Effects of placental mTOR alteration on the pancreatic beta cell mass of embryonic day 17.5 mice

A Thesis

SUBMITTED TO THE FACULTY OF THE UNIVERSITY OF MINNESOTA

BY

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IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF

MASTER OF SCIENCE IN STEM CELL BIOLOGY

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May 2022

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ACKNOWLEDGEMENT

I thank Dr. Emilyn Alejandro (University of Minnesota), my project advisor. I'll start by stating, if "above & beyond" was a person, Dr Alejandro would be this person. She has such an admirable zeal and drive for the work she does as a scientist and mentor, and I do not regret joining her lab. Immediately I joined her lab in January 2021, we established defined expectations to help achieve the goals and deadlines we set. Watching and learning from her over the months, and seeing how she enjoys her work, has greatly inspired me, and ignited my passion to make impact through medical research. She has pushed me to be a better scientist and person. Her mentoring, recommendations and guidance have been nothing short of excellent. I truly am honored to work with her!

I thank the members of the Alejandro lab, namely, Brian Akhaphong, Alicia Wong, Megan Beetch, Seokwon Jo, Eric Gustafson, Grace Chung, and Briana Clifton, for their training, intellectual contributions, and assistance in the lab throughout this project. The lab has been a place of intellectual, technical, and personal growth for me, and I can say that it's been a pleasure to work with each of these lab members.

I acknowledge the Integrative Biology and Physiology (IBP) department and the University of Minnesota Imaging Centers (https://med. umn.edu/uic) for imaging and microscope training, which allowed me to obtain many of the images in this thesis.

I thank God for strength and ability to carry out this research. I couldn't have gone through the years without Him, and for this, I am ever grateful. I thank my immediate family [Abigail (my sister), Isaac (my brother), Nene (mum) and Bayode (dad) Oribamise], my extended family, and my friends from Nigeria and the USA for their never-ending support and encouragement throughout this project.

I thank the Regenerative Medicine Minnesota (RMM) for funding this project.

DEDICATION

I dedicate this thesis to the many patients suffering from Type 2 diabetes. It is truly a global menace that needs to be well-studied and understood in order to develop a cure for it. I also dedicate my thesis to researchers all over the world that have spent years attempting to manage and develop cures for Type 2 diabetes. Your works have greatly contributed to the current knowledge and therapies being developed.

ABSTRACT

The fetal environment during development is a strong determinant for health in adulthood. Pancreatic beta cells development *in-utero* has an important role in the predisposition to Type 2 Diabetes (T2D). The pancreatic endocrine cells are highly sensitive to nutrient flux during development and very early in life. Clinical studies suggest that signaling of mechanistic target of rapamycin (mTOR) in the placenta regulates fetal birth weight and the offsprings' metabolic course, partly by modulating maternal to fetal nutrient flux.

This study aims at exploring the link between placental mTOR activity and beta cell mass at embryonic day (e) 17.5, a gestational period in which beta cells become more abundant in the pancreatic islets. Pancreatic beta cells are known to be impacted by amino acid (AA) levels during development. *We hypothesized that alteration of mTOR in the placenta will affect AA transporters (which are essential for amino acid transport for beta cell growth in-utero), thus affecting the gestational growth and development of beta cells in the offspring.* Within beta cells, AAs promote mTOR signaling, which regulates cell growth, proliferation, and protein synthesis. We tested our hypothesis by genetically deleting mTOR or activating mTORC1, via deletion of its negative regulator, Tuberous Sclerosis Complex (TSC2), in placental trophoblast cells using placental-specific Cre recombinase, and by characterizing the effects of placental mTOR on pancreas morphology at e17.5. Interestingly, we revealed that the placental-specific knock out of mTOR led to an increase in protein expression of amino acid transporter SNAT-1 and beta cell mass in e17.5 female offspring. However, the placental-specific knock out of TSC2 showed no effect on total beta cell mass of e17.5 embryos. These results suggest that placental mTOR may regulate AA flux to the growing pancreas, but additional experiments are needed to better understand amino acid placental functions and its effects on pancreatic progenitors and beta cell development.

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LIST OF ABBREVIATIONS

mTOR: mechanistic target of rapamycin

TSC2: Tuberous Sclerosis Complex 2

DM: Diabetes Mellitus

AA: Amino acid

SNAT: Sodium-coupled Neutral Amino acid Transporter

LAT: L-type amino acid transporter

HSP90: heat shock protein 90

T2D: Type 2 Diabetes

EMT: epithelial-mesenchymal transition

Wnt: Wingless-related integration site

BMP: Bone Morphogenetic Protein

FGF: Fibroblast growth factor

IUGR: Intrauterine growth restriction

GFP: Green Fluorescent Protein

CYP19: cytochrome P450 enzymes

PCR: Polymerase chain reaction

DNA: Deoxyribonucleic Acid

WT: Wild type

KO: Knock out

ATP: adenosine triphosphate

Rheb: Ras homolog enriched in brain

GAP: GTPase-activating protein

Pdx1: Pancreatic and duodenal homeobox 1

AMPK: Adenosine monophosphate-activated protein kinase

1.0. CHAPTER 1: INTRODUCTION

1.1. Type 2 diabetes

Among a few other classifications, Diabetes Mellitus (DM) is classified into Type 1 diabetes (T1D), which is characterized by pancreatic beta cells' obliteration by the immune system, and Type 2 diabetes (T2D) which is a hyperglycemic state that is associated with insulin resistance and obesity. T2D accounts for 90-95% of all diabetes cases worldwide. It is the most frequently occurring type of diabetes, as greater than 34.2 million people in the United States of America (USA) and 350 million people worldwide are affected by this disease (CDC, 2020). In fact, T2D has been described as a worldwide epidemic. A robust study by Mayer-Davis *et al*. in 2017 investigated about 11,245 youths with T1D (0-19 years old) and 2846 with T2D (10-19 years old) from five research locations in the USA. Findings from this study reported that the relative annual increase in the incidence of T2D was 4.8%, compared to that of T1D, which was 1.8%, after adjusting for age, sex, and race or ethnicity (Mayer-Davis *et al*., 2017). Such epidemiologic studies shed light on the high possibility of further increase of T2D cases in the coming years, thus emphasizing the need for more studies to identify the risk factors for this disease.

Although T2D can be developed any time from childhood to adulthood (Lee *et al*., 2019, Carrillo-Larco *et al*., 2019), it has been mostly reported in middle-aged and older adults (CDC, 2020). Several symptoms have been reported to clinically manifest in patients with T2D, including mild symptoms like polydipsia, polyphagia, polyuria, and unhealing sores. These slowly progress over the course of years to more severe clinical manifestations including nephropathy, neuropathy, hyperglycemic diabetic coma, disorders of the immune systems, amongst a host of other chronic and acute complications (Lee *et al*., 2019). Many diabetes patients are also diagnosed with cardiovascular-related symptoms including diabetes-related heart disease, peripheral artery disease (PAD), coronary artery disease (CAD) (Carrillo-Larco *et al*., 2019). In fact, Einarson *et al*.'s review of 57 articles from North America, Western Pacific/China and Europe revealed that atherosclerosis was observed in 29.1%, heart failure in 14.9%, myocardial infarction in 10%, coronary heart disease in 21.2%, angina in 14.6% and stroke in 7.6% of the 4,549,481 T2D patients investigated; and overall, cardiovascular diseases were reported to affect about 32.2% and cause 9.9% of deaths among these T2D patients (Einarson *et al*., 2018). Another note-worthy manifestation of T2D is obesity, as T2D

patients have been reported to either be obese or have a higher percentage of body fat (Leitner *et al*., 2017). There is currently no cure for T2D, it is thus critical to explore modifiable risk factors that increase the probability of developing T2D. Animal studies in rodents have shown that the *in-utero* environment is important for pancreatic progenitor development and eventual adult beta cell mass (Petrik *et al*., 1999, Dumortier *et al*., 2007, Alejandro *et al*., 2014, 2020). The offspring of animals fed with low diets were shown to have reduced beta cell mass at birth, hyperglycemia and glucose intolerance in adulthood (Alejandro *et al*., 2014), insulin resistance with increased age (Alejandro *et al*., 2020), reduced insulin secretion in adulthood (Petrik *et al*., 1999), which are major manifestations of T2D. Furthermore, human studies such as those carried out by Barker *et al*., (Barker *et* al., 1993) showed that men and women with T2D in adulthood had reduced fetal growth and birthweight, which have been shown by animal studies of Intra-uterine growth restriction (IUGR) to be strongly correlated with reduced beta cell mass (Limesand *et al*., 2005). Such animal studies and human epidemiology studies have led to the fetal origins of health and disease which suggest that a subgroup of people have increased predisposition to T2D because of their exposure to impaired nutrient availability during development which result in reduced birth weight and very possibly reduced beta cell mass.

In T2D, loss of beta cell mass, as shown in **Figure 1**, leads to an impairment in the pulsatility and secretion of insulin, resulting in an interference in the crosstalk between glucagon and insulin within the islet, this further results in inadequate inhibition of glucagon release by pancreatic alpha cells. In addition, the reduced insulin pulsatility affects insulin's primary action of stimulating glucose uptake by cells of the adipose tissue and muscles and impairs insulin signaling in the liver, leading to increased glucose release into the circulation. This glucose secretion by the liver is exacerbated by the amplified glucagon concentrations. These then further result in hyperglycemia observed in T2D patients. It is important to note that there is a vicious cycle between beta cell mass, function, hyperglycemia and increased secretory demand for insulin production that is apparent in T2D. This emphasizes the need for studies that can identify factors that cause this reduced beta cell mass.

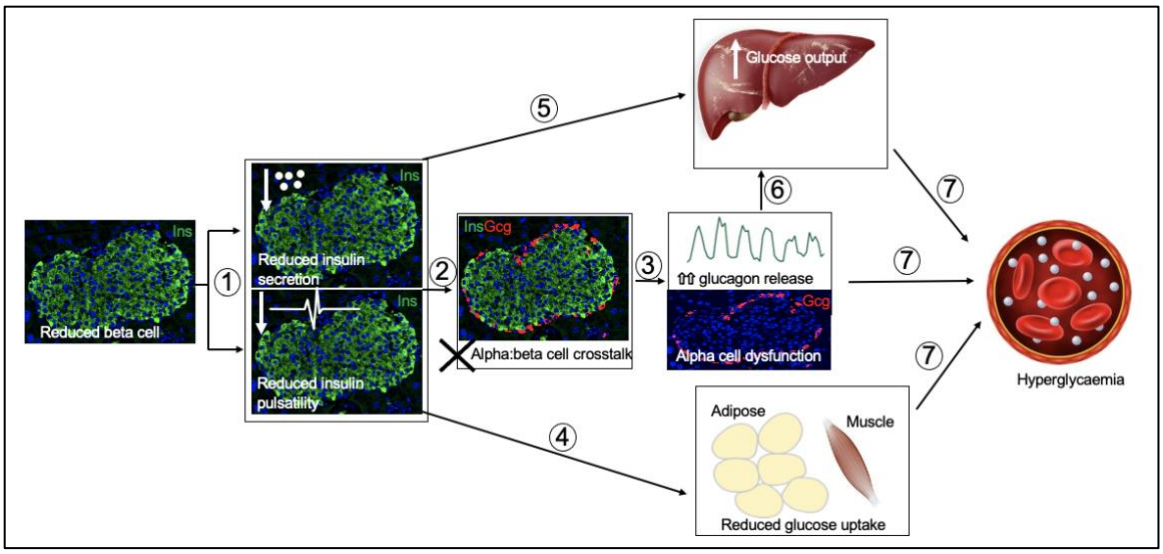

Figure 1: Reduced beta cell mass leads to hyperglycemia

Reduced beta cell mass results in impaired insulin pulsatility and secretion (2) This leads to an interruption in the insulin-glucagon crosstalk within the islet. (3) This disruption leads to inadequate inhibition of glucagon release by alpha cells. (4) In addition, this reduced insulin pulsatility affects insulin's primary action of stimulating glucose uptake by cells of the adipose tissue and muscles and (5) impairs insulin signaling in the liver, leading to increased glucose release by the liver. (6) The liver's glucose secretion is exacerbated by the amplified glucagon levels. (7) A combination of these ultimately results in hyperglycemia in type 2 diabetes patients. Schematic inspired by Meier & Bonadonna, 2013

1.2. Pancreas development in mice

The developmental periods of life are critical for the eventual growth of an organism. It is thus very important to appreciate the physiological developmental of the pancreas in mice. Thorough developmental biology studies of the endocrine pancreas, greatly assisted by gene deletion and overexpression studies, lineage tracing, fate-mapping studies, gene sequencing, cell sorting, and array studies in mice have greatly informed our understanding of the sequence of events controlling the differentiation of cells from the foregut endoderm into fully differentiated pancreatic beta cells during development (Jørgensen *et al*., 2007, Dassaye *et al.,* 2016, Morgani & Hadjantonakis, 2020). Early during development in mice, the embryoblast, also known as the blastocyst's inner cell mass, re-organizes, through the process of gastrulation, from a one-dimensional layer of epithelial cells (blastula) into a multi-layered embryo with a 3-layer body plan

(gastrula) that includes the endoderm, ectoderm and mesoderm. A thorough review by Morgani & Hadjantonakis (2020) reports that at e5.5, before gastrulation, the mouse embryo consists of the primordium of the embryo proper which is the embryonic epiblast, the primordium of the yolk sac which is the visceral endoderm, and the primordium of the placenta which is the extraembryonic ectoderm. At the embryo's distal tip is a thickening of the visceral endoderm (VE) called the distal VE that acts as one of the embryo's specialized signaling centers. By e6.25, which is the onset of mouse gastrulation, this specialized signaling center has moved anteriorly to become the anterior VE. Cells within the epiblast's proximal posterior area receive instructions from Wnt, Nodal, Bone morphogenetic protein (BMP) and Fibroblast growth factors (FGF) signals and then delaminate from the primitive streak to go through an epithelial-mesenchymal transition (EMT). Subsequently, cells that exit the distal epiblast through the anterior primitive streak generate the axial mesoderm and definitive endoderm (Morgani & Hadjantonakis, 2020). The endodermis is later converted to a primitive gut tube structure. Studies by Jørgensen *et al.* further indicate that the gut tube closes because of signals from GATA-binding transcription factor 4, the matrix metallopeptidase 2 (MMP2), Furin and various BMPs. Importantly, both the medial and lateral endoderm express pancreatic transcription factors Pdx1, Nkx6-1, Nkx6-2, and Nkx2-2 prior to the complete gut tube closure and prior to the visibility of the pancreatic tissue anatomy. Studies have described a vast number of molecular markers for pancreatic cell types and stages, the first of which is Pdx1, which is the most specific and earliest gene expressed in the primordium of the pancreas and can identify pancreas specification prior to any visible morphological pancreas patterning (Ahlgren *et al*., 1996, Li *et al*., 1999). Interestingly, other factors such as Hlxb9 (Hb9), Hex (Hex), Onecut1 (HNF6), Tcf2 (vHNF1, HNF1β), and Foxa2 (HNF3β) are known to be expressed before Pdx1 expression in the mouse pancreas but, because these genes are more widely expressed, they cannot be regarded as specific pancreas markers. It has been shown that as development continues, co-expression of Pdx1, Hb9, Nkx6-1, and Nkx2-2 delineates the epithelial common pancreatic progenitor cells (Memon *et al*., 2018) and a complex and sophisticated interplay between various signaling pathways in the pancreatic mesenchyme is fundamental for growth.

The adult pancreatic islets (Islets of Langerhans) contain five distinct endocrine cell types: Beta cells (Insulin-producing cells) in the islet's core, with Alpha (Glucagon-producing cells), Delta (Somatostatin-producing cells), F cells (Pancreatic polypeptide producing cells) at the periphery and a small proportion of Epsilon cells (Ghrelin-producing

cells) dispersed within the islets. The beta cells are important as they play the important role of glucose maintenance. They carry out this function by secreting insulin in the presence of high nutrients such as glucose. In a physiological condition, when a person ingests food, glucose is up taken by glucose transporters on the membrane and pushed to the cytosol where adenosine triphosphate (ATP) is produced by the process of glycolysis and cellular respiration in the mitochondria. The increased level of ATP/ADP ratio then causes the ATP-sensitive potassium channel to close, preventing potassium ion from leaving the cells. This leads to membrane depolarization and opening of voltage-gated calcium channel, thus allowing the entry of calcium into the cells. This calcium leads to the exocytosis of insulin-containing vesicles and the subsequent release of insulin into the circulation. The released insulin then performs two important functions of stimulating glucose uptake in the peripheral tissues and converting glucose to glycogen for hepatic storage. The alpha cells also play an important role in glucose homeostasis via their secretion of glucagon which converts stored glycogen in the liver back to glucose during a fasting state when blood glucose is reduced. The joint function of the beta cells and alpha cells thus helps to maintain plasma glucose levels. Studies such as that carried out by Stanger *et al*., 2007, have shown that the final pancreas' and beta cell size are determined by the original number of progenitors present during development. In this study, conditional ablation of $Pdx1^+$ progenitor cells was carried out at different timepoints in pregnant mice and the offspring' pancreas and beta cell mass were markedly reduced as a result. This sheds light on the importance of the developmental stages in the eventual growth, development and health of the pancreas, which the current study aims to explore.

These progenitor cells are sensitive to nutritional status and stressors during development. Some of such stressors have been reported through studies by Petrk *et al.,* in 1999 that showed that low protein diet fed to pregnant mice during gestation resulted in a reduction in beta cell mass at birth caused mainly by reduced beta cell replication during development and increased apoptosis during lactation (Petrik *et al.,* 1999). More than a decade later, similar studies by Alejandro *et al*. confirmed that such a model of nutrient depletion causes neonatal beta cell mass reduction, glucose intolerant offspring upon adulthood and insulin resistance later in life (Alejandro *et al*., 2014, Alejandro *et al*., 2020). Similarly, when calorie was restricted during the gestation period of pregnant rats in Dumortier *et al*.'s study, some observed manifestations included decreased neonatal beta cell mass caused by impaired beta cell neogenesis because of the reduced number of $Pdx1^+$ and $Ngn3^+$ progenitors in animals from the same group (Dumortier *et al.,* 2007).

These data suggest that the beta cells are particularly sensitive to nutrient flux early in life. Indeed, glucose and amino acids have been shown to impact beta cell development (Filhoulaud *et al*., 2009, Elghazi *et al.,* 2017). In addition to these animal studies, human epidemiologic studies have also pointed to the fact that there is a subgroup of people that are susceptible to T2D and insulin resistance because of their exposure to malnourished states and stressors during fetal stages (Barker *et al*., 1993). This concept is known as fetal reprogramming.

1.3. The role of the intra-uterine environment in T2D development: mTOR and amino acid transporters' potential role in fetal reprogramming

The known development origins of health and disease (DOHaD) concept adopts that environmental influences during development, especially throughout fetal growth, have the potential to predispose a person to diseases after birth and during early and late adulthood. This concept has been backed up by studies that showed that, with oral glucose challenge, adult offspring whose mothers had been exposed to mid and late gestation malnutrition due to famine had decreased glucose tolerance (Ravelli *et al*., 2001). In fact, the well-known "Barker hypothesis", a hypothesis that explores the developmental origins of disease, states that adult diseases emerge from fetal adaptation caused by malnutrition (Philips *et al*, 1993, Barker *et al*., 2002, Hoy *et al.,* 2019). This is because organisms hold a capability to respond to exterior indicators by modifying their phenotype across development to suit their milieu. Poor maternal nutrition during pregnancy may indicate to the fetus that there is post-natal scarcity of nutrients, making the fetus acclimatize its metabolic trajectory to match an environment of reduced and inadequate available energy. Upon birth, when the baby's postnatal environment does not match the prenatal milieu it's been exposed and conditioned to, this results in maladaptation that consequently leads to the onset of metabolic disorders like T2D and obesity (Eriksson *et al*., 2003). A study by Barker *et al.* in 1993 which followed up 673 men and women into adulthood. These men and women were born with lower birthweight and the follow up study showed that they had 10 times greater risk of developing metabolic syndrome than those with normal birthweights. This study concluded that hypertension and T2D both originate from *in-utero* sub-optimal development. (Barker *et al.,* 1993). Such metabolic, endocrine or cardiovascular adaptations have the potential to cause permanent changes to the associated organ systems and increase risk factors of pathophysiology and diseases such as insulin resistance and T2D. As discussed above, the pancreatic

beta cells have been shown to have structural changes in response to sub-optimal *in-utero* environment. Previous studies have also shown that maternal nutrient supply and fetal placental transport function are two primary determinants of fetal nutrition (Kozuki *et al*., 2015, Gaccioli & Lager, 2016). Having established the role of nutrient supply in the *in-utero* environment on fetal growth, it is important to explore how these nutrients are transported. This is through amino acid (AA) transporters situated in the trophoblast of the placenta. It is well established that nutrients are critical in the regulation of beta cell mass and function. They are a source of energy and serve as signaling molecules to impact cell growth and differentiation. In addition to glucose and lipids, amino acids are a major source of nutrients in fetal growth and metabolism as they establish and prescribe the protein required by the developing fetus and serve as substrate source for production of energy (Wu *et al*., 2018, Gao *et al.,* 2020).

Two well-characterized AA transporters are L-type amino acid transporter (LAT) and Sodium-coupled Neutral Amino Acid Transporter (SNAT). LAT functions as an exchanger for the uptake of essential amino acids like leucine, while SNAT transports neutral amino acids and usually localizes to the maternal facing membrane of the placental syncytiotrophoblast. There are studies in humans that show that there is an active accumulation of amino acids (AAs) across the syncytiotrophoblast, the placenta's transport & hormone-producing epithelium (Cetin *et al*., 1996). This is possible because of the coordinated action of accumulative, exchanging, and facilitated amino acid transporters that reside in the maternal and fetal epithelial membranes. These amino acid transporters function in various ways. For example, accumulative AA transporters such as SNAT drive net uptake from maternal or fetal blood into placental trophoblast and consequently initiate high neutral and charged amino acid concentrations in the cell. Exchangers such as LAT utilize a mechanism that moves accumulated AAs from the trophoblast's cytosol and allows for a placental influx of essential amino acids. Finally, with the diffusion of AAs down their concentration gradient through the action of facilitated transporters, AAs can flow out from the cytosol of the trophoblast into the fetal circulation, resulting in net uptake of AAs by the fetus. There are functional regulators of amino acid transport including but not restricted to steroid hormones, insulin growth factors, availability of oxygen and nutrients (Vaughan, Rosario & Jansson, 2017).

With respect to nutrient availability, an ideal candidate that regulates this amino acid supply and receipt is mechanistic target of rapamycin (mTOR) kinase which possesses the ability to couple growth factors and nutrients to promote cellular growth.

mTOR, a serine/threonine kinase which exists in two complexes, one complex bound to raptor (mTOR complex 1) and another bound to rictor (mTOR complex 2), is a major regulator of trophoblast apical plasma membrane transport. Previous works by Roos *et al*. in 2007, Jansson *et al*. in 2013, Rosario *et al*. in 2016 have described the ability of mTOR to positively maintain amino acid systems L, A, β through its downstream phosphorylation of ribosomal S6 kinase (S6K1 and 2) and 4E-binding protein (4EBP1 and 2). With inhibition of either of the mTOR complexes, there is a reduction in SNAT2 abundance as shown by Rosario *et al.* 2016. This is because of mTOR's role in inhibiting the translation of NEDD4-2 whose function is to ubiquitinate and target the SNAT and LAT transporters to the proteasome (**Figure 2**)**.** With this inhibition, there is thus a preservation in the available placental AA transporters. Upstream of mTOR, the mTOR complex 1 (mTORC1) signaling pathway is negatively regulated by Tuberous Sclerosis Complex (TSC2) (**Figure 2**).

1.4. mTOR and TSC2 pathways

Genetic studies done in *Drosphila* were the pioneering studies that suggested what roles TSC2 played in the mTOR pathway. One of such studies was carried out by McManus and Alessi, who reported TSC2's function of inhibiting p70 ribosomal protein S6 kinase (McManus & Alessi, 2002). These results were confirmed in mammalian cells (Inoki *et al*., 2002; Tee *et al*., 2002). With respect to the mechanism of TSC2's effect on mTOR, TSC2 consists of one leucine zipper domain, two coiled-coil domains and one GTPase-accelerating protein (GAP) domain, among its other putative domains. TSC2 has been shown to function as a GAP towards Ras homolog enriched in brain (Rheb), which is a small G protein. GAPs' known effect on activated G proteins is to cause termination of their downstream signaling event. In accordance, TSC2's GAP domain is known to increase GTP hydrolysis on Rheb, leading to downregulation of its downstream effects. GTP-bound, activated Rheb normally results in increased mTOR activation, thus an inhibition of Rheb in the presence of TSC2 leads to mTOR reduction (**Figure 2**)**.**

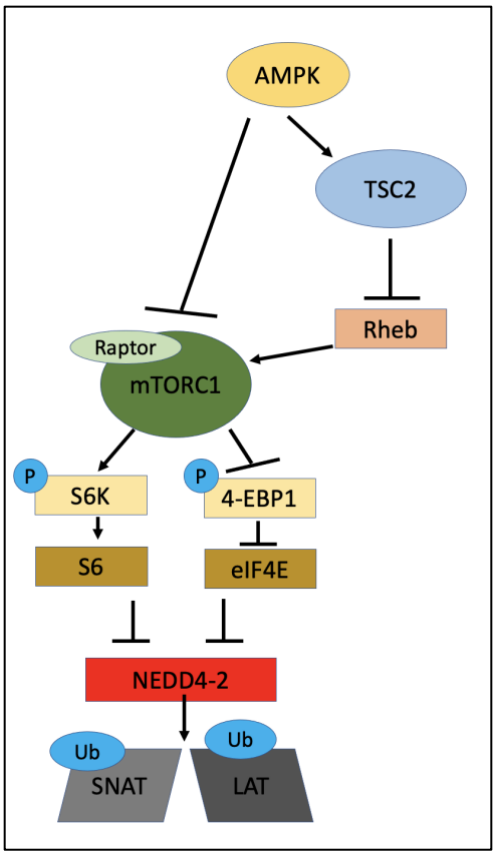

Figure 2: mTOR and TSC2 pathways

TSC2 acts upstream of mTOR. mTOR in the placenta integrates signals of growth factors and nutrients from the mother to ensure modulation of nutrient transport to the fetus. This modulation is through the inhibition of NEDD4-2 which differentially ubiquitinates AA.

1.5. Hypothesis

In this study, we hypothesized that placental mTOR acts to regulate amino acid transport in the placenta during gestation. Thus, with knock out of mTOR in the placenta, we hypothesized that there will be reduced amino acid transport to the developing fetus. Because amino acids (AA) are the major source of nutrients for fetal growth and metabolism, we hypothesize that this reduced amino acid supply could lead to reduced fetal weight and reduced mTOR activity in the pancreas. This is because within beta cells, AAs promote mTOR signaling, which regulates cell growth, proliferation, and protein synthesis. This reduced pancreatic mTOR activity within the beta cells could further and consequently lead to reduced beta cell mass in embryonic day (e) 17.5 mice (**Figure 3**). We have chosen to investigate this phenotype at embryonic day (e) 17.5 because it is about this time point within the 2nd and 3rd trimesters in mice that beta cells begin to

become more abundant in the developing islets compared to other endocrine cells (Johansson *et al*., 2007, Jørgensen *et al., 2007*).

To test the current hypothesis, we generated placental mTOR loss (mTOR KO) or gain (TSC2 KO) of function in preclinical murine models and analyzed the effect of the knockout on the expression levels of amino acid transporters, fetal weights and beta and alpha cell masses of the growing embryos at e17.5 (**Figure 3**). The results from this study benefit the understanding of general islet biology, beta cell mass, and factors that influence these phenotypes. Findings from this study will be essential for both the T2D and the stem cell biology fields. For the former, understanding the mechanisms that control beta cell mass could potentially lead to the development of curative therapies for T2D. For the latter, exploring factors responsible for beta cell development *in-utero* could potentially inform the stem cell biology field for the development of induced pluripotent stem cell (iPSC)-derived beta cells *in vitro*, all of which will be beneficial to the patients with this disease.

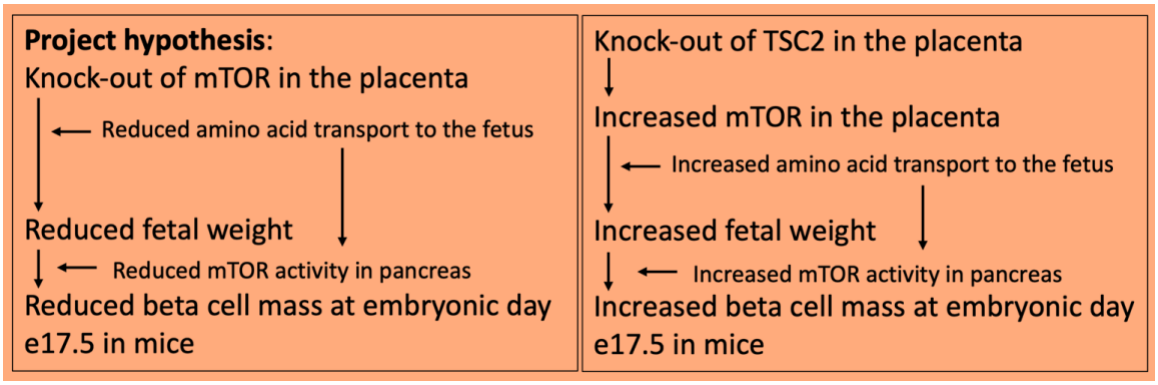

Figure 3: Project hypothesis

2.0. CHAPTER 2: METHODS

2.1. Study approval

All animal studies were approved by the Institutional Animal Care and Use Committee (protocol 1806-36072A) at the University of Minnesota.

2.2. Generation of mouse models with genetic alteration of mTOR function in the placenta

As similarly described by Akhaphong *et al*., (2021), the mice used for this study were generated on a mixed background, they were housed in groups on a 14:10 light-dark

cycle having ad libitum access to standard diet. To generate mouse models with loss of placental mTOR signaling in the placenta, female mice with trophoblast-specific Cre recombinase transgene driven by the CYP19 promoter with loxP-flanked sites in the *mTOR* gene (Cyp19Cre+ $mTOR^{flox/flox}$) were bred with male mice $mTOR^{flox/flox}$ without Cre as shown in **Figure 4**. The resulting generation were homozygous placental CYP19Cre+ $mTOR^{flox/flox}$ (referred to as mTOR KO) heterozygous CYP19Cre+ $mTOR^{flox/wt}$ (mTOR Het), CYP19Cre- $mTOR^{flox/flox}$, $mTOR^{flox/wt}$ (littermate controls). "mTOR Het" cohorts were not included in the analysis of this study because of lack of sufficient animals with this genotype. Ai6 mice which express robust ZsGreen1 fluorescence, a variant of Green fluorescent protein (GFP), following Cre-mediated recombination driven by the CAG promoter (CAG-ZsGreen1) were further bred with the mTOR mice to generate mice which express ZsGreen upon Cre-mediated recombination in their placenta (e.g., mTOR CypCre+;CAG). The primers used for CYP19Cre and floxed mTOR are listed in **Table 1**. Purchase of $mTOR^{flox/flox}$ and Ai6 (CAG-ZsGreen1) mice was from the Jackson Lab while provision of CYP19Cre mice was made by Dr. G. Leone of Ohio State University, USA.

Primer names		Primer sequence
CYP19cre	Forward	GAC CTT GCT GAG ATT AGA TC
	Reverse	GAC GAT GAA GCA TGT TTA GCT GGC C
mTOR floxed	Forward	TTT AGG ACT CCT TCT GTG ACA TAC ATT TCC T
	Reverse	TTA TGT TTG ATA ATT GCA GTT TTG GCT AGC AGT
TSC2 floxed	Forward	AAG CAG GTC TGC AGT G
	Reverse	ACA ATG GGA GGC ACA TTA CC
SRY	Forward	TTG TCT AGA GAG CAT GGA GGG CCA TGT CAA
	Reverse	CCA CTC CTC TGT GAC ACT TTA GCC CTC CGA

Table 1: Table of primers used for genotype and gender determination

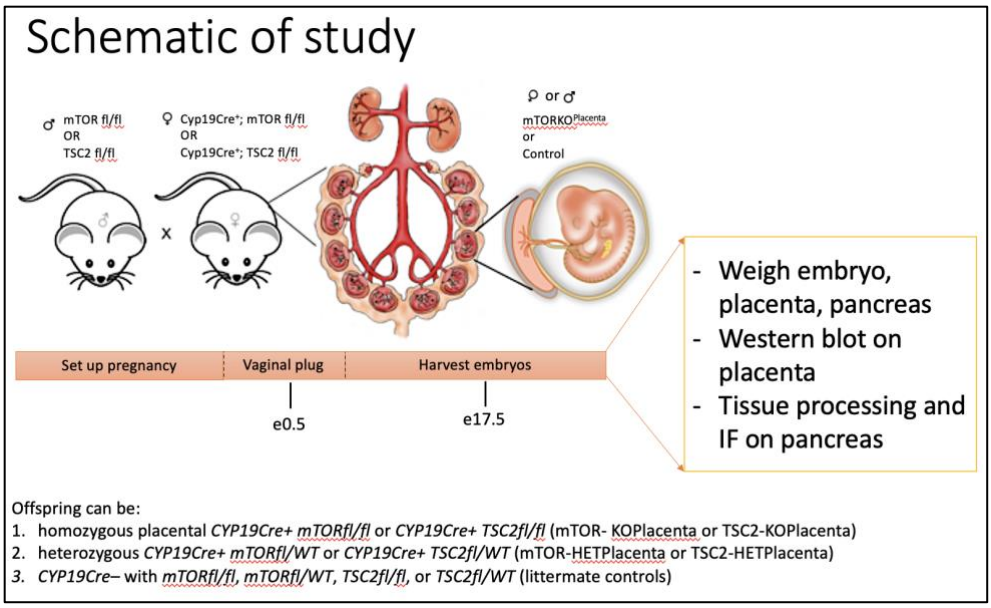

Figure 4: Schematic of breeding study

2.3. Generation of mouse models with genetic alteration of TSC2 function in the placenta

As similarly described by Akhaphong *et al*., (2021), to generate mouse models with gain of placental mTOR signaling in the placenta, female mice with trophoblast-specific Cre recombinase transgene driven by the CYP19 promoter was crossed with male mice that had loxP-flanked sites in the *TSC2* gene as shown in **Figure 4**. The resulting generation are homozygous placental CYP19Cre+ $TSC2^{flox/flox}$ (referred to as TSC2 KO) heterozygous CYP19Cre+ $TSC2^{flox/wt}$ (TSC2 Het), CYP19Cre- $TSC2^{flox/flox}$, $TSC2^{flox/wt}$ (littermate controls). “TSC2 Het” cohorts were not included in the analysis of this study because of lack of sufficient animals with this genotype. Ai6 (CAG-ZsGreen1) mice were further bred with the TSC2 mice to show recombination in the placenta as described in the “Generation of mouse models with genetic alteration of MTOR function in the placenta” section above. The primers used for CYP19Cre and floxed TSC2 are listed in **Table 1**. Purchase of $TSC2^{flox/flox}$ was also from the Jackson Lab.

When the fetuses were harvested, they were washed in PBS, each fetus was made dry of surrounding liquid and weighed on a sensitive weighing balance. From the harvested offspring, tail samples were collected for DNA, placenta samples were snap-frozen in liquid nitrogen, stored at -80C and later used for western blot analysis, pancreas samples were formalin-fixed and processed for immunofluorescence staining (**Figure 4**).

2.4. DNA extraction, Polymerase Chain Reaction (PCR), Gel electrophoresis

During harvest, the tails were collected on ice and kept at -20^{o}C until DNA extraction. 50ul of alkaline lysis buffer was added to the tails which were further subjected to DNA extraction by heating at 95^{o}C for 30 minutes. Thereafter, an equal volume of Neutralization buffer (50ul) was added to the heated tails. The protocols for making the buffers are in **Table 2**

The extracted DNA was kept at -20^{o}C until PCR. PCR was carried out by adding the extracted DNA to the master mix containing water, 10X Taq Reaction buffer, $MgCl_2$ (25mM), dNTP mix (10mM), the forward and reverse primers (**Table 1**), Taq Polymerase. The kit used was New England BioLabs kit (#B9014S).

The PCR cycling conditions used for:

- SRY genotyping was 95^{o}C x 30 seconds, (95^{o}C x 30 seconds, 65^{o}C x 30 seconds, 68^{o}C x 30 seconds) x 30 cycles, 68^{o}C x 5 minutes, 4^{o}C until the samples were run on the gel.
- mTOR flox genotyping was 94^{o}C x 2 minutes, (94^{o}C x 20 seconds, 62^{o}C x 20 seconds, 72^{o}C x 40 seconds) x 30 cycles, 72^{o}C x 5 minutes, 4^{o}C until the samples were run on the gel.
- Cyp19-cre genotyping was 95^{o}C x 3 minutes, 95^{o}C x 30 seconds, 58^{o}C x 30 seconds, 72^{o}C x 30 seconds) x 35 cycles, 72^{o}C x 3 minutes, 4^{o}C until the samples were run on the gel.

The PCR products were subjected to gel electrophoresis in 2% agarose gel in 1X Tris-acetate-EDTA (TAE) buffer with Ethidium bromide (VWR, #97062-736) at 90 volts, 400mAmps, 50minutes and bands were imaged using the Biorad Gel Doc Xr Imaging System.

Alkaline Lysis Reagent (pH of Alkaline Lysis Reagent =~12)		
Reagents	**Final Concentration**	**Amount for 200 mL**
Sodium Hydroxide (RPI, #S24000-1000.0)	25 mM	200 mg
Ethylenediaminetetraacetic acid (EDTA) (Sigma-Aldrich, #EDS-100G)	0.2 mM	14.88 mg
Neutralization Reagent (pH of Neutralization Reagent =~5)		
Reagent	**Final Concentration**	**Amount for 200 mL**

Tris-Hydrochloride (Cat no. 30-069)	40 mM	1.3 g

Table 2: Preparation of buffers for DNA extraction

2.5. Pancreas tissue collection and processing

After setting up timed pregnancies in the late afternoon, vaginal plug observation the next morning was recorded as embryonic day 0.5 (e 0.5). In the morning of the 17th day, e17.5, the dams were sacrificed, and pancreas were carefully harvested under a microscope. The harvested pancreas were immersed in 3.75% formalin for 5 hours, then 70% Ethanol overnight, then the tissues were processed and embedded. Embedded blocks were sectioned at a thickness of 5 µm. All areas of the embedded blocks from the e17.5 tissues were sectioned at a thickness of 5µm. Three (3) sections were collected onto 1 slide and sections were allowed to adhere unto the slides at 37^{o}Celsius until staining. About 40-60 slides resulted from 1 pancreas. Of these, 5-6 slides from various equally distributed parts of the tissue were stained (e.g., slides 5, 15, 25, 35, 45 from 50 slides generated from one tissue) and served as a representation of the respective tissue.

2.6. Immunostaining

Sections on the slides were deparaffinized in 2 washes of Citrisolv (fisher scientific, #04-355-121) for 5 & 10 minutes respectively, dehydrated in 100%, 95%, 75% Ethanol for 3 minutes each. Antigen retrieval was then carried out using tissue microwave at 95^{o}C two times for 4 minutes each. The tissues were then permeabilized in Triton X (ThermoFischer, #28313), blocked using Roche blocking buffer (Roche, Milipore Sigma #11096176001), and co-stained overnight (14-18hrs) with Insulin and Glucagon primary antibodies (**Table 3**) to determine the cells that are positive for these hormones. Primary antibody was washed with Phosphate Buffered Saline + 0.1% Tween 20 (PBST) (3 times for 5 minutes each), incubated for 1.5hrs at 37^{o}C in secondary antibody and washed again with PBST (2 times for 5 minutes each). Tissues on slides were washed with DAPI once for 3 minutes on a rocking platform to stain the nuclei. Antifade mounting media without DAPI was used to mount. All murine samples were visualized and imaged using either Nikon ECLIPSE NI-E microscope or Keyence microscope at the indicated magnifications (10x magnification for the whole pancreas and 20x magnification for individual islets). Imaging for beta cell mass was not carried out at the same intensities because intensity was not measured, as the aim was to determine what cells did or did not express the

respective hormone, irrespective of the intensity at which they were expressed. Beta and alpha cell mass analysis were carried out using FijI (http://imagej.nih.gov/ij/).

Target	Catalog Number	Primary antibody Concentration	Secondary antibody concentration
Insulin (Guineapig)	Sigma I8510	1:10,000	1:400 (Anti-Guineapig FITC, Jackson Immunoresearch #106-005-003)
Glucagon (Rabbit)	Abcam ab92517	1:10,000	1:400 (Anti-Rabbit Cy3, Jackson Immunoresearch #111-005-144)

Table 3: Table of antibodies used for immunofluorescence staining

2.7. Beta and alpha cell mass quantification

Upon imaging, quantifications of the beta and alpha cell area were carried out using FijI (http://imagej.nih.gov/ij/). The 10x images of the whole pancreas were opened on the software, thereafter the images were manually cleaned on the software to exclude non-acinar areas of the pancreas, thereafter the cleaned images were converted to grey scale to allow the FijI software recognize the respective hormone-positive areas. The Fiji software then quantified the insulin-positive cell areas, glucagon-positive cell areas, the total pancreas area. These values were exported unto a Microsoft Excel spreadsheet, wherein the values of the 5-6 tissues per animal are averaged and the insulin-positive to total pancreas ratio was multiplied by the pancreas weight of the animal to obtain its beta cell mass. Similarly, the glucagon-positive to total pancreas ratio was multiplied by the pancreas weight of the animal to obtain its alpha cell mass.

2.8. Western blotting

Whole placenta were snap-frozen in liquid nitrogen immediately after harvest from e17.5 embryos and stored at -80^{o}C until analysis. Snap-frozen tissues were slightly thawed on regular ice, and 50-80mg of tissues were homogenized in complete RIPA (containing protease inhibitor, phosphatase inhibitor, and 1% SDS) using Benchmark D1000 homogenizer. Homogenized tissues were sonicated, vortexed at 2000rpm for 15 minutes and centrifuged at 16,000 x g for 15 minutes, 4^{o}C. Resulting supernatants transferred to new tubes and are the isolated protein (Protein lysates). Protein lysates were quantified using Pierce™ BCA® Protein Assay Kits and Reagents (Thermo

Scientific). 50-80ug protein were denatured at 95^{o}C, 5mins and separated by electrophoresis using SDS on 1.5mm NuPAGE 4-12% Bis-Tris or 6% Tri-Glycine Protein gels. Transfer of proteins from gel to Immun-Blot® PVDF Membrane (Bio-Rad #1620177) was carried out overnight (18hr-20hrs) at 18V, 4^{o}C using the BioRad power source. Membranes were cut according to each proteins' molecular weights and blocked at room temperature for 1 hour using 5% blocking grade buffer (Bio-Rad, #CX25215) in TBS 0.1% + Tween 20 (TBST) (Sigma). The blocked blots were incubated with primary antibody overnight at 4^{o}C, washed with TBST 3 times for 5mins at Room temperature, incubated with HRP-conjugated secondary antibody at room temperature for 1hour, washed again as described above and developed the blots with the WesternBright Quantum Kit (Advansta, #K-12042-D21). All washes and incubations were performed on a rocking platform. The bands were imaged using the Li-Cor Odyssey Fc Imaging System. Bands were analyzed by densitometry and normalized to HSP90, beta actin or vinculin as indicated in the result. Antibodies used, concentrations and catalogue numbers are listed in **Table 4.**

Target	**Antibody name**	**Catalog Number**	**Primary AB Concentration**	**Secondary AB concentration**
mTOR	mTOR (7C10) Rabbit mAb	CST 2983S	1:1000	1:1,000 (Anti-rabbit IgG, HRP-linked Antibody, CST #7074)
HSP90	HSP90 Rabbit Ab	CST 4874	1:3000	1:5000 (Anti-rabbit IgG, HRP-linked Antibody, CST #7074)
Tuberin/TSC2	Tuberin/TSC(D93F12) XP® Rabbit mAb	CST 4308S	1:1000	1:10,000 (IRDye 800CW anti-Rabbit, Licor # 926-32211)

p-s6 (S240/244)	Phospho-S6 Ribosomal Protein (Ser240/244) (D68F8) XP® Rabbit mAb	CST 5364S	1:1000	1:2,000 (Anti-rabbit IgG, HRP-linked Antibody, CST #7074)
p-S6 Kinase	Phospho-p70 S6 Kinase (Thr389) (108D2) Rabbit mAb	CST 9234	1:1000	1:3,000 (Anti-rabbit IgG, HRP-linked Antibody, CST #7074)
Vinculin	Vinculin (E1E9V) XP (R) Rabbit mAb	Protein-tech 66305-I-Ig	1:3000	1:5,000 (Anti-rabbit IgG, HRP-linked Antibody, CST #7074)
SNAT-1	Sc-137032 Anti-SNAT1 Antibody (H-9)	Santa-cruz sc-137032	1:500	1:10,000 (HRP anti-Rabbit abcam #ab6721)
LAT1/SLC7A5	SLC7A5 Polyclonal antibody	Protein tech 13752-1-AP	1:1000	1:1000 (Anti-rabbit IgG, HRP-linked Antibody, CST #7074)
LAT4/SLC43A2	SLC43A2 Polyclonal Antibody	Invitrogen PA5-23571	1:1000	1:1000 (Anti-rabbit IgG, HRP-linked

				Antibody, CST #7074)
beta-actin	B Actin (D6A8) Rabbit mAb	CST 8457S	1:1000	1:3000 (Anti-rabbit IgG, HRP-linked Antibody, CST #7074)

Table 4: Table of antibodies used for western blotting

2.9. Statistical Analysis

Data analysis was carried out using two-tailed Mann-Whitney t-test or Welch's t-test. P value of <0.05 was considered as statistically significant. Sex was considered as a variable and data were segregated.

3.0. CHAPTER 3: RESULTS

3.1. Validation of TSC2 and mTOR models

To explore the hypothesis that placental mTOR alteration influences beta cell mass during development, we generated a mouse model of mTOR deletion in the placenta by using the Cre recombinase transgene driven by the human CYP19 gene. We also generated a mouse model of mTORC1 overexpression by carrying out TSC2 deletion in the placenta also utilizing the Cre recombinase transgene driven by the human CYP19 promoter. Primary studies by Wenzel & Leone in 2007 and further validation-based studies by Lopez-Tello J *et al.* in 2019 and Akhaphong *et al*. in 2021 showed the effectiveness of this technique to induce placental trophoblast-specific alteration of genes.

To determine the genotype of the collected embryos, DNA extractions were carried out on the tail of the collected e17.5 embryos, Polymerase Chain Reaction (PCR) was performed on the extracted DNA and the PCR products were subjected to gel electrophoresis. **Figure 5A** thus shows the results of the gel electrophoresis wherein the "mTOR floxed gel" showed the embryos that had the mTOR gene floxed. The "Cyp19-Cre gel" showed which of the mTOR floxed embryos were CYP19-Cre positive upon looking at the Rosa26 locus for the Cyp-19 construct. The bands in the A' and B' lanes on the "Cyp19 gel" represent a Cyp19-Cre positive animal used as a positive control and a Cyp19-Cre negative animal used as a negative control respectively. The experimental

samples were thus identified as Cyp19-cre positive or negative based on their expression as compared to the controls (A' and B' on the Cyp19 gel). For example, if some mouse samples expressed CYP19-Cre bands, albeit much fainter than the mTOR KO, they are considered mTOR control based on the expression of the Cyp19-Cre positive control. In the current study, gender of the animals was considered in our comparisons for thoroughness. The sex-determining region Y (*SRY*) gene encodes the SRY protein and initiates testis differentiation in placental mammals, thus its expression is indicative of a male gender (Berta *et al*., 1990). Thus, also in **Figure 5A**, the animals were identified as SRY positive (male) or negative (female). Appropriate positive and negative controls were used for proper analysis of the data. Similarly, gel electrophoresis from PCR products of tail extracts from TSC2 mice were used to determine which TSC2 embryos expressed the CYP19 Cre transgene and were male or female (data not shown).

Because it is important to investigate the efficacy of the CYP19 Cre to cause changes in the translated protein, western blotting was carried out to assess mTOR or TSC2 protein in the respective models. **Figure 5B** shows the raw western blot data depicting the reduction in mTOR in the mTORKO^{placenta}. It is important to note that while the mTOR bands in the control lanes are barely visible because of antibody sensitivity in the western blot assay, the bands were quantifiable and thus quantified using FIJI showing a statistically significant reduction of mTOR in the placenta (**Figure 5C**). **Figure 5D** shows a reduction of the TSC2 protein in the TSC2KO^{placenta} compared to littermate controls. Also, the quantification of the TSC2 bands, as seen in **Figure 5E** shows that this reduction was significant compared to the littermate control. It is important to note that a complete loss of TSC2 or mTOR protein in the placenta is not expected, because the Cre-mediated recombination of the genes took place in the trophoblast of the placenta and not the whole placenta, but because the whole placenta was used for the western blot, the bands for the mTORKO^{placenta} and TSC2KO^{placenta} still show some protein, albeit reduced compared to the littermate controls without any placental gene alterations (mTORcontrol^{placenta} and TSC2control^{placenta}).

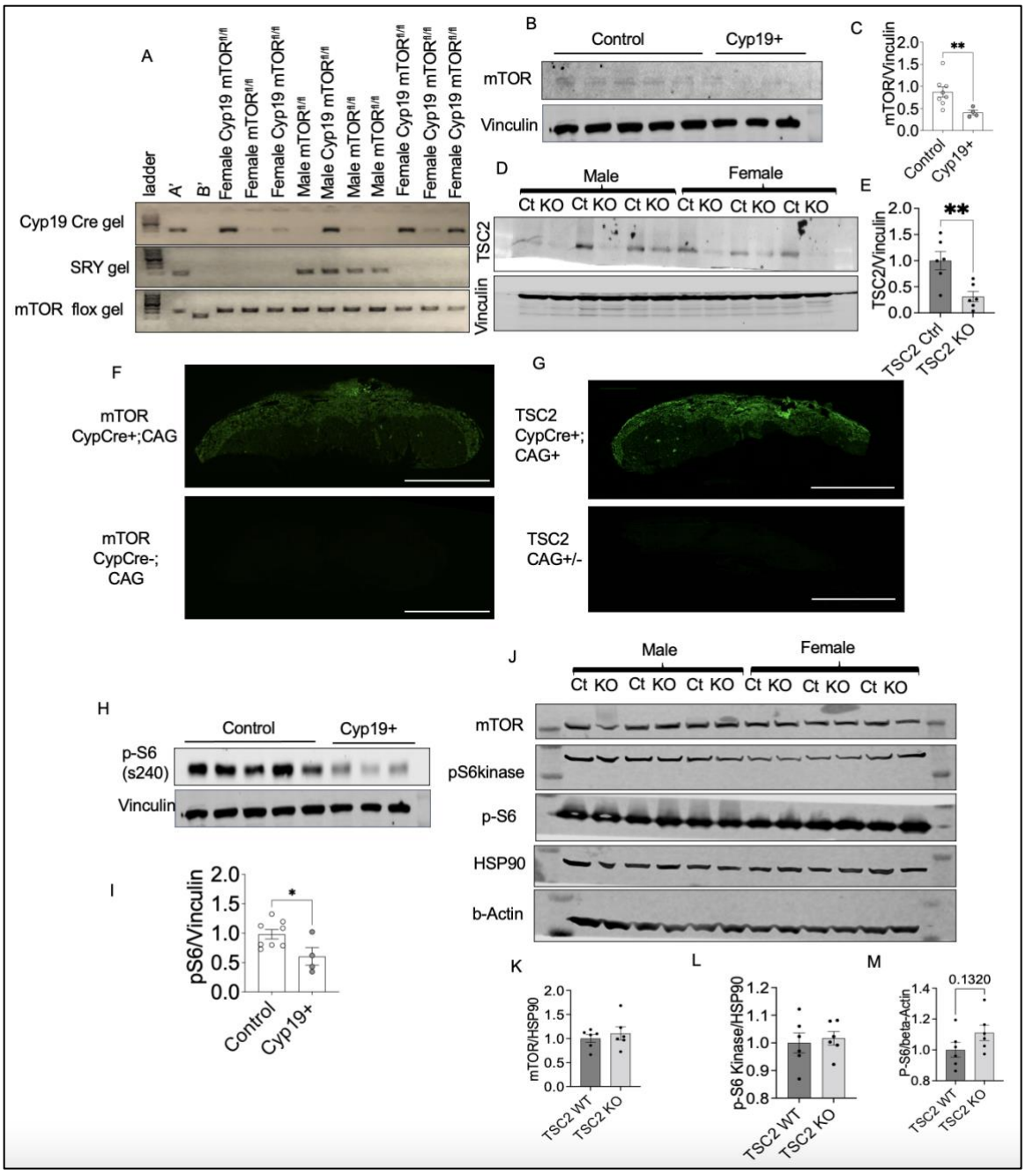

Figure 5: Validation of mTOR and TSC2 transgenic models

(A) Gel electrophoresis showing the gender and genotype of e17.5 mice following DNA extraction and PCR. A' in Cyp19, SRY, mTOR gels are positive controls Cyp19-Cre+, Male, mTOR f/f controls respectively, B' in Cyp19, SRY, mTOR gels are negative controls Cyp19-Cre-, Female, Wildtype controls respectively. (B) Raw western blots showing that mTOR is reduced in the placenta of mTORKO^{Placenta} mice. (C) Quantification of the bands in B showing that mTOR is reduced in the placenta of mTORKO^{Placenta} mice (D) Raw western blots showing that TSC2 is reduced in the placenta of TSC2KO^{Placenta} mice. Ct are

the TSC2 controls, KO are the TSC2 KO placenta. (E) Quantification of the bands in D showing that TSC2 is reduced in the placenta of TSC2KO^{Placenta} mice (F) ZsGreen is expressed in mTOR CypCre+:CAG only. (G) GFP is expressed in TSC2 CypCre+:CAG only (H) Raw western blot showing that mTOR's downstream target, phospho-S6 is reduced in the placenta of mTORKO^{Placenta} mice. (I) Quantification of the bands in H showing that p-S6 is reduced in the placenta of mTORKO^{Placenta} mice (J) Raw western blot showing that mTOR, p-S6kinase and p-S6 are unchanged in the placenta of TSC2KO^{Placenta} mice compared to TSC2KO^{Placenta} mice. (K-M) Quantification of the bands in J showing that mTOR, p-S6kinase and p-S6 are unchanged in the placenta of TSC2KO^{Placenta} mice compared to TSC2KO^{Placenta} mice. Error bars represent mean ± SEM. Scale bars in images are 200 μm. *P < 0.05

To further validate the placental gene alteration, using ZsGreen, a green fluorescence protein reporter line, specificity of CYP19-Cre (CAG-ZsGreen) in the placenta of the mTORKO^{placenta} and not the control littermates was confirmed as shown in **Figure 5F**. Similarly, specificity of CYP19-Cre in the placenta of the TSC2KO^{placenta} embryos and not the control littermates was confirmed as shown in **Figure 5G**. To explore if the downstream signaling of the mTOR proteins was also affected by the reduction of the mTOR and TSC2 placental proteins, downstream targets in the mTOR pathway were probed for via western blot. Phosphorylation of ribosomal protein S6 (p-S6), a downstream target and an indication for activity of mTORC1 (Brunn *et al*., 1996, Annovazzi *et al*., 2009), was reduced in the placenta lysates of the mTORKO^{placenta} as shown in **Figure 5H** (raw blot) **and Figure 5I** (quantification of bands). This indicates a functional efficacy of the knockdown of *mTOR* in the placenta. Primary studies by Inoki *et al*. in 2002 and Zhan *et al*. in 2019 revealed the role of TSC2 as a negative regulator of mTORC1 signaling (Inoki *et al*., 2002, Zhan *et al*., 2019). To investigate the functional efficacy of *TSC2* knockdown in our model, western blots were also carried out on several markers in the mTOR pathway. Interestingly, although after seeing a significant reduction in TSC2 protein in **Figure 5D&E**, using the same lysates to perform a western blot, mTOR, and mTORC1's downstream markers, p-S6 kinase and p-S6, were observed to be unchanged (**Figure 5J**). The quantifications of these proteins are shown in **figures 5K** (mTOR), **5L** (p-S6 kinase), **5M** (p-S6). One caveat to note in this experiment is that these phosphorylated proteins, phospho-S6 kinase and phsopho-S6, were normalized to HSP90 and beta actin respectively, which are acceptable housekeeping genes. However, an even better loading

control for p-S6 kinase and p-S6 would be total S6 kinase and total S6 respectively which were not probed for in this blot. While we observe TSC2 being reduced in these same lysates with no effect on mTORC1's downstream targets, it would have been interesting to also explore the levels of Rheb, the immediate recipient of TSC2's GAP function. The results so far indicate that the gene editing of mTOR and TSC2 showed perturbation in the respective genes and proteins (mTOR and TSC2), with perturbation in the downstream signaling of mTOR but not TSC2.

3.2. Beta and alpha cell mass analysis for mTOR and TSC2 model

Upon observing these placental gene and protein changes, next, to test our hypothesis that genetic alteration of mTOR in the placenta will adversely affect the beta cell mass in offspring, the sectioned tissues were immuno-stained for both Insulin and Glucagon to determine the cells that are positive for these hormones. Upon imaging, quantifications of the beta and alpha cell area were carried out using FijI as described in the methods. **Figure 6A** shows 20x representative images of Insulin & Glucagon co-stained islets from male mTORcontrol^{placenta} and mTORKO^{placenta} mice. Upon quantification of the whole pancreas imaged at 10x magnification, we interestingly observed no changes in the beta cell mass between the male mTORcontrol^{placenta} and mTORKO^{placenta} mice (**Figure 6B**) even after we'd observed perturbations of mTORC1's downstream target, 22hosphor-s6. Studies by Yin *et al*., (2020) showed that islet-specific knock-out of Raptor, one of the binding partners of mTORC1, led to reduced beta and increased alpha cell masses. Thus, we thought it'd be interesting to analyze alpha cell mass changes in our model. **Figure 6C** showed that there was no change in the alpha cell mass resulting from placental mTOR alteration.

Interestingly, in the female cohort, which underwent the same tissue processing and staining procedures as the male cohort of animals, we observed that there was an increase in the beta cell mass of the mTORKO^{placenta} mice compared to the mTORcontrol^{placenta} mice (**Figure 6D, 6E**). Although, conflicting with our hypothesis that reduced placental mTOR will lead to reduced beta cell mass, we found this to be an interesting piece of data especially because of the observed reduced placental mTOR in our model as seen in **Figures 5B** and **5H**. There was no observable or statistically significant difference between the alpha cell mass of mTORcontrol^{placenta} and mTORKO^{placenta} (**Figure 6F**). Also, to answer the question of whether mTOR alteration affected the sex comparisons, we compared the beta cell masses of the male to the female

cohorts in the controls (**Figure 6G**). Using that as a standard of normalcy, we also compared the male and female beta cell masses in the mTORKO^{placenta} animals and found no change. Similarly, the alpha cell mass levels were unchanged (**Figure 6H**). In the TSC2 models, **Figures 6I-N** showed that beta cell mass and alpha cell mass were unchanged between TSC2control^{placenta} and TSC2KO^{placenta} in both male and female cohort. This doesn't come as a surprise because of the unchanged levels of mTORC1's downstream targets, p-s6 and p-s6 kinase, in the placenta of the TSC2control^{placenta} and TSC2KO^{placenta} mice (**Figure 5J-M**).

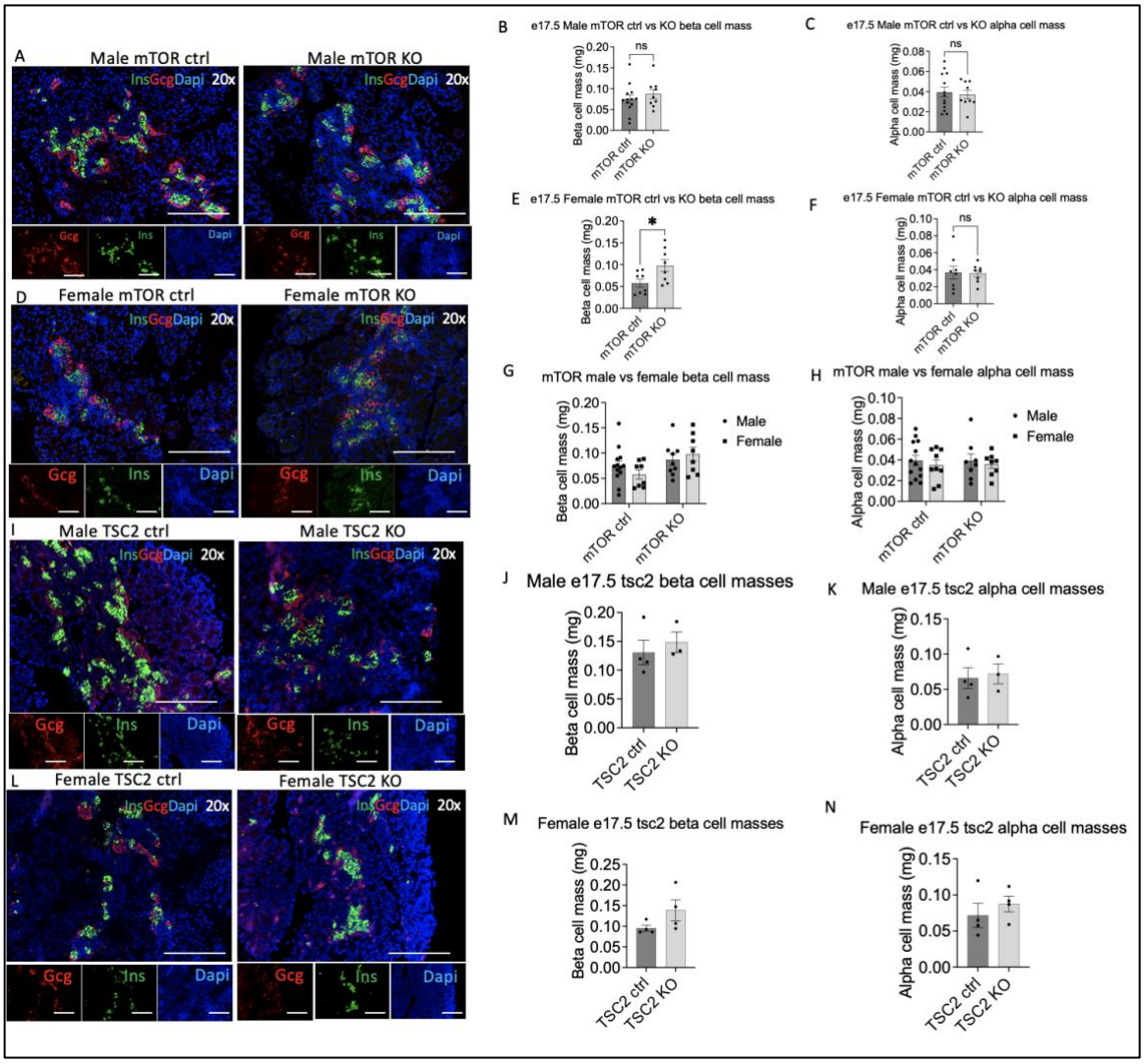

Figure 6: Effects of mTOR alteration on beta and alpha cell masses

(A) Representative images of islets from male mTORcontrol^{placenta} and mTORKO^{placenta} e17.5 mice taken at 20x magnification (B) Graphs showing beta cell mass of male mTORcontrol^{placenta} and mTORKO^{placenta} upon quantification of 10x pancreas. (C) Graphs

showing alpha cell mass of male mTORcontrol^{placenta} and mTORKO^{placenta} upon quantification of 10x pancreas (D) Representative images of islets from female mTORcontrol^{placenta} and mTORKO^{placenta} e17.5 mice taken at 20X magnification (E) Graphs showing beta cell mass of female mTORcontrol^{placenta} and mTORKO^{placenta} upon quantification of 10x pancreas. (F) Graphs showing alpha cell mass of male mTORcontrol^{placenta} and mTORKO^{placenta} upon quantification of 10x pancreas (G) Graphs showing beta cell mass of male mTORcontrol^{placenta} and mTORKO^{placenta} of male vs female cohorts upon quantification of 10x pancreas (H) Graphs showing alpha cell mass of male mTORcontrol^{placenta} and mTORKO^{placenta} of male vs female cohorts upon quantification of 10x pancreas (I) Representative images of islets from male TSC2control^{placenta} and TSC2KO^{placenta} e17.5 mice taken at 20X magnification (J) Graphs showing beta cell mass of male TSC2control^{placenta} and TSC2KO^{placenta} upon quantification of 10x pancreas (K) Graphs showing alpha cell mass of male TSC2control^{placenta} and TSC2KO^{placenta} upon quantification of 10x pancreas (L) Representative images of islets from female TSC2control^{placenta} and TSC2KO^{placenta} e17.5 mice taken at 20X magnification (M) Graphs showing beta cell mass of female TSC2control^{placenta} and TSC2KO^{placenta} upon quantification of 10x pancreas (N) Graphs showing alpha cell mass of male TSC2control^{placenta} and TSC2KO^{placenta} upon quantification of 10x pancreas. Error bars represent mean ± SEM. *P < 0.05. Scale bars in images are 200 μm

3.3. Fetal, placental and pancreas weight measurements in mTOR and TSC2 models

Various studies (e.g., Yung *et al*., 2008, Chen *et al*., 2015, Hung *et al*., 2017) have shown that reduced birthweight is strongly correlated with reduced placental mTOR level. Roos *et al.* found that one of the indicators for mTORC1 activity, phsopho-S6K1, was significantly reduced in placentas collected from IUGR-related human pregnancy complications. Similarly, in 2015, Chen *et al.* obtained placental tissues from 25 IUGR complicated pregnancies and found that mTOR activity was markedly reduced in the homogenates of these IUGR placentas compared to placenta from gestational age-matched pregnancy with normal growth. These studies strongly suggest a relationship between mTOR signaling and fetal growth, and this relationship is further explained by placental mTOR's ability to regulate amino acid transport to the growing fetus. This led us to ask whether there are changes in the fetal weight of the e17.5 mice with placental mTOR and TSC2 alterations. Interestingly, as we observed for the beta and alpha cell masses, **Figure 7A** shows the fetal weight between male mTORcontrol^{placenta} and

mTORKO^{placenta} and revealed no significant change. Similarly, there were no observable changes in the fetal weight between the female mTORcontrol^{placenta} and mTORKO^{placenta} as seen in **Figure 7B**. This data suggests that the mTOR alteration in the placenta in this cohort had no effect on the weight of the fetuses. Interestingly, some studies have also reported increased mTOR expression (Aiko *et* al., 2014) and unchanged mTOR expression (Jansson *et al*., 2006, Ma *et al*., 2011, Fahlbusch *et al*., 2015) in rat, ewes and human IUGR models. In the placental TSC2 model, it is expected that TSC2 alteration activates mTOR signaling thus leading to increased fetal growth in the TSC2KO^{placenta} animals. Studies by Jansson *et al*., 2013 showed that there is activation of the mTOR pathway and amino acid transporters in overweight babies from obese mothers. **Figure 7C** showed that this was not the case in our model, as fetal weight was unperturbed between the male TSC2control^{placenta} and TSC2KO^{placenta}. Similarly, the fetal weight was also unchanged between the female TSC2control^{placenta} and TSC2KO^{placenta} in **Figure 7D**. This again suggests that the TSC2 alteration was not effective to cause mTOR activation and consequent fetal weight increase.

Because the genetic alteration was placental specific, we explored the possibility of weight perturbation more locally in the placenta. Akhaphong *et al*. (2021) showed a reduction in placental weight in female mice that had placental-specific mTOR deletion. We also thus compared the placental weights of mTORcontrol^{placenta} and mTORKO^{placenta} in male (**Figure 7E**) and female (**Figure 7F**) mice and observed no difference. Similarly, the TSC2control^{placenta} and TSC2KO^{placenta} models for male (**Figure 7G**) and female (**Figure 7H**) showed no difference. With mTOR's ability to regulate nutrient transport to the developing fetus and organs thus regulating the pancreatic mTOR activity to facilitate pancreatic growth (**Figure 3**), we also thought to explore the role of placental mTOR alteration on pancreas weight. At the time of dissection, the embryos were placed under a microscope to magnify the view of the embryonic organs, and embryonic pancreas were meticulously dissected. The dissected pancreas for each animal was then weighed and compared between the genotypes of the mice. **Figure 7I** showed that there was no difference in the pancreas weights of male mTORcontrol^{placenta} and mTORKO^{placenta}. The pancreas weight of the female mTORKO^{placenta} also was unchanged compared to the mTORcontrol^{placenta}, although there seemed to be a slight upward trend (**Figure 3J**). Same results of unchanged pancreas weight phenotypes were observed in the TSC2 models (**Figures 3K, 3L**).

In addition to investigating the sex-specific changes in the weight parameters, we asked the question of whether the metabolic health of the dams (who experienced placental mTOR *in-utero*) caused changes in the weight parameters between male and female. To answer this question, we first investigated what a normal comparison between male and female would be, that is, in a cohort where both parents had no genetic alteration (called the WT-WT embryos) In the first pair of vertical bars of **Figures 7M, N, O**, we observed the normal weight comparison of the fetus, placenta and pancreas. Using this comparison as a landmark, we observed that the weights of male versus female were also unchanged in the mTOR and TSC2 animals with and without CYP19 (**Figures 7M-O)**. These data are indicative of a null-effect of the placental alteration on the weights of the fetus, placenta and pancreas.

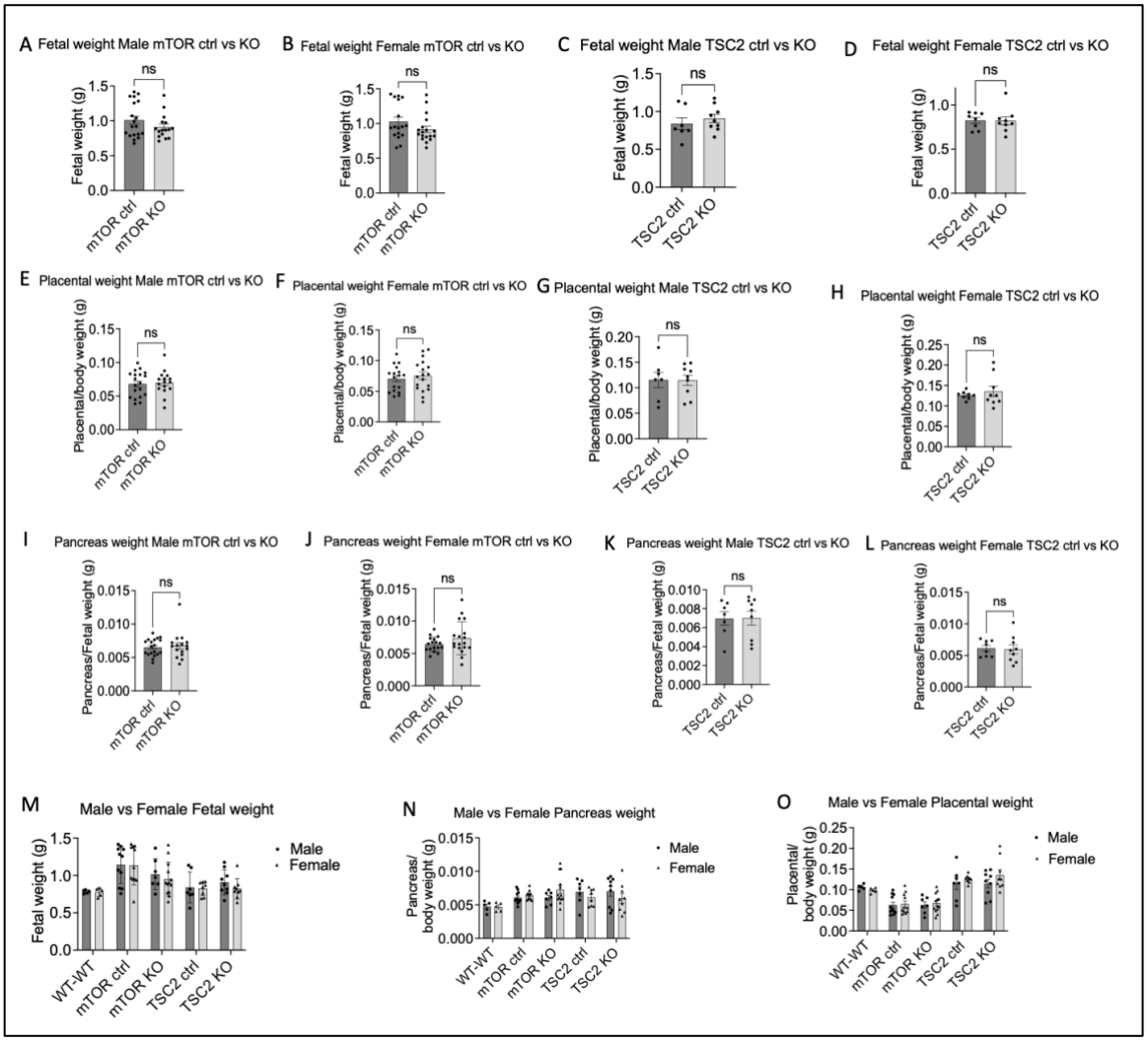

Figure 7: Effects of placental mTOR alteration on fetal, placental, pancreas weights

(A-O) Unchanged effects across all the comparisons as indicated in the title of each graph and described in the text. Error bars represent mean ± SEM.

3.4. Effects of mTOR and TSC2 on amino acid transporters

As a result of the null effects we observed in the fetal weight, placental weight, pancreas weight, and because of the increased beta cell mass of the mice with placental mTOR alteration, we asked whether we had truly perturbed placental mTOR's function on the amino acid transporters that transport amino acids for fetal growth and metabolism. The gestation period is an important time during development that greatly affects the growth and development of the offspring, both globally and metabolically. The mTOR complexes have been shown to play significant roles in regulating this growth and development through their regulation of amino acid exchange between the mother and fetus. Works by Rosario *et al*., 2016 have shown that with inhibition of mTOR complex 1 in primary human trophoblast cells, there is reduction in the abundance of amino acids in the placenta. This group reported that mTORC1 inhibition increased Nedd4-2 expression, promoted ubiquitination and decreased the plasma membrane expression of SNAT2 and LAT1, consequently resulting in decreased cellular amino acid uptake in the *ex vivo* human placenta.

Having shown perturbation of mTOR and TSC2 gene and protein levels in the placenta of our models and having observed the effects in beta cell mass and pancreas weights, we further probed for the protein abundance of these amino acid transporters in the corresponding placental lysates via western blot. Despite the reduction in mTOR and p-S6 protein levels observed in the mTORKO^{placenta}, **Figure 8A** showed that there was no change in the System L amino acid transporter isoform, LAT1, in the placenta of the mTORKO^{placenta} embryos compared to mTORcontrol^{placenta}. **Figures 8B** and **C** show the result of the quantified bands from the raw blots in the female and male cohorts respectively. Another transporter, an isoform of the Sodium-coupled Neutral Amino acid Transporter, SNAT1, was probed for (**Figure 8D)**, and interestingly, the raw western blots and its quantifications showed that there were unchanged levels of SNAT1 in the male placenta (**Figure 8E)** but an increase in SNAT1 in the female mTORKO^{placenta} compared to the littermate controls (**Figure 8F**). This increase in SNAT1 observed in the female mTORKO^{placenta} was particularly interesting because of the increased beta cell mass that had been observed also in the female mTORKO^{placenta} (**Figure 6E**). This suggests that there is a strong link between SNAT1 amino acid transporter and beta cell

mass. It is important to note that while the SNAT1 bands were smeared on the blot (**Figure 8D**), the bands between 50-70kDa were accurately quantified to obtain the quantifications in **Figure 8E** and **8F.** Further experiments, for example, Immunohistochemistry (IHC) will need to be performed on the placenta of these female mTORKO^{placenta} mice to further validate the observed western blot results.

These unchanged and increased amino acid transporter levels in the mTORKO^{placenta} despite observing reduced mTOR levels suggest the possibility of an adaptation by the placenta to the altered mTOR levels. In addition, in the TSC2 placentas, there were no observable changes in the quantified protein levels of LAT1 and LAT4 transporters (**Figure 8G-I**). Similarly, SNAT1 was probed for, but bands were hardly observed and unquantifiable because of problems with antibodies, and thus that piece of data was inconclusive and not shown in this thesis, thus, an IHC will also need to be performed on the placenta of these mice to measure their SNAT1 levels. These unchanged levels of the TSC2 placenta are unsurprising, given the unchanged levels in mTOR signaling observed in **Figure 5J-M**. NEDD4-2 was not probed for in these lysates, but it would have been interesting to find out if NEDD4-2 was perturbed. The results suggest that mTOR and TSC2 perturbation in the placenta do not result in the hypothesized changes in the placental amino acids, this may be due to adaptive changes in the placenta (in the case of the mTOR models) and null-effects on mTOR signaling (in the TSC2 models).

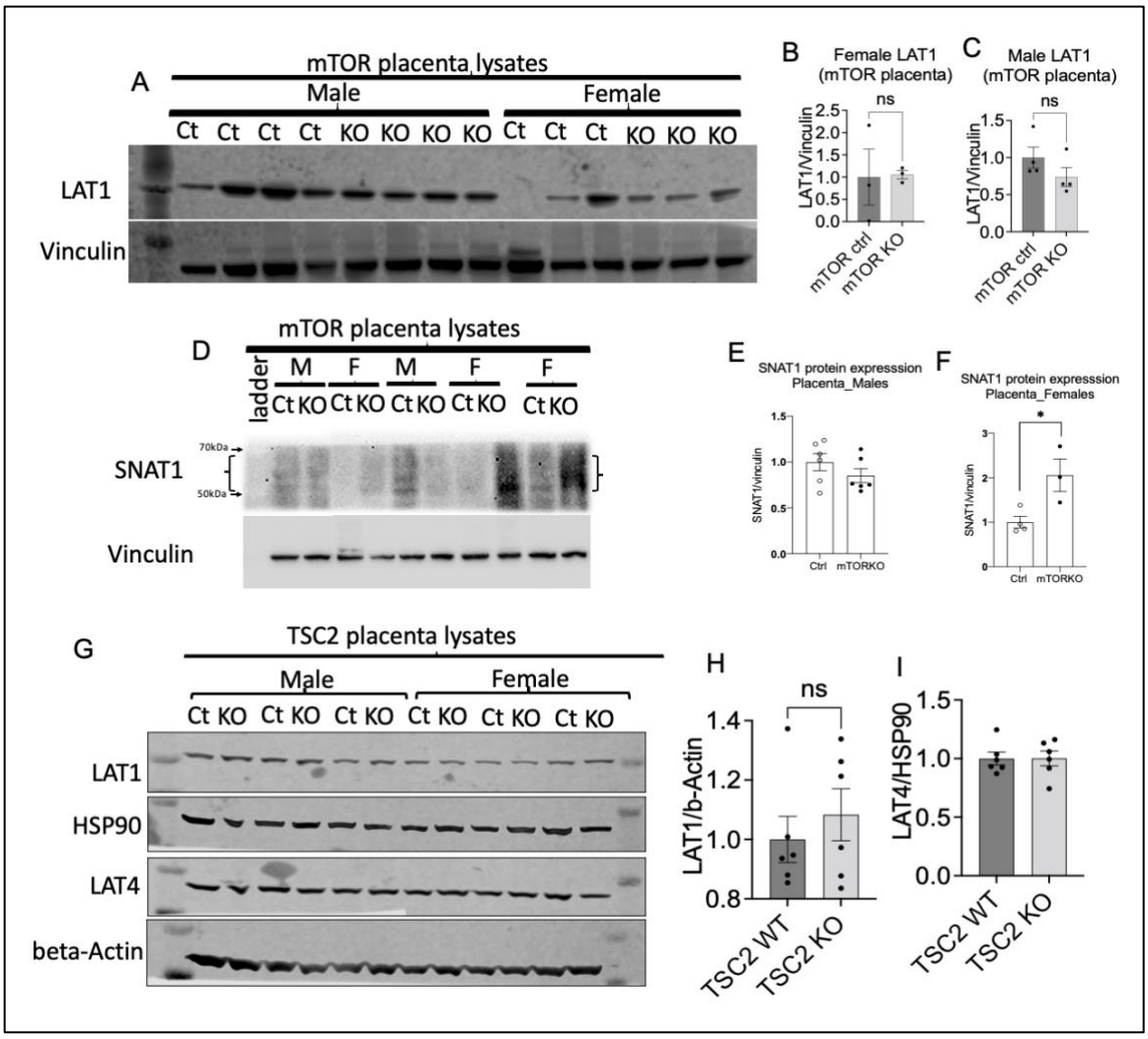

Figure 8: Effects of mTOR and TSC2 perturbations on amino acid transporters

(A) Raw western blots showing LAT1 level in mTORKO^{Placenta} vs mTORcontrol^{Placenta} (B-C) Quantifications show that LAT1 in unchanged in female and male mTORKO respectively (D) Raw western blots showing SNAT1 levels in mTORKO^{Placenta} vs mTORcontrol^{Placenta} (E) SNAT1 is unchanged in male mTORKO^{Placenta} compared to mTORcontrol^{Placenta} (F) SNAT1 is increased in female mTORKO^{Placenta} compared to mTORcontrol^{Placenta} (G) Western blots showing LAT1 & LAT4 levels in TSC2KO^{Placenta} vs control. HSP90 and beta-actin are used as housekeepers (H-I) Quantifications show that LAT1 and LAT4 are unchanged in TSC2KO^{Placenta} compared to TSC2control^{Placenta} Error bars represent mean ± SEM. *$P < 0.05$

4.0. CHAPTER 4: DISCUSSION

Although several models of maternal malnutrition e g., low protein diet, calorie restriction, have been studied, the role of placental mTOR activity on offspring beta cell mass *in vivo* has not been extensively explored. Although mTOR activity in placental explants has been correlated with fetal growth and amino acid transport, the role of this nutrient and hormone-sensitive signaling pathway on the development of beta cells has not been explored using *in vivo* preclinical models. More importantly, it's role on beta cell mass at embryonic day 17.5, when beta cells become more abundant in the mouse islets, has not been interrogated. In the current study, using mouse model of loss- (mTOR model) and gain-of-function (TSC2 model) of placental mTOR, we investigated the role of placental mTOR on beta and alpha cell masses at e17.5 via mTOR's role as a nutrient sensor and regulator of amino acid transporters during gestation.

4.1. Unchanged placental mTORC1 signaling upon alteration of placental TSC2 gene and protein

After validation of the genetic alteration by gel electrophoresis, western blot and trophoblast-specific CAG-ZsGreen expression, we found that there was efficient knockdown in the mTOR model both locally (mTOR reduction) and functionally (p-S6 reduction), but this was not the case in the TSC2 model where we observed TSC2 protein reduction but no reduction of the downstream effectors. While this came as a surprise, we speculate that the unchanged mTOR activity could be a result of a compensation by adenosine monophosphate-activated protein kinase (AMPK) which is upstream of TSC2 and acts as a direct negative regulator on mTORC1 (**Figure 2**). Thus, there is a possibility that, while TSC2 plays its role of mTORC1 activation, that activation is inhibited by another factor (AMPK) acting directly on mTORC1. This thus emphasizes the need for other approaches to enhance mTOR expression and activity as opposed to the disinhibition strategy by TSC2 alteration used in the current study, for example, using recombinant adeno-associated virus9 (rAAV9) vectors to overexpress the gene as has been done by Ding *et al*. in 2016, or carrying out Cre-inducible overexpression of the gene as has been shown by Li *et al.,* 2011.

Because deletion of TSC2 did not lead to increased mTORC1 activity in our model, we also speculate a possibility of the role of nutrient flux in mTORC1 activation. In other

words, the ingestion of food by the pregnant dam on the morning of the harvest could have led to a haphazard regulation of mTORC1 irrespective of TSC2's effects or non-effect compared to if the different dams used have been subjected to the same food intake especially closer to the hours of harvest.

A study by Rachdi *et al*., although with a beta cell-specific deletion of TSC2, reported increased pS6 kinase, pS6 and beta cell mass expansion (Rachdi *et al.* (2008), but these studies phenotypes were assessed and observed in the mice at adulthood (8-week old mice) and not at the embryonic periods of beta cell specific TSC2 disruption. The concept that has been observed in beta cells might also apply to placental cells in our KO model. In other words, there is activation of mTOR signaling in the beta cells when it is necessary. Thus, the TSC2 model mice have the capacity to have increased mTORC1 but measuring mTORC1 at the basal levels may not reveal its activity on beta cell until that activity is needed in certain abnormal physiological, for example, when the animals are fasted, or glucose challenged. In other words, the placental cells might not have had mTOR pathways activated in our TSC2 KO mice since the nutritional status of the animals was not compromised, so the disinhibition might not have caused any significant changes in mTOR activity. This may be the reason why we did not observe changes in response at birth and at embryonic stages. Also, in our study, it would have been interesting to probe for Rheb, to understand what effect the GAP domain of the placental TSC2 disruption had on its direct target.

4.2. Impact of mTOR alteration on amino acid transporters

In the mTOR-KO model, where it is expected that the amino acid transporters are reduced or dysfunctional, there is also an expected reduction in the amino acids being transported to the fetus, thus leading to reduced mTOR activity in the fetus and its organs, which should result in fetal, organ size and beta cell mass reduction. This expectation and thus, hypothesis, stemmed from studies on human placental explants that revealed mTOR's ability to carry out this function through its interaction with NEED4-2. Although our group has previously shown with greater number of numbers of animals, that deleting placental mTOR causes reduced placental and body weight at e17.5 and P0, this was not the case in the current cohort of mice as I observed no changes in placental and bodyweight at e17.5. Further, we observed an unchanged level in LAT1 amino acid transporter, and interestingly observed an increase in SNAT1 amino acid transporter in the mTORKO^{Placenta} female. This may be due to an adaptation by the placenta to meet the

nutrient demand by the fetus. The placenta can sense both maternal nutrient levels and fetal demand and has the ability to adapt and adjust to maintain adequate supply. Such adaptive placental responses have been well reported by Jones *et al*., 2006, where they describe the adaptive regulation of SNAT 1 and SNAT 2 in the placenta, and Aiko *et al.* who observed an adaptive response of amino acid transporters System L and mTOR in the placenta of IUGR patients. A similar adaptive response that showed upregulation of transporters has also been described in tumor cells to compensate limiting nutrient conditions in the cells. (Commisso *et al*., 2013). More evidence has suggested an adaptive model of transport during gestation. For example, Coan *et al.* in 2010 revealed that under limiting conditions, such as undernutrition of pregnant mice, which is similar to the mTOR alteration in our mice model, there are morphological and functional adaptations in placental parameters to support fetal growth. Interestingly, this adaptation may be sex specific, with female placenta demonstrating more resilience (Nuggent *et al*., 2018; Barke *et al*., 2019).

We thus raise the possibility that an adaptive response may account for the unchanged levels in LAT1 amino acid transporter and increased female SNAT1 amino acid transporter observed in our model. This SNAT1 increase was only observed in the female but not in the male placenta, raising the possibilities of differences in a sex-specific sensing of nutrients during gestation. It will also be worthwhile to validate the results observed by western blot through IHC staining of SNAT1 in the male and female placenta of the mTORcontrol^{placenta} and mTORKO^{placenta} mice. Here, we've investigated the levels of amino acid transporters. Another important experiment to carry out would be to track the direct transport of certain amino acids during gestation. For example, investigating the transport of Leucine, as was performed by Coan *et al*., 2010 and Akhaphong *et al*., 2021, where radiolabeled isotopes were used to measure the unidirectional flow of nutrients from maternal to fetal placental interface, would shed more light on the transporter efficiency of LAT1 which transports leucine. A missing piece in our study was not investigating NEDD4-2 which has been identified and characterized as the mediator between mTOR levels and amino acid transporters levels (Hatanaka *et al*., 2006, Rosario *et al*., 2016), and not investigating other SNAT isoforms (e.g., SNAT2, SNAT4). These would have been more informative for the current study to understand why mTORC1's downstream targets were reduced but the expected effects on amino acid transporters was not observed.

4.3. Impact of placental mTOR alteration on beta cell mass in the offspring

In the placental mTOR-KO model, the hypothesized reduction in the amino acid tranport to the fetus is further expected to lead to a reduction in global fetal growth and mTOR activity in the metabolic tissues of the fetus, thus a reduction in beta cell mass. Furthermore, TSC2 alterations observed in the TSC2-KO model is hypothesized to lead to a consequent increase in beta cell mass via mTOR's regulation of amino acid transport. However, in the TSC2 model, we observed no change in the beta cell mass and in the mTOR model, we observed no change in the beta cell mass and an increase in the beta cell mass in females.

The null effects of placental TSC2 alterations on beta cell mass did not come as a surprise because in the TSC2-KO model, having observed TSC2 alteration, there was no observation of an increase in mTORC1 downstream targets and thus a consequent null effect on the amino acid transporter levels. In addition, one caveat to the beta cell mass levels is the number of animals in each cohort which was about 3-4 in number. Although, this is an acceptable number of animals to show significance, perhaps, more animals in each group would have further tightened the wide-spread data points observed in each bars.

With respect to the mTOR-KO model, the adaptive response proposed to occur with the placental amino acid transporters which led to an unchanged level in these transporters explains the levels of beta cell mass observed in the e17.5 male pancreas. It is important to note that there was an increase in beta cell mass in the female mice, this coincides with the increase observed in SNAT1 also in the female mice. Where there was an unchanged amino acid transport in male mTOR models, we also observed unchanged levels in beta cell mass. This suggests the possibility of a link between placental amino acid transport levels and beta cell mass of offspring. One experiment that can be of great resource to better understanding these mechanisms of increased and unchanged beta cell mass would be exploring the mTOR activity through phosphorylated S6 (p-S6) immunofluorescent staining in beta cells, as amino acid transport affects the pancreatic mTOR activity which is what would directly affect the beta cell mass. Also, investigating proliferation by ki67 staining in the pancreas would be a useful experiment to understand if there is increased proliferation of beta cell mass in those female mTORKO^{placenta} animals compared to their littermate controls.

One other caveat to such studies is the consideration of maternal health. Although it was appropriate to use littermate controls, it would be helpful to compare the data with

true WT, where the dams had no experience of placental mTOR deficiency. For example, in our model, the female dam is Cyp19-Cre positive, thus an mTORKO^{placenta} animal, previously shown to have increased metabolic dysfunction in high-fat diet (Akhaphong *et al*., 2021). Thus, it is possible that she may have had metabolic dysfunction during pregnancy that can alter the mTORcontrol^{placenta} offspring. This raises the possibility that her offspring who, even though they are Cyp19-Cre negative, may still have some metabolic changes that are significantly different from offspring (called WT-WT) of true wildtype parents whose metabolism mimics a more physiologic state. Thus, carrying out an experiment to compare placental and metabolic parameters between mTORcontrol^{placenta} mice and WT-WT mice would also be a beneficial future direction.

5.0. CHAPTER 5: CONCLUSION

The findings from this study revealed that although there was a knockout of the genes of interest in our models, the amino acid transporter levels and placental functioning was not dramatically changed as expected based on past human placental explant studies. This may be due to several other biochemical changes in the placenta that mask the significant downstream effects of the gene modification in *in vivo* models. We also observed a possible link between beta cell mass and amino acid transporter levels, which will need to be further investigated by other experiments. This study further highlights a greater understanding of the *in-utero* factors that could affect beta cell growth and development. This is of great importance because better exploration of these factors could help in better prevention of Type 2 diabetes.

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