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# Specific Aim 3

**Elucidate the consequences of placental nutritional stress driven by mTORC1 hyperactivation on placental role.** *In utero* exposures play a significant role in molding offspring health according to the Developmental Origins of Health and Disease (Wadhwa *et al.*, 2009). The fetus can therefore sense maternal status and adapt accordingly. Pregnancy is associated with increased maternal weight and elevated cortisol levels (Abrams *et al.*, 1995; Jung *et al.*, 2011). Of concern, maternal obesity and maternal cortisol, aside from the natural progression of pregnancy, influence the fetus gravely through perturbing placental function and impairing healthy fetal growth. The placenta is the direct and only site of communication between mother and fetus during *in utero* development (Brett *et al.*, 2014). The placenta is the rate-limiting step for fetal nutrient and gas acquisition (Brett *et al.*, 2014). Additionally, the placenta plays an important endocrine role to promote fetal growth and nutrient supply (Malassine *et al.*, 2003). The placenta is highly regulated to ensure adequate growth of the fetus in normal pregnancies (Napso *et al.*, 2018).

Maternal obesity has long term effects on both mother and child. Children of mothers with class III obesity are at 2.32 times higher risk of being large for gestational age regardless of other pregnancy complications (Kim *et al.*, 2016). Children of obese mothers are at higher risk of developing non-communicable diseases like hypertension, insulin resistance and diabetes later in life with higher odds of 3.84 and 3.0 of developing childhood overweight and cardiometabolic profile, respectively, as early as six years of age (Leddy *et al.*, 2008; Samuelsson *et al.*, 2008; Mingrone *et al.*, 2008; Gaillard *et al.*, 2014; Williams *et al.*, 2014; Stubert *et al.*, 2018). Alarmingly, data collected from 47 states in the United States show that more than 50% of pregnant women were either obese or overweight in 2014[[1]](#footnote-1) (Branum *et al.*, 2014). The exact mechanisms by which the offspring health is affected in response to *in utero* exposures remain elusive. In maternal obesity, placental nutrient transport and endocrine function are believed to be suboptimal leading to unhealthy fetal growth (Leddy *et al.*, 2008; Kipmen-Korgun *et al.*, 2012; Waffarn & Davis, 2012; Gaccioli *et al.*, 2013*a*). To model excessive nutritional stress, such as that of maternal obesity, I will use a genetic model of mTORC1 hyperactivation in the placenta using a trophoblast-specific driver. With this model, I will determine placental hormone production and evaluate nutrient transport, along with offspring metabolic health. My hypothesis is that mTORC1-hyperactivation as a model of obesity will increase placental and fetal development rate through alterations in placental nutrient transport and endocrine function and will cause impaired offspring health. To test this hypothesis, I will examine a) how placental mTORC1 hyperactivation affects placental development and function and b) how mTORC1 hyperactivation affects fetal and offspring development and health.

# Rationale and Background

## Murine Placental Development and Physiology

The definitive structure of the mouse placenta is (Malassine *et al.*, 2003). The placenta encompasses two sides, an arc-shaped surface facing the maternal side and another flat surface facing the fetal side (Georgiades *et al.*, 2002). The mouse placenta has three distinct compartments, a decidual maternal zone which is the outermost compartment, a fetal-derived junctional zone that mediates placental endocrine function, and a fetal-derived labyrinth zone that comprises the majority of the placenta and is the main site for nutrient and gas exchange (Woods *et al.*, 2018). Three exchange barriers exist moving inwards from the decidua to the fetal compartment including two syncytiotrophoblast layers (in the labyrinth layer) and one fetal endothelial cell layer (Georgiades *et al.*, 2002). The two syncytiotrophoblast barriers comprise the microvillous membrane facing the maternal circulation and the basal membrane facing fetal circulation (Brett *et al.*, 2014). Figure 1 represents the mouse placenta (Bronson & Bale, 2016).

At midgestation, placental invasion of the maternal uterine cavity occurs to allow maternal blood flow into the placental cavity (Malassine *et al.*, 2003; Woods *et al.*, 2018). This invasion permits direct nutrient uptake from the maternal circulation to the fetus through the placenta. Prior to this invasion, the embryo acquires nutrients from the yolk sac, the initial placental structure that absorbs nutrients from maternal circulation (Malassine *et al.*, 2003; Woods *et al.*, 2018).

### Figure 1: Diagram representing the mouse placental cell types and zones from (Bronson & Bale, 2016)



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## Maternal Obesity Prevalence and Risk

Obesity in pregnancy is on the rise in the Unites states with an 11.3% increase from 2005 till 2014 in pregnant women, accounting for 7.3% of the global burden of obesity for 2014 (Chen *et al.*, 2018). In a large cohort study of 287213 participants, 24.3% of pregnant women were obese and 9.6% had severe obesity at the time of their first antenatal appointment booking (Sebire *et al.*, 2001). Increasing maternal BMI had higher odds of having gestational diabetes, prolonged hospital postnatal stay, emergency cesarean section, offspring birthweight higher than the 90% percentile, stillbirth, and other antenatal, maternal and fetal complications (Sebire *et al.*, 2001). In another cohort study of 96801 participants, 18.2% of women were overweight and 10.1% were classified as obese pre-pregnancy (Baeten *et al.*, 2001). Pre-pregnancy overweight and obesity were had elevated risk of preeclampsia and eclampsia, gestational diabetes, and delivering preterm (Baeten *et al.*, 2001). Pre-pregnancy obesity had significantly increased risk of infant death within the first year of life (Baeten *et al.*, 2001). Furthermore, pre-pregnancy obesity is positively associated with large-for-gestational-age deliveries (Ehrenberg *et al.*, 2004).

## Role of Placental mTORC1

mTORC1 is a crucial nutrient sensor that plays a role in integrating maternal and fetal signals to ensure adequate nutrient transport to the fetus through the placenta (Wen *et al.*, 2005; Roos *et al.*, 2007; Mparmpakas *et al.*, 2012; Jansson & Powell, 2013). Expression of mTORC1 was detected in the syncytiotrophoblasts of human placentas (Roos *et al.*, 2007). To validate the role of mTORC1 in the placentas, several studies showed its importance in regulating amino acid transport. Rapamycin treatment of human term-placentas at 100nM caused reduced system L transport activity suggesting a relationship between mTORC1 function and leucine transport (Roos *et al.*, 2007). Furthermore, in placentas of intrauterine growth restriction (IUGR), mTORC1 protein expression was upregulated by 51%, while its expression was downregulated in placentas from large-for-gestational-age deliveries (Roos *et al.*, 2007). Inhibition of mTORC1 by rapamycin in human term-placental extracts reduced placental radiolabeled amino acids transported by system A, system L and taurine transporters (Roos *et al.*, 2009*a*). Rapamycin caused reductions in mRNA expression of LAT1 and TAUT, transporters of system L and taurine, respectively, despite no changes in transporter protein expression (Roos *et al.*, 2009*a*). Additionally, placental explant rapamycin treatment alone showed reduced system A, system L, and taurine transporter activity in the presence of 16mM of glucose suggesting an mTORC1-dependent mechanism (Roos *et al.*, 2009*b*). Insulin increased system A and system L activity in human placental explants, while rapamycin addition ablated the stimulatory effects of insulin (Roos *et al.*, 2009*b*). Furthermore, when glucose availability was reduced, system L transport was reduced and rapamycin addition did not further change the reduced activity of system L suggesting that glucose availability influences an mTORC1-dependent regulatory mechanism (Roos *et al.*, 2009*b*). Glucose availability did not affect System A transport in the absence of rapamycin suggesting that mTORC1 prevented changes in activity, but when mTORC1 was inhibited in the presence of rapamycin, decreased glucose availability increased system A activity suggesting an mTORC1-independent mechanism of compensation in case of hypoglycemia (Roos *et al.*, 2009*b*). These findings suggest that the changes in transporter activity through mTORC1 is mediated by glucose availability, since system L activity decreased (Roos *et al.*, 2009*b*). Taurine transporter activity increased with reduced glucose availability, and this increase was still evident in the presence of rapamycin, indicating an mTORC1-independent mechanism in conditions of hypoglycemia (Roos *et al.*, 2009*b*).

The role of mTORC1 in placental glucose transport is not yet thoroughly studied (Winterhager & Gellhaus, 2017). The first study showing a link between mTORC1 and glucose transport demonstrated that inhibition of mTORC1 in JEG-3 human choriocarcinoma cell line caused significant reductions in GLUT3 mRNA and protein expression (Xu *et al.*, 2015). In addition to the findings from Xu et al (Xu *et al.*, 2015), the results from Roos et al. demonstrate an effect of glucose concertation on mTORC1 signaling (Roos *et al.*, 2009*b*), suggesting a potential feedback mechanism between mTORC1, glucose availability, and placental glucose transport.

Additionally, as mTORC1 has been shown to affect placental amino acid transport, fatty acids have been suggested to differentially influence placental amino acid uptake through mTORC1 (Lager *et al.*, 2014). Incubation of human term-placentas with oleic acid (18:1) showed increased system A amino acid transport along with increased phosphorylated mTORC1 and 6SK1 expression (Lager *et al.*, 2014). Incubation of cells with docosahexaenoic acid (22:6) reduced placental system A and system L amino acid transport and reduced phosphorylated mTOC1 and 4E-BP1 expression (Lager *et al.*, 2014). The effects of mTORC1 on placental fatty acid transport has not been assessed to my knowledge.

Given that mTORC1 activity in other tissues is affected by multiple factors including glucose concertation, amino acid availability and fatty acid concentrations (Gupta & Jansson, 2019), it is presumed that placental mTORC1 is similarly affected by maternal nutrient availability (Gupta & Jansson, 2019).

Additionally, pregnant mice treated with rapamycin at embryonic day (E) 11.5 every 12 hours had fetal lethality at E16.5 with severe fetal growth restrictions (Hennig *et al.*, 2017*a*). Pregnant mice treated with rapamycin at E15.5 every 12 hours until delivery, had reduced offspring weight at postnatal day 1 but unaffected fetal lethality (Hennig *et al.*, 2017*a*). This mTORC1 inhibition was evident in fetal tissue (lung, heart, and kidneys) at PND1, but placental mTORC1 activity was not assessed. Rapamycin treatment may have had a pronounced effect on the placenta to lead to fetal lethality when treatment was initiated at E11.5 sine the placenta was less developed that that treated at E15.5 (Hennig *et al.*, 2017*a*).

Furthermore, homozygous blastocysts that do not express mTORC1 have arrested growth at E5.5 and definite lethality of mTORC1 -null embryos by E11.5 (Gangloff *et al.*, 2004), while another study with mTORC1-null blastocysts showed resorption and fetal lethality by E8.5 demonstrating the crucial role of mTORC1 in placental and fetal proliferation and survival (Murakami *et al.*, 2004). Similar to mTORC1 inactivation, embryonic mTORC1 hyperactivation in rats using *Tsc2* deletion showed 0% fetal viability at E13.5 with abnormal brain development (Rennebeck *et al.*, 1998). Furthermore, *Tsc1-*null mouse embryos, similar to *Tsc2-*null mice, showed embryonic lethality at early midgestation and were much smaller than the control embryos indicating developmental delays (Yagi *et al.*, 1990; Kwiatkowski *et al.*, 2002).

## Placental and Fetal Development in Maternal Obesity

Additionally, obesity is further associated with increased cortisol levels as pregnancy progresses (Lucassen & Cizza, 2012).

Due to the prevalence of obesity and corticosteroid treatments during pregnancy, we will focus primarily on these two *in utero* perturbances and their effect on placental role. We will examine the mechanisms by which maternal obesity and stress influence the offspring health through altering the maternofetal interface and placental nutrient transport and endocrine function (Singh *et al.*, 2012; Díaz *et al.*, 2014; Dimasuay *et al.*, 2016). Determining the exact mechanisms by which maternal obesity and stress affect placental function will allow us to develop future treatments that rescue the effects of maternal obesity and corticosteroid-induced stress on the offspring. We will test the hypothesis that placental nutrient transport and endocrine function are impaired when the maternal milieu is compromised by obesity or increased maternal stress ultimately influencing the health of the developing fetus.

**Specific Aim 1: Determining the effects of maternal diet-induced obesity on placental transport of nutrients and endocrine function.** The specific mechanisms that mediate placental transport of macronutrients in conditions of maternal obesity remain elusive. Our hypothesis is that maternal obesity increases the flux of glucose and lipids from the maternal compartment to the fetal compartment and leads to an impaired placental hormonal function. Reduced placental growth hormone has been associated with intrauterine growth restriction (Koutsaki *et al.*, 2011) and thus we believe that increased levels may contribute to fetal macrosomia. To test this we will determine a) how maternal obesity affects mRNA and protein expression of macronutrient transporters in the placenta b) the flux of macronutrients from the maternal to placenta and fetal compartment *in vivo* and *in vitro* and c) changes in the placental growth hormone secretions.

**Rationale:** Although maternal obesity is shown to have negative outcomes on offspring health, the exact mechanisms modulating these outcomes remain unclear. There has been a focus on diabetes and pregnancy outcomes showing inconsistent results regarding placental system A transport of amino acids (Gaccioli *et al.*, 2013*a*). The function of the placenta in the context of maternal obesity requires further investigation as different models of obesity in animals have shown dissimilar maternal and placental phenotypes (Gaccioli *et al.*, 2013*a*). Maternal obesity exacerbates the natural state of maternal insulin-resistance via increased placental hormonal function. Placental hormonal function further promotes a catabolic maternal state and reduces maternal insulin sensitivity (Leddy *et al.*, 2008). Mechanisms thought to be responsible for the placental and fetal phenotypes in maternal obesity include the increased lipid transport to the placenta and fetus considering the increased insulin resistant state of the mother (Leddy *et al.*, 2008). The mammalian target of rapamycin 1 (mTORC1) is thought to play an active role in promoting placental anabolic function and increased fetal supply of nutrients by orchestrating changes in nutrient transport. According to Jansson et al., a diet high in fat and sugar showed increased activation of mTORC1, and increased placental insulin and insulin-like growth factor1 signaling pathways. (Jansson *et al.*, 2013). This aim will determine the effects of maternal obesity on placental function and will help elucidate some of the potential mechanisms underlying these changes.

**Is mTORC1 the main driver of altered placental nutrient flux in maternal obesity?** Recent studies have highlighted the role of mTORC1 in promoting fetal nutrient acquisition (Jansson *et al.*, 2013; Hennig *et al.*, 2017*b*). Rapamycin treatment during late gestation in mice reduced pup birth weight and caused placental insufficiency mimicking intrauterine growth restriction (Hennig *et al.*, 2017*b*).

## Effect of Obesity on Placental Nutrient Transporters

### Glucose Transporters

Glucose transport across the mammalian placenta is thought to occur mainly via GLUT1 and is complemented by GLUT3 (Hahn & Desoye, 1996; Hahn *et al.*, 1999). GLUT1 and GLUT3 are the most extensively studied transporters in the placenta.

### Amino Acid Transporters

Amino acid concentrations are higher in the fetal umbilical vein than in the mother’s circulation showing a need for active transport of amino acids through the placenta (Cetin *et al.*, 1996). Several transport systems exist on the placental membrane including System A for alanine, serine, proline, and other neutral amino acids, System ASC for alanine, serine and cysteine, and anionic amino acids, System L for leucine, isoleucine, valine, tyrosine, and other neutral amino acids, System N, for neutral and cationic amino acids, system β, system y+, and other systems (Regnault *et al.*, 2002; Gaccioli *et al.*, 2015; Vaughan *et al.*, 2017). System A is sodium-dependent and allows transport of small non-branched amino acids like alanine and glycine (Jones *et al.*, 2006), and its activity is strongly related to fetal growth with evidence suggesting that system A activity being negatively associated with the severity of IUGR (Glazier *et al.*, 1997; Vaughan *et al.*, 2017). Hence, despite the presence of multiple placental amino acid transport systems, system A was the main studied system in most of the currently available research.

### Fatty Acid Metabolism

Lipoprotein lipase (LPL) is present on the placental microvillous membrane and plays a crucial role in lipid metabolism (Huter *et al.*, 1997). Its activity comprises the first step of placental transfer of fatty acids from mother to fetus by breaking down maternal triglycerides into fatty acids that can then be transported across the placenta (Huter *et al.*, 1997). Low-density lipoprotein (LDL) receptor is also located on the microvillous membrane of the placenta and is important for uptake of LDL from the maternal circulation to the fetus through the placenta (Huter *et al.*, 1997).

**Expected Results and Alternate Approaches.** Based on the central role of mTORC1 in the development of many tissues we predict placental mTORC1 activation, as a model of nutritional stress will *increase* placental size, specifically of the labyrinthine zone but that fetii will be otherwise normal. The hypothesis that chronic mTORC1 activation will not be lethal in fetus is supported by conditional fetal knockouts of *Tsc1* in heart, brain, liver, and kidneys all of which are carried to term (Anderl et al., 2011; Meikle et al., 2007; Malhowski et al., 2011; Inoki et al., 2011; Bissler et al., 2019; Kayyali et al., 2015). As with Aim 1A, these studies are powered to detect a >50% reduction in viability at each time point. If *Cyp19a1-Cre* or *Sox2-Cre* driven *Tsc1* knockout mice are viable this will be a valuable tool to understand chronic outcomes of early mTORC1 activation including metabolic disease risk. If mice are inviable, our studies will be able to determine the stage at which viability is lost, and test if mTORC1 inhibition could rescue these defects. We expect to be able to evaluate mTORC1-activated placentae at least at an early stage, can compare these to currently available transcriptomic data from fetal overnutrition studies (Gabory et al., 2012). Similar to Aim 1A, our within-dam design allows us to control for the maternal environment but precludes definitive evaluation of communication between mTORC1-activated placentae and maternal health.

## Effect of Obesity on Placental mTORC1 Function

Pre-pregnancy body mass index (BMI) was positively correlated with placental mTORC1 activity and birth weight (Jansson *et al.*, 2013). System A and system L amino acid transporter activity was unchanged with increased maternal pre-pregnancy BMI, but system A SNAT2 protein expression was positively associated with offspring birth weight suggesting that increased amino acid uptake may contribute to increased birthweight and fetal overgrowth in maternal obesity (Jansson *et al.*, 2013). Additionally, in a mouse model of maternal obesity induced by a high fat high sugar diet, E18.5 placental weight was unchanged but fetal weight increased by 18% (Rosario *et al.*, 2016). Placental insulin and mTORC1 signaling were significantly increased, suggesting a potential increased placental amino acid transport contributing to the increased fetal weight (Rosario *et al.*, 2016). In rats, (Gaccioli *et al.*, 2013*b*)mTORC1 is activated under conditions of nutrient excess in many tissues, including the placenta and is associated with both placental and fetal size (Jansson et al., 2013; Rosario et al., 2016; Sati et al., 2016; Song et al., 2017) DAVEE

## Effect of Obesity on Placental Endocrine Function

## Effect of Obesity on Offspring

Maternal obesity is positively associated with childhood risk of developing metabolic syndrome in large-for-gestational-age babies (Boney *et al.*, 2005). Indeed, maternal pre-pregnancy obesity was the strongest determinant of childhood obesity at 6-11 years of age (Catalano *et al.*, 2009*a*). Furthermore, maternal obesity was positively correlated with fetal body fat percentage and fetal insulin resistance whereby maternal obesity caused significant increase in neonatal body fat percentage, neonatal fat mass, placental weight, and umbilical cord insulin levels (Catalano *et al.*, 2009*b*). Fetuses of mothers who had obesity were more insulin resistant than fetuses of lean mothers using umbilical cord blood at delivery (Catalano *et al.*, 2009*b*). Additionally, preschool childhood obesity was positively associated with maternal pre-pregnancy BMI (Whitaker, 2004). Children born to obese mothers were two times more likely to be LGA, and LGA was further predictive of early childhood obesity (Whitaker, 2004).

# Experimental Design

To determine the effects of mTORC1 hyperactivation on the placenta and the fetus, we will develop a placenta-specific *Tsc1* knockout (KO) model. To isolate placental from fetal and maternal mTORC1 signaling, our knockout model will ablate Tsc1 conditionally in the placenta. To my knowledge, this is the first time such a model has been generated. To generate the *Tsc1*-KO, we will use the Cre-loxP recombination technology. We will leverage the fact that placental tissue is primarily fetal derived, so the genotype of the offspring will dictate the genotype of most of the placenta. The breeding scheme is represented in Figure 3. First, female mice with homozygously flanked *Tsc1* gene exons 17 and 18 of *Tsc1* will be crossed with a male having placental driver *Cyp19a1-CreTg/+* (Wenzel & Leone, 2007).This *Cyp19a1-Cre* has been also used elsewhere to generate a placental knockout model (Wieczorek *et al.*, 2019). This cross will generate wild-type (WT) and heterozygous (Het) offspring at a 1:1 ratio. The expected timeline between this first breed and the second one is 9-12 weeks. The offspring of this first cross will be bred (WT x Het) to generate the parental strains for this experiment. Briefly, this cross will yield a combination of knockout *Tsc1* fl/fl;*Cyp19a1-CreTg/ +*, conditionally heterozygous *Tsc1* fl/+;*Cyp19a1-CreTg/+* , and wild-type *Tsc1* fl/fl ; *Cyp19a1-Cre* +/+ , *Tsc1* fl/+ ; *Cyp19a1-Cre* +/+ , *Tsc1* +/+ ; *Cyp19a1-Cre* +/+ , or *Tsc1* fl/fl ; *Cyp19a1-Cre* Tg/+ (no Cre transgene) at an expected ratio of 1:2:5 with the knockout and wild-type (*Tsc1* fl/fl ; *Cyp19a1-Cre* +/+ only) animals only being used for further breeding. The expected timeline for this second cross to generate mature offspring capable of breeding is also 9-12 months. The final parental breed of WT x KO will generate our placental KO model. The final offspring generated from the next generation will all have the floxed allele with the Cre (KO) or without (WT). The offspring generated from the last main parental breed will either be WT with intact placentas or knockout with placental KO and a phenotypically WT embryo.

To determine how mTORC1-hyperactivation model of obesity affects placental function, we will use n=X WT females and Y WT and KO males /per group 8 week-old C57BL/6 virgin mice from the parental strain (shown in Figure 2). At 6-weeks, mice will be single-housed to allow for acclimatization prior to mating then will be randomized into one of the following four groups, to assess placental morphology (at E14.5) and effects on offspring (after delivery). The experimental design is represented in Figure 4. Pending these results, other groups may be evaluated at different gestation timepoints.

Cohort A Groups:

1. *WT till E14.5:* WT female mated with WT male on water and normal chow diet until midgestation at embryonic day 14.5
2. *KO till E14.5*: WT female mated with KO male on water and normal chow diet until midgestation at embryonic day 14.5

Cohort B Groups:

1. *WT till Weaning*: WT mother mated with WT male on water and normal chow diet until delivery and weaning
2. *KO till Weaning*: WT female mated with KO male on water and normal chow diet until delivery and weaning

All groups will have *ad libitum* access to normal chow diet and water. WT female mice will be mated with age-matched WT or KO male mice after 2 weeks of acclimatization to being single-housed. A copulatory plug will be checked daily to identify E0.5 day. Mice will be mated with age-matched males immediately after acclimatization while having *ad libitum* access to water. We will check for the presence of copulatory plugs daily. Males will be removed from the cage after a copulatory plug is detected. Dams from all groups will undergo body mass assessment three times weekly using magnetic resonance to assess body composition. Water and food intake will be recorded weekly.

For groups of Cohort A that will be sacrificed prior to delivery (E14.5), placental and fetal extractions will occur midgestation at E14.5, since by midgestation, the placenta is fully developed and mature. Briefly, the dams will be anesthetized using a vaporizer during the placental and fetal extraction. Litter size will be determined per dam and will account for potential resorbed placentas. Placental and fetal weights will be collected. Placentas will be snap frozen in liquid nitrogen while some will be embedded in paraffin for histology. Molecular studies on placental samples will be conducted to determine protein expression.

For the groups of Cohort B that will deliver their pups at E21.5, survival and birth rates will be noted. Pups will be sexed and culled to 2 at PND2.5. The offspring will be weighed at PND0.5, PND7.5, 14.5, and at 21.5. Pups will be weaned based on sex and genotype. The weaned pups will have *ad libitum* access to normal chow diet and water. Their water and food intake will be assessed weekly. They will further undergo body composition analysis by echoMRI at weaning and weekly thereafter till 6 weeks of age. At the age of 6 weeks, offspring insulin sensitivity will be assessed by an insulin tolerance test (ITT) followed by sacrifice and tissue collection of fat pads 3 days after the ITT. Offspring fat pads (gWAT and iWAT) will be collected and weighed to determine adiposity.

### Figure 2: Schematic diagram representing TSC1/mTORC1 pathway in KO and WT placenta



### Figure 3: Diagram representing the breeding method to generate the knockout placenta



### Figure 4: Diagram representing the experimental design and respective timeline



# Methods

## Food Intake

Food will be weighed when the treatment starts and throughout the experiment. The weight of the dam’s food will be recorded three times weekly every Monday, Wednesday, and Friday. Food will also be weighed at delivery for the dam. Food will be topped off to ~400g weekly every Friday. Food intake will be calculated as:

If the dam is single housed or with nursing pups:

(the new added total food weight - the last measurement’s food weight) / # of days between measurements

If more than one adult mouse is in the cage (when the male is breeding in the same cage, or when weaned offspring are housed together), food intake will be calculated as follows:

(the new added total food weight - the last measurement’s food weight) \* #of days between measurements / sum of days spent by each mouse in that cage between measurements

## Body Composition

Mice will be weighed by using dynamic weighing to capture accurate weight using a digital scale. The weight will be recorded along with the mouse ear tag number. The mouse will be gently placed in the MRI tube with the plunger slightly compressing along the mouse body to ensure it cannot move during the measurement. Fat, lean, free water and total water mass (g) will be recorded for each animal.

## Sacrifice and Tissue Collection

Dams of groups E14.5 will be sacrificed on the respective dates based on their treatment group. Dams will be anesthetized using an isoflurane vaporizer. Toe punches will be performed to ensure that the mouse is under anesthesia. A midline incision of the skin from the rectum to the diaphragm will be made while the mouse is still alive and anesthetized using the vaporizer. The uterine horn will be exposed and placental and fetal excision will begin in order along the uterine horn starting from the side (closer to the ovaries). The amniotic sac for each pup will be ruptured using fine scissors. The placenta will be detached from the maternal tissue and the umbilical cord then weighed and immediately snap frozen or cryopreserved and in paraffin for future molecular and histological studies. Fetuses will be weighed after removal from the amniotic sac then they will be immediately sacrificed by decapitation using surgical scissors. After the complete extraction of tissue, dams will be euthanized while under anesthesia by cardiac exsanguination.

Offspring of dams that will be allowed to deliver and nurse (groups of E21.5) will be dissected at 6 weeks of age. Offspring will be first anesthetized using isoflurane drop jar. Offspring will be sacrificed using isoflurane drop jar. Cervical dislocation will be performed as a secondary measure to confirm euthanasia. We will dissect the offspring fat pads by a midline incision of the skin from the rectum to the diaphragm, extract inguinal and gonadal white adipose tissue. Inguinal white adipose tissue (iWAT) will be collected from the mouse right side first by pulling the peritoneum away from the skin. Inguinal fat will be carefully extracted, weighed then snap frozen in liquid nitrogen for further molecular studies. Right gonadal white fat tissue (gWAT) will be collected next by scraping the fat along the gonads (ovaries or testis), weighed, and then snap frozen in liquid nitrogen in 2ml tubes. The fat pads will be stored at a temperature of -80C.

## Insulin Tolerance Test

Weaned offspring in groups water or dexamethasone till delivery from cohort A (pre-gestation) and cohort B (at conception) will undergo an insulin tolerance test (ITT) being challenged with 1 U/kg body weight after a 6-hour fast with *ad libitum* access to water. The effects of antenatal glucocorticoid exposure on offspring adolescent insulin sensitivity will be determined. Briefly, after the fast, the tail will be cut to allow for blood sampling via AccuCheck Advantage Glucometer. Tail vein blood will be immediately measured at 0minutes after the 6-hour fast to denote fasting blood glucose. Mice will be injected by a syringe into the interperitoneal cavity with the appropriate insulin dosage. The timer will be set as to allow for blood collection every 15 minutes. Blood will be collected at 5, 30, 45, 60, 75, 90 and 120 minutes after injection. After the ITT is done, mice will have *ad libitum* access to normal chow diet and water again. These data will be analyzed by mixed linear models of glucose at each time point.

## Real time qPCR

Using the placental tissues collected from the dams, we will assess RNA expression of macronutrient transporters and endocrine hormones. RNA samples will be prepared from the mouse tissues using the PureLink RNA Mini Kit. Briefly, tissues will be cut to ~50mg samples that will be homogenized and treated to collect the RNA. The RNA will be quantified using a nanodrop. Later, first strand cDNA will be synthesized from the purified RNA samples using High Capacity cDNA Reverse Transcription Kit. The cDNA samples will be diluted and added to the clear 384 well plate in triplicates. A Primer/SYBR Green mix will be prepared for each primer. Briefly, we will use sequence-specific primers to amplify GLUT1, GLUT3, GLUT4, SNAT1, SNAT2, SNAT4, LPL, GDF15 and IGF-II using primer pairs (forward and reverse). This will allow us to assess the overall endocrine and transport function of the placentas of Dex- and Water-treated dams. PCR will be performed for *Sry* to determine the sex of the placentas/fetuses using a piece of the placenta or fetal tails, respectively.

## Genotyping

Maternal and fetal genotyping will be conducted to confirm the GR KO or WT genotype of the dams and fetuses/placentas. To genotype the dams, DNA extraction from tail clips will be done. qPCR analysis of the *Tsc1* and *Cyp19a1*-Cre gene will be conducted to determine presence of the floxed alleles. For fetal/placental genotyping, fetal tail will be entirely clipped for DNA analysis to confirm the presence of Cre and floxed alleles*.*

## Western Blotting

Using the placentas collected at E14.5, mTORC1 activity will be assessed. Validation of glucocorticoid receptor ablation will be validated from collected placentas. Briefly, a portion of the sample will be boiled and loaded into different wells with a ladder control. Proteins will transfer to nitrocellulose overnight. The matrix will be stained for total protein using Revert total protein and scanned by LiCOR to normalize against total protein. Samples will be incubated with the primary then the secondary antibodies. Briefly, antibodies against total and phosphorylated mTORC1 targets (S6K, 4EBP1, S6) and regulators (Akt, IRS and TSC2) and antibodies against GR will be used.

## Histology

Placentas collected from control and experimental at E14.5 will be embedded in paraffin and stained at the Rogel Cancer Center’s Tissue and Molecular Pathology. Slides will be blindly assessed for labyrinth thickness and area.

Expected Results

## **Aim 3.1:** How does placental mTORC1 activity affect placental development, fetal growth, and fetal survival?

## **Aim 3.2:** How does placental mTORC1 hyperactivation affect the expression of placental nutrient transporter expression?

## **Aim 3.3:** How does mTORC1 signaling affect placental endocrine function?

## **Aim 3.4:** How does placental mTORC1 hyperactivation affect offspring survival, weight, body composition, and insulin sensitivity?

# Potential Pitfalls and alternate Approaches (Aims 3.1-3.4)

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