagon secretion, cholinergic receptor activation is stimulatory to both (1, 29). Carbachol activates cholinergic muscarine 3 receptors on alpha cells (17), as well as beta cells, while 2-DG stimulates both sympathetic adrenergic and parasympathetic cholinergic neurotransmission to the islet. Intravenous injection of carbachol resulted in a significantly blunted acute glucagon response in Foxc2 transgenic mice compared to wild type mice, despite having higher basal glucagon (Figure 4D, G). 2-DG administration resulted in significantly impaired acute glucagon responses in Foxc2 transgenic mice (Figure 4E, H), as well as impaired total glucagon secretion (Figure 4E).

Foxc2 transgenic mouse islets have normal responses to parasympathetic and sympathetic neurotransmitter analogues ex vivo. As the insulin and glucagon secretory responses to 2-DG were impaired in Foxc2 transgenic mice in vivo, but the insulin secretory response to carbachol was normal, it was unclear as to whether the defect in hormone secretion was due to altered transmission of neural signals to the islet, altered response of the islets to neurotransmitter stimuli or both. If neural signaling to the islet is impaired in vivo, then isolated islets should respond normally to stimulation by neurotransmitters in vitro if post receptor signaling and function is unaltered. We therefore next performed in vitro insulin secretion experiments in islets isolated from wild type and Foxc2 transgenic mice. Similar to the insulin secretion in vivo during hyperglycemic clamps, islets from Foxc2 transgenic mice had slightly, but not significantly, lower insulin secretion in response to 11.1 mmol/l glucose (Fig. 5A). However, more importantly, islets from wild type and Foxc2 transgenic mice displayed similar increases in insulin secretion in response to the parasympathomimetic carbachol (Fig. 5B). Furthermore, glucose-stimulated insulin secretion was suppressed to similar extents by the \( \alpha_2 \)-receptor agonist clonidine in wild type and transgenic mice (Fig. 5B). Arginine stimulates insulin secretion independently of any receptor activation and, in the
presence of arginine, carbachol stimulated and clonidine inhibited insulin secretion to similar extents in both wild type and Foxc2 transgenic mice (Fig. 5B).

**Foxc2 transgenic mice have smaller islet size compared to wild type mice.** Although it was clear that there was low basal and glucose stimulated insulin secretion and impairments in glucagon secretion in response to autonomic neurotransmission, it was unclear whether this was due to alterations in islet morphology. We therefore next investigated islet morphology in Foxc2 transgenic mice. Immunohistochemical analysis of pancreatic sections showed that the core-mantle structure, typical of rodent islets, was unaltered in Foxc2 transgenic mice (Figure 6A). However, evaluation of islet areas in wild type and Foxc2 transgenic mice revealed significantly smaller total areas of islets from Foxc2 transgenic mice (Figure 6B). This was associated with a significantly lower insulin positive area of islets of Foxc2 transgenic mice (Figure 6C) but not the glucagon positive area (Figure 6D).

**DISCUSSION**

It has been over 30 years since a number of studies demonstrated that insulin resistant subjects have a compensatory increase in insulin secretion (10, 11, 23). Despite much research on this area since then, relatively few studies have demonstrated the logical reverse that increased insulin sensitivity is compensated for by decreased insulin secretion. A few studies have been performed on elite athletes as a model of increased insulin sensitivity, each showing reduced fasting and stimulated insulin secretion in response to oral or intravenous glucose or arginine (6, 35). After bariatric surgery an increase in insulin sensitivity after weight loss is met with a decrease in insulin secretion (18). This inverse adaptation would functionally be a safeguard against increased risk for hypoglycemia in high insulin sensitivity. It is known that a degree of expansion of functional beta cell mass partly explains the mechanism by which insulin resistance is compensated for by increased insulin secretion. A
mechanism for how the body adapts to increased insulin sensitivity has not been described to
date. The findings of this study suggest that the metabolic response to increased systemic insulin
sensitivity is a decrease in basal and glucose stimulated insulin secretion at physiological
glucose levels. This decreased insulin secretion is therefore appropriate for the ambient level
of insulin sensitivity. The islets have smaller insulin positive areas in response to increased
insulin sensitivity just as insulin positive islet area increases in response to insulin resistance
(7). When challenged with high glucose there was no difference in insulin secretion between
wild type and transgenic mice but when challenged with supra-physiological amounts of
glucose, there was a greater insulin secretory response in transgenic mice suggesting that
there is no functional impairment of the beta cell population resulting from the high level of
insulin sensitivity and the beta cells are “rested” therefore primed to give a greater response to
an extreme glucose challenge.

The regulation of islet hormone secretion by the nervous system involves autonomic nerves,
sensory nerves and neuropeptides (1). Sympathetic nervous signaling results in decreased
insulin secretion and increased glucagon secretion, as energy in the form of glucose needs to
be rapidly mobilized from stores under conditions of acute stress (24). Parasympathetic
nervous signaling to the islet occurs via the vagal nerve (5). Parasympathetic vagal
stimulation results in the secretion of the major islet hormones insulin, glucagon, somatostatin
and pancreatic polypeptide (4, 12). In addition to autonomic signals, there are sensory nerves
innervating the islet which affect islet hormone secretion (38). Similar to sympathetic nervous
signaling, sensory nervous activation and the release of calcitonin gene-related peptide
(CGRP) within the islet is inhibitory to insulin secretion and stimulatory to glucagon secretion
(2).
As tools for investigating autonomic stimuli in vivo, we used the cholinergic agonist carbachol and the autonomic activator 2-DG, which is a non-metabolized glucose analogue which through competition with glucose uptake and intracellular phosphorylation creates intracellular glycopenia (14, 42). Since central nervous system is highly dependent on glucose availability, systematic administration of 2-DG results in neuroglycopenia (31) which activates the autonomic nervous system, as evidenced by increased plasma catecholamine levels and pancreatic sympathetic activation (20). This in turn results in increased glucagon and insulin secretion as we previously have demonstrated in mice (24, 26), and since these responses are inhibited by both muscarinic blockade and alpha-adrenergic blockade (25), it is clear that both the parasympathetic and sympathetic branches of the autonomic nervous system are activated by 2-DG in mice. We observed increased basal glucagon secretion but impaired glucagon secretion in response to cholinergic or autonomic stimuli. There appeared to be a dysregulation between autonomic nervous signaling and the islet, as the suppressive action of adrenergic signals on insulin secretion and the stimulatory effect of adrenergic and cholinergic signals on glucagon secretion, were impaired in Foxc2 transgenic mice. This could be due to an increase in the basal sympathetic activity in response to the extreme insulin sensitivity seen in Foxc2 transgenic mice. Foxc2 transgenic mice had decreased basal insulin and increased basal glucagon which would be expected if sympathetic signaling to the islet were increased. In fact, in response to 2-DG, plasma insulin in wild-type mice was initially suppressed to the same level as that of Foxc2 transgenic mice at baseline, whereas plasma insulin was completely unchanged immediately after 2-DG injection in Foxc2 transgenic mice. This suggests that Foxc2 transgenic mice already had a high level of sympathetic nervous signaling to the islet. When islets from Foxc2 transgenic mice were stimulated with parasympathetic or sympathetic neurotransmitter analogues in vitro, the insulin and glucagon secretory responses were normal. This confirms that altered neural signaling in vivo, not
altered islet function, is the main contributor to the secretory phenotype of Foxc2 transgenic mice.

The finding of altered islet responses to nervous inputs in the lean, highly insulin sensitive Foxc2 transgenic mice should not be a complete surprise. Nearly 30 years ago Peterson and colleagues demonstrated an inverse relationship between body fat and autonomic nervous activity (34). Subsequent studies have demonstrated that caloric restriction and/or exercise result in increased insulin sensitivity in parallel with an increase in autonomic nervous activity (32).

Mechanistically, the overexpression of the FOXC2 transcription factor in adipose tissue results in increases in brown adipose tissue mass and white adipose tissue with brown adipose characteristics (15). There are a number of other transgenic mouse models that show increased brown adipose tissue characteristics and energy expenditure and these are also associated with increased insulin sensitivity (16, 19, 33). However the dramatic increase in insulin sensitivity seen in Foxc2 transgenic mice and other models cannot be explained by increased energy dissipation in brown and white adipose tissue alone. Bartness and colleagues have recently described the neural innervation and signaling not only to the brown adipose tissue but also from the brown adipose tissue to centers in the brain (39). The signaling from the brown adipose tissue was mediated via sensory nerves and fed back to the autonomic nervous system at the level of the hindbrain (39). This suggests that the increased mass and metabolic activity of brown adipose tissue of Foxc2 transgenic mice could itself be activating autonomic nervous signaling independent of the insulin sensitivity status. There are some limitations to the study that are worth mentioning. This study was only carried out in female mice, as previous studies have demonstrated a significantly greater islet hormone response to autonomic nervous stimulus by 2-DG in females than in males (27). Furthermore, the high degree of insulin sensitivity in the Foxc2 transgenic mouse model is much less pronounced in
males (data not shown), making them less suitable for a study on islet adaptation to high degrees of insulin sensitivity. The reasons for this gender difference are not known at present. Another limitation of our study is the lack of definitive information about the absolute beta cell mass in this model of extreme insulin sensitivity.

To our knowledge, our study is the first to demonstrate that chronically increased insulin sensitivity affects islet hormone secretion by a mechanism involving alterations to islet morphology and the basal activation state of the branches of the autonomic nervous system. Future studies will need to be performed to determine whether autonomic nervous signaling affects islet hormone secretion in highly insulin sensitive humans.

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DISCLOSURES

The authors have nothing to disclose with regards to this study.

AUTHOR CONTRIBUTIONS

B.O. designed and performed experiments, data analysis and wrote the manuscript. S.E. generated and provided the Foxc2 transgenic mouse line and wrote the manuscript. MR
performed the immunohistochemistry. B.A. designed experiments, performed data analysis
and wrote the manuscript. All of the authors approved of the final version of the manuscript.

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**FIGURE LEGENDS**

**Figure 1.** Bodyweight, fasting glucose and insulin and insulin sensitivity measured by euglycemic-hyperinsulinemic clamp in wild type and Foxc2 transgenic mice

(A) Body weight in four month old female wild type (WT) and Foxc2 transgenic (Foxc2TG) (n = 26-29). Mice were fasted for 4 (n = 14-20) or 16 (n = 8-9) hours and plasma glucose (B) and insulin (C) were determined. Euglycemic-hyperinsulinemic clamp experiments were carried out on non-fasted anesthetized mice (n = 5-7 mice per group). (D) Insulin was infused at a constant rate and glucose at a variable rate to obtain a steady state blood glucose value of 6.5 mmol/l between 60 and 90 minutes post infusion start. (E, F) The rate of glucose infusion was recorded for all time points and the glucose infusion rate (GIR) was calculated during the 30 minute steady state period between 60 and 90 minutes. Data are presented as mean ± SEM. Asterisks indicate the probability level of random difference between the wild type and Foxc2 transgenic mice * p < 0.05, ** p < 0.01, *** p < 0.001.

**Figure 2.** Dynamic insulin secretion in vivo determined by hyperglycemic clamp
Plasma glucose (A, B, C) and insulin (D, E, F) in mice fasted five hours and given a bolus injection of D-glucose followed by variable infusion of glucose to reach target blood glucose of 11.1 mmol/l (A, D, G), 16.7 mmol/l (B, E, H) or 33.3 mmol/l (C, F, I) and which was maintained at a steady state for 90 minutes. The acute insulin response to glucose (∆AIR) was calculated as described in Experimental procedures. n = 7-11 per group. Data are presented as mean ± SEM. Asterisks indicate the probability level of random difference between the wild type and Foxc2 transgenic mice * p < 0.05, ** p < 0.01, *** p < 0.001.

Figure 3. Plasma glucose and insulin in response to non-glucose secretagogues

Plasma glucose and insulin in mice fasted five hours and given single intravenous injections of L-Arginine (A, D, G) (n = 4), Carbachol (B, E, H) (n = 6-10) or 2-deoxy D-glucose (C, F, I) (n = 11-12). The acute insulin responses (∆AIR) (G, H, I) to each secretagogue were calculated as described in Experimental procedures. Data are presented as mean ± SEM. Asterisks indicate the probability level of random difference between the wild type and Foxc2 transgenic mice * p < 0.05, ** p < 0.01, *** p < 0.001.

Figure 4. Plasma glucagon after fasting or injection of non-glucose secretagogues

Plasma glucagon in mice fasted for 4 (A) or 16 (B) hours (n = 11-14 per group). Plasma glucagon in mice fasted for five hours and given single intravenous injections of (C) L-Arginine (n = 4), (D) Carbachol (n = 6) or (E) 2-deoxy D-glucose (n = 11). The acute glucagon responses to each secretagogue were calculated as described in Experimental procedures. Data are presented as mean ± SEM. Asterisks indicate the probability level of random difference between the wild type and Foxc2 transgenic mice * p < 0.05, ** p < 0.01, *** p < 0.001.

Figure 5. Insulin and glucagon secretion in isolated islets from wild type and Foxc2 transgenic mice
(A) Insulin secretion in response to varying glucose concentrations. (B) The fold-change in insulin secretion in response to neural (carbachol 100 µM, clonidine 1 µM) and non-neural (arginine 10 mM) factors in the presence of 11.1 mmol/l glucose. (C) Glucagon secretion in response to glucose alone and in the presence of neural and non-neural factors. (D) The fold-change in glucagon secretion in response to neural and non-neural factors in the presence of 11.1 mmol/l glucose. n = 3 mice per genotype.

Figure 6. Islet morphology and cellular composition in wild-type and Foxc2 transgenic mice

(A) Islet fluorescent immunohistochemistry from Foxc2 transgenic (TG) and wild-type (WT) mice fed normal kilocaloric diets (NK). Top panel- staining with antibodies against GLUT2 (green). Bottom panel- staining with antibodies against insulin (red) and glucagon (green). Scale bar represents 50 µm. (B) Total islet area (C) insulin positive islet area (D) glucagon positive islet area. n = 4 mice per group. Data are presented as mean ± SEM. Asterisks indicate the probability level of random difference between the wild type and Foxc2 transgenic mice * p < 0.05, ** p < 0.01
A. Glucose (mmol/l) and Insulin Secretion (pg/hr/islet) for WT and Foxc2 TG:

B. Glucose (mmol/l) and Glucagon Secretion (pg/hr/islet) for WT and Foxc2 TG:

C. Insulin Secretion (fold change) and Glucagon Secretion (fold change) for WT and Foxc2 TG:

- Carbachol
- Clonidine
- Arginine
- Arginine + Carbachol
- Arginine + Clonidine
A

WT
Foxc2
TG

0
5000
10000
15000
20000

Total Islet Area (m²)

B

WT
Foxc2 TG

0
5000
10000
15000
20000

Total Islet Area (μm²)

C

WT
Foxc2 TG

0
5000
10000
15000
20000

Insulin Positive Area (μm²)

D

WT
Foxc2 TG

0
5000
10000
15000
20000

Glucagon Positive Area (μm²)