LIVER FOXN3 INCREASES PANCREATIC α-CELL MASS WITHOUT ALTERING HEPATIC AMINO ACID UTILIZATION
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ABSTRACT
Insulin injection into peripheral tissues inefficiently controls elevated fasting blood glucose in type 1 diabetes mellitus (DM1) patients. Peripheral insulin lacks the signaling of healthy β-cell insulin, which directs adjacent pancreatic α-cells to stop production of glucagon, a hormone releasing glucose from the liver into the blood. Since peripheral insulin injection to treat DM1 poorly inhibits pancreatic glucagon secretion, fasting blood glucose remains persistently elevated in DM1. Glucagon receptor antagonism (GRA) therapies aim to reduce glucagon activity by blocking glucagon receptors in the liver, thereby increasing the efficacy of injected insulin. GRA is currently unviable since it promotes dysregulated α-cell proliferation and glucagon secretion, in the long-term. Similar α-cell growth and blood glucagon levels were identified in animal models expressing the transcriptional repressor protein FOXN3 in the liver. This paper aims to characterize mice with the liver-specific deletion of Foxn3 to determine whether the gene’s expression is necessary for pancreatic α-cell growth, and whether its deletion mitigates adverse effects of GRA therapy. Results validated that liver-specific deletion of Foxn3 causes α-cell expansion and decreases glucose utilization during fasting, resulting in elevated blood glucose. Mechanistically, Foxn3 is shown to act independently from GRA since its expression does not alter liver amino acid metabolism and losing Foxn3 in the liver does not reduce dysregulated α-cell proliferation and glucagon secretion during GRA therapy. Future insights into liver FOXN3-directed activation of pancreatic α- and β-cell development in a diabetic mouse may elucidate mechanisms of glucagon action in the absence of pancreatic insulin signaling.
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INTRODUCTION

Roughly 100,000 Americans, a majority of which are under the age of 20, are diagnosed yearly with insulin production deficiency that defines type 1 diabetes mellitus (DM1) [29]. Fasting blood glucose is hormonally regulated by insulin, which stimulates the liver to store excess glucose, and glucagon, which releases stored glucose in the liver [1]. DM1 is characterized by hyperglycemia resulting from decreased insulin secretion in pancreatic β-cells and increased glucagon secretion in pancreatic α-cells [1]. Both these hormones must be reciprocally regulated in the pancreas to normalize blood glucose levels; however, only a significant minority of patients achieve glycemic targets for safe metabolic control [30]. Currently, DM1 is treated by administering only insulin into peripheral tissues, which reduces blood glucose, but fails to decrease pancreatic glucagon secretion [21]. An alternative to excessive insulin dosing is minimizing glucagon’s effect on the liver. Consequently, treatments blocking the action of glucagon via glucagon receptor antagonism (GRA) have successfully improved hyperglycemia and DM1 glycemic control, in the short-term [3]. Long-term interference with glucagon receptor action, however, promotes detrimental increases in liver fatty acid content and causes further α-cell proliferation [4], [5]. An improved understanding of liver glucagon action and its effects on α-cell regulation is paramount to optimizing GRA therapies for DM1.

Increased liver glucagon secretion in DM1 is strongly associated with specific genes that increase fasting blood glucose, and provide alternative targets for reducing glucagon action. Human genomics studies point to a gene associated with increased fasting blood glucose, independent of fasting insulin level, implying a baseline increase in glucagon secretion [6], [7]. Among non-diabetic subjects, a single nucleotide variant rs8004664 in the first intron of the FOXN3 gene in human liver hepatocytes was associated with fasting blood glucose [8]. Importantly, this variant was significantly and independently associated with increased fasting blood glucose, but not insulin levels in humans [8]. Hepatic FOXN3 acts as a transcriptional repressor protein that binds DNA to down-regulate expression of MYC, encoding a master transcriptional regulator of glucose utilization during fasting [9]-[11]. By decreasing human MYC mRNA expression to limit hepatic glucose utilization during fasting, FOXN3 may increase the glucose available for release into circulation [11].

Probing FOXN3 action in the liver has verified the protein’s role in increasing α-cell proliferation, and increased glucagon secretion, leading to higher fasting blood glucose [11]-[12]. Additionally, glucagon-stimulated degradation of FOXN3 in mouse hepatocytes suggests a reciprocal feedback relationship between FOXN3 expression and glucagon secretion [13]. Critically, mouse models expressing FOXN3 showed an increase in serum amino acids and reduced hepatic amino acid utilization, consistent with the downregulation of protein products controlled by MYC [13]. Elevated serum amino acids are also hallmarks of extended GRA treatment, where excess hepatic amino acids in the blood promote pancreatic signaling that results in α-cell growth [14]-[16].

Both FOXN3 expression and glucagon receptor inactivation through GRA treatment promote insulin-independent increases in glucagon and α-cell proliferation [12], [23]. This functional similarity raises the possibility that liver FOXN3 recruits free amino acids to direct α-cell growth in the same way as GRA [14]-[16]. However, the degree to which GRA treatment intersects with FOXN3 regulation of glucose and amino acid utilization in a fasting state has not been explored.

Using a liver-specific Foxn3-deletion mouse model (Foxn3^{LKO}), we aim to clarify how liver Foxn3 regulates amino acid metabolism to promote α-cell proliferation in the pancreas. We
also seek to learn how losing liver Foxn3 can impact the effectiveness of GRA therapy. The absence of Foxn3 in the model will allow us to quantify changes in differential gene expression for protein targets involved in the catabolism and transport of amino acids in the liver, relative to a wildtype control. Foxn3<sup>LKO</sup> mice will be subject to extended treatment with antagonistic glucagon receptor antibody to determine the effect of GRA therapy on α-cell proliferation and liver amino acid profile. These studies broadly explore the role of inter-organ signaling between the liver and pancreas in establishing dysregulated glucagon secretion in DM1. The findings motivate exploring future regulatory nodes that might make GRA therapy a more practical long-term treatment for DM1.
Type 1 diabetes mellitus (DM1) is caused by the destruction of insulin-producing β-cells, resulting in a lack of glucose utilization in the body [1]. Logically, the first successful treatment aimed to subcutaneously inject patients with regular doses of insulin to restore vital glucose utilization and prevent the body from utilizing ketone bodies as a primary energy source, which causes dangerous blood acidification [1]. Initial pancreatic extracts were not pure and their effects included an initial spike in blood glucose, which corresponded to the first observation of pancreatic glucagon’s effects [31]. Glucagon was identified as a hormone responsible for increasing glucose supply by upregulating glycogen breakdown and gluconeogenesis in the liver [31]. It is secreted from pancreatic α-cells, which are immediately juxtaposed to β-cells in pancreatic islets. These cells’ proximity allows healthy insulin secretion to tightly regulate glucagon action and further prevent blood glucose from rising uncontrollably [32]. Since DM1 patients lack insulin secretion in the pancreas, this feedback is critically absent, leading to chronically elevated glucagon secretion [32]. Long-term glucagon secretion contributes to elevated blood glucose levels and is part of the reason a majority of DM1 patients fail to meet goals for satisfactory glycemic control [30]. Since the precise ratio of glucagon to insulin is nearly impossible to control by peripheral injections of insulin alone, alternative therapies have been proposed to minimize the effect of glucagon in DM1.

Recently, glucagon receptor antagonism (GRA) therapies have been explored in clinical trials as a supplement to standard insulin therapy in DM1 [3]. The principle is to introduce glucagon receptor-blocking monoclonal antibodies, which interfere with glucagon binding and action [21]. Short-term effects of the therapy are beneficial in lowering blood glucose in DM1 to alleviate hyperglycemia [3]. Additionally, DM1 patients see lower required insulin doses and improved blood glucose control [3]. Over longer periods of treatment, however, DM1 patients see increased expression of amino acid transporters in the liver, higher serum amino acid and serum glucagon levels [4], [5]. Here, a liver-α-cell axis is identified where amino acids can respond to glucagon secreted from the liver, promoting growth factors that trigger α-cell hyperplasia [22]. Importantly, further data shows that GRA reduced amino acid utilization in the liver, which in turn resulted in an increase in blood amino acids [23]. These increased amino acids are sensed as excess nutrients by a mechanistic target of rapamycin (mTOR) signaling complex in α-cells, which promotes further expansion of α-cells in the islet [24], [25]. It makes sense that blocking the action of glucagon would stimulate the body to produce more hormone by increasing the number of α-cells. The potential for dysregulated α-cell growth is one of the major barriers to implementing GRA as a pharmacological treatment for DM1. Therefore, a better model of how metabolic control in the liver influences α-cell growth in the pancreas is imperative to improving the safety of GRA treatments.

Understanding the role of the transcriptional repressor protein FOXN3 in the liver as a regulator of fasting blood glucose, may provide insight into pathways with effects similar to GRA. Through a genome-wide association study of non-diabetic patients, it was found that a single nucleotide variation rs8004664 was significantly and independently associated with fasting blood glucose [11]. Carriers of this variation showed greater expression of the transcriptional repressor protein FOXN3 in liver tissue and elevated fasting blood glucose [8], [11]. It had been shown that zebrafish and mouse embryos deficient for foxn3/Foxn3 were not viable due to craniofacial defects, posing a challenge in developing a loss-of-function model [17], [18]. When foxn3 transcript was suppressed in mature zebrafish, mutants showed decreased fasting blood glucose and glucagon levels, and fewer α-cells [11]. Conversely, when human FOXN3 transcript was
overexpressed, zebrafish showed increased fasting blood glucose and glucagon, and greater numbers of α-cells in the principal islet [11]. These studies identified the c-MYC gene, encoding a master transcriptional regulator of glucose utilization during fasting, as the primary target of FOXN3 [11]. Cellular (c)-MYC acts as a cancer-promoting gene that greatly increases glucose utilization in the liver [33]. Healthy levels of FOXN3 work to limit excess expression of c-MYC and prevent uncontrolled cell proliferation in the liver. As a result, FOXN3 acts independently of insulin to reduce glucose utilization in the liver and raise fasting blood glucose [12].

Further studies were carried out in liver cell culture and in vivo mouse liver to show that glucagon stimulates the proteasomal degradation of FOXN3 mRNA [12]. This reciprocal feedback with glucagon suggested that FOXN3 might form part of a pathway between the liver and pancreatic α-cells that controls glucagon secretion [19]. The relationship was explored in a mouse model where liver Foxn3 is decreased in mature mice via a liver-tropic virus encoding silencing RNAs for the gene [20]. Mice with downregulated Foxn3 characteristically had decreased fasting glucose and increased c-Myc expression, without changes in insulin or glucagon [20]. An important new observation was that amino acid transporters and catabolic enzymes involved in the utilization of amino acids were decreased significantly in the livers of these mice [20]. Therefore, it was implied that any proposed mechanism should incorporate an altered amino acid metabolism as one of the driving factors for α-cell growth and increased glucagon secretion.

Directly comparing independent models for the influence of liver FOXN3 and GRA therapy on increasing pancreatic α-cell growth alludes to their similarities. There is no evidence whether FOXN3 promotes the buildup of serum amino acids associated with GRA therapy, or whether it achieves the same effects by an alternate mechanism. Either way, the suggestion that FOXN3 action is functionally similar to GRA treatment is supported [12]. Consequently, it may be a viable target for future clinical studies seeking to ameliorate the detrimental long-term effects of GRA therapy for DM1.

Fig. 1. The left model identifies glucagon regulation in the absence of FOXN3. MYC is permitted to transcribe genes involved in healthy glucose utilization during fasting. Normal amino acid transferase and catabolic enzyme activity are noted in the liver, leading to healthy α-cell islets producing glucagon. The right model identifies glucose regulation in the presence of FOXN3. MYC is inhibited, leading to dysregulated glucose utilization during fasting. Amino acid transferase and enzymatic activity in the liver is increased to recruit free amino acids to pancreatic α-cells to stimulate glucagon production.
α-cell islets to promote hyperplasia and hyperglucagonemia. Glucagon works in negative feedback to degrade FOXN3.

The proposed mechanism (Fig. 1) incorporates information on the action of FOXN3 across multiple studies and implicates the utilization of amino acids as the missing element in the current literature. Conceptually, this model is supported by studies of interrupted signaling in glucagon-deficient animal models and GRA-based therapies [16]. Since these models propose hyperglucagonemia and α-cell hyperplasia modulated by altered amino acid metabolism in the liver, there is likely some intersection with FOXN3’s function [12]. While whole-body loss of FOXN3 expression is not possible, a model limited to the liver will be sufficient to examine this gene’s effects on increasing α-cell growth, specifically [12].
METHODS

A liver-specific Foxn3-deletion mouse model (Foxn3\textsuperscript{LKO}) was verified and investigated to determine how liver Foxn3 regulates amino acid metabolism to promote α-cell proliferation in the pancreas. Mature mice from this cohort were treated with antagonistic glucagon receptor antibody (GcgR mAb) over four weeks to simulate extended glucagon receptor antagonism (GRA) therapy. Alterations in α-cell proliferation and liver amino acid expression were monitored in the context of losing liver Foxn3.

A. Materials

Since Foxn3 is required for craniofacial development, a whole-body gene knockout mouse is not viable [18]. Mice with directional LoxP gene recognition sites flanking both sides of the essential exon 2 in the Foxn3 gene (Foxn3\textsuperscript{flox/flox}) were obtained from the European Mouse Mutant Archive (Munich, Germany). LoxP sequences are unlikely to occur in the mouse genome by chance and act as a binding site for Cre recombinase to excise the flanked gene from DNA. Breeding Foxn3\textsuperscript{flox/flox} mice with liver-specific Albumin-Cre mice (Comparative Medical Center, Salt Lake City, Utah) causes near-total loss of functional full-length Foxn3 mRNA in the livers of homozygous offspring (Foxn3 LKO). All mouse studies obtained advance approval from the Institutional Animal Care and Use Committee of the University of Utah.

Deletion of Foxn3 exon 2 was verified by isolating RNA from 0.5 mm mouse tail clippings using Thermo Fisher RNA Extraction Products (Waltham, Massachusetts) RNA concentration was quantified using UV-Vis spectrophotometry. Equivalent amounts of complementary DNA were generated and amplified for the isolated RNA using the reverse-transcriptase polymerase chain reaction (RT-PCR) from the Thermo Fisher cDNA Synthesis Kit. RNA primers for LoxP and Albumin-Cre genes were combined with generated cDNA to determine the relative expression of each gene via fluorescence detected in real-time quantitative PCR (qPCR). Threshold (Ct) values for fluorescence for each sample were normalized to \textit{ActB} expression in the liver, and fold change between groups was calculated using the \(\Delta\Delta\text{Ct} \) method [25]. Liver-specific knock-out Foxn3\textsuperscript{LKO} mice (N=8) expressed both genes, while the control group Foxn3\textsuperscript{loxp/loxp} mice (N=8) only expressed LoxP.

For further verification of liver-specific deletion, RNA was isolated from terminally-collected livers and subject to qPCR analysis for Foxn3 and the \textit{Myc} target of the translated protein Foxn3. Protein was isolated from liver samples and identified using gel electrophoresis-based Bio-Rad Laboratories’ Western blotting kit (Hercules, California) in conjunction with antibodies specific to Foxn3 and Myc proteins (Thermo Fisher, Waltham, Massachusetts). Foxn3/Foxn3 expression was verified against mouse intestine tissue control expressing a normal amount of both the protein and gene.

B. Foxn3\textsuperscript{LKO} Characterization

Metabolic function in Foxn3\textsuperscript{flox/flox} and Foxn3\textsuperscript{LKO} mouse livers was assessed through analysis of fasting blood glucose, dynamic endocrine tests, and serum glucagon and insulin concentration in 16-week-old mice. All blood glucose measurements are conducted on overnight (12 hour) fasted mice at least 10-weeks of age, drawing blood from the cut caudal vein with a Bayer Contour glucometer (Whippany, New Jersey), unless otherwise specified. During dynamic endocrine tests, serum glucose was measured following the intraperitoneal injection of body weight-dependent saline solutions of glucose (3mg/kg), insulin (0.05 U/kg), glucagon (0.1 mg/kg), pyruvate (2 mg/kg), and arginine (2 g/kg). All mice (N=16) included in the study were male and
fed normal chow diets. Blood glucose was measured at 0, 15, 30, 45, 60 and 120 minutes after injection with glucose, insulin, and pyruvate; blood glucose was measured at 0, 5, 10, 15 and 20 minutes after injection with glucagon and arginine. Approximately 50 µL of blood was collected at 0- and 15-minutes post-injection with glucose and arginine, and from all mice, once, following overnight fasting. Samples were centrifuged at 5000 rpm to isolate 10 µL blood serum for downstream quantification of serum insulin using the Insulin Ultra-Sensitive Assay Kit (cisbio, Codolet, France) or serum glucagon using the Glucagon Serum Kit (cisbio). Fasting levels of glucagon and insulin, as well as changes in their secretion during treatment with glucagon and arginine are effective indicators of α- and β-cell function [26].

Following the conclusion of the above studies, we fasted mice overnight and euthanized by respiratory exposure to 5% Isoflurane, USP (Akorn Animal Health, Lake Forest, Illinois) and cervical dislocation. We harvested pancreases, livers, and blood (by cardiac puncture in the ventricle) from Foxn3LKO and control mice during dissection. All samples were flash-frozen in liquid nitrogen and stored at -80°C, unless otherwise specified. Pancreatic tissue was used to quantify α- and β-cell proliferation by immunohistochemistry, while serum and liver tissues were assessed for changes in genetic expression or metabolic intermediates of amino acid metabolism.

Cut samples of pancreatic tissue (~0.1 g) were fixed in 4% formaldehyde and later transferred to a 70% ethanol solution. Samples were submitted to the ARUP Research Histology Lab (Salt Lake City, Utah) and embedded in optimal cutting temperature (OCT) compound before frozen sectioning on a cryomicrotome to a thickness of 3-30 µm. Non-adjacent sections were stained using anti-glucagon and anti-insulin hematoxylin counterstain antibodies (Thermo Fisher, Waltham, Massachusetts) as markers for α- and β-cells, respectively. Sections adhered to charged microscope slides, which were imaged using the ZEISS Axioscan 7 Microscope and ZEN 3.2 image-processing software (Oberkochen, Germany). We conducted three independent, unbiased counts of islet cells in all mice, reporting the mean count, normalized to the sample weight.

Intrahepatic concentrations of amino acids were determined to evaluate whether Foxn3 increases the uptake of amino acids in the liver. Gas chromatography-mass spectrometry (GC-MS) measurements from 100 mg of frozen liver samples were conducted by Metabolomics, Proteomics, and Mass Spectrometry Core at the University of Utah (Salt Lake City, Utah). Serum samples were assessed by liquid chromatography in tandem with mass spectrometry (LC-MS/MS) from 300 µL serum samples processed by ARUP Laboratories (Salt Lake City, Utah). Fold increases between amino acid concentration between fasting Foxn3LKO and control samples were measured and normalized to the sample mass in both cases. In conjuction, total RNA was extracted from Foxn3LKO and control mouse livers using Illumina TruSeq Stranded Total RNA Sample Prep Kit (San Diego, California) to prepare samples for bioinformatic analysis [27]. Differential gene expression, relative to the control group, was computed using DESeq2 software and reported as a mean fold-change between groups [28]. We were interested in increased expression of genes coding for enzymes and transporters involved in the catabolism and transport of glucose and amino acids in Foxn3LKO mice.

C. Glucagon Receptor Antagonism

The following study was conducted with a separate cohort of Foxn3LKO (N=8) and control Foxn3loxp/loxp (N=8) mice to determine whether prolonged treatment with glucagon receptor inhibitor antibody (GcgR mAb) increased α-cell proliferation in the absence of Foxn3. Foxn3LKO and control Foxn3loxp/loxp ten-week-old mature mice were treated with GcgR mAb-4 (Eli Lilly, Indianapolis, Indiana) at a dose of 10 mg/kg of body weight. Injections were administered intraperitoneally, once weekly, for four weeks. Dynamic endocrine tests were performed between
the fifth and seventh weeks, as described above, including all relevant blood collection for quantifying serum insulin and glucagon. After seven weeks, mice were euthanized, with liver and pancreas tissue harvested during dissection. Glucagon- and insulin-positive cell mass, corresponding to α- and β-cells in pancreatic tissue, was quantified as described previously.

D. Statistical Analysis

For measurements involving biological replicates, results are reported as mean ± standard error of the mean (SEM). Biological replicates are plotted as scatterplots with superimposed lines representing mean ± SEM. Based on mean ± SEM plasma glucagon in a prior study, we estimate that a sample size of 8-10 animals per control group, and per GcgR treatment group would provide 80% power to detect a 10% difference, assuming a type I error rate of 5% (α=0.05). At this appropriate power level, significance between non-normally distributed sample groups of mice is evaluated using a two-tailed Student’s t-test. Time series data are compared by evaluating area under the curve (plotted as a bar chart of mean ± SEM), as appropriate when no time points are missing data and variability (SEM) for each group is shown.
RESULTS

A. Liver-Specific Deletion of Foxn3

Novel liver-specific deletion of the mouse Foxn3 gene in this study needed verification to determine whether the appropriate relationship was investigated. We confirmed a significant reduction in Foxn3 mRNA transcript expression in the liver tissue of Foxn3LKO mice (Fig. 2), while wildtype livers showed expression similar to control intestine tissue where no change was observed (not pictured). Consequently, we infer the decreased function of the Foxn3 protein product in LKO liver tissue. This loss of function is evidenced by higher expression of the c-Myc mRNA, whose transcription Foxn3 otherwise restricts (Fig. 2B). Greater liver Foxn3 expression continues to be characterized by higher fasting blood glucose (Fig. 2C), remaining consistent with prior results of single nucleotide variation in the rs8004664 allele associated with FOXL3.

![Fig. 2](image_url)

**Fig. 2.** Liver-specific deletion of Foxn3 in mice is verified to decrease Foxn3 and c-Myc mRNA transcript abundance in the liver, causing a decrease in fasting blood glucose. qPCR identifies the abundance of [A] Foxn3 and its mechanistic target [B] c-Myc mRNA transcript extracted from liver tissue. Expression is normalized to β-actin (ActB) expression, found to be stable across all eukaryotic cell types. [C] Overnight fasting blood glucose of mice. Wildtype (Foxn3^flox/flox_) mice are shown in black (N=7) and Foxn3LKO mice in red (N=9). Data are shown as mean ± SEM with indicated significance for a two-tailed Student’s t-test: * is P<0.05, ** is P<0.01, NS is not significant at these levels.

B. Foxn3LKO Characterization

The project’s primary aim was to determine how disruption of Foxn3-regulation in the liver influences islet cell development and hormonal secretion in the pancreas. Foxn3LKO mice showed a significant decrease in both glucagon secretion (Fig. 3B) and α-cell islet density in the pancreas (Fig. 3D). Proliferation of β-cells was not implicated in Foxn3-influenced pancreatic cell growth. However, both cell types were quantified in the study to allow for unbiased α-cell counting that considers islet size in the mice. Foxn3LKO mice showed no difference in insulin secretion (Fig. 2A) but experienced significantly diminished β-cell islets (Fig. 2C), indicating proliferation of both islet cell types may occur in tandem.
Fig. 3. Liver-specific deletion of Foxn3 causes decreased fasting serum glucagon associated with a decrease in α-cell mass, while decreased β-cell mass produced no change in serum insulin. ELISA-based quantification of overnight fasting serum concentrations of [A] insulin and [B] glucagon in mature mice. [C] α-cells were quantified from glucagon-positive staining of non-adjacent sections of pancreatic tissue, with [D] β-cells quantified from insulin-positive staining of separate non-adjacent pancreatic sections. Wildtype (Foxn3\textsuperscript{flox/flox}) mice are shown in black (N=7) and Foxn3\textsuperscript{LKO} mice in red (N=9). Data are shown as mean ± SEM with indicated significance for a two-tailed Student’s t-test: * is P<0.05, ** is P<0.01.

The most significant finding was that these decreases in glucagon secretion and islet cell density occurred independently of an altered amino acid metabolism in the Foxn3-deficient liver. There was no change in the serum amino acid profile for LKO mice (Fig. 4A), suggesting that mTOR-dependent blood amino acid nutrient sensing does not reduce islet proliferation in these mice. mRNA transcript expression in liver tissue showed no significant change in amino acid transferase enzymes, involved in the movement of amino acids from the tissue (Fig. 4B). Additionally, there was no significant change in amino acid concentrations in the liver tissue itself (Fig. 4C). Downregulated in Glucokinase (Gck) mRNA expression and glucose-6-phosphate and fructose-6-phosphate metabolic intermediates in Foxn3\textsuperscript{LKO} mice suggests an increase in glycolysis and/or gluconeogenesis when Foxn3 is expressed in the liver (Fig. 4B-C). Increases in these metabolic processes would predict the increased fasting blood glucose seen in wildtype mice (Fig. 2C).
Fig. 4. There is no change in amino acid utilization during fasting in the livers of Foxn3LKO mice, with some downregulation of enzymes and intermediates of glucose metabolic pathways. [A] Amino acid concentrations in the serum of fasting wildtype (Foxn3^{flox/flox}) mice black (N=7) and Foxn3^{LKO} mice in red (N=9). Data are shown as mean ± SEM with no significance at P<0.05 for Student’s t-test. Negative decadic logarithm of significance value for Student’s t-test as a function
of the binary logarithm of fold change from Foxn3\textsuperscript{LKO} to wildtype mice for [B] liver gene expression (\textit{Gck} indicates glucokinase) and [C] liver metabolites (including amino acids, intermediates of glycolysis/gluconeogenesis, and fatty acid pathway intermediates). Significant values showing at least two-fold change are highlighted on the appropriate plot.

Significant results from dynamic endocrine testing show Foxn3\textsuperscript{LKO} mice having a delayed increase in blood glucose in response to glucose injection, and greater increase in blood glucose following arginine injection (Fig. 5A-D). Increasing pyruvate means to provide greater substrate for gluconeogenesis, which should increase measured blood glucose over time. There is no difference in pyruvate response (Fig. 5C) but this does not, by itself, indicate constant gluconeogenesis between groups due to the molecule’s various roles in metabolism. Arginine is an amino acid implicated in stimulating β-cell insulin production. Foxn3\textsuperscript{LKO} mice display higher levels of insulin secretion, but no significant change in secretion during the test duration (Fig. 5D-E). A higher-magnitude insulin response in Foxn3\textsuperscript{LKO} mice runs contrary to their significantly lesser number of β-cells (Fig. 3C) and contrary to their continuously elevated blood glucose over the test duration (Fig. 5D). Neither group experienced significantly different insulin secretion in response to glucagon injection (Fig. 5F). Healthy Foxn3\textsuperscript{LKO} mice experience a decreased sensitivity to insulin’s effects, but not glucagon.
**Fig. 5.** Liver-specific deletion of *Foxn3* blunts the increase in blood glucose following glucose injection and increases insulin secretion following β-cell stimulation with arginine injection. Change in blood glucose over time for intraperitoneal injection with [A] glucose (GTT), [B] insulin (ITT), [C] pyruvate (PTT), and [D] arginine (AST). Serum insulin concentration at [E] time 0- and 15-minutes post arginine injection and [F] time 0- and 30-minutes post glucagon injection. Wildtype (*Foxn3* ^{flox/flox}) mice are shown in black (N=7) and *Foxn3* ^{LKO} mice in red (N=9). Data are shown as mean ± SEM with indicated significance for a two-tailed Student’s t-test: * is P<0.05, ** is P<0.01.
C. Glucagon Receptor Antagonism

Assessment of the effectiveness of GcgR Ab-4 treatment in the context of Foxn3 liver deficiency depended on metrics related to increased proliferation of islet cells. In healthy Foxn3<sup>LKO</sup> mice, the numbers of both α- and β-cells were significantly decreased (Fig. 3C-D); however, there is no difference in cells between both islets following GRA (Fig. 6A-B). Islet counts suggest that decreased liver Foxn3 inhibits cell proliferation in the healthy control state, without GRA treatment. Blood glucagon values prior to GRA mirror the significant difference seen in healthy controls (Fig. 3A/5E), while the nonsignificant difference after treatment (Fig. 6F) suggests decreased liver Foxn3 promotes increased glucagon secretion. In both the pre- and post-treatment comparisons, there is no difference in the amount of serum insulin measured (Fig. 6C-D).
Fig. 6. Following extended treatment with GcgR Ab-4, there is no significant change in α- and β-cell mass and associated serum glucagon and insulin levels, when Foxn3 is deleted in the liver. ELISA-based quantification of overnight fasting serum concentrations of insulin [C] before and [D] after treatment with GcgR Ab-4, and glucagon [E] before and [F] after treatment with GcgR Ab-4. [A] α-cells were quantified from glucagon-positive staining of non-adjacent sections of

• Foxn3^{flox/flox}
• Foxn3^{LKO}
pancreatic tissue, with β-cells quantified from insulin-positive staining of separate non-adjacent pancreatic sections. Wildtype (Foxn3^flox/flox) mice are shown in black (N=7) and Foxn3^LKO mice in red (N=9). Data are shown as mean ± SEM with indicated significance for a two-tailed Student’s t-test: * is P<0.05, ** is P<0.01.

Dynamic endocrine testing for Foxn3^LKO mice was conducted to supplement data for islet quantification under GRA and remained consistent with most pre-treatment levels for blood glucose response (Fig. 7A-D). Overall, levels of blood glucagon prior, during, and after GRA treatment (Fig. 7F) support prior literature that glucagon receptor inactivation increases fasting blood glucose. Treatment with GcgR Ab-4 was successful in increasing the insulin response to glucagon injection in both LKO and wildtype mice (Fig. 7G). However, arginine injection only significantly increased wildtype insulin secretion (Fig. 7E), suggesting β-cell production of insulin in LKO mice may be impaired.
**Fig. 7.** Following extended treatment with GcgR Ab-4, Foxn3^{LKO} mice have decreased levels of serum glucagon and exhibit normal insulin secretion. Change in blood glucose over time for intraperitoneal injection with [A] glucose (GTT), [B] insulin (ITT), [C] pyruvate (PTT), and [D] arginine (AST). Serum insulin concentration at [E] time 0- and 15-minutes post arginine injection and [G] time 0- and 30-minutes post glucagon injection. [F] Reflects the change in fasting blood glucagon prior, immediately before and after GRA treatment. Wildtype (Foxn3^{floxflox}) mice are shown in black (N=7) and Foxn3^{LKO} mice in red (N=9). Data are shown as mean ± SEM with indicated significance for a two-tailed Student’s t-test: * is P<0.05, ** is P<0.01.
Elevated fasting blood glucose in type 1 diabetes mellitus (DM1) responds inefficiently to insulin injection, since a lack of pancreatic insulin signaling elevates blood glucagon, causing further glucose release from the liver [30]. Glucagon receptor antagonism (GRA) is a treatment aiming to minimize glucagon’s effects by blocking glucagon receptors in the liver, but may cause dysregulated α-cell proliferation [4], [5]. Similar α-cell growth was observed in animal models expressing the transcriptional repressor protein FOXN3 in the liver [11], [12]. We characterized mice with the liver-specific deletion of Foxn3 to determine whether the gene’s expression is necessary for α-cell development in the pancreas, and whether its deletion mitigates shortcomings of GRA therapies. As expected, Foxn3 expressed in the liver causes α-cell expansion (Fig. 3D) and decreases glucose utilization during fasting (Fig. 4), resulting in elevated blood glucose. Critically, Foxn3 is shown to act independently from GRA since its expression does not alter liver amino acid metabolism (Fig. 4), and losing Foxn3 in the liver does not inhibit α-cell growth resulting from GRA therapy (Fig. 6A). Continued investigation of liver FOXN3-mediated pancreatic α- and β-cell development may elucidate mechanisms of glucagon action when fasting, integral to the pathology of DM1.

Before evaluating the role of liver Foxn3 in regulating glucagon action in our mouse model, it was important to verify that a liver-specific deletion was consistent with the gene’s expected function. Liver tissue indicated decreased expression of Foxn3 (Fig. 2A) mRNA transcript with increased expression of Foxn3’s transcriptionally repressed target, c-Myc (Fig. 2B), in the liver-specific knockout mice (Foxn3LKO). c-Myc is a gene implicated in increasing glucose utilization in the liver [33], thereby decreasing fasting blood glucose. Augmented function of unrepressed c-Myc in Foxn3LKO mice is supported by their lower fasting blood glucose (Fig. 2C). Previous models of foxn3/Foxn3 suppression, in zebrafish and mice, attribute this decreased fasting blood glucose to diminished levels of serum glucagon and fewer α-cells in the pancreatic islets, without changes in insulin secretion [11], [20]. Accordingly, Foxn3LKO mice exhibit lower fasting levels of blood glucagon (Fig. 3B) and fewer α-cells in the pancreas (Fig. 3D), relative to Foxn3flox/flox (WT) controls. These data are coupled with no significant changes in fasting plasma insulin (Fig. 3A) to validate Foxn3LKO as model for describing the influence of liver Foxn3 on glucagon action. Diverging from prior findings, decreased numbers of β-cells in Foxn3LKO mice (Fig. 3C) were not anticipated, and their effect is not clearly examined in our studies. In response to intraperitoneal arginine injection in the arginine stimulation test (AST), Foxn3LKO mice have a higher sustained blood glucose (Fig. 5A), without a corresponding decrease in insulin secretion (Fig. 5E). This test was designed to stimulate β-cell insulin secretion, suggesting a persistently elevated blood glucose response may indicate diminished β-cell function. Meanwhile, Foxn3LKO mice have a blunted increase in blood glucose during the glucose tolerance test (GTT) (Fig. 5A), suggesting improved β-cell insulin secretion. Such conflicting outcomes can partially be explained by the presence of glucagon action as a confounding variable. Any change in blood glucose cannot be fully attributed to either glucagon or insulin, since both hormones’ secretion is tightly controlled by signaling in adjacent α- and β-cells [32]. Certainly, Foxn3LKO mice support that liver Foxn3 increases fasting blood glucose and serum glucagon, while promoting both α- and β-cell growth (Fig. 5).

Our larger goal, beyond validating Foxn3LKO mice as a model, is to determine whether liver Foxn3 recruits free amino acids to direct α-cell proliferation. Both mice deficient for glucagon receptors in the liver or treated with glucagon receptor-blocking antibodies exhibit excess glucagon and α-cell growth [14], [34]. By implying Foxn3 function follows a similar mechanism,
we expected to observe decreased levels of serum amino acids, decreased liver expression for genes involved in amino acid catabolism, and higher levels of amino acids remaining in livers for Foxn3LKO mice [14], [23], [24], [34]. For all these parameters, our results suggest liver Foxn3 does not alter liver amino acid metabolism to increase pancreatic islet growth in a manner similar to GRA. Comparisons of serum amino acids in Foxn3LKO mice to WT controls with expressed Foxn3 show no significant difference on terminally collected serum (Fig. 4A). Quantification of mRNA transcript in the liver shows no genes involved in amino acid catabolism or transport are significantly downregulated in Foxn3LKO mice (Fig. 4C). Likewise, a profile of liver metabolites shows no significant difference and change in amino acids or factors associated with the mechanistic target of rapamycin (mTOR) pathway leading to α-cell growth, between Foxn3LKO and WT mice (Fig. 4B). The only significant decreases observed in the Foxn3LKO mouse liver are concentrations of glucose-6-phosphate (G6P), fructose-6-phosphate (F6P) and expression of enzyme glucokinase (Gck) (Fig. 4). These changes are consistent with the role of augmented c-Myc in increasing glucose utilization in the liver via upregulation of glycolysis [10]. c-Myc action alone, however, does not justify the increase in pancreatic islet mass resulting from liver Foxn3 expression. We conclude liver Foxn3 drives increased fasting serum glucagon levels and pancreatic islet growth, without influencing amino acid utilization in the liver.

Since liver Foxn3 and GRA both significantly stimulate pancreatic α-cell growth, the second aim of our study determines whether losing liver Foxn3 can mitigate α-cell growth in GRA treatment. Prior to treatment with the glucagon receptor antibody (GcgR Ab4), the second cohort of Foxn3LKO mice also had decreased serum glucagon (Fig. 6E). Following treatment, there was no significant difference in serum glucagon, serum insulin, α- and β-cells between Foxn3LKO mice relative to WT (Fig. 6). These data suggest α-cell expansion in response to GRA persists for all mice, regardless of Foxn3 expression in the liver. Importantly, β-cell function continues to be normal for both groups during treatment with GcgR Ab-4. Mice show an almost identical insulin secretion response to reduce blood glucose during glucagon or arginine injection in GTT and ATT, respectively (Fig. 7). Liver Foxn3 expression does not appear to compound the natural increase in serum glucagon or α-cell growth occurring as a result of GcgR Ab4 treatment. Critically, deleting Foxn3 in the liver does not mitigate the harms of GRA treatment by minimizing α-cell growth in a non-diabetic mouse model.

A key limitation in our study design is that it attempts to characterize a novel Foxn3LKO mouse model, while concurrently determining the mechanism by which Foxn3 regulates α-cell growth. The GRA-inspired mechanism of increased amino acid export from the liver driving α-cell growth assumes Foxn3LKO mice are identical to prior animal models in the literature [11], [12], [20]. Novel observations in Foxn3LKO mice, such as decreased β-cells, shed some doubt on the proposed mechanism before any studies were conducted. Strong similarities in glucagon action between GRA and FOXN3 expression exist, but amino acid utilization is not the common factor leading to α-cell growth. In the context of the Foxn3LKO model, a stronger hypothesis might be to consider a mechanism by which liver Foxn3 drives α- to β-cell conversion. Studies have shown mature β-cells to dedifferentiate and later redifferentiate into α-cells based on cues from glucagon action in the pancreas [35], [36]. Similar limitations apply when interpreting results from Foxn3LKO mice treated with GcgR Ab-4; the study was likely confounded by changes in β-cell development concurrent with α-cell growth in GRA treatment. Critically, the study was also lacking a negative control group of mice that could distinguish the effects of GcgR Ab-4 injection alone. Two additional groups of Foxn3LKO and WT mice should have been included in the study and given a sham injection of saline over the study duration. Such a modification requires additional
experimental groups, but separates the effects of liver Foxn3 deletion from glucagon receptor inactivation.

Moving forward, a major change in the model for liver FOXN3 regulation of glucagon metabolism must be the incorporation of β-cell development observed in Foxn3LKO mice. An inducible diabetic Foxn3LKO mouse eliminates β-cells in mature mice, to limit insulin signaling in the pancreas [32]. Therefore, diabetic mice might clarify Foxn3’s direct influence on α-cells and glucagon secretion, alone, in the pancreatic islets. It remains unlikely, however, that amino acid buildup in serum will continue to be implicated as the underlying mechanism for α-cell growth in the new model. Studies catering to alternate mechanisms of α-cell growth, such as α-to-β-cell differentiation, might offer more valuable insight into how liver Foxn3 influences fasting glucagon action.

Another avenue to explore is the influence of diet on glucagon action in Foxn3LKO mice. Studies show that human FOXN3 mRNA transcript in transgenic zebrafish livers is regulated by nutrients available to the animal [11]. Feeding mice a high-fat, low nutrient diet may stimulate liver Foxn3 expression to increase glucagon secretion and islet growth to a lesser degree. Importantly, this relationship between low-nutrient diets, increased glucagon secretion, and loss of insulin sensitivity may provide insight in treating related type-2 diabetes mellitus [12].

These current studies demonstrate that liver Foxn3 significantly increases fasting blood glucose, glucagon, and pancreatic α- and β-cell mass, without altering amino acid utilization in the liver. Additionally, loss of Foxn3 in the liver during GRA does not decrease serum glucagon and α-cell growth, occurring as negative consequences of blocking glucagon action. The greatest progress from our study involves validating the Foxn3LKO mouse as a mostly consistent model for FOXN3 regulation of fasting blood glucose. Better understanding the role of β-cell development and alternate mechanisms of islet expansion are immediate goals that demonstrate the potential of novel Foxn3LKO mice. Diabetes is a disease fundamentally tied to dysregulated fasting blood glucose. Further elucidating the mechanistic role of FOXN3 in fasting blood glucose regulation may enhance its significance as an alternative therapeutic target in diabetes treatment and pathology.
REFERENCES


