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# Energy Homeostasis During Fasting and Fed States: Foxa2 Regulates Hepatic Amino Acid Uptake

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**ENERGY HOMEOSTASIS DURING FASTING AND FED STATES:**

**FOXA2 REGULATES HEPATIC AMINO ACID UPTAKE**

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by

Vivian M. Lee

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# ENERGY HOMEOSTASIS DURING FASTING AND FED STATES: FOXA2 REGULATES HEPATIC AMINO ACID UPTAKE

Vivian M. Lee, Ph.D.
The Rockefeller University 2007

## ABSTRACT

In MODY3 patients, as well in the experimental mouse model, the Tcf1 -/-mouse, there is a defect in arginine-induced insulin secretion.  In this thesis, we examined the role of arginine transporters in the insulin secreting cells in arginine-induced insulin secretion.  We first characterized arginine uptake by MIN6 cells as having a Km of 102.6µM and being partially Na-dependent and entirely Cl-dependent.  We then examined Tcf1 -/- pancreatic islets, which are defective for arginine-induced insulin secretion.  Using gene expression array analysis and semi-quantitative RT-PCR analysis on pancreatic islets from Tcf1 +/+ and Tcf1 -/- mice, we found eight arginine transporters expressed in the pancreatic islets with only two transporters, mNAT3 and CAT3, regulated by Tcf1 in the islets.  Using siRNA-mediated knockdown of both transporters, we found that these transporters are not required for arginine-induced insulin secretion in MIN6 cells.  We conclude that there is a high level of redundancy for arginine transport into insulin-secreting cells.

We also examined the regulation of mNAT3 in the liver.  The liver is the main site for gluconeogenesis during fasting, and has been shown to increase amino acid uptake during periods of low nutrient intake to increase its substrate

pool for glucose production. We found that mNAT3 is upregulated during fasting, and that this response is abolished with insulin. Using mice injected with adenovirus expressing either Foxa2 or GFP as a control, we found that Foxa2 mediates this insulin-sensitive increase in hepatic mNAT3 expression. Through electrophoretic mobility shift assays and chromatin immuno-precipitation experiments, we found that Foxa2 binds to promoter elements of the mNAT3 gene, Slc38a4. We also showed these promoter elements to be important for Foxa2 transactivation using luciferase reporter gene assays. In addition, with liver perfusion experiments using mice infected with adenovirus, we found increased arginine uptake by livers overexpressing Foxa2. Thus, we have identified Foxa2 as a mediator for increased hepatic amino acid uptake during fasting.

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I am deeply indebted to my mentor, Markus Stoffel. My years in his laboratory have been ones where I have learned the most about science and medicine, as well as the practice of both. It has been a privilege to work with someone whose genuine enthusiasm for science and the application of it I very much admire.

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I owe everything to my parents, Lou-Chuang and Li-her Lee, and my sister, Jane, for I cannot even imagine where I would be right now if it were not for their love, support, and inspiration by example, which I have enjoyed all my life. I would also like to thank Matteo Ruggiu, the consummate scientist, for being a constant reminder of all things great in my life.

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**CHAPTER 1**

**INTRODUCTION TO ISLET STUDIES**

*1.1 Introduction*

Insulin is a polypeptide hormone that coordinates energy fuel metabolism and generation in different tissues of the body. It is produced by the beta-cells of the islets of Langerhans, which are clusters of cells that make up 1% of the pancreas. Insulin is secreted by the beta-cells in response to increases in blood glucose, certain amino acids, and fatty acid levels. Thus, insulin secretion is a marker for the "fed" state; accordingly, its metabolic effects are anabolic, leading to synthesis of glycogen from glucose, triacylglycerols from fatty acids, and protein from amino acids. In a "fasted" state, insulin levels are low in the body. The body responds by maintaining glucose levels through glycogen breakdown and gluconeogenesis, as well as by generating ketone bodies through lipolysis and fatty acid oxidation, in order to maintain a steady source of energy for all the tissues in the body.

In this thesis, we examined the relationship between insulin and amino acid transport. As described before, amino acids stimulate the secretion of insulin. In fact, before the oral glucose tolerance test, insulin secretion defects were formerly diagnosed in the clinic by a test where a bolus of arginine was administered and the resulting levels of insulin in the blood were measured. Despite the fact that arginine-induced insulin secretion has been long-established

and used as a diagnostic tool in the clinic, little is known about the mechanism and the molecules involved. We endeavored to identify genes that may be involved in arginine-stimulated insulin secretion to gain a better understanding of the insulin secretion process. Given the current global epidemic of type 2 diabetes, which is characterized by insulin resistance and/or abnormal insulin secretion, we were interested to learn about alternative pathways leading to insulin secretion, in hopes that they may either shed light on the pathogenesis of diabetes or be manipulated for therapeutic purposes.

We also looked at the relationship of insulin signaling and amino acid transport in the liver in our study. As described before, when the body is in a "fasted" state, insulin levels are low. This triggers the body to increase glucose production, either through glycogen breakdown or by gluconeogenesis. The liver is the site of 90% of gluconeogenesis in the body. Amino acids are used as substrates for gluconeogenesis. Thus, to address the increase in amino acid consumption for gluconeogenesis, the liver needs to increase the uptake of amino acids during periods of fasting. The molecular mechanisms linking insulin action to hepatic amino acid transport were examined in this thesis, and are described later in Chapters 4 through 6.

*1.2  Diabetes mellitus*

Diabetes is an endocrine disorder in which there is a deficiency in insulin action. There are two main forms of diabetes: type 1 diabetes is characterized by

an absolute insulin deficiency due primarily to an autoimmune-mediated destruction of pancreatic beta-cells, while type 2 diabetes, which comprises about 90% of diabetes cases, is characterized by insulin resistance and/or abnormal insulin secretion (Zimmet et al., 2001). The number of people worldwide with either type of diabetes was estimated at 150 million in 2001. The number of affect people was also predicted to increase to 220 million in 2010 and 300 million in 2025. The increase in the prevalence of diabetes is mostly due to an epidemic of type 2 diabetes, which is strongly associated with a sedentary lifestyle and obesity (Zimmet, 1999). Sharp rises in the incidence of type 2 diabetes have been observed in both developed and developing countries (Zimmet, 1999).

People with diabetes are susceptible to a variety of complications that cause morbidity and premature death, including accelerated atherosclerosis, retinopathy, nephropathy, neuropathy, and foot ulcers. In fact, diabetic retinopathy is the leading cause of blindness in the United States; in addition, diabetic nephropathy is the leading cause of end-stage renal disease in the U.S. Because of the morbidity and mortality associated with diabetes, a lot of focus has been placed prevention of complications. In a study performed by the Diabetes Control and Complications Trial Research Group in 1993, it was established that intensive control of blood glucose levels was useful in the prevention of the retinopathy, neuropathy, and nephropathy associated with diabetes.

In addition to the treatment of diabetes, much attention has also been placed on the prevention of diabetes. Many studies have been directed at identifying people who are at an increased risk of developing type 2 diabetes. Most of these studies have focused on individuals with impaired glucose tolerance (IGT). Subjects are given an oral glucose load, and blood glucose levels are determined two hours following the glucose administration. Individuals with IGT have blood glucose levels intermediate between normal and diabetic individuals. In addition, people with increased fasting glucose levels have also been shown to have a greater risk of developing type 2 diabetes.

*1.3 Maturity-onset diabetes of the young (MODY)*

The understanding of the progression of diabetes also requires an understanding of the mechanisms that control insulin secretion and mediate insulin actions. In the study of insulin secretion, animal model systems become especially important since it is difficult to take pancreatic islet biopsies from patients to study insulin secretion. In order to understand the changes that occur in diabetes, diabetic animal models are also important for comparison. Type 2 diabetes is generally a multifactorial disease, associated with ethnicity and gender, as well as advanced age, making it difficult to model in an animal system (Groop, 1997). However, there also exists a form of diabetes that is caused by mutations in a single gene, called maturity-onset diabetes of the young (MODY). Since diabetes is caused by a single gene mutation in MODY, the MODY genes

have been used as targets for genetic mouse manipulations to generate animal models of diabetes.

MODY accounts for 1-2% of all diabetes cases, and is characterized by development of diabetes early in life (often before the age of 25) and autosomal dominant inheritance. So far, six genes have been identified, which lead to MODY when mutated (Table 1): hepatocyte nuclear factor 1α (HNF1α, TCF1), hepatocyte nuclear factor 1β (HNF1β, TCF2), hepatocyte nuclear factor 4 (HNF4), pancreatic and duodenal homeobox gene 1 (PDX1), neuroD1, and glucokinase (Frayling et al., 2001; Fajans et al., 2001). The glucokinase gene encodes an enzyme that catalyzes the first step in glycolysis, which is essential for generating the metabolic signal to stimulate insulin secretion in response to glucose (Matchinsky, 2000). All of the other MODY genes encode transcription factors that are important in multiple metabolic pathways (Shih and Stoffel, 2002).

Table 1. MODY-related genes and the clinical phenotypes associated with their mutation.

| MODY Type | Gene | Product function | % of MODY | Clinical feature |
|---|---|---|---|---|
| MODY1 | HNF4α | transcription factor | 3 | diabetes; microvascular complications; decreased serum levels of triglycerides, apolipoproteins AII and CIII, and Lp(a) lipoprotein |
| MODY2 | GCK | glucose sensing | 14 | impaired glucose tolerance, diabetes |
| MODY3 | TCF1 | transcription factor | 69 | diabetes; microvascular complications; renal glycosuria; increased sensitivity to sulfonylurea drugs |
| MODY4 | PDX1 | transcription factor | < 1 | diabetes |
| MODY5 | TCF2 | transcription factor | 3 | diabetes; renal cysts and other abnormalities of renal development; progressive non-diabetic renal dysfunction; genital abnormalities in (females) |
| MODY6 | NEUROD1 | transcription factor | < 1 | diabetes (severity unknown) |
| MODY X | unknown | unknown | 11 | |

Studies of the MODY genes and their function in animal models have uncovered many diverse processes that are essential for proper maintenance levels. These processes range from mechanisms by which pancreatic beta-cells detect glucose, to neuroendocrine tissue development during embryogenesis, to beta-cell hyperplasia as an adaptive response to maintain normal glucose levels during physiological growth or in obesity (Matchinsky, 2005; Sander and German, 1997; Akpinar et al. 2005).

*1.4 MODY3 and TCF1*

Maturity-onset diabetes of the young type 3 (MODY3) is the most common of all the MODYs, accounting for 70% of all MODY cases. MODY3 is caused by mutations in the TCF1 gene. TCF1 encodes a homeodomain transcription factor composed of an amino-terminal dimerization domain, a POU-homeobox DNA-binding domain, and a carboxy-terminal transactivation domain (Mendel and Crabtree, 1991). Tcf1 is expressed in the liver, kidney, intestines, and pancreatic islets (Blumenfeld et al., 1991). There is no defect in insulin action in MODY3 patients; thus, impaired beta-cell function appears to be the primary cause of diabetes in these patients (Fajans et al., 2001). MODY3 patients demonstrate a defect in glucose- and arginine-induced insulin secretion as well as hypersensitivity to sulfonylurea therapy. In addition, MODY3 patients may also have glycosuria, resulting from decreased renal reabsorption of glucose (Menzel et al., 1998; Pontoglio et al., 2000). Depending on glycemic control, these

patients may suffer the full spectrum of diabetic complications, including blindness and kidney failure (Fajans et al., 2001).

Tcf1 has been genetically knocked out in mice (Pontoglio et al., 1996; Lee et al., 1998). Unlike in humans, where a single defective Tcf1 allele leads to disease, the Tcf1 +/- mice have no detectable phenotype. Tcf1 -/- mice have been shown to have a phenotype of defective glucose- and arginine-induced insulin secretion and hypersensitivity to sulfonylureas, as well as dwarfism, renal Fanconi-like syndrome, and defects in bile acid and plasma HDL-cholesterol metabolism (Pontoglio et al., 1996; Lee et al., 1998; Pontoglio et al., 1998; Shih et al., 2001). When normalized to pancreatic weight, the insulin content of the Tcf1 -/- mice was 60% that of wild-type littermates (Pontoglio et al., 1998). However, insulin secretion in response to arginine by Tcf1 -/- islets was about 13% of the insulin response found in Tcf1 +/+ islets; a similar decrease in insulin secretion by Tcf1 -/- was found with glucose stimulation (Pontoglio et al., 1998). Thus, decreased insulin content cannot account for the decrease in insulin secretion by the Tcf1 -/- islet. Other parts of the insulin secretion pathway need to be examined.


*1.5 Insulin secretion mechanisms*

Insulin secretion by pancreatic beta-cells is a physiologic response to changes in their environment (for example, changes in the extracellular glucose concentration), that has been adapted to maintain fuel homeostasis in the entire

organism. To stimulate insulin secretion, glucose is first taken up and metabolized by the pancreatic beta-cell. Glycolysis leads to an increase in the ATP/ADP ratio that leads to closure of ATP-sensitive potassium channels. The ATP-sensitive potassium channels are normally open for the positively charged potassium ions to leave the cell, following the electrochemical gradient. Upon their closure, potassium is sequestered inside the cell, which leads to depolarization of the cell membrane. Calcium is then released into the cytoplasm via voltage-dependent calcium channels, which in turn leads to extracellular release of insulin from the insulin-containing granules of the cell.

In addition, the metabolism of glucose leads to an increase in cAMP, from the cyclization of the generated ATP by adenylate cyclases. This increase in cAMP leads to activation of protein kinase A (PKA). PKA inactivates ATP-sensitive potassium channels, leading to increased membrane depolarization. In addition, both PKA and cAMP-regulated guanine nucleotide exchange factor II (cAMP-GEFII) increase mobilization of calcium from intracellular stores, which further increases the release of insulin granules.

The gastrointestinal tract also secretes hormones as a physiologic response to changes in food intake, which allow the body to adapt to maintain fuel homeostasis. The glucose-dependent insulinotropic polypeptide (GIP) and the glucagon-like peptide 1 (GLP-1) have been the most studied of these gut-derived peptide hormones. These peptide hormones which cause an augmentation of the

insulin secretion response after oral glucose intake compared with intravenous glucose intake are termed incretin hormones.

GIP is a peptide secreted from K-cells that are found in the small intestinal mucosa, mostly in the duodenum (Mortensen et al., 2003). GIP plasma concentration is increased 10- to 20-fold following meal ingestion, with secretion stimulated by carbohydrates and lipids. Interaction between GIP and its receptor on islet cells, a type II G protein-coupled receptor, causes an increase in cAMP levels that lead to increased insulin secretion via the mechanisms described above.

GLP-1 is secreted by L-cells of the intestinal mucosa. Binding of GLP-1 to the GLP-1 receptor, a member of the same family as the GIP receptor, causes activation of adenylate cyclase resulting in an increase in cAMP formation, as with GIP (Mayo et al., 2003; Holz, 2004). GLP-1 also stimulates gene transcription and biosynthesis of insulin (Fehmann and Habener, 1992), as well as transcription of other genes involved in insulin secretion, such as glucokinase and GLUT2 (Buteau et al., 1999). In addition, GLP-1 has been shown to stimulate beta-cell proliferation and inhibit beta-cell apoptosis (Stoffers et al., 2000; Xu et al., 1999; Farilla et al., 2003; Li et al., 2003). Studies with a GLP-1 receptor antagonist suggest that GLP-1 plays a significant role in controlling post-prandial glucose levels with oral glucose intake in rats (Kolligs et al., 1995; Wang et al., 1995), as well as in humans (Edwards et al., 1999).

Amino acids also enhance insulin secretion from pancreatic beta-cells. In particular, L-leucine, L-glutamine, L-alanine, and L-arginine have been studied for their insulinotropic effects. Leucine is transaminated to alpha-ketoisocaproate and enters the TCA cycle via acetyl-CoA, thus increasing ATP production (Panten et al., 1972). In addition, leucine increases mitochondrial metabolism by allosterically activating glutamate dehydrogenase (GDH) in the islets (Sener and Malaisse, 1980; Gylfe, 1976). In contrast, glutamine alone does not stimulate insulin secretion or potentiate insulin secretion induced by glucose (Sener et al., 1982); however, it enhances the insulin secretion induced by leucine (Sener and Malaisse, 1980). It is believed that this is due to the allosteric activation of GDH by leucine, which increases entry of glutamine into the TCA cycle. Alanine is also believed to stimulate insulin secretion through metabolism and entry into the TCA cycle (Brennan et al., 2002). In addition, studies in insulin-secreting cell lines suggest that alanine can also stimulate insulin secretion electrogenically, due to co-transport with $Na^+$ leading to membrane depolarization (Dunne et al., 1990; McClenaghan et al., 1998).

Although arginine can be metabolized into components of the glycolytic pathway, the mechanism of arginine-induced insulin secretion does not rely on metabolism of arginine since non-metabolizable analogs of arginine can also stimulate insulin secretion (Smith et al., 1997). Arginine-induced insulin secretion also is not related to the generation of nitric oxide, although arginine is also a substrate for nitric oxide synthase, as the nitric oxide synthase inhibitor, L-

NMMA, does not inhibit insulin secretion (Smith et al., 1997; Henningsson and Lundquist, 1998). Instead, it is believed that arginine stimulates insulin secretion by directly depolarizing the cell through its entry, since it is a basic amino acid carrying a positive charge (Figure 1). In agreement with this theory, it has been shown that arginine cannot further stimulate insulin secretion in maximally depolarized islet cells. Furthermore, arginine analogs that still carry a positive charge, such as the nitric oxide inhibitor L-NMMA, can also stimulate insulin secretion (Smith et al., 1997; Sener et al., 2000).
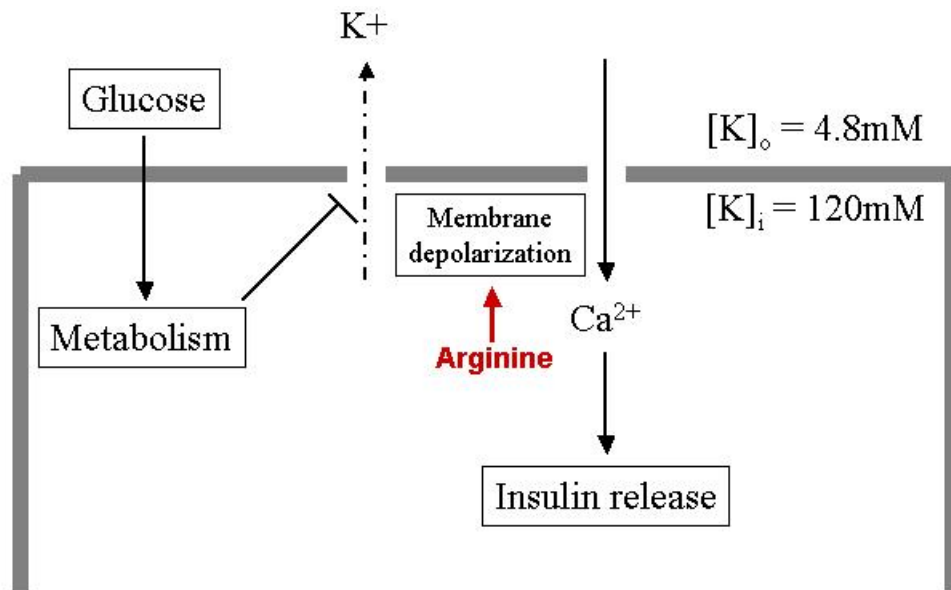


Figure 1. Schematic diagram of the insulin secretion pathway. Glucose enters the cell and is metabolized. Metabolic products of glucose close membrane potassium channels, leading to decreased efflux of potassium. The resulting membrane depolarization opens calcium channels that allow calcium to enter the cytoplasm. The increase in intracellular calcium triggers insulin release from intracellular granules. Arginine is believed to stimulate insulin secretion by membrane depolarization upon entry into the cell.

*1.6  Insulin secretion defects in the Tcf1 -/- pancreatic islets*

As described before, Tcf1 -/- mice have a defect in glucose-induced insulin secretion.  Through whole-cell voltage clamp experiments, it was found that the ability of glucose to induce closure of ATP-dependent potassium channels is significantly reduced in Tcf1 -/- beta-cells compared to Tcf1 +/+ and +/- beta-cells (Dukes et al., 1998).  In contrast, the ability for membrane depolarization to trigger calcium influx is intact in the Tcf1 -/- islets (Pontoglio et al., 1998).  Thus, the defect in Tcf1 -/- insulin secretion pathway lies upstream of membrane depolarization.

Tcf1 -/- mice also have a defect in arginine-induced insulin secretion.  In contrast to glucose, which depolarizes the beta-cell via closure of ATP-dependent potassium channels, arginine is believed to depolarize the beta-cell itself upon entry.  Since the insulin secretion pathway downstream of membrane depolarization is intact in Tcf1 -/- beta-cells, the defect in arginine-induced insulin secretion may be due to a decrease in arginine uptake.  Thus, the study of arginine transporters and their regulation by Tcf1 may be the key to identifying the molecular players involved in arginine-induced insulin secretion and understanding the Tcf1 -/- beta-cell pathology.


*1.7  Arginine transporters*

Arginine transport into cells can be mediated by four established classes of cationic amino acid transporters: system y+, system y+L, system b0+, and

system B0+ (Deves and Boyd, 1998). These classes have been defined based on ion dependency, substrate specificity, and relative affinity. In addition to these four systems, there is also a member of system N that has is reported to transport arginine; however, arginine transport by this transporter has not been well-characterized. The general characteristics of each family is outlined in Table 2.

Table 2. Systems of amino acid transporters that mediate arginine transport.

| Class | Gene | Activity | Tissue distribution | Link to disease |
|-------|------|----------|---------------------|-----------------|
| System y+ | SLC7A1 | Na-independent cationic transport | ubiquitous except for liver | |
| | SLC7A2 | Na-independent cationic transport | liver, muscle, pancreas | |
| | SLC7A3 | Na-independent cationic transport | thymus, ovary, testis, brain | |
| | SLC7A4 | unknown | brain, testis | |
| System y+L | SLC7A6/SLC3A2 | Na-independent cationic transport; Na-dependent neural transport | brain, small intestine, testis, heart, kidney, lung, liver | |
| | SLC7A7/SLC3A2 | Na-independent cationic transport; Na-dependent neural transport | small intestine, kidney, lung | lysinuric protein intolerance (LPI) |
| System b0+ | SLC7A9/SLC3A1 | Na-independent cationic and neutral transport | small intestine, kidney, pancreas | type B cystinuria |
| System B0+ | SLC6A14 | Na- and Cl-dependent cationic and neutral transport | lung, stomach, intestine (low) | |
| System N | SLC38A4 | unknown | liver | |

System y+ is made up of four members: CAT1, CAT2, CAT3, and CAT4, which are encoded by Slc7a1, Slc7a2, Slc7a3, and Slc7a4, respectively. In contrast to members of the other classes, these transporters are selective for cationic amino acids only (Verrey et al., 2004). Each transporter has 14 putative transmembrane domains and is glycosylated. Cationic amino acid transport mediated by these transporters is Na-independent. Within this family, CAT1, CAT2, and CAT3 are more closely related to each other (~60% identity) than to

CAT4 (~40% identity) (Verrey et al., 2004). The function of CAT4 has been under debate: while it was first characterized as being able to mediate cationic amino acid transport (Sperandeo et al., 1998), other groups have not been able to detect this activity (Wolf et al., 2002).

System y+L activity is mediated by heterodimeric transporters, comprised of a heavy chain subunit, 4F2hc, encoded by Slc3a2, linked via disulfide bonds to a light chain subunit, either y+LAT-2 or y+LAT-1, encoded by Slc7a6 and Slc7a7, respectively. The light chain subunits contain 12 putative membrane-spanning domains and are not glycosylated. The heavy chain subunit, 4F2hc, is a single membrane-spanning glycoprotein, and is required for surface expression of the light chains (Wagner et al., 2001). These transporters mediate Na-independent transport of cationic amino acids, as well as the Na-dependent uptake of neutral amino acids (Deves et al., 1992; Torrents et al., 1998). While the physiological role of y+LAT-2 is not clear at present, mutations in y+LAT-1 have been found to be the underlying cause of the hereditary lysinuric protein intolerance disease (Shoji et al., 2002; Sperandeo et al., 2005).

System b0+ activity is mediated through a heterodimer of b0+AT (encoded by Slc7a9) and rBAT (encoded by Slc3a1). It is characterized by Na-independent transport of cationic and neutral amino acids. The heterodimer is primarily expressed in the small intestine and the kidney, where it is localized to the basolateral membrane, as well as in the pancreas (Bertran et al., 1993). Like y+LAT-1 and y+LAT-2, b0+AT has 12 putative transmembrane domains. rBAT,

similar to 4F2hc, has only one transmembrane domain and is glycosylated. Mutations in rBAT have been linked to cystinuria, a hereditary disease that involves defective transepithelial transport of cystine and dibasic amino acids in the kidney and intestine (Calonge et al., 1994).

System B0+ activity is mediated by ATB0+ (encoded by Slc6a14), a member of the neurotransmitter transporter family, and is characterized by Na- and Cl-dependent cationic and neutral amino acid transport. It has been shown that the SLC6A14 is associated with obesity through genetic screening of obese Finnish families (Suviolahti et al., 2003). It was hypothesized that the transporter may be important for increasing the availability of tryptophan, which is used to synthesize serotonin, a neurotransmitter that increases satiety. This would be due either to an increase of amino acid absorption from the intestinal tract, or to increased uptake by target cells. In addition, ATB0+ is believed to play a key role in transepithelial transport of arginine in airway cells, which is important for providing a substrate for nitric oxide synthase to airway cells, as well as regulating the amino acid concentration in airway surface fluids (Galietta et al., 1998; Rotoli et al., 2005).

mNAT3 (encoded by Slc38a4) was first characterized by Sugawara et al. as an amino acid transporter expressed mainly in the liver. The transporter was named ATA3, since they had classified it originally as a system A amino acid transporter (2000). The group further characterized the transporter as mediating both neutral and cationic amino acid transport, with a higher affinity for cationic

amino acids (Hatanaka et al., 2001). However, uptake at different doses and kinetic studies of cationic amino acid transport was not performed in these studies. Slc38a4 was later classified as a system N amino acid transporter, due to its inability to transport methylaminoisobutyric acid, which is a characteristic of the system A transporters. In addition, in these later studies there was no detectable transport of cationic amino acids by mNAT3 (Gu et al., 2003). Thus, the ability of mNAT3 to mediate cationic transport is unclear.

*1.8 Experimental study*

We were interested in understanding the mechanism of arginine-induced insulin secretion and the molecules involved. Tcf1 -/- mice provided us with a unique model to identify molecules that are involved in arginine uptake by pancreatic beta-cells. Tcf1 -/- mice have a defect in arginine-induced insulin secretion and functional analysis of the Tcf1 -/- beta-cells have demonstrated that this defect is upstream of membrane depolarization. Since arginine is believed to stimulate insulin secretion by direct depolarization of the beta-cell upon uptake, a defect in arginine-induced insulin secretion may be cause by decreased arginine uptake.

We hypothesized that the defective arginine-induced insulin secretion in Tcf1 -/- mice is due to a defect in arginine uptake. In this thesis, we sought to identify the transporters that may be involved in uptake of arginine by beta-cells. We characterized arginine uptake by insulin secreting cells, as well as arginine

transport by the Slc38a4 gene product, mNAT3, which had not been well-characterized but was believed to mediate cationic amino acid transport. In addition, we examined the expression and regulation by Tcf1 of arginine transporters in insulin-secreting cells through both gene expression arrays analysis and RT-PCR screening.

**CHAPTER 2 -- RESULTS PART I**

*2.1  Characterization of arginine uptake by MIN6 cells*

We hypothesized that cellular entry of arginine into the pancreatic islet cell and the subsequent membrane depolarization were sufficient to stimulate insulin secretion.  Since arginine transport is the key step in arginine-induced insulin secretion, we first sought to characterize arginine uptake by insulin-secreting cells.  Radioactive ligand uptake assays were performed on monolayers of mouse insulinoma cells (MIN6).

*2.1.1  Timecourse of arginine uptake by MIN6 cells*

We assayed the amount of arginine uptake by MIN6 cells over ten minutes using uptake buffer containing 10μM arginine.  Uptake of $^3$H-arginine in Tyrode's solution was found to be linear over four minutes (Figure 2). Thereafter, arginine uptake assays were measured after two minutes.

Figure 2. Timecourse for arginine uptake by MIN6 cells. Uptake was initiated by the addition of H3 arginine to MIN6 monolayers at 37°C and measured over 10 minutes in Tyrode's solution. Data are mean ± SE, n=6.

### 2.1.2 *Dose-dependence of arginine uptake by MIN6 cells*

We assayed ^{3}H-arginine uptake with arginine concentrations ranging from 10nM to 20mM in the uptake buffer. Uptake was measured after two minutes. We found that MIN6 cells take up arginine in a dose-dependent manner (Figure 3).
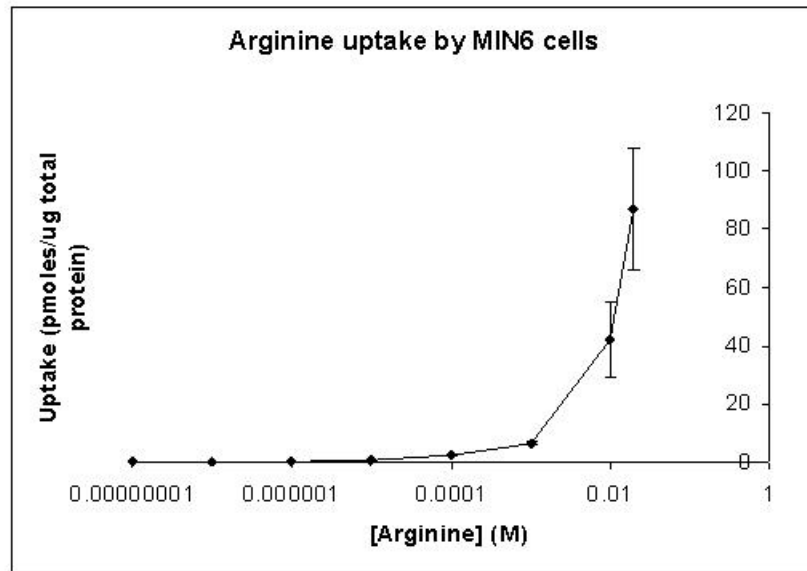
Figure 3. Dose-dependent uptake of arginine by MIN6 cells. MIN6 monolayers were incubated with varying concentrations of arginine in Tyrode's solution for two minutes. Data are mean ± SE, n=6.

### 2.1.3  Kinetics of arginine uptake by MIN6 cells

To determine the Michaelis-Menten constant, Km, and the maximum velocity, Vmax, of arginine uptake by MIN6 cells, we measured the uptake of arginine after two minutes with varying concentrations of arginine. Inverse velocity was plotted against inverse arginine concentration to obtain a Lineweaver-Burk plot (Figure 4). The inverse y-intercept, taken as Vmax, was 0.154 pmoles/µg total protein per second. By multiplying the Vmax to the slope of the line, we calculated the Km of MIN6 arginine uptake to be 102.6µM.

20
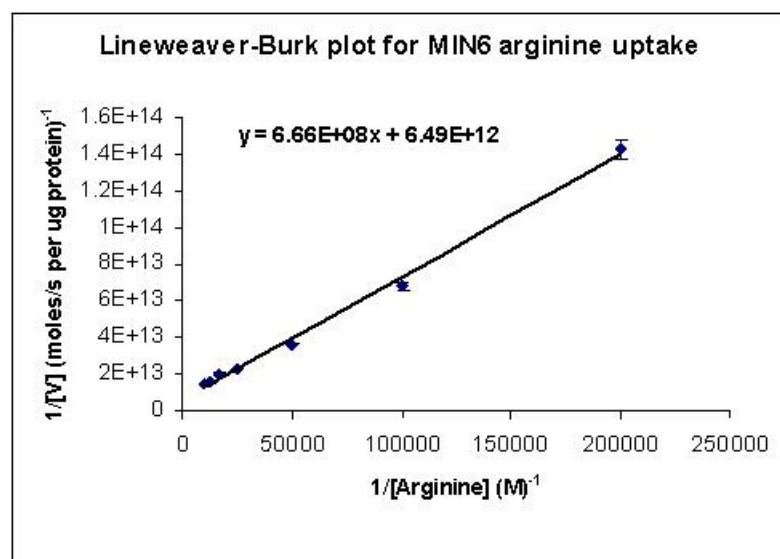
Figure 4. Lineweaver-Burk plot for MIN6 arginine uptake. MIN6 monolayers were incubated with varying concentrations of arginine in Tyrode's solution for two minutes. Inverse velocity was plotted vs. inverse arginine concentration. Data are mean ± SE, n=3.

### 2.1.4 *Effect of ion substitution on arginine uptake by MIN6 cells*

In addition to determining the kinetics of arginine uptake by MIN6 cells, we wanted to further characterize uptake based on Na- and Cl-dependence. To address this, we modified the uptake buffer to be either Na- or Cl-free and determined the effect on arginine uptake. For the Na-free experimental buffer, we replaced NaCl with choline chloride and used KOH instead of NaOH to adjust the pH. For the Cl-free experimental buffer, we replaced Cl with gluconate for NaCl, KCl, and $CaCl_2$; $MgCl_2$ was replaced with $MgSO_4$. We found a 37% decrease in arginine uptake with the Na-free buffer, while substitution of

Cl lead to no significant change in uptake (p>0.05 by student's t-test). Thus, arginine uptake by MIN6 cells is partly Na-dependent and is Cl-independent.
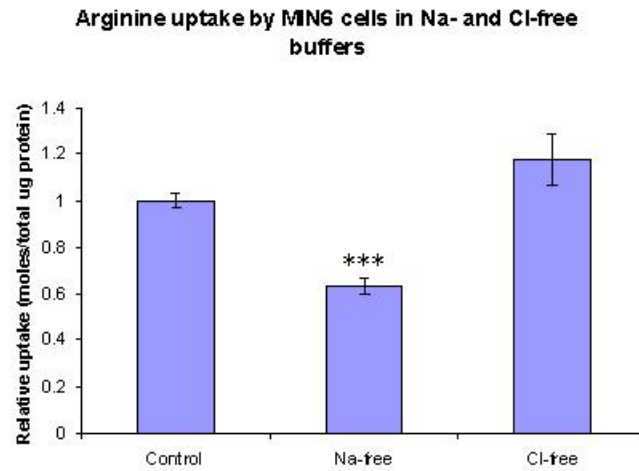


Figure 5. Na- and Cl-dependence of arginine uptake by MIN6 cells. Radioactive ligand uptake assays were performed in Na-free and Cl-free Tyrode's solution to determine the effect of ion substitution on arginine uptake by MIN6 cells. Data are mean ± SE, n=6. ***: p<0.001 by Student's t-test.

## 2.2 Gene expression array analysis of Tcf1 +/+ and Tcf1 -/- pancreatic islets

Since arginine-induced insulin secretion depends on the depolarization upon cellular entry, we hypothesized that the arginine-induced insulin secretion defect in the Tcf1 -/- mice may be due to a defect in arginine uptake. Thus, by studying the transport of arginine into insulin-secreting cells and the transporters that mediate it, we may better understand the molecular basis of this phenotype. To identify any factors that may contribute to the defect in insulin secretion in the Tcf1 -/- mice, we analyzed gene expression in pancreatic islets

using Affymetrix oligonucleotide expression probe arrays, MG-U74A, MG-U74B, and MG-U74C.

Two RNA samples were generated from pancreatic islets of either Tcf1 +/+ or Tcf1 -/- mice.  The RNA from the islets of roughly fifty mice was pooled to generate each RNA sample.  Two probe sets were made from each RNA sample, and each probe was analyzed on separate chips.  Gene expression analysis values differed by less than 2% for the two probe sets generated from the Tcf1 +/+ RNA, and by less than 10% for the two probes generated from the Tcf1 -/- RNA, verifying consistency in probe generation and in gene chip hybridization.  Comparison of gene expression levels between Tcf +/+ and -/- samples were performed using Microarray Suite 4.0.

Since Tcf1 is known to be a transcriptional activator, only genes that were expressed at higher levels in Tcf1 +/+ islets were considered as potential direct targets of Tcf1.  We identified the amino acid transporter gene, Slc38a4, which encodes mNAT3, as a potential target of Tcf1 through the gene chip screen with approximately 15.8-fold increased expression in the Tcf1 +/+ samples compared to Tcf1 -/- samples (Table 3).  Five other genes of arginine transporters were represented on the gene chips: Slc7a1, Slc7a2, Slc7a3, Slc7a7, and Slc7a9.  All of these other transporters represented on the microarray showed too low levels of expression to demonstrate a difference in expression levels between Tcf1 +/+ and Tcf1 -/- samples upon data analysis, except for Slc7a7, which had "decreased" levels in the Tcf1 +/+ samples.

Table 3. Gene expression array results for arginine transporters in Tcf1 +/+ and Tcf1 -/- pancreatic islets. Data were analyzed using Microarray Suite 4.0.

| GenBank | Gene | Tcf1 -/- | Abs Call | Tcf1 +/+ | Abs Call | Difference | Fold Change |
|---------|------|----------|----------|----------|----------|------------|-------------|
| M26687 | Slc7a1 | -63.4 | Absent | -22.9 | Absent | no change | n/a |
| L03290 | Slc7a2 | -45.2 | Absent | 137 | Marginal | no change | n/a |
| U70859 | Slc7a3 | -337.5 | Absent | -256.2 | Absent | no change | n/a |
| AJ012754 | Slc7a7 | 171.2 | Present | 8.2 | Absent | decreased | ~-8.2 |
| AJ249198 | Slc7a9 | 55.6 | Absent | -26.9 | Absent | no change | n/a |
| AI648998 | Slc38a4 | 54.8 | Absent | 1302.3 | Present | increased | ~15.8 |

## 2.3  Characterization of arginine transport by Slc38a4

The Slc38a4 gene product, mNAT3, was originally characterized by Hatanaka et al. as being able to mediate transport of cationic amino acids, including arginine (2001). However, a rigorous demonstration of arginine uptake and a characterization of the transport kinetics were absent from this study. Thus, we performed radioactive ligand uptake studies to investigate whether mNAT3 does indeed transport arginine and to define the kinetics of this transport.

### 2.3.1  Timecourse of arginine transport by mNAT3

We studied the transport activity of mNAT3 by overexpressing it in COS-7 cells. COS-7 were transfected with either an mNAT3 expression vector or with an empty pcDNA3 vector. Overexpression of mNAT3 protein was verified by Western blot (Figure 6A). Radioactive ligand uptake assays were performed on

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both mNAT3-transfected and pcDNA3-transfected COS-7 cells in parallel. mNAT3-mediated transport was calculated by subtracting the uptake by pcDNA3-transfected cells from that of the mNAT3-transfected cells. We measured the uptake of $^3$H-arginine over 20 minutes with an uptake buffer containing 10µM arginine. We determined that arginine uptake is linear over this time period. Thereafter, arginine uptake was measured after two minutes.
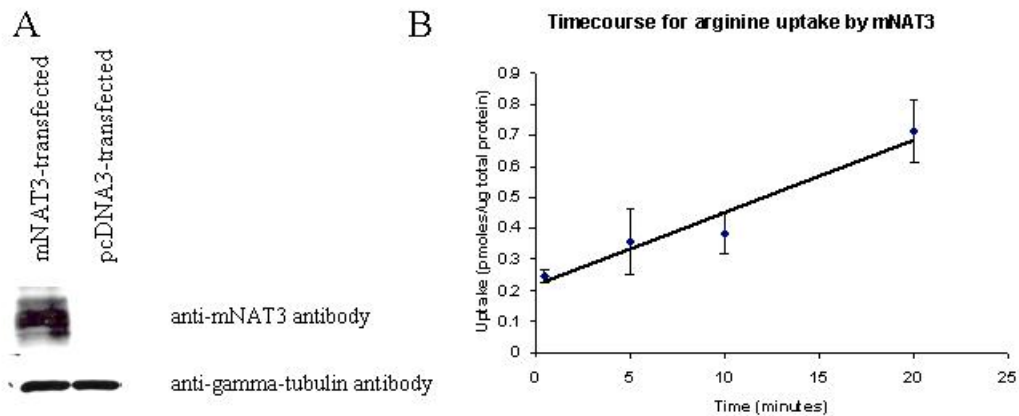


Figure 6. Arginine uptake measurements in COS-7 cells overexpressing mNAT3. Radioactive ligand uptake assays were performed on COS-7 monolayers that had been transfected either with an mNAT3 expression vector or with an empty vector. A. Western blot of transfected COS-7 protein lysates with either mNAT3 or gamma-tubulin antibody; B. Timecourse or arginine uptake by mNAT3 in COS-7 cells. mNAT3-mediated uptake was calculated by subtracting the uptake by empty vector-transfected cells from mNAT3-transfected cells. Data are mean ± SE, n=3.

### 2.3.2 Dose-dependence of arginine transport by mNAT3

Arginine uptake by COS-7 cells overexpressing mNAT3 was assayed with buffers containing various amounts of arginine, ranging from 10nM to 20mM.

Uptake was assayed after two minutes, and the ^{3}H-arginine uptake by pcDNA-transfected COS-7 cells was subtracted from the uptake of mNAT3-transfected cells to obtain the arginine uptake due to mNAT3. We determined that arginine uptake mediated by mNAT3 is dose-dependent (Figure 7).

**Arginine uptake mediated by mNAT3**
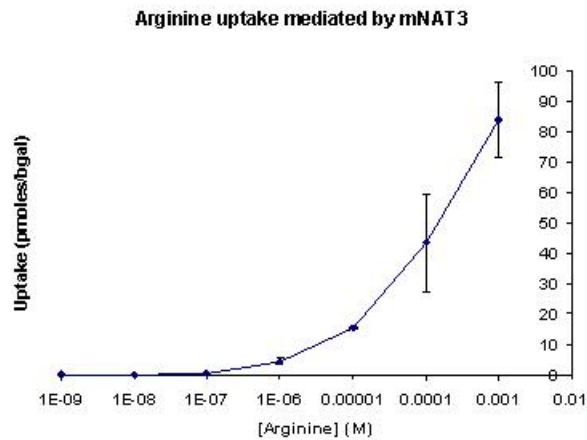


Figure 7. Dose-dependent uptake of arginine mediated by mNAT3 in COS-7 cells. Transfected COS-7 monolayers were incubated with varying concentrations of arginine in Tyrode's solution for two minutes. mNAT3-mediated arginine uptake was calculated by subtracting $H^3$-arginine uptake by pcDNA3-transfected COS-7 cells with that of mNAT3-transfected cells. Data are mean ± SE, n=6.

### 2.3.3  *Kinetics of arginine transport by mNAT3*

We measured the mNAT3-mediated uptake of arginine with varying concentrations of arginine and plotted the inverse velocity against inverse arginine concentration to obtain a Lineweaver-Burk plot (Figure 8). From this

plot, we determined the Vmax of mNAT3-mediated arginine uptake to be 0.039 pmoles/s per μg total protein. The Km was calculated to be 53μM.
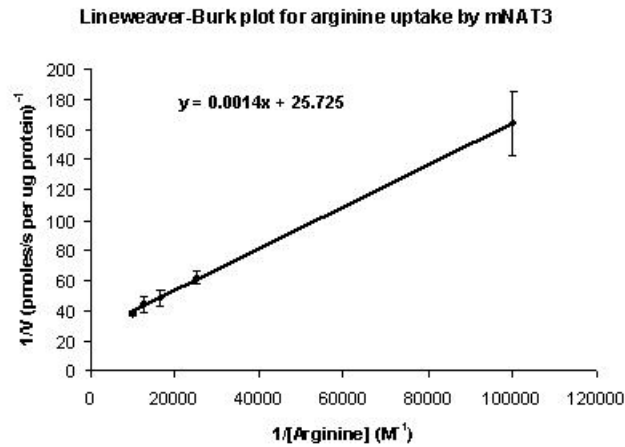


Figure 8. Lineweaver-Burk plot for mNAT3-mediated arginine transport. Transfected COS-7 monolayers were incubated with varying concentrations of arginine in Tyrode's solution for two minutes. mNAT3-mediated arginine uptake was calculated by subtracting $H^3$-arginine uptake by pcDNA3-transfected COS-7 cells with that of mNAT3-transfected cells. Inverse velocity was plotted vs. inverse arginine concentration. Data are mean ± SE, n=6.

*2.3.4 Effect of ion substitution on arginine transport by mNAT3*

We also measured mNAT3-mediated arginine uptake in COS-7 cells using Na- and Cl-free buffers to determine whether this transport is Na- or Cl-dependent. In the absence of sodium, we found that arginine uptake by mNAT3 was decreased by 55%. In the absence of chloride, arginine uptake was decreased by 32%. Thus, arginine transport by mNAT3 is partly both Na- and Cl-dependent.

**Na- and Cl-dependence of arginine transport by mNAT3**
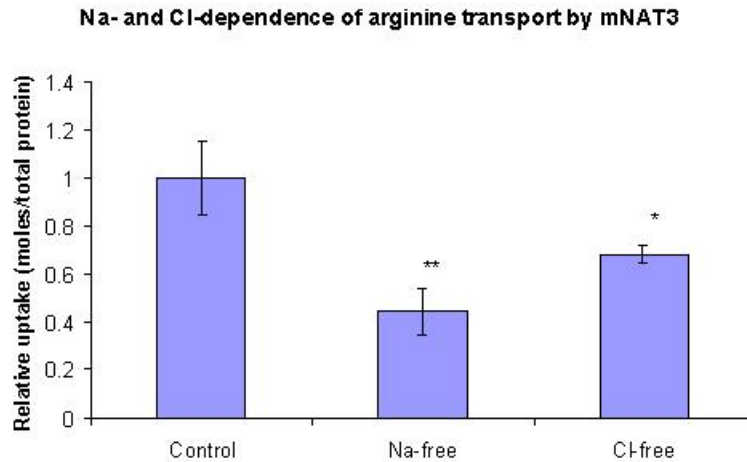


Figure 9. Na- and Cl-dependence of mNAT3-mediated arginine uptake by COS-7 cells. Radioactive ligand uptake assays were performed in Na-free and Cl-free Tyrode's solution to determine the effect of ion substitution on arginine uptake by COS-7 cells overexpressing mNAT3. Data are mean ± SE, n=6. *: $p<0.05$, **: $p<0.01$ by student's t-test.

## 2.4  Tissue expression of arginine transporters

We established that mNAT3 is a transporter of arginine. To better understand its role in arginine uptake in insulin-secreting cells, we wanted to see which, if any, other arginine transporters were expressed in insulin-secreting cells. We generated a cDNA tissue panel, to be able to look at relative RNA expression levels of the various amino acid transporters in different tissues. Using gene-specific primers, we performed RT-PCR with the tissue panel for each arginine transporter. We found that of the eleven known arginine transporters, eight were expressed in islets and MIN6 cells (Figure 10). In

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addition to Slc38a4, insulin-secreting cells were determined to also express the Slc7a1, Slc7a2, Slc7a3, Slc7a4, Slc7a6, Slc7a7, and Slc3a2 genes.
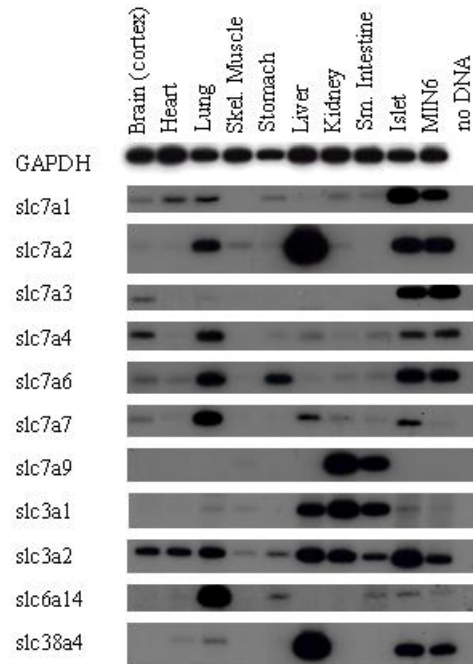


Figure 10. Expression of arginine transporters in various tissues. RT-PCR was used to determine where each arginine transporter is expressed.

## 2.5 Arginine transporter expression in Tcf1 +/+ and -/- pancreatic islets

Since we were interested in determining whether the arginine-induced insulin secretion defect in Tcf1 -/- mice could be due to a defect in arginine uptake by the insulin-secreting cells, we sought to determine which of these eight arginine transporters were regulated by Tcf1. Using semi-quantitative RT-PCR, we examined the expression levels of each arginine transporter in a pancreatic

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islet cDNA panel from Tcf1 +/+ and Tcf1 -/- mice. Of the eight arginine transporters expressed in the islets, we found only Slc7a3, Slc7a7, and Slc38a4 to be expressed at significantly different levels in Tcf1 +/+ and Tcf1 -/- islets (Figure 11). In good correlation to the results of the gene expression array results, Slc38a4 was found to be downregulated in the Tcf1 -/- islets compared to Tcf1 +/+. In similar agreement to the gene expression array results, we found Slc7a7 to be upregulated in the Tcf1 -/- islets. While the expression levels of Slc7a3 were too low to be able to yield a comparison between Tcf1 +/+ and -/- expression levels in the gene chip study, we found through RT-PCR that Slc7a3, like Slc38a4, is downregulated in the Tcf1 -/- islets.
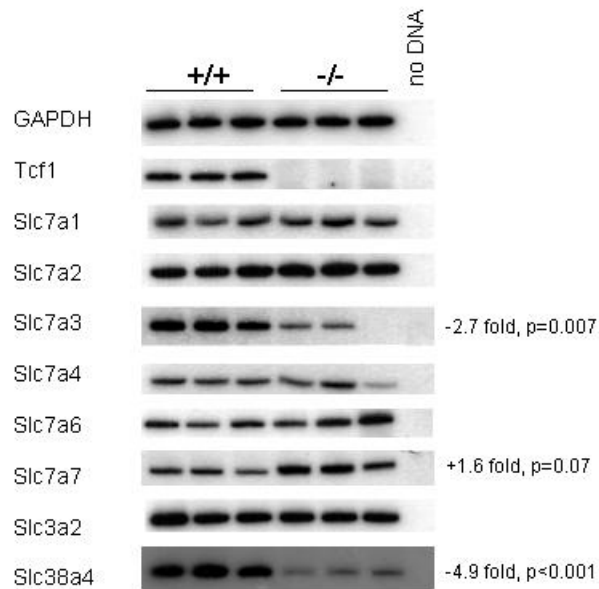


Figure 11. Tcf1 regulation of arginine transporters in pancreatic islets. Semi-quantitative RT-PCR was used to assay expression levels of arginine transporters in Tcf1 -/- mice compared to wild-type littermate controls. GAPDH expression levels were used as a control. p values were calculated using Student's t-test.

*2.6 Arginine uptake and insulin secretion with Slc38a4 (mNAT3) and Slc7a3 (CAT3) knockdown*

We were interested to know whether a defect in arginine uptake by insulin-secreting cells was the cause of the defect in arginine-induced insulin secretion in Tcf1 -/- mice. Since mNAT3 and CAT3 were the only arginine transporters whose gene expression was downregulated in Tcf1 -/- islets, we decided to knockdown expression of these two transporters in MIN6 cells to see if we could affect arginine uptake and insulin secretion.

*2.6.1 Knockdown of Slc38a4 (mNAT3) in MIN6 cells*

We electroporated two siRNAs designed to target Slc38a4 into MIN6 cells to test the effect of Slc38a4 knockdown. We confirmed knockdown of mNAT3 by Western blot (Figure 12).
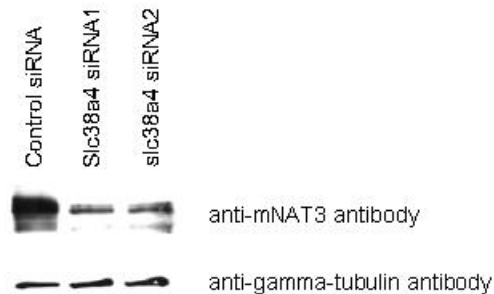


Figure 12. mNAT3 knockdown in MIN6 cells. Two siRNAs targeting Slc38a4 were electroporated into MIN6 cells. Protein lysates were harvested 72 hours post-electroporation and examined by SDS-PAGE. Anti-gamma-tubulin antibody was used as a control.

Radioactive ligand uptake assays with $^3$H-arginine were performed on MIN6 cells to compare arginine transport in cells electroporated with Slc38a4

siRNA with cells electroporated with a control siRNA. We found a small but significant decrease in arginine uptake by MIN6 cells with Slc38a4 siRNA (Figure 13). Insulin secretion in response to arginine and leucine were also measured and was found to be unaffected by mNAT3 knockdown (Figure 14).
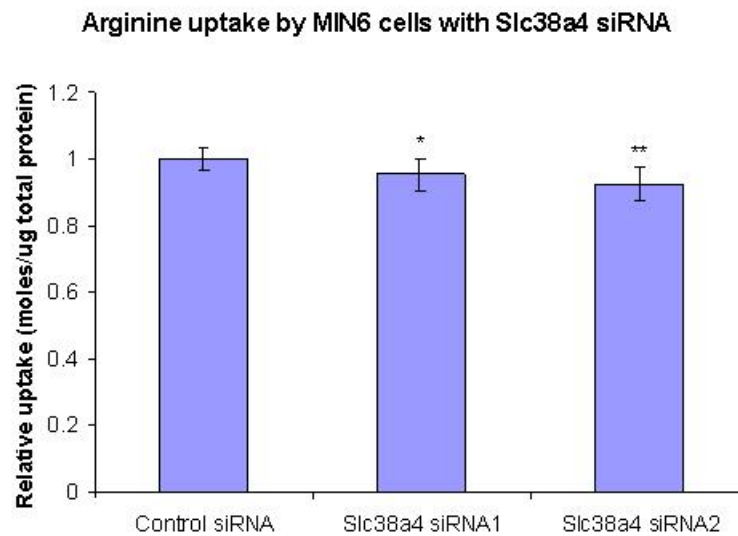


Figure 13. Arginine uptake with mNAT3 knockdown in MIN6 cells. Two siRNAs targeting Slc38a4 were electroporated into MIN6 cells. Radioactive ligand uptake assays were performed on MIN6 monolayers 72 hours post-electroporation. Data represent mean ± SE, n=9. *: p<0.05; **: p<0.01 by student's t-test.

**Insulin secretion in response to arginine and leucine by MIN6 cells electroporated with Slc38a4 RNAi**
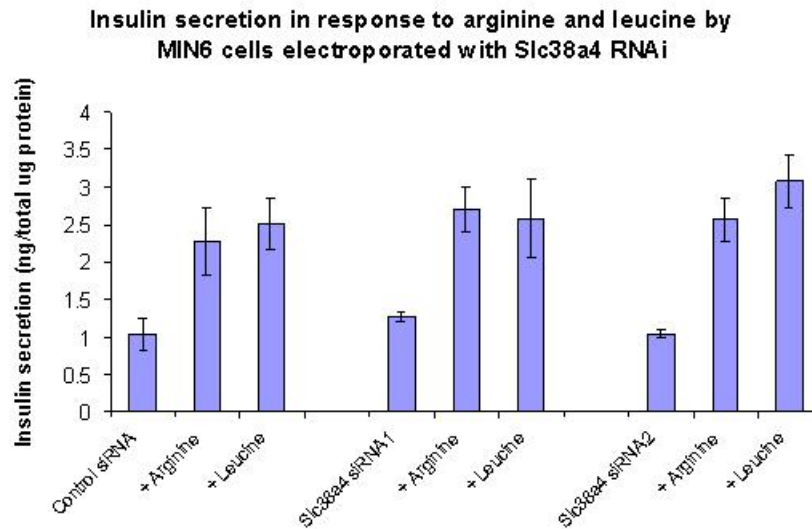
Figure 14. Insulin secretion with mNAT3 knockdown in MIN6 cells. Two siRNAs targeting Slc38a4 were electroporated into MIN6 cells. Insulin secretion assays were performed 72 hours post-electroporation. Krebs-Ringer Buffer (KRB) containing 3.3mM glucose was used for basal insulin secretion measurements. MIN6 cells were stimulated with either 20mM arginine or 20mM leucine. Data represent mean ± SE.

*2.6.2  Knockdown of Slc7a3 (CAT3) in MIN6 cells*

Two siRNAs designed to target Slc7a3 were electroporated into MIN6 cells. Knockdown of Slc7a3 in electroporated cells was confirmed by semi-quantitative RT-PCR (Figure 15A). We found that Slc7a3 knockdown led no significant change in arginine uptake (Figure 15B). There was also no change in insulin secretion in response to arginine and leucine with Slc7a3 knockdown (Figure 16).

A



B

**Arginine uptake by MIN6 cells with Slc7a3 siRNA**
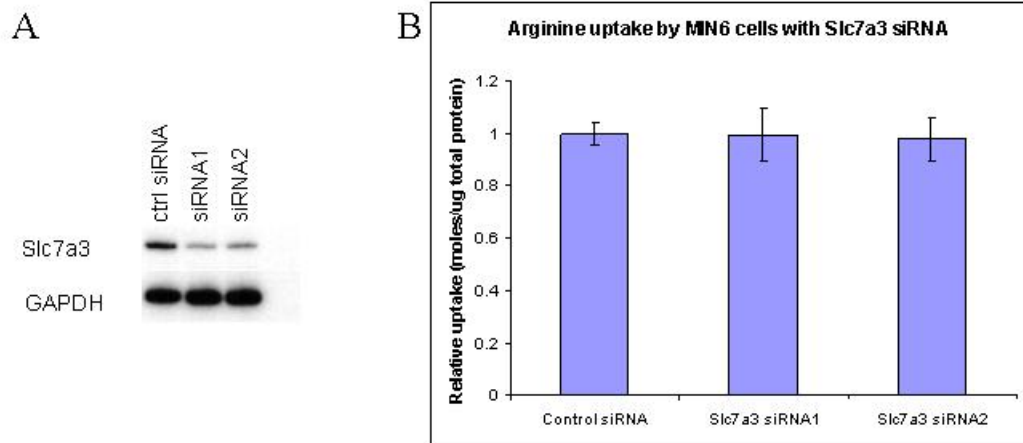
Figure 15. Arginine uptake measurements in MIN6 cells electroporated with Slc7a3 siRNA. Radioactive ligand uptake assays were performed on MIN6 monolayers that had been electroporated with either Slc7a3 siRNA or a control siRNA. A. Semi-quantitative RT-PCR to show knockdown of Slc7a3 in MIN6 cells, 72 hours after electroporation; B. Radioactive arginine uptake by electroporated MIN6 cells, measured 72 hours after electroporation. Data are mean ± SE, n=6.



**Insulin secretion in response to arginine and leucine by MIN6 cells with Slc7a3 siRNA**
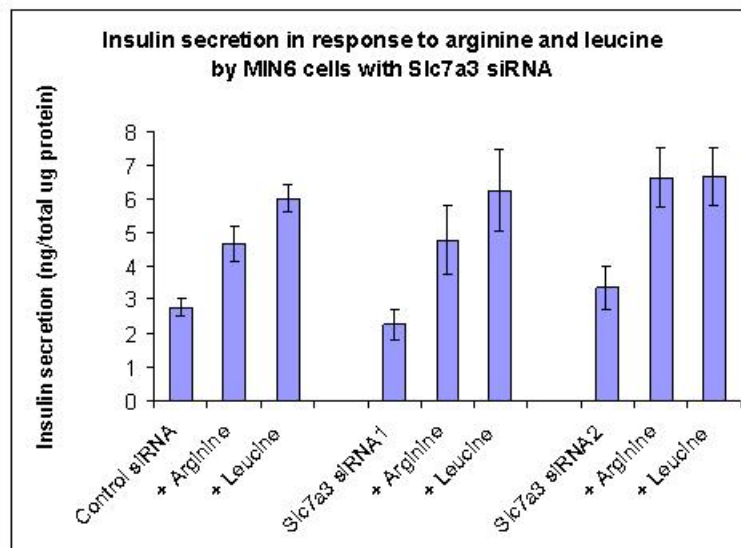
Figure 16. Insulin secretion with Slc7a3 knockdown in MIN6 cells. Two siRNAs targeting Slc7a3 were electroporated into MIN6 cells. Insulin secretion assays were performed 72 hours post-electroporation. Krebs-Ringer Buffer (KRB) containing 3.3mM glucose was used for basal insulin secretion measurements. MIN6 cells were stimulated with either 20mM arginine or 20mM leucine. Data represent mean ± SE.

## 2.6.3 Knockdown of both Slc38a4 (mNAT3) and Slc7a3 (CAT3)

We also electroporated MIN6 cells with both Slc38a4 and Slc7a3 siRNA and examined arginine uptake and insulin secretion. Using the maximal amount of siRNA for electroporation, we were unable to detect a change arginine uptake by cells with both Slc38a4 and Slc7a3 siRNA compared with cells with control siRNA. When we performed insulin secretion assays on these cells, we found no change in glucose or leucine-induced insulin secretion, and a small increase in arginine-induced insulin secretion in the cells electroporated with Slc38a4 and Slc7a3 siRNAs (Figure 17).
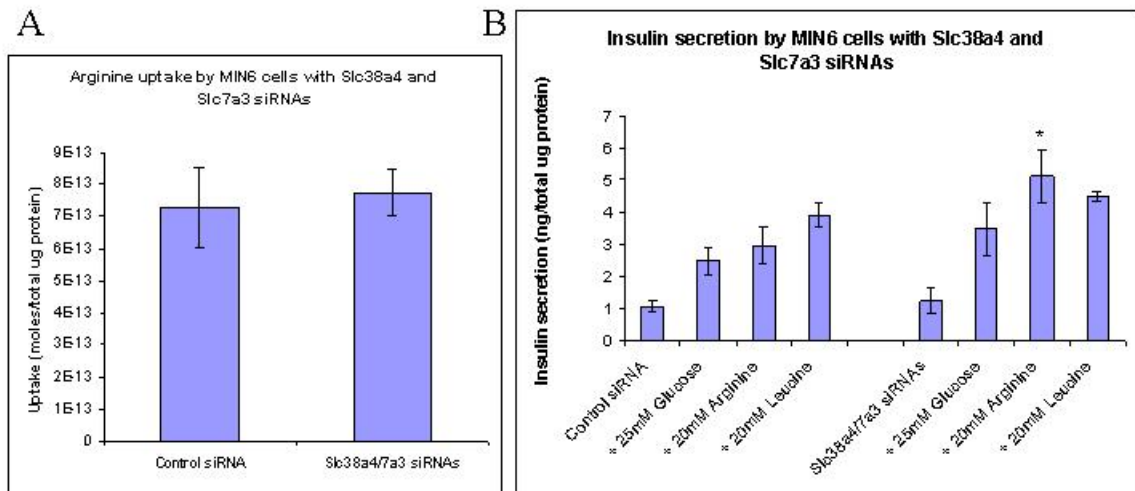


Figure 17. Knockdown of both Slc38a4 and Slc7a3 in MIN6 cells. A. Arginine uptake assays were performed 72 hours post-electroporation of the siRNAs. Data represent mean ± SE; B. Insulin secretion assays were performed 72 hours post-electroporation. Krebs-Ringer Buffer (KRB) containing 3.3mM glucose was used for basal insulin secretion measurements. MIN6 cells were stimulated with either 25mM glucose, 20mM arginine or 20mM leucine. Data represent mean ± SE.

# CHAPTER 3 – DISCUSSION PART I

## 3.1 *Characterization of arginine uptake by insulin-secreting cells*

In this study, we identified eight arginine transporters to be expressed in pancreatic islet tissue. All of these transporters, with the exception of y+LAT-1 encoded by the Slc7a7 gene, are also expressed in MIN6 cells, a mouse insulinoma cells that constitute a more beta-cell-specific test sample. Thus, we believe that y+LAT-1 may be exclusively expressed in the non-beta-cells of the pancreatic islets, such as the glucagon- or somatostatin-producing cells of the islet.

The expression of seven different arginine transporters in MIN6 cells suggests that arginine uptake may be a highly redundant function in insulin-secreting cells. We also characterized the uptake of arginine by insulin-secreting cells using radioactive ligand uptake studies with MIN6 cells. We determined the Km for arginine uptake by MIN6 cells to be 102.6µM. This value for uptake kinetics is consistent with the notion that all seven arginine transporters expressed may be active in arginine uptake by insulin-secreting cells. The reported Km values for the seven arginine transporters we have identified to be expressed in MIN6 cells range from 43µM to 120µM, with the exception of the CAT2A transporter encoded by Slc7a2 (Kim et al., 1991; Habermeier et al., 2003; Hosokawa et al., 1997; Pfeiffer et al. 1999; Wells et al., 1992). The CAT2A transporter has a Km of 3mM, over ten-fold higher than the Km values of

arginine uptake by the other expressed transporters and of arginine uptake by MIN6 cells (Habermeier et al., 2003). This significantly lower substrate affinity suggests that CAT2A may not have a large role in arginine uptake by insulin-secreting cells.

We also determined that arginine uptake by MIN6 cells is partly Na-dependent. This finding is particularly striking because of the seven transporters expressed in MIN6 cells, all have been reported to be Na-independent for arginine transport except for mNAT3 encoded by the Slc38a4 gene, which we had characterized in this study as having partial Na-dependence. This suggests that mNAT3 normally has a significant role in arginine uptake by MIN6 cells. However, our experiments with siRNA-mediated knockdown of mNAT3 expression in MIN6 cells have demonstrated that mNAT3 activity is not crucial for proper arginine uptake and arginine-induced insulin secretion. This further demonstrates the redundancy of arginine transport activity and the ability for the other arginine transporters to compensate for the loss of activity of one.

The redundancy of arginine transport function, which is directly linked to arginine-induced insulin secretion activity, is particularly interesting considering the clinical use of the arginine bolus test, in which a bolus of arginine is delivered to patients intravenously and resultant plasma insulin levels are determined. This test was commonly used in the clinic to diagnose insulin secretion defects, before the oral glucose tolerance test was developed. Not much was then understood about the mechanism of arginine-induced insulin secretion and what

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the test was measuring exactly. Recent studies that have shown that arginine-induced insulin secretion is due to electrogenic uptake of arginine into the cells have allowed the possibility that arginine-induced insulin secretion defects may result from defects in arginine uptake. Our study, which shows seven arginine transporters that are expressed in insulin secreting cells and that are able to maintain normal levels of arginine uptake and arginine-induced insulin secretion with knockdown of two arginine transporters, suggests that arginine uptake in insulin-secreting cells is highly robust. Thus, it is highly unlikely that any defect in insulin production from the arginine bolus test would be due to a defect in arginine uptake. Instead, a defect would most likely be due to a decrease in insulin content, which is closely linked to islet mass within the pancreas.

*3.2 Implications for arginine-induced insulin secretion in Tcf1 -/- mice*

Of the eight arginine transporter genes found to be expressed in islets, only Slc38a4 and Slc7a3 were found to be regulated by Tcf1, using semi-quantitative RT-PCR analysis of pancreatic islet tissue. Using siRNAs to knock down expression of these two transporter genes in MIN6 cells, we found that decreased expression of these genes had no effect on either arginine uptake or insulin secretion. Thus, we were unable to mimic the insulin secretion defects found with Tcf1 deficiency with knockdown of these two transporters. These results suggest that mNAT3 and CAT3 activity is not crucial to maintain normal arginine uptake and arginine-induced insulin secretion in insulin-secreting cells.

We cannot exclude the possibility that the level of siRNA-mediated knockdown in our study was insufficient to see the effects of total loss of activity by these transporters. It may be possible that the loss of transport activity by mNAT3 and CAT3 causes the defect in arginine-induced insulin secretion in the Tcf1 -/- mice, but that this cannot be modeled using siRNAs in MIN6 cells due to insufficient transporter knockdown in the MIN6 cells. It is important to note, however, that even in the Tcf1 -/- islets, there is not complete knockdown of either transporter.

If loss of mNAT3 and CAT3 activity is not the cause of the arginine-induced insulin secretion defect in the Tcf1 -/- mice, it is possible that there are other yet unidentified arginine transporters that are expressed in insulin-secreting cells and are regulated by Tcf1, which we have not studied. It is also possible that there is post-transcriptional regulation of arginine transporter expression and/or function that is affected in the Tcf1 -/- islets of the other arginine transporters. mNAT3 and CAT3 were identified as the only arginine transporters downregulated in Tcf1 -/- pancreatic islets through RT-PCR of a cDNA panel from islets of Tcf1 +/+ and Tcf1 -/- mice. Since Tcf1 is well-characterized as a transcriptional activator, we sought to identify genes regulated by Tcf1 at the transcriptional level. However it is possible that Tcf1 may either directly or indirectly affect the protein expression or activity of arginine transporters by a mechanism as yet unidentified. Thus, impaired activity of another arginine transporter, which has no decrease in cDNA expression levels

in the Tcf1 -/- islets, may be the cause of defective arginine-induced insulin secretion in the Tcf1 -/- mice.

To begin examining this possibility, it would be useful to systematically design siRNAs targeting the other arginine transporters expressed in MIN6 cells, and assay changes in arginine uptake and insulin secretion. While this would not identify what causes the arginine-induced insulin secretion defect in Tcf1 -/- mice, it would identify any transporters with activity that is important for uptake and insulin-secretion function.

*3.3 Tcf1 as a master regulator of transporters*

We have demonstrated that Tcf1 regulates the expression of amino acid transporters, encoded by the genes Slc7a3, Slc7a7, and Slc38a4. As we were unable to mimic the arginine-induced insulin secretion defect with knockdown of Slc7a3 and Slc38a4, it is possible that Tcf1 regulates arginine uptake through regulation of additional transporters. In addition to the defect in arginine-induced insulin secretion, other characteristics of the Tcf1 -/- mouse suggest an important role for Tcf1 in regulating transmembrane transport of various compounds.

Tcf1 -/- mice also have elevated plasma bile acid levels (Shih et al., 2001). This was found to be due to decreased expression of bile acid transporters on the basolateral membrane of hepatocytes, leading to decreased uptake of bile acids from the blood into the liver. Bile acid transporter genes that have been shown

to be regulated by Tcf1 include Slc10a1, Slc21a3, Slc21a5, and Slc21a6 (Shih et al., 2001; Jung et al., 2001). In addition, Tcf1 -/- mice also have increased urinary and fecal bile acid excretion, which is believed to be secondary to decreased bile acid uptake in the ileum as well as in the kidneys from decreased expression of the bile acid transporter Slc10a2 expressed in these tissues (Shih et al., 2001).

Tcf1 -/- mice also exhibit a renal Fanconi-like syndrome (Pontoglio et al., 1996). This is characterized by a defect in reabsorption of glucose, phosphate, and amino acids in the renal tubules, leading to glucosuria, phosphaturia, and aminoaciduria. Tcf1 has been shown to regulate expression of the glucose transporter Slgt2, as well as sodium/phosphate cotransporters Npt1 and Npt4 (Pontoglio et al., 2000; Cheret et al., 2002). In addition to the Tcf1 -/- mouse model, MODY3 patients are also characterized by severe glucosuria, due to reduced glucose reabsorption in the renal proximal tubule. Thus, the relevance of Tcf1 in regulating transmembrane transport exists not only in the mouse model, but also in actual MODY3 patients.

# CHAPTER 4

# INTRODUCTION TO LIVER STUDIES

## 4.1 Overview of insulin action

As discussed in Chapter 1, insulin is a polypeptide hormone that is secreted in response to increases in blood glucose, amino acids, and fatty acids levels. Once secreted into the bloodstream, it is able to signal to different organs to adjust their fuel metabolism to maintain homeostasis in the organism. Thus, in large part due to the action of insulin, plasma glucose remains in a range between 4mM and 7mM in normal individuals, despite periods of fasting and feeding (Saltiel and Kahn, 2001). The metabolic effects of insulin are generally anabolic: as it signals a "fed" state in the organism, it promotes the synthesis of glycogen from glucose, triacylglycerols from fatty acids, and protein from amino acids. Insulin also stimulates the insertion of glucose transporters into the cell membrane in tissues, such as muscle, to allow for glucose uptake by these tissues. Since glucose is available from external sources, the organism does not need to generate glucose; thus, gluconeogenesis and glycogenolysis are shut off by insulin. In the absence of insulin when nutrient intake is low, the body acts to maintain glucose levels through both glycogen breakdown and de novo synthesis of glucose. In addition, the body generates ketone bodies through lipolysis and fatty acid oxidation, as an energy source for tissues.
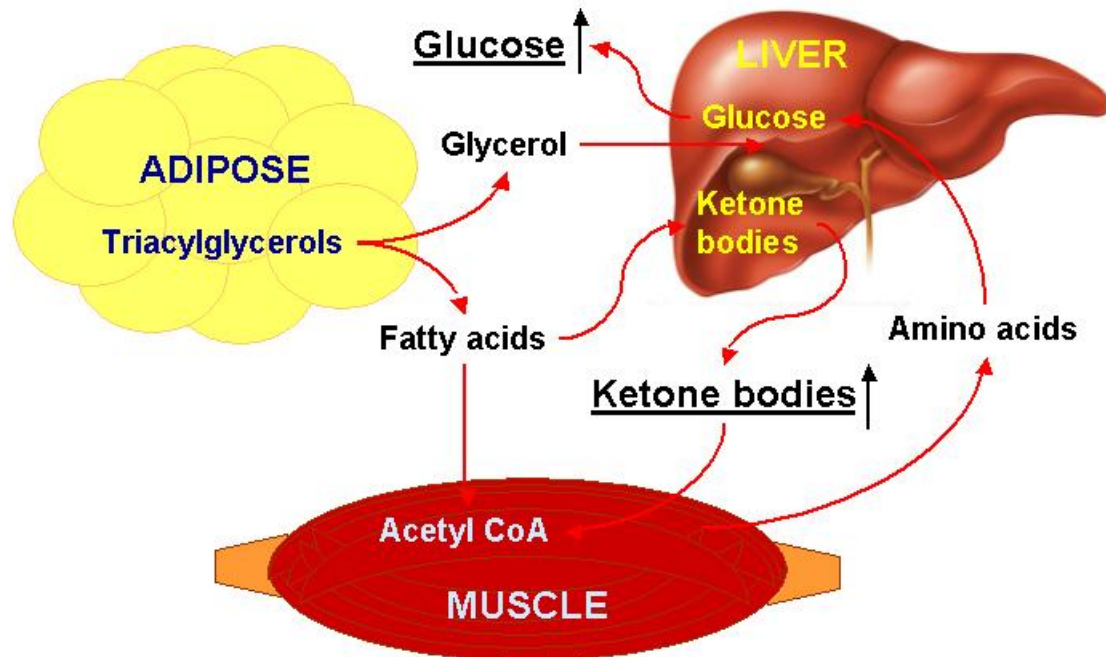
Figure 18. Tissue metabolism with low insulin. During periods of low nutrient intake, plasma insulin levels are low. The body responds by increasing β-oxidation of fatty acids, ketogenesis, to be used as an energy source by tissues, and gluconeogenesis, to maintain plasma glucose levels as another energy source for tissues.

A major target for insulin action is the liver. When insulin levels are low, the liver becomes a site of hepatic glycogen breakdown, gluconeogenesis, β-oxidation, and ketogenesis (Nordlie et al., 1999; Pilkis et al., 1992; Laffel, 1999). While gluconeogenesis also occurs in the kidneys, the liver is the site of 90% of gluconeogenesis in the body. Hepatic glucose production is a key mechanism for providing the organism with glucose during periods of fasting, and is affected by the levels of available glucogenic substrates and the activity of regulatory enzymes. Insulin is known to inhibit gluconeogenesis predominantly by decreasing the expression of genes or by changing the activity (by changing

phosphorylation states) for key enzymes in the gluconeogenesis pathway (Barthel and Schmoll, 2003).  Insulin also inhibits β-oxidation of fatty acids and promotes lipogenesis, mainly through regulating the transcription of genes for enzymes involved in those pathways.  We will discuss the molecular mechanisms of these insulin-regulated effects.

*4.2 Insulin receptor and insulin receptor substrates*

Insulin mediates its actions by binding to its receptor on target tissues. The insulin receptor is a member of a subfamily of receptor tyrosine kinases that includes the insulin-like growth factor-I receptor (IGFIR) and the insulin receptor-related receptor (IRR) (Patti and Kahn, 1998).  Two alpha-subunits and two beta-subunits make up the insulin receptor (reviewed by De Meyts and Whittaker, 2002). The alpha-subunits are entirely extracellular and contain the hormone-binding domains.  The alpha-subunits are each linked to a beta-subunit and to each other by disulfide bonds (White and Kahn, 1994).  The beta-subunits, which contain the ATP-binding and tyrosine kinase domains, pass through the cellular membrane.  In the absence of ligand, the alpha-subunit inhibits the tyrosine kinase activity of the beta-subunit.  Upon insulin binding to the alpha-subunit, this repression of the beta-subunit kinase activity is released.  The two beta-subunits of the insulin receptor then transphosphorylate each other, leading to a conformational change that further increases kinase activity (Patti and Kahn, 1998).

There are at least 11 intracellular substrates of the insulin receptor and the IGF1R kinases. Of these, six belong to the family of insulin-receptor substrate (IRS) proteins. Other substrates include Grb2-associated binder-1 (Gab1), Cas-Br-M ecotropic retroviral transforming sequence homologue (Cbl), adaptor protein with a pleckstrin homology (PH) and Src homology 2 (SH2) domain (APS), and the SH2-containing protein (Shc) isoforms (Pessin and Saltiel, 2000).

The IRS proteins have both PH domains and phosphotyrosine-binding (PTB) domains. The PTB domains allow the IRS proteins to have high binding affinities for the activated insulin receptors. The PH domains allow for binding to membrane phospholipids, and have also been found to be important for activation of downstream signaling molecules (Vainshtein et al., 2000). In addition, the IRS proteins contain up to 20 potential tyrosine-phosphorylation sites, allowing for interactions with other molecules containing SH2 domains once they are phosphorylated by the insulin receptor tyrosine kinase. IRS-1 and IRS-2 are widely expressed and implicated in regulation of metabolism. IRS-3 is mainly expressed in the adipocytes and brain; IRS-4 is expressed primarily in embryonic tissues; IRS5/DOK4 is ubiquitously expressed, but most highly in the liver and kidney; and IRS6/DOK5 is most highly expressed in skeletal muscle (Taniguchi et al., 2006; Cai et al., 2003).

While the IRS proteins are highly homologous to each other, knockout and knockdown studies have shown that they do not perform redundant roles. Biochemical studies have shown that IRS proteins have different binding

affinities for various SH2 proteins (Sun et al., 1997), providing at least one explanation for their differential functions. In a study using adenovirus-mediated RNA interference to knockdown IRS-1 and IRS-2 expression specifically in the livers of wild-type mice, it was found that knockdown of IRS-1 was associated with a increase in gene expression of gluconeogenic enzymes glucose-6 phosphatase (G-6-P) and phosphoenolpyruvate carboxykinase (PEPCK), and a trend toward increased blood glucose levels (Taniguchi et al., 2005). In contrast, knockdown of IRS-2 in the liver resulted in increased hepatic lipid accumulation. While IRS-1 and IRS-2 are implicated in different insulin signaling effects, they also play complementary roles and are able to compensate for the loss of the other to some extent. For example, the upregulation of gluconeogenic enzymes observed with knockdown of IRS-1 was less than that observed with concommitant knockdown of IRS-2. The hepatic knockdown of both IRS-1 and IRS-2 resulted in systemic insulin resistance, glucose intolerance, and hepatic steatosis (Taniguchi et al., 2005).

*4.3 Insulin-regulated signaling pathways*

Insulin binding to the insulin receptor leads to activation of two separate signal transduction cascades: 1. the mitogen-activated protein kinase (MAPK) cascade; and 2. the phsophatidylinositol 3-kinase (PI3K) cascade (Figure 19).
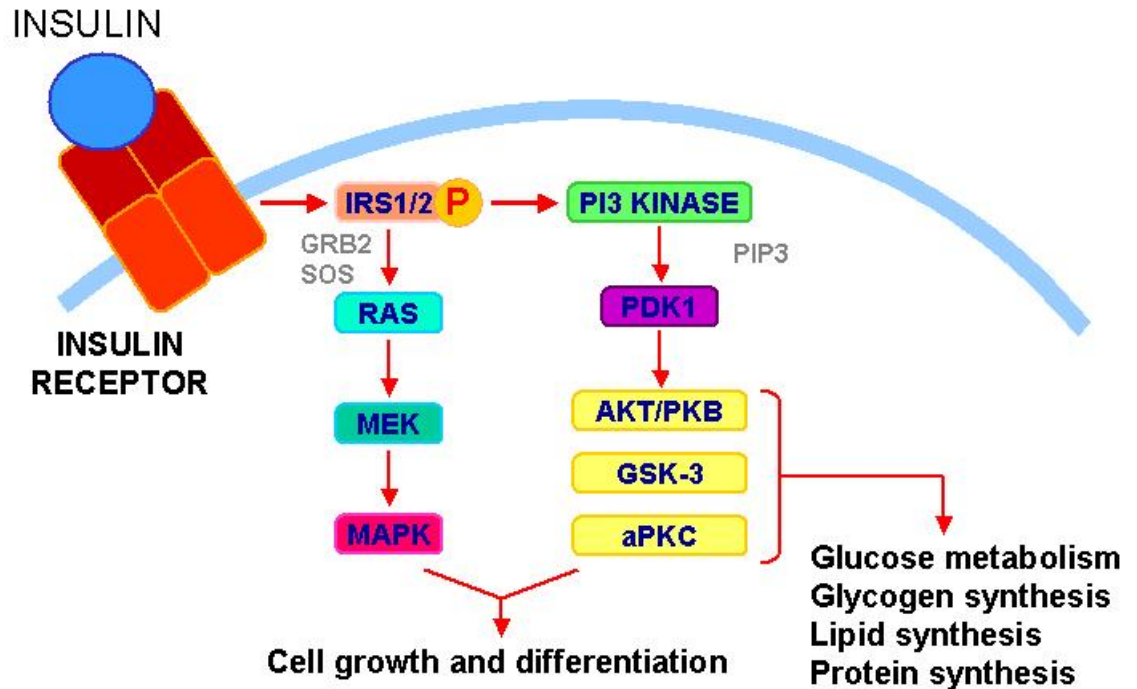
Figure 19. Main pathways of insulin signaling.

The adaptor molecule growth-factor-receptor-bound protein 2 (Grb2) binds to phophorylated IRS proteins. Grb2 constitutively associates with son-of-sevenless (Sos), the guanine nucleotide exchange factor for Ras, which is located on the plasma membrane. Binding of Grb2 to phosphorylated IRS proteins brings Sos to the plasma membrane to Ras, activating its GTPase, which then stimulates Raf-1. Raf-1 is a serine/threonine kinase that activates mitogenic effector kinase (MEK), which in turn activates MAPK (Myers et al., 1994). Activation of this pathway by phorbol myristate acetate (PMA) was found to suppress the transcription of the PEPCK and G-6-P genes (Schmoll et al., 2001; Gabbay et al., 1996). However, neither pharmacological inhibitors of MAPK (PD-

98059, PD-184352, and U-0126) nor overexpression of dominant negative mutants of Ras affects the regulation by insulin of PEPCK and G-6-P gene expression (Agati et al., 1998; Schmoll et al., 2001, Gabbay et al., 1996; Sutherland et al., 1998). Thus, the physiological importance of the MAPK pathway in insulin-regulated hepatic metabolism is unclear.

PI3K is comprised of a regulatory subunit and a catalytic subunit. Kinase activity of the enzyme requires interaction of the SH2 domains in the regulatory subunit with phosphotyrosines in the IRS proteins. PI3K catalyzes the formation of phospholipid $PIP_3$ via phosphorylation of $PI(4,5)P_2$. Proteins with PH domains that bind $PIP_3$ are then recruited to the cell membrane, where they are activated. Among those proteins activated via the PI3K pathway are members of the AGC superfamily of serine/threonine protein kinases, such as 3-phophoinositide-dependent protein kinase 1 (PDK1), which phosphorylates and activates the serine/theonine kinase Akt/PKB, glycogen synthase kinase-3 (GSK-3), as well as atypical forms of protein kinase C, aPKCs, such as PKCλ and PKBξ. Many transcription factors are regulated via the PI3K signaling pathway. Pharmacological inhibitors of PI3K (wortmannin and LY-294002) can abolish the insulin-induced suppression of PEPCK and G-6-P gene expression (Agati et al., 1998; Dickens et al., 1998). Adenovirus-mediated overexpression of a dominant negative mutant of the PI3K regulatory subunit, which cannot bind to the catalytic subunit, increases PEPCK and G-6-P gene expression and hepatic

glucose production (Miyake et al., 2002). Thus, the PI3K pathway is a key mediator of hepatic insulin effects.

*4.4 Transcription factors involved in insulin-regulated hepatic metabolism*

Many of insulin's effects in the liver are mediated through transcriptional regulation of genes involved in metabolic processes. A number of transcription factors and coactivator proteins have been identified as being involved in the insulin signaling process. In this section, we will describe the sterol response element-binding protein (SREBP)-1c, the liver X-activated receptor (LXR)α and LXRβ, and peroxisome proliferator-activated receptor-γ coactivator-1 (PGC-1). The forkhead members of transcription factors, which may be regulated by insulin via the PI3K pathway, will be discussed in the next section.

Through activation of PI3K, insulin stimulates transcription of the SREBP-1c gene in hepatocytes, as well as in adipose and muscle cells (Foufelle and Ferre, 2002). In addition, activation of Akt/PKB in vitro was found to be sufficient for upregulation of SREBP-1c gene expression (Fleischmann and Iynedjian, 2000). Adenovirus-mediated overexpression of SREBP-1c has been found to decrease gene expression of PEPCK in vitro and in vivo (Chakravarty et al., 2001; Becard et al., 2001). SREBP-1c overexpression also decreases hyperglycemia in diabetic mice (Becard et al., 2001). SREBP-1c can also increase transcription of lipogenic genes, such as acetyl-CoA synthetase, fatty acid synthase, acetyl-CoA carboxylase (Edwards et al., 2000; Osborne, 2000). Thus, by increasing

expression of SREBP-1c, insulin can decrease gluconeogenesis and increase lipogenesis.

Insulin signaling also induces expression of LXRs. LXRα, which is expressed mainly in liver, kidney, and intestines, and LXRβ, which is expressed ubiquitously, belong to the nuclear receptor superfamily (Edwards et al., 2002). The endogenous activating ligands of the LXRs are oxysterols, allowing the factors to sense changes in cholesterol levels. In fact, LXRα has been found to be important for the response to administration of cholesterol-enriched diets in mice (Peet et al., 1998). LXRs form heterodimers with the retinoic acid receptor-α (RXR) to bind to response elements in their target genes, activating expression of genes involved in lipogenesis, bile acid formation, and cholesterol transport, such as fatty acid synthase, cholesterol 7α-hydroxylase, and ABCA1. In addition, LXR has been shown to also increase expression of SREBP-1c. LXR agonists can decrease blood glucose in rodent models, at least in part through suppression of gluconeogenic enzyme expression (Cao, et al., 2003; Stulnig et al., 2002). This may be through direct interaction of LXR with these target genes, or through an indirect mechanism, such as through regulation of SREBP-1c.

PGC-1 is a coactivator protein that is positively regulated by glucagon via cAMP. In vitro studies using PGC-1 promoter-reporter gene plasmids transfected into HepG2 cells show that insulin suppresses PGC-1 promoter activity and that this suppression can be mimicked with coexpression with Akt/PKB (Daitoku et al., 2003). In addition, these in vitro studies suggest that

PGC-1 is a direct target of Foxo1, a forkhead transcription factor that is inactivated by Akt/PKB. PGC-1 acts to increase gluconeogenesis, by increasing transcription of gluconeogenic enzymes.

*4.5 Forkhead family of transcription factors*

Foxo1 and Foxa2 are two other transcription factors that are regulated by insulin. Foxo1 and Foxa2 are both members of the forkhead family of transcription factors. Forkhead proteins are a large family of functionally diverse transcription factors, which are involved in a variety of cellular processes, such as cranial and neural development, immune regulation, pancreatic and liver development, as well as liver metabolism (Lehmann et al., 2003; Coffer and Burgering, 2004; Lee, et al., 2004; Lee et al., 2005; Nakae et al., 2002; Wolfrum et al., 2004).

The original *fork head* (*fkh*) gene was identified in *Drosophila melanogaster*, through a screen of embryonic lethal mutants. It was found to be required for terminal pattern formation in the embryo (Weigel et al., 1989). The year following the identification of *fork head*, a novel group of liver-specific transcription factors was identified whose members have DNA-binding domains with a high degree of homology to that of the *fkh* gene product (Lai et al., 1990). This family was called the hepatic nuclear factor 3 (HNF3) family. Since then, many transcription factors with this forkhead motif have been identified in a whole range of organisms, from yeast to humans.

More than a hundred members of the forkhead transcription factor family have been identified to date, leading to a change in the nomenclature (Kaestner et al., 2000). Fox (for "forkhead box") is now used to name all chordate forkhead transcription factors, followed by a letter denoting its subfamily based on phlyogenetic analysis (not based on function). Each gene from a subfamily of Fox proteins is then identified by a number. Fox proteins all share a highly conserved DNA-binding domain, which forms a butterfly-like structure when bound to DNA, giving it the description "winged helix" (Clark et al., 1993). Since most of the amino acids within this DNA-binding domain are conserved, it is not understood how Fox transcription factors have different DNA sequence specificity. In contrast to the highly conserved DNA-binding domain, the transactivation or transrepression domains of the Fox family have little homology.

Foxo1 and Foxa2 are forkhead family transcription factors that are regulated by insulin. Foxo1 promotes gluconeogenesis (Nakae et al., 2001; Nakae et al., 2002), while Foxa2 regulates fatty acid metabolism (Wolfrum et al., 2004). Insulin signaling results in activation of Akt/PKB, which then phosphorylates Foxo1 and Foxa2 (Brunet et al., 1999; Biggs et al., 1999; Wolfrum et al., 2003). Phosphorylation leads to nuclear exclusion of these transcription factors, thus inhibiting their transcriptional activity (Figure 20).
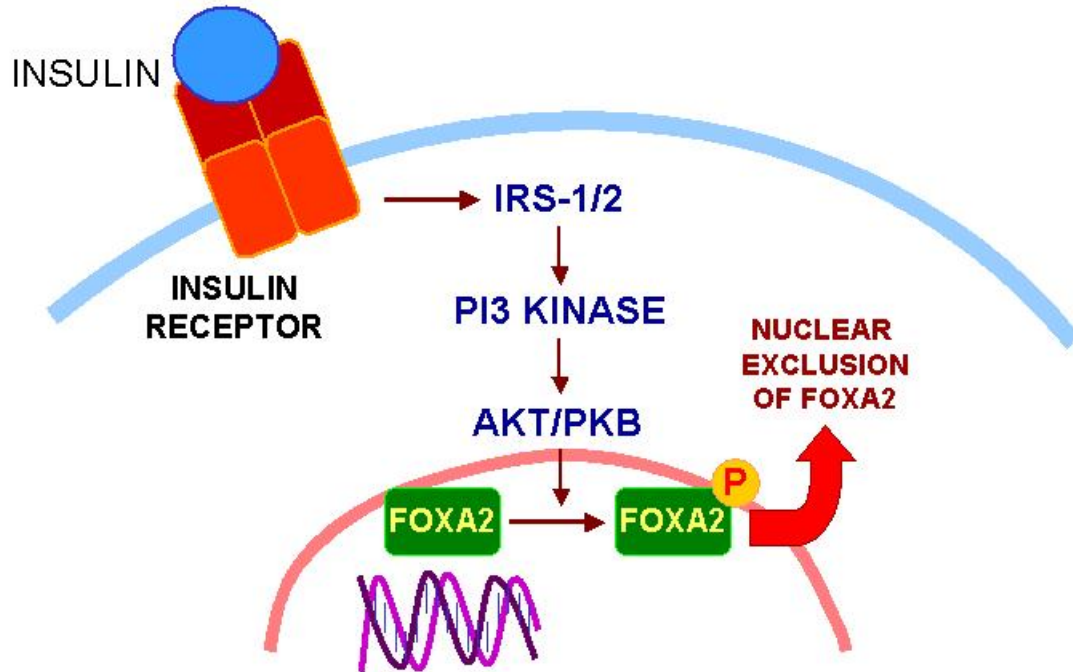
Figure 20. Mechanism of insulin-mediated nuclear exclusion of Foxa2 in liver tissue. The activated insulin receptor phosphorylates IRS proteins which leads to activation of PI3 kinase. Active PI3 kinase results in phosphorylation and activation of other kinases, including Akt/PKB, which leads to phosphorylation and subsequent nuclear exclusion of Foxa2.

A mutant form of Foxa2 was generated, changing the phosphorylated threonine at position 156 to alanine. This resulted in a constitutively active form of Foxa2, which could remain localized in the nucleus even with insulin present (Wolfrum et al., 2004). When adenovirus expressing this constitutively active form of Foxa2, called Foxa2 (T156A), was injected into hyperinsulinemic mice, where endogenous Foxa2 is permanently inactive due to the high amounts of insulin present, decreases in hepatic triglyceride content, increases in plasma triglyceride levels, and increases in ketone bodies in the plasma were noted. On the other hand, haploinsufficient Foxa2 +/- mice have decreased levels of

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ketogenesis and β-oxidation (Wolfrum et al., 2004). The data suggest that Foxa2 is an important insulin-regulated mediator of lipid metabolism, actively promoting ketogenesis and β-oxidation during fasting when insulin levels are low.

### 4.6 Insulin signaling and amino acid uptake

Insulin promotes an anabolic state in the organism, leading to increased amino acid uptake by muscle cells for protein synthesis (Biolo et al., 1995). In the absence of insulin, or in insulin-resistant states, muscle breakdown increases without this anabolic stimulus. In fact, age-related insulin resistance has recently been proposed to be a cause of the loss of muscle mass and strength (sarcopenia) observed in elderly individuals (Rasmussen et al., 2006). Because of this regulation, during times of fasting, when insulin in not present, protein synthesis is decreased and less amino acids are taken up by skeletal muscle.

In contrast, studies have shown that hepatic amino acid uptake and metabolism increases in the liver during periods of fasting (Low et al., 1992; Grofte et al., 1999; Gu et al., 2005). This effect helps to provide more amino acids to the liver cells, which need to increase gluconeogenesis during fasting. It was found that hepatic nitrogen clearance, a measure of hepatic amino acid metabolism, decreases with a hyperinsulinemic and euglycemic clamp in normal and healthy individuals, suggesting that insulin plays a direct role in regulation (Grofte et al., 1999). However, with a hyperinsulinemic and hypoglycemic

clamp, hepatic nitrogen clearance increases despite the high insulin levels, suggesting that there is another level of regulation that hypoglycemia may trigger, to counteract the effect of insulin.

While these effects of fasting and feeding on hepatic amino acid uptake and metabolism have been long known, the molecular mechanisms governing these processes had not been studied until recently with a study on a system N amino acid transporter, SNAT3, encoded by Slc38a3 (Gu et al., 2005). In this study, it was found that SNAT3 expression is downregulated by insulin. This downregulation could be blocked by the PI3K inhibitor, LY294002, but not by the MAPK inhibitor, PD98059. This data not only demonstrate that insulin that regulate expression of an amino acid transporter, but that it is through the PI3K signaling pathway that insulin induces this effect.

*4.7 Experimental study*

We had identified the Slc38a4 gene, encoding the amino acid transporter mNAT3, as being expressed in pancreatic islet cells and regulated by the transcription factor Tcf1. We had also shown that Slc38a4 is highly expressed in the liver as well. In this study, we analyzed the regulation of Slc38a4 by Tcf1 in the liver as well as the pancreatic islets. We also hypothesized that Slc38a4 may be regulated by metabolic states and endeavored to identify the molecular basis for this regulation.

# CHAPTER 5 -- RESULTS PART II

## 5.1 Tcf1 regulation of Slc38a4 in the pancreatic islets and the liver

We examined the expression and regulation of Slc38a4 in the liver, where it was strongly expressed in addition to in the pancreatic islets (Figure 10). By semi-quantitative RT-PCR using liver tissue from Tcf1 +/+ and -/- mice, we found that Slc38a4 is also regulated in the liver, but not as strongly as in the pancreatic islets (Figure 21). We were interested in this difference in Tcf1 regulation, and wanted to discover the basis for this.
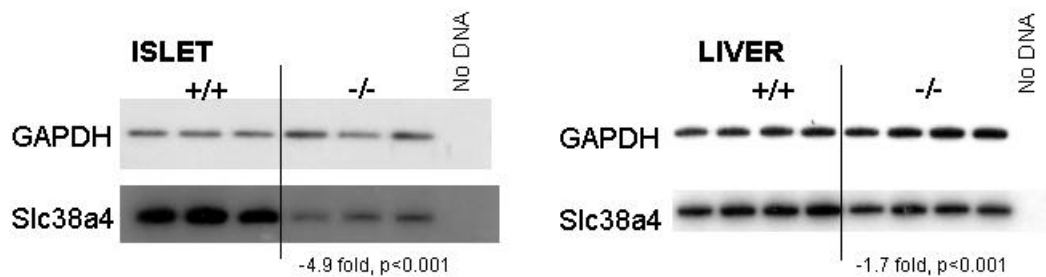


Figure 21. Slc38a4 is regulated by Tcf1 in both pancreatic islet and liver tissues. Semi-quantitative RT-PCR was performed with a panel of either islet or liver cDNA isolated from Tcf1 +/+ and -/- mice. Relative expression was measured using the Scion Image software.

## 5.2 Alternative transcriptional start sites for Slc38a4

One possibility we examined for the difference in Tcf1 regulation of Slc38a4 was differential transcriptional start sites. We performed 5′ RACE on RNA from pancreatic islets. Sequencing of the 5′ end of the islet Slc38a4 transcript revealed an alternative first and second exon of Slc38a4, compared

with the Slc38a4 mRNA sequence from liver cDNA libraries (Genbank accession no. BC024123). These two transcript isoforms differ only in their 5′ untranslated region; the open reading frames are identical, and hence, they encode identical proteins (Figure 22A). RT-PCR using isoform-specific primers showed that while the liver contains both transcript isoforms of Slc38a4, the islet contains only one isoform, which we termed the "islet" isoform (Genbank accession no. AY027919) (Figure 22B). We termed the originally identified Slc38a4 transcriptional isoform the "liver" isoform.
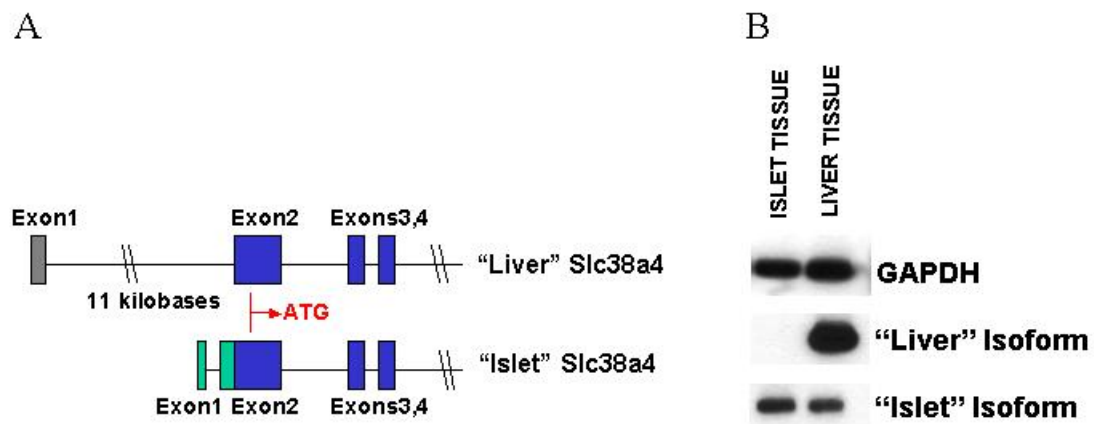


Figure 22. Slc38a4 has two distinct transcriptional start sites. Sequencing of the 5′ end of the Slc38a4 transcript from pancreatic islet tissue showed a different transcriptional start site from the originally identified Slc38a4 transcript from liver. A. Schematic representation of the Slc38a4 gene organization showing the two transcriptional start sites; B. RT-PCR of liver and islet cDNA with isoform-specific primers to determine expression of each isoform in the tissues.

### 5.3 Tcf1 regulation of both transcriptional isoforms of Slc38a4

We investigated if both transcriptional isoforms of Slc38a4 were regulated by Tcf1. To address this question, we performed RT-PCR of the two isoforms in

a panel of liver Tcf1$^{+/+}$ and Tcf1$^{-/-}$ mice. We found that the "islet" isoform of Slc38a4 (which is present in both liver and islet) is strongly regulated by Tcf1, while the "liver" isoform is regulated as well, but to a lesser degree (Figure 23).
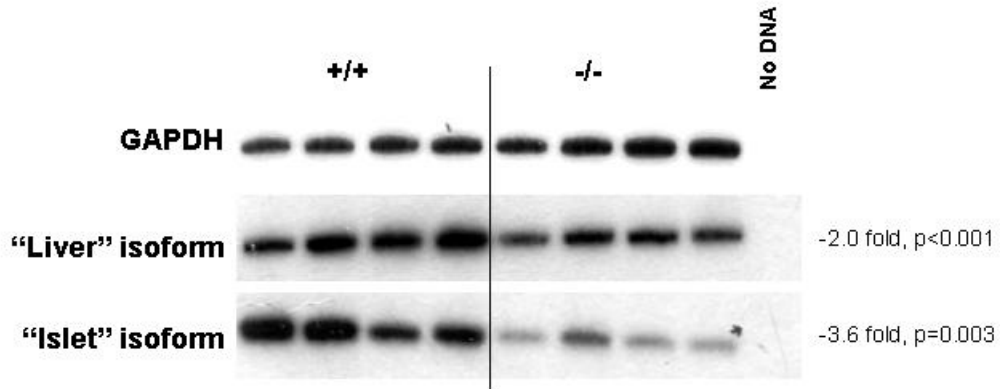


Figure 23. Both transcriptional isoforms of Slc38a4 are regulated by Tcf1. Semi-quantitative RT-PCR of a panel of cDNA from the liver of Tcf1 +/+ and -/- mice using Slc38a4 isoform-specific primers shows regulation of both isoforms by Tcf1.

## 5.4 Regulation of Slc38a4 in the liver by metabolic states and insulin

The liver responds to fasting by increasing the uptake of amino acids, which are then used as carbon skeletons for the generation of glucose. Since we had shown that Slc38a4 mediated the uptake of arginine into cells, as well as neutral amino acids (shown by other groups), we questioned whether it could have a role in the hepatic response to fasting. We generated a panel of liver cDNA from mice that had been fasted overnight, fed control mice, and overnight-fasted mice that had been injected with 10ng insulin by portal vein injection.

We found that Slc38a4 expression is upregulated during fasting. In addition, using isoform-specific primers, we found that both Slc38a4 transcript isoforms were upregulated by fasting (Figure 24A). Furthermore, this upregulation of Slc38a4 was dependent on the absence of insulin, since it is abolished with the injection of insulin into the overnight-fasted mice. Interestingly, this upregulation of Slc38a4 by fasting was not observed in the pancreatic islets (Figure 24B).
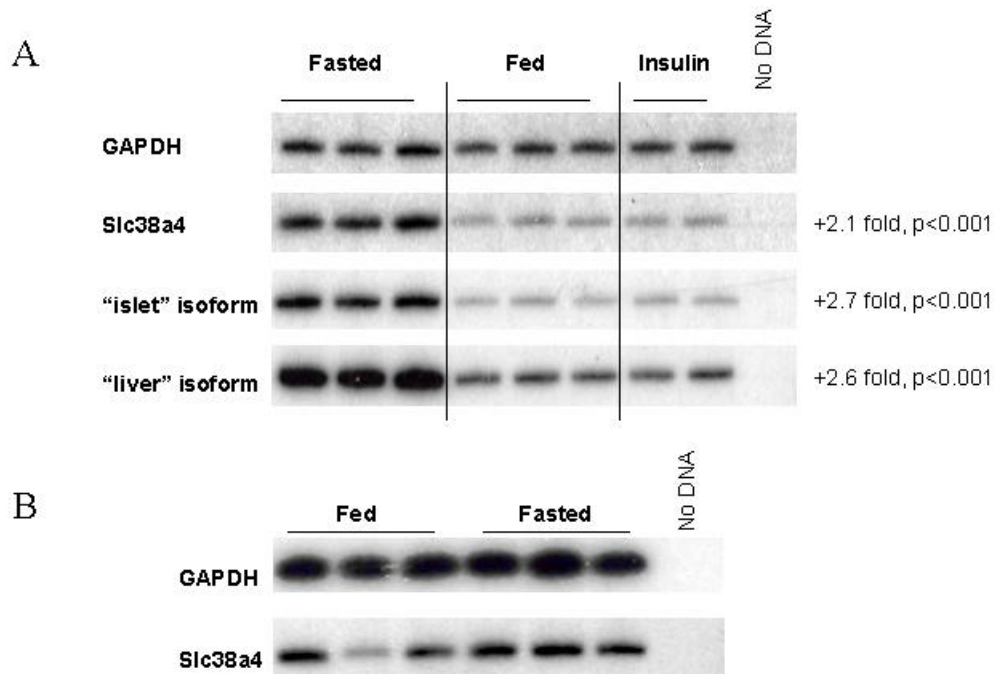


Figure 24. Slc38a4 regulation by fasting and by insulin in the liver of C57Bl/6J mice. C57Bl/6J mice were either fasted overnight, fed (control), or fasted and injected with 10ng insulin. Liver cDNA was isolated and RT-PCR analysis was performed to examine Slc38a4 expression. A. Slc38a4 is upregulated in the liver during fasting, and this upregulation is abolished with the injection of insulin; B. Slc38a4 is not significantly upregulated by fasting in the pancreatic islets.

*5.5 Slc38a4 is regulated by Foxa2*

We then examined Foxa2 as a potential regulator of this upregulation of Slc38a4 during fasting. In the liver, Foxa2 is negatively regulated by insulin. Insulin signaling stimulates a kinase cascade that leads to phosphorylation of a threonine residue of Foxa2, leading to Foxa2 nuclear exclusion. Thus, during fasting, when insulin levels are low, Foxa2 is present in the nucleus and active; when insulin is present, Foxa2 is phosphorylated and shuttled out of the nucleus. This shuttling of Foxa2 does not occur in the pancreatic islets, where Foxa2 is not insulin-responsive. Since Foxa2 is a transcriptional activator that is inhibited by insulin in the liver, but not in islet, we considered Foxa2 as a candidate regulator of Slc38a4, which we found to be upregulated in the liver during fasting, but not in pancreatic islets.

To study Foxa2 regulation, we used mice that were injected with either a GFP-expressing adenovirus, or an adenovirus that expresses a constitutively active form of Foxa2, which has the phophorylated Thr residue mutated to Ala. Through RT-PCR of a liver cDNA panel from overnight-fasted GFP-adenovirus- and Foxa2 (T156A)-adenovirus-infected C57Bl6 mice, we found upregulation of Slc38a4 with Foxa2. We also looked for Foxa2 regulation of Slc38a4 expression in hyperinsulinemic and diabetic mouse models, transgenic mice overexpressing Srebp-1c in adipose tissue (*Srebp-1c*) and *ob/ob* mice, where Foxa2 is constutively inactive due to nuclear exclusion. We found that infection of these mice with adenovirus expressing constitutively active Foxa2 at nuclear levels that are

similar to the fasted state led to even more pronounced upregulation of Slc38a4. In addition, we found that expression of both transcript isoforms of Slc38a4 are regulated by Foxa2 in all mouse models. Thus, we have found that Foxa2 is a mediator of the insulin-responsive regulation of Slc38a4.
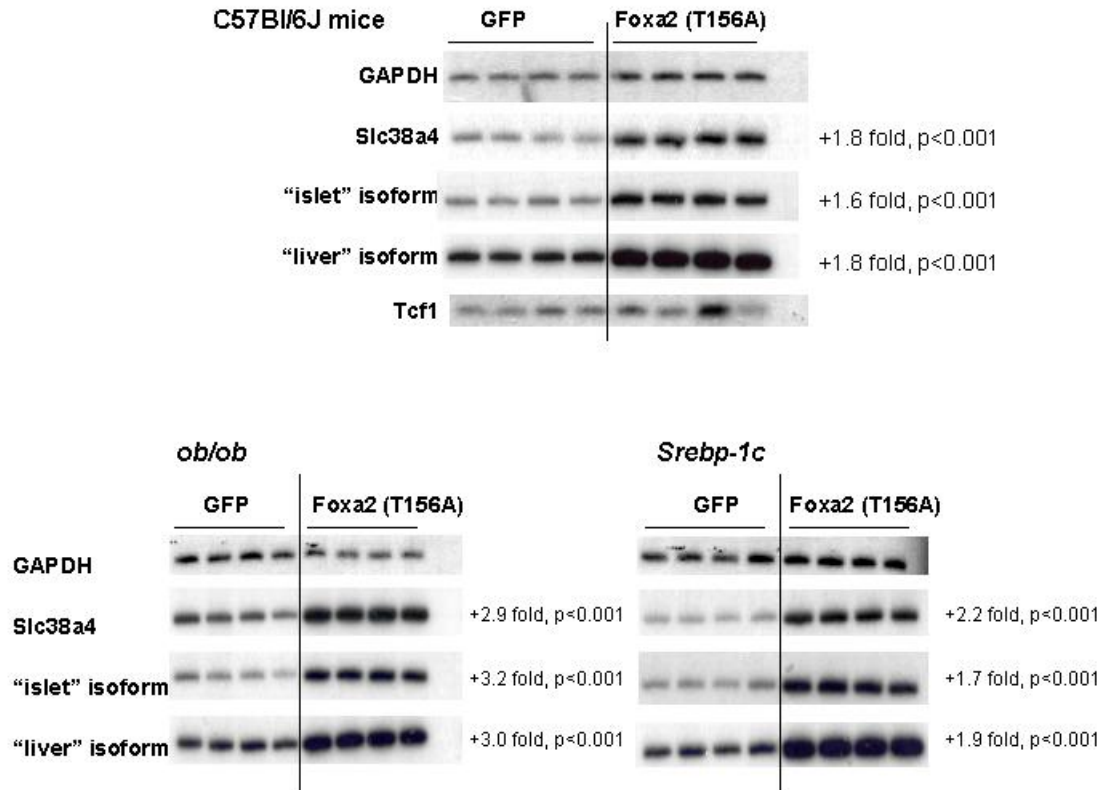


Figure 25. Slc38a4 regulation by Foxa2. Mice (C57Bl/6J control, *ob/ob*, *Srebp-1c* transgenic mice) were injected with either GFP-adenovirus or Foxa2 (T156A)-adenovirus. Liver cDNA was generated, following an overnight fast, and analyzed by RT-PCR for Slc38a4 expression.

*5.6 Identification of Tcf1 and Foxa2 regulatory elements in the Slc38a4 "islet" isoform promoter*

We wanted to determine the promoter elements responsible for Tcf1 and Foxa2 regulation. We began by analyzing the Slc38a4 "islet" isoform promoter,

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using the TFSEARCH program (ver.1.3). This revealed a putative binding site for both Tcf1 and Foxa2 at position –70 in the "islet" isoform promoter. In addition, when we compared this to the human SLC38A4 gene sequence, we found that this promoter element is conserved in both mouse and human (Figure 26).



Foxa2/Tcf1 site at -70

CTTCCTT**TGTCAATAATTTACTTTT**G Mouse
CTTCCT TGTCAA AATTTACTTTTG
CTTCCTC**TGTCAACAATTTACTTTT**G Human

Exon 1

Figure 26. Putative Tcf1 and Foxa2 binding site in the Slc38a4 "islet" isoform promoter. Sequence analysis of the Slc38a4 "islet" promoter using the TFSEARCH ver.1.3 software revealed this putative binding site. Comparison with the sequence of the human SLC38A4 gene revealed that this site is located in a region of sequence conservation.

We generated luciferase reporter gene plasmids to investigate this putative binding site. We found transactivation by both Tcf1 and Foxa2 when we co-transfected expression plasmids for these transcription factors along with a luciferase reporter gene plasmid with the Slc38a4 "islet" isoform promoter. The transcriptional activity of the luciferase reporter gene is reduced when the binding site is selectively mutated (Figure 27).

A

**Transactivation of Slc38a4 "islet" promoter by Tcf1**



B

**Transactivation of Slc38a4 "islet" promoter by Foxa2 (T156A)**
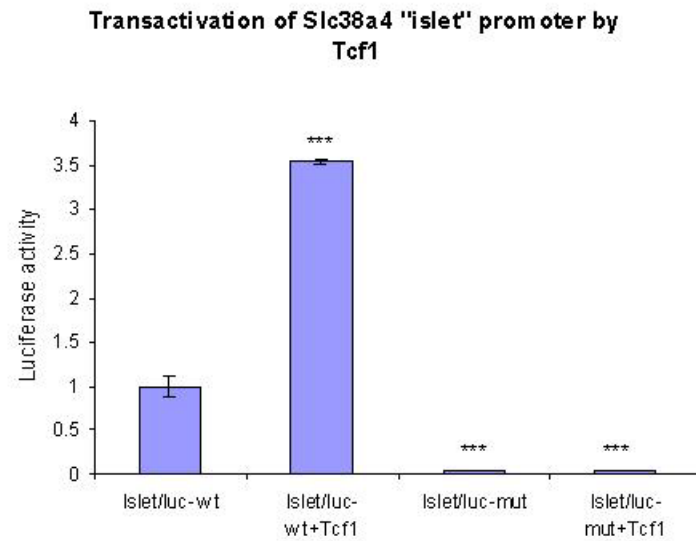


Figure 27. Mutation of the Tcf1/Foxa2 site leads to decreased transactivation by both transcription factors in a luciferase reporter gene assay. Reporter gene plasmids were generated with 600 bases of the Slc38a4 "islet" promoter sequence, with and without mutation at the putative Tcf1/Foxa2 binding site. Reporter gene plasmids were co-transfected with either pcDNA3 (control) or expression vectors for either Tcf1 or Foxa2 (T156A). Equal amounts of a CMV-LacZ plasmid was also transfected to normalize for transfection efficiency. Luciferase activity was normalized for β-galactosidase activity for all experiments. A. Slc38a4 "islet" promoter reporter gene plasmids cotransfected with Tcf1; B. Slc38a4 "islet" promoter reporter gene plasmids cotransfected with Foxa2. Data are represented as mean ± SE, n=6. **: $p<0.01$, ***: $p<0.001$ by Student's t-test, comparing pcDNA3-transfected with either Tcf1- or Foxa2-transfected samples.

In addition, we performed electrophoretic mobility shift assays to determine whether Tcf1 and Foxa2 bind directly to this site. We generated 32P-labeled double-stranded oligonucleotides containing the putative Tcf1/Foxa2 binding site. Incubating this probe with Hepa 1-6 (mouse liver cell line) extract, we were able to see specific radioactive band shifts corresponding to Tcf1 and Foxa2 binding (Figure 28). Using cold oligonucleotides containing known Tcf1 and Foxa2 sites, we were able to inhibit the radioactive band shifts in a specific manner, while a cold probe containing the mutated binding site was unable to inhibit the shift even at 100-fold excess. The binding of both Tcf1 and Foxa2 to the site was further demonstrated by supershifting their respective bands using anti-Tcf1 and anti-Foxa2 antibodies. An anti-c-jun antibody was also used as a negative control, which was unable to supershift any bands.

Figure 28. EMSA using oligonucleotides for the putative Tcf1/Foxa2 binding site in the Slc38a4 "islet" promoter. The Tcf1 competitor was a cold oligonucleotide containing a Tcf1 binding site from the Tmem27 mouse promoter (Akpinar et al., 2005). The Foxa2 competitor was a cold oligonucleotide containing the Foxa2 consensus binding site. The mutant competitor was a cold oligonucleotide of the original probe, with the binding site mutated. Poly-dIdC was also added to each reaction to quench non-specific binding to the oligonucleotides.

*5.7 Identification of Tcf1 and Foxa2 regulatory elements in the Slc38a4 "liver" isoform promoter*

We performed a similar analysis of the Slc38a4 "liver" promoter to determine the promoter elements important for Tcf1 and Foxa2 regulation of this isoform. Sequence analysis revealed two Tcf1 binding sites at positions –1000 and –40 in the "liver" isoform promoter (Figure 29). A Foxa2 binding site was

also identified in the promoter at position -220.   By comparing the mouse

promoter sequence with the sequence upstream of the human SLC38A4 gene, we

found that these elements are located in regions of sequence conservation
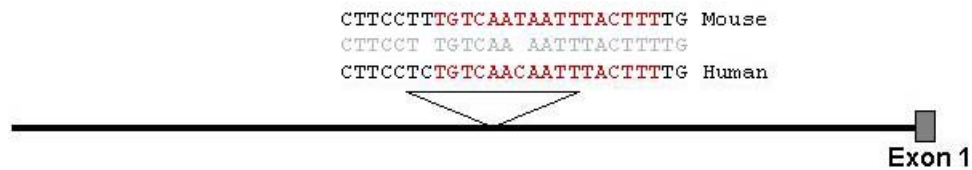
between mouse and human.
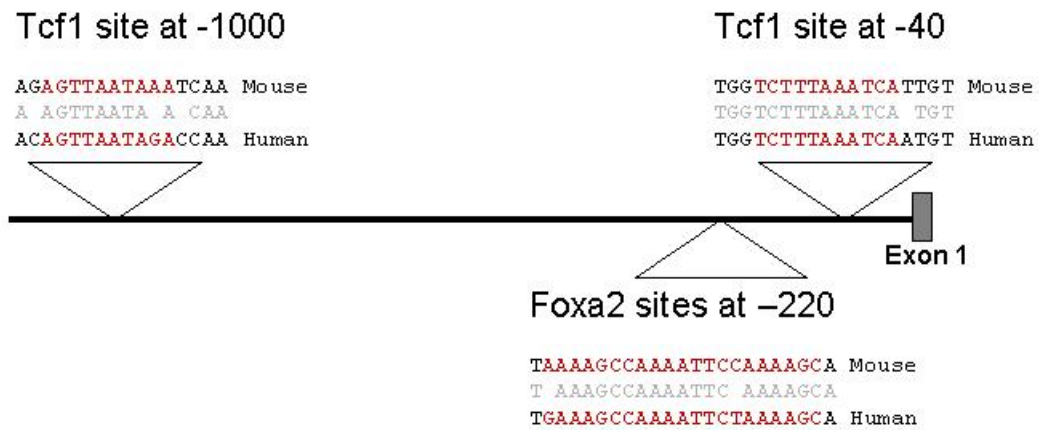


Figure 29. Putative Tcf1 and Foxa2 binding sites in the Slc38a4 "liver" promoter. Sequence analysis of the Slc38a4 "liver" promoter using the TFSEARCH ver.1.3 software revealed these putative binding sites. Comparison with the sequence of the human SLC38A4 gene revealed that these sites are located in a regions of sequence conservation between mouse and human.

We performed luciferase reporter gene assays with the Slc38a4 "liver"

promoter, and compared levels of transactivation when we mutated the various

Tcf1 and Foxa2 sites (Figure 30).

A    **Transactivation of mutant Slc38a4 "liver" promoters by Tcf1**



B    **Transactivation of Slc38a4 "liver" promoter by Foxa2 (T156A)**
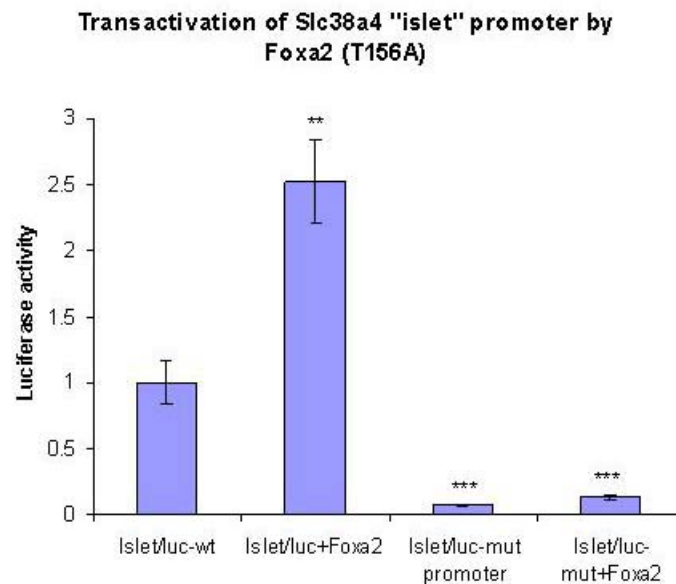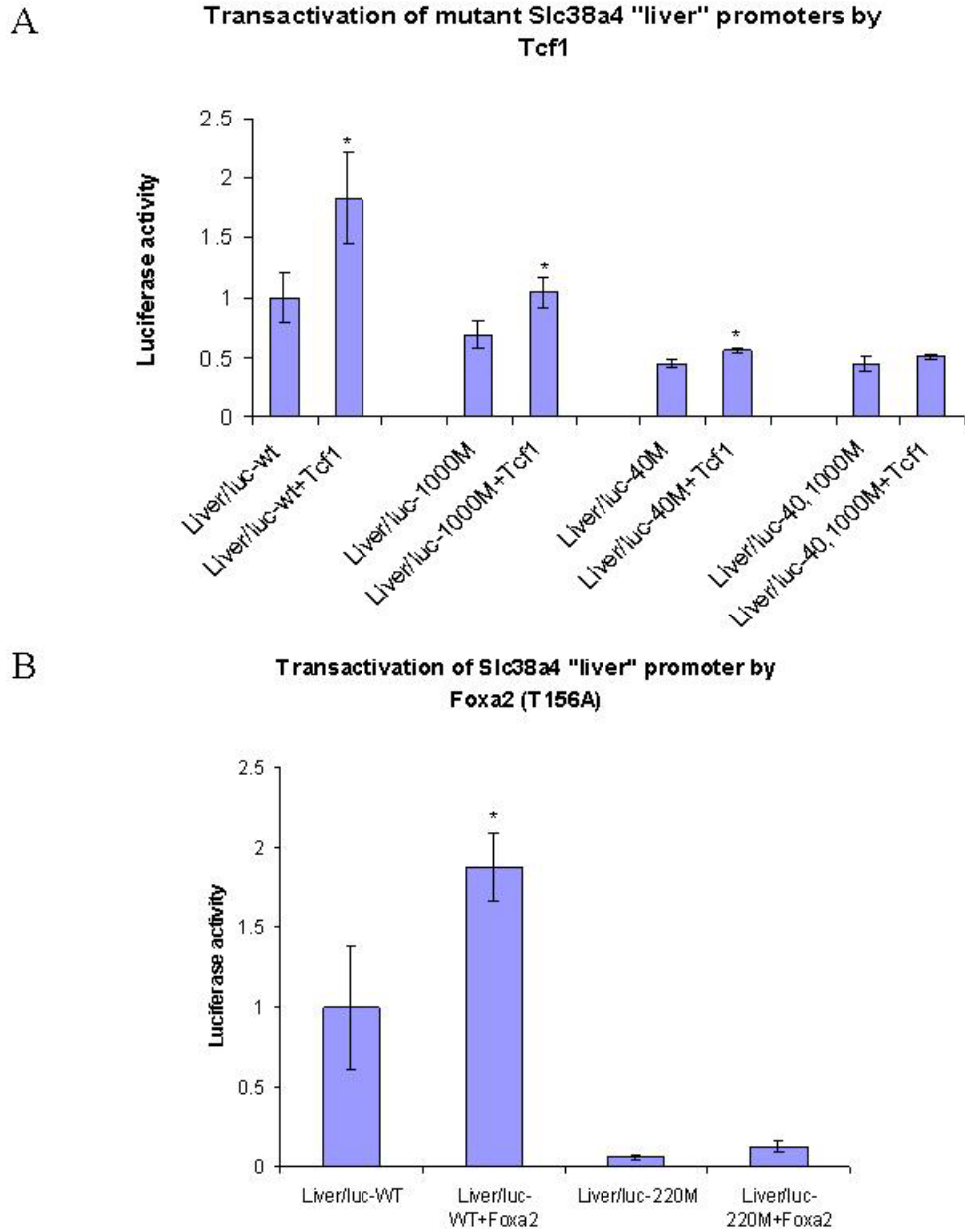


Figure 30. Mutation of the Tcf1 and Foxa2 sites leads to decreased transactivation by both transcription factors in a luciferase reporter gene assay. Reporter gene plasmids were generated with 1200 bases of the Slc38a4 "liver" promoter sequence, with and without mutations at the putative Tcf1 and Foxa2 binding sites. Reporter gene plasmids were co-transfected with either pcDNA3 (control) or expression vectors for either Tcf1 or Foxa2 (T156A). Equal amounts of a CMV-LacZ plasmid was also transfected to normalize for transfection efficiency. Luciferase activity was normalized for β-galactosidase activity for all experiments. A. Slc38a4 "liver" promoter reporter gene plasmids cotransfected with Tcf1; B. Slc38a4 "liver" promoter reporter gene plasmids cotransfected with Foxa2. Data are represented as mean ± SE, n=6. *: p<0.05 by Student's t-test, comparing pcDNA-transfected samples with Tcf1- or Foxa2-transfected samples.

We found that mutating the putative Tcf1 binding site at position –1000 or –40 significantly decreases the level of transactivation by Tcf1 (Figure 30A). When we mutate both sites, we eliminate all transactivation by Tcf1. We also found that mutating the Foxa2 binding site at position –220 also decreases the transactivation by Foxa2 in luciferase reporter gene assays (Figure 30B).

EMSA analysis was also performed using radioactively labeled oligonucleotides of all three of these putative binding sites in the Slc38a4 "liver" promoter, to determine whether Tcf1 or Foxa2 directly bind to these sites (Figure 31). We were able to see radioactive band shifts for the oligonucleotide probes for each site with the addition of Hepa 1-6 cell extract. With the addition of anti-Tcf1 and anti-Foxa2 antibodies, we were able to demonstrate that Tcf1 directly binds to the probe of the position –40 and –1000 Tcf1 binding sites, and that Foxa2 directly binds to the probe of the position –220 Foxa2 binding site. We also found a strong band shift with the –1000 Tcf1 binding site, and found that this is due to direct binding by Foxa2 by supershifting this band with anti-Foxa2 antibodies; however, through luciferase reporter gene assays, we found that this site is not important for Foxa2 transactivation, as mutation of the putative Foxa2 site does not affect luciferase expression. The specificity of the radioactive band shift by either Tcf1 or Foxa2 binding to the oligonucleotide probes was also demonstrated by the inhibition of this shift by the addition of cold probes containing known Tcf1 and Foxa2 sites.
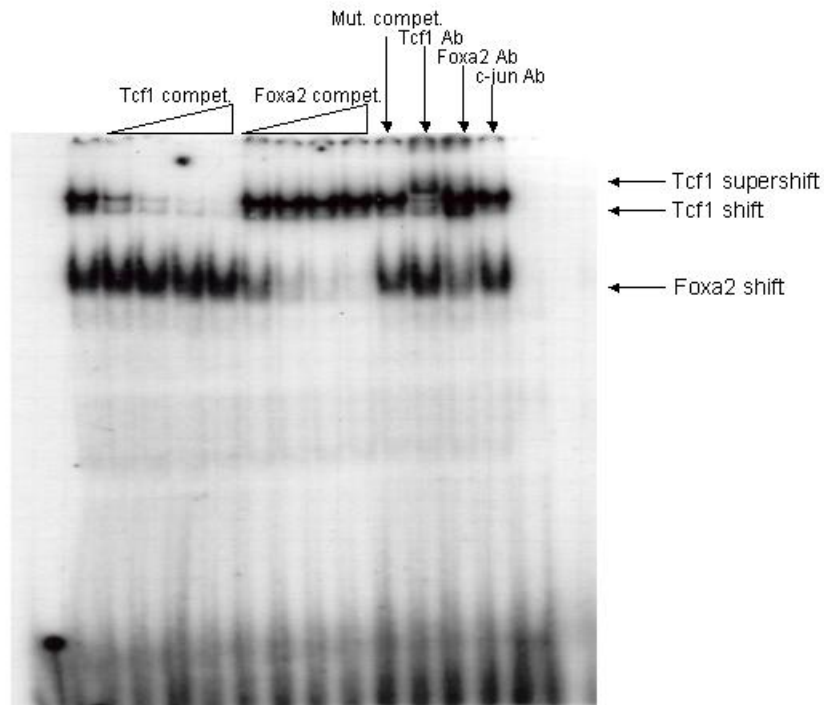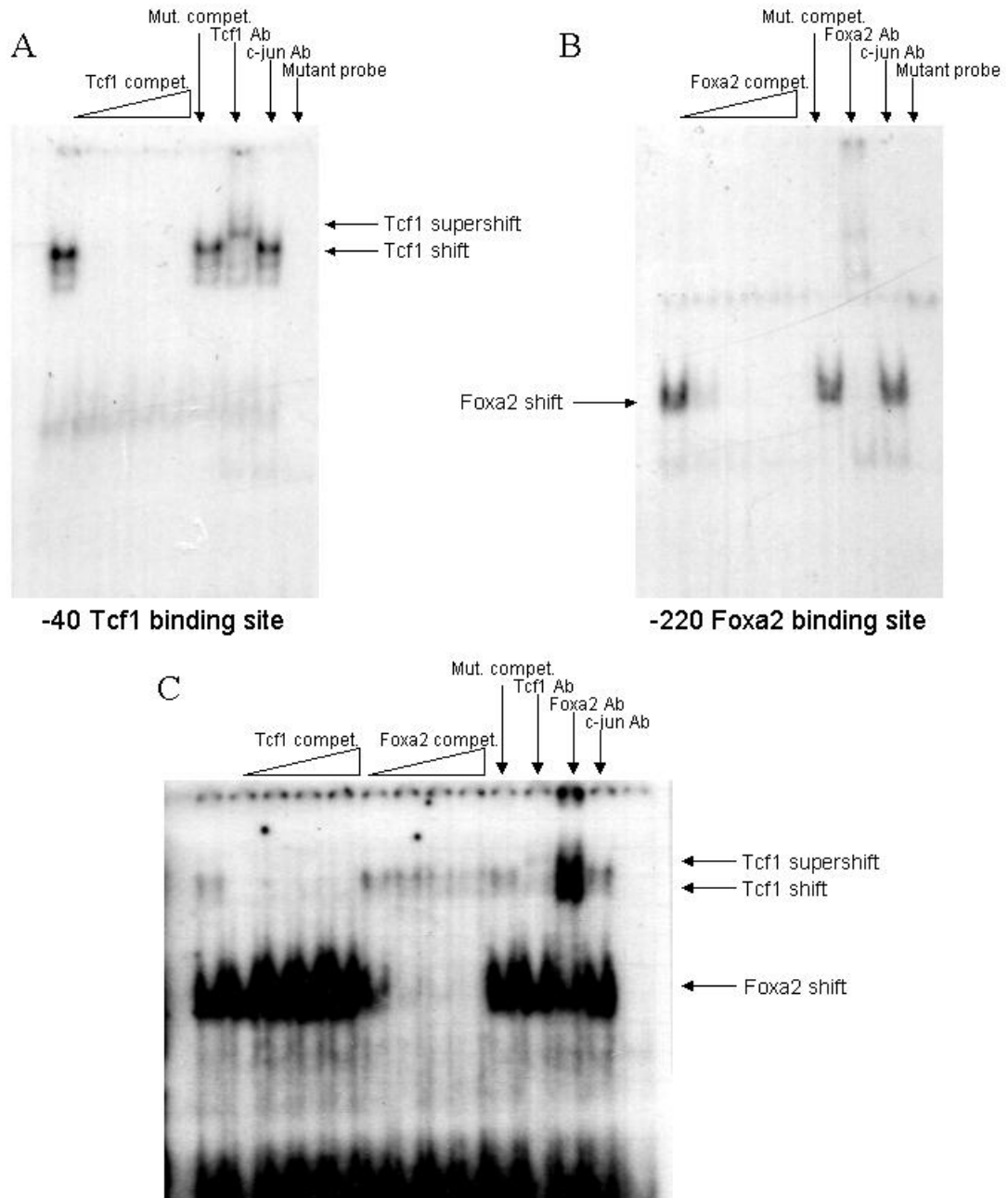
Figure 31. EMSA using oligonucleotides for the putative Tcf1 and Foxa2 binding sites in the Slc38a4 "liver" promoter. The Tcf1 competitor was a cold oligonucleotide containing a Tcf1 binding site from the Tmem27 mouse promoter (Akpinar et al., 2005). The Foxa2 competitor was a cold oligonucleotide containing the Foxa2 consensus binding site. The mutant competitor was a cold oligonucleotide of the original probe, with the binding site mutated. Poly-dIdC was also added to each reaction to quench non-specific binding to the oligonucleotides. A. Probe contains the putative Tcf1 binding site at position −40 in the "liver" isoform promoter; B. Probe contains the putative Foxa2 binding site at position −220; and C. Probe contains the putative Tcf1 binding site at position −1000.

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*5.8 Synergistic transactivation of Slc38a4 by Tcf1 and Foxa2*

We were interested to see if Tcf1 and Foxa2 have a synergistic effect on transactivation of Slc38a4. To determine this, we performed luciferase reporter gene assays, co-transfecting both Tcf1 and Foxa2 expression vectors with the reporter gene plasmids containing either the Slc38a4 "islet" or "liver" isoform promoters. We found significantly higher transactivation through both the Slc38a4 "islet" and "liver" isoform promoters with Tcf1 and Foxa2, than we found by co-transfecting either factor singly with the promoter-reporter gene plasmids (Figure 32). Thus, Tcf1 and Foxa2 are able to transactivate through both Slc38a4 promoters in a synergistic manner.

## Synergistic transactivation of Slc38a4 promoters by Tcf1 and Foxa2
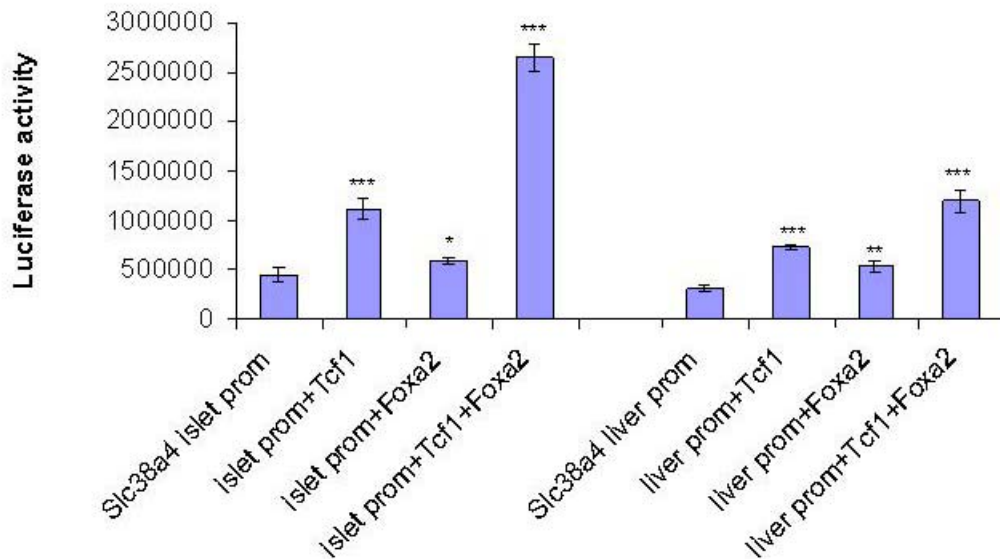


Figure 32. Tcf1 and Foxa2 act synergistically to transactivate through the Slc38a4 "islet" and "liver" isoform promoters. Promoter-reporter gene plasmids were co-transfected with expression vectors of Tcf1 and Foxa2 either singly, or together. Equal amounts of a CMV-LacZ plasmid was transfected in each experiment to normalize for transfection efficiency. Luciferase activity was normalized by β-galactosidase activity. Data are represented as mean ± SE. *: $p<0.05$; **: $p<0.01$; and ***: $p<0.001$ by Student's t-test, comparing pcDNA3-transfected samples with Tcf1- or Foxa2-transfected samples.

### 5.9  In vivo binding of Foxa2 to the Slc38a4 promoters

We wanted to see whether Foxa2 binds to the Slc38a4 promoters in vivo. We performed chromatin immunoprecipitation analysis on liver tissue from C57Bl/6J and *ob/ob* mice infected with either Foxa2 (T156A)-adenovirus or GFP-adenovirus.  Through chromatin immunoprecipitation with an anti-Foxa2 antibody, we found that Foxa2 interacts with the promoters of both Slc38a4 isoforms in fasted C57Bl/6J mice and the *ob/ob* mice infected with the Foxa2

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(T156A)-adenovirus (Figure 33). However, Foxa2 does not interact with these promoters in *ob/ob* mice infected with the GFP-adenovirus. In addition, Foxo1, a related transcription factor, does not interact with either promoter in any of the conditions tested, as we are unable to amplify any of these promoter regions after chromatin immunoprecipitation with Foxo1.
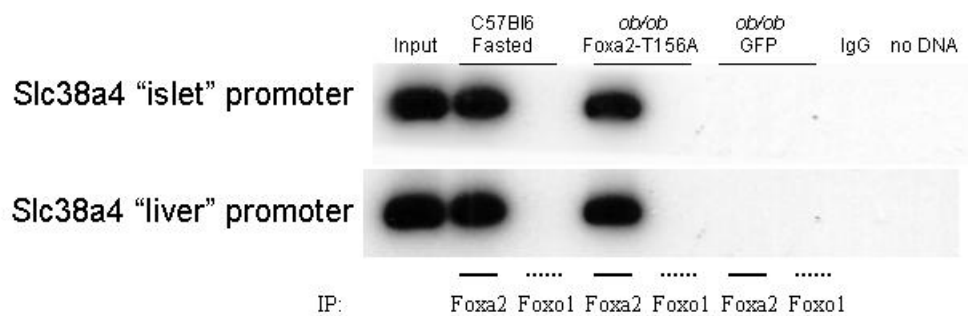


Figure 33. Foxa2 binds to the Slc38a4 promoters in vivo. Chromatin immunoprecipitation analysis was performed on liver tissue from C57Bl/6J and *ob/ob* mice infected with either Foxa2 (T156A)-adenovirus or GFP-adenovirus, using either anti-Foxa2 or anti-Foxo1 antibodies, or rabbit IgG control. Foxa2 was found to interact with both Slc38a4 isoform promoters in the liver cells of C57Bl/6J mice and *ob/ob* mice infected with Foxa2-adenovirus. Foxa2 does not interact with these promoters in the GFP-adenovirus-infected *ob/ob* mice. Foxo1 does not interact with these promoters in any of these conditions.

*5.10 Expression of Foxa2 (T156A) increases arginine uptake by perfused mouse livers*

Since we had shown Foxa2 regulation of an amino acid transporter gene, we wanted to demonstrate that Foxa2 activation does indeed lead to a change in amino acid uptake in vivo. For this experiment, we infected C57Bl/6J mice with either adenovirus that expresses the constitutively active Foxa2 (T156A) or GFP-

adenovirus as a control. We perfused the liver of these mice with Krebs-
Henseleit buffer containing ^{3}H-labeled arginine to assay the uptake of arginine
by these livers. We found significantly increased uptake of arginine by the livers
of mice infected with the Foxa2 (T156A)-adenovirus (Figure 34).

### Arginine uptake by perfused livers
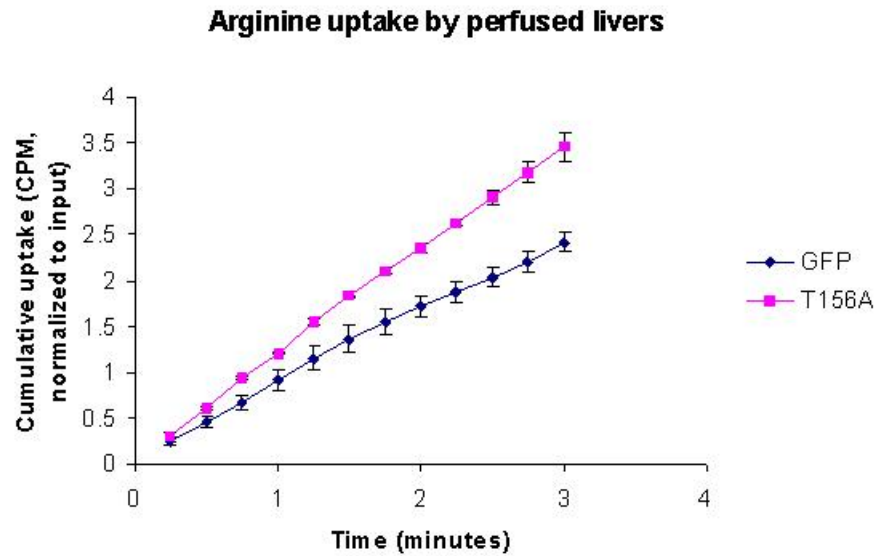


Figure 34. Foxa2 leads to increased arginine uptake by perfused mouse livers. Mice were infected with
either Foxa2 (T156A)-adenovirus or GFP adenovirus. Livers were perfused with Krebs-Henseleit buffer
containing 5mM glucose, 20ng/mL insulin, and 300μM 3H-arginine. Uptake was measured by
subtracting the radioactivity of the outflow from the input. Cumulative uptake was normalized to the
CPM of the input solution for each experiment. Data are represented as mean ± SE, n=3.

# CHAPTER 6 – DISCUSSION PART II

## 6.1 *Alternative splicing as a mechanism for differential regulation of Slc38a4*

In this study, we examined the regulation of the Slc38a4 gene, which we had previously identified as being highly regulated by the transcription factor, Tcf1, in pancreatic islets.  We found that Slc38a4 was also regulated by Tcf1 in liver tissue as well; however, the level of regulation in the liver was less than that observed in the pancreatic islets.  We identified alternative transcriptional start sites leading to two transcript isoforms for Slc38a4.  While the liver expresses both Slc38a4 transcripts, islet tissue expresses only one transcript.  Furthermore, while both transcripts were found to be positively regulated by Tcf1, the "islet" transcript was more strongly upregulated by Tcf1.  We believe that these data together suggest that the differential expression of these two transcript isoforms and their differences in Tcf1 regulation may be the basis of the regulatory differences observed between the liver and islet expression of Slc38a4.

The existence of alternative transcripts for the Slc38a4 gene is interesting, however not entirely surprising.  Recent studies show that alternative splicing seems to be the rule for human genes rather than the exception, which explains how the number of human proteins outnumbers that of genes several-fold. Increasingly more studies have shown how alternative transcripts may also mediate tissue-specific regulation.  The regulation of Slc38a4 in the pancreatic beta-cell and in the liver that we have observed is such an example.  Yet another

example is the HNF4α gene.  HNF4α gene expression in pancreatic endocrine and exocrine cells is dependent on Tcf1, while HNF4α expression is not affected by the absence of Tcf1 in the liver.  Similar to our findings with Slc38a4, it was shown that HNF4α has two alternate tissue-specific promoters, one of which is mainly expressed in the islet and is highly regulated by Tcf1, while the other is expressed in the liver and is not affected by Tcf1 (Boj et al., 2001).

In addition to having distinct regulatory properties through the alternative promoters, the two Slc38a4 transcripts may have different functional properties conferred by their sequence differences.  The alternative transcripts of the Slc38a4 gene that we have identified do not differ in sequence in their translated regions and encode proteins of identical sequence.  However, it is possible that these transcripts may still differ in their gene product activity through their sequence differences within the 5'-untranslated regions (UTR).  An intriguing example of activity regulation through the 5'-UTR is the Slc7a1 gene, which encodes the Cat1 amino acid transporter.  It has been shown that the mRNA for the Slc7a1 gene contains an internal ribosome entry site (IRES) in its 5'-UTR (Fernandez et al., 2001).  With amino acid starvation, general cap-dependent protein synthesis is inhibited (Pain, 1994); however, the IRES-mediated translation of the Slc7a1 mRNA increases under conditions of amino acid starvation, providing a mechanism by which cells may preferentially translate only mRNAs that are essential for cell survival during periods of limited nutrient supply (Fernandez et al., 2001).

It would be interesting to test the hypothesis that the 5′-UTRs of the two alternative transcripts of Slc38a4 may yield different translational properties. For example, one may perform similar studies as Fernandez et al. (2001), and clone the two Slc38a4 5′-UTRs into bicistronic mRNA, where one reporter gene is translated through a cap-dependent scanning mechanism, while a second reporter gene encoded downstream of the 5′-UTR insert is translated only if it is preceded by an IRES. Differences in 5′-UTR function may also be elucidated with cell lysate fractionation to purify polysomes and the transcripts associated with the polysomes. One may then perform RT-PCR to determine the presence of either the "islet" or "liver" Slc38a4 transcripts to see if there is a difference in their association with the ribosomes. These studies would elucidate potential differences in transcript interactions within the 5′-UTRs that may have translational consequences.

While increasingly more examples of alternative transcripts and promoters are being discovered to mediate tissue-specific expression and regulation, it is still unclear what mechanism causes a transcript to be predominantly expressed in one tissue rather than another. Using the Slc38a4 gene as an example, the complete absence of the "liver" transcript in pancreatic islet tissue is a striking observation. It would be interesting to study potential mechanisms for this phenomenon, particularly the possibility of differences in histone structure in the two different tissues. This may be elucidated with additional chromatin immunoprecipitation assays with antibodies to acetylated histones and with

antibodies to un-acetylated histones to test for binding of the "islet" and "liver" promoter sequences in the two tissues. It is possible that differences in histone structure and modification may be a general mechanism for tissue-specific transcript silencing.

*6.2 Regulation and function of Slc38a4 in the liver*

In this study, we have also shown that Slc38a4 expression is upregulated in the liver of overnight fasted mice, and that this upregulation is inhibited by insulin. Through in vitro and in vivo experiments, we have also demonstrated that Slc38a4 is a direct target of the transcription factor, Foxa2, as well as Tcf1. Furthermore, we have shown that Foxa2 overexpression in the liver can increase arginine uptake in liver perfusion studies, thus correlating the increase in Slc38a4 gene expression with increase Slc38a4 transporter activity.

Our study is the first to identify an insulin-regulated transcription factor to regulate a member of the Slc38a family. Previous work by Gu et al. (2005) had shown regulation of Slc38a3 to also be negatively regulated by insulin on a molecular level via the PI3-kinase pathway. It is possible that Foxa2 may mediate the insulin-sensitive gene expression of Slc38a3, as well as other transporter genes.

We have found that both Tcf1 and Foxa2 regulate both alternative transcripts of Slc38a4, and that their transactivation is synergistic. We also showed that Tcf1 expression does not change with fed or fasted states, while it

has been shown that Foxa2 activity is dependent on the absence of insulin signaling. Our results suggest that while both transcription factors are important for Slc38a4 expression, they have distinct regulatory functions. Tcf1 plays a role mainly in mediating basal expression levels of Slc38a4, while Foxa2 modulates Slc38a4 expression with changes in the metabolic state of the organism and insulin levels.

Foxa2 has already been shown to mediate other metabolic pathways in the liver, dependent on the metabolic state of the organism. For example, it was demonstrated that Foxa2 mediates the increased hepatic fatty acid oxidation that occurs during fasting, which provides energy from fat stores for the organism while other energy sources are not available (Wolfrum et al., 2004). We have now shown that Foxa2 may also mediate the liver response to fasting by increasing amino acid uptake, which may be broken down to carbon skeletons for the generation of glucose, as well as used for protein renewal.

In the *ob/ob* and *Srebp-1c* mouse models, Foxa2 is permanently excluded from the nucleus and inactive, due to the hyperinsulinemia (Wolfrum et al., 2004). In contrast, a related forkhead family transcription factor, Foxo1, shows resistance to insulin and may remain in the nucleus driving transcription of its target genes. Foxo1 is normally responsible for increasing expression of genes involved in gluconeogenesis during fasting. Thus, development of Foxo1 insulin resistance in hyperinsulinemic states results in exacerbation of hyperglycemia from inappropriate activation of the gluconeogenic pathways. The insulin

sensitivity of Foxa2 in type 2 diabetic and hyperinsulinemic models may also contribute to the disease state: permanently inactive Foxa2 leads to decreased fatty acid oxidation, which leads to hepatic steatosis that may also increase insulin resistance. However, in this study, we have shown another arm of Foxa2 function that may help alleviate the type 2 diabetic state. With hyperinsulinemia inactivating Foxa2, amino acid uptake will be lower, decreasing the amount of amino acid-derived substrates for gluconeogenesis. Thus, Foxa2 nuclear exclusion in the type 2 diabetic and hyperinsulinemic models helps to decrease the gluconeogenesis that is being inappropriately driven by insulin-resistant Foxo1.

# CHAPTER 7 – EXPERIMENTAL PROCEDURES

## 7.1 Experimental animals

All animal models were housed in Laboratory of Animal Research Center (LARC), a pathogen-free animal facility at the Rockefeller University. The animals were maintained on a 12-hour light/dark cycle and fed a standard rodent chow. Genotyping of transgenic mice was performed on DNA isolated from three-week old mice by PCR (Lee et al., 1998).

## 7.2 Cell culture

MIN6 cells were cultured with DMEM medium containing 25mM glucose, 15% fetal bovine serum, and 5μM 2-mercaptoethanol. COS-7, Hepa 1-6, and HepG2 cells were cultured with DMEM medium containing 25mM glucose and 10% fetal bovine serum. Collagenized plates were used for culturing HepG2 cells. All cells were maintained in a humidified incubator at 5% $CO_2$.

## 7.3 In vitro radioactive ligand uptake assay

MIN6 monolayers were plated at a concentration of 500,000 cells/well in six-well plates and assayed for arginine uptake the following day. On the day of the assay, MIN6 monolayers were washed and pre-equilibrated in control Tyrode's solution (135mM NaCl, 5mM KCl, 1.8mM CaCl2, 1mM MgCl2, 10mM Hepes, and 5.6mM glucose, pH 7.4) at 37°C for 30-40 minutes. To assay arginine

uptake, the cells were incubated with Tyrode's solution containing $^3$H-arginine for a fixed amount of time. Cells were then washed twice with ice-cold control Tyrode's solution and lysed with RIPA buffer (50mM Tris, 1% NP-40, 0.25% Na-deoxycholate, 150mM NaCl, 1mM EDTA, pH 7.4) for 20 minutes on ice. Cell lysates were measured for $^3$H radioactivity content as well as protein content for normalization. For ion dependence experiments, the Tyrode's buffer used for uptake assays was made substituting either Na with choline, or Cl with gluconate. For the uptake experiments, all incubations and washes were timed to the second.

Assays for the mNAT3 protein transport activity were performed in COS-7 cells. COS-7 cells were transfected using Fugene 6 at 50% confluency in six-well plates with either mNAT3 expression vector or with pcDNA3.1 (Invitrogen) as a control empty vector. Radioactive ligand uptake assays were performed two days post-transfection. Uptake assays were performed as described for MIN6.

## 7.4 *Pancreatic islet isolation*

Pancreatic islets were isolate from 6 to 8-week old mice. The mouse pancreas was first perfused with Hank's Buffered Salt Solution (HBSS) through the bile duct. Perfused pancreata were dissected out and placed in a solution of HBSS containing 1-1.5mg/mL collagenase, type XI (Sigma) and digested at 37$^o$C for 10-12 minutes. Digested pancreata were washed two times with HBSS. The tissue was then applied to a Ficoll gradient (25%, 23%, 20.5%, and 11% layers)

and centrifuged at 800 x g for 10 minutes.  Islet tissue at the interface between the 23% and 20.5% layers was taken and washed once with HBSS before being resuspended in culture medium.  Islets were cultured overnight in RPMI medium containing 11.1mM glucose and 10% fetal bovine serum.  Total RNA was extracted from picked islets using TRIzol reagent (Gibco-BRL).

For experiments examining gene expression in the islets during fasting and fed states, pancreatic islets were isolated as above.  However, islets were resuspended in cold HBSS following the final HBSS wash, and never cultured in the RPMI medium.  Islets were then picked directly from the HBSS suspension and used immediately for RNA extraction with TRIzol.

## 7.5  Gene expression array analysis

RNA was pooled from 20 Tcf1 +/+ and 30 Tcf1 -/- mice.  A small aliquot of each sample was analyzed on a denaturing formaldehyde gel to assess RNA sample integrity.  RNA was purified using RNeasy columns (Qiagen), and then used for cDNA synthesis using the Superscript Choice cDNA Synthesis Protocol (GibcoBRL), except that an HPLC-purified T7-Promoter-dT24 primer (Genset) was used to initiate the first-strand reaction.  Biotin-labeled cRNA was synthesized from T7-cDNA using the RNA transcript labeling kit Bio Array (Enzo), supplemented with biotin 11-CTP and biotin-16-UTP (Enzo) as specified in the Affymetrix technical manual.  The sample was cleaned with RNeasy columns (Qiagen) to remove free nucleotides and quantitated

spectrophotometrically. Biotin-labeled cRNA was fragmented in 40mM Tris (pH 8.0), 100mM KOAc, and 30mM MgOAc for 30 minutes at 94°C. Hybridization samples were prepared according to the Affymetrix manual with a final concentration of fragmented cRNA of 0.05µg/µL.

Affymetrix Genechip MG-U74A, MG-U74B, and MG-U74C probe arrays were prehybridized with 100mM MES (Sigma) pH 6.6, 1M[Na+], 20mM EDTA, and 0.01% Tween-20 (Sigma) for 10 minutes at 45°C. Hybridization samples were heated to 99 C for 5 minutes, equilibrated to 45 C for 5 minutes, and centrifuged at 14,000 x g for 5 minutes to sediment any particulate matter. The prehybridization solution was removed and the sample was added to the probe array and allowed to hybridize for 16 hours, rotating at 60 rpm at 45 C. Probe arrays were subsequently washed and stained according to the Affymetrix technical manual in an Affymetrix fluidics station. The arrays were scanned using a Hewlett-Packard confocal laser scanner and visualized using Affymetrix Genechip 3.1 software. Comparison of gene expression levels between the different groups were carried out using software Microarray Suite 4.0.

*7.6  RNA interference*

Synthetic siRNAs were synthesized by Proligo. Sequences were designed to target Slc38a4 and Slc7a3. The sequences targeting Slc7a3 are: siRNA1 5′-GUAUUGUGAUCUCGAUGUUTT-3′, and siRNA2 5′-GCGUUCUCAUCCUCA GAUATT-3′. The sequences targeting Slc38a4 are: siRNA1 5′-GAUGCCGAAA

GUCAGAAGUTT-3', and siRNA2 5'-CCAUCUACAGCGAGCUUAATT-3'. siRNAs were transfected into MIN6 cells by electroporation using the Nucleofector Kit V (Amaxa), according to the manufacturer's protocol. For electroporation into $3\text{-}5\text{x}10^6$ cells, 0.5 nmoles of siRNA was used.

### 7.7 Protein immunoblotting

We prepared cell extracts for immunoblotting by lysing in RIPA buffer for 20 minutes on ice, and centrifuging at 13,000 x g to remove debris. Samples were loaded onto SDS-glycine 4-15% polyacrylamide gradient gels (Biorad) and transferred onto a nitrocellulose membrane (Schleicher & Schuell) by electroblotting. Membranes were first incubated with TBS with 0.1% Tween 20 (Sigma) and 5% milk for one hour at room temperature. Membranes were then incubated with primary antibodies overnight at 4°C. The antibody to mNAT3, the Slc38a4 gene product, (a generous gift from Dr. J. X. Jiang, Department of Biochemistry, University of Texas Health Science Center) was used at a concentration of 1:3000. We used anti-gamma-tubulin antibody (Sigma) as a loading control at a concentration of 1:10,000. Membranes were then washed three times for five minutes each with TBS with 0.1% Tween 20 (TBST) before incubating with TBST with 5% milk and either anti-mouse (for gamma-tubulin detection) or anti-rabbit (for Slc38a4 protein detection) HRP-conjugated antibodies (Calbiochem).

*7.8  In vitro insulin secretion and measurement*

MIN6 cells were washed once and preincubated for 30 minutes in Krebs Ringer Buffer (KRB; 135mM NaCl, 1.5mM CaCl$_2$, 0.5mM KH$_2$PO$_4$, 3.6mM KCl, 0.5mM MgSO$_4$, 2mM NaHCO$_3$, 10mM Hepes, 0.1% BSA, pH 7.38) containing 2.5mM glucose.  Cells were then washed once and incubated for one hour with KRB containing 3.3mM glucose for basal insulin secretion measurements, 3.3mM glucose plus 20mM arginine or leucine, or 25mM glucose for stimulated insulin secretion measurements.  Insulin secretion assay samples were taken from each well following the incubation and centrifuged for five minutes at 400 x g to pellet any loose cells in the sample.  The supernatant was carefully taken off and measured for insulin content using an insulin radioimmunoassay kit (Linco Research).

*7.9  Vectors*

The mNAT3 expression vector was generated by cloning the cDNA for Slc38a4 (Genbank accession no. BC024123) into the pcDNA3.1 vector (Invitrogen).  The reporter plasmid Islet/luc-wt was generated by first performing PCR to amplify a 600-bp fragment of the promoter for the Slc38a4 "islet" isoform (Genbank accession no. AY027919), using primers with the sequences TGCTCACTATGTTCTTAGCAGGTG and GGGTCTTTTAAGCTGA TTGCACGCC.  The amplified fragment was cloned into the vector, pCR®II-TOPO using the TOPO TA cloning kit from Invitrogen, according to the

manufacturer's instructions. Plasmids with the promoter fragment in a forward orientation were selected after sequencing. The insert was then cut out of the pCR®II-TOPO vector with the restriction enzymes *Kpn* I and *Xho* I and ligated into the pGL2-Enhancer vector (Promega) cut with the same enzymes.

The reporter gene plasmid Islet/luc-mut is identical to Islet/luc-wt, except that the sequence at position –74 to –65 has been mutated from AATAATTTAC to GTGTATCTCT. The reporter plasmid Liver/luc-wt was generated by first performing PCR to amplify a 1200-bp fragment of the promoter for the Slc38a4 "liver" isoform (Genbank accession no. BC024123), using primers with the sequences GAAAGATGACAATTAAGCAGCCAA and CTGAACTTTTTGGAA AGCAAGACA. The amplified fragment was cloned into the pCR®II-TOPO vector using the TOPO TA cloning kit from Invitrogen. The insert was then cut out of the pCR®II-TOPO vector with the restriction enzymes *Kpn* I and *Xho* I and ligated into the pGL2-Enhancer vector (Promega) cut with the same enzymes. The reporter gene plasmids with mutated Tcf1 binding sites in the "liver" promoter are identical to Liver/luc-wt except that Liver/luc-1000M has the sequence from -1000 to -992 changed from TTAATAAAT to CCAATAAGG, Liver/luc-40M has the sequence from -45 to -42 changed from ATTG to CGCG, and Liver/luc-40,1000M has both of the previously described sequence exchanges. The reporter gene plasmids with mutated Foxa2 binding sites in the "liver promoter" are also identical to Liver/luc-wt except that Liver/luc-220M

has the sequence from –225 to –209 changed from AAAGCCAAAATTCCAAA to CCAGCCACCATTCCACC.

## 7.10  Transient transfections and luciferase assay

Fugene 6 reagent (Roche) was used to transfect HepG2 cells, according to the manufacturer's directions.  The total transfected DNA amount per well in a six-well plate was 0.5µg, using equal amounts of the luciferase reporter construct, transcription factor expression vector, and CMV-LacZ plasmid. Luciferase activity was measured two days following transfection and was normalized for transfection efficiency, assayed by β-galactosidase activity (Shih et al., 2001).

## 7.11  Whole cell extract preparation for electrophoretic mobility shift assays

HepG2 cells were grown to confluency in 150mm tissue culture dishes. Cells were washed once with ice-cold PBS and scraped into 3mL PBS.  Cells were centrifuged at 4,000 x g for four minutes and resuspended in two volumes of high-salt extraction buffer consisting of 400mM KCl, 20mM Tris pH 7.5, 20% glycerol, 2mM DTT, 1x Complete TM Protease Inhibitors (Boehringer Mannheim), and 20ug/mL aprotinin (Sigma).  Cell lysis was performed by freezing and thawing.  Cellular debris was removed by centrifugation at 15,000 x g for ten minutes at 4°C.  Whole cell extracts were stored at -80°C.

*7.12 Electrophoretic mobility shift assay (EMSA)*

EMSA analysis was performed as described previously (Shih et al., 2001) with minor modifications. Each assay contained 10μg of whole cell extracts and 2μg poly-dIdC (Sigma), sonicated to be 200-400 basepairs in length, in binding buffer (20mM Hepes pH 7.9, 10% glycerol, 150mM NaCl, 1mM DTT). Whole cells extracts were incubated with $^{32}$P-labeled double-stranded oligonucleotide probes containing the wild-type or mutant Tcf1 or Foxa2 binding sites in Slc38a4 promoters. For the Slc38a4 islet promoter Tcf1/Foxa2 binding site, probe sequences were: WT 5′-TCTTCCTTTGTCAATAATTTACTTTTGT, and Mut 5′-TCTTCCTTTGTCGTGTATCTCTTTTTGT-3′. For the Slc38a4 liver promoter Tcf1 binding sites, probe sequences were: -1000 WT 5′-ATCTCAGAGTTAATAAA TCAAATGGGCACAA-3′, -1000 Mut 5′-ATCTCAGAGCCAATAAGGCAAATG GGCACAA-3′, -40 WT 5′-GGTCTTTAAATCATTGTCTTGCTTTCCA-3′, and –40 Mut 5′-GGTCTTTAAATCCGCGTCTTGCTTTCCA-3′. For the Slc38a4 "liver" promoter Foxa2 binding sites, probe sequences were: -220 WT 5′-GCCAAAATTCCAAAAGCAAATAGAAATT-3′, and -220 Mut 5′- GCCACCA TTCCACCAGCAAATAGAAATT-3′. The cold competitor for Tcf1 binding was an unlabeled double-stranded oligonucleotide containing the Tcf1 binding site in the TMEM27 promoter (sequence: 5′-GGAGATTTTCGTAAATAACTGACA-3′; Akpinar et al., 2005). The cold competitor for Foxa2 binding was an unlabeled double-stranded oligonucleotide containing the Foxa2 consensus site (sequence: 5′-GTTGACTAAGTCAATAATCAGAATCAG-3′). Supershifts were carried out

with an anti-Tcf1 antibody (Geneka Biotechnology Inc.) or an anti-Foxa2 antibody (generous gift from Dr. J. E. Darnell).  As a control, anti-c-jun antibody was used (Santa Cruz Biotechnology).

For each reaction, 10μg whole cell extract was first incubated at room temperature for ten minutes in binding buffer with 2μg poly-dIdC.  Cold inhibitors and antibodies were then added to the reactions, and all samples were incubated for another five minutes at room temperature.  Radioactive probes were finally added to each reaction, and the samples were incubated for another 15 minutes at room temperatures.  Samples were then loaded onto a 4% polyacrylamide gel (0.25X TBE) and run at 200-250V.


## 7.13  *Chromatin immunoprecipitation*

Chromatin immunoprecipitation was performed on liver tissue from C57Bl/6J mice and *ob/ob* mice that had been infected with either Foxa2 (T156A)-adenovirus or GFP-adenovirus ($10^{11}$) via tail vein injection ten days prior to the experiment.  Liver tissue was homogenized in phosphate-buffered saline (PBS; Sigma) containing 1x Complete TM Protease Inhibitors (Boehringer Mannheim), and cross-linked with 1% formaldehyde for ten minutes at room temperature.  Cross-linking was stopped with the addition of glycine to a final concentration of 0.125M.  Cells were washed twice with PBS containing protease inhibitors.  The Chromatin Immunoprecipitation Assay Kit from Upstate Cell Signaling Solutions (Lake Placid, NY) was used for the rest of the procedure, according to

the manufacturer's instructions. Anti-Foxa2 antibody and anti-Foxo1 antibody (Cell Signaling) were used for immunoprecipitation. Primers were used to amplify the Slc38a4 "islet" promoter region from –180 to –1, and the Slc38a4 "liver" promoter region from –230 to –1.

### 7.14 *Liver perfusion and radioactive ligand uptake assay*

Mice were infected with either Foxa2 (T156A)-adenovirus or GFP-adenovirus ($10^{11}$ particles per mouse) ten days before the perfusion experiment. Mice were fasted overnight, and anesthetized with intraperitoneal injection of pentobarbitone sodium (60mg/kg body weight). The portal vein and the inferior vena cava were cannulated. The liver was perfused with Krebs-Henseleit (KH) buffer (119 mM NaCl, 4.7 mM KCl, 2.5 mM $CaCl_2$, 1.2mM $MgSO_4$, 1.2mM $KH_2PO_4$, 25mM $NaHCO_3$, 5mM glucose) with 20ng/mL insulin, equilibrated with 95% $O_2$/5% $CO_2$. The rate of perfusion was maintained at 1 mL/minute. Once blood was flushed out of the liver with KH buffer, we began a timed perfusion with KH buffer with 20ng/mL insulin and 300uM arginine labeled with $^3$H. The outflow was collected and radioactivity levels were measured. Uptake was measured by subtracting the CPM from the outflow from the input perfusion buffer CPM. Cumulative uptake was normalized by the radioactivity of the input perfusion buffer.

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