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# Specific Aim 3

**Elucidate the consequences of placental nutritional stress driven by mTORC1 hyperactivation on placental role.**

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). In cases of maternal nutritional stress by obesity, placental nutrient transport and endocrine function are believed to be suboptimal leading to unhealthy fetal growth (Leddy *et al.*, 2008; Gaccioli *et al.*, 2013*a*). 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 maternal obesity has been linked to placental mTORC1 function (Wen *et al.*, 2005; Roos *et al.*, 2007; Mparmpakas *et al.*, 2012; Jansson & Powell, 2013). 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).

### mTORC1 and Placental Amino Acid Transport

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.

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*).

### mTORC1 and Placental Glucose Transport

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.

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.

### mTORC1 and Fatty Acid Transport

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). Additionally, fatty acid transporter, FABP4 has been localized in trophoblasts and in the endothelial cells (Scifres *et al.*, 2011), but it remains less studied in context of obesity. FATP1 fatty acid transporter protein has also been localized at the microvillous and basal plasma membranes of the placenta (Campbell *et al.*, 1998), but remains less studied in context of obesity.

In humans, newborns born to mothers with pre-pregnancy obesity had higher total cholesterol and LDL plasma levels (Dubé *et al.*, 2012). Fatty acid-binding proteins, needed for fatty acid storage and metabolism in the placenta, showed reduced mRNA expression of FABP1 and reduced protein expression of FABP1 and FABP3 (Dubé *et al.*, 2012). Furthermore, placental LPL activity was increased in placentas of obese women compared to their lean counterparts (Dubé *et al.*, 2012). This data suggests that placental lipid transport is affected by maternal obesity, but further studies are needed to better assess this phenomenon.

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 mTORC1 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).

### mTORC1 and Fetal Lethality

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). 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). 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).

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).

## Obesity Effects on Placental Development and Function and Fetal Weight

Some human studies have demonstrated that maternal obesity causes increased placental weight and overgrowth compared to placentas form lean women (Ouyang *et al.*, 2013; Leon-Garcia *et al.*, 2016; Rosado-Yépez *et al.*, 2019), while other studies did not show placental overgrowth with maternal obesity (Kovo *et al.*, 2015). It is well established, however, that maternal obesity is positively associated with birthweight (Sebire *et al.*, 2001; Ehrenberg *et al.*, 2004; Ouyang *et al.*, 2013; Acosta *et al.*, 2015; Kovo *et al.*, 2015; Leon-Garcia *et al.*, 2016; Rosado-Yépez *et al.*, 2019).

In humans, 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).

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). Maternal obesity in mice fed a high fat, high sugar diet prior to mating to achieve a 25% increase in body weight have increased fetal weights but unaffected placental weights (Rosario *et al.*, 2015). Protein expression of SNAT2 and LAT1 were significantly increased but no changes in SNAT4 or LAT1 were detected (Rosario *et al.*, 2015). Consistent with the increased expression of SNAT2 and LAT1, *ex vivo* trophoblast uptake of radiolabeled amino acids by system A and system L was increased by 1.9 and 2.1 folds, respectively (Rosario *et al.*, 2015).

In rats, maternal obesity induced by 7 weeks of high-fat diet feeding prior to mating and throughout pregnancy showed increased fetal weight at E21 along with significantly increased placental mTORC1 signaling (Gaccioli *et al.*, 2013*b*). There was no detected change in placental weight or transporter expression of GLUT1, GLUT3, SNAT2, SNAT4, FATP4, and FATP6 and LPL, but SNAT1 protein expression was reduced in the placentas from dams on high-fat diet (Gaccioli *et al.*, 2013*b*).

Mice fed a high-fat diet but that did not develop obesity at mating show increased fetal weights at E18.5 and increased placental mTORC1 signaling (Jones *et al.*, 2009). Increased fetal weight was attributed to the increased placental transport of radiolabeled glucose and system A amino acid *in vivo* (Jones *et al.*, 2009). *Ex vivo*, placental GLUT1 and SNAT2 protein expression was markedly increased despite no change in GLUT3 and SNAT4 expression (Jones *et al.*, 2009). Rats fed a high-fat diet starting at E2 and throughout pregnancy who did not develop obesity had increased mTORC1 signaling evident by increased ratio of phosphorylated to total 4E-BP1 in male placentas but not in female placentas (Song *et al.*, 2017). Placental weights were unchanged as labyrinth zone thickness was decreased and decidual thickness increased (Song *et al.*, 2017). Additionally, GLUT3 and SNAT2 mRNA expression was increased only in male placentas despite no changes in male fetal weights, but there were no changes in GLUT1 or SNAT1 expression (Song *et al.*, 2017).

## Effect of Obesity on Placental Endocrine Function

Growth differentiation factor 15 is produced in the placenta, and changes are associated with a variety of complications including miscarriage, nausea and hypertension (Tong *et al.*, 2004; Chen *et al.*, 2016; Petry *et al.*, 2018). There are no studies assessing placental GDF15 activity in response to maternal obesity. Placental GDF15 levels are positively correlated with maternal and fetal GDF15 levels, suggesting that the placenta is the primary source of this hormone during pregnancy (Sugulle *et al.*, 2009).

## 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). In humans, maternal obesity was associated with early adulthood development of obesity and insulin resistance, even if the offspring had a normal birthweight (Mingrone *et al.*, 2008). Finally, 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; Mingrone *et al.*, 2008; Gaillard *et al.*, 2014; Williams *et al.*, 2014; Stubert *et al.*, 2018).

# 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 3). 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, 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?

I expect that mTORC1 hyperactivation in the placenta will cause increased fetal and placental weight, and the labyrinth zone will have increased thickness and area. This is supported by evidence from increased human placental weights in maternal obesity (Ouyang *et al.*, 2013; Leon-Garcia *et al.*, 2016; Rosado-Yépez *et al.*, 2019). It is worthy to mention that not all studies found increased placental weight in maternal obesity (Kovo *et al.*, 2015). Additionally, a study showed that labyrinth area was increased in a mouse model of obesity induced by high-fat diet (Hayes, 2012). The predicted increased fetal weight is supported by maternal diet-induced obesity in rodents (Rosario *et al.*, 2015, 2016). I predict fetal survival to be unaffected. This is supported by unaltered litter size in mouse model of obesity (Rosario *et al.*, 2016).

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

Since GLUT1 and GLUT3 are the most important glucose transporters in rodents, I hypothesize that our exposure will cause upregulation of GLUT1 and GLUT3 supported by reduced GLUT3 expression in JEG-3 human choriocarcinoma cell line with mTORC1 inhibition (Xu *et al.*, 2015) and increased placental GLUT1 expression in non-obese mice fed a high-fat diet (Jones *et al.*, 2009).

As System A amino acid transporter is primarily associated with fetal growth, System A activity will be studied in our model. I predict SNAT2 expression, but not SNAT4, to be increased supported by increased placental SNAT2 expression in obese mice but no changes in SNAT4 (Rosario *et al.*, 2015). I predict no change in SNAT1 expression as shown by no change in SNAT1 mRNA expression in human placental extracts treated with rapamycin (Roos *et al.*, 2009*a*).

It is worth noting that not all studies are in agreement regarding transporter expression with Gacciolli et al. detecting no change in placental weight or transporter expression of GLUT1, GLUT3, SNAT2, SNAT4 in the placentas from obese rats fed a high-fat diet with reductions in SNAT1 expression (Gaccioli *et al.*, 2013*b*).

Additionally, I predict placental LPL activity to be increased as it was increased in placentas of obese women compared to their lean counterparts (Dubé *et al.*, 2012).

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

I hypothesize GDF15, an anorexic hormone, to be upregulated in mTORC1-hyperactivated placentas. Given that GDF15 levels are increased in muscle with activated mTORC1 activity (Tang *et al.*, 2019; Stephenson *et al.*, 2019) and reduced in plasma after rapamycin treatment (Khan *et al.*, 2017), and since placental mTORC1 is hyperactivated in our model, then GDF15 levels should increase accordingly.

Placental *Igf2* is predicted to be upregulated since mouse model of maternal obesity showed increased insulin signaling along with increased mTORC1 signaling (Rosario *et al.*, 2016). Furthermore, mTORC1 is suggested to positively affect expression of Igf-II (Erbay *et al.*, 2003), and was increased in female placentas of rats fed a high-fat diet during gestation (Song *et al.*, 2017).

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

It is well established that maternal obesity is positively associated with birthweight (Sebire *et al.*, 2001; Ehrenberg *et al.*, 2004; Ouyang *et al.*, 2013; Acosta *et al.*, 2015; Kovo *et al.*, 2015; Leon-Garcia *et al.*, 2016; Rosado-Yépez *et al.*, 2019), and therefore I hypothesize that offspring weight will be higher at PND0.5, 7.5, 14.5, 21.5 and weekly after weaning till 6 weeks of age with increased body fat mass, consistent with human data showing increased childhood obesity at 6-11 years of age (Catalano *et al.*, 2009*a*).

Offspring at 6 weeks of age are expected to be insulin resistant since maternal obesity was positively correlated with fetal insulin resistance (Catalano *et al.*, 2009*b*), and maternal obesity was associated with early adulthood development of obesity and insulin resistance (Mingrone *et al.*, 2008).

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

It is possible that our placental mTORC1 hyperactivation may prove lethal, as this model has not been generated or described before. In that case, we will use a different parental strain of Hets (Het x Het) to generate a partial knockout that may prove viable. If the mice are viable, this model will prove useful in determining the exact mechanism at which mTORC1 acts in the placenta in the absence of all the confounding variables that maternal obesity poses.

# Appendix A: Summary Table of Compiled Studies Examining Effects of Maternal Obesity / mTORC1 on Placental/Fetal Development and Health

|  |  |  |
| --- | --- | --- |
| Paper | Methods/Exposure | Results |
| (Roos *et al.*, 2009*a*) | Human term-placental extracts used to measure 14CMeAIB, 3HTaurine, and 3HLeucine uptake when incubated with 100nM rapamycin | Rapamycin reduced TAUT, system A and system L transport  Rapamycin reduced expression of pS6K and p4E-BP1, downstream effectors of mTORC1.  Protein expression of SNAT2, SNAT4, LAT2, and TAUT was unaffected by rapamycin.  mRNA expression of LAT1 and TAUT were reduced whereas that of SNAT1, SNAT2, SNAT4, and LAT2 was unchanged with rapamycin. |
| (Roos *et al.*, 2007) | Human term-placental extracts from AGA, LGA and SGA/IUGR deliveries incubated with radiolabeled amino acids transported by system A, system L and taurine. | Expression of mTOR was detected in the syncytiotrophoblasts.  Rapamycin 100nM reduced system L activity but not system A or taurine activity.  mTORC1 expression was upregulated by 51% along with 45% reduction of pS6K in IUGR placentas, downregulated by 39% with no change in pS6K in LGA placentas. |
| (Roos *et al.*, 2009*b*) | Human term-placental explants used to determine amino acid transport with varying glucose concentrations, insulin, IGF1, and rapamycin. | Rapamycin reduced system L activity in presence of 16mM glucose standard glucose concentration).  Reductions in glucose concentrations from 16 to 4.5 and 0.5 caused decreased system L activity in a dose-dependent manner, but adding rapamycin did not cause further reductions to the activity at the lower glucose concentrations.  Reductions in glucose concentrations did not affect system A activity, but at the lower glucose concentrations (4.5 and 0.5 mM) rapamycin increased its system A activity in a dose-dependent manner compared to cells incubated with rapamycin and 16mM glucose.  Taurine transporter activity was increased with lower glucose concentrations in presence and absence of rapamycin, though at the respective concentrations (at 16 and 0.5mM glucose), rapamycin reduced activity.  In 16mM glucose, insulin increased System A and system L activity, but this increase was abolished when insulin and rapamycin were both added.  IGF1 increased system A activity only but this was abolished with rapamycin +IGF1 incubation.  Protein expression of pS6K was significantly reduced only when glucose levels were lowest at 0.5mM but expression as unchanged between 16 and 4.5mM. p4E-BP1 expression was unchanged at all three glucose concentrations.  AMPK and REDD1 expression was unchanged at all glucose concentrations. |
| (Xu *et al.*, 2015) | JEG-3 human choriocarcinoma cell line used to determine GLUT3 expression | Treating cells with rapamycin reduced GLUT3 mRNA expression by 60% and reduced protein expression by 28%  Raptor knockdown to inhibit mTORC1 reduced GLUT3 mRNA expression by 41% and reduced protein by 50%. |
| (Lager *et al.*, 2014) | Human term-placental extracts used to assess amino acid uptake using isotope-labeled tracers when incubated with saturated and unsaturated fatty acids (DHA 22:6 polyunsaturated, OA 18:1 monounsaturated, PA16:0 saturated). | DHA reduced system A and system L amino acid uptake and reduced phosphorylated mTORC1, reduced p4E-BP1 expression with no effect on pS6K1 (reduced mTORC1 signal).    DHA+OA incubation increased system A amino acid uptake, but did not affect system L. Had no effect on phosphorylated mTORC1, p4E-BP1, or pS6K1 (no effect on mTORC1).  OA increased system A uptake but did not affect system L. Oa increased phosphorylated mTORC1, and increased pS6K1 but did not affect 4E-BP1 expression (increased mTORC1 activity).  PA did not cause changes in amino acid uptake. PA did not affect mTORC1 signaling or downstream targets. |
| (Hennig *et al.*, 2017) | Pregnant mice treated with subcutaneous injections of rapamycin (5mg/kg body weight) every 12 hours starting at E15.5 until delivery  Treatment of rapamycin starting at E11.5 | Offspring of dams treated with rapamycin at E11.5 every 12 hours died at E16.5 and had severe growth restriction and malformations.  Using mice treated at E15.5:  PND1 offspring tissue (heart, kidney, and lung) showed reduced mTORC1 verifying fetal mTORC1 inhibition.  Rapamycin treatment caused reduced offspring weight at PND1 with reduced heart weight by 34.5%. Kidney weight was reduced by 19.7%  mTORC1 inhibition at E15.5 till delivery had no effect on fetal lethality. |
| (Jansson *et al.*, 2013) | Placentas from term-pregnancies with available pre-pregnancy maternal BMI | Pre-pregnancy body mass index (BMI) was positively correlated with placental mTORC1 activity and birth weight.  System A and system L amino acid transporter activity was unchanged with increased maternal pre-pregnancy BMI.  System A SNAT2 protein expression was positively associated with offspring birth weight |
| (Rosario *et al.*, 2016). | Mouse model of obesity fed high fat,high sugar diet starting at 13 weeks of age and for 4-6 weeks prior to mating to establish 25% increase in weight. Dams were maintained on their control or experimental diets during pregnancy. | E18.5 experimental fetuses had 18% increase in weight.  No difference in litter size.  Placental weights at E18.5 were the same.  Placental pS6 and 4E-BP1 had increased phosphorylation by 150 and 89%, respectively indicating increased placental mTORC1 signaling.  Placental AMPK, upstream mTORC1 inhibitor, had reduced phosphorylation by 75%.  Placental insulin/IGF-I signaling was increased with higher phosphorylated IRS1 and Akt by 50% and 90%, respectively. |
| (Gaccioli *et al.*, 2013*b*) | Rats fed high fat diet at 6 weeks of age for 7 weeks then mated. Rats maintained on diet throughout pregnancy.  Trophoblast plasma membranes were assessed for LPL activity and for amino acid transport activity. | E21 fetal weight increased significantly  Fetal blood glucose was higher but not significant  Fetal plasma triglyceride, plasma insulin, and plasma leptin were significantly higher.  E21 placental weight was not significantly different.  Placental mTORC1 signal increased as shown by increased phosphorylation of downstream targets.  Placental AMPK phosphorylation was significantly reduced (AMPK is inhibitor of mTORC1).  System A: decreased SNAT1 protein expression in HF placentas, and unchanged SNAT2 and SNAT4.  No change in placental glucose transporters: GLUT1, 3 and 9.  No change in LPL or fatty acid transporter expression (FATP4 and FATP6). |
| (Jones *et al.*, 2009) | 8-week old mice fed high fat diet for 8 weeks prior to mating and during pregnancy.  *In vivo*  and *ex vivo p*lacental studies to assess glucose and amino acid transporter activity. | E18.5 fetuses had 43% increased weights.  Placental weight was not different.  *In vivo* transport showed increased placental clearance of radiolabeled glucose by 5-fold and amino acid (MeAIB) by 10-fold in HF placentas.  HF placentas had increased GLUT1 and SNAT2 protein expression *ex vivo.* SNAT4 and GLUT3 were unaffected. |
| (Song *et al.*, 2017) | Rats fed a high fat diet starting at E2 and throughout gestation. | On E21, placental total thickness was unchanged, but labyrinth thickness was reduced and inversely, decidual thickness increased. Placental weight was not affected.  Fetal weight was reduced only in females of HF dams, no change in males.  GLUT3 mRNA expression increased in male placentas of HF diet, but not in female placentas.  GLUT1 and SNAT1 were unchanged in male and female placentas.  SNAT2 mRNA expression was increased in male placentas only but unchanged in female placentas.  mTORC1 signaling was increased in male placentas as evident by reduced total 4E-BP1 levels which caused increased ratio of p4E-BP1:total 4E-BP1. No change in other downstream targets of mTORC1 and no change in mTORC1 signal in female placentas.  *Igf2* mRNA expression was increased in female placentas. |
| (Rosario *et al.*, 2015) | Mice fed high fat, high sugar diet starting at 13 weeks of age. Mice were then mated after a 25% increase in body weight was achieved, maternal obesity.  Trophoblast plasma membranes isolated for glucose and amino acid transport activity. | E18.5 fetal weight was increased by 18%, no change in placental weights.  Placental weights unchanged.  SNAT2 protein expression increased in TPM of HF dams. No change in SNAT4 expression. System A amino acid uptake was increased by 1.9 fold.  LAT1 protein expression increased (system L amino acid transporter), but no change in LAT2. System L amino acid uptake increased by 2.1 fold.  GLUT1 and GLUT3 protein expression was increased in HF dam placentas. |
| (Dubé *et al.*, 2012) | Womn prospectively enrolled in study with varying pre-pregnancy BMI. Placentas collected at term delivery to assess LPL activity and linoleic acid uptake. | Newborns of obese mothers had increased total cholesterol and LDL plasma levels, but had similar TG and FFA.  Placental expression of FABP1 mRNA and protein was reduced in placentas of obese women.  FABP3 mRNA expression was unchanged, but FABP3 protein was reduced in placentas of obese women.  LPL protein and mRNA expression was unchanged, but LPL activity was increased in 3rd trimester placentas from obese women compared to lean. |

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1. The prevalence of obesity in the United States has been estimated at 39.8% for adults in 2015-2016. Data obtained from National Health and Nutrition Examination Survey, National Center for Health Statistics, December 2017 <https://www.cdc.gov/nchs/data/factsheets/factsheet_nhanes.pdf> [↑](#footnote-ref-1)